Mitochondrial GSH replenishment as a potential therapeutic approach for Niemann Pick type C disease

Sandra Torres\textsuperscript{a,b,1}, Nuria Matías\textsuperscript{a,b,1}, Anna Baulies\textsuperscript{a,b}, Susana Nuñez\textsuperscript{a,b}, Cristina Alarcon-Vila\textsuperscript{a,b}, Laura Martínez\textsuperscript{a,b}, Natalia Nuño\textsuperscript{a,b}, Anna Fernande\textsuperscript{a,b}, Joan Caballer\textsuperscript{a,b}, Thierry Levade\textsuperscript{c}, Alba Gonzalez-Franquesa\textsuperscript{d}, Pablo García-Roves\textsuperscript{d}, Elisa Balboa\textsuperscript{e}, Silvana Zanlungo\textsuperscript{e}, Gemma Fabrias\textsuperscript{f}, Josefa Casas\textsuperscript{f}, Carlos Enrich\textsuperscript{g,h}, Carmen García-Ruiz\textsuperscript{a,b,h,i}, José C. Fernández-Checa\textsuperscript{a,b,i,e}

\textsuperscript{a} Cell Death and Proliferation, Institute of Biomedical Research of Barcelona (IIBB), CSIC, Barcelona, Spain
\textsuperscript{b} Liver Unit, Hospital Clinic I Provincial de Barcelona, IDIBAPS and CIBERehd, Barcelona, Spain
\textsuperscript{c} Institut National de la Santé et de la Recherche Médicale (INSERM) UMR1037, Centre de Recherches en Cancérologie de Toulouse, Toulouse, France
\textsuperscript{d} Diabetes and Obesity Research Laboratory, Institut d’Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS) and Spanish Biomedical Research Centre in Diabetes and Associated Metabolic Disorders (CIBERDEM), Barcelona, Spain
\textsuperscript{e} Departamento de Gastroenterología, Pontificia Universidad Católica de Chile, Santiago, Chile
\textsuperscript{f} Institute of Biomedical Research of Barcelona (IIBB), CSIC, Barcelona, Spain
\textsuperscript{g} Research Center for ALPD, Keck School of Medicine, University of Southern California, Los Angeles, CA, United States
\textsuperscript{h} Departament de Biologia Cel·lular, Immunologia i Neurociències, Facultat de Medicina, Universitat de Barcelona, 08036 Barcelona, Spain
\textsuperscript{i} Department of Biotechnology, Immunology and Neurosciences, Faculty of Medicine, University of Barcelona, 08036 Barcelona, Spain
\textsuperscript{j} Research Unit on BioActive Molecules (RUBAM), Department of Organic Chemistry, Institut d’Investigacions Químiques i Ambientals de Barcelona, Conselleria de Universitats i Recerca, Barcelona, Spain
\textsuperscript{k} Centre de Recerca Biomèdica CELLEX, Institut d’Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), 08036 Barcelona, Spain

**ABSTRACT**

Niemann Pick type C (NPC) disease is a progressive lysosomal storage disorder caused by mutations in genes encoding NPC1/NPC2 proteins, characterized by neurological defects, hepatosplenomegaly and premature death. While the primary biochemical feature of NPC disease is the intracellular accumulation of cholesterol and gangliosides, predominantly in endolysosomes, mitochondrial cholesterol accumulation has also been reported. As accumulation of cholesterol in mitochondria is known to impair the transport of GSH into mitochondria, resulting in mitochondrial GSH (mGSH) depletion, we investigated the impact of mGSH recovery in NPC disease. We show that GSH ethyl ester (GSH-EE), but not N-acetylcysteine (NAC), restored the mGSH pool in liver and brain of Npc1\textsuperscript{-/-} mice and in fibroblasts from NPC patients, while both GSH-EE and NAC increased total GSH levels. GSH-EE but not NAC increased the median survival and maximal life span of Npc1\textsuperscript{-/-} mice. Moreover, intraperitoneal therapy with GSH-EE protected against oxidative stress and oxidant-induced cell death, restored calbindin levels in cerebellar Purkinje cells and reversed locomotor impairment in Npc1\textsuperscript{-/-} mice. High-resolution respirometry analyses revealed that GSH-EE improved oxidative phosphorylation, coupled respiration and maximal electron transfer in cerebellum of Npc1\textsuperscript{-/-} mice. Lipidomic analyses showed that GSH-EE treatment had no effect in the profile of most sphingolipids in liver and brain, except for some particular species in brain of Npc1\textsuperscript{-/-} mice. These findings indicate that the specific replenishment of mGSH may be a potential promising therapy for NPC disease, worth exploring alone or in combination with other options.

**Keywords:**
Ceramide
Sphingolipids, Mitochondrial GSH
Cerebellum
Hepatosplenomegaly
Lysosomal disorders

1. Introduction

Niemann-Pick type C (NPC) disease is an inherited lysosomal storage disorder that results in neurodegeneration, liver disease and premature death \cite{1,2}. The juvenile and most common form of the disease presents with progressive learning defects and ataxia and is typically diagnosed between 6 and 15 years of age. Mutations in genes encoding NPC1 and NPC2 proteins are causally linked to the pathology.

**Abbreviations:** CDX, 2-hydroxypropyl-β-cyclodextrin; GCS, glucosylceramide synthase; GSH-EE, GSH ethyl ester; mGSH, mitochondrial GSH; NAC, N-acetylcysteine; NPC, Niemann Pick type C disease; SMS, sphingomyelin synthase

* Corresponding authors at: Cell Death and Proliferation, Institute of Biomedical Research of Barcelona (IIBB), CSIC, Barcelona, Spain.

E-mail addresses: cgrbam@iibb.csic.es (C. Garcia-Ruiz), checa229@yahoo.com (J.C. Fernández-Checa).

These authors contributed equally to the work.

http://dx.doi.org/10.1016/j.redox.2016.11.010
Received 13 October 2016; Received in revised form 31 October 2016; Accepted 14 November 2016
Available online 20 November 2016
2213-2317 © 2016 The Authors. Published by Elsevier B.V.
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/BY-NC-ND/4.0/).
Specifically, NPC1 mutations account for 95% of cases but also alterations in NPC2 gene have been found [2,4]. NPC1 is a transmembrane protein that binds cholesterol in its luminal N-terminal domain to allow its export, while NPC2 resides in the lysosomal lumen and transfers cholesterol to NPC1 [5]. NPC1 deficiency in mice reproduces many of the deficits seen in NPC patients, including neurological defects and ataxia by 6–7 weeks of age and reduces the maximal life span to about 10–12 weeks [2,4]. Loss of function of NPC1 causes an accumulation of free cholesterol in lysosomes and late endosomes [3,4], thereby altering intracellular transport of cholesterol. Accumulation of glycosphingolipids, especially GM2 and GM3 gangliosides, has been reported in brain of mouse models and patients [6]. NPC disease in patients and Npc1−/− mice is also characterized by abnormal mitochondrial function and subsequent oxidative stress [7–11]. However, the contribution of these observations to NPC disease is not well understood. For instance, treatment with antioxidants such as N-acetylcysteine (NAC), vitamin E or vitamin C, has shown little impact in modifying NPC pathology, exerting a modest effect (5–10%) in the extension of life span in Npc1−/− mice [7,12,13].

Impaired egress and subsequent accumulation of cholesterol in endolysosomes is the primary consequence of defective NPC1 function and is considered a major pathogenic mechanism of NPC disease [2,4]. Moreover, increased levels of cholesterol in mitochondria from liver and brain of Npc1−/− mice have been reported as well as [10,14,15] but the role of this event in NPC disease has not been explored. Moreover, mitochondrial cholesterol accumulation is known to decrease mitochondrial GSH (mGSH) stores by impairing cytosolic GSH transport into mitochondria [16,17]. Therefore, our aim was to address the impact of mGSH replenishment in Npc1−/− mice and fibroblasts from patients with NPC disease on the susceptibility to oxidant-induced cell death and NPC pathology. We show that GSH ethyl ester (GSH-EE) restores the mGSH pool and this event confers resistance to oxidative stress and cell death, improves cerebellar mitochondrial function and NPC pathology with a significant increase in the median survival and maximal life span of Npc1−/− mice. These findings reveal a key role of mGSH depletion in the progression of this lysosomal disorder and suggest that GSH-EE may be a promising approach for the treatment of NPC disease worth of exploring alone or in combination with existing options.

2. Materials and methods

2.1. Npc1−/− mice and fibroblasts from patients with NPC disease

Npc1−/− mice (NPC1NIH, BALB/cJ strain) were obtained from The Jackson Laboratories. At the time of weaning (21 days), mice were genotypically identified by PCR using DNA prepared from tail-tips and international laws and policies.

Cultured human skin fibroblasts from control individuals (HSF; GM5659D) and patients with NPC disease obtained from the Laboratoire de Biochimie Metabolique, Institut Federatif de Biologie (CHU Toulouse, France) and from Coriell Institute for Medical Research (GM03123, NJ, USA) were grown at 37 °C in 5% CO2.

Mitochondrial fraction was isolated from liver and brain samples from Npc1−/− mice with or without GSH-EE treatment. Tissue homogenates were pelleted, washed in PBS, and

2.3. Cell viability assays

Cell viability was performed based on trypan blue exclusion, double staining with Hoechst 33258 as described previously [18] or by the release of Glutathione-S-Transferase as detailed in Supplemental materials and methods.

2.4. Motor coordination testing

All behavioral tests were conducted during the light cycle phase in an enclosed behavior room. Same animals were used for two motor tests, hanging test and beam transversal test, and results were evaluated and analyzed by an investigator blinded to the groups as described [19]. Briefly, hanging test was used to assess neuromuscular and locomotor development. Mice were placed on an iron wire and had to suspend their body weight with their forelimbs to avoid falling and to aid in progression around the rod. The latency to fall down and the ability to grip the wire was scored as follows: 0, mice fall immediately; 1, grips the wire with forelimbs; 2, grips the wire with forepaws and tries to support itself with its hind paws; 3, grips the wire with 3 or 4 paws; 4, grips the wire with 4 paws and twists its tail around the wire; 5, grips the wire with 4 paws, twists its tail around the wire and moves to the pole. The maximum time permitted was 60 s. Beam transversal test allows the quantification of motor deficit by measuring the time spent to arrive to the platform once all four paws of the animals are in contact with the beam (escape latency, EL), the time spent before falling (tumbled down latency, TDL) and the number of errors (NE) committed for the animals in each beam. The width of the beam was 2.5 cm and the shape was rectangular.

2.5. Mitochondria isolation and GSH determination

Mitochondrial fraction was isolated from liver and brain by Percoll density centrifugation as described previously [20–22]. Mitochondrial enrichment and integrity were ascertained by the specific activity of succinic dehydrogenase and by the acceptor control ratio determined as the ADP-stimulated oxygen consumption over its absence using a Clark oxygen electrode with glutamate/malate or succinate as substrates for respiratory sites for complexes I or II. Final mitochondrial fraction was devoid of contamination by ER, plasma membrane, recycling endosomes and lysosomes as assessed by the levels of Bip/GRP78, Na+/K+ ATPase a1, Rab11 and acid phosphatase levels, respectively. Alternatively, primary mouse hepatocytes and fibroblasts were fractionated into cytosol and mitochondria by digitonin permeabilization as described previously [23]. In some cases, for the determination of mGSH levels the isolation buffer (250 mM succrose, 20 mM HEPES, 1 mM EDTA, 1 mM EGTA, 1.0% (w/v) BSA, 25 micromol/100 mL protease inhibitor mixture, pH7.4) was supplemented with 1 mM DTT as described [24]. GSH levels in homogenates and mitochondrial fraction were determined by HPLC as described [21].

2.6. Lipidomic sphingolipid analysis

Mass spectrometry analysis of lipid species was performed in liver and brain samples from Npc1−/− mice with or without GSH-EE treatment. Tissue homogenates were pelleted, washed in PBS, and...
transferred to glass vials. Sphingolipid extracts, spiked with internal standards (N-dodecanoylsphingosine, N-dodecanoylceramide, N-dodecanoylsphingosylphosphorylcholine and C17-dihydro-sphingosine, 0.2 nmol each), were prepared as described [25]. The instrument consisting of a Waters Aquity UPLC system connected to a Waters LCT Premier orthogonal accelerated time of flight mass spectrometer (Waters, Millford, MA), operated in positive electrospray ionisation mode. Full scan spectra from 50 to 1500 Da were acquired and individual spectra were summed to produce data points each 0.2 s. Mass accuracy and reproducibility were maintained by using an independent reference spray by the LockSpray interference. The analytical column was a 100 mm x 2.1 mm i.d., 1.7 µm C8 Aquity UPLC BEH (Waters). The two mobile phases were phase A: water/formic acid (500/1 v/v); phase B: methanol/formic acid (500/1 v/v), both also contained 5 mM ammonium formate. A linear gradient was programmed— 0.0 min: 80% B; 3 min: 90% B; 6 min: 90% B; 15 min: 99% B; 18 min: 99% B; 20 min: 80% B. The flow rate was 0.3 mL/min. The column was maintained at 30 °C. Quantification was carried out using the extracted ion chromatogram of each compound, using 50mDa windows. The linear dynamic range was determined by injecting standard mixtures.

2.7. Tissue and fibroblasts respirometry

Fresh liver and cerebellum samples from Npc1+/+ and Npc1−/− mice treated with GSH-EE were homogenized mechanically with a PBI Schredder System (Pressure Biosciences, South Easton, MA, USA) [26] in MR05 respiratory media (sucrose, 110 mM; potassium lactobionate, 60 mM; EGTA, 0.5 mM; MgCl2·6H2O, 3 mM; taurine, 20 mM; KH2PO4, 10 mM; HEPES, 20 mM; BSA, 1 g/l; pH 7.1 at 37 °C). Homogenates were introduced in the 2 mL chamber of an Oroboros-2k™ respirometer (Oroboros® Instruments GmbH Corp, Austria) to perform high-resolution respirometry studies. To assess mitochondrial respiration through complex I (CI) malate (2 mM), pyruvate (5 mM) and glutamate (10 mM) were added (leak state) subsequently ADP+MgCl2 (5 mM) and cytochrome c (10 mM) were titrated (coupled CI). Cytochrome c was added to assess the integrity of the membrane to rule out damage (permeabilization) of the outer mitochondrial membrane during sample preparation. To study complex II (CII), rotenone (0.5 mM) was added to inhibit CI before the addition of succinate (10 mM). To study uncoupled CII, oligomycin (2.5µM) was added to inhibit ATP synthase (or complex V, CV). Coupled CII respiration was calculated by subtracting the uncoupled CII respiration from the CII respiration. To study the electron transport system capacity (ETS), subsequently the protonophore carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) was added in followed titrations until reaching the maximal respiration. Finally, antimycin A, a complex III inhibitor, was added to inhibit respiration. The residual oxygen consumption remaining after antimycin A addition was subtracted from all previous respiratory states. Protein levels were measured in the liver and cerebellum homogenates for the normalization of the different respiratory states.

Fibroblast from control subjects and patients with NPC disease were culture to determine Routine (R) respiration. To study uncoupled respiration, oligomycin was added to inhibit ATP synthase. Coupled respiration was calculated by subtracting the uncoupled respiration from the routine respiration. To study ETS capacity, FCCP was titrated until reaching maximal respiration. In this case, oxygen consumption values were normalized by number of cells.

2.8. Statistical analyses

Statistical analyses were performed using GraphPad Prism 6 (Graphpad Software Inc). Unpaired Student’s t-test (two tailed) was performed between two groups and one or two-way ANOVA followed by Tukey’s Multiple Comparison test were used for statistical comparisons between three or more groups. Kaplan-Meier survival curves were plotted using the GraphPad Prism and the log-rank test was undertaken to determine the statistical significance. The corresponding number of experiments is indicated in the figure legends. Data in graphs are shown as mean ± s.e.m.

3. Results

3.1. GSH-EE treatment but not NAC restores mGSH levels in liver and brain from Npc1−/− mice and fibroblasts from NPC patients

Besides endolysosomes, cholesterol has been shown to accumulate in hepatic and brain mitochondria of Npc1−/− mice [10,14,15]. Consistent with the impairment of mitochondrial GSH transport by cholesterol [15–17,27], liver and brain mitochondria from Npc1−/− mice exhibited decreased mGSH levels compared to Npc1+/+ mice (Fig. 1A, B). Similar findings were observed in mitochondria from fibroblasts of NPC patients (Fig. 1C), which also exhibit increased mitochondrial cholesterol loading [9]. To address the potential impact of mGSH depletion in NPC disease, we first examined strategies to recover mGSH levels. The pool of mGSH derives from the transport of cytosolic GSH into the mitochondrial matrix [17,28]. Although NAC provides cysteine, the rate-limiting amino acid needed for GSH neosynthesis in the cytosol, mitochondrial cholesterol accumulation impairs the transport of the newly synthesized cytosolic GSH into mitochondria due to the cholesterol-dependent decrease of mitochondrial membrane fluidity, as reported in steatohepatitis, anthrax infection and Alzheimer’s disease [14,16,27,29–32]. GSH ethyl ester (GSH-EE), on the other hand, freely crosses membrane bilayers and diffuses into mitochondria resulting in mGSH replenishment. Indeed, GSH-EE has been shown to increase mGSH levels in cerebral cortex from newborn rats [33] and to protect against TNF-induced hepatocellular apoptosis and diet-mediated steatohepatitis [16,17,32]. Therefore, we examined the effect of in vivo treatment of Npc1−/− mice with GSH-EE. As seen, intraperitoneal (i.p.) treatment of Npc1−/− mice at postnatal day 7 (P7) every 12 h with GSH-EE restored mGSH levels in liver and brain mitochondria (Fig. 1A, B) as well as in isolated hepatocytes from Npc1−/− mice (Fig. 1D). Similar replenishment of mGSH by GSH-EE was observed in fibroblasts from patients with NPC disease (Fig. 1C). In line with these findings, GSH-EE increased total GSH levels in Npc1−/− mice as well as in fibroblasts of NPC patients (Fig. 1E-H). In contrast, in vivo treatment with NAC at P7 failed to replenish mGSH stores in affected organs of Npc1−/− mice or fibroblasts from NPC patients (Fig. 1A-D), although it efficiently increased total GSH levels, in agreement with previous findings [7]. GSH-EE treatment, however, did not prevent the accumulation of cholesterol in mitochondria.
isolated from liver and brain from \textit{Npc1}^{-/-} mice (21 ± 3 vs 25 ± 4 μg cholesterol/mg protein in liver mitochondria from \textit{Npc1}^{-/-} and \textit{Npc1}^{-/-}+GSH-EE groups, respectively, and 35 ± 6 vs 32 ± 5 μg cholesterol/mg protein in brain mitochondria from \textit{Npc1}^{-/-} and \textit{Npc1}^{-/-}+GSH-EE groups, respectively), suggesting that the recovery of mGSH by GSH-EE is independent of cholesterol-mediated impairment in the transport of GSH into mitochondria. These findings show that unlike NAC, GSH-EE is capable of restoring mGSH pool in \textit{Npc1}^{-/-} mice and in fibroblasts from NPC patients.

3.2. Intraperitoneal GSH-EE therapy improves NPC pathology and extends the survival of \textit{Npc1}^{-/-} mice

Premature death is a characteristic feature of NPC disease, and hence we next examined the survival of \textit{Npc1}^{-/-} mice following GSH-EE and NAC treatment in vivo. Although somewhat lower than reported in some studies [34,35], the median survival and maximal life span of our vehicle-treated \textit{Npc1}^{-/-} colony were in line with previous reports [19,36,37]. As seen, intraperitoneal GSH-EE therapy significantly extended the median survival and increased the maximum life span of \textit{Npc1}^{-/-} mice with respect to vehicle-treated \textit{Npc1}^{-/-} mice (Fig. 2A), similar to the effect we observed following subcutaneous treatment of \textit{Npc1}^{-/-} mice with 2-hydroxypropyl-β-cyclodextrin (CDX), an agent that extracts cholesterol from membrane bilayers [34], or CDX plus GSH-EE (Torres et al, manuscript in preparation). Moreover, GSH-EE-treated \textit{Npc1}^{-/-} mice maintained a significantly higher body weight compared to vehicle-treated \textit{Npc1}^{-/-} mice, while \textit{Npc1}^{-/-} mice treated with NAC exhibited somewhat lower body weight gain (Fig. 2B). Consistent with the inability of NAC to replenish mGSH levels, intraperitoneal NAC therapy did not have a significant impact in the survival of \textit{Npc1}^{-/-} mice (Fig. 2A), in agreement with previous observations [7].

As NPC disease causes neurodegeneration, with characteristic signs of cerebellar ataxia, and liver disease, we next addressed the pathological impact of mGSH replenishment by GSH-EE. GSH-EE treatment improved motor coordination assessed by the hanging and beam transversal tests (Fig. 2C, D). Consistent with this outcome, the levels of calbindin, a calcium-binding protein critical for the precision of motor coordination [38], were reduced in cerebellar Purkinje cells from \textit{Npc1}^{-/-} mice while GSH-EE treatment significantly increased calbindin expression (Fig. 2E). Moreover, \textit{Npc1}^{-/-} mice exhibited age-dependent liver injury assessed by serum ALT levels that was evident 7 weeks after birth (Supplementary Fig. 1). H & E analyses indicated alterations in liver parenchyma with the presence of inflammatory foci, bigger and more irregular hepatocytes with foamy cytoplasm, suggesting lipid accumulation (Fig. 2F). In addition, compared to vehicle-treated \textit{Npc1}^{-/-} mice GSH-EE therapy at P7 significantly decreased liver damage as indicated by H & E analyses (Fig. 2G, H) and decreased levels of serum transaminases (Fig. 2I, J). Thus, these findings underscore that GSH-EE therapy ameliorates NPC pathology and increases the survival of \textit{Npc1}^{-/-} mice.

3.3. GSH-EE treatment protects against oxidative stress in \textit{Npc1}^{-/-} mice and fibroblasts from patients with NPC disease

Given the lack of therapeutic effect of NAC in the survival of \textit{Npc1}^{-/-} mice, we next examined potential mechanisms underlying the beneficial effect of mGSH replenishment by GSH-EE treatment. As NPC disease is characterized by oxidative stress [7,11], we first examined the susceptibility to oxidant-induced cell death. As seen, GSH-EE treatment protected isolated hepatocytes from \textit{Npc1}^{-/-} mice and fibroblasts from NPC patients against hydrogen peroxide-induced cell death (Fig. 3A, B). In line with these findings, in \textit{vivo} treatment of \textit{Npc1}^{-/-} mice with GSH-EE decreased oxidative stress in liver and cerebellum as revealed by reduced carbonylated proteins (Fig. 3C, D). Moreover, GSH-EE administration attenuated the increase of MitoSOX™ fluorescence in hepatocytes isolated from \textit{Npc1}^{-/-} mice (Fig. 3E), indicating reduced generation of superoxide anion, while GSH-EE treatment increased mitochondrial membrane potential (Fig. 3F). Furthermore, GSH-EE therapy significantly increased ATP levels in liver and cerebellum from \textit{Npc1}^{-/-} mice (Supplementary Fig. 2), suggesting improved energy status. Furthermore, \textit{Npc1}^{-/-} mice exhibited increased caspase-3 processing to active fragment in liver and cerebellum and GSH-EE treatment decreased caspase 3 activity (Fig. 3G, H). Moreover, increased plasma levels of cholesterol oxidation products have been identified as sensitive and specific markers of NPC disease [39]. In line with these findings, we observed increased plasma levels of the oxysterols cholestane 3β, 5α, 6β-triol and 7-ketocholesterol, which were differentially affected by GSH-EE treatment (Supplementary Fig. 3). NPC disease most severely affects Purkinje cells of the cerebellum. As seen, nitrotyrosine immunohistochemical analysis of cerebellar frozen sections indicated marked nitrotyrosylation in granule and Purkinje cells from \textit{Npc1}^{-/-} mice compared to \textit{Npc1}^{-/-} mice (Fig. 3I). Interestingly, GSH-EE treatment reduced protein nitrotyrosylation in cerebellar slices from \textit{Npc1}^{-/-} mice (Fig. 3J). Thus, these findings indicate that GSH-EE therapy attenuates the susceptibility to oxidative stress and cell death in NPC disease.

3.4. GSH-EE therapy improves mitochondrial function in cerebellum from \textit{Npc1}^{-/-} mice

As mitochondria are one of the main culprits of ROS overgeneration leading to oxidative stress, we next evaluated the effects of GSH-EE treatment in mitochondrial function and morphology. High-resolution respirometry analyses were performed in homogenates from cerebellum of \textit{Npc1}^{-/-} mice with or without i.p. GSH-EE treatment. Oxygen consumption rate assessed at the leak state (no ADP, state 2 respiration) in the presence of malate and glutamate (complex I substrates) was similar in the cerebellum of \textit{Npc1}^{-/-} and \textit{Npc1}^{-/-} mice and was not affected upon GSH-EE treatment (Fig. 4A). However, oxidative phosphorylation through complex I in the presence of ADP was reduced in cerebellum of \textit{Npc1}^{-/-} mice, and this effect was recovered by GSH-EE treatment (Fig. 4A). GSH-EE treatment caused similar effects when the respiratory capacity was evaluated in the presence of rotenone (complex I inhibitor) and succinate (complex II substrate) and with the addition of the ATP synthase inhibitor oligomycin and the pronotophore FCCP to monitor coupled respiration and the maximal electron transfer System (ETS), respectively (Fig. 4B). A similar outcome was observed in liver homogenates of \textit{Npc1}^{-/-} mice compared to \textit{Npc1}^{-/-} mice, but unlike in cerebellum, GSH-EE treatment failed to restore leak respiration, oxidative phosphorylation via complex I and II as well as coupled and ETS respiration via complex II (Supplementary Fig. 4).
**A**
Hepatocytes

**B**
Fibroblasts

**C**
Liver carbonyl proteins

**D**
Cerebellum carbonyl proteins

**E**
MitoSOX fluorescence intensity

**F**
TMRE fluorescence intensity

**G**
Liver Caspase-3/β-actin

**H**
Cerebellum Caspase-3/β-actin

**I**
Nitrotyrosilation, DAPI
Fig. 4). Fibroblasts from patients with NPC disease showed a significant reduction in mitochondrial performance at routine, coupled and ETS respiratory states compared to fibroblasts from healthy subjects (Supplementary Fig. 5). These findings of mitochondrial respiratory rates in liver and cerebellum correlate with the expression of mitochondrial complexes (Fig. 4C). Blue native electrophoresis revealed significant downregulation of supercomplexes 1+3+4+1V2 and, particularly, complex IV in liver but not cerebellum of Npc1-/− mice that was not reversed by GSH-EH treatment, with modest differences observed regarding complex CV levels in cerebellum (Fig. 4C). Western blot analyses revealed decreased expression of the mitochondrial DNA (mttDNA)-encoded subunit of the CIV cytochrome c oxidase I in liver but not cerebellum that was not prevented by GSH-EH treatment (Supplementary Fig. 6). Moreover, electron microscopy analyses showed autophagic vacuoles of electron-dense materials, mitochondrial morphological alterations predominantly in liver with decreased mitochondrial number and increased length (Supplemental Fig. 7) without a significant effect following GSH-EH treatment. In contrast to the liver, the mitochondrial structure and number in cerebellum was similar between Npc1+/+ and Npc1−/− mice and GSH-EH treatment increased the mitochondrial length in Npc1−/− mice (Fig. 4D, E). Overall, these findings point to reversible functional mitochondrial alterations in cerebellum of Npc1−/− mice, which are largely restored by GSH-EH treatment, suggesting the involvement of an oxidative stress-dependent mechanism of mitochondrial dysfunction. The mitochondrial alteration in liver of Npc1−/− mice likely reflects structural defects with decreased expression of mtDNA-encoded subunits and assembly of mitochondrial complexes that are refractory to GSH-EH administration.

3.5. Effect of GSH-EH therapy in the sphingolipidomic profile of liver and brain from Npc1−/− mice

In addition to cholesterol, NPC disease is also characterized by the accumulation of sphingolipids in the affected organs [6]. Therefore, we next addressed whether GSH-EH treatment induced major changes in the sphingolipidomic profile in liver and brain of Npc1−/− mice. As seen, mass spectrometry analysis revealed increased levels of specific molecular species of ceramide, sphingomyelin, monohexosylceramide (glucoyl and galactosylceramides), dihexosylceramide (lactosylceramides) and sphingosine in liver of Npc1−/− mice (Fig. 5). The increase of sphingolipids in brain of Npc1−/− mice was evident for ceramide C18:0, dihexosylceramides C18GGCer and C20GGCer and sphingosine (Fig. 6). In contrast to these findings, the levels of specific sphingomyelin (C22SM, C24SM, C24:1SM and C24:2SM) and, particularly, the most predominant monohexosylceramide species (C18GCar, C20GCar, C22GCar, C24GCar and C24:1GCar) decreased in brain of Npc1−/− mice compared to Npc1+/+ mice (Fig. 6), in agreement with previous findings [6]. These changes in sphingolipid homeostasis were accompanied by unchanged expression of glucosylceramide synthase (GCS) and sphingomyelin synthase (S extremes) 2 in liver and brain, while that of SMS 1 decreased in liver (Fig. 7). Interestingly, the sphingolipidomic profile of the liver from Npc1−/− mice treated with GSH-EH was similar to that of vehicle-treated Npc1−/− mice (Fig. 5). Moreover, GSH-EH treatment did not significantly change the profile of ceramide, dihexosides species or sphingosine levels in brain of Npc1−/− mice, except for the normalization of specific molecular species of sphingomyelin and monohexosylceramides in the brain of Npc1−/− mice (Fig. 6). These findings underscore that GSH-EH therapy has a modest and selective effect on the sphingolipid profile in Npc1−/− mice.

4. Discussion

The primary biochemical feature of NPC disease is the accumulation of free cholesterol, predominantly in endolysosomes, due to defective NPC1 function. Also described but poorly characterized is the increase of cholesterol in mitochondria in the affected organs of Npc1−/− mice. As mitochondrial cholesterol accumulation depletes the mGSH pool [14,15], we examined for the first time the impact of mGSH replenishment in NPC disease. Our findings show a differential outcome between NAC and GSH-EH in the compartmentalization of GSH in NPC disease. Incubation of hepatocytes from Npc1−/− and fibroblasts of NPC patients with NAC and GSH-EH results in a significant increase in total GSH levels. Moreover, consistent with the ability to cross the blood brain barrier [14,33], in vivo treatment with both precursors increased total GSH levels in liver and brain homogenates from Npc1−/− mice, in agreement with previous findings [7]. However, unlike GSH-EH, NAC failed to restore the mGSH pool despite significantly increasing total GSH levels. The inability of NAC to increase mGSH in NPC disease is in line with findings in liver and alveolar type II cells following chronic alcohol drinking [20,23,40], which causes increased mitochondrial cholesterol loading [41,42] and subsequent impairment in the transport of GSH from the cytosol into mitochondria through disruption of membrane dynamics. Interestingly, we show that the proficiency of NAC to restore total GSH levels but not the mGSH pool had little impact in the survival of Npc1−/− mice or NPC pathology, in line with previous studies [7,43]. Accordingly, short-term NAC administration to NPC patients revealed no significant effects on oxidative stress [7]. In addition, vitamin C treatment did not significantly modify disease progression nor increased life span of Npc1−/− mice [13]. Thus, while oxidative stress is a characteristic feature of NPC disease, treatment with antioxidants or replenishment of total GSH levels is not an efficient approach to modify disease progression.

The present findings provide evidence that selective restoration of mGSH following GSH-EH therapy has a promising therapeutic impact in NPC disease, significantly preserving calbindin levels, improving motor coordination and increasing the survival of Npc1−/− mice. While GSH-EH treatment increased mGSH levels in both liver and cerebellum, the normalization of mitochondrial function was only observed in the latter, which paralleled the improvement of Purkinje cell survival and protection against oxidative stress, likely mediating the enhanced motor coordination and increased survival. Recent observations indicated that the hepatic re-expression of NPC1 in Npc1−/− mice corrects the liver phenotype of the disease, although, this outcome had no impact in improving neurological symptoms or life-span extension.
Fig. 5. Mass spectrometry analyses of hepatic sphingolipid profile of Npc1<sup>−/−</sup> mice. Liver samples from Npc1<sup>+/−</sup> mice and Npc1<sup>−/−</sup> mice treated with saline or GSH-EE at p7 every 12 h for 6 weeks were processed for lipidomic analyses of ceramide (A); sphingomyelin (B); monohexosides (C); dihexosides (D) species and sphingosine (E) levels. Values are the mean ± SEM of 5 mice per group. P < 0.05 vs. Npc1<sup>+/−</sup> or Npc1<sup>−/−</sup> samples as indicated.

Fig. 4. High-resolution respirometry of cerebellum from Npc1<sup>−/−</sup> mice treated with GSH-EE. Cerebellar homogenates of Npc1<sup>+/−</sup> mice or Npc1<sup>−/−</sup> mice treated with saline or GSH-EE were analyzed in Oroboros-2k<sup>™</sup> respirometer. (A) For mitochondrial respiration through complex I, malate 2 mM, pyruvate 5 mM and glutamate 10 mM were added with or without ADP+MgCl<sub>2</sub> (5 mM) to determine respiration at coupled or leak states, respectively. Data are presented as means ± SEM (n=3 to 7, P < 0.05 vs. Npc1<sup>+/−</sup> or Npc1<sup>−/−</sup> mice, one-way ANOVA and Tukey’s Multiple Comparison Post-test). (B) Oxidative phosphorylation through complex II was determined using succinate (10 mM) as substrate in the presence of rotenone (0.5 mM). Uncoupled respiration through complex II was determined in the presence of oligomycin (2.5 µM) and the electron transport System capacity was determined in the presence of FCCP. Coupled respiration was calculated by subtracting the uncoupled respiration from the respiration through complex II. Data are presented as means ± SEM (n=3 to 7, P < 0.05 vs. Npc1<sup>+/−</sup> or Npc1<sup>−/−</sup> mice, one-way ANOVA and Tukey’s Multiple Comparison Post-test). (C) Blue native Page electrophoresis of liver and cerebellum Npc1<sup>+/−</sup> or Npc1<sup>−/−</sup> mice treated or not with GSH-EE to detect OXPHOS complexes with Total Mitoprobe OXPHOS Rodent antibody cocktail. Images are representative of 4 replicates per group. (D) Electron microscopy analyses of cerebellum from Npc1<sup>+/−</sup> or Npc1<sup>−/−</sup> mice with or without GSH-EE that were transcardially washed with cold saline before perfusion with 2.5% glutaraldehyde. Images were acquired with a Gatan Orius digital camera and are representative of 5 replicates per group. (E) Mitochondrial number and length of cerebellar samples were quantified from images of ultrathin sections from (D) acquired by moving at random across the EM grid (two grids per animal) and analyzed using the iTEM program and ImageJ software. Data are presented as means ± SEM (n=4 to 7, P < 0.05 vs. Npc1<sup>+/−</sup>, one-way ANOVA and Tukey’s Multiple Comparison Post-test).
These findings along with our observations suggest that the neurological alterations rather than the liver phenotype determine the overall progression of the juvenile form of the disease. Moreover, our findings indicate that GSH-EE treatment does not affect the lipidomic profile of most sphingolipid species in liver and brain of Npc1−/− mice, except for specific sphingomyelin and monohexosylceramide species in brain of Npc1−/− mice. However, the relevance of the latter findings remains to be further investigated, as the role of the disruption of sphingolipid homeostasis in NPC disease is poorly understood. For instance, although accumulation of gangliosides, especially GM2 and GM3, has been reported in brain of Npc1−/− mice [6], GM3 synthase deletion has been shown to worsen NPC disease resulting in accelerated premature death of Npc1−/− mice despite decreasing GM3 levels [44]. In light of these findings, it seems unlikely that the effect of GSH-EE in the normalization of specific brain sphingolipid species may have contributed to the improvement in the neurological phenotype and increased survival of Npc1−/− mice.

Currently, there are no FDA-approved treatments for NPC disease. Given the promising effects of GSH-EE shown here and exploiting the fact that it crosses the blood brain barrier, it may be worth exploring its therapeutic efficacy of intraperitoneal or subcutaneous CDX administration in ameliorating NPC symptoms in patients. Therefore, since recent findings indicated that the intracisternal CDX administration prevents cerebellar dysfunction and delays premature death in feline NPC disease [52], it may be worth investigating whether GSH-EE combined with intrathecal injection of CDX via lumbar puncture may exhibit a potentiating effect in the treatment of NPC disease. Although to our knowledge there are no studies addressing the suitability of GSH-EE treatment for human therapy, the present findings suggest this approach may be worth exploring in patients with NPC disease.

In summary, the present findings provide new insights on the pathogenesis of this lysosomal storage disorder, with the identification of mGSH depletion as an important player. In addition, since mitochondrial cholesterol increase causes the depletion of mGSH, further understanding the mechanism underlying the accumulation of cholesterol in mitochondria may be of relevance in NPC disease. In this regard, we have observed increased expression of StARD1 levels in liver and brain of Npc1−/− mice by an ER stress-independent mechanism, and this outcome is accompanied by a significant upregulation of MLN64 [53,54], these findings suggest that StARD1 and MLN64 could account for the accumulation of cholesterol in mitochondria and the expected mGSH depletion.

[37]. These findings along with our observations suggest that the neurological alterations rather than the liver phenotype determine the overall progression of the juvenile form of the disease. Moreover, our findings indicate that GSH-EE treatment does not affect the lipidomic profile of most sphingolipid species in liver and brain of Npc1−/− mice, except for specific sphingomyelin and monohexosylceramide species in brain of Npc1−/− mice. However, the relevance of the latter findings remains to be further investigated, as the role of the disruption of sphingolipid homeostasis in NPC disease is poorly understood. For instance, although accumulation of gangliosides, especially GM2 and GM3, has been reported in brain of Npc1−/− mice [6], GM3 synthase deletion has been shown to worsen NPC disease resulting in accelerated premature death of Npc1−/− mice despite decreasing GM3 levels [44]. In light of these findings, it seems unlikely that the effect of GSH-EE in the normalization of specific brain sphingolipid species may have contributed to the improvement in the neurological phenotype and increased survival of Npc1−/− mice.

Currently, there are no FDA-approved treatments for NPC disease. Given the promising effects of GSH-EE shown here and exploiting the fact that it crosses the blood brain barrier, it may be worth exploring its therapeutic efficacy of intraperitoneal or subcutaneous CDX administration in ameliorating NPC symptoms in patients. Therefore, since recent findings indicated that the intracisternal CDX administration prevents cerebellar dysfunction and delays premature death in feline NPC disease [52], it may be worth investigating whether GSH-EE combined with intrathecal injection of CDX via lumbar puncture may exhibit a potentiating effect in the treatment of NPC disease. Although to our knowledge there are no studies addressing the suitability of GSH-EE treatment for human therapy, the present findings suggest this approach may be worth exploring in patients with NPC disease.

In summary, the present findings provide new insights on the pathogenesis of this lysosomal storage disorder, with the identification of mGSH depletion as an important player. In addition, since mitochondrial cholesterol increase causes the depletion of mGSH, further understanding the mechanism underlying the accumulation of cholesterol in mitochondria may be of relevance in NPC disease. In this regard, we have observed increased expression of StARD1 levels in liver and brain of Npc1−/− mice by an ER stress-independent mechanism, and this outcome is accompanied by a significant upregulation of MLN64 [53,54], these findings suggest that StARD1 and MLN64 could account for the accumulation of cholesterol in mitochondria and the expected mGSH depletion.
Funding

The work was supported by grants SAF-2014-57674-R, SAF-2015-69944-R from Plan Nacional de I+D, Spain, Fundació Marató de TV3, La Mutua Madrileña, PI11/0325 (META) grant from the Instituto Salud Carlos III, and by the support of CIBEREHD; the center grant P50-AA-11999 Research Center for Liver and Pancretic Diseases funded by NIAAA/NIH; and support from AGAUR of the Generalitat de Catalunya 2014-SGR785.

Competing interests

The authors declare no competing financial interests.

Acknowledgments

We want to thank Drs. Vicente Ribas and Fabian Arenas for helpful discussions.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.redox.2016.11.010.

References
