Glucosylsphingosine Promotes α-Synuclein Pathology in Mutant GBA-Associated Parkinson’s Disease

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Glucocerebrosidase 1 (GBA) mutations responsible for Gaucher disease (GD) are the most common genetic risk factor for Parkinson’s disease (PD). Although the genetic link between GD and PD is well established, the underlying molecular mechanism(s) are not well understood. We propose that glucosylsphingosine, a sphingolipid accumulating in GD, mediates PD pathology in GBA-associated PD. We show that, whereas GD-related sphingolipids (glucosylceramide, glucosylsphingosine, sphingosine, sphingosine-1-phosphate) promote α-synuclein aggregation in vitro, glucosylsphingosine triggers the formation of oligomeric α-synuclein species capable of templating in human cells and neurons. Using newly generated GD/PD mouse lines of either sex [Gba mutant (N370S, L444P, KO) crossed to α-synuclein transgenics], we show that Gbu mutations predispose to PD through a loss-of-function mechanism. We further demonstrate that glucosylsphingosine specifically accumulates in young GD/PD mouse brain. With age, brains exhibit glucosylceramide accumulations colocalized with α-synuclein pathology. These findings indicate that glucosylsphingosine promotes pathological aggregation of α-synuclein, increasing PD risk in GD patients and carriers.

Key words: α-synuclein; GBA; glucosylsphingosine

Significance Statement

Parkinson’s disease (PD) is a prevalent neurodegenerative disorder in the aging population. Glucocerebrosidase 1 mutations, which cause Gaucher disease, are the most common genetic risk factor for PD, underscoring the importance of delineating the mechanisms underlying mutant GBA-associated PD. We show that lipids accumulating in Gaucher disease, especially glucosylsphingosine, play a key role in PD pathology in the brain. These data indicate that ASAH1 (acid ceramidase 1) and GBA2 (glucocerebrosidase 2) enzymes that mediate glucosylsphingosine production and metabolism are attractive therapeutic targets for treating mutant GBA-associated PD.
sist mainly of aggregated α-synuclein (Spillantini et al., 1997). While aggregated α-synuclein present in Lewy bodies is fibrillar, recent studies have suggested that soluble oligomers are more toxic, largely contributing to PD-related neurodegeneration (Winner et al., 2011).

Mutations in GBA, encoding lysosomal glucocerebrosidase 1 (GCase1), are the most common genetic risk factor for PD (Sidransky et al., 2009). Biallelic mutations in GBA cause Gaucher disease (GD), a lysosomal storage disorder (Tsuji et al., 1987, 1988). The most common GCase1 mutations are N370S and L444P, accounting for 70% of disease alleles (Charron et al., 2000; Grabowski et al., 2014). Both GD patients and heterozygous carriers are at increased risk for PD, with higher risk in homozygous individuals (~20-fold vs 5-fold, respectively) (Bultron et al., 2010; Alcalay et al., 2014). Hence, understanding the mechanistic links between GCase1 mutations and PD is of importance. GD patients develop Lewy body pathology, underscoring the importance of α-synuclein aggregation in GD-associated PD (Wong et al., 2004). However, it is unclear how GCase1 mutations contribute to PD pathology. Indeed, several contradictory mechanisms have been proposed. It has been suggested that mutant GCase1 physically interacts with and induces acceleration of α-synuclein aggregation in lysosomes (Yap et al., 2011), whereas another study showed that GCase1 loss-of-function-induced lysosomal dysfunction causes α-synuclein aggregation in lysosomes, further perturbing its function (Mazzulli et al., 2011). GCase1 catalyzes the conversion of glucosylceramide (GlcCer) to glucose and ceramide. The primary defect in GD is the accumulation of GlcCer in lysosomes and is seen most prominently in macrophages. GlcCer is alternatively processed to glucosylsphingosine (GlcSph), which can readily exit the lysosome (see Fig. 1A) (Elledge, 2006; Hein et al., 2007; Ferraz et al., 2016). As GlcCer builds up, it spills over into the cytosol (Hein et al., 2007). GlcCer and GlcSph are well established as primary storage lipids in GD (Nilsson and Svennerholm, 1982). These cytosolic sphingolipids are hydrolyzed by nonlysosomal GCase2 (GBA2; not deficient in GD) (Yildiz et al., 2006), to ceramide, sphingosine (Sph), and sphingosine-1-phosphate (SIP). Ceramide does not accumulate due to hydrolysis by neutral ceramidase, resulting in secondary accumulation of Sph and SIP in GD. Together, lipids accumulating peripherally in GD are GlcCer, GlcSph, Sph, and SIP (Mistry et al., 2014). There is also evidence that loss of GCase1 activity leads to lysosomal dysfunction (Bae et al., 2015). While this cascade has been demonstrated in peripheral organs, presently it is not clear whether similar events occur in the brain.

Here, we propose that GlcCer and its metabolites are critical players in mutant GBA-associated PD. We show that sphingolipids accumulating in GD accelerate α-synuclein aggregation in vitro, with potent effects of GlcSph and Sph in inducing pathology. α-synuclein species capable of templating in human cells and neurons. Using newly generated Gba mutant (N370S, L444P) and knock-out (KO) mouse models crossed with an α-synuclein transgenic PD mouse, we show that GCase1 deficiency promotes α-synuclein pathology. Importantly, we show that GlcSph accumulates in the brain of young GD mice, consistent with GlcSph being the toxic lipid species in mutant GBA-associated PD through initiation of pathological α-synuclein aggregation.

Materials and Methods

**Protein expression.** Human WT α-synuclein, along with the point mutants A30P and A53T, was purified as previously described (Chandra et al., 2003).

**Liposome preparation.** Liposomes were prepared using 75% phosphatidylycholine (PC) and 25% experimental lipid. For circular dichroism experiments seen in Figure 1, liposomes were prepared to a total concentration of 138 μM, or 34.5 μM of experimental lipid. For circular dichroism experiments seen in Figure 6, liposomes were prepared to an experimental concentration of 0.1, 1, and 10 μM of GlcSph with a constant 30 μM PC for all conditions and PC control. Experimental lipids include GlcSph (Matreya 1306), SIP (Sigma S9666), PC (Avanti Polar Lipids 840051P), ceramide (Avanti Polar Lipids 860052P), and Sph (Avanti Polar Lipids 860537P). Lipid was solubilized in 2:1 chloroform/methanol and dried in a glass tube using a gaseous nitrogen stream. Dried lipids were hydrated in 0.1 M phosphate buffer, pH 7.4 at 37°C for 1 h, then vortexed for 5 s to form a liposome suspension. Liposomes were sonicated in a 2510 Branson ultrasonic cleaner bath for 30 min at 25°C, put through 2 freeze/thaw cycles, then bath sonicated for another 60 min. Liposome size was limited to 100 nm using a liposome extruder and used immediately. In the case of GM1 (Avanti Polar Lipids, 860065P), liposomes were not prepared using PC, as GM1 is soluble in aqueous solutions.

**Circular dichroism.** Circular dichroism was performed on a Chirascan spectrophotometer (wavelength range: 190–250, step size: 1A, repeats: 3). Samples were prepared by mixing 138 μM total lipid (or 34.5 μM experimental lipid) to 13.8 μM α-synuclein protein (lipid/protein = 10:1) and protein/lipid mixtures were incubated at 37°C, shaking at 1000 rpm. Circular dichroism was measured on freshly prepared samples and subsequently daily for 5 d. Spectra were analyzed for secondary structure content following data acquisition using the DichroWeb CONTINLL database, reference set 7 (Provencher and Glöckner, 1981; van Stokkum et al., 1990; Lobley et al., 2002; Whitmore and Wallace, 2004, 2008).

**Electron microscopy.** The 3 μL α-synuclein/lipid sample (prepared as described for circular dichroism) was added to carbon-coated grids (Electron Microscopy Sciences CF400-Cu) for 2 min at room temperature. Grids were washed once in 2% uranyl acetate, incubated in fresh 2% uranyl acetate for 1 min, blotted, and allowed to air dry. Imaging of samples was performed on an FEI Tecnai G2 Spirit TWIN (T12).

**Atomic force microscopy (AFM).** AFM was performed on a Bruker Dimension Fastscan AFM using ScanAsyst Peak Force Tapping imaging mode. SCANASYST-AIR tips (Bruker Scientific) were used for imaging. The 4 μL of α-synuclein/lipid sample was added to freshly cleaved mica (Electron Microscopy Sciences 71856-01-10) and incubated at room temperature for 1 min. Mica was washed twice with 50 μL demineralized water to remove excess sample. Samples were then dried using nitrogen gas and imaged immediately after. Aggregate length was quantified using ImageJ software, with n = 2 images per condition.

**Aggregation assays in stem cells (iPSCs).** Differentiation of induced human neurons. Induced human neurons were produced through the forced expression of transcription factor neurogenin-2 in iPSCs, as previously
described (Zhang et al., 2013). The Ngn2-IPSC line contains a single copy of a doxycycline-inducible mouse neurogenin2 cassette (which also contained a CAG promoter driving a third-gen Tet transactivator) into the safe harbor AAVS1 locus of the parent WTC11 IPSC line. All induced neurons were used at least 30 d following differentiation.

Aggregation assay in induced human neurons. iPSCs were plated at a density of 100 cells/cm² in 12-well plates, differentiated as outlined above, and allowed to grow in culture for 30 d; 0.2 mg/ml liberase or monomeric α-synuclein was put through 3 freeze/thaw cycles and sonicated in a 2510 Branson ultrasonic cleaner bath for 10 min; 50 µl of sample was added to 200 µl OptiMEM (Invitrogen), whereas 10 µl of GenePORTER reagent was added to 200 µl OptiMEM in a separate tube. Following 5 min incubation, the two mixtures were combined and allowed to incubate for 20 min at room temperature. The protein mixture was then added to the media over the induced neurons and allowed to incubate at 37°C for 7 d. Cells were then scraped and run on a Western blot for analysis of intracellular α-synuclein levels.

Generation of mouse lines. Production of all GD lines is summarized in Figure 4. The insertion of a Neo cassette between exons 7 and 8 of Gba resulted in a complete Gba KO, which was rescued in the skin using the K14-Cre as described by Mistry et al. (2010). Rescue in the skin prevented lethality, as Gba KO in the skin caused a disruption in the skin permeability barrier resulting in severe dehydration and early death. Gba mutant mice were generated by knocking in either Gba N370S (exon 9) or L444P (exon 10; L444P mice were a gift from Dr. Richard L. Proia) (quint et al., 2007). Homogenates were then put through 3 freeze/thaw cycles and the supernatant and 2

GCase1 and GCase2 activity assay. A modified form of a previously established GCase assay was used in this paper (Mistry et al., 2010), with the following changes: decrease in buffer pH, disuse of sodium taurocholate detergent, and the use of CBE and NB-DNJ inhibitors to determine baseline activity levels. All noted changes bettered assay accuracy. The insertion of a Neo cassette between exons 7 and 8 of Gba resulted in a complete Gba KO, whereas those unable to hang for this amount of time were considered healthy, whereas those that found dead were included in the analysis. Mouse behavior was characterized using the hanging grip test to assess motor phenotype. Mice able to hang for the complete 200 min are considered healthy, whereas those unable to hang for this amount of time exhibit motor phenotypes.

Immunohistochemistry. Brains were postfixed in 4% PFA overnight, incubated in 30% sucrose, and frozen in OCT. Brains were sectioned into 30 µm free-floating sections using a Leica cryostat. Sections were washed in PBS, permeabilized in 0.5% Triton X-100 in PBS, and blocked in 0.1% Triton X-100 and 2% goat serum in PBS for 30–60 min at room temperature. Sections were then incubated in primary antibody in blocking solution overnight at 4°C, washed in PBS, and incubated in secondary antibody in blocking solution for 1 h at room temperature.

Whole spinal columns were postfixed in Cal-Rite decalifying/fixation solution (Thermo Fisher Scientific S5501) for 2 d. Spinal cords were dissected out, incubated in 30% sucrose for 2 d, and frozen in OCT. Spinal cords were then sectioned into 7 µm sections onto X-tra slides (Leica Biosystems 3800200) and dried for 10 min. Slides were then washed in PBS for 5 min to remove OCT, permeabilized in 0.5% Triton X-100 in PBS, and blocked in 0.1% Triton X-100 and 2% goat serum in PBS for 30–60 min at room temperature. Slides were then incubated in primary antibody in blocking solution overnight at 4°C, washed in PBS, and incubated in secondary antibody in blocking solution for 1 h at room temperature.

All samples were imaged using a Zeiss Laser Scanning Microscope 710. Primary antibodies include α-synuclein (BD Biosciences 610786, RRID: AB_398107 at 1:500), CD68 (Bio-Rad MCA1957, RRID: AB_322219 at 1:500), glucosylceramide (Glycobiotec RAS0011 at 1:100), and phosphorylated α-synuclein (Wako 015-25191, RRID: AB_2537218 at 1:1000). Goat AlexaFlour secondary antibodies were used at 1:500 dilution for each primary antibody sample. Nissl stain. Spinal cords for Nissl stain were treated and sectioned as outlined above. After drying, slides were washed in PBS 2 times for 5 min, washed in water 1 time for 1 min, incubated in 0.1% cresyl violet/1% acetic acid for 20 min, washed in water 2 times for 5 min, then dipped in 90%, 95%, and 100% ethanol. Slides were then cleaned in xylene 2 times, mounted using Permount (Fisher Scientific SP-15), and allowed to dry overnight. Slides were imaged using an AmScope B490B-M Digital Compound Microscope.

Lipidomics. Lipidomics were completed as previously described (Mistry et al., 2014). Briefly, brain tissue samples were analyzed using an API 4000 triple-quadrupole mass spectrometer interfaced with an Agilent 1200 HPLC.

Differential extraction. Total brain homogenate was serially extracted using detergents as outlined by Zhang et al. (2012). Serial extraction resulted in a soluble fraction (S1) and an SDS-insoluble fraction (P3), which were then analyzed by Western blot for phosphorylated α-synuclein levels.

Dot blot on brain homogenate. A total of 5 µl of the soluble fraction (S1) from mouse brains serially extracted using detergents (see Differential detergent extraction) was blotted onto nitrocellulose membrane and allowed to dry for 1 h under vacuum. Membranes were blocked at room temperature for 1 h, incubated in primary anti-oligomer A11 antibody

200 µl of precooled stop solution (0.5 µM glycine-NaOH, pH 10.6) was added. Measurements were taken with a standard plate reader (366 nm excitation, 445 nm emission). Standard curves were created using 8 concentrations of 4-methylumbelliferone standard ranging from 1 to 8 µM prepared in 1% DMSO.

GCase1 levels were determined by subtracting GCase2 and background GCase activity levels (as determined through use of inhibitors: conundertol, ephox, CBE for Gba1 and N-butyl-deoxyinosinimycin, NB-DNJ for Gba2). Similarly, GCase2 levels were determined by subtracting GCase1 and background GCase activity levels (as determined through use of inhibitors). All inhibitors and baseline levels were prevalidated through the evaluation of Gba KO and Gba2 KO mice as controls.

Characterization of mouse weight, behavior, and survival. Mouse weight was recorded monthly on a cohort containing 163 mice. Survival of all mouse lines was recorded over a 24 month period. Mice with severe dermatitis were censored, whereas mice suffering from end-stage motor phenotypes or those that were found dead were included in the analysis. Mouse behavior was characterized using the hanging grip test to assess motor phenotype. Mice able to hang for the complete 200 min are considered healthy, whereas those unable to hang for this amount of time exhibit motor phenotypes.
Assessment of lumbar motor neurons in spinal cord (see Fig. 5B). Two-tailed t tests were used to test significance relative to WT. N and p values: WT/WT: N = 3; /WT: N = 2, p = 0.489; /KO: N = 5, p = 0.144; SNCA<sup>T5</sup>: N = 2, p = 0.002; /WT SNCA<sup>T5</sup>: N = 2, p = 0.009; /KO SNCA<sup>T5</sup>: N = 3, p = 0.001.

Assessment of microgliosis recruitment in SNCA<sup>T5</sup> mice (see Fig. 5D). Two-tailed t tests were used to test significance relative to WT. N and p values: WT/WT: N = 3; /WT: N = 2, p = 0.315; /KO: N = 1, no p values. SNCA<sup>T5</sup>: N = 2, p = 0.005; /KO SNCA<sup>T5</sup>: N = 3, p = 0.001.

Motor phenotype onset to death in GD/PD mice (see Fig. 5H). Two-tailed t tests were used to test significance of GD/PD early death mice relative to WT mice: N = 10 for both groups. p value relative to PD = 1.64 × 10⁻⁴.

Lipidomics on mouse brain (see Fig. 6A–D). Two-tailed t tests were used to test significance relative to WT. N for all analyses: WT/WT: 3, L444P/ WT: 3, N37OS/WT: 2, KO/WT: 2, L444P/KO: 2, N37OS/KO: 6, KO/KO (GlcSph Only): 1. p value for GlcSph levels: L444P/WT = 0.234, N37OS/WT = 0.313, KO/WT = 0.608, L444P/KO = 3.35 × 10⁻⁴, N37OS/KO = 0.002.

GlcSph-derived β-sheets α-synuclein (see Fig. 6F). Two-tailed t tests were used to test significance relative to WT; N = 3 for all samples. p value relative to PC: GlcSph-1 = 0.243, GlcSph-1h = 0.472, GlcSph-10 = 0.004.

Concentration of lysosomal fractions (see Fig. 6L). Two-tailed t tests were used to test significance of Gba<sup>k0</sup> relative to WT; N = 3 for both groups. p value relative to WT = 0.031.

GlcCer standard curve (see Fig. 6J). Standard curve with samples containing 0, 0.2, 0.5, 1, and 2 μg GlcCer were plotted and yielded an R² = 0.99.

GlcCer levels in brain by antibody (see Fig. 7B, C). Two-tailed t tests were used to test significance relative to WT/WT. For Figure 7B, N and p values: anticipated death: N = 3; early death: N = 3, p = 0.013. For Figure 7C, N and p values: WT/WT: N = 3; SNCA<sup>T5</sup>: N = 3, p = 0.624; /WT SNCA<sup>T5</sup>: N = 4, p = 0.783; /KO SNCA<sup>T5</sup>: N = 3, p = 0.010.

Phosphorylated α-synuclein S129 levels by antibody (see Fig. 7D, H). One-tailed t tests were used to test significance relative to WT/WT. For Figure 7D, N and p values: WT/WT: N = 3; SNCA<sup>T5</sup>: N = 3, p = 0.010; /WT SNCA<sup>T5</sup>: N = 4, p = 0.036; /KO SNCA<sup>T5</sup>: N = 3, p = 0.010. For Figure 7H (16 kDa), N and p values versus WT/WT: SNCA<sup>T5</sup>: N = 3, p = 4.120 × 10⁻⁴; /KO SNCA<sup>T5</sup>: N = 3, p = 0.008. For Figure 7I (37 kDa), N and p values versus WT/WT: SNCA<sup>T5</sup>: N = 3, p = 0.090; /KO SNCA<sup>T5</sup>: N = 3, p = 0.002.

Aggregated α-synuclein levels by ELISA (see Fig. 7I). Two-tailed t tests were used to test significance relative to WT/WT. N and p values: WT/WT: N = 3; SNCA<sup>T5</sup>: N = 3, p = 0.016; /KO SNCA<sup>T5</sup>: N = 3, p = 0.014; p values versus SNCA<sup>T5</sup>: /KO SNCA<sup>T5</sup> = 0.034.

GBA2 activity levels in mouse brain (see Fig. 8A). Two-tailed t tests were used to test significance relative to young cohort. N and p values: WT/WT: N = 8, 3-month, 9 1-year mice, p = 0.507; /WT: N = 26 3-month, 9 1-year mice, p = 0.038; /KO: N = 16 3-month, 8 1-year mice, p = 0.398.

GBA2 protein levels in mouse brain (see Fig. 8C). Two-tailed t tests were used to test significance relative to young cohort. Six mice per genotype were used, with 3 young (age 3 months) and 3 old (age 1 + year) mice. p values: WT/WT = 0.193, /WT = 0.021, /KO = 0.991.

Results

Sphingolipids accumulating in GD promote α-synuclein aggregation into distinct pathologic species in vitro

To determine whether the sphingolipids that accumulate peripherally in GD, GlcCer, GlcSph, Sph, and S1P interact with α-synuclein and influence its aggregation, we used circular dichroism to track the secondary structure of α-synuclein in the presence of respective lipids. To mimic a cellular setting, we prepared and incubated with purified human WT α-synuclein. PC alone serves as a negative control as it does not alter the conformations of...
α-synuclein, whereas GM1 is a negative control for inducing an α-helical conformation (Martinez et al., 2007). Circular dichroism spectra were measured immediately after mixing and subsequently daily for 5 d. On day 0, α-synuclein was unstructured in the presence of all lipids, except GM1 and BMP, both of which induced an α-helical conformation upon mixing (Fig. 1B, C, F). Secondary structure was analyzed for the emergence of β-sheets and fibrils, indicative of transition from unstructured, monomeric α-synuclein to oligomers or fibrils. After 5 d of incubation, α-synuclein samples containing GD-associated sphingolipids were mainly β-sheeted (Fig. 1B, D, E), whereas samples with PC and Cer remained unstructured and samples with GM1 gangleoside and BMP remained α-helical (Fig. 1B, D, F). Of the four GD sphingolipids, GlcCer was the slowest to induce β-sheet structure formation (Fig. 1E, G, H). Incubation with GlcCer has been previously shown to accelerate α-synuclein aggregation, supporting our findings (Mazzulli et al., 2011). These results clearly show that all sphingolipids that accumulate in GD can interact with WT α-synuclein to accelerate its aggregation into oligomeric and/or fibrillar states. We obtained similar results with PD mutants of α-synuclein (A53T, A30P) (Fig. 1G, H).

To differentiate between oligomer and fibril formation in the circular dichroism samples, we performed electron microscopy and AFM to visualize the α-synuclein aggregates formed in the presence of our experimental lipids (day 5 samples). Using electron microscopy, we found α-synuclein fibrils in samples containing GlcCer, Sph, and S1P, whereas unstructured protein was present in other samples (Fig. 2A). The fibrils were typical of those seen when α-synuclein alone is aggregated in vitro (Fig. 2A) or purified from transgenic mice (Recasens et al., 2014; Peelaerts et al., 2015). Interestingly, we did not see long fibrils in the presence of GlcSph (Fig. 2A), even though GlcSph induced β-sheet structure as measured by circular dichroism (Fig. 1B, D, E). To further elucidate the type of α-synuclein aggregates formed in the presence of GlcSph, we imaged these samples with AFM. Intriguingly, we discovered oligomers in the GlcSph sample (Fig. 2B). We also confirmed the presence of long fibrils in the GlcCer and S1P samples using this method, and identified that Sph induces a heterogenous mixture of oligomers and fibrils (Fig. 2B). When we measured the length of the α-synuclein species, we can clearly differentiate the ones formed by GlcSph and Sph (Fig. 2C), which have a median length of 0.603 and 0.582 μm (Fig. 2D). These findings demonstrate a direct role of GlcCer, GlcSph, Sph, and S1P in promoting α-synuclein aggregation into different types of species, further suggesting that some of these sphingolipids may be more pathological than others within the nervous system.

α-Synuclein species produced by GlcSph and Sph have distinctive aggregation propensity in mammalian cell culture and human neurons

We established an intracellular α-synuclein aggregation assay in HEK293T cells based on previous literature (Luk et al., 2009),
Briefly, we added preformed α-synuclein aggregates to the culture media of HEK293T cells overexpressing α-synuclein-GFP, facilitated internalization, and monitored templating of endogenous α-synuclein-GFP into intracellular, GFP-positive aggregates (Fig. 3A). This assay was used to evaluate the relative pathogenicity of aggregated species formed following α-synuclein incubation with GD-associated sphingolipids. We found that preformed aggregated α-synuclein species formed with GlcSph and Sph produced significantly more internal, GFP-positive aggregates than with GlcCer and S1P, related lipids, and controls (Fig. 3A). Notably, the templating propensity of the preformed aggregated species correlated with the oligomeric α-synuclein structures observed by AFM in the samples containing GlcSph and Sph (Fig. 2B–D). Although we cannot exclude the possibility that oligomeric α-synuclein species are taken up more efficiently, it would still suggest that size of α-synuclein species formed is a determinant of its pathogenicity. These results show a specific effect of GlcSph and Sph in inducing the formation of oligomeric α-synuclein species that are capable of pathologic templating.

To confirm these results in a neuronal model, we used human neurons derived from iPS cells. Differentiation was induced through neurogenin 2 expression, generating excitatory neurons, as described previously (Zhang et al., 2013). Consistent with previous reports, the human neurons show localization of α-synuclein at presynaptic puncta and MAP2-positive dendrites. Similar to our experiments in HEK293T cells, we added exogenous preformed GD sphingolipid-induced α-synuclein species and assessed their effects on internal α-synuclein aggregation. By Western blotting, we could show that samples with GlcSph and Sph specifically promoted endogenous α-synuclein aggregation, whereas samples with GlcCer, S1P, and control lipids resulted in only monomeric α-synuclein (Fig. 3B). The aggregation observed is unlikely to be remnants of exogenous α-synuclein species as we do not see any aggregates in GlcCer and S1P samples, even though they are likely to be stable. These results in neuronal cells replicate our findings in HEK293T culture, verifying that oligomeric α-synuclein species formed with GlcSph and Sph are more pathogenic in hu-
man neurons than α-synuclein species formed by other experimental lipids.

New long-lived mouse models for GD/PD

To test the role of altered sphingolipid metabolism in mutant GBA-associated PD, we generated new GD mouse lines. Previously produced germline homozygous Gba KO mice as well as homozygous N370S or L444P mice exhibited disruption of skin permeability barrier that caused neonatal lethality (Xu et al., 2003). Therefore, we generated conditional Gba KO mice and Gba mutant knock-in (N370S, L444P) mice, which are rescued by expressing Gba only in skin (Fig. 4A–C), similar to mice generated by Enquist et al. (2007). The knock-in mice were bred to Gba KO to generate Gba mutant allelic on a WT and KO background. Significantly, these mice do not die due to skin permeability issues, allowing us to study age-related phenotypes. The homozygous Gba KO (GbaKO/KO) and knock-in (GbaL444P/KO, GbaN370S/KO) mice are viable, fertile, and grossly normal with the expected experimental lipids.

GlcSph accumulates early in mutant GBA-associated PD

To evaluate the altered sphingolipid metabolism in the brains of GD/PD mice, we quantified GD associated sphingolipid levels in the brains of a young (3-month) GD/PO mouse cohort by mass spectrometry. We found that GlcSph levels are significantly increased in homozygous GbaL444P/KO, GbaN370S/KO, and GbaKO/KO mice at 3 months of age (Fig. 6B). We do not observe any change in these values in SNCA+PD mice, suggesting that α-synuclein transgenic expression does not influence GlcSph levels. Notably, GlcCer, Sph, and S1P levels were not altered at 3 months (Fig. 6A,C,D), even though it has been established that these lipids eventually increase in the plasma of PD patients (Dekker et al., 2011; Mistry et al., 2014). These results are consistent with a previous report indicating that GlcCer does not accumulate in the brain at an early age in a neuropathic GD mouse model; GlcCer was shown to significantly increase only by 6 months of age (Dai et al., 2016), suggesting that accumulation of GlcCer lags behind that of GlcSph in the brain. However, given the high baseline levels of GlcCer relative to other sphingolipids assays, it is possible that smaller, localized increases are not detectable until levels increase significantly above endogenous levels. To address this possibility, we completed a lysosomal fractionation of mouse brain and found no differences between GlcCer levels in fractions from WT and GD mouse brain enriched for lysosomes (Fig. 6G–K).

Next, we investigated the potency of the observed brain GlcSph concentrations in our in vitro aggregation assay. We determined that incubation with GlcSph at concentrations seen in homozygous GD brains results in the formation of similar oligomeric α-synuclein species (Fig. 6E). The effect of GlcSph on α-synuclein aggregation was also shown to be concentration dependent by circular dichroism (Fig. 6F). Together, these findings strongly suggest that GlcSph can potentely enhance pathological α-synuclein aggregation in the brain.
GD/PD mouse mortality correlates with level of sphingolipid accumulation in brain

Survival of GD/PD mice was assessed over the course of 24 months. Only mice suffering from end-stage motor phenotypes associated with PD or those that were found dead were included in this analysis. The ages at death of SNCATg, heterozygous Gba/WTSNCATg, and homozygous Gba/KOSNCATg mice were collected and plotted as frequency distributions (Fig. 7A). A Gaussian curve was fitted to the SNCATg distribution, indicating values within 1, 2, and 3 SDs of the mean age of death (16.2 months). This curve was overlaid onto the frequency distributions for heterozygous Gba/WTSNCATg and homozygous Gba/KOSNCATg mice, allowing for visual comparison between genotypes (Fig. 7A).

Although all values of the SNCATg line fall within 2 SDs of the SNCATg mean, a small fraction of heterozygous Gba/WTSNCATg and a larger fraction of homozygous Gba/KOSNCATg values fall farther than 2 SDs to the left of the SNCATg mean (Fig. 7A). These data show that a subset of the GD/PD mice exhibit premature morbidity relative to the anticipated age of death of SNCATg mice, suggesting an acceleration of PD phenotypes and pathology due to contributions from Gba mutation. This distribution mirrors the patient population, in which GBA mutation confers a risk for PD onset in a dose-dependent manner. Our GD/PD mouse lines also capture the imperfect penetrance of the GBA phenotypes to PD manifestation in humans.

As a means of investigating the molecular differences between the early death and anticipated death groups, we immunostained brain sections from age-matched homozygous Gba/KOSNCATg early death mice and homozygous Gba/KOSNCATg anticipated death mice, using an antibody against GlcCer (Fig. 7B) (Doering et al., 2002; D’Angelo et al., 2007). We discovered an approximately fivefold increase in GlcCer levels in the early death group relative to the anticipated...
death group (Fig. 7B), strongly correlating PD-related morbidity to increased GD sphingolipid levels in brain.

These results indicate that the accumulation of GD-related sphingolipids in the brain may be an important factor in prompting the early death observed in homozygous Gba−/−SNCA+/+ mice. Interestingly, we noticed that only homozygous GbaL444P/KO/SNCA+/+ and GbaL444P/KO/SNCA−/−, and not GbaL444P/KO/SNCA−/−, contributed to the early death group. These findings suggest that morbidity may be inversely correlated to Gba protein level in brain, as homozygous GbaN370S/KO/SNCA−/− were previously found to have ~50% residual protein levels (Fig. 4D). This finding is consistent with clinical data that the N370S mutation has a lower odds ratio for PD than L444P (Gan-Or et al., 2008).

Aged GD/PD mice exhibit α-synuclein pathology in regions of GlcCer accumulation

Using the early death homozygous GbaL444P/SNCA−/− mice and WT, SNCA−/−, and GbaL444P/SNCA−/− controls, we further investigated relative levels of GlcCer and α-synuclein pathology in brain. As noted in our comparative studies of early and anticipated death scenarios, multiple brain regions showed increased GlcCer levels in homozygous early death mice (Fig. 7C,F). Together with our lipidomic analyses, these data support an age-dependent accumulation of GlcCer in the brain. Here we show representative images of GlcCer staining in the CA3 region in hippocampus. Quantitative immunofluorescence showed that homozygous GbaL444P/KO/SNCA−/− and GbaL444P/KO/SNCA−/− mice have ~10-fold GlcCer accumulation relative to WT levels (9.8 ± 2.99 vs 1.2 ± 0.11; p = 0.0096).

We and others have shown that SNCA−/− PD mice exhibit α-synuclein pathology, which is associated with increased insolubility of the protein (Chandra et al., 2005; Gallardo et al., 2008). To monitor α-synuclein pathology, we immunostained the above cohort of GD/PD brain sections with an antibody against Ser129 phosphorylated α-synuclein, a marker of α-synuclein pathology (Saito et al., 2003). As expected, SNCA−/− PD mice show
Figure 6. GlcSph accumulates early in mouse brain: **A**, GlcCer; **B**, GlcSph; **C**, Sph; **D**, S1P levels in GD/PD brains. N = 2–6 per genotypes, except Gba KO (N = 1); age of mice = 2–3 months. p value relative to WT/WT for GlcSph levels: L444P/WT 0.234, N370S/WT 0.313, KO/WT = 3.350 × 10^-5, N370S/KO = 0.002. **E**, AFM images of α-synuclein incubated with 0.1, 1, and 10 μM GlcSph with PC control on day 0 and 7. Scale bars, 1 μm. **F**, Percentage β-sheeted content of α-synuclein in the presence of 0.1, 1, and 10 μM GlcSph on day 0 and day 7. N = 3 for all samples. p value relative to PC: GlcSph-0.1 = 0.243, GlcSph-1 = 0.472, GlcSph-10 = 0.004. **p < 0.01** (two-tailed Student’s t test). **G**, Subcellular fractionation showing lysosomes on the top fraction (F1) and mitochondria on the bottom fraction between 27% and 23% density interfaces (F2). Two smaller bands are seen between F1 and F2 (not taken). **H**, Western blot shows enrichment of lysosomes in F1. Cathepsin D, ATP6V1a, and LAMP1 were used as lysosomal markers; Cathepsin D and ATP6V1a can be seen in the homogenate, supernatant, and lysosomal fraction, but not in the mitochondrial fraction. LAMP1 can be seen predominantly in the lysosomal fraction. **I**, Relative protein levels in lysosomal fractions were determined by BCA. GBA KO lysosomes (11.38 mg/ml) were found to have almost 3 times as much protein as WT lysosomes (4.27 mg/ml), a likely indicator of expected lysosomal enlargement and dysfunction in the GBA KO mice. p value /KO relative to WT 0.031. **p < 0.05** (two-tailed Student’s t test). **J**, Dot blot shows relative GlcCer levels using a GlcCer antibody (Glycobiotech). Blotted GlcCer lipid shows that the dot blot method is robust and quantitative, with a standard curve with R² = 0.99. **K**, GlcCer levels from brain homogenate, lysosomal fraction, and mitochondrial fraction were analyzed using the dot blot method in **J**. No difference between WT and GBA KO was found in any of the fractions, including total brain homogenates confirming the lipidomic results in A. However, there was a clear enrichment of GlcCer in the mitochondrial fraction relative to the others.
Figure 7. GD/PD mice exhibit α-synuclein pathology and GlcCer accumulation. A, Histograms of age of death of SNCA<sup>Tg</sup> mice (N = 12), heterozygous GBA<sup>WT/WT</sup> SNCA<sup>Tg</sup> (N = 36), and homozygous GBA<sup>KO/KO</sup> SNCA<sup>Tg</sup> (N = 18). Gaussian curve fitted to SNCA<sup>Tg</sup> histogram superimposed over all three histograms, showing differences in distributions; 1, 2, and 3 SDs from the mean shown through progressively darker shading under Gaussian curve. B, Quantification of GlcCer levels in the CA3 region of hippocampus in homozygous GBA<sup>KO/KO</sup> SNCA<sup>Tg</sup> brain of early death and anticipated death mice (N = 3 mice per group). N and p values relative to anticipated death: anticipated death: N = 3; early death: N = 3, p = 0.013. C, Quantification of GlcCer levels in the CA3 region of hippocampus in GD/PD mice cohorts. Values normalized to 1 for GBA<sup>WT/WT</sup>. N and p values relative to WT/WT: WT/WT: N = 3; SNCATg: N = 3, p = 0.624; /WT SNCATg: N = 4, p = 0.016; /KO SNCATg: N = 3, p = 0.010. D, Relative Ser129 phosphorylated α-synuclein levels in the CA3 region of the hippocampus in the same brains. N and p values relative to WT/WT: WT/WT: N = 3; SNCATg: N = 3, p = 0.004; /WT SNCATg: N = 4, p = 0.016; /KO SNCATg: N = 3, p = 0.010. E, Correlation of GlcCer and Ser129 phosphorylation α-synuclein levels across the different genotypes. r<sup>2</sup> = 0.827, Pearson's product moment-correlation p = 1.618 × 10<sup>−5</sup>. N = 3 or 4 per genotype; age of mice = 8–11 months. F, Representative brain sections stained with antibodies to GlcCer and Ser129 phosphorylated α-synuclein. Scale bar, 250 μm. G, Western blot of soluble fraction of mouse brain homogenate following differential detergent extract in age-matched GBA<sup>WT/WT</sup>, SNCA<sup>Tg</sup>, and GBA<sup>KO/KO</sup> SNCA<sup>Tg</sup> exhibiting early death, probing for phosphorylated α-synuclein S129. H, Quantification of G, focusing on expected bands at 16 and 37 kDa. 16 kDa: N and p values versus WT/WT: SNCA<sup>Tg</sup>: N = 3, p = 4.120 × 10<sup>−4</sup>; /KO SNCA<sup>Tg</sup>: N = 3, p = 0.008. 37 kDa: N and p values versus WT/WT: SNCA<sup>Tg</sup>: N = 3, p = 0.002. I, Dot blot of soluble fraction of mouse brain homogenate following differential detergent extract in age-matched GBA<sup>WT/WT</sup>, SNCA<sup>Tg</sup>, and GBA<sup>KO/KO</sup> SNCA<sup>Tg</sup> exhibiting early death, using anti-oligomer antibody A11. J, Quantification of ELISA assay probing for the presence of aggregated α-synuclein in total brain homogenate from age-matched GBA<sup>WT/WT</sup>, SNCA<sup>Tg</sup>, and GBA<sup>KO/KO</sup> SNCA<sup>Tg</sup> exhibiting early death. N and p values relative to WT/WT: WT/WT: N = 3; SNCA<sup>Tg</sup>: N = 3, p = 0.014; /KO SNCA<sup>Tg</sup>: N = 3, p = 0.014; p values versus SNCA<sup>Tg</sup>: /KO SNCA<sup>Tg</sup> = 0.034.
increased Ser129 phosphorylated α-synuclein levels in the brain, but homozygous GbaL444P/KO-SNCATg and GbaKO/KO-SNCATg mice developing early phenotypes show even larger increases (Fig. 7D, F). To determine the relationship between GlcCer and Ser129 phosphorylated α-synuclein levels, we measured and compared relative intensities. As seen in Figure 7E, GlcCer and Ser129 phosphorylated α-synuclein levels are linearly correlated ($r^2 = 0.82$, Pearson’s product moment-correlation $p = 1.618 \times 10^{-7}$). To confirm this finding, we determined the average Mander’s coefficient M2 expressing overlap between the two channels as 0.841, suggesting that GlcCer accumulation, and likely the other GD sphingolipids, are triggers for α-synuclein Ser129 phosphorylated pathology. We further confirmed the presence α-synuclein pathology in homozgyous, phenotypic GbaL444P/KO-SNCATg and GbaKO/KO-SNCATg mouse brain using Western blotting, showing the appearance of a soluble oligomeric α-synuclein species in GbaL444P/KO-SNCATg and GbaKO/KO-SNCATg mice, which was not present in SNCATg mouse brain (Fig. 7G,H). These results were verified using dot blot with an anti-oligomer antibody and a commercial ELISA kit against oligomeric and aggregated α-synuclein (Fig. 7I,J).

Finally, we examined the two key enzymes that regulate GlcSph levels: acid ceramidase and Gba2. Quantitative Western blotting showed that neither acid ceramidase nor Gba2 levels change with Gba genotype (Fig. 8B,D). When we examined these two enzymes in aged mice, we observed that, whereas acid ceramidase levels were unaltered, both Gba2 activity and protein levels decreased with age (Fig. 8A, C, E). Decreasing Gba2 is likely to contribute to the further accumulation of GlcSph in an age-dependent manner in our mouse lines and is consistent with clinical data (Ferraz et al., 2016).

**Discussion**

Although it is firmly established that GBA mutations, even in the heterozygote carrier state, are a major genetic risk factor for PD, the molecular mechanism(s) underlying the association of GBA mutations and PD are poorly understood. Hitherto, the focus in the field has been on GD mutant GCase1 itself and its interactions with α-synuclein, focusing mainly on gain-of-function mechanisms, or lysosomal dysfunction to describe the nexus between GBA mutations and PD. These studies are hard to reconcile with the subcellular localization of GCase1 (lysosomal) and α-synuclein (presynaptic), and do not explain the striking incremental risk of PD associated with null or severe GBA alleles (i.e., 84G ins G mutation, which leads to frame shift and premature protein truncation) compared with mild mutations (N370S) (Gan-Or et
Effects of Gaucher Sphingolipid Lipid Accumulation on α-Synuclein Pathology

Figure 9. Model of how GD-related sphingolipids impact α-synuclein pathology. Deletion or GD mutations in GBA leads to accumulation of GlcSph in the cytosol of neurons. GlcSph directly interacts with α-synuclein to promote its aggregation into distinct pathogenic oligomeric species. These pathogenic species further template intracellular α-synuclein aggregation and may have the capacity to spread to neighboring neurons. With age, GlcCer also accumulates, and there is a decrement of Gba2 and lysosomal enzymes, exacerbating α-synuclein pathology and promoting α-synuclein pathology. Deletion or GD mutations in GBA leads to accumulation of GlcSph in the cytosol of neurons. GlcSph directly interacts with α-synuclein to promote its aggregation into distinct pathogenic oligomeric species. These pathogenic species further template intracellular α-synuclein aggregation and may have the capacity to spread to neighboring neurons. With age, GlcCer also accumulates, and there is a decrement of Gba2 and lysosomal enzymes, exacerbating α-synuclein pathology and promoting α-synuclein pathology. Deletion or GD mutations in GBA leads to accumulation of GlcSph in the cytosol of neurons. GlcSph directly interacts with α-synuclein to promote its aggregation into distinct pathogenic oligomeric species. These pathogenic species further template intracellular α-synuclein aggregation and may have the capacity to spread to neighboring neurons. With age, GlcCer also accumulates, and there is a decrement of Gba2 and lysosomal enzymes, exacerbating α-synuclein pathology and promoting α-synuclein pathology. Deletion or GD mutations in GBA leads to accumulation of GlcSph in the cytosol of neurons. GlcSph directly interacts with α-synuclein to promote its aggregation into distinct pathogenic oligomeric species. These pathogenic species further template intracellular α-synuclein aggregation and may have the capacity to spread to neighboring neurons. With age, GlcCer also accumulates, and there is a decrement of Gba2 and lysosomal enzymes, exacerbating α-synuclein pathology and promoting α-synuclein pathology. 

al., 2008). Although it is possible the two proteins interact when α-synuclein is degraded at lysosomes, all present evidence suggests that α-synuclein forms cytosolic or synaptic aggregates, not intralysosomal aggregates (Okazaki et al., 1961). Lysosomal dysfunction is likely to be an important contributor in the link between GD and PD, but not the sole factor. Only GD and GBA mutants consistently increase risk of PD across the multiplicity of lysosomal diseases. A unique feature of GD, compared with other lysosomal disorders, is the accumulation of GlcCer and its bioactive metabolite GlcSph generated via activation of an alternative metabolic pathway involving acid ceramidase. Therefore, the premise of our studies is that GlcCer and GlcSph, accumulating due to a loss of GCase1 function, play a key role in mutant GBA-associated PD (Fig. 9). Accordingly, we have examined these sphingolipids that accumulate in GD as effectors of α-synuclein toxicity. Importantly, these sphingolipids are present in the cytosol where α-synuclein aggregates, making them feasible mediators of α-synuclein pathology.

We determined that all four sphingolipids (GlcCer, GlcSph, Sph, 5-1-P) promote the aggregation of WT α-synuclein into β-sheeted conformations in vitro (Fig. 1). Similar results were obtained when these sphingolipids were added to mutant α-synuclein, with the exception of GlcCer, which did not promote aggregation (Fig. 1G,H). These findings suggest that GlcCer’s metabolites, rather than GlcCer alone, may play an integral role in influencing pathological α-synuclein aggregation. AFM imaging and templating assays supported this notion, revealing that GlcSph and Sph induced the formation of oligomeric α-synuclein species capable of templating endogenous α-synuclein into aggregates in mammalian culture and human neurons (Fig. 3). Together, our in vitro results implicate GlcCer downstream metabolites as potential mediators of α-synuclein toxicity in GBA-associated PD.

Molecular characterization of homozygous GD/PD mice developing early phenotypes further validated our in vitro findings. Lipidomic analysis of young homozygous GD/PD mice brains revealed that only GlcSph accumulates initially, highlighting the importance of the oligomeric α-synuclein species formed by this sphingolipid in mediating toxicity. Furthermore, homozygous, phenotypic GD/PD mice show a clear correlation between GlcCer and α-synuclein pathology and the appearance of a novel, soluble aggregated α-synuclein species in GD/PD brain relative to controls (Fig. 7). The sum of these experiments implicates GlcSph, which was found to produce toxic oligomeric α-synuclein species in vitro and accumulated in homozygous GD/PD mouse brain, in mediating PD risk in GD patients. This conclusion is supported by previous literature, in which GlcSph was suggested to mediate brain pathology in neuropathic forms of GD, implicating a role in neurodegeneration (Nilsson and Svennerholm, 1982; Orviskiy et al., 2002; Schueier et al., 2003). Notably, we and others have shown massive accumulation of GlcSph in the plasma of GD patients with biallelic GBA mutations (Dekker et al., 2011; Murugesan et al., 2016), and recent studies have also reported the accumulation of GlcSph in the substantia nigra of sporadic PD patients (Rocha et al., 2015a). GlcSph has also emerged as a promising biomarker for GD, highlighting its specificity and abundance in GD patients (Dekker et al., 2011; Mirzaian et al., 2013). These clinical data underscore the relevance of our finding that GlcSph levels mediate the risk for developing PD.

The detailed characterization and phenotyping of our long-lived homozygous GD/PD mice have allowed us to investigate the mechanism by which GBA mutations confer risk for PD development. Our molecular characterization of GD/PD brain and survival data support a loss-of-function mechanism in Gba-linked PD. We showed that GlcCer accumulation and α-synuclein aggregation are correlated in brains of both GbaL444P/KO/SCNA78 and GbaKO/KO/SCNA78 lines (Fig. 7), suggesting that GBA L444P mutations result in a loss of function of GCase1. This is consistent with the finding that GbaL444P/KO brains lack GCase1 expression and enzymatic activity similar to GbaKO/KO (Fig. 4D,E). In contrast, the GbaN370S/KO/SCNA78 did not phenocopy GbaKO/KO/SCNA78 and did not contribute to the mice in the homozygous GD/PD early death group. This difference in survival is likely attributed to the N370S mutation being a trafficking mutant with 50% residual protein expression (Fig. 4D). This seems likely, as in clinical studies of PD stratified by GBA mutation, N370S had the lowest odds ratio for risk (2.2) compared with L444P (odds ratio 5.3) and other severe mutations (odds ratio 7.9) (Gan-Or et al., 2008). Some heterozygous GD/PD mice...
GlcCer in GBA-linked PD brains, and GlcSph accelerates the age-related decline in lysosomal GCase1 activity (et al., 2014). Importantly, heterozygote carriers of GBA mutations were shown to have age-related decline in lysosomal GCase1 activity (et al., 2015). Surprisingly, even in non–GBA-associated PD, reduced GCase1 activity has been described in the substantia nigra of autopsy brains of PD patients (Gegg et al., 2012). Furthermore, GCase1 activity decreases with age in normal individuals (Rocha et al., 2015a).

Here, we propose a model where GlcSph accumulates before GlcCer in GBA-linked PD brains, and GlcSph accelerates the aggregation of α-synuclein into pathological species (Fig. 9). We show that these aggregated α-synuclein species are capable of self-templating into Lewy body-like pathology. This is compounded by a decrease in lysosomal protein degradation capacity with age (Bae et al., 2015) or by a decrease in lysosomal catabolic activity, which in turn leads to increased α-synuclein levels. These two contributions exacerbate aggregation of α-synuclein, leading to the eventual demise of the neuron (Fig. 9).

Our cumulative data point to ASAHI1 (acid ceramidase) and GBA2 (glucocerebrosidase 2) as new therapeutic targets for the preventative and acute treatment of mutant GBA-associated PD. These novel targets have emerged as alternatives to CNS-penetrant recombinant GCase1 enzyme replacement therapy and inhibition of GlcCer synthase (substrate reduction therapy), both of which potently reduce GlcCer and its metabolites and are currently under investigation.

References


