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N-butyl-deoxynojirimycin delays motor deficits, cerebellar microgliosis, and Purkinje cell loss in a mouse model of mucopolidosis type IV

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Abstract

Mucopolidosis type IV (MLIV) is a lysosomal storage disease exhibiting progressive intellectual disability, motor impairment, and premature death. There is currently no cure or corrective treatment. The disease results from mutations in the gene encoding mucopolin-1, a transient receptor potential channel believed to play a key role in lysosomal calcium egress. Loss of mucopolin-1 and subsequent defects lead to a host of cellular aberrations, including accumulation of glycosphingolipids (GSLs) in neurons and other cell types, microgliosis and, as reported here, cerebellar Purkinje cell loss. Several studies have demonstrated that N-butyl-deoxynojirimycin (NB-DNJ, also known as miglustat), an inhibitor of the enzyme glucosylceramide synthase (GCS), successfully delays the onset of motor deficits, improves longevity, and rescues some of the cerebellar abnormalities (e.g., Purkinje cell death) seen in another lysosomal disease known as Niemann-Pick type C (NPC). Given the similarities in pathology between MLIV and NPC, we examined whether miglustat would be efficacious in ameliorating disease progression in MLIV. Using a full mucopolin-1 knockout mouse (*Mcoln1*^{-/-}), we found that early miglustat treatment delays the onset and progression of motor deficits, delays cerebellar Purkinje cell loss, and reduces

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Author Contributions

LB and SW designed the experiments, and LB performed the experiments and compiled the data. Data analysis was carried out by LB, SW, JS and KD. Mass spectrometry was performed by JS, LK, and JL, and analyzed by JS, LK, JL, and LB. Retinopathy western blot analysis was performed by YG. The document was written and compiled by LB, with consult and editing by SW, JS, KD and other authors.

cerebellar microgliosis characteristic of MLIV disease. Quantitative mass spectrometry analyses provided new data on the GSL profiles of murine MLIV brain tissue and showed that miglustat partially restored the wild type profile of white matter enriched lipids. Collectively, our findings indicate that early miglustat treatment delays the progression of clinically relevant pathology in an MLIV mouse model, and therefore supports consideration of miglustat as a therapeutic agent for MLIV disease in humans.

Graphical abstract

Keywords

mucopolipidosis type IV; miglustat; lysosomal storage disease; small molecule therapy; mucolipin-1; Purkinje cells; glycosphingolipids

Introduction

Mucopolipidosis type IV (MLIV, OMIM #252650) is an autosomal recessive, lysosomal storage disorder (Bach et al., 1975; Bargal et al., 2000; Tellez-Nagel et al., 1976). Typically, the disease clinically presents at 1–2 years of age and over time leads to severe and progressive intellectual disability, motor and speech deficits, progressive retinopathy leading to blindness, systemic pathologies, and a shortened lifespan (Amir et al., 1987; Schiffmann et al., 2014; Venugopal et al., 2007). There is currently no corrective therapy or cure for MLIV. The prevalence of MLIV is relatively high in the Ashkenazi Jewish population with an estimated carrier frequency of 1:100, but can also occur in the general population (Altarescu et al., 2002; Bargal et al., 2001, 2000; Edelman et al., 2002). The disease results from mutations in the gene encoding mucolipin-1 (*MCOLN1*), a 65 kDa protein also known as TRPML1 that serves as a transmembrane transient receptor potential (TRP) channel found in the limiting membrane of late endosomes and lysosomes (Bach, 2005; Bargal et al., 2000; Ye et al., 2004). *MCOLN1* is localized to chromosome 19p13.2–13.3 with both major and minor MLIV-causing mutations identified in *MCOLN1*, as well as over 20 independent mutations (Bargal et al., 2001, 2000; Slaugenhaupt et al., 1999; Sun et al., 2000; Venugopal et al., 2007; Wakabayashi et al., 2011). These mutations range from causing reduced mRNA expression to yielding nonfunctional protein (Sun et al., 2000; Wakabayashi et al., 2011). Mucolipin-1 is ubiquitously expressed with highest expression in the brain and kidney (Bach, 2005; Cheng et al., 2010) and its loss leads to systemic disease accompanied by severe neurological pathology.

The function of mucolipin-1 is not fully understood, but it is known that it serves a critical role in pH regulation and as a nonselective ion channel permeable to calcium, iron, and other cations (Bach, 2005; Cheng et al., 2010; Dong et al., 2009, 2008; Soyombo et al., 2006). Endosomal/lysosomal calcium egress through this TRP channel appears to have an important function in key cellular processes requiring a local, lysosomal calcium source, such as the fusion of organelles during autophagy and lysosomal exocytosis (Curcio-Morelli et al., 2010; LaPlante et al., 2006; Lima et al., 2012; Medina et al., 2015). The loss of mucolipin-1 and resulting defects in these and possibly other processes lead to a host of MLIV pathologies, including storage of cellular substrates like glycosphingolipids (GSLs) normally targeted to the lysosome for degradation, dysregulation of lysosomal pH, and the buildup of autophagosomes (Bach et al., 1975; Micsenyi et al., 2009; Venkatachalam et al., 2008; Venugopal et al., 2007; Wong et al., 2015; Ye et al., 2004). In addition, loss of mucolipin-1 secondarily leads to other abnormalities such as widespread gliosis, dysmyelination, and Purkinje cell abnormalities (Grishchuk et al., 2014; Micsenyi et al., 2009; Schiffmann et al., 2014) and death as further reported here. Numerous MLIV disease models have been generated that recapitulate cellular pathologies, including murine, *Drosophila*, and *C. elegans* models (Lima et al., 2012; Schaheen et al., 2006; Venkatachalam et al., 2008; Venugopal et al., 2007). The MLIV mouse model (*Mcoln1*^{-/-} mice) displays progressive behavioral and locomotor deficits, including hind-limb clasping, abnormal gait, and cognitive impairment (Grishchuk et al., 2014; Venugopal et al., 2007). As the mechanism linking loss of mucolipin-1 to these abnormalities is not fully established, this knowledge gap yields a major obstacle to developing a direct therapy for this disorder.

Substrate reduction therapy (SRT) has shown success in certain lysosomal diseases, such as Type 1 Gaucher, where enzyme replacement therapy (ERT) is not appropriate for all patients, and Sandhoff and Niemann-Pick type C (NPC) diseases where ERT or gene replacement therapy is not currently available (Aerts et al., 2006; Cox, 2005; Di Rocco et al., 2012; Pastores, 2006; Pastores and Barnett, 2003; Patterson et al., 2007; Stein et al., 2012; Tallaksen and Berg, 2009; Wortmann et al., 2009; Zervas et al., 2001b). SRT aims to improve overall cellular function by reducing the synthesis of those substrates which accumulate as storage (Aerts et al., 2006; Cox, 2005). These studies have shown success particularly with one compound, N-butyl-deoxynojirimycin (NB-DNJ), otherwise known as miglustat, a small imino sugar that is an inhibitor of the GSL synthetic enzyme known as glucosylceramide synthase (GCS) (Elstein et al., 2004; Jeyakumar et al., 1999; Pastores, 2006; Pastores and Barnett, 2003; Platt et al., 1997a, 1994; Stein et al., 2012; Zervas et al., 2001b). Located in the ER and Golgi, GCS catalyzes the first committed step along the GSL pathway (Platt et al., 1997a). In the context of SRT, this enzyme acts upstream of substrates accumulating in type 1 Gaucher and Sandhoff diseases and contributing to storage in NPC disease (Pastores and Barnett, 2003; Platt et al., 1994; Zervas et al., 2001a, 2001b). Importantly, in murine and feline NPC models, miglustat successfully delayed Purkinje cell loss, cerebellar gliosis, the progression of motor deficits linked to cerebellar pathology, and improved longevity (Davidson et al., 2009; Stein et al., 2012; Zervas et al., 2001b). Given these findings, we hypothesized that miglustat may reduce the cerebellar abnormalities in MLIV and could represent a viable therapy that may improve the quality of life in MLIV patients. In order to investigate this hypothesis, we examined the effects of miglustat in

Mcoln1^{-/-} mice (Venugopal et al., 2007). We found that miglustat successfully delays motor deficits, improves the profile of specific lipid classes in the cerebellum, and delays cerebellar microgliosis and cerebellar Purkinje cell loss. We believe these data indicate that miglustat warrants further investigation as a treatment for MLIV patients.

Materials and Methods

Mouse Generation and Administration of Miglustat

Heterozygous (*Mcoln1*^{+/-}) mice were crossed to obtain littermate wild type (*Mcoln1*^{+/+}) and mutant (*Mcoln1*^{-/-}) mice, which were then genotyped prior to postnatal day 10 (P10) (Venugopal et al., 2007). Cohorts of male and female *Mcoln1*^{+/+} and *Mcoln1*^{-/-} littermate mice were then randomly assigned for treatment with miglustat or saline vehicle starting at P10. Using a miglustat solution of 30 mg/ml saline, mice received 300 mg miglustat/kg body weight or the equivalent volume of normal saline. These were administered daily via intraperitoneal (IP) injection from P10-P21, followed by IP injection three times a week after P21 and until the end of the study. A total of 47 mice were used in this treatment study: WT saline n=14, WT miglustat n=13, *Mcoln1*^{-/-} saline n=8, *Mcoln1*^{-/-} miglustat treated n=12. Additional mice were used as untreated *Mcoln1*^{-/-} controls for western blot analyses and CD68 counts following the conclusion of the drug treatment cohort. All studies were performed in consideration of high standards of scientific rigor (Landis et al., 2012) and in accordance with the Albert Einstein College of Medicine's IACUC and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Behavioral Studies

Behavioral assessments were made at timepoints selected to represent pre-symptomatic (7–15 for open field/object placement, 15–16 wks for balance beam), early symptomatic (26 wks), and end-stage disease (32 wks) based on previous reports in *Mcoln1*^{-/-} mice (Grishchuk et al., 2014; Micsenyi et al., 2009; Venugopal et al., 2007). Mice were given a randomized number to blind the tester to genotype and treatment, and their tails color-coded per cage during testing to represent each blinded number. All behavioral work was performed within the Albert Einstein College of Medicine Animal Phenotyping Core Facility and according to detailed published protocols (Dere et al., 2007; Ennaceur and Delacour, 1988; Stanley et al., 2005):

Open field—Mice were placed in a 41×41 cm open field testing box devoid of objects with orientation markers pasted to the sides of the box. Biobserve video tracking software was used to track and quantify the mouse's movements within the arena, including the total track-length explored, entries into the center arena, time spent in the center arena, and track-length in the center arena. The center of the arena was set at 17×17 cm for each trial. The number of rears were manually counted in a blinded fashion to the tester, with a "rear" being considered when the mouse lifted its body upward and both front paws were elevated from the floor. The task was performed over a 6 minute window and analyzed in both 3 minute bins and the total 6 minutes.

Object Placement—Mice were placed in the open field box containing 2 identical objects. The amount of time spent exploring each object was manually timed over a 5 minute window. The mouse was then removed from the arena for a 16 minute window, during which one object was moved to a new location. The mouse was then placed back in the arena and the amount of time spent at each object timed manually over a 3 minute testing window, with exploration of the objects including sniffing and/or touching the object in question. A preference for the newly placed object was deemed as greater than or equal to 55% exploration of the novel object compared to the unmoved object.

Balance beam—Three beams were utilized, 30mm (wide), 24mm (medium), and 18mm (narrow) diameter beam. Beams were raised using tripods and stabilized, with hides available at the end of the beam to promote passage across the beam. A light gradient was generated to stimulate the mouse moving from the start (bright) of the beam to the end of the beam (dark). Covered bins were located underneath the beams as a safety precaution. Prior to first testing, mice were trained on a 3 inch wide-set beam to promote passage across the beam. During testing, mice were run through the narrow, medium, then wide beams and the number of slips counted manually, with the observer blinded to genotype and treatment.

Tissue Collection

At end-stage timepoints (ranging from 7–9 months for the MLIV mouse model), mice were administered an overdose of ketamine/xylazine, and once insensate, desanguinated with saline and transcardially perfused with 4% paraformaldehyde. Following desanguination and prior to paraformaldehyde perfusion, tissue for western blot and mass spectrometry analyses was collected and flash frozen with liquid nitrogen and stored at -80°C . Following perfusion, tissues were collected and stored in 4% paraformaldehyde for 24 hours, then transferred to 0.1M phosphate buffer at 4°C .

Total Lipid Extraction for ESI-MS/MS

Liquid nitrogen flash frozen samples containing $\frac{1}{2}$ cerebrum and $\frac{1}{2}$ cerebellum with adjacent brain stem from miglustat and saline treated WT and *Mcoln1*^{-/-} mice (4 each) were first sectioned (30 μm) using a Leica cryostat microtome. Sections were subsequently homogenized in MilliQ water, at a concentration of 5–10% tissue by weight, with a Branson cup horn homogenizer. The extraction was performed by a modified method as described previously (Natori et al., 1988). Briefly, 250 μl of homogenate was transferred to a 15 ml glass tube and was extracted sequentially in solvent mixtures of increasing polarity. In the first step, 2 ml of C:M:W (2 parts chloroform: 1 part methanol: 0.1 part MilliQ water; v/v/v) was added and the mixture was vortex-mixed for 90 minutes (30s vortex and 10 minute pause) and left standing for 30 minutes. Samples were then centrifuged at $400\times g$ for 15 minutes to remove protein/tissue precipitate and both the upper water phase and the lower organic solvents were collected and removed from protein/tissue precipitate and transferred to a new glass tube after each step. In the second step, 1 ml of C:M:W (1:1:0.1; v/v/v) mixture was added to remaining tissue precipitate and the samples were vortex-mixed for 50 minutes and left standing for 10 minutes. Liquid phases from both steps were consequently combined. Lastly, 1 ml of C:M:W (1:2:0.1; v/v/v) was used. Samples were vortex-mixed for 60 minutes, left overnight at 4°C , vortex-mixed and left standing for 60 minutes. All the

resultant liquid phases were combined with those previously collected and all were evaporated under the liquid nitrogen stream. The remaining homogenate was used to determine the protein concentration. Lipid extracts were reconstituted in C:M (2:1; v/v) to reach the final protein concentration of 1 µg/µl and stored at −20° C.

Lipid Analyses by ESI-MS/MS

Lipids were quantified as previously described (Hlková et al., 2012; Kuchar et al., 2009) using an AB/MDS SCIEX API 4000 tandem mass spectrometer equipped with an ESI ion source and coupled to an Agilent HPLC 1290 series. Samples for electrospray ionization tandem mass spectrometry (ESI-MS/MS) analyses were prepared from lipid extracts corresponding to 40 µg protein aliquots for gangliosides and sulfatides and 5 µg protein aliquots for other lipids. Aliquots were evaporated and dissolved in 1 ml of methanol for gangliosides and sulfatides and in 1 ml of methanol with 5 mM NH₄COOH for other lipids. Individual samples (20 µl) were introduced by flow injection (flow rate 50 µl/min of the methanol mobile phase). Ceramide (Cer), ceramide monohexoside (CMH, represents both GlucosylCer and GalactosylCer), ceramide dihexoside (CDH), Gb3Cer, Gb4Cer and sphingomyelin (SM) were electrosprayed in the positive ion mode to form [M+H]⁺ ions. Generated ions were analyzed by selected reaction monitoring (SRM) of precursor ions and the common ceramide fragment with 264 m/z for ceramide and glycosphingolipids or 184 m/z for sphingomyelin were used. Gangliosides and sulfatides were electrosprayed in negative mode to generate [M−H][−] ions. SRM transition pairs used precursor ions of analyzed lipids and common sialic acid product ion with 290 m/z for gangliosides and HSO₄[−] with 97 m/z for sulfatides. To quantitate, the following internal standards were added to the samples: C17:0 ceramide (Avanti Polar Lipids), C15:0 GalCer for CMH (Matreya), C16:0-D₃ LacCer for CDH (Matreya), C17:0 Gb3Cer for Gb3Cer and Gb4Cer (Matreya), C17:0 sphingomyelin (Avanti Polar Lipids), C18-D₃ GM1 (Avanti Polar Lipids), C18-D₃ GM2 C18-D₃ GM3 (Avanti Polar Lipids) and C12:0 sulfatide (Avanti Polar Lipids). All samples were measured in technical replicates and average values were calculated for each sample.

Immunofluorescence Staining and Tissue Labeling

Fixed tissues were sectioned (coronal for cerebrum and sagittal for cerebellum) at 35 µm thickness using a Leica V100S vibratome (McGlynn et al., 2004). For immunofluorescent staining, tissues were first blocked for 90 minutes at 4°C with shaking (10% goat serum, 0.02% saponin, 1× PBS). Sections were then incubated with primary antibody in diluent solution (5% normal goat serum, 0.02% saponin, 1× PBS) overnight at 4° C. Primary antibody(ies) included anti-GM2 (1:50, mouse IgM, cell culture supernatant produced in-house by Dr. K. Dobrenis using the 10–11 hybridoma line from Progenics Pharmaceuticals, Tarrytown, NY USA), anti-CD68 (1:500, rat monoclonal antibody, AbD Serotec MCA1957), anti-GFAP (1:1000, rabbit polyclonal antibody, Sigma G9269), and anti-Calbindin-D 28K (1:500, mouse monoclonal antibody, Sigma C9848). Following primary incubation, sections were washed four times for 10 minutes at room temperature, then incubated with species-specific secondary antibodies, conjugated to Alexa Fluor 488 or 546 (1:250, in diluent solution described above), for 60 min at room temperature. Tissues were

then washed four times in 1× PBS and mounted with Prolong Antifade solution with or without DAPI (Invitrogen).

Confocal Imaging

Confocal imaging was performed with a Zeiss Meta 510 Duo V2 laser scanning confocal microscope using Plan-Apochromat 20×/0.8, C-Apochromat 40×/1.2 water UV-VIS-NIR, and Plan-Apochromat 63×/1.4 oil DIC objectives and 351 nm, 488 nm, 543 nm and 633 nm laser lines. Acquisition channels were combined to produce merged images, and analyzed using ImageJ software and MetaMorph software (Molecular Devices). To evaluate and control for autofluorescence, 35 µm sections were mounted directly without any processing and imaged under the same conditions as stained sections, using the same bandwidths and filter sets.

Cell Counts

Following immunofluorescence labeling for CD68, CD68 positive (CD68+) cells were counted using an upright Olympus epifluorescence microscope equipped with StereoInvestigator (MBF Bioscience). Sagittal sections of cerebellar lobules were outlined lobule by lobule and x,y,z dimensions obtained to determine the volume of each lobule counted (Sillitoe and Joyner, 2007). Data were compiled as anterior, central, posterior, and nodular zones (Sillitoe and Joyner, 2007) such that lobules I–II, III, and IV–V were grouped as the anterior zone, lobules VI–VII were grouped as the central zone, lobule VIII as the posterior zone, and lobules IX and X were grouped as the nodular zone (Sillitoe and Joyner, 2007). An area of dorsal neocortex was outlined for cerebral cell counts, assuming equal extent of microglial presence across dorsal neocortical layers in end stage tissue. CD68+ cells were then counted manually blinded to genotype and treatment. Cell counts were normalized per arbitrary unit volume.

Western Blot Analysis

Cerebellar Tissue—Tissues were homogenized in ice cold lysis buffer: 50 mM TrisHCl (pH 7.5), 150 mM NaCl, 1% Igepal, 1% sodium deoxycholate, 1% sodium dodecyl sulfate, 1% Triton X100, and Complete Mini Protease Inhibitor Cocktail (Roche 11-836-153-001) using a Dounce homogenizer, then passed through a 27-gauge needle. Protein concentrations were quantified using a Pierce BCA Protein Assay Kit (ThermoFisher Scientific), and 15 µg of protein per sample was added to lysis buffer along with 4× Laemmli Sample Buffer plus β-mercaptoethanol and boiled for 5 minutes. Samples were run on a SDS-PAGE 12% Tris-Glycine gel in a BioRad Mini Protean Cell at 100 volts for 75 minutes, then transferred to a PVDF membrane using a BioRad Mini TransBlot Cell at 0.35 amps for 60 minutes at 4° C. Membranes were blocked for 60 minutes in 5% milk in Tris-buffered-saline with 0.1% Tween 20 (TBST), then incubated with primary antibodies (anti-calbindin 1:1000, rabbit polyclonal antibody, Millipore AB1778; anti-beta actin 1:3000, rabbit polyclonal antibody, Sigma A2066), diluted in 1% BSA, overnight at 4° C. The blot was rinsed in TBST four times for 10 minutes each, then incubated with secondary: polyclonal donkey anti-rabbit IgG conjugated with IRDye 800CW (1: 10,000, LiCor 925-32212) for 60 minutes at room temperature. The blot was then rinsed in TBST four times for 10 minutes and imaged using

an Odyssey CLx imaging station equipped with Image Lite Studio Software. Quantification was performed with Image Lite Studio Software. The intensity of bands for calbindin were normalized to those of beta-actin to correct for loading differences.

Retinal Tissue—Ocular tissue was homogenized in radio-immunoprecipitation assay buffer (RIPA, Boston BioProducts) supplemented with 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA) and a protease inhibitor cocktail (Roche 11873580001). Homogenates were centrifuged at 5000 rpm for 10 minutes at 4°C, then supernatants were collected and protein concentration determined using Pierce BSA Protein Assay kit (ThermoFisher Scientific). Proteins (20µg) were separated by SDS-PAGE on a 12% Bis-Tris gel in 3-(N-morpholino) propanesulfonic acid (MOPS) buffer (Invitrogen) and transferred onto nitrocellulose membrane (ThermoFisher Scientific) for immunodetection. The following primary antibodies were used: anti-rhodopsin (1:1000, mouse monoclonal, Chemicon MAB5356) and anti-β-actin (1:5000, rabbit polyclonal, Abcam ab8227). After incubation with primary antibody, the secondary antibodies were applied: polyclonal goat anti-mouse or goat anti-rabbit IgG conjugated with IRDye 680 or IRDye 800 both from LI-COR. Protein bands were visualized using the Odyssey Infrared Imaging System and analyzed by Odyssey v3.0 software (LI-COR). The intensity of bands for rhodopsin were normalized to beta-actin.

Statistical Analyses and Graphical Representation

All data were compiled and statistically analyzed using GraphPad Prism Software Version 7.0a (Figures 1(C–E)–6) and JMP Software from SAS Version 13.0.0 (Figure 1A–B). To determine the statistical significance of behavioral data, repeated measures (RM) ANOVA (genotype × treatment × time point) was performed and a difference was deemed as significant where $p < 0.05$. When RM ANOVA detected a difference, a following ANOVA at each timepoint (by genotype and treatment) was performed and differences deemed significant where $p < 0.05$. Where appropriate, post-hoc parametric Tukey-Kramer HSD pairwise comparisons at each timepoint were then performed. Western blot band measurements and cell counts were analyzed using an unpaired t-test with Welch's correction. Statistical analyses of mass spectrometry data were performed using two-way ANOVA (genotype and treatment), followed by Tukey's multiple comparisons tests where appropriate. For all data, significant differences obtained in pairwise comparisons are noted in graphical representations between WT saline - *Mcoln1*^{-/-} saline, *Mcoln1*^{-/-} saline - *Mcoln1*^{-/-} miglustat, WT saline - *Mcoln1*^{-/-} miglustat, and WT saline - WT miglustat. Significant differences are denoted as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. For all graphs, mean ± standard error is plotted and were prepared using Graphpad Prism Software.

Results

Miglustat delays motor deficits in *Mcoln1*^{-/-} mice

In order to assess changes that may be occurring with chronic miglustat therapy, we performed behavioral analyses throughout the course of our study on “pre-symptomatic” mice (7–15 weeks), at the onset of visible disease phenotypes (26 weeks), and nearing end-

stage disease (32 weeks). Based on previous findings of motor deficits in *Mcoln1*^{-/-} mice, we were particularly interested in the motor and exploratory activity of our cohort (Grishchuk et al., 2014). Many lysosomal diseases display motor phenotypes indicative of cerebellar abnormalities, such as diminished motor coordination, and there is evidence that miglustat can ameliorate these changes (Davidson et al., 2009; Zervas et al., 2001b). In NPC disease as well as other mouse models demonstrating Purkinje cell loss, reduced performance on a balance beam (i.e., more slips) is also observed (Barski et al., 2003; Davidson et al., 2009; Stein et al., 2012). *Mcoln1*^{-/-} mice had significantly impaired performance on wide, medium, and narrow beams (increasing difficulty), compared to WT mice, and worsened over time (Fig. 1A, Fig. S1A–B). Miglustat treated *Mcoln1*^{-/-} mice also displayed deficits at the first time point of balance beam assessment (15–16 weeks) compared to WT mice, but importantly they did not worsen over time. Indeed, drug treated *Mcoln1*^{-/-} mice displayed a significantly better performance than control *Mcoln1*^{-/-} mice by 32 weeks on all balance beams, and showed no significant difference from WT controls by 26 and 32 weeks on the wide balance beam (Fig. 1A, Fig. S1A–B).

We next observed mice within an open field to assess any changes to exploratory activity, anxiety, and general motor ability. *Mcoln1*^{-/-} mice showed a trend towards executing less rears within the open field compared to WT mice at both early ages and nearing end-stage disease (Fig. 1B), yet explored similar lengths of track to WT mice (Fig. S1F). Miglustat treated *Mcoln1*^{-/-} mice appeared to sustain rearing activity closer to WT levels at late stages (Fig. 1B). *Mcoln1*^{-/-} mice also had similar behavior to WT mice within the center of the arena, including the percent track explored within the center arena, entries into the center, and the duration of time spent in the center arena (Fig. S1C–E). Behavior in the center arena is a useful estimate of anxiety, wherein less entries into, time spent, or track explored in the center are indicators of increased anxiety (Lau et al., 2008). Here we also found that miglustat treated *Mcoln1*^{-/-} mice explored significantly less center arena track than both WT and *Mcoln1*^{-/-} saline treated mice (Fig. S1E).

We also asked whether there were any changes to cognitive ability in *Mcoln1*^{-/-} treated or saline treated mice. To test spatial memory retention, we utilized a novel object placement task to assess the ability of our cohort to recognize a newly placed object. No differences were detected between WT and *Mcoln1*^{-/-} saline treated mice, or *Mcoln1*^{-/-} saline treated mice compared to miglustat treated *Mcoln1*^{-/-} mice (Fig. S1G). No significant changes were detected in weight over time with miglustat treatment as well (Fig. S2).

Given what we know of balance beam performance being linked to cerebellar Purkinje cell function, and from miglustat trials in NPC disease where miglustat delayed cerebellar-mediated motor deficits alongside a delay in Purkinje cell loss, the detected changes in motor coordination are likely reflective of improved Purkinje cell health in miglustat treated *Mcoln1*^{-/-} mice. To probe this question further, we set out to quantify Purkinje cell health within our cohort.

Miglustat delays Purkinje cell loss in *Mcoln1*^{-/-} mice

To assess Purkinje cell loss in *Mcoln1*^{-/-} mice and if there was any protection with miglustat therapy, we performed western blot analysis of calbindin-D 28K (henceforth called

calbindin-D), a protein known to be enriched in Purkinje cells within the cerebellum and detectable by western blot (Barski et al., 2003). *Mcoln1*^{-/-} mice exhibited diminished calbindin-D levels compared to age matched WT levels (Fig. 1C). Though published studies of cerebellar tissue from end-stage *Mcoln1*^{-/-} mice report evidence of Purkinje cell disease and associated axonal spheroid formation suggestive of Purkinje cell loss (Micsenyi et al., 2009), here we provide the first quantitative evidence of Purkinje cell loss in *Mcoln1*^{-/-} mice (Fig. 1C). Importantly, miglustat treated *Mcoln1*^{-/-} mice had significantly higher levels of calbindin-D compared to saline treated *Mcoln1*^{-/-} mice, yet also tended to have lower, but not significantly different, calbindin-D levels than WT mice (Fig. 1C). Our western blot analyses are consistent with evidence of partially restored Purkinje cell health in miglustat treated *Mcoln1*^{-/-} mice detected via immunofluorescent staining for calbindin-D (Fig. 1D–E). Together, these findings support miglustat delaying Purkinje cell death in the MLIV mouse model.

Miglustat reduces cerebellar microgliosis in *Mcoln1*^{-/-} mice

In other lysosomal diseases such as NPC, Purkinje cell loss occurs alongside microgliosis and is delayed with miglustat treatment (Stein et al., 2012). Even further, these changes occur in a lobule-specific pattern. In NPC, Purkinje cell loss and microgliosis begins in the anterior and posterior zones, and progress through to the central zones, resulting in the anterior and posterior zones having more extensive atrophy of the molecular and PC layers than the central, and even more so, nodular zones (Elrick et al., 2010; Ko et al., 2005; Sarna and Hawkes, 2003). In MLIV disease, it is possible that a similar process occurs if the reduced Purkinje cell number seen here is due indeed to cell autonomous degeneration. If microgliosis occurs at least in part in response to Purkinje cell death, we would expect to see a similar anterior to nodular progression of microgliosis over the course of MLIV disease as well. To obtain zone-specific information on the pattern of microgliosis in MLIV and determine if there is any improvement with miglustat treatment, we utilized CD68 as a marker for activated microglia/brain macrophages and counted the number of CD68-positive (CD68+) cells per unit volume within each lobule. Each lobule was then grouped into zones, wherein the anterior zone contained lobules I–II, III, and IV–V, the central zone contained lobules VI–VII, the posterior zone contained lobule VIII, and nodular zones contained lobules IX and X (Sillitoe and Joyner, 2007). Our data show a pattern in which *Mcoln1*^{-/-} mice exhibit microgliosis in all cerebellar zones, with the anterior zone having more extensive microgliosis than the central, posterior, and nodular zones (Fig. 2C). In the anterior zone, miglustat treated *Mcoln1*^{-/-} mice show statistically significantly fewer CD68+ cells than control *Mcoln1*^{-/-} mice, and in the central and posterior zone numbers of CD68+ cells were no longer significantly greater than WT mice in *Mcoln1*^{-/-} mice receiving miglustat (Fig. 2C).

Mcoln1^{-/-} mice have been described previously to exhibit age-dependent cerebral and cerebellar microgliosis and astrogliosis (Grishchuk et al., 2014; Micsenyi et al., 2009), which is consistent with our CD68 stains in late-stage cerebellum (Fig. 2A, Fig. 1D–E). We next asked if the abnormal cerebral microgliosis and astrogliosis might be improved by miglustat as well. Immunofluorescent stains in the dorsal neocortex for CD68+ cells and GFAP+ astrocytes were consistent with previous reports of microgliosis and astrogliosis in

Mcoln1^{-/-} mice (Fig. 2B). While miglustat treated *Mcoln1*^{-/-} mice appeared to exhibit reduced intensity of staining for both GFAP and CD68 and perhaps a reduction in the number of GFAP+ processes (Fig. 2B), quantitative cell counts of CD68+ microglia showed no reduction in these activated cell types within dorsal neocortex (Fig. 2D). Our observations indicate that while microgliosis is reduced in the cerebellum with miglustat, there is likely no reduction to the number of CD68+ microglia in the cerebrum. Further study aimed at understanding any potential impact miglustat may have on microglial and astrocytic phenotype based on morphologic and other antigenic criteria may shed further light on these findings.

Miglustat does not improve retinopathy in *Mcoln1*^{-/-} mice

Given previous findings of miglustat particularly impacting cerebellar pathology (Stein et al., 2012) as opposed to other areas of the central nervous system, a major question was whether or not miglustat would have any impact on retinal pathology in MLIV. As mentioned previously, one of the most devastating symptoms in MLIV is retinopathy leading to blindness in adolescence (Goldin et al., 2008; Venugopal et al., 2007). To test if miglustat had an effect on rod degeneration, we assessed rhodopsin levels in ocular tissue via western blot analysis as a marker of retinal health (Fig. 3). Rhodopsin levels were decreased in *Mcoln1*^{-/-} mice compared to WT (Fig. 3A), and were not affected by miglustat treatment. These data suggest that miglustat does not delay or improve retinopathy in *Mcoln1*^{-/-} mice (Fig. 3B).

Quantitative mass spectrometry analyses of GSL profiles of MLIV brain tissue show that miglustat partially restores a WT profile of lipids enriched in white matter

Mass spectrometry analysis of sphingolipids in cerebellar and cerebral fractions from *Mcoln1*^{-/-} and WT mice along with their miglustat treated counterparts provided novel data on both the lipid profile of *Mcoln1*^{-/-} mice and miglustat's impact on the cerebrum versus cerebellum (plus adjacent brainstem). Lipids both upstream and downstream of the GCS-mediated step in GSL synthesis were analyzed (Fig. 4A–D, 5A–C), including a comprehensive isoform analysis for each lipid studied (Fig. 4E–I, 5D–H and complete list of isoforms analyzed in Fig. S3–5). Total lipid classes are plotted in Figures 4B–D and 5A–C. Quantification of lipids enriched in both cerebral and cerebellar white matter, such as ceramide monohexoside (CMH), ceramide dihexoside (CDH), sphingomyelin, and sulfatides revealed that *Mcoln1*^{-/-} mice trended towards lower levels of CDH, sulfatides, and CMH (Fig. 4B, 5A). Importantly, these lipids tended to be closer to WT levels in *Mcoln1*^{-/-} mice with miglustat treatment in the cerebellum, but not cerebrum (Fig. 4B, 5A). Isoform analysis revealed that the major isoforms detected of these lipids, such as C24:1 CMH and C24:1 sulfatide, were significantly decreased in *Mcoln1*^{-/-} cerebral fractions and unchanged with miglustat (Fig. 5F, G, Fig. S3 for full list of isoforms analyzed). In contrast to the cerebrum, C24:1, CMH and C24:1 sulfatide levels were lower than WT in the cerebella of saline treated *Mcoln1*^{-/-} mice, and were improved by miglustat treatment in *Mcoln1*^{-/-} mice in the cerebellum without reaching the level of WT mice (Fig. 4G–H). Given that these lipids are abundant in myelin sheaths these changes are consistent with the previously reported dysmyelination in the MLIV cerebrum and cerebellum (Grishchuk et al., 2014; Schiffmann

et al., 2014). Further, our data suggest miglustat may delay such white matter pathology in the cerebellum (Fig. 4G–H).

We next focused our analysis specifically on complex gangliosides, a class of lipids that are documented to contribute to the heterogeneous storage accumulation characteristic of MLIV disease. Given our findings of miglustat's ability to delay Purkinje cell death, cerebellar microgliosis, and motor deficits, we set out to gain further information on the mechanism by which these phenotypes are improved. As miglustat is a GCS inhibitor and has been shown to provide moderate decreases in storage content in specific glycosphingolipid-accumulating lysosomal diseases previously, such as in Sandhoff, Tay-Sachs and NPC models (Jeyakumar et al., 1999; Platt et al., 1997a; Stein, et al., 2012), we set out to investigate if miglustat reduced storage content in *Mcoln1*^{-/-} mice. MLIV disease in patients has been characterized by accumulation of complex gangliosides (Bach, et al., 1975; Tellez-Nagel, et al, 1976) and detailed analysis in *Mcoln1*^{-/-} mice have demonstrated accumulation of GM2, GD3, and GM3 gangliosides, and some storage and redistribution of GM1, with these changes occurring in a largely non-overlapping intracellular vesicular pattern (Micsenyi et al., 2009). Given the prevalent storage of GM2 in MLIV, we examined GM2 storage in cerebrum using immunofluorescent staining, however, GM2 labeling did not show an overt visible reduction in *Mcoln1*^{-/-} mice receiving miglustat (Fig. 6A). Mass spectrometry analysis of the major isoform of GM2 revealed that both miglustat and saline treated *Mcoln1*^{-/-} mice had significantly elevated GM2 levels in cerebrum and cerebellum compared to WT (Figs. 4C, 5B, and 6B–C, with full list of isoforms analyzed in Fig. S4C–D) and indeed, miglustat treatment appeared to further elevate GM2 in the treated mutant mice. Based on these findings, we conclude that GM2 levels in *Mcoln1*^{-/-} mice were not reduced by miglustat treatment and indeed, one possibility is that the tendency for miglustat-treated *Mcoln1*^{-/-} mice to exhibit slightly higher levels of GM2 may be a result of Purkinje cell rescue.

A second glycolipid reported to contribute to neuronal storage in cortical neurons in MLIV disease (Micsenyi, et al., 2009), GM1 ganglioside, was detected at significantly lower levels in *Mcoln1*^{-/-} mice compared to WT mice in cerebella but not cerebra (Fig. 4C, I, Fig. 5B, H, Fig. S4A–B). Given the role of GM1 ganglioside in comprising neuronal and axonal membranes, we believe this decrease may be reflective of white matter abnormality in the cerebellum of *Mcoln1*^{-/-} mice. GM1 levels were significantly elevated in miglustat treated *Mcoln1*^{-/-} mice compared to saline treated *Mcoln1*^{-/-} mice in the cerebellum (Fig. 4C, I). Interestingly, WT miglustat treated mice also tended to have slightly elevated levels of GM1 (Fig. 5B, H) in the cerebrum compared to WT saline treated mice.

No significant changes were detected in total levels of globo-series glycolipids in *Mcoln1*^{-/-} mice compared to WT in either cerebra or cerebella (Figs. 4D, 5C, Fig. S5C–D). While total ceramide levels largely remained unchanged in the cerebra and cerebella of *Mcoln1*^{-/-} mice compared to WT (Figs. 4D, 5C), upon isoform analysis we found a tendency for there to be an elevated ratio of short chain ceramide isoforms (C18:0) to long chain ceramide isoforms (C24:1) in both *Mcoln1*^{-/-} cerebra and cerebella (Fig. 4E–F, Fig. 5D–E, Fig. S5). While the role of ceramides in regulating cell death is complex, the ratio of these ceramides can be indicative of apoptotic signaling, in which C18:0 ceramide tends towards pro-apoptosis, and C24:1 ceramide tends towards anti-apoptosis (Grösch et al., 2012). One possibility is this

elevated ratio of C18:0 to C24:1 may be reflective of cellular death occurring in both cerebrum and cerebellum (Grösch et al., 2012). Importantly, this ratio is closer to WT levels with miglustat treatment in the cerebellum, but remained unchanged in the cerebrum (Fig. 4F, 5E). Of note, both saline and miglustat treated *Mcoln1*^{-/-} mice had elevated levels of C18:0 ceramide (Fig. 5D) compared to WT saline treated mice in the cerebrum.

Discussion

Our data indicate that miglustat treatment of *Mcoln1*^{-/-} mice successfully delays impairments in cerebellar-mediated motor coordination, Purkinje cell death, and cerebellar microgliosis. We report the first quantitative evidence of Purkinje cell degeneration in *Mcoln1*^{-/-} mice. Given previous findings demonstrating that there is no apparent neuronal loss in the MLIV cerebrum (Grishchuk et al., 2014), our findings are consistent with Purkinje cells being a degenerating cell population in MLIV disease. We found that miglustat improved the lipid profile of specific lipids in *Mcoln1*^{-/-} mice, particularly those lipids known to be most abundant in white matter. These benefits came despite no detectable overall reduction of GSL storage examined. Our findings suggest miglustat may represent a viable, albeit limited, therapeutic intervention for human MLIV disease, a condition presently without any corrective therapy.

Our study demonstrates that *Mcoln1*^{-/-} mice exhibit deficits in cerebellar-mediated motor function, and that these deficits can be improved with early miglustat treatment. Our findings through mass spectrometry show miglustat improves the cerebellar but not cerebral profile of lipids enriched in white matter, and our western blot data are consistent with a delay in Purkinje cell loss. Taking together these results, we posit that the benefits to motor phenotypes in *Mcoln1*^{-/-} mice are linked to miglustat slowing Purkinje cell death in our *Mcoln1*^{-/-} cohort and thereby improving cerebellar function. Questions remain as to precisely how miglustat slows death in this particular cell population, and additionally, what is initially responsible for Purkinje cell loss in MLIV disease. Our findings demonstrate miglustat does not provide protection to cell death in the retina, another area known to undergo degeneration in MLIV (Venugopal et al., 2007). One possibility is that miglustat specifically interferes with the pathways leading to Purkinje cell death, or alternatively that it acts on an as of yet unidentified pathway that leads to improvements in Purkinje cell health. Future studies directed at understanding the nature of Purkinje cell death in MLIV (i.e., whether it is autonomous versus non-autonomous cell death) and miglustat's impact on Purkinje cells may shed much needed light on both the mechanisms of this drug's action as well as neurodegenerative pathways that may be important therapeutic targets in MLIV.

Our collective findings also raise interesting questions regarding the mechanism by which miglustat delays development of the MLIV phenotype. Previous reports of improvements with miglustat to lysosomal disease phenotypes, particularly in the cerebellum, also came despite only moderate decreases in detected storage levels (Davidson et al., 2009; Jeyakumar et al., 1999; Platt et al., 1997a; Stein et al., 2012; Zervas et al., 2001b). One possibility is that altered levels of lipid species detected via mass spectrometry and immunofluorescent analyses represents changes occurring prior to the first administration of miglustat, as in vivo the drug only partially decreases the synthesis of GSLs, and therefore would not be

expected to reduce existing storage or correct the cellular defects that lead to storage. Another possibility is that the benefit of miglustat in delaying specific lysosomal disease symptoms comes at least in part through another mechanism(s) other than slowing GSL storage. It has been shown that reducing GBA2 activity either pharmacologically or genetically in clinical models of NPC delays disease phenotypes in a manner similar to miglustat (Marques et al., 2015). Importantly, while miglustat was originally purported as an inhibitor of GCS with an IC_{50} in the micromolar range (Platt et al., 1994; Ridley et al., 2013), it has also been discovered to be a very effective inhibitor of the hydrolysis of glucocerebroside by the non-lysosomal enzyme GBA2, with an IC_{50} down in the nanomolar range. Furthermore this enzyme (GBA2) is highly expressed in cerebellar Purkinje cells (Ridley et al., 2013). Additional credence to the hypothesis that GBA2 inhibition contributes to miglustat's delays in select lysosomal disease phenotypes is provided by findings suggesting that GBA2 is elevated in NPC and Type 1 Gaucher disease. In Type 1 Gaucher disease, over-activity of GBA2 is likely compensatory to the primary GBA1-deficit (Marques et al., 2015). In NPC disease, GBA2 was found to be elevated with unknown etiology, possibly in response to storage imbalance of cholesterol and glycosphingolipids (Marques et al., 2015). While inhibition of GBA2 in lysosomal diseases where its activity is pathologically elevated may be an underlying contributing mechanism of miglustat action, there is as of yet, no evidence of elevated GBA2 activity in MLIV. Future studies aimed at assessing potential perturbations to GBA2 expression or activity in MLIV may provide a better understanding of miglustat's action in this disease. Further, if miglustat's benefit is through mechanisms involving GBA2, this would be consistent with the apparent therapeutic confinement miglustat has to the cerebellum as opposed to other brain areas and organ systems (Marques et al., 2015). Interestingly, the elevations we saw in cerebellar GM1 and GM2 levels via mass spectrometry could also be consistent with inhibition of GBA2 with miglustat (Ashe et al., 2011). However, as compelling as this argument is, GBA2 inhibition alone may also not fully explain the mechanism behind miglustat's impact on clinical symptoms. Studies exploring loss of function mutations in GBA2 in mouse models have demonstrated that these mice also exhibit ataxia, suggesting that preservation of this enzyme's activity is important for maintaining cerebellar health (Hammer et al., 2013). It would be important to determine the specific contribution of GCS inhibition, GBA2 inhibition, or potentially a combination of both, to improving clinical MLIV disease. Such studies could further optimize pharmacological agents available to inhibit GCS and GBA2 as well as other currently unidentified targets, and potentially improve patient outcomes.

In addition to the mechanism by which miglustat delays cerebellar Purkinje cell death, another question that remains unanswered following our study is how miglustat impacts inflammation and the etiology of activated microglia in MLIV. While we found extensive microgliosis in the cerebellum of the untreated mutant mice, particularly in the anterior zone, and a decrease in cerebellar microgliosis with miglustat, neocortical microgliosis does not have a clear reduction with miglustat treatment (Fig. 2). In NPC disease, microgliosis also occurs in both cerebrum and cerebellum (Stein et al., 2012), and in the cerebellum progresses alongside Purkinje cell loss over the course of disease (Ko et al., 2005). Given the timing of the pathological progression of these phenotypes, previous studies have explored the possibility that diseased microglia mediate Purkinje cell death (Ko et al., 2005). These

studies have concluded however that Purkinje cell death, at least in NPC disease, occurs autonomously, therefore indicating that the microglial changes occur either secondary to Purkinje cell loss or due to intrinsic changes as a result of genetic deficiency (Elrick et al., 2010; Ko et al., 2005). Given the similarity in Purkinje cell degeneration and microgliosis in MLIV to NPC disease, these findings may help promote study on the etiology of both pathologies in MLIV. In the case of MLIV disease, previous reports have demonstrated that microgliosis and astrogliosis in cerebra and cerebella are an early disease event in the MLIV mouse model, and in the cerebrum occurs without apparent neuronal loss (Grishchuk et al., 2014). Future studies establishing a timeline of microgliosis and Purkinje cell death may be an important objective to further understand the cause of microgliosis in this disease, particularly in the cerebellum versus cerebrum. Furthermore, while we did not find any significant changes to cerebral microglial number with miglustat, we did observe a trend towards reduction in the intensity of immunostaining of GFAP+ and CD68+ cells in cerebrum. One possibility is that this is a result of changes to the level of activation of microglial and reactive astroglial cells. Previous studies utilizing the GCS inhibitor Genz-682452 have demonstrated a reduction in central nervous system gliosis in the neuronopathic Gaucher mouse model, suggesting inhibition of GCS may impact inflammatory pathways outside of the cerebellum (Marshall et al., 2016). Interestingly, studies exploring miglustat's inhibition of GBA2 activity in a model of cystic fibrosis have even shown that the drug inhibits the inflammatory response to infection in bronchial epithelial cells, suggesting a more direct link between miglustat and inflammatory processes (Loberto et al., 2014). Further comprehensive study aimed at assessing miglustat's impact on inflammatory events would be an important next step to understanding the benefit miglustat could have on gliosis in neurodegenerative diseases.

Importantly, we also observed some behavioral effects present in both miglustat treated groups compared to their saline treated counterparts. Our behavioral data indicate that WT miglustat treated mice tended to exhibit more slips on the narrow balance beam compared to their saline treated counterparts at 15–16 wks of age (Fig. 1A). This may be due to variable activity in younger mice, as miglustat treated WT mice performed comparably to saline treated WT mice on the medium and wide beams at all ages and on the narrow beam at later timepoints. Alternatively, it could be reflective of increased activity/hyperactivity following miglustat treatment (Fig. 1A, Fig. S1A–B), (Sprott and Eleftheriou, 2009). Indeed, miglustat treated mice had a non-significant tendency to explore more track in the open field at later timepoints, which could support miglustat increasing activity (Fig. S1F). We however do not anticipate any hyperactivity induced by miglustat to mask the benefit it has in delaying motor deficits. Miglustat treated WT mice did not display a difference in balance beam performance at later timepoints (Fig. 1A, Fig. S1A–B), where it is clear that miglustat provides a benefit to *Mcoln1*^{-/-} mice, nor did miglustat treated WT mice have significantly impacted motor behavior in the open field (Fig. 1B, Fig. S1C–F). Our data suggest that miglustat may increase anxiety-like behaviors, as we found tendencies towards decreased time spent and track explored in the center of the open field by both WT and *Mcoln1*^{-/-} miglustat treated mice compared to their saline treated littermates (Fig. S1D–E).

The primary goal of this study was to determine if miglustat would be efficacious for the treatment of MLIV disease. To date, miglustat as well as next generation compounds similar

to miglustat that target GCS activity have been explored for the treatment of numerous lysosomal diseases, including Gaucher, Fabry, Tay-Sachs, Sandhoff, and NPC (Ashe et al., 2015, 2011; Jeyakumar et al., 1999; Marshall et al., 2016; Platt et al., 1997a; Stein et al., 2012; Zervas et al., 2001b). While miglustat is not known to act directly on the mucopolipin-1 protein or to compensate for its loss, previous studies have shown that it does improve the pathologies that occur in NPC disease that are also known to occur in MLIV, such as microgliosis, Purkinje cell loss, motor coordination deficits, and premature death. Miglustat is FDA approved for the treatment of type 1 Gaucher disease, and approved in the European Union for the treatment of both Gaucher and NPC. It is relatively well tolerated by patients, crosses the blood brain barrier, and has established dosage and safety information (Elstein et al., 2004; Pastores, 2006; Platt et al., 1997b; Stein et al., 2012). In the case of MLIV, patients are often diagnosed in infancy when clinical phenotypes may have already progressed (Amir et al., 1987; Grishchuk et al., 2014; Wakabayashi et al., 2011). It is important to make this distinction as we assess the potential benefit of miglustat to MLIV patients and other disease models exhibiting some degree of cerebellar pathology, as evidence suggests that miglustat delays disease phenotypes, rather than reversing existing pathologies. For example, miglustat treated *Mcoln1*^{-/-} mice exhibited deficits on the balance beam already at early ages (Fig. 1A, Fig. S1A–B), but performed stably over time. This suggests miglustat can delay motor phenotypes, but does not reverse or prevent the phenotype present at 7–15 weeks of age. Continuing studies that more directly address the loss of mucopolipin-1 and intervene at earlier stages of disease may be fruitful in combination with miglustat treatment to further ameliorate MLIV symptoms.

Conclusions

Miglustat is the first drug that in preclinical studies has demonstrated benefit, albeit partial, towards MLIV disease. Our data suggest miglustat may delay disease symptoms for MLIV patients. Importantly, miglustat provides these benefits despite its mechanism not directly addressing the disease-causing loss of mucopolipin-1. Our hope is this study will promote further optimization of compounds similar in action to miglustat, and foster studies aimed at better understanding miglustat's mechanism for the potential application to MLIV and other patients suffering from related lysosomal disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

NB-DNJ	N-butyl-deoxynojirimycin
GCS	glucosylceramide synthase
GSL	glycosphingolipid
CMH	ceramide monohexoside
CDH	ceramide dihexoside
GBA2	glucocerebrosidase
NPC	Niemann-Pick type C
SRT	substrate reduction therapy

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Highlights

- Miglustat treatment delayed motor deficits in a mouse model of MLIV.
- Miglustat treatment delayed Purkinje cell loss in a mouse model of MLIV.
- Miglustat reduced cerebellar gliosis in a mouse model of MLIV.
- No change in GM2 storage was detected with miglustat in MLIV mice.
- Miglustat improved the MLIV cerebellar profile of white matter enriched lipids.

Figure 1. Miglustat delays motor deficits and Purkinje cell loss in *Mcoln1*^{-/-} mice

A) Number of slips on a narrow balance beam (18mm) are plotted over time. RM ANOVA (genotype × treatment × time) showed significant effects of genotype, genotype × treatment, time × genotype, and time × treatment. Significant effects were found by ANOVA at 15–16 wks for genotype and treatment, at 26 wks for genotype and genotype × treatment, and at 32 wks for genotype, treatment, and genotype × treatment. Significant differences obtained from post hoc pairwise comparisons are shown with asterisks (see end of legend for key). Note that at all time points treated and saline treated *Mcoln1*^{-/-} mice had significantly more

slips than WT, and at the final time point treated *Mcoln1*^{-/-} mice showed significantly fewer slips than saline treated ones. B) Number of rears executed in the open field (OF) over a 6 minute window are plotted. Significant effects were detected by RM ANOVA and by ANOVA at 7–15 wks for genotype, but significant differences were not found in any post hoc pairwise comparisons. All pairwise comparisons were made at each time point, with significant differences denoted between WT saline - *Mcoln1*^{-/-} saline, *Mcoln1*^{-/-} saline - *Mcoln1*^{-/-} miglustat, WT saline - *Mcoln1*^{-/-} miglustat, and WT saline - WT miglustat wherever detected. Mouse numbers are shown in nested tables as WS (WT saline), WM (WT miglustat), MS (*Mcoln1*^{-/-} saline), and MM (*Mcoln1*^{-/-} miglustat). C) Calbindin levels are plotted normalized to actin loading controls, statistically analyzed by unpaired t-test with Welch's correction). D) Low magnification (20×) images of immunofluorescent stains for calbindin and CD68 in lobule VIII cerebellar sections. Arrows denote gaps marking Purkinje cell absence. Max intensity projection of equivalent number of optical slices for each image. E) High magnification (63×) images of calbindin and CD68 stained lobule III cerebellar sections, max intensity projections as in (D). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Asterisks show level of significance between WT saline group and group denoted by symbol next to asterisk, or between groups specified by brackets.

Figure 2. Cerebellar and cerebral gliosis in *Mcoln1*^{-/-} mice with and without treatment
 A) Sample images of CD68 stained WT, *Mcoln1*^{-/-}, and miglustat treated *Mcoln1*^{-/-} cerebellar tissues from lobule I. Max intensity projection of equivalent number of optical slices for each image. B) Sample images of CD68 and GFAP stained WT, *Mcoln1*^{-/-} saline treated, and miglustat treated *Mcoln1*^{-/-} tissues from dorsal neocortex. Single plane image shown in each panel. C) Number of CD68+ cells per unit normalized volume in anterior, central, posterior, and nodular zones of the cerebellum are plotted. *Mcoln1*^{-/-} samples include both saline treated and untreated mice, WT samples include both miglustat and

saline treated samples. n=6 per zone, n=5 for posterior zone *Mcoln1*^{-/-} saline. D) Number of CD68+ cells per unit volume in sections of dorsal neocortex. n=5. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, unpaired t-test with Welch's correction.

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Figure 3. Miglustat does not rescue retinopathy in *Mcoln1*^{-/-} mice

A,B) Western blot analysis of rhodopsin levels in ocular tissue normalized to actin loading controls for WT versus *Mcoln1*^{-/-} saline treated mice (A), and saline treated *Mcoln1*^{-/-} mice versus *Mcoln1*^{-/-} miglustat treated (B) is plotted. WT n=4, 2 replicates each.

Mcoln1^{-/-} saline and miglustat treated, n=3, 2 replicates each. *p<0.05, unpaired t-test with Welch's correction.

Figure 4. Cerebellar lipid analysis via mass spectrometry reveals alterations in the *Mcoln1*^{-/-} lipid profile with improvements to specific lipid classes upon miglustat treatment

A) Schematic of GSL synthesis pathway and abbreviations used. Purported point of action of miglustat denoted in red. B–D) Total levels detected for each lipid listed in cerebellum, including those enriched in white matter (B), complex gangliosides (C), and ceramide & globo-series glycolipids (D). E–I) Major (ie, most prevalent) isoforms detected are plotted for each lipid (complete isoform analysis plotted in Supp. Fig.3–5.), including ceramides (E), ratio of ceramides C18:0 to C24:1 are plotted in F), CMH (G), sulfatides (H), and GM1 (I). n=4 per group. Differences were detected by two-way ANOVA for genotype in total CMH,

total CDH, total sulfatides, total GM2, C24:1 CMH, and C24:1 sulfatide; for treatment in the ratio of C18:0/C24:1 ceramide; and for both genotype and treatment in total GM1 and C18:0 GM1. Where appropriate, post hoc pairwise comparisons were made with significant differences detected between WT saline - *Mcoln1*^{-/-} saline, *Mcoln1*^{-/-} saline - *Mcoln1*^{-/-} miglustat, WT saline - *Mcoln1*^{-/-} miglustat, and WT saline - WT miglustat denoted. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

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Figure 5. Cerebral lipid analysis in *Mcoln1*^{-/-} mice reveals changes to the *Mcoln1*^{-/-} lipid profile not corrected with miglustat

A–C) Total levels of lipids enriched in cerebral white matter, complex gangliosides, and ceramide & globo-series glycolipids are plotted as in Fig. 4. D–H) Major isoforms detected are plotted for each lipid as in Fig. 4 (complete isoform analysis plotted in Supp. Fig. 3–5), including ceramides (D, ratio of ceramides C18:0 to C24:1 are plotted in E), CMH (F), sulfatides (G), and GM1 (H). n=4 per group. Differences were detected by two-way ANOVA for genotype in total CMH, total CDH, total sulfatides, total GM2, total GM3, C18:0 Cer, C24:1 Cer, C24:1 CMH, and C24:1 sulfatide; for treatment in total GM1, total Gb3Cer, total Gb4Cer, the ratio of C18:0/C24:1 ceramide, and C18:0 GM1. Pairwise comparisons were made when appropriate, with significant differences between WT saline - *Mcoln1*^{-/-} saline, *Mcoln1*^{-/-} saline - *Mcoln1*^{-/-} miglustat, WT saline - *Mcoln1*^{-/-} miglustat, and WT saline - WT miglustat denoted. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 6. Assessment of GSL storage in *Mcoln1*^{-/-} cerebrum and cerebellum

A) Immunofluorescent detection of GM2 in neocortical cerebral tissue. Max intensity projection of equivalent number of slices for each image. B,C) Lipid analysis via mass spectrometry of the major isoform (i.e. most prevalent) of GM2 in both cerebrum (B) and cerebellum (C). n=4 per group. Differences detected in both cerebrum and cerebellum in genotype by ANOVA and results of post hoc pairwise comparisons noted. All pairwise comparisons were made using Tukey's multiple comparisons tests when appropriate, with significant differences between WT saline - *Mcoln1*^{-/-} saline, *Mcoln1*^{-/-} saline - *Mcoln1*^{-/-} miglustat, WT saline - *Mcoln1*^{-/-} miglustat, and WT saline - WT miglustat denoted.

*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.