Research paper

Systemic enzyme delivery by blood-brain barrier-penetrating SapC-DOPS nanovesicles for treatment of neuronopathic Gaucher disease

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A R T I C L E  I N F O

Article History:
Received 6 November 2019
Revised 6 March 2020
Accepted 10 March 2020
Available online 10 April 2020

Keywords:
Neurodegenerative disease
Central nervous system
SapC-DOPS nanovesicles
Enzyme replacement therapy
Neuronopathic Gaucher disease
Acid-\(\beta\)-glucosidase

A B S T R A C T

Background: Enzyme replacement therapy (ERT) can positively affect the visceral manifestations of lysosomal storage diseases (LSDs). However, the exclusion of the intravenous ERT agents from the central nervous system (CNS) prevents direct therapeutic effects.

Methods: Using a neuronopathic Gaucher disease (nGD) mouse model, CNS-ERT was created using a systemic, non-invasive, and CNS-selective delivery system based on nanovesicles of saposin C (SapC) and dioleoylphosphatidylserine (DOPS) to deliver to CNS cells and tissues the corrective, functional acid \(\beta\)-glucosidase (GCase).

Findings: Compared to free GCase, human GCase formulated with SapC-DOPS nanovesicles (SapC-DOPS-GCase) was more stable in serum, taken up into cells, mostly by a mannose receptor-independent pathway, and resulted in higher activity in GCase-deicient cells. In contrast to free GCase, SapC-DOPS-GCase nanovesicles penetrated through the blood-brain barrier into the CNS. The CNS targeting was mediated by surface phosphatidylserine (PS) of blood vessel and brain cells. Increased GCase activity and reduced GCase substrate levels were found in the CNS of SapC-DOPS-GCase-treated nGD mice, which showed profound improvement in brain inflammation and neurological phenotypes.

Interpretation: This first-in-class CNS-ERT approach provides considerable promise of therapeutic benefits for neurodegenerative diseases.

Funding: This study was supported by the National Institutes of Health grants R21NS 095047 to XQ and YS, R01NS 086134 and UH2NS092981 in part to YS; Cincinnati Children’s Hospital Medical Center Research Innovation/Pilot award to YS and XQ; Gardner Neuroscience Institute/Neurobiology Research Center Pilot award to XQ and YS; Hematology-Oncology Programmatic Support from University of Cincinnati and New Drug State Key Project grant 009ZXD01207-205 to XQ.

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1. Introduction

Gaucher disease (GD) is a lysosomal storage disease with a frequency of 1/50,000 to 100,000 in general population \cite{1, 2}. In GD, \(\text{GBA}1\) mutations lead to defective acid \(\beta\)-glucosidase (GCase) function and the accumulation of its substrates, glucosylceramide (GluCer) and glucosylsphingosine (GluSph), resulting in multi-organ dysfunctions \cite{1, 3}. Typical manifestations of GD type 1 include visceral, hematologic and bone disease reflected by hepatosplenomegaly, anemia, thrombocytopenia and osteo-penia/-porosis. In comparison, types 2 and 3 exhibit these visceral signs and also early onset progressive neuronopathic disease, i.e., nGD, with primary brain pathology characterized by neuronal necroptosis and inflammation \cite{1, 4, 5}. Life expectancy is about 1–2 years for GD type 2 patients, and up to 30–40 years for those with GD type 3. \(\text{GBA}1\) mutations have also been identified as the most common genetic risk factor for Parkinson and Lewy Body diseases \cite{6–8}.

Approved therapies for GD include enzyme replacement therapy (ERT; e.g., Imiglucerase, Velaglucerase alfa and Taliglucerase alfa) \cite{9} and substrate reduction therapies (SRT; Miglustat and Eliglustat) \cite{10}. These therapies have positive effects on the visceral manifestations of the GD variants \cite{11, 12}, but none have direct effects on the central nervous system (CNS) signs and symptoms \cite{12–14}. A new

https://doi.org/10.1016/j.ebiom.2020.102735

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Research in the context

Evidence before this study

Gaucher disease is a rare genetic disease caused by the mutations of GBA1 gene encoding lysosomal enzyme, acid β-glucosidase (GCase), and is classified as visceral (Type 1) or neuronopathic (Types 2 and 3). A major limitation of FDA approved enzyme replacement therapy (ERT) is failure to cross the blood-brain barrier (BBB). Therefore, the currently available treatments are only effective on the visceral manifestations of Gaucher disease and are completely ineffective for Types 2 and 3 neuronopathic central nervous system (CNS) variants that present often early in life with high mortality. A novel complex, saposin C (SapC) and dioleoylphosphatidylserine (DOPS) nanovesicles, has the ability to cross the BBB and selectively target neuronal tissue, providing a biological vehicle for delivering GCase into the CNS.

Added value of this study

We demonstrate that a systemic CNS-selective delivery system using SapC-DOPS nanovesicles supplies functional GCase to a variety of tissues, especially the brain. SapC-DOPS-GCase, as a novel therapeutic approach, corrects GCase deficiency in CNS cells and tissues and shows efficacy in reducing CNS inflammation and neurological phenotypes in a mouse model of neuronopathic Gaucher disease. Our study establishes a new mechanism of CNS targeting of SapC-DOPS through a specific phosphatidylserine receptor and the lymphatic circulation. This CNS-selective delivery system using SapC-DOPS nanovesicles provides a new strategy for treating neuronopathic Gaucher disease.

Implications of all the available evidence

This therapeutic approach utilizing SapC-DOPS nanovesicles to deliver the enzyme into the brain will advance CNS disease treatment in neuronopathic Gaucher disease. Although this study was focused on a rare disease, there may be ramifications for similar but vastly more common conditions such as Parkinson disease in which decreased GCase activity has been documented in the patients’ brains. As without improvements in treatment neuronopathic Gaucher disease will remain lethal, SapC-DOPS-GCase has the potential to translate rapidly to improved patient care.
assay, serum, cells and visceral tissues (Figs. 1, 2, 3F, S2; Tables S1 and S2). The brain PS (BPS) system (0.5 \mu M BPS) is used to determine SapC’s effect on brain GCase activity by homogenizing the brains in 1X PBS and incubated in 0.5 \mu M BPS (Figs. 3D and 5B). The GCase activity assay was carried out as described\[33\]. Protein concentrations of cells and tissues were determined by the BCA assay using bovine serum albumin (BSA) as a standard. In the cell-free assays, the dilution of GCase (Velaglucerase alfa, 1 mg/mL) was 1:2000. The final concentration of polysorbate 20 was ~ 4 nM which has no impact on GCase activity[34].

2.4. Cell culture

Fibroblasts from Gba1 null (Gba1\(^{-/-}\)), 4 L;C* (see below) and SapC deficient (C\(^{-/-}\)) mice [35-37] and human GD types 2 and 3 patient fibroblasts (GM1260: GBA1 L444P/P415R and GM877: GBA1 L444P)

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**Fig. 1.** SapC-DOPS activates GCase. (a) SapC-DOPS and SapC similarly activate GCase in a cell- free assay. DOPS is a control. SapC, 0 to 500 nM; DOPS, 0 - 4500 nM. Free GCase incubated with SapC-DOPS or SapC for 30 min before determining the GCase activity. (b) SapC-DOPS increases GCase activity in fibroblasts from SapC-deficient mice (4L;C* and SapC-/-). The cultured cells were incubated with 100 nM SapC-DOPS for 30 min before harvesting for GCase activity assay. Student’s t-test. ***, p < 0.0001. (c) Kd of GCase interaction with SapC-DOPS compared to SapB-DOPS. (d) Kd of SapC interaction with DOPS compared to SapB with DOPS. The Kd values were determined by microscale thermophoresis (MST) using Affinity Analysis v2.1.3 software. (e) Functional domain of SapC. GCase activity in 4L;C* fibroblasts treated with WT SapC, mutant SapCs or WT SapB formulated with DOPS-GCase. The cells were incubated for 5 days with two changes of fresh medium with the formulation. One-Way ANOVA with Tukey’s multiple comparison test. ***, p < 0.05. The samples were assayed in triplicates/experiment of 2 - 3 independent experiments.
Coriell Institute, Camden, NJ) were cultured in DMEM with 10% FBS. Transformed Gba1<sup>-/-</sup> neurons, kindly provided by Dr. Ellen Sidransky at NIH [38], were maintained in Neurobasal Medium (Gibco, Carlsbad, CA). Macrophage mannose receptor positive, J774E macrophage cell lines were maintained with 10% FCS/DMEM as described [39]. For GCase uptake experiments, the cells (1 x 10<sup>6</sup> cells/well) were incubated for 24 or 48 h, or 5 days (with two changes of fresh medium with the formulation) with equal amounts of GCase protein (80 µg/mL) as SapC-DOPS-GCase or as free GCase freshly prepared in 0.1 M CP buffer (pH5.6). GCase activity and protein concentration were determined [33]. The cells cultured on chamber slides were processed for lysosomal localization of GCase by immunofluorescence using Lamp2a as a lysosomal marker. For mannose receptor-mediated GCase uptake experiments: J774E and Gba1<sup>-/-</sup> neuron

Fig. 2. SapC-DOPS nanovesicles preserve GCase. Cellular GCase activity (a) and GCase protein (b) of mouse Gba1<sup>-/-</sup> fibroblasts that were incubated for 24 or 48 h with SapC-DOPS-GCase and GCase, respectively. SapC-DOPS-GCase incubated cells showed higher levels of GCase activity and protein than GCase incubated cells. (c) GCase activity of human GD2 and GD3 fibroblasts, GM1260 (L444P/P415R) and GM877 (L444P/L444P), incubated with SapC-DOPS-GCase for 24 h. (d) Human GCase (green) detected in the lysosome (Lamp2a, red) of Gba1<sup>-/-</sup> fibroblasts incubated with SapC-DOPS-GCase and GCase for 24 h. The cells with SapC-DOPS-GCase showed higher PCC (Person correlation coefficient) compared to GCase incubated cells (n=8–14 cells), indicating more GCase targeting to lysosomes in SapC-DOPS-GCase incubated cells compared to free GCase incubated cells. Scale bar =100 µm for all images. (e) Effect of mannan on GCase uptake. J774E macrophages and Gba1<sup>-/-</sup> neurons were incubated with 80 µg GCase/mL of SapC-DOPS-GCase or free GCase in the presence and absence of mannan (2 mg/mL). All the cells used for the assays were washed prior to collection. The data presents net GCase activity (increased GCase activity level minus basal/endogenous GCase activity level). The samples were assayed in triplicates/experiment of 2–3 independent experiments.

L444P, Coriell Institute, Camden, NJ) were cultured in DMEM with 10% FBS. Transformed Gba1<sup>-/-</sup> neurons, kindly provided by Dr. Ellen Sidransky at NIH [38], were maintained in Neurobasal Medium (Gibco, Carlsbad, CA). Macrophage mannose receptor positive, J774E macrophage cell lines were maintained with 10% FCS/DMEM as described [39]. For GCase uptake experiments, the cells (1 x 10<sup>6</sup> cells/well) were incubated for 24 or 48 h, or 5 days (with two changes of fresh medium with the formulation) with equal amounts of GCase protein (80 µg/mL) as SapC-DOPS-GCase or as free GCase freshly prepared in 0.1 M CP buffer (pH5.6). GCase activity and protein concentration were determined [33]. The cells cultured on chamber slides were processed for lysosomal localization of GCase by immunofluorescence using Lamp2a as a lysosomal marker. For mannose receptor-mediated GCase uptake experiments: J774E and Gba1<sup>-/-</sup> neuron
Fig. 3. Tissue distribution of SapC-DOPS-GCase. (a to e) 4 L;C* and WT mice at 38 days of age were i.v. infused with one bolus injection of SapC-DOPS-GCase, vehicle (CP buffer) or free GCase (54.6 mg/kg SapC-DOPS and 0.4 mg/kg GCase; 0.4 mg/kg free GCase). Tissues were collected 3 h post injection or as indicated. (a to c) GCase protein detected by immunoprecipitation followed with immunoblot in 4 L;C* (a) and WT (b) brains with SapC-DOPS-GCase. (c) GCase protein detected in the 4 L;C* brains and livers at 1 to 24 h post injection with SapC-DOPS-GCase. (d) GCase activity increased in SapC-DOPS-GCase treated brains. (e) GCase protein detected in the 4 L;C* liver. Human GCase (20 ng) is a loading control in A and E. (f) GCase activity in 4 L;C* mouse tissues at 49 days of age treated with SapC-DOPS-GCase, free GCase or vehicle-CP buffer (2 h post i.p. injection of, 2 x injection/2 h). SapC-DOPS-GCase was distributed to liver, spleen, lung, lymph nodes and bone marrow cells. SapC-DOPS-GCase-treated mice have significantly higher activity in those tissues than free GCase-treated mice. (g-k) Distribution of human GCase in brain regions of 4 L;C* mice at 38 days of age treated with SapC-DOPS-GCase or free GCase (3 h post one bolus i.v. injection). (g) Human GCase (green) co-stained with VCAM 1 (red) positive endothelium by immunofluorescence (IF) in SapC-DOPS-GCase treated 4 L;C* mouse brain. Scale bar is 20 μm. (h) Human GCase (green) detected by IF in the lysosome (Lamp2a, red) of brain cells. No GCase is detected in free GCase treated 4 L;C* brains. Image magnifications are 400x. (i) Panel: representative images of GCase protein (green) detected by IF in astrocytes (red, GFAP), neurons (red, NeuN) and microglia (red, CD68) in SapC-DOPS-GCase-treated 4 L;C* mouse brains. Scale bar = 10 μm for all images. (j) The graph shows the percentage of brain cells containing GCase. (k) Human GCase positive cells (green stars) determined by IF were distributed in 4 L;C* mouse brain regions that show inflammation stained with anti-CD68 (brown). Hematoxylin stains nuclei (blue). Scale bar is 3 mm. n = 3 mice, replicated assays of 2-3 independent experiments, n > 5 images/mouse. Student’s t-test: *, p < 0.05; **, p < 0.01; *** , p < 0.001.
cells were treated with a mannose receptor inhibitor, mannann (2 mg/mL) overnight. The cells were collected and washed with glycin (0.5%) saline to remove the surface attached GCase prior to GCase activity measurement. All the cells used in the activity assay were washed prior to collection.

2.5. SapC-DOPS-GCcase serum/medium stability

Freshly formulated SapC-DOPS-GCcase in CP buffer (pH 5.6) was tested for its stability in serum (pH 7.4) from wild type (WT) mice and DMEM medium (pH 7.2, 10% FCS) over 8 h. GCcase activities were measured at 0, 1, 2, 4, 6 and 8 h with the Tc/Tx system. The same amount of free GCcase was set as a control to compare its activity versus SapC-DOPS-GCase at same condition. The assays were repeated twice, each in triplicates. 

2.6. Immunoprecipitation (IP) of GCcase

IP was performed using Dynabeads® protein G kit to quantify human GCcase protein in the mice. Protein G was prepared by cross-linking the trapping antibody, rabbit anti-human GCcase [40] using bis(sulfosuccinimidyl) substrate (BS3, Thermo-Fisher). Mouse brain or liver lysates (1 mg protein from nGD mice and 10 mg protein from WT mice) were mixed with 80 μL of protein G beads cross-linked with anti-GCcase antibody and incubated at 4°C overnight. GCcase was eluted from the beads using glycine, pH ~7.2 - 8 and analyzed by immunoblot. Following elution, the beads were denatured with SDS buffer and showed no detectable GCcase.

2.7. Immunoblotting

The cells and tissues were homogenized in Mammalian-Protein Extraction Reagent (M-PER) and subjected to electrophoresis on 4–12% NuPAGE gels. The proteins were transferred to PVDF membranes using an iBlot 2 gel transfer device (Life Technologies). The 4 L;C* mice were treated with SapC-DOPS-GCase, free GCcase or vehicle at 10 μL/kg body weight. Starting day 21, when the tail vein was not accessible, the formulation was administered by daily intraperitoneal (i.p.) injections at dose of total SapC-DOPS (109.2 mg/kg) with GCcase (0.8 mg/kg). Tail vein of intravenous (i.v.) injection started at day 28, 3 times per week, at a dose of total SapC-DOPS (54.6 mg/kg) and GCcase (0.4 mg/kg). The parallel control mice were either not injected, injected with vehicle (CP buffer or saline), free GCcase in CP buffer (i.p-0.8 mg/kg, i.v.--0.4 mg/kg) or SapC-DOPS (i.p-109.2 mg/kg, i.v.- 54.6 mg/kg). In some experiments, the mice were administered with acute i.p. or i.v. dosing as indicated in the figure legend. Mouse body weights were recorded daily. The mice were monitored for survival and assessed for phenotype development during the treatment and used for analysis of GCcase activity, protein and substrate, and brain pathology. The non-4 L;C* littermates (4 L;WT and 4 L;C+) do not show abnormal behavior or pathology and have a normal life span [15, 36]. The strain and age-matched WT mice and non-4 L;C* littermates were used as controls. We did not observe gender differences in preliminary studies; age and strain matched mice of both genders were included in all the analyses. All mice were housed under pathogen-free conditions in the animal facility according to IACUC approved protocol (2018–0056) at Cincinnati Children’s Hospital Research Foundation.

2.9. CellVue maroon (CVM) in K14-VEGFR3-ig

The K14-VEGFR3-ig mouse is a brain microlymphatic mouse model. K14-VEGFR3-ig mice were obtained from Drs. Kari Alitola (University of Helsinki, Finland) and Melody Swartz (Institute for Molecular Engineering, University of Chicago, Chicago, IL) [41, 42]. WT and K14-VEGFR3-ig mice with engrafted orthotopic brain tumors (100,000 LLC-GFP cells) were treated with 250 μL SapC-DOPS-CVM [SapC = 0.4 mg/mL, DOPS = 0.082 mg/mL, CVM = 0.04 μg/mL] via the tail vein at 12 days of age. The brains of SapC-DOPS-CVM administered mice were collected 24 h post i.v. injection. CVM signal was observed by IVIS imaging (λEx = 640 nm; λEm = 700 nm, 0.1 s) as described [32].

2.10. Glycosphingolipid analysis

Glucosylceramide (GluCer) and glucosylsphingosine (GluSph) in cells and tissues were analyzed at the Medical University of South Carolina Lipidomics Shared Resource: Analytical Unit. The concentration of GluCer and GluSph in the tissues was normalized to mg tissue weight, and in the cells, was normalized by mg protein of the cell lysate [43].

2.11. Immunohistochemistry

Brain tissues were collected after transcardiac perfusion with saline and fixed in cold 4% paraformaldehyde (PFA) for processing frozen blocks. CD68 and GFAP monoclonal antibody staining was as described [44]. CD68 and GFAP signals were quantified using Fiji for ImageJ [44].

2.12. Fluoro-Jade C (FJC) staining

FJC (AG325, Millipore, MA) is a polyanionic fluorescein derivative which selectively binds to degenerative neurons for evaluation of neurodegeneration [45]. Frozen brain sections were air-dried and dipped in 80% ethanol/1% sodium hydroxide, 70% ethanol, and 0.06% potassium permanganate for 5, 2, and 10 min, respectively. The sections were rinsed with distilled water and then incubated with 0.0004% FJC in 0.1% acetic acid for 20 min. FJC staining was detected under a fluorescent microscope at 480 nm excitation and 525 nm emission. Images were acquired through a 20x objective with a Zeiss Apotome 200 M, and the fluorescence of FJC-positive cell signals was quantified using Fiji for ImageJ [44].
2.13. Immunofluorescence

The mouse tissues and the cells on chamber slides were fixed with 4% PFA, permeabilized with 0.3% Triton X-100 in PBS, and quenched with 0.05 M NH₄Cl. The cells were blocked with 1.5% BSA and 1.5% non-fat milk in PBS at room temperature for 1 hr. Frozen PFA fixed tissue sections were treated with 0.3% Triton X-100 in PBS and blocked with 1.5% BSA and 10% goat serum in PBS. Primary antibodies were applied to the cells or tissue sections and incubated overnight at 4 °C. Dilutions of primary antibodies were as follows: rabbit anti-human GCase (1:200); rat anti-Lamp2α or rat anti-Lamp1 (1:250); rat anti-mouse LYVE1 monoclonal (1:250); mouse anti- GAP monoclonal (1:250); mouse anti-NeuN monoclonal (1:500); and mouse anti-CD68 monoclonal (1:250). Following washing with PBS plus 0.05% Tween-20 (10 min, 3 times), the samples were incubated for 1 hr at room temperature with secondary antibodies; goat anti-rabbit-FITC (1:500), goat anti-rat or anti-mouse- Texas Red (1:500). The samples were washed as above and mounted with Anti-fade mounting medium with DAPI. Fluorescence signals were acquired with a Zeiss Apotome 200 M. Signals from the images were quantified by Fiji for Image [44].

2.14. Behavioral testing

Sensorimotor function was assessed in the mice by gait analyses as previously described [46]. Hindlimb clamping was tested by tail suspension. The mouse was lifted away from all surrounding objects by grasping tail at the base. Hindlimb position was monitored for 30 secs and scored. Score 0 is when hindlimbs are consistently splayed outward, away from the abdomen. Score 1 is when one hindlimb is retracted toward the abdomen for >50% of 30 secs. Score 2 is when both hindlimbs are partially retracted toward the abdomen for >50% of 30 secs. Score 3 is when hindlimbs are entirely retracted and touching the abdomen for >50% of 30 secs. The mice were tested with 2 trials at each time point, 10 mins, apart between trials.

2.15. Lactadherin administration and CVM quantitation

4 L:C mice at 42–43 days of age were i.v. injected with 100 μL lactadherin (20 μg/mouse, n = 5), BSA (20 μg/mouse, n = 5) or PBS (100 μg, n = 2). Thirty min later, each mouse was given SapC-DOPS-CVM (200 μL/mouse) by i.v. injection. Mice were euthanized 3 h after the SapC-DOPS-CVM injection and perfused with saline followed by 4% PFA. Brains and meninges were collected for ex-vivo IVIS imaging and post-fixed in 4% PFA for 24 h followed by 30% sucrose for histological studies. CVM signal was observed by IVIS imaging (λEx = 640 nm; λEm = 700 nm; Exposure = 0.5 s) as described [30]. Age, strain and sex matched 4 L:C mice and Non-4 L:C littermate (4 L:WT) control mice were injected i.v. with 100 and 200 μL bovine lactadherin-FITC (83 μg/mL in PBS). The brain sections were stained with rat-anti-mouse CD68 monoclonal antibody (MCA1957, Bio-Red, 1:200), anti-Tuj1 or anti-VCAM1 antibodies with secondary antibody conjugated with Texas Red (1:500), respectively. Images of brain sections were acquired with a Nikon C-plusr2 microscope and quantification of fluorescence signals on these images were analyzed by ImageJ.

2.16. Statistical analysis

The data were analyzed by Student’s t-test or ANOVA test, and Log-Rank (Mantel-Cox) test for survival using GraphPad Prism. Figure bars present as mean±SEM.

3. Results

3.1. Preparation of SapC-DOPS-GCase formulation

Both GCase and SapC are lysosomal proteins that bind phospholipids in the cell membrane [47], thereby providing a rationale to formulate SapC-DOPS with GCase. Since SapC can enhance GCase activity [25, 48], the preservation of this SapC function was first determined in SapC-DOPS nanovesicles. GCase was incubated with various concentrations of SapC-DOPS or SapC and the activity was measured. As shown in Fig. 1a, activation of GCase by SapC-DOPS is comparable to that by SapC alone. Using SapC-deficient fibroblasts [mouse SapC<sup>−/−</sup> and mouse 4 L:C<sup>−/−</sup> harboring V394L/V394L Gba1 (4L) and sapc<sup>−/−</sup> (C′) homoygosity] [36, 37], SapC-DOPS enhanced GCase activity by 1.3 fold in 4 L:C<sup>−/−</sup> and 1.2 fold in SapC<sup>−/−</sup> fibroblasts (Fig. 1b). Thus, SapC’s activation function on GCase is preserved in SapC-DOPS nanovesicles both in vitro and ex vivo.

GCase is rapidly inactivated by neutral pH, but acidic (lysosomal) pH 5.5 preserves GCase activity [49]. SapC-DOPS with GCase was formulated in CP buffer (pH 5.6) by routine extrusion technology (Fig. S1a). To determine the maximal GCase that can be carried by SapC-DOPS, the stoichiometry of GCase vs. SapC-DOPS and composition of SapC-DOPS were developed by individually optimizing 1) GCase and 2) DOPS in the formulation. Each formulation was centrifuged to harvest GCase bound to SapC-DOPS, recovered in the pellet vs. unbound free GCase in the supernatant. GCase activity and protein of each bound and free fraction were determined. When SapC (0.1 mM) and DOPS (0.9 mM) were mixed with various amounts of GCase (0.7 to 1.3 μM), the highest GCase activity and protein levels were found in the bound form when 1.3 μM GCase was used (Fig. S1b and Table S1). To optimize the DOPS, we tested ratios of fixed SapC (0.1 mM) vs. various amounts of DOPS (0.5–1.1 mM) with 1.3 μM GCase. Free and bound GCase activities and protein are shown in Fig. S1c and Table S2. These results demonstrate that 0.1 mM SapC: 0.7–0.9 mM DOPS is optimal with 1.3 μM GCase in the formulation. This SapC-DOPS-GCase formulation was used in following cell and mice experiments.

3.2. Characterization of SapC-DOPS-GCase formulation

A strong GCase and SapC interaction occurs in the presence of unsaturated, acidic phospholipids [25, 48]. Microscale thermophoresis (MST) was used to determine the interaction/binding of GCase with SapC-DOPS (Supplementary Methods). MST detects the changes in the hydration shell, charge or size of molecules by measuring changes of the mobility of molecules in microscopic temperature gradients [50]. Using fluorescent NBD-phosphatidylserine (PS) as probe in DOPS, GCase interacted with SapC-DOPS with a binding constant Kd = 29.35 ± 1.91 nM (Fig. 1c and Table S3). As a positive control, the interaction of SapC with DOPS has a Kd = 13.45 ± 0.9 nM (Fig. 1d and Table S3). There were no detectable interactions between GCase and DOPS, saposin B (SapB)-DOPS or SapC-DOPC (phosphatidylcholine), or between SapB and DOPS, under the same experimental conditions (Table S3). Thus, GCase has specific binding affinity for SapC-DOPS.

The number of SapC and GCase molecules in each DOPS nanovesicle is estimated based on the simple geometry and average size measurements for DOPS liposomes (Supplementary Methods). Each DOPS nanovesicle contains an estimated 3.31 × 10⁷ DOPS molecules. In optimized SapC-DOPS-GCase formulation, about 75% GCase bound on SapC-DOPS, thus, there are approximately 3.68 × 10⁴ SapC and 3.95 × 10⁴ GCase molecules in each DOPS nanovesicle. SapC contains fusogenic/binding (NH2-region) and GCase activation (COOH-region) domains [22, 51]. To determine the formulation with maximal uptake of GCase, SapC-DOPS, SapB-DOPS, and SapC mutant-DOPS were compared by measuring the changes in GCase activity in 4 L:C<sup>−/−</sup> fibroblasts following incubation with these.
nanovesicles (Fig. 1e). Data from our previous studies show that mutant SapC (K13A) has intact GCase activation, but defective PS binding activity, mutant SapC (Q48N) does not activate GCase, but has PS membrane fusogenic/binding activity and mutant SapC (K23A) has neither GCase activation nor PS binding activities [22, 51]. SapB, as a negative control, does not have fusogenic/binding or GCase activation functions. These results and our previous studies [22, 51] indicate that SapC in the DOPS nanovesicle retains its GCase activation function in addition to its fusogenic lipid binding activity potentially allowing it to target PS-exposed brain cells (see below).

3.3. SapC-DOPS-GCase formulation preserves GCase activity and facilitates cell uptake

Serum stability of GCase in the SapC-DOPS-GCase formulation was determined by measuring GCase activity in WT mouse sera or culture medium at 0 to 8 h and compared to free GCase. The half-life or EC50 values showed that SapC-DOPS-GCase is 3 times more stable than free GCase in serum and 1.6 times more in culture medium (Fig. S2).

To determine whether SapC-DOPS improves GCase functionality, SapC-DOPS-GCase [SapC/DOPS (1:9) mM with 80 µg/mL GCase] was assayed for GCase activity and tested in mouse fibroblasts derived from Gba1−/− newborn mice that have no functional GCase [35]. SapC-DOPS-GCase and free GCase with equal amounts of GCase protein were added to Gba1−/− fibroblasts. The cells with SapC-DOPS-GCase had 4-fold more GCase activity at 24 h and 15-fold more at 48 h than the cells incubated with free GCase (Fig. 2a). Cells incubated with SapC-DOPS-GCase had higher total cellular GCase protein levels than those with free GCase at 24 h and 48 h after loading (Fig. 2b). Increased GCase activity and protein were detected 4 h after loading. Human GD types 2 and 3 patient fibroblasts [52] that have low levels of residual GCase activity also showed an increase of GCase activity when incubated with SapC-DOPS-GCase (Fig. 2c).

SapC-DOPS-GCase taken up by the Gba1−/− fibroblasts was targeted into lysosomes (Fig. 2d), whereas this was less obvious with free GCase. The co-localization of GCase with the lysosomal marker, Lamp1, was determined by Pearson Correlation Coefficient (PCC) in the acquired image using Zeiss image analysis software (Fig. S3). The cells with SapC-DOPS-GCase showed higher PCC (0.70 ± 0.08) compared to the cells with free-GCase (PCC = 0.41 ± 0.06). These results demonstrate that human GCase formulated with SapC-DOPS is targeted to, and is functional in GD fibroblasts. SapC-DOPS preserves GCase activity and likely protects GCase from denaturation, therefore facilitating cellular uptake and lysosomal localization.

3.4. Effect of mann on uptake of SapC-DOPS-GCase

Oligo-mannosyl-terminated oligosaccharides on GCase (e.g., velaglucerase alfa) promote preferential uptake into myeloid-lineage cells expressing the mannose receptor and neurons [53-55]. To determine if cell uptake of SapC-DOPS-GCase is dependent on the mannose receptor, uptake was assessed with J774E macrophages that express the macrophage mannose receptor and transformed mouse Gba1−/− neurons in the presence or absence of mannin, a competitor for the mannose receptor. Both cell types were incubated with equal GCase, either as free GCase or as SapC-DOPS-GCase. The cells were washed thoroughly before the activity assay and confirmed by immunofluorescence that there was no significant GCase binding on the cell surface. The cells with SapC-DOPS-GCase showed more GCase activity than those with free GCase (Figs. 2e and 2f). Mannin blocked ~50% of free GCase uptake, but only ~20% of SapC-DOPS-GCase in either macrophages or neurons (Figs. 2e and 2f). These data suggest that SapC-DOPS-GCase enters the cells largely through routes other than the mannose receptor, e.g., direct cell membrane fusion.

3.5. Tissue distribution and CNS targeting of SapC-DOPS-GCase

As mentioned above, mannose-terminated oligosaccharides promote cellular uptake of GCase via the mannose receptor. However, several organs are poorly or inaccessible to these agents, including the brain, alveoli of the lungs, and lymph nodes [12, 56]. To assess the ability of SapC-DOPS-GCase to enter the brain and visceral tissues, 4 Lc4 mice were intravenously (i.v.) or intraperitoneally (i.p.) infused with equal amounts of protein (equal activity) of free GCase or SapC-DOPS-GCase. Their activities were kept constant in each experiment. In acute experiments, tissue GCase protein and activities were determined at 3 h, or as indicated in Fig. 3, post injection. Brain accessible SapC-DOPS-GCase was confirmed by immunoblot. Human GCase protein in the brain lysates was immunoprecipitated using our specific rabbit anti-human GCase antibody (no cross-reactivity with mouse GCase) followed by immunoblotting with goat anti-human GCase (Fig. 3a). In comparison, human GCase protein was detected in the liver of free GCase and SapC-DOPS-GCase injected mice (Fig. 3e). SapC-DOPS-GCase was also delivered into WT mice brains, but at much lower levels than nGD mice (Fig. 3b). GCase protein in 4 Lc4 brains was detected up to the end point (24 h post-injection) of the experiment (Fig. 3c). Mice injected with SapC-DOPS-GCase showed GCase activity in the brain, but those injected with free GCase did not, indicating that SapC-DOPS allowed GCase access into the brain (Fig. 3d).

In visceral tissues, GCase activity was determined in SapC-DOPS-GCase i.p.-injected mice. Higher GCase activity was detected in the liver, spleen, lung, lymph nodes and bone marrow of SapC-DOPS-GCase injected mice than free GCase-injected mice suggesting that SapC-DOPS protects GCase from rapid clearance by the reticuloendothelial system. It also protects GCase from pH inactivation in serum as evidenced by the long half-life of 0.68 h in the serum (Fig. S2). Liver lysate showed greater incorporation of SapC-DOPS-GCase than free GCase to liver (Figs. 3e and 3f). By contrast, in comparison to free GCase injected tissues, SapC-DOPS-GCase-injected mice had increased GCase activity in lymph nodes and lung (Fig. 3f).

In SapC-DOPS-GCase-treated 4 Lc4 brains, GCase was observed lining the endothelium of blood vessels in the treated 4 Lc4 brain (Fig. 3g). GCase was trafficked into the lysosome in the brain cells of the 4 Lc4 mice that had been administered SapC-DOPS-GCase, but not free GCase (Fig. 3h). GCase signals were in ~6% of neurons, 6–18% of astrocytes and 8–18% microglia cells counted in each brain region (Fig. 3i and j). GCase target cells were distributed mainly in the cortex, midbrain, brain stem and thalamus (Fig. 3i and k). GCase was co-localized with SapC in treated mouse brain cells demonstrating that GCase is delivered by SapC-DOPS nanovesicles into the brain (Fig. S4). These results confirm that SapC-DOPS-GCase entered the brain cells when infused i.v., a clinically relevant, non-invasive procedure for GD treatment.

3.6. SapC-DOPS-GCase mitigated neuronal phenotypes in nGD mouse model, 4 Lc4 mice

The 4 Lc4 model is viable analog of human nGD that have progressive accumulation of substrates and CNS manifestations [36, 57]. 4 Lc4 mice has a Gba1 mutation V394L/V394L and lack of SapC [36]. 4 Lc4 the mice used in this study are in the C57BL/6J background with a median life span of 56 days. SapC-DOPS-GCase was administered to 4 Lc4 mice for evaluation of in vivo efficacy. The SapC-DOPS-GCase formulation used for mice injection was routinely assayed for GCase activity and a confirmed ~80% GCase activity remained prior to use. In preliminary studies, SapC-DOPS-CVM and SapC-DOPS-GCase were delivered into young (13 day old) 4 Lc4 brains by i.p. injection. Before day 28, when the tail vein was not accessible, the formulation was administered by daily i.p. injections. Tail vein i.v. injection started at day 28 and 3 times per week. The treatment started when disease signs appeared around 21 days.
Treatment effect was evaluated in 4L;C* mice by neurological phenotype, inflammation, neurodegeneration, and substrate reduction. 4L;C* mice developed age-related hindlimb clasping behavior. 4L;WT littermates that have normal phenotype are used as controls to assess clasping behavior change in 4L;C* mice with treatment. The treated 4L;C* mice showed significantly delayed hindlimb clasping (Fig. 4a). 4L;C* mice developed hindlimb paralyses with abnormal gait, a sensorimotor function deficit. Compared to 4L;C* mice who received vehicle, SapC-DOPS-GCase treated 4L;C* mice had significantly improved stride length by gait analysis at 50 and 55 days of age (Fig. 4b). The SapC-DOPS-GCase treatment significantly prolonged 4L;C* survival to a median of 64 days vs. 56 days for untreated mice; a 14% extension compared to untreated 4L;C* or 15% compared to free GCase and Vehicle-4L;C* mice (Fig. 4c). Because free GCase
does not cross the BBB, as expected. 4 L;C* mice administered with free GCase did not have improved survival and compared to the untreated and vehicle groups (Fig. 4c). The treatment also reduced the substrate, glucosylceramide, accumulation in the brains of 4 L;C* mice (Fig. 4d). Brain inflammation was analyzed by anti-CD68 staining on activated microglial cells and anti-GFAP staining on activated astrocytes. SapC-DOPS-GCase treatment significantly mitigated brain inflammation in 4 L;C* mice in the cortex, thalamus, midbrain and brainstem regions (Figs. 4e and S5a). Neurodegeneration, assessed by Fluoro-Jade C (FJC), an anionic fluorescent dye as a marker of degenerating neurons, was significantly diminished in SapC-DOPS-GCase treated 4 L;C* brain stem, midbrain and thalamus regions (Fig. S5b). These data demonstrate improved neuronopathic phenotype in nGD mice by SapC-DOPS-GCase treatment.

3.7. SapC-DOPS’s in vitro and in vivo efficacy

4 L;C* mice have SAPC deficiency so the efficacy of SapC-DOPS to replace SapC in 4 L;C* mice was evaluated. Increased GCase activity in SapC-DOPS treated 4 L;C* mouse fibroblasts indicates the uptake of SapC is functional (Fig. 1b). Compared to control mice, i.v. injected SapC-DOPS fluorescently labeled with CVM primarily localized to the thalamus of 4 L;C* mice (Fig. 5a); this brain region exhibits inflammatory cells in this model [36, 46]. In the control, 4 L;WT mice that do not show brain inflammation, low level of CVM was detected (Fig. 5a), suggesting the accessibility of SapC-DOPS into non-inflamed normal brain. To evaluate SapC-DOPS’s in vivo efficacy, 4 L;C* mice were treated with SapC-DOPS, which increased brain GCase activity (Fig. 5b) and survival (Fig. 5c). Both SapC-DOPS and SapC-DOPS-GCase improved abnormal gait at 45 days of age in 4 L;C* mice (Fig. 5d). While SapC-DOPS alone had small effects on survival of 4 L;C* mice, SapC-DOPS-GCase treated 4 L;C* mice showed longer survival and improved gait (Figs. 5c and 5d), signifying enhanced efficacy when GCase was added to the SapC-DOPS nanovesicles.

3.8. Phosphatidylserine-mediated brain targeting of SapC-DOPS nanovesicles

SapC, a membrane-associated protein, has a favorable binding affinity for unsaturated, negatively charged PS. To determine if SapC-DOPS targeting of 4 L;C* brains is dependent on PS, 4 L;C* mice were i.v. injected with lactadherin or BSA prior to SapC-DOPS-CVM. Lactadherin is a PS-specific binding protein that acts through its C2 domain (Lact-C2) [58]. SapC and Lact-C2 both bind PS through its negatively charged head group [47, 59]. Lactadherin cannot enter intact cells so it binds PS only on the outside of the cells [60]. We have previously demonstrated that lactadherin can block SapC-DOPS entry into glioblastoma (brain cancer) cells by shielding the cell surface PS, both in vitro and in vivo [30]. The CVM signals were detected in the control, BSA injected 4 L;C* mice, but were significantly reduced in lactadherin injected mice (Figs. 6a and 6b). Brain cell types that lactadherin binds were determined in the 4 L;C* mice i.v. administered with lactadherin-FITC. The lactadherin signals were on active microglial cells (CD68*), neurons (TuJ1*) and endothelium (VCAM1*) (Fig. 6c), but largely absent on astrocytes (GFAP*). Lactadherin signals were enriched in a dose-dependent manner in the inflammatory regions that have strong CD68 signals (Fig. 6d). To test whether lactadherin directly inhibits the PS-binding activity of SapC-DOPS nanovesicles, we treated SapC-DOPS-CVM with lactadherin at 37 °C for 30 mins, and collected the pellet after ultracentrifugation to remove free lactadherin. The mixture in the pellet showed the surface PS-binding activity on human brain tumor cells (Gli36) at the same level as the non-lactadherin treated SapC-DOPS-CVM by flow cytometry analysis (Fig. S6). This indicates that free lactadherin cannot significantly reduce the PS-binding effect of SapC-DOPS-CVM. These data support a PS-mediated pathway via BBB for SapC-DOPS targeting into 4 L;C* brain and especially inflamed regions.

3.9. Lymphatic pathway is involved in SapC-based nanovesicles targeting to CNS

To determine the pathway of SapC-DOPS targeting to CNS, SapC-DOPS-CVM was i.v. injected to WT mice. The CVM had a different distribution in brain meninges compared to lectin-stained blood vessels (Fig. S7a), indicating SapC-DOPS does not accumulate in these blood vessels. However, GCase was detected in mouse brain meninges and co-localized with LYVE1 (lymphatic vessel marker) in 4 L;C* mice 24 h after i.v. administration of SapC-DOPS-GCase (Fig. S7b). CVM infiltrated in lymph nodes in mice i.v. administered SapC-DOPS-CVM (Fig. S7c). Lymphatics-defective transgenic mice, K14-VEGFR3-Ig, develop a lymphedema-like phenotype characterized by edema, swelling of feet and dermal fibrosis but they survive to adulthood despite the lack of lymphatic vessels in several tissues [41, 42]. There is regeneration of the lymphatic vasculature in some tissues but not in the brain where microlymphatics are absent in adult K14-VEGFR3-Ig mice [41, 42]. To test whether the vesicle accumulation in brain is based on the meninges-mediated transport mechanism, we generated orthotopic brain tumors (10 × 10^5 LLC-GFP cells) in K14-VEGFR3-Ig mice. Long-term SapC-DOPS accumulation was dramatically reduced in brain tumors of these mice (Fig. S7d). Our data indicate that the meningeal-lymphatics are required for SapC-based vesicle uptake in diseased brains and suggest that SapC-based nanovesicles target the CNS through the blood-lymphatic loop [61].

4. Discussion

There is a major unmet medical need for a GD treatment with direct effects on the CNS. Herein, we developed a novel strategy using BBB-penetrating SapC-DOPS nanovesicles through a non-invasive, intravenous administration, to transport functional GCase into the nGD brain which resulted in therapeutic effects. Formulating GCase into SapC-DOPS preserved GCase function and enhanced stability and uptake into cells, primarily via mannos receptor-independent pathways. We also demonstrate that SapC-DOPS-GCase crosses the BBB of normal and inflamed brains via a mechanism that requires PS-targeting (see diagram in Fig. S8). In vivo efficacy of SapC-DOPS-GCase in a mouse model of nGD showed significant improvement in CNS inflammation, neurodegeneration, survival and neurological phenotype even when the treatment started around onset of disease signs, demonstrating the therapeutic value of SapC-DOPS-GCase in lessening the nGD.

We have an evidence of binding and activation properties of SapC with GCase [25]. In this study, GCase binding to SapC-DOPS revealed a Kd of 29 nM indicating a tight interaction of GCase with SapC-DOPS nanovesicles. In the SapC-DOPS-GCase formulation, GCase is sheltered by the lipid bilayer that allows slow release of GCase and protects it from rapid denaturation by the neutral pH of plasma or culture media [49] (Figs. 1 and S2), and delays clearance into the reticuloendothelial system (Fig. 3f). EC50 of GCase in this formulation is three times more than free GCase in serum (Fig. S2). Thus, SapC-DOPS-GCase provides a more stable ERT formulation than current ERT agents.

GCase therapeutics are mannos-terminated recombinantly-produced human enzyme and its cell uptake is preferential for the mannos receptor [53,54]. Reduced mannos receptor-dependency of SapC-DOPS-GCase suggests that the GCase has surface and interior membrane occupancy of the SapC-DOPS nanovesicles. Thus, significant amounts of GCase are delivered through fusion of SapC-based vesicles with the cell and organelle membranes. Consequently, SapC-DOPS-GCase can access a wider variety of cell types allowing the enzyme into the organs that are inaccessible by ERT, such as lymph nodes, lung and brain (Fig. 3) [12,56]. While ERT has no effect on
lymph nodes in GD type 3 patients [12], SapC-DOPS-GCase accesses lymph nodes (Fig. 3f) and may provide an option for GD patients with massive mesenteric or other lymphadenopathy. Phosphatidylserine (PS) is normally found on the inner, ‘invisible’ leaflet of the plasma membrane. SapC, a membrane-associated protein, has a favorable binding affinity with unsaturated, negatively polarized phospholipids.
charged PS. Multiple lines of evidence indicate that inflamed and damaged cells (i.e., apoptotic or necrotic cells) often have abnormal surface PS [62,63]. PS-selective SapC-based nanovesicles are taken up by the brain, especially by PS-exposed enriched in inflamed-brains, by crossing compromised BBB in several neuronal disease mouse models including brain tumor, multiple sclerosis, and epilepsy [30]. As SapC-DOPS targeting is blocked by pre-treatment with the PS-specific binding protein, Lact-C2, such nanovesicles must directly interact...
and fuse with the PS-exposed cell membranes and cell surface PS as a major, specific target for SapC-DOPS-GCase (Fig. S7a). PS is a general surface lipid biomarker of inflammatoty cells that is suitable for targeted therapy and diagnosis using SapC-based nanovesicles as well as other PS-selective agents.

Nanoscale lipid-based vesicles are taken up by the lymphatic system [64,65]. Indeed, SapC-DOPS nanovesicles or SapC-DOPS-GCase significantly accumulated in mouse spleen, lymph nodes, and liver (Figs. 3 and S7). In prior studies, we have also demonstrated that the nanovesicle was associated with macrophages and neutrophils in inflammatory arthritic joints [29]. Meningeal lymphatics are a reservoir in the CNS blood-lymph circulatory loop [61]. Here, the long-term accumulation of the systemically administrated fluorescent SapC-DOPS vesicles in meningeal tissues (Figs. S7a and S7b) was shown and this signal was significantly reduced in the animals with a defective meningeal lymphatic system (Fig. S7d). In addition, the SapC-DOPS-GCase nanovesicles are co-localized with inflammatoty cells (microglia/macrophage) from brain (Fig. 4). These findings suggest that SapC-DOPS-GCase may be co-transported by inflammatoty cells from the spleen and/or lymph nodes into nGD brains via a compromised blood-lymph circulatory loop (Fig. S8b). This type of loop warrants further investigation.

SapC activates GCase to achieve maximal enzymatic function both in vitro and in vivo [22, 23, 25, 36] and, importantly can induce some GCase mutants to achieve near normal GCase activity [33]. This enhanced activity of mutant GCase stimulated by SapC-DOPS may be therapeutic in selected GD patients who have specific “susceptible” mutations. The GCase activation domain of SapC is localized in the carboxyl-terminal region [22, 66] and the primary physiological function of SapC has been identified from lysosomal storage diseases caused by deficiencies of SapC in humans and mice [28, 37, 67]. Seven cases of a GD-like disease linked to mutations in SapC have been reported in Europe and China [27, 28, 68, 69]. Therefore, therapeutic SapC-DOPS may benefit GD due to defective GCase as well as nGD with SapC deficiency. Our data showed that SapC-DOPS enhances GCase activity ex vivo and in vivo (Fig. 5). Notably, SapC-DOPS treatment improved survival and gait abnormalities in 4L:C* mice even when the treatment was started at the age when disease signs appears, demonstrating its CNS efficacy, but SapC-DOPS-GCase showed better effects.

Although ERT is the current standard of care for GD, the available ERT agents do not access particular organs, e.g. brain, lung alveoli, and lymph nodes, and are unable to treat the associated inflammation in such organs [12,70]. Additionally, available ERT agents have no direct effects on nGD since the BBB blocks CNS access to the enzyme. The BBB-penetrating SapC-based nanovesicles developed here delivered exogenous functional GCase into the CNS and establish a potential brain-specific enzymatic therapy for nGD. SapC-DOPS-GCase allows intravenous administration of enzyme treatment for the CNS disease, which has advantages over more invasive procedures, e.g. intracerebral and intrathecal delivery of biologicals into the CNS. SapC-DOPS-GCase provides more benefits than conventional ERT by stabilizing GCase and delivering enzyme into more organs, e.g. brain, lung and lymph nodes that are inaccessible by conventional ERT. Our studies have shown that functional GCase transported by SapC-DOPS nanovesicles is taken up by the inflamed CNS but also by normal brain, which allows SapC-DOPS-GCase treatment at the stages with and without inflammatory breach of BBB. The delivered GCase distributes to the affected regions and reduces substrate accumulation in nGD, subsequently mitigating the phenotype and pathology. While disease improvement was achieved when the treatment started around onset of disease signs, there is a possibility that treatment initiation at a younger age, e.g., postnatal 3 days in this model, could significantly improve the efficacy [15,71]. The basis for the selective CNS regional uptake of SapC-DOPS-GCase in the 4LC* mice requires additional investigations. The positive outcomes from these preclinical evaluations in the mouse model of nGD strongly support the translational potential of SapC-DOPS-GCase for GD treatment.

In summary, we developed SapC-DOPS-GCase as a novel therapeutic approach for GD therapy and potentially other lysosomal storage diseases or neurodegenerative disorders. SapC-DOPS-GCase is more stable than conventional ERT and accessible to both visceral and CNS organs, providing a potentially first ERT for managing both CNS and visceral symptoms. This study demonstrates a new mechanism of CNS targeting of SapC-DOPS via a PS-mediated and lymphatic circulation system. SapC-DOPS is a GMP-grade biological and has exhibited a superb safety profile in a Phase 1 clinical trial (ClinicalTrials.gov Identifier: NCT02859857) [72, 73]. The ERT of velaglucerase alfa used in this study is a FDA approved drug for GD treatment. nGD will remain lethal without improvements in treatment but since both of the biological entities have good safety profiles, SapC-DOPS-GCase has the potential to rapidly translate to improved patient care. Although this study was focused on a rare disease, there may be ramifications for similar but more common diseases such as Parkinson disease since decreased GCase activity has been documented in the Parkinson diseased brain, even in patients without GBA1 mutations [74, 75].

Declaration of Competing Interest

The authors claim a provisional patent application has been filed on March 10, 2020. Consistent with current Cincinnati Children’s Hospital Medical Center policies, the development and commercialization of this technology has been licensed to Bexion Pharmaceuticals, LLC, in which X. Qi, holds (< 5%) equity interest.

Acknowledgements

Shire/Takeda provided Velaglucerase alfa for this study. Drs. Kari Alitola and Melody Swartz provided K14-VEGFR3-Ig mice. Dr. Ellen Sidransky provided mouse Gba1−/− neurons. This study was supported by the National Institutes of Health grants R21NS 095047 to XQ and R01 NS 086134 and UH2 NS092981 in part to YS; Cincinnati Children’s Hospital Medical Center Research Innovation/Pilot award to YS and XQ; Gardner Neuroscience Institute/Neurobiology Research Center Pilot award to XQ and YS, Hematology-Oncology Programmatic Support from University of Cincinnati and New Drug State Key Project grant number 009ZXX09102-205 to XQ.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2020.102735.

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