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Glucocerebrosidase haploinsufficiency in A53T α -synuclein mice impacts disease onset and course

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Abstract

Mutations in *GBA1* encountered in Gaucher disease are a leading risk factor for Parkinson disease and associated Lewy body disorders. Many *GBA1* mutation carriers, especially those with severe or null *GBA1* alleles, have earlier and more progressive parkinsonism. To model the effect of partial glucocerebrosidase deficiency on neurological progression *in vivo*, mice with a human A53T α -synuclein (*SNCA*^{A53T}) transgene were crossed with heterozygous null *gba* mice (*gba*^{+/-}). Survival analysis of 84 mice showed that in *gba*^{+/-}/*SNCA*^{A53T} hemizygotes and homozygotes, the symptom onset was significantly earlier than in *gba*^{+/+}/*SNCA*^{A53T} mice (p-values 0.023–0.0030), with exacerbated disease progression (p-value < 0.0001). Over-expression of *SNCA*^{A53T} had no effect on glucocerebrosidase levels or activity. Immunoblotting demonstrated that *gba* haploinsufficiency did not lead to increased levels of either monomeric SNCA or insoluble high molecular weight SNCA in this model. Immunohistochemical analyses demonstrated that the abundance and distribution of SNCA pathology was also unaltered by *gba* haploinsufficiency. Thus, while the underlying mechanism is not clear, this model shows that *gba* deficiency impacts the age of onset and disease duration in aged *SNCA*^{A53T} mice, providing a valuable resource to identify modifiers, pathways and possible moonlighting roles of glucocerebrosidase in Parkinson pathogenesis.

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Keywords

α -synuclein; Parkinson disease; Gaucher disease; Mouse model; Glucocerebrosidase; Aggregates

1. Introduction

Parkinson disease (PD), a common neurodegenerative disorder associated with aging, is characterized pathologically by intraneuronal Lewy bodies and the loss of dopaminergic neurons in the substantia nigra [1]. Clinically, patients manifest with resting tremors, bradykinesia, rigidity and postural instability [2], as well as non-motor symptoms including cognitive impairment [3]. Identifying genetic contributions to Parkinson disease has illuminated molecular mechanisms underlying disease pathogenesis [4,5], particularly the gene encoding alpha-synuclein (*SNCA*), a major component of Lewy bodies. The Ala53Thr (A53T) mutation in *SNCA*, initially identified in rare families with Parkinson disease [6] increases its propensity to aggregate [7,8].

The genetic landscape of Parkinson disease now includes nearly thirty genes [9]. Heterozygous mutations in *GBA1*, the gene mutated in Gaucher disease, encoding the lysosomal enzyme glucocerebrosidase (GCase, EC 3.2.1.45) are the most common genetic risk factor for Parkinson disease as well as dementia with Lewy bodies, often associated with an earlier onset and more severe cognitive and non-motor symptoms [10,11]. Severe *GBA1* mutations, such as loss of function mutations c.84insG and IVS2+1G > A, confer a higher risk for Parkinson disease and a more progressive course compared to milder mutations like N409S (N370S) [12,13]. Many groups have investigated the relationship between *SNCA* levels and GCase deficiency or inhibition, but only a few have utilized animal models [14–17], including mice treated with the GCase inhibitor conduritol- β -epoxide (CBE) [16,18]. To further investigate the link between *GBA1* mutations and Parkinson disease, we sought to develop an *in vivo* mouse model recapitulating the accelerated Parkinson disease course observed in loss-of-function *GBA1* mutation carriers, by crossing a *SNCA*^{A53T} transgenic mouse that develops severe motor impairment and neuronal *SNCA* inclusions [19], with heterozygotes for a functionally null *gba* allele [20]. While homozygous null *gba* mice die perinatally, heterozygotes, with 40–50% of wildtype GCase enzyme activity, develop normally [20], and mimic patients with Parkinsonism carrying loss-of-function *GBA1* mutations. Unlike the CBE treated models, these mice age naturally, enabling the analysis of factors associated with aging.

2. Materials and methods

2.1. The generation of *gba*^{+/-}/*SNCA*^{A53T} mice

In this study, *gba*^{+/-} mice [20] were crossed with mice with a human *SNCA*^{A53T} transgene [19]. *gba*^{+/-}/*SNCA*^{A53T} homozygotes and hemizygotes, along with their *gba*^{+/-} and *gba*^{+/+}/*SNCA*^{A53T} controls, were followed for two years. All housing and breeding of mice were performed under NHGRI Animal Care and Use Committee-approved protocols.

2.1.1. Mouse background—*gba* knock-out (*gba*^{tm1Nsb}; maintained as heterozygotes) and transgenic *SNCA*^{A53T} (Tg(Prnp-*SNCA**A53T)83Vle) mice were originally on mixed backgrounds (C57BL/6 X FVB/N and C57BL/C3H, respectively). Both lines were backcrossed against the C57BL/6 J (B6) background, with matings being informed by genetic markers. The Mouse Medium Density (MD) Linkage panel (1449 SNPs; Illumina) was used to define blocks of heterozygosity. At each subsequent generation, pups were genotyped for the presence of the mutation (*gba*−) or transgene (*SNCA*^{A53T}) and were assessed using progressively more focused panels of microsatellite markers within these heterozygous blocks. Markers, first chosen at intervals of < 5 Mb within each heterozygous block, were selected at decreased intervals as heterozygosity diminished with each generation. The two male pups with the highest levels of homozygosity were mated to B6 females at each generation. This process was repeated in excess of ten generations for *gba*^{tm1Nsb}, and five generations for Tg(Prnp-*SNCA**A53T) mice, at which time the Y chromosome was fixed by mating a female mutant/transgenic pup with a B6 male mouse.

2.1.2. Mouse genotyping—Genomic DNA was prepared from tail biopsies as previously described [21]. The *SNCA* primers used for PCR amplification to confirm the presence of human *SNCA*, and neo primer set used to screen for the *gba* null allele are shown below.

SNCA-F 5′-TGC CTG TGG ATC CTG ACA AT-3′.

SNCA-R 5′-GGG GAG GGG CAA ACA ACA GA -3′.

NEO-1F 5′-ACA GAC AAT CGG CTG CTC TGA TGC -3′.

NEO-2R 5′-CTC GTC AAG AAG GCG ATA GAA GGC-3′.

2.1.3. Evaluation of *SNCA* copy number—To distinguish hemizygous from homozygous *SNCA*^{A53T} mice, transgene copy-number was evaluated using TaqMan (applied Biosystems, Foster City, CA) Copy Number real-time PCR assays. Two FAM-labeled probes were used to measure the human *SNCA* in genomic DNA: Hs05969230_cn in exon 2 and Hs02236645_cn in exon 7. The mouse *Tfrc* gene assay (VIC-labeled; 4,458,366) was used as an endogenous control. Assays were performed according to the manufacturer's directions, using 20 ng of genomic DNA. Reactions were run on a StepOnePlus Real-Time PCR system and data were analyzed using the StepOne Software v2.3. Relative quantitation was calculated using the 2^{−CT} method. Heterozygote and homozygote controls were included in each panel of reactions.

2.1.4. Phenotyping of mice—Six groups of mice were followed: wildtype controls (*gba*^{+/+}), *gba* null heterozygotes (*gba*^{+/-}), *SNCA*^{A53T} hemizygotes with and without the null *gba* allele ((*gba*^{+/-}//*SNCA*^{A53T}) and (*gba*^{+/+}//*SNCA*^{A53T})) and *SNCA*^{A53T} homozygotes with and without the null *gba* allele ((*gba*^{+/-}//*SNCA*^{A53T/A53T}) (*gba*^{+/+}//*SNCA*^{A53T/A53T})). An equal number of male and female mice were included in this study. Weight was recorded weekly from age 6 months. Once a 13% drop in weight was observed, measurements continued at least biweekly. Mice were monitored for gait abnormalities, hunching or arching of the back, abnormal grooming, and urinary retention, and symptoms were recorded or filmed. Mice were euthanized when total limb paralysis and/or bladder dysfunction developed, together with the analogous aged-matched controls.

2.2. Tissue collection and processing

2.2.1. Murine tissues—Mice were deeply anesthetized and perfused with PBS, followed by 70% ethanol/150 mM NaCl. Brains and spinal cords were dissected out and further fixed in 70% ethanol/150 mM NaCl followed by paraffin infiltration [19] for imaging. For RNA and protein studies, brain and other tissues were collected and immediately snap-frozen in liquid N₂.

2.2.2. Human brain samples—Total protein from five cortical brain samples collected from autopsies performed at the NIH Clinical Center or from the Massachusetts General Hospital Brain Bank was extracted in RIPA buffer. Samples included a normal control, two patients with type 1 Gaucher disease (genotype N370S/N370S), one with and one without Parkinson disease, a c.84insG heterozygote with parkinsonism, and a patient with Parkinson disease without *GBA1* mutations. Each subject was male and above age 55 years. Samples were processed as described for mouse brain samples.

2.3. Protein extraction and evaluation

2.3.1. Protein extraction—Total protein was extracted from forebrain, midbrain and total mouse brain samples using two different extraction buffers: 1:10 (w/v) citrate-phosphate buffer (pH 5.4, 0.25% Triton X-100 and protease inhibitor cocktail) and RIPA buffer (Thermo Scientific, Waltham, MA). Samples were homogenized on ice, sonicated for 10 s, and centrifuged at 5000 rpm for 10 min.

2.3.2. Glucocerebrosidase levels—The lysates in citrate-phosphate buffer were used for GCase activity and immunoblotting using a fluorescent activity-based probe specific for GCase (MDW933) [22]. 15 µg of midbrain and forebrain lysate and 1.2 µM imiglucerase (Genzyme, Cambridge, MA), used as a control, were incubated with 100 nM of the GCase-specific MDW933 fluorescent probe at 37° C for 90 min and run on a 4–20% Criterion: TGX™ gel (Bio-Rad laboratories). The results were analyzed using an excitation wavelength of 488 nm and emission of 520 nm to measure the fluorescent signal in the gel [23].

2.3.3. Separation into soluble and insoluble protein—Brain lysates in RIPA buffer were used for western immunoblotting for monomeric *SNCA*, and other lysosomal proteins. Extracted protein was also fractionated into soluble and insoluble forms. The entire brain from symptomatic *SNCA*^{A53T} mice with and without *gba*^{+/-}, aged-matched non-symptomatic mice and control mice (wt/wt, and wt/*gba*^{+/-}) were homogenized in 5 volumes of Triton X-100 buffer (1% Triton X-100, 125 mM NaCl, 20 mM NaCl, 5 mM EDTA, 1.5 mM MgCl₂, 10% glycerol) supplemented with protease and phosphatase inhibitors (Thermo Scientific) using a glass Teflon Dounce homogenizer. The brain lysates were centrifuged at 100,000 g for 90 min at 4°. The collected supernatant was considered the soluble fraction. The pellet was extracted in 2.5 volumes of 2% SDS, 50 mM Tris-HCl, 125 mM NaCl, 1 mM EDTA and protease and phosphatase inhibitors, followed by sonication with a probe sonicator at 40% amplitude, and then boiled for 15 mins. The re-suspended pellet was centrifuged at 25000 g for 25 min at 25 °C. The supernatant was considered the insoluble fraction. The protein concentration was determined by BCA assay (ThermoScientific).

2.3.4. Western blotting and antibodies—The SDS-soluble and insoluble fractions and whole lysate from brain samples from symptomatic and control mice were run on Western immunoblots to evaluate levels of monomeric, soluble and HMW SNCA. GCase levels were also compared to samples from age-matched, pre-symptomatic and control mice. Twenty µg of protein was incubated at 70 °C with DTT for 10 min. Protein was loaded onto BioRad Criterion TGX precast gels (Hercules, CA) and run for 45 min at 200 V. Protein was transferred using the BioRad Turbo Transfer system with the default mixed molecular weight setting onto a PVDF membrane. The membrane was dried between filter paper for at least 30 min, rehydrated in methanol and blocked before probing. SNCA (1:1000, SC-7011, S129 and SNCA-211) (Santa Cruz, CA) primary antibodies were incubated for 1 h in 5% milk following 1 h of blocking. Secondary antibodies were prepared in 1:3000 dilutions in 5% milk. HRP conjugate beta-actin (ab20272) (abcam, Cambridge, England) was incubated for 1 h in 5% BSA following 1 h of blocking. Westerns were developed using ECL reagent and imaged. Antibodies used included antibody to Cathepsin-D, (Abcam, ab 52,832, 1/1000); Cathepsin-B (Abcam, ab 137662, 1/1000); Cathepsin-L (Abcam, ab 6314, 1/1000); LAMP1 (Abcam, ab 25630, 1/1000); GCase (Sigma, G4171, 1/1000) (St. Louis, MO) and LIMP-2 (Santa Cruz, SC 133127, 1/1000).

2.4. Enzymatic activity

2.4.1. Glucocerebrosidase activity—GCase activity was measured using a standard fluorometric assay [24]. Protein extracts were incubated for 15 min at 37 °C with 100 µM CBE (Sigma Aldrich) to subtract non-specific GCase activity. Assay buffer, composed of 10 mM 4-methylumbelliferyl-β-D-glucopyranoside (Sigma Aldrich) in pH 5.4 citrate-phosphate buffer, was added to CBE-treated and untreated samples and incubated for 1.5 h at 37 °C. The reaction was terminated with stop solution (1 M NaOH and 1 M glycine), and measured fluorescence was normalized to protein concentrations measured with a Pierce BCA protein assay kit (Thermo Scientific).

2.4.2. Cathepsin-D activity assay—Cathepsin D (CAT D, (EC 3.4.23.5) activity was measured using a fluorogenic CAT D substrate (MCA-GKPILFFRLK(Dnp)-D-R-NH₂) (Calbiochem, #219360) (Long beach, CA). Briefly, 7 µg brain samples from wt/wt, *gba*^{+/-} and *SNCA*^{A53T} hemizygotes with and without the null *gba* allele were homogenized in buffer (50 mM sodium acetate with 20 mM sodium chloride at pH = 5) and freeze-thawed four times to generate lysates. The lysates were incubated with the substrate at a final concentration of 20 µM in buffer. Reactions were performed in polypropylene, 384-well flat-bottom microplates (781,209, Greiner Bio-one) (Monroe, North Carolina) containing 50 µL buffer incubated at 25 °C using a microplate reader (Tecan Infinite M200 Pro). For CAT-D absorbance (excitation and emission wavelengths at 328 and 393 nm, respectively) was recorded as a function of time.

2.5. Quantification of glucosylsphingosine and glucosylceramide

Glycosphingolipids (glucosyl- and galactosyl-sphingosines, glucosyl- and galactosyl-ceramides) were extracted from mouse forebrain and midbrain regions. Tissues, homogenized with PBS buffer. *N, N*-di-methyl-galactosylsphingosine and galactosylceramide (d18:1 8:0), were used as internal standards prior to the

glycosphingolipid extraction. Quantification of glucosylsphingosines and glucosylceramides was performed using the API 4000 LC-MS-MS system (Applied Biosystems) with a Varian Metasil AQ C-18 column. Isomer separation of glucosylsphingolipids was performed using a Supelco HILIC column. A positive ion electrospray method using MRM was utilized for both analyses [25]. Data were normalized to the total protein content in samples, and was analyzed by a Student's two-tailed *t*-test (represented as mean $\pm$ SEM).

2.6. Pathological assessments

2.6.1. Evaluation of SNCA inclusion pathology—To evaluate the extent and distribution of SNCA inclusion pathology, brains from both symptomatic and control mice were processed and assessed by immunocytochemistry and immunofluorescence. Tissue sections were stained with: anti-pSer129 SNCA antibody 81A [26], anti-pSer129 SNCA antibody EP1536Y (Abcam), anti-SNCA antibody Syn 506 [27,28] anti-p62/sequestrome-1 (SQSTM1; Proteintech) (Chicago, IL) and anti-tyrosine hydroxylase antibody (TH; Millipore) (Fisher Scientific). Quantification of SNCA pathology burden area was performed as described [29]. Briefly, tissue sections stained with EP1536Y were loaded into ImageScope TM software and the burden was calculated by dividing the number of positive pixels by the total area. The images were randomized, coded, and analyzed blinded to the experimental conditions. Two-tailed *t*-tests and one-way analysis of variance (ANOVA) with post-hoc Dunnett's multiple comparison tests were performed in GraphPad Prism v5.03 software.

Quantification of HMW SNCA in the insoluble protein fraction (from 37 to 66 kDa) was performed using image J and were normalized to levels of monomeric SNCA.

2.6.2. Evaluation of proteinase K resistant inclusions—Proteinase K (PK) digestion was performed on paraffin embedded brain sections from symptomatic mice using a modified protocol [30]. Sections were rehydrated and subsequently digested with 50 μ g/ml PK (Roche Molecular Biochemicals, Mannheim, Germany) in PK buffer (10 mM Tris-HCl, pH 7.8, 100 mM NaCl, 0.1% Nonidet-P40) for 1 h at 55 °C. Following this, immunohistochemical staining using the anti-pSer 129 antibody EP1536Y (Abcam) was conducted as previously described [29], with the PK digestion in lieu of steam based antigen exposure.

2.7. RNA evaluations

2.7.1. RNA extraction—RNA was extracted from frozen midbrain samples from five symptomatic *gba*^{+/-}//*SNCA*^{A53T/A53T} and five *gba*^{+/+}//*SNCA*^{A53T/A53T} mice and age-matched controls using TriZol (Fischer Scientific).

2.7.2. Quantitative gene expression analysis—RNA levels were measured using the following probes, FAM-labeled probes, Hs01103383 m1(*SNCA*), Mm00484705_g1 (*gba*), Mm00484700_m1(*gba*, Mm00515586_m1(*ctsd*), and Mm00446977_m1(*scarb2*) (Applied Biosystem) were used to quantify the exogenous human *SNCA* and four other related genes in total mouse mRNA. The mouse *Tfrc* gene assay (VIC-labeled; Mm0044194_m1) was used as an endogenous control for these experiments. Total RNA (2 μ g) was reverse

transcribed using the Applied Biosystem High Capacity cDNA Reverse Transcription Kit (Invitrogen) following the manufacturer's instructions. For the duplex, real-time assay, 1X master mixes were prepared for each gene probe with water, 20X *Tfrc*- endogenous control probe, 20XFAM labeled gene probe, and 2X TaqMan Universal PCR Master Mix (Invitrogen). In a 96-well plate, 2 μ L of cDNA and 18 μ L of master mix were plated in triplicate. The assay was run on the StepOnePlus Real-Time PCR system and the data analyzed with StepOne Software v2.3 via the 2^{-CT} method.

2.8. Statistical analysis

Statistical analyses were performed using SAS statistics software version 9.14, and the Type I Error rate for statistical significance level was set at $P < 0.05$. The length of lifespan in *gba*^{+/+}//*SNCA*^{A53T} and *gba*^{+/-}//*SNCA*^{A53T} mice was evaluated using *Kaplan-Meier* survival analysis. The severity of symptoms was analyzed by comparing the interval between initial weight loss and when symptomatic animals required euthanasia. A Student's *t-test* was used to evaluate mean differences in the time intervals between the groups, reporting the observed *p*-value. Statistical analysis of the protein data was performed using GraphPad prism 5 software, * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$ and **** $p < 0.00001$. Data were evaluated as mean $\pm$ SEM. *t-test* or ANOVA were used to compare means of different groups of samples.

3. Results

3.1. The symptom onset and progression is faster in *SNCA*^{A53T} mice with *gba* haploinsufficiency

gba^{+/-} and *SNCA*^{A53T} lines were backcrossed to B6 mice to reduce the effect of genetic background. The two models were then crossed, aged, and over 200 mice with genotypes including *gba*^{+/-}//*SNCA*^{A53T} and *gba*^{+/-}//*SNCA*^{A53T/A53T} and their controls (wt/wt, *gba*^{+/-}, *gba*^{+/+}//*SNCA*^{A53T} and *gba*^{+/+}//*SNCA*^{A53T/A53T}) were followed for two years, monitoring their weight and the onset and progression of motor symptoms. In symptomatic mice, weight loss of over 13% was the first symptom, followed by arching of the back, impaired axial rotation, limb paralysis and bladder dysfunction. Survival analysis of 84 symptomatic mice, showed that *gba*^{+/-}//*SNCA*^{A53T} hemizygous and homozygous mice exhibited symptoms on average 9.76 weeks and 4.04 weeks earlier than *gba*^{+/+}//*SNCA*^{A53T} hemizygous and homozygous controls, respectively ($p < 0.023$, 0.0030), (Fig. 1A, B, Table 1). The rate of progression between initial onset of symptoms until euthanasia was 2.4 times more rapid in *gba*^{+/-}//*SNCA*^{A53T/A53T} mice ($p < 0.0001$) and 4.1 times more rapid in *gba*^{+/-}//*SNCA*^{A53T} hemizygotes ($p < 0.0001$) compared to *gba*^{+/+}//*SNCA*^{A53T} mice (Fig. 1, Table 2).

3.2. Differences in protein and RNA expression in mice with and without *gba* haploinsufficiency

To investigate the effect of over-expression of *SNCA*^{A53T} on GCase and other related proteins, we evaluated GCase activity, RNA expression and protein levels. Symptomatic mice were compared to littermates with the same genotype without overt symptoms (referred to as pre-symptomatic). Western blots of forebrain and midbrain lysates (N = 5) demonstrated an approximate 50% reduction in GCase levels in mice with a *gba*^{+/-}

genotype compared to *gba*^{+/+} (Fig. 2A–D). However, there were no significant differences in GCase levels in symptomatic *gba*^{+/-}//*SNCA*^{A53T} and *gba*^{+/+}//*SNCA*^{A53T} mice compared to *gba*^{+/-} and wt/wt controls, respectively (Fig. 2A–D). RNA quantification showed no influence of exogenous *SNCA*^{A53T} on *gba* expression (Supplementary Fig. 1). GCase activity, measured in forebrain and midbrain samples, matched the corresponding GCase levels (Fig. 2E). Quantitative RNA assays for prosaposin, (*psap*), the GCase activator saposin-C, and *scarb-2*, encoding LIMP-2, which transports GCase from the ER to lysosomes, were unaffected by *SNCA*^{A53T} over-expression (Supplementary Fig. 1). LIMP-2 levels (Fig. 2J, K) were similar in all groups. *SNCA* expression in both *gba*^{+/+}//*SNCA*^{A53T} and *gba*^{+/-}//*SNCA*^{A53T} mice was 40-fold higher than in controls (Supplementary Fig. 1). We next evaluated LAMP1 (a member of the LAMP complex reflecting lysosomal density) and specific cathepsins (CATs) involved in *SNCA* degradation. Protein levels of LAMP1 (Fig. 2F, I) and CAT-B (Fig. 2K) and L (Fig. 2L) in all mice were the same as wildtype. No differences were observed in either RNA expression of CAT-D (Supplementary Fig. 1) or cleaved CAT-D species among the different genotypes (N = 3) (Fig. 2F–H). CAT-D activity, was similar in midbrain and whole brain in symptomatic *gba*^{+/+}//*SNCA*^{A53T} and *gba*^{+/-}//*SNCA*^{A53T} mice (Supplementary Fig. 2).

3.3. Evaluation of human *SNCA*^{A53T} over-expression in murine brain samples

To determine whether the over-expression of human *SNCA*^{A53T} contributed to the earlier symptom onset and severity in *gba*^{+/-}//*SNCA*^{A53T} mice, we analyzed levels of monomeric, soluble and insoluble *SNCA* in brain from mice with the different genotypes.

3.3.1. Evaluation of monomeric *SNCA*—*SNCA*^{A53T} mice with and without *gba* haploinsufficiency had similar levels of the monomeric *SNCA* in protein lysates from forebrain, midbrain and whole brain (Fig. 3A–F). Levels were also comparable in symptomatic and age-matched pre-symptomatic mice (Fig. 3E, F). Quantification of endogenous *SNCA* in controls (wt, *gba*^{+/-}), and exogenous *SNCA*^{A53T} in mice with and without *gba*^{+/-}, differed significantly ($p < 0.0001$), but there was no effect of the null *gba* allele on levels of monomeric *SNCA* (Fig. 3F).

3.3.2. Analysis of soluble and insoluble *SNCA*—Levels of monomeric *SNCA* in the soluble (Fig. 3G, H) and in the insoluble brain fractions from *SNCA*^{A53T} hemizygotes (Fig. 3I, J) were significantly higher in symptomatic mice than in pre-symptomatic mice ($p < 0.05$ – 0.001). To exclusively evaluate human *SNCA*, we used the human *SNCA*211 antibody, which also showed higher levels of *SNCA* once mice became symptomatic, regardless of genotype (Fig. 3I). However, we consistently observed less aggregated *SNCA* in symptomatic *gba*^{+/-}//*SNCA*^{A53T} mice compared to *gba*^{+/+}//*SNCA*^{A53T} mice, ($p < 0.05$) (N = 3), (Fig. 3I, K).

3.4. Evaluation of fractionated *SNCA* in human brain samples

To determine whether levels of high molecular weight (HMW) *SNCA* in this mouse accurately reflected *SNCA* in human brain, we evaluated *SNCA* levels in cortex from a control, a patient with idiopathic Parkinson disease, patients with Gaucher disease with and without Parkinson disease, and a c.84insG Gaucher carrier with parkinsonism (analogous to

murine genotype *gba*^{+/-}). Total monomeric SNCA was clearly present in all cases, but was lowest in the idiopathic Parkinson disease sample (Fig. 3L). However, blotting the soluble and insoluble fractions (Fig. 3M, N) suggested that this patient had more of the HMW and oligomeric forms of SNCA than those with *GBA1* mutations.

3.5. Assessment of SNCA pathology

To assess the presence and distribution of SNCA inclusion pathology, symptomatic homozygous and hemizygous *SNCA*^{A53T} mice with and without the null *gba* allele were analyzed by immunocytochemistry and immunofluorescence analyses with antibodies EP1536Y and 81A to SNCA phosphorylated at Ser129 (a marker of SNCA inclusion pathology) [31], anti-SNCA antibody Syn506 (preferentially detects SNCA in pathological inclusions) [28] or anti-p62/sequestosome (an autophagic adaptor and ubiquitin-like protein) (Fig. 4A). Quantification of SNCA inclusion density in the pons of symptomatic mice (N = 17; 4–5 per genotype) (Fig. 4B), assessed by a blinded observer, showed no significant effect of the *gba*^{+/-} allele on the density of SNCA pathology. Like mice with wildtype *gba* [19], *SNCA*^{A53T} mice with a *gba*^{+/-} allele had SNCA inclusions in the midbrain, but not in TH-positive neurons (Fig. 4C). p62, SNCA and tyrosine hydroxylase (TH) levels in midbrain from symptomatic *SNCA*^{A53T} mice, evaluated by western blotting, showed no effect of the *gba*^{+/-} allele (N = 3) (Fig. 4D–G). All symptomatic mice analyzed had widespread SNCA inclusion pathology in the brainstem, midbrain, hypothalamus and spinal cord regions, with more sparse pathology in the cerebellum and motor cortex (Fig. 4H, Supplementary Fig. 3A, B). Pre-symptomatic mice were also evaluated, but no SNCA inclusions were observed (Supplementary Fig. 3D). Paraffin embedded brain sections of brainstem and midbrain from symptomatic mice with and without the *gba*^{+/-} allele (N = 3) were digested with proteinase K and stained for pSer129 SNCA. Proteinase K resistant inclusions were seen in a similar density and distribution in both groups (Supplementary Fig. 3C).

3.6. Glucosylsphingosine and glucosylceramide levels in murine brain samples

Mass spectrometry was performed to evaluate whether the null *gba* allele in *SNCA*^{A53T} mice impacted brain glucosylsphingosine or glucosylceramide levels. No significant differences in lipids levels were noted, as expected in heterozygote animals (N = 5) (Fig. 5).

4. Discussion

Identifying the mechanistic basis for the association between *GBA1* and the synucleinopathies continues to be a challenge, and additional pathways, genes and/or epigenetics may play a role [9,32]. An appropriate animal model would greatly facilitate our understanding of this complicated relationship. Previous attempts at modeling *GBA1*-associated parkinsonism in the mouse used the inhibitor CBE, which inactivates GCase [16,18], but fails to represent the natural development of Parkinson disease, an age-related disorder. Studying L483P (L444P) mice over-expressing *SNCA*^{A53T}, it was shown that the mutated GCase interfered with degradation and caused accumulation of SNCA in cultured primary neurons, although the mice had few symptoms [15]. By deleting one *gba* allele in a transgenic mouse overexpressing *SNCA*^{A53T}, we sought to create a model that, upon aging, developed SNCA aggregates in neurons. This *in vivo* model with *gba* haploinsufficiency

mimics the situation encountered in carriers of functionally null mutations, like c.84insG, IVS2+1G > A or recombinant alleles, who develop parkinsonism. The aging mice developed symptoms gradually, as occurs in patients with parkinsonism. Survival analysis of 84 mice with different genotypes (Fig. 1A, B and Tables 1 and 2), demonstrated that absence of one *gba* allele in *SNCA*^{A53T} homozygotes and hemizygotes resulted in earlier and more severe symptoms. Lipidomic analyses of mouse brain samples showed no increase in glucosylsphingosine or glucosylceramide, indicating that the more severe clinical course was not due to substrate storage. Symptomatic *gba*^{+/-}/*SNCA*^{A53T} and *gba*^{+/-} mice showed similar GCase levels and activity in midbrain and forebrain, (Fig. 2A–E). These findings differ from other mouse models where overexpression of SNCA resulted in reduced GCase levels and activity [17]. Studies in neuronal models and iPSC-derived neurons from a patient with *SNCA* triplication also indicated that excess SNCA inhibited GCase activity, while subsequent substrate accumulation enhanced SNCA aggregation [33,34]. In our mice, over-expressed SNCA^{A53T} did not have a similar impact on GCase or its substrates.

Surprisingly, immunoblotting suggested that *gba*^{+/-}/*SNCA*^{A53T} mice have less insoluble HMW and oligomeric forms of SNCA [35] than mice without a null *gba* allele (Fig. 3I, K). Furthermore, the distribution of SNCA pathology and abundance of SNCA aggregates throughout the neuroaxis were evaluated in multiple brain region, revealing that results in symptomatic *SNCA*^{A53T} homozygotes and hemizygotes with and without the null *gba* allele were not significantly different (Fig. 4H). There were also indications of less HMW SNCA in brain autopsy samples from patients with *GBA1*-associated parkinsonism (Fig. 3L–N) [36]. These results emphasize the need to evaluate both monomeric and aggregated forms of SNCA in studies of human and mouse brain, as the level of monomeric SNCA does not reflect the actual pathology. Furthermore, factors modifying SNCA such as phosphorylation, ubiquitination, alternative splicing [37,38] could contribute to the altered forms of SNCA observed. Levels of the different species of CAT-D, a protein thought to reduce SNCA aggregation [39], as well as CAT-D activity at two timepoints, did not differ in *gba*^{+/-}/*SNCA*^{A53T} mice (Fig. 2D, E). Thus, the impact of mutant GCase in Parkinson pathogenesis may not be directly related to its enzymatic activity or that of CAT-D. In the aging brain, other factors such as oxidative stress, variation in RNA expression, protein modifications and epigenetics may alter pathways involving GCase and SNCA levels or function.

Our finding that *gba* haploinsufficiency accelerates disease onset and exacerbates symptom progression in this mouse corresponds to the clinical observation of an earlier age of Parkinson disease onset and, at times, more progressive course in carriers of *GBA1* mutations. However, the basis for these findings remains enigmatic. Since *gba* haploinsufficiency did not result in significantly enhanced SNCA levels or abundance of SNCA pathology in terminal mice, another, yet unknown secondary function of GCase protein in distinct neuronal populations may initiate or contribute to the earlier and more severe symptoms in *gba*^{+/-}/*SNCA*^{A53T} mice. This is supported by a study of dopaminergic neurons from aged mice with a conditional *GBA1* knock-out, which had no motor dysfunction and no midbrain accumulation of endogenous SNCA [40]. It has been proposed that deficient GCase promotes cellular cascades contributing to earlier Parkinson pathogenesis and synaptic dysfunction [16]. It is also possible that GCase can influence the formation of prion-like SNCA amyloid seeds that initiate inclusion formation and/or the

subsequent prion-like spread of SNCA inclusion pathology [31,41] or its cell-to-cell transmission as previously suggested [42]. Further cellular and molecular evaluations using this model may facilitate the identification of modifier genes, or other factors impacting the association between *GBA1* and parkinsonism or reveal a moonlighting role for GCase in neuronal cells.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ymgme.2017.11.001>.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CAT	cathepsin
CBE	conduritol- β -epoxide
ER	endoplasmic reticulum
<i>gba</i>^{+/-}	heterozygous null mouse <i>gba</i> allele
GCase	glucocerebrosidase
GD	Gaucher disease
HMW	high molecular weight
LAMP2	lysosomal membrane protein 1
LIMP2	lysosomal integral membrane protein 2
PD	Parkinson disease
SNCA	α -synuclein
<i>SNCA</i>^{A53T}	mutant human A53T α -synuclein
TH	tyrosine hydroxylase

References

1. Forno LS. Neuropathology of Parkinson's disease. *J Neuropathol Exp Neurol*. 1996; 55:259–272. <http://dx.doi.org/10.1097/00005072-199603000-00001>. [PubMed: 8786384]
2. Hoehn MM, Yahr MD. Parkinsonism: onset, progression and mortality. *Neurology*. 1967; 17:427–442. [PubMed: 6067254]
3. Goldman JG, Postuma R. Premotor and nonmotor features of Parkinson's disease. *Curr Opin Neurol*. 2014; 27:434–441. <http://dx.doi.org/10.1097/WCO.0000000000000112>. [PubMed: 24978368]
4. Thomas B, Beal MF. Parkinson's disease. *Hum Mol Genet*. 2007; 16:R183–R194. <http://dx.doi.org/10.1093/hmg/ddm159>. [PubMed: 17911161]
5. Nalls MA, Pankratz N, Lill CM, Do CB, Hernandez DG, Saad M, et al. Large-scale meta-analysis of genome-wide association data identifies six new risk loci for Parkinson's disease. *Nat Genet*. 2014; 46:989–993. <http://dx.doi.org/10.1038/ng.3043>. [PubMed: 25064009]
6. Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, et al. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science*. 1997; 276:2045–2047. [PubMed: 9197268]
7. Conway KA, Harper JD, Lansbury PT. Accelerated in vitro fibril formation by a mutant α -synuclein linked to early-onset Parkinson disease. *Nat Med*. 1998; 4:1318–1320. <http://dx.doi.org/10.1038/3311>. [PubMed: 9809558]
8. Giasson BI, Uryu K, Trojanowski JQ, Lee VM. Mutant and wild type human alpha-synucleins assemble into elongated filaments with distinct morphologies in vitro. *J Biol Chem*. 1999; 274:7619–7622. [PubMed: 10075647]
9. Singleton A, Hardy J. The evolution of genetics: Alzheimer's and Parkinson's diseases. *Neuron*. 2016; 90:1154–1163. <http://dx.doi.org/10.1016/j.neuron.2016.05.040>. [PubMed: 27311081]
10. Sidransky E, Nalls MA, Aasly JO, Aharon-Peretz J, Annesi G, Barbosa ER, et al. Multicenter analysis of glucocerebrosidase mutations in Parkinson's disease. *N Engl J Med*. 2009; 361:1651–1661. <http://dx.doi.org/10.1056/NEJMoa0901281>. [PubMed: 19846850]
11. Brockmann K, Srulijes K, Pfleiderer S, Hauser AK, Schulte C, Maetzler W, et al. *GBA*-associated Parkinson's disease: reduced survival and more rapid progression in a prospective longitudinal study. *Mov Disord*. 2015; 30:407–411. <http://dx.doi.org/10.1002/mds.26071>. [PubMed: 25448271]
12. Gan-Or Z, Giladi N, Rozovski U, Shifrin C, Rosner S, Gurevich T, et al. Genotype-phenotype correlations between *GBA* mutations and Parkinson disease risk and onset. *Neurology*. 2008; 70:2277–2283. <http://dx.doi.org/10.1212/01.wnl.0000304039.11891.29>. [PubMed: 18434642]
13. Thaler A, Gurevich T, Bar Shira A, Gana Weisz M, Ash E, Shiner T, et al. A “dose” effect of mutations in the *GBA* gene on Parkinson's disease phenotype. *Parkinsonism Relat Disord*. 2017; 36:47–51. <http://dx.doi.org/10.1016/j.parkreldis.2016.12.014>. [PubMed: 28012950]
14. Sardi SP, Clarke J, Kinnecom C, Tamsett TJ, Li L, Stanek LM, et al. CNS expression of glucocerebrosidase corrects-synuclein pathology and memory in a mouse model of Gaucher-related synucleinopathy. *Proc Natl Acad Sci U S A*. 2011; 108:12101–12106. <http://dx.doi.org/10.1073/pnas.1108197108>. [PubMed: 21730160]
15. Fishbein I, Kuo YM, Giasson BI, Nussbaum RL. Augmentation of phenotype in a transgenic Parkinson mouse heterozygous for a Gaucher mutation. *Brain*. 2014; 137:3235–3247. <http://dx.doi.org/10.1093/brain/awu291>. [PubMed: 25351739]
16. Ginns EI, Mak SKK, Ko N, Karlgren J, Akbarian S, Chou VP, et al. Neuroinflammation and α -synuclein accumulation in response to glucocerebrosidase deficiency are accompanied by synaptic dysfunction. *Mol Genet Metab*. 2014; 111:152–162. <http://dx.doi.org/10.1016/j.ymgme.2013.12.003>. [PubMed: 24388731]
17. Yang J, Hertz E, Zhang X, Leinartaitė L, Lundius EG, Li J, et al. Overexpression of α -synuclein simultaneously increases glutamate NMDA receptor phosphorylation and reduces glucocerebrosidase activity. *Neurosci Lett*. 2016; 611:51–58. <http://dx.doi.org/10.1016/j.neulet.2015.11.023>. [PubMed: 26610904]
18. Rocha EM, Smith GA, Park E, Cao H, Graham AR, Brown E, et al. Sustained systemic Glucocerebrosidase inhibition induces brain α -synuclein aggregation, microglia and complement

- C1q activation in mice. *Antioxid Redox Signal*. 2015; 23:550–564. <http://dx.doi.org/10.1089/ars.2015.6307>. [PubMed: 26094487]
19. Giasson BI, Duda JE, Quinn SM, Zhang B, Trojanowski JQ, Lee VMY. Neuronal alpha-synucleinopathy with severe movement disorder in mice expressing A53T human alpha-synuclein. *Neuron*. 2002; 34:521–533. [PubMed: 12062037]
 20. Tybulewicz VLJ, Tremblay ML, LaMarca ME, Willemsen R, Stubblefield BK, Winfield S, et al. Animal model of Gaucher's disease from targeted disruption of the mouse glucocerebrosidase gene. *Nature*. 1992; 357:407–410. <http://dx.doi.org/10.1038/357407a0>. [PubMed: 1594045]
 21. Zangala T. Isolation of genomic DNA from mouse tails. *J Vis Exp*. 2007:e246.
 22. Witte MD, Kallemeijn WW, Aten J, Li KY, Strijland A, Donker-Koopman WE, et al. Ultrasensitive in situ visualization of active glucocerebrosidase molecules. *Nat Chem Biol*. 2010; 6:907–913. <http://dx.doi.org/10.1038/nchembio.466>. [PubMed: 21079602]
 23. Westbroek W, Nguyen M, Siebert M, Lindstrom T, Burnett RA, Aflaki E, et al. A new glucocerebrosidase-deficient neuronal cell model provides a tool to probe pathophysiology and therapeutics for Gaucher disease. *Dis Model Mech*. 2016; 9:769–778. <http://dx.doi.org/10.1242/dmm.024588>. [PubMed: 27482815]
 24. Peters SP, Coyle P, Glew RH. Differentiation of beta-glucocerebrosidase from beta-glucosidase in human tissues using sodium taurocholate. *Arch Biochem Biophys*. 1976; 175:569–582. [PubMed: 958319]
 25. Praggastis M, Tortelli B, Zhang J, Fujiwara H, Sidhu R, Chacko A, et al. A murine Niemann-pick C1 I1061T knock-in model recapitulates the pathological features of the most prevalent human disease allele. *J Neurosci*. 2015; 35:8091–8106. <http://dx.doi.org/10.1523/JNEUROSCI.4173-14.2015>. [PubMed: 26019327]
 26. Waxman EA, Giasson BI. Specificity and regulation of casein kinase-mediated phosphorylation of alpha-synuclein. *J Neuropathol Exp Neurol*. 2008; 67:402–416. <http://dx.doi.org/10.1097/NEN.0b013e31816fc995>. [PubMed: 18451726]
 27. Duda JE, Giasson BI, Mabon ME, Lee VMY, Trojanowski JQ. Novel antibodies to synuclein show abundant striatal pathology in Lewy body diseases. *Ann Neurol*. 2002; 52:205–210. <http://dx.doi.org/10.1002/ana.10279>. [PubMed: 12210791]
 28. Waxman EA, Duda JE, Giasson BI. Characterization of antibodies that selectively detect alpha-synuclein in pathological inclusions. *Acta Neuropathol*. 2008; 116:37–46. <http://dx.doi.org/10.1007/s00401-008-0375-1>. [PubMed: 18414880]
 29. Rutherford NJ, Sacino AN, Brooks M, Ceballos-Diaz C, Ladd TB, Howard JK, et al. Studies of lipopolysaccharide effects on the induction of α -synuclein pathology by exogenous fibrils in transgenic mice. *Mol Neurodegener*. 2015; 10:32. <http://dx.doi.org/10.1186/s13024-015-0029-4>. [PubMed: 26223783]
 30. Neumann M, Kahle PJ, Giasson BI, Ozmen L, Borroni E, Spooren W, et al. Misfolded proteinase K-resistant hyperphosphorylated α -synuclein in aged transgenic mice with locomotor deterioration and in human α -synucleinopathies. *J Clin Invest*. 2002; 110:1429–1439. <http://dx.doi.org/10.1172/JCI15777>. [PubMed: 12438441]
 31. Uchihara T, Giasson BI. Propagation of alpha-synuclein pathology: hypotheses, discoveries, and yet unresolved questions from experimental and human brain studies. *Acta Neuropathol*. 2016; 131:49–73. <http://dx.doi.org/10.1007/s00401-015-1485-1>. [PubMed: 26446103]
 32. Aflaki E, Westbroek W, Sidransky E. The complicated relationship between Gaucher disease and parkinsonism: insights from a rare disease. *Neuron*. 2017; 93:737–746. <http://dx.doi.org/10.1016/j.neuron.2017.01.018>. [PubMed: 28231462]
 33. Mazzulli JR, Xu YH, Sun Y, Knight AL, McLean PJ, Caldwell GA, et al. Gaucher disease glucocerebrosidase and α -synuclein form a bidirectional pathogenic loop in synucleinopathies. *Cell*. 2011; 146:37–52. <http://dx.doi.org/10.1016/j.cell.2011.06.001>. [PubMed: 21700325]
 34. Mazzulli JR, Zunke F, Isacson O, Studer L, Krainc D. α -Synuclein-induced lysosomal dysfunction occurs through disruptions in protein trafficking in human midbrain synucleinopathy models. *Proc Natl Acad Sci*. 2016; 113:1931–1936. <http://dx.doi.org/10.1073/pnas.1520335113>. [PubMed: 26839413]

35. Tsika E, Moysidou M, Guo J, Cushman M, Gannon P, Sandaltzopoulos R, et al. Distinct region-specific alpha-synuclein oligomers in A53T transgenic mice: implications for neurodegeneration. *J Neurosci*. 2010; 30:3409–3418. <http://dx.doi.org/10.1523/JNEUROSCI.4977-09.2010>. [PubMed: 20203200]
36. Choi JH, Stubblefield B, Cookson MR, Goldin E, Velayati A, Tayebi N, et al. Aggregation of α -synuclein in brain samples from subjects with glucocerebrosidase mutations. *Mol Genet Metab*. 2011; 104:185–188. <http://dx.doi.org/10.1016/j.ymgme.2011.06.008>. [PubMed: 21742527]
37. Kalia LV, Kalia SK. α -Synuclein and Lewy pathology in Parkinson's disease. *Curr Opin Neurol*. 2015; 28:375–381. <http://dx.doi.org/10.1097/WCO.0000000000000215>. [PubMed: 26110807]
38. Visanji NP, Brothie JM, Kalia LV, Koprich JB, Tandon A, Watts JC, et al. α -Synuclein-based animal models of Parkinson's disease: challenges and opportunities in a new era. *Trends Neurosci*. 2016; 39:750–762. <http://dx.doi.org/10.1016/j.tins.2016.09.003>. [PubMed: 27776749]
39. Qiao L, Hamamichi S, Caldwell KA, Caldwell GA, Yacoubian TA, Wilson S, et al. Lysosomal enzyme cathepsin D protects against alpha-synuclein aggregation and toxicity. *Mol Brain*. 2008; 1:17. <http://dx.doi.org/10.1186/1756-6606-1-17>. [PubMed: 19021916]
40. Soria FN, Engeln M, Martinez-Vicente M, Glangetas C, López-González MJ, Dovero S, et al. Glucocerebrosidase deficiency in dopaminergic neurons induces microglial activation without neurodegeneration. *Hum Mol Genet*. 2017; 26:2603–2615. <http://dx.doi.org/10.1093/hmg/ddx120>. [PubMed: 28520872]
41. Guo JL, Lee VMY. Cell-to-cell transmission of pathogenic proteins in neurodegenerative diseases. *Nat Med*. 2014; 20:130–138. <http://dx.doi.org/10.1038/nm.3457>. [PubMed: 24504409]
42. Bae EJ, Yang NY, Song M, Lee CS, Lee JS, Jung BC, et al. Glucocerebrosidase depletion enhances cell-to-cell transmission of α -synuclein. *Nat Commun*. 2014; 5:4755. <http://dx.doi.org/10.1038/ncomms5755>. [PubMed: 25156829]

Fig. 1. Symptom severity and lethality in the different groups of mice

Kaplan-Meier plot depicting age at death for 84 mice with different genotypes. Mice were sacrificed when they were unable to move (A) *SNCA*^{A53T} homozygotes (B) *SNCA*^{A53T} hemizygotes. Red (solid) line indicates mice with wt *gba* and blue (dashed) line mice with a null *gba* allele (*gba*^{+/-}).

SE= standard error and CI= confidence interval (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Evaluation of GCase and related genes

(A, C) Western blot showing levels of GCase in forebrain (A) and midbrain (C) samples from *SNCA^{A53T}* heterozygotes with and without the null *gba* allele using the MDW933 probe. (B, D) Quantification of GCase levels in mouse brain samples. (B) Forebrain, (N = 5 for each genotype) and (D) midbrain (N = 3 for each genotype). (E) Glucocerebrosidase activity in forebrain and midbrain samples (N = 3 for each genotype). (F) Western blot analyses showing levels of pro-CAT-D with cleaved forms and LAMP1 in whole brain samples. (G, H, I) Quantification (N = 3) of heavy chain (G) and cleaved (H) CAT-D and LAMP1 (I). (J) Western blot showing levels of LIMP-2, CAT-B and CAT-L. (K, L, M) Quantification of Western blots (N = 3) for CAT-B (K), CAT-L (L) and LIMP-2 (M). Graph Pad Prism 7 was used to analyze the data. Statistical significance was determined by comparing the means of different groups using *t*-test or one-way ANOVA, **p* < 0.05, ***p* < 0.001.

Fig. 3. Analysis of human SNCA in mouse brain

Western blot of monomeric SNCA in lysates from forebrain (A, B) and midbrain (C, D) in symptomatic *SNCA*^{A53T} mice with and without *gba*^{+/-} and controls (wt/wt & *gba*^{+/-}) (N = 2, except, for *gba*^{+/-}//*SNCA*^{A53T} where N = 3). (E, F) Western blot and quantification of monomeric SNCA in whole brain homogenates. Age-matched pre-symptomatic (~) mice are also shown (N = 3). (G) Monomeric SNCA in the soluble protein fraction. Blot is also probed with antibody 211 which is specific for human SNCA. (H) Quantification of soluble monomeric SNCA (N = 3). (I) Insoluble SNCA, showing both the monomeric and HMW forms. (J) Quantification of insoluble monomeric SNCA (N = 3). (K) Semi-quantification of HMW SNCA (quantifying SNCA between 37 and 66 kDa) in symptomatic mice with and without the null *gba* allele, N = 3. (L, M, N) Immunoblottings of monomeric and HMW forms of SNCA in human cortex from: 1-normal control; 2-patient with type 1 Gaucher disease; 3-patient with type 1 Gaucher disease and Parkinson disease; 4- c.84insG carrier with Parkinson disease and 5-patient with Parkinson disease without *GBA1* mutations. β -actin was used as a loading control. (L) Monomeric SNCA in human whole brain lysates. (M, N) Monomeric and high molecular weight SNCA. (M) Soluble protein fraction. (N) Insoluble protein fraction.

Fig. 4. Assessment of SNCA pathology

(A) Representative images of immunofluorescence analysis depicting SNCA inclusion pathology in the pons of symptomatic *SNCA*^{A53T} hemizygote (top two panels) or *SNCA*^{A53T} homozygote (lower two panels) mice with *gba*^{+/+} or *gba*^{+/-} using anti-p62 antibody and Syn 506. Merged images counterstained with 4,6-diamidino-2-phenylindole (DAPI) are shown (right). (B) Quantitative analysis of SNCA burden in the pons using antibody Syn 506. (C) Representative images of immunofluorescence analysis depicting SNCA inclusion pathology in the midbrain of symptomatic *SNCA*^{A53T} hemizygous or homozygous mice with *gba*^{+/+} or *gba*^{+/-} using anti-TH and Syn 506. (D) Immunoblotting of three proteins, p62, SNCA and TH, evaluated by immunofluorescence and their quantification (E, F, G), N = 3. (H) Immunohistochemical analysis depicting pathology in the hypothalamus, spinal cord, cerebellum and motor cortex in symptomatic *SNCA*^{A53T} hemizygous or *SNCA*^{A53T} homozygous mice with *gba*^{+/+} or *gba*^{+/-} using antibodies to pSer129 α-syn (EP1536Y or 81A). Scale bar 50 μm.

Fig. 5. Analyses of glucosylceramide and glucosyl-sphingosine in mouse brain samples
 Glucosylceramide (GlcCer) isoforms, analyzed in forebrain (A) and midbrain (B) of control and *SNCA*^{A53T} mice with and without the *gba* null allele; N = 5.
 Glucosylsphingosine (GlcSph) levels in forebrain (C) and midbrain (D) of controls and *SNCA*^{A53T} mice with and without the *gba* null allele; N = 5. Graph Pad Prism 7 was used to analyze the data. Statistical significance was determined by comparing the means of different groups using *t*-test or one -way ANOVA.

Table 1

Age of mice when euthanasia was required.

Genotype	Sample size	Mean age (month)	SE	95% CI	P-value
<i>SNCA</i> ^{A53T} homozygotes					
<i>gba</i> ^{+/-} // <i>SNCA</i> ^{A53T/A53T}	13	11.65	0.22	11.19, 12.10	< 0.0030
<i>gba</i> ^{+/-} // <i>SNCA</i> ^{A53T/A53T}	14	10.64	0.21	10.20, 11.08	
<i>SNCA</i> ^{A53T} hemizygotes					
<i>gba</i> ^{+/-} // <i>SNCA</i> ^{A53T/wt}	45	18.64	0.53	17.59, 19.79	< 0.0230
<i>gba</i> ^{+/-} // <i>SNCA</i> ^{A53T/wt}	12	16.20	0.91	14.39, 18.02	

SE = standard error and CI = confidence interval.

Table 2

Interval of symptoms progression.

Genotype	Sample size	Time to euthanize (day)	SE	95% CI	P-value
<i>SNCA</i> ^{A53T} homozygotes					
<i>gba</i> ^{+/+} // <i>SNCA</i> ^{A53T/A53T}	13	1.962	0.1831	1.563, 2.361	<0.0001
<i>gba</i> ^{+/-} // <i>SNCA</i> ^{A53T/A53T}	14	0.7857	0.1358	0.4923, 1.079	
<i>SNCA</i> ^{A53T} hemizygotes					
<i>gba</i> ^{+/+} // <i>SNCA</i> ^{A53T/wt}	45	3.667	0.206	3.246, 4.088	<0.0001
<i>gba</i> ^{+/-} // <i>SNCA</i> ^{A53T/wt}	12	0.889	0.266	0.4923, 1.079	

SE = standard error and CI = confidence interval.