

The association between lysosomal protein glucocerebrosidase and Parkinson's disease

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Abstract. – BACKGROUND: In recent years, mutations in glucocerebrosidase gene (GBA), which encodes the lysosomal enzyme glucocerebrosidase (GCase) deficient in Gaucher disease (GD), were found to be the most widespread genetic for the development of Parkinson disease.

AIM: In this work, we investigated the possibility of a biological linkage between GCase and alpha-synuclein.

MATERIALS AND METHODS: siRNA was used to knockdown the GBA, then the related proteins such as alpha-synuclein were detected, additionally, the mutations of GBA were also detected. We also provide evidence that a mouse model of Gaucher disease (GBAD409H/D409H) to detect the gene types of GBA.

RESULTS: The results showed functional knockdown (KD) of GBA in neuroblastoma cells culture causes a significant accumulation of alpha-synuclein and alpha-synuclein-mediated neurotoxicity. Furthermore, KD of GBA in rat primary neurons expressing the A53T mutation of alpha-synuclein, decreases cell viability. In addition, we observed that overexpression of several GBA mutants (N370S, L444P, D409H, D409V) significantly raised human alpha-syn levels of vector control. Glucosylceramide (GlcCer), the GCase substrate, influenced formation of purified a-syn by stabilizing soluble oligomeric intermediates. We also provide evidence that a mouse model of Gaucher disease (GBAD409H/D409H) exhibited alpha-syn aggregates in substantia nigra, cortex and hippocampus regions. ELISA analysis showed a significant rise in membrane-associated α -syn and western blot analysis showed that two forms of alpha-syn oligomers were present in brain homogenates from the hippocampus D409H mice.

CONCLUSIONS: These studies support the contention that both WT and mutant GBA can cause Parkinson disease-like alpha-synuclein pathology.

Key Words:

Glucocerebrosidase, α -synuclein, Parkinson disease, siRNA, Pathology.

Introduction

Gaucher disease, the most common lysosomal storage disease, is an inherited recessive autosomal metabolic defect due to a deficiency of the lysosomal enzyme glucocerebrosidase (GCase), encoded by GBA1 gene^{1,2}. Glucocerebrosidase (GCase) is a lysosomal enzyme that hydrolyses the beta-glycosidic linkage of glucosylceramide (GlcCer), a ubiquitous sphingolipid present in the plasma membrane of mammalian cells, originating ceramide and glucose³. The human GBA gene is located on chromosome 1q21 and comprises 11 exons and 10 introns spanning over 7kb⁴.

Clinical, genetic and pathological studies demonstrate that mutations in GBA, are risk factors for Parkinson disease and related disorders⁵. Parkinson disease, the second most common neurodegenerative disease after Alzheimer disease (AD), characterized by the presence of Lewy bodies and the loss of dopaminergic neurons in the substantia nigra pars compacta⁶. Numerous susceptibility genes have been shown to predispose for PD. This work has lead to the discovery of mutations in SNCA, PARK2, PINK1, PARK7 and LRRK2 as causes of primary parkinsonism and/or PD⁷. While the identification of these loci has been important, mutation of these genes is responsible for a relatively small proportion of PD cases⁸. In recent years, mutations in GBA1 gene were found to be the most widespread genetic for the development of Parkinson disease.

The first associations of the glucocerebrosidase enzyme with parkinsonism were discovered through careful clinical observation of people affected by Gaucher disease (GD), who in several cases developed Parkinson's disease^{9,10}. Furthermore, carriers of GBA mutations, particularly family members of patients with GD have displayed an increased rate of parkinsonism. Subsequently, these findings were confirmed by studies

in larger cohorts of patients with PD. Patients with parkinsonism as well as other Lewy body disorders have at least a five-fold increase in the number of carriers of *GBA* mutations as compared to age matched controls¹¹. In addition, *GBA* mutations are more frequent in patients with Lewy body disorders (LBD)¹².

Many findings suggest that *GBA* protein and α -synuclein are implicated in a common cellular pathway and different hypothesis have been created to explain the linkage between them, including protein aggregation, prion transmission, lipid accumulation and impaired autophagy, mitophagy or trafficking. Each model has inherent limitations, and a second-hit mutation might be essential⁵.

The link between *GBA* mutations and the risk of developing PD is now being explored at the cellular level. In this article, we investigated the potential role of GCase and GC in neurodegeneration and the different theories proposed to explain this association.

Materials and Methods

Cell Culture

SH-SY5Y cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium containing 10% fetal bovine serum (FBS), 1% streptomycin and penicillin. The cells were differentiated in neurobasal media supplemented with B-27 and 40 mM retinoic acid for 7 days. All maintained in a 37°C, 5% CO₂, fully humidified incubator, passed twice or thrice weekly, and prepared for experimental procedures when in log-phase growth.

RT-PCR

RNA was extracted from human neuroblastoma cells (SHSY5Y), RAN Stat 60 (Testest, Friendswood, TX, USA) according to manufacturer's instructions. The cDNAs were prepared by reverse transcription (Superscript III; Invitrogen Co, Carlsbad, CA, USA). PCR was performed using the ABI PRISM 7000 Sequence Detection System (Santa Rosa, CA, USA) and primers. The CT of the *a-syn* gene was calibrated against that of the reference gene *HPRT* (hypoxanthine-phosphoribosyltransferase 1).

Preparation of Primary Mesencephalic Cultures

Primary midbrain cultures were prepared via dissection of day 17 embryos obtained from

pregnant Sprague-Dawley rats (Harlan, Indianapolis, IN, USA). The cells were plated on poly-L-lysine-treated 48-well plates at a density of 163,500 cells per well. Five days after plating, the cells were treated with cytosine arabinofuranoside (20 mM, 48 h) to inhibit the growth of glial cells. At this stage (i.e. 7 days *in vitro*), the glial cells accounted for 50% of the total cell population, and the neurons appeared differentiated with extended processes.

Lentiviral and Adenoviral Transductions

Primary cultures (7 days *in vitro*) were transduced with lentivirus and/or adenovirus in the presence of polybrene (6 mg/ml). The transductions were carried out for 72 h at a multiplicity of infection (MOI) of 3 in the case of A53T adenovirus and *GBA* shRNA (short hairpin RNA) lentivirus. The cells were then treated with fresh media for an additional 24 h and analyzed by immunocytochemistry. Control samples consisted of untransduced primary rat midbrain cultures.

Immunofluorescence Assay

Treated cells were fixed in freshly prepared 4% paraformaldehyde in phosphate buffered saline (PBS) for 30 min at room temperature and then permeabilized with 0.2% Triton X-100 for 10 min. After blocking with 4% bovine serum albumin (BSA), slides were incubated with IKK α antibody (dilution, 1:100) for overnight at 4°C, washed three times in PBST and reacted with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (dilution, 1:100) for 1 h and counterstained for nuclei with 10 μ g/mL 4,6-diamidino-2-phenylindole (DAPI) for 10 min. Cells were then washed, mounted, and examined under a laser scanning confocal microscope.

Animals

In our biochemical studies, we used mice with D409H mutant knocking ba1 alleles and their WT (wild type) littermates. The generation of mouse models with reduced enzymatic GCase activity and their characterization were previously reported. All animal procedures and care methods were approved by the according Ethics Committee.

Histochemistry

Midbrain sections were immunostained using antibodies against α -syn (Syn-1, Transduction Laboratories, Lexington, KY, USA) or glial acidic fibrillary protein (GFAP, Chemicon, Temecula,

CA, USA). Sections were then incubated with a FITC-conjugated species-specific secondary antibody and mounted onto slides as previously described.

Statistical Analysis

All the data were confirmed from three independent experiments. The values were expressed as means $\pm$ SD. $p < 0.05$ was considered statistically significant. Statistical analysis was performed using SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA). Difference between two groups was analyzed by two-tailed Student's t test, and that between three or more groups was analyzed by one-way ANOVA multiple comparisons. Difference with $p < 0.05$ (*) or $p < 0.01$ (**) was considered statistically significant.

Results

Depletion of GCase Increases α -synuclein Levels in SH-SY5Y Cells

To test the hypothesis that inhibition of GCase would elicit changes in cellular α -synuclein level, we evaluated the protein by using Western blot analysis in SH-SY5Y cells differentiated to the neuronal phenotype. We performed siRNA knockdown of GBA in differentiated SH-SY5Y cells, GCase shRNA-mediated KD (knockdown) by lentiviral infection resulted in a 60% reduction in GCase protein levels compared to control, and an accumulation of glucosylceramide (GC) (Figure 1A). We hypothesized that endogenous α -synuclein protein may accumulate in neurons infected with GCase shRNA. Notably, knockdown of GBA was

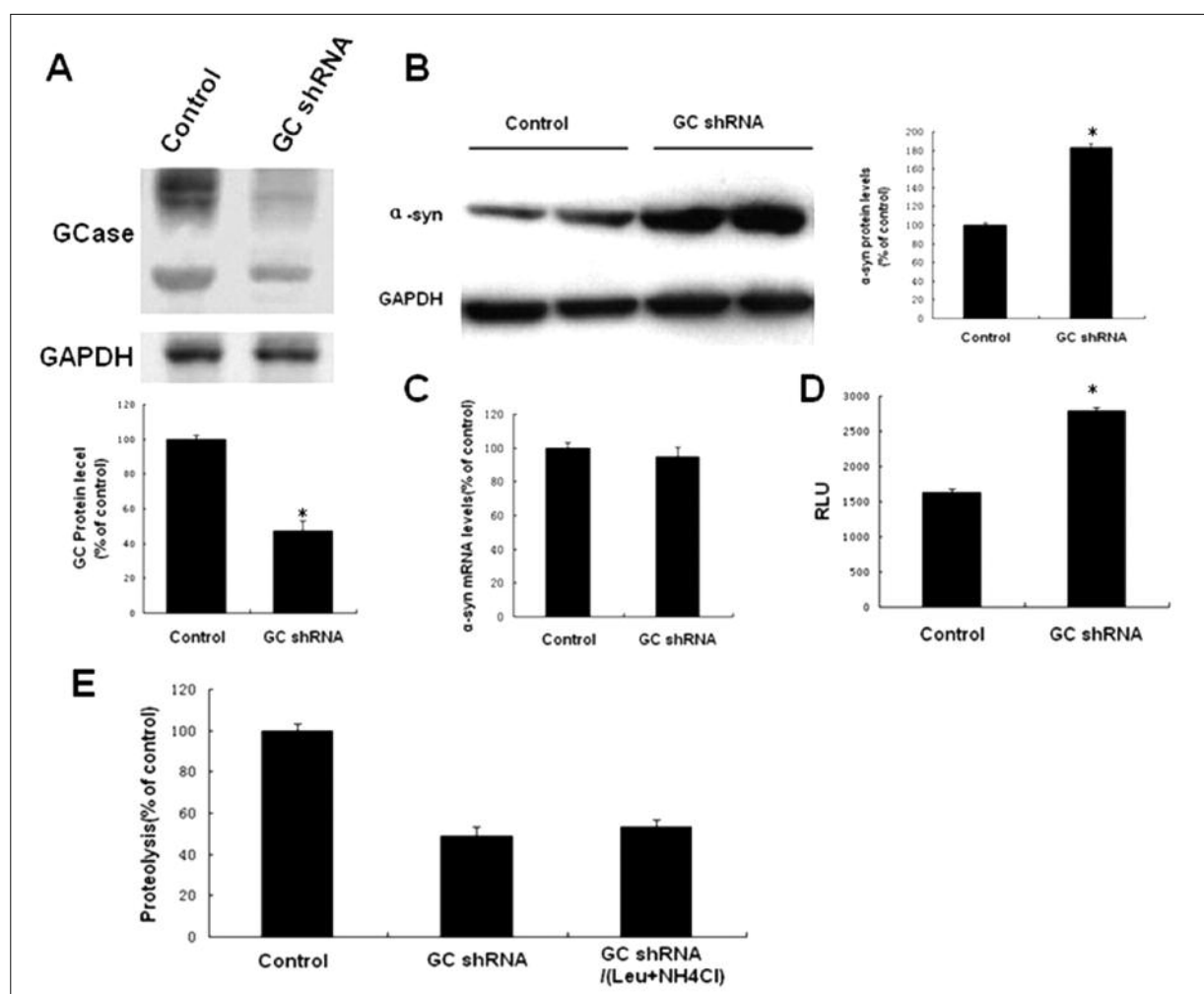


Figure 1. Depletion of GCase increases α -syn levels and compromises protein degradation. **A**, KD of GCase protein in cortical neurons by GCase shRNA is shown by western blot. **B**, α -syn expression analysis in GCase shRNA cells. Quantification is shown right ($n = 4$, $*p < 0.01$). **C**, Expression of α -syn mRNA was measured using RT-PCR in differentiated SH-SY5Y cells. **D**, α -syn-mediated neurotoxicity in GCase shRNA cells. **E**, Proteolysis of long-lived proteins in neurons assessed at 12 hr.

accompanied by a significant increase in α -synuclein protein levels (Figure 1B). Indeed, GCase KD increased the steady-state levels of α -synuclein by 1.8-fold relative to controls. In order to determine whether increased levels of the protein were due to enhanced transcription, RT-PCR was performed to measure transcript levels in SH-SY5Y. No change in α -synuclein gene expression was detected. These findings indicate that increased α -synuclein levels observed resulted from compromised protein degradation (Figure 1C). We next determined the effect of GCase KD on α -synuclein-mediated neurotoxicity. Reducing GBA expression consistently increased toxicity in SH-SY5Y cells, as indicated by increased adenylate kinase release (Figure 1D). In addition, we determined whether GCase KD affects a lysosomal degradation pathway, neurons were treated with the well-established lysosomal inhibitors ammonium chloride (NH_4Cl) and leupeptin. These compounds did not additively inhibit the proteolysis in GCase shRNA-treated cells, indicating that GCase KD affects a lysosomal-mediated pathway (Figure 1E). These cell culture results suggest a synergistic effect whereby an increase in α -synuclein combined with a decrease in GBA results in increased cytotoxicity.

GBA Reduction Increases Neurotoxicity in Rat Midbrain Neurons Overexpressed A53T- α -synuclein

We also investigated whether the interaction of α -synuclein and GBA was present for the A53T

mutation in α -synuclein. In this experiment, we examined how the reduction in endogenous GBA influences α -synuclein-mediated toxicity in cultured primary rat midbrain neurons expressing the A53T form of α -synuclein, an *in vitro* model of PD. Primary rat midbrain neuron cultures consist of 4-5% tyrosine hydroxylase positive (TH+) cells, which have been described as being exquisitely sensitive to α -synuclein levels in PD. 96 hours after transduction, we assessed relative dopaminergic cell viability by immunocytochemistry for TH, a marker of dopaminergic neurons. Transduction with the A53T- α -synuclein adenovirus or with either GAK shRNA virus alone did not affect dopaminergic cell viability compared with untransduced cultures. However, simultaneous exposure to the A53T- α -synuclein adenovirus and either of the GBA shRNA dramatically reduced the number of TH+ neurons relative to MAP2+ (microtubule-associated protein 2) neurons, suggesting that α -synuclein neurotoxicity is triggered or enhanced by the combination of overexpression of mutant SNCA (synuclein alpha) and reduction in GBA (glucosidase beta acid) (Figure 2A, B). These results indicating that GBA reduction plays an important role in cell toxicity when A53T mutant- α -synuclein is overexpressed.

Mutant GBA Proteins Contribute to α -synuclein Aggregation

GBA genotyping studies on various cohorts of Parkinson's disease patients showed an increased

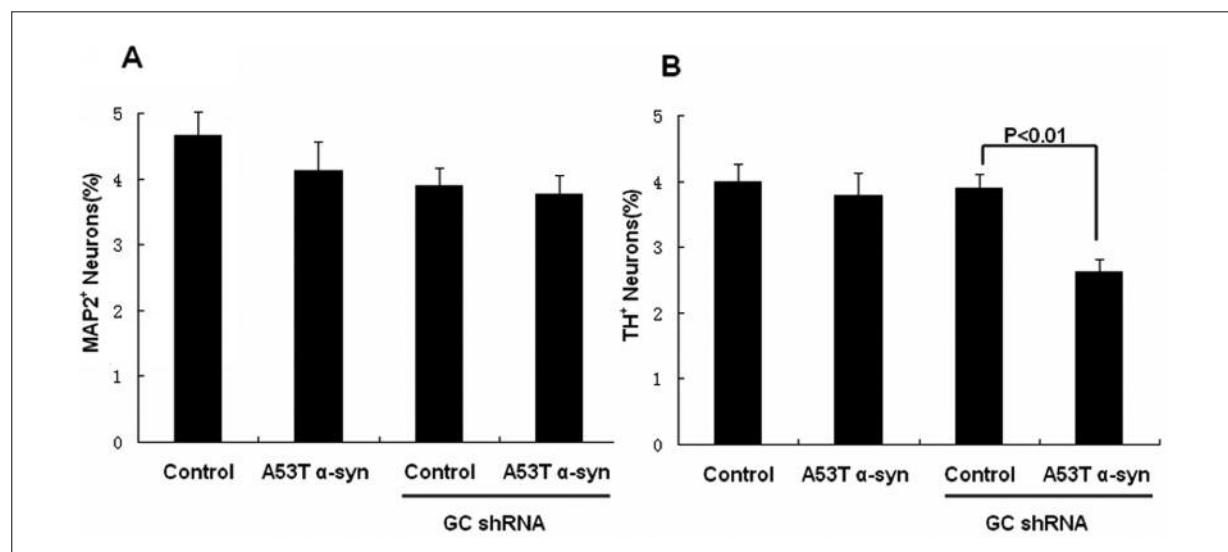


Figure 2. The effect of GCase KD in rat midbrain neurons overexpressed A53T- α -syn. Primary midbrain cultures were transduced with A53T adenovirus (MOI=3) with or without lentivirus encoding GBA shRNA (MOI=3). Control cells were incubated in the absence of virus.

frequency of GBA mutations. GBA mutations have been described that probably result in unstable or misfolded mutant protein¹³, this could contribute to the enhanced aggregation of α -synuclein by a direct or indirect interaction between GCcase and α -synuclein. We explored whether mutant GC might be involved in the process of α -synuclein inclusion formation. We next examined the effect of GBA mutants including N370S, L444P, D409V, and D409H, which are associated with Gaucher disease, PD and DLB. Among the 4 variants tested, all of them significantly raised α -syn levels, albeit to varying degrees. Among

them, the D409H variant generated the largest accumulation of α -synuclein (Figures 3A, B). Goker-Alpan, et al¹⁴ reported α -synuclein-positive Lewy bodies are found in the brains of patients with GBA mutations and PD, we decided to investigate the association in cellular level. Immunofluorescence analysis revealed that GCcase was colocalized with the lysosomal marker, LAMP-1 (lysosomal-associated membrane protein 1). Double-label immunofluorescence showing GCcase (green) and α -synuclein (red), GCcase and α -syn colocalized as shown in the overlay panels (Figure 3C) The result supported

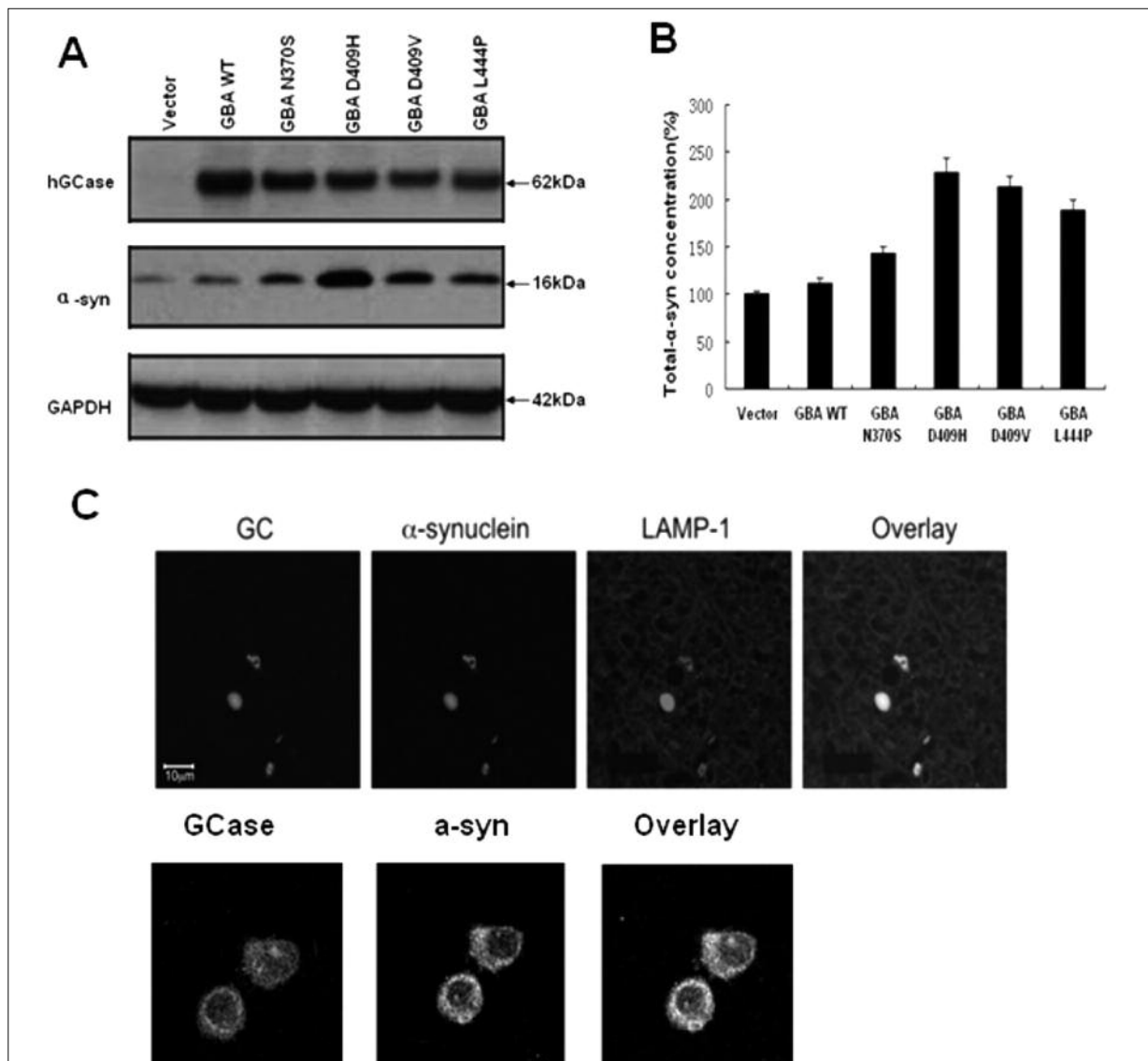


Figure 3. Effects of mutant GCase expression on α -syn levels. **A**, GCase and α -syn analysis by western blot in cells transiently transfected with GBA-encoding cDNA. **B**, Cell lysates were corrected for equal total protein content and subjected to quantification of soluble SNCA by sandwich enzyme-linked immunosorbent assay. **C**, Neurons were infected with GCase shRNA. Constructs and cellular distribution patterns of LAMP1, GCase α -syn were assessed by immunocytochemistry.

if GBA mutations have an enhanced effect on α -synuclein pathology. It is possible that mutant GCase may contribute to impaired α -synuclein clearance in the lysosome.

Mechanisms Reserch of GBA Mutations and Parkinsonism

Multiple systems and organelles can be affected by α -syn accumulation. The increased α -syn levels and toxicity that occur with GCase depletion may result from generalized lysosomal inhibition or may be due to alterations in GlcCer lipid metabolism. To distinguish between these two possibilities, we inhibited lysosomal protein degradation with leupeptin in WT α -syn-expressing H4 (histamine receptors) neurons and

assessed neurotoxicity. We found that leupeptin treatment did not enhance α -syn-mediated neurotoxicity (Figure 4A). We next assessed by western blotting the level of the microtubule-associated protein 1 light chain 3 (LC3), a highly specific autophagosomal marker. During autophagosome formation, the LC3-I cytosolic form is converted into lipid conjugated LC3-II form, whose amount correlates with the number of autophagosomes. Western blot analysis indicated a comparable increase in the levels of LC3-II and by leupeptin treatment or GCase KD (Figure 4B). Biochemical analysis revealed an increase of T-insoluble α -syn in leupeptin-treated cells but no change in the amount of T-soluble α -syn (Figure 4C). Thus, despite similar effects on the total α -syn levels

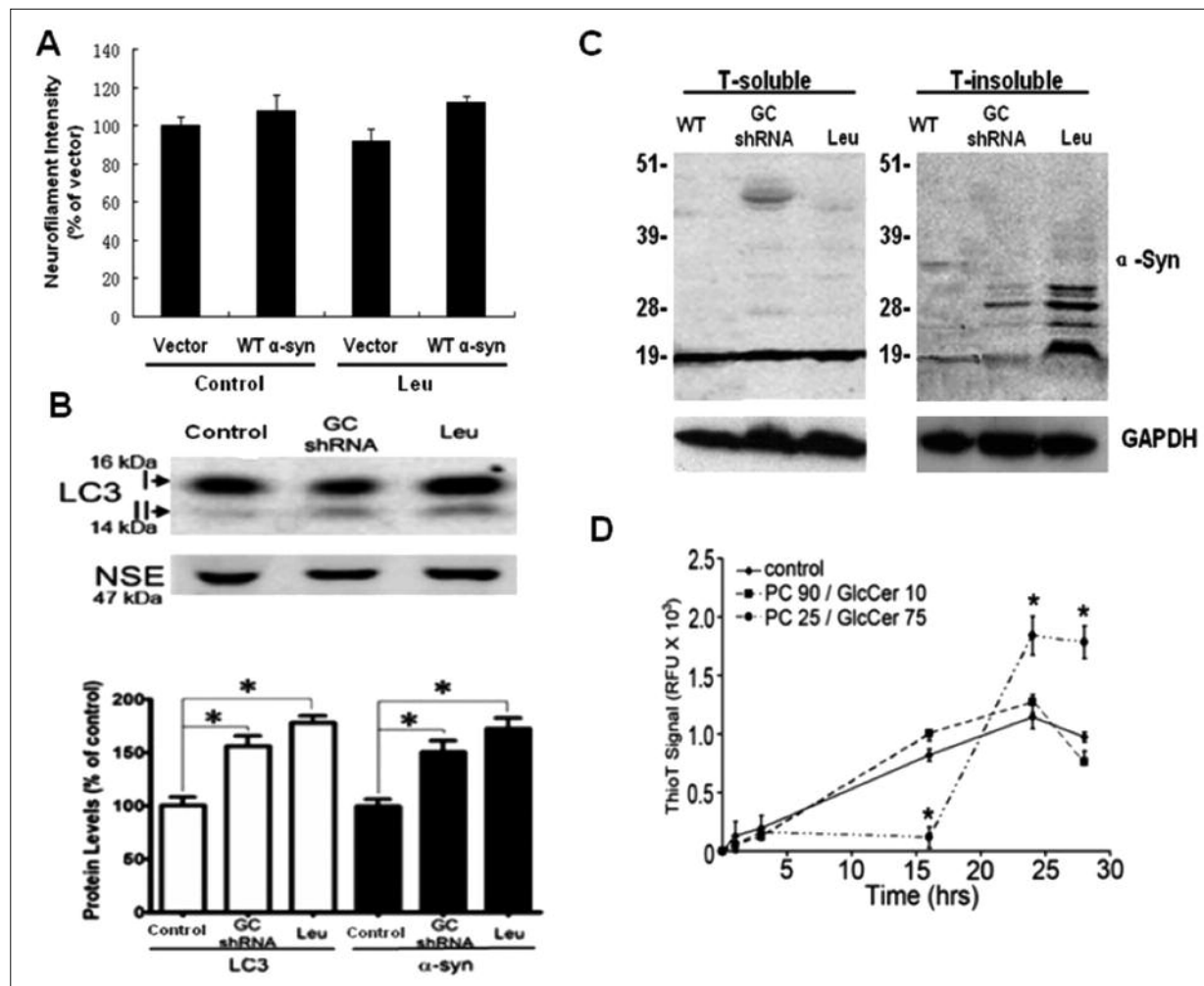


Figure 4. **A**, Neurotoxicity assessment by neurofilament immunostaining in either empty vector (vect) or WT α -syn infected cells with or without leupeptin treatment ($n = 4$). **B**, Western blot of LC3-II upon GCase knockdown or leupeptin treatment. NSE was used as a loading control. **C**, Western blot of T-soluble and T-insoluble α -syn in GCase knockdown or leupeptin treatment cells. **D**, Purified α -syn was incubated with mixtures of PC and GlcCer at pH 5.0, 37°C and amyloid formation was assessed by thioflavin T fluorescence (relative fluorescence units [RFU], $n = 4$, $*p < 0.01$).

by leupeptin or GCase KD, only GCase KD increased the steady-state levels of soluble α -syn.

We next examined whether GlcCer directly influences the *in vitro* aggregation of recombinant α -syn. Lipid dispersions made of mixtures of purified GlcCer and brain phosphatidylcholines (PCs) were incubated with α -syn at acidic conditions to simulate a lysosome like environment *in vitro*. Our experiments revealed that acidic reactions containing lipid dispersions made of 90% PC and 10% GlcCer (PC90/GlcCer10) did not significantly influence the fibril formation of α -syn compared to control reactions containing α -syn alone. However, increasing the amount of GlcCer to 75% while keeping the total lipid amount constant (PC25/GlcCer75) altered the kinetic profile of α -syn fibril formation by delaying the formation of insoluble thioT-positive α -syn fibrils (Figure 4D).

Endogenous Rise of α -synuclein in the Brain of Homozygous D409H GBA Mice

As GCase KD and GlcCer metabolic pathway appears to affect the levels and aggregation of α -syn in neuronal cultures and *in vitro*, we next ex-

amined whether GCase depletion and GlcCer accumulation affect 12-mo-old levels and solubility *in vivo*. For this, brain tissues from a previously described GD mouse model (GBA^{D409H/D409H}) were analyzed to determine whether endogenously expressed α -syn protein levels were elevated. Serial sagittal brain sections from 12-mo-old D409H mice were examined by immunofluorescence microscopy to provide an overview of brain α -syn distribution. D409H mice showed positive α -syn immunoreactivity in multiple brain regions including the substantia nigra (SN), cortex (Ctx), as well as hippocampus (Figure 5A). Immunofluorescence analysis revealed the presence of α -syn accumulations in the form of punctated structures, whereas WT mice showed a normal neuropil staining pattern expected for α -syn. Large α -syn particles ($\geq 5 \mu\text{m}$) were observed in cerebral cortex substantia nigra and of 12-mo-old D409H mice, in comparison to the hippocampal region.

We next focused on hippocampus, a region characteristically affected in humans with DLB or advanced PD and in Gaucher disease patients with Parkinsonism. Brain homogenates from the

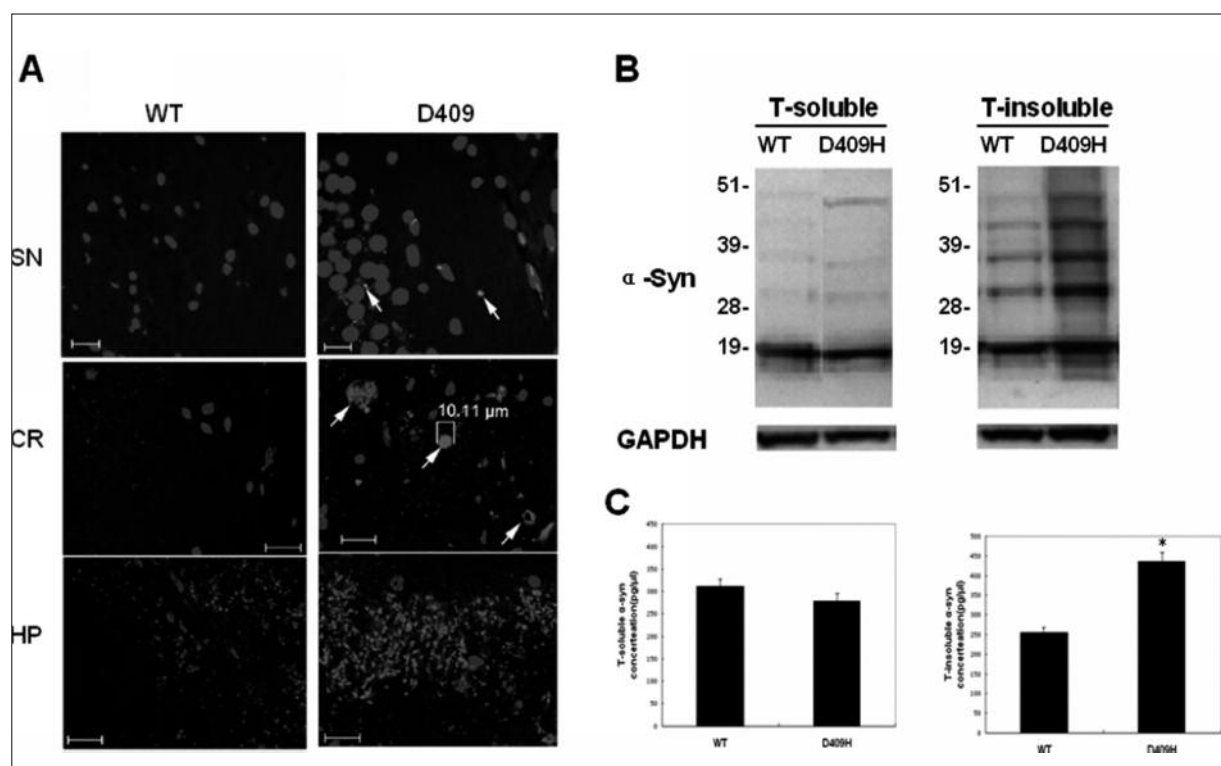


Figure 5. α -syn in mouse brain. **A**, Dual-label immunohistochemistry was performed in coronal brain sections detect α -syn. **B**, Western blot of T-soluble and T-insoluble α -syn in 12-mo-old D409H mice. GAPDH was used as a loading control. MW markers are indicated in kDa. **C**, T-soluble and T-insoluble α -syn were quantified by sandwich enzyme-linked immunosorbent assay.

hippocampus of WT and D409H gba mice, α -syn immunoreactivity was assessed by Western blot analysis. Immunoblots for α -syn showed the presence of monomers in the hippocampus of WT mice. However, two forms of α -syn oligomers were present in the hippocampus of D409H mice (Figure 5B). Using a sandwich ELISA pair that was optimized for the quantification of mouse α -syn, we found that homozygous D409H gba mice showed a trend for lower α -syn concentrations in the T-soluble fraction accompanied by a significant rise in membrane-associated α -syn from hippocampi when compared with their WT littermates (Figure 5C). Taken together, these data are consistent with our cell culture and *in vitro* data.

Discussion

Parkinson's disease is a progressive neurodegenerative disorder that was first described by James Parkinson in 1817. Genetic research in the past decade has changed the view of PD from an archetypical nongenetic disease to one having a clear genetic basis in a percentage of patients¹⁵. Past work has evidenced the link between Gaucher disease and the synucleinopathies Parkinson disease and dementia with Lewy bodies, that display abnormal fibrillation and an accumulation of proteinaceous, insoluble α -syn in neurons and glia, indicating a shared cellular pathology for the handling and clearance of α -syn¹⁶.

α -syn is a small, intrinsically unfolded cytoplasmic protein which is highly expressed in the central nervous system and concentrated in presynaptic terminals representing 0.5-1% of the total cytosolic protein in brain¹⁷. Previous studies of brain samples from subjects with GBA-associated synucleinopathies demonstrated that glucocerebrosidase is a component of α -syn positive intraneuronal inclusions¹⁴. So we evaluated the relationship between GBA and α -synuclein by manipulating GBA expression affects α -syn protein levels and α -synuclein-mediated toxicity, respectively in SH-SY5Y cells, which are dopaminergic neuroblastoma cells used as a model for PD, and primary rat neuronal cells transduced with the virus expressing α -synuclein with the A53T mutation. In both models, knock-down of GBA expression resulted in dramatic increases in cell death. Specifically, in the SH-SY5Y cells, siRNA knockdown of GBA in-

creased toxicity and overall α -synuclein protein levels. In the primary neuronal cultures, expression of shRNA for GBA reduced the survival of TH+ dopaminergic neurons. From these data we infer that down-regulation of GBA enhances the preferential toxicity of A53T α -synuclein to dopaminergic neurons.

Here, we demonstrated in cellular experiments that mutations in the GCase influence SNCA processing and its steady-state levels. Interestingly, GCase was colocalized with the lysosomal marker, LAMP-1. There is considerable evidence implicating defects in the lysosome-autophagy pathway in neurodegenerative disorders. Furthermore, α -synuclein can be selectively translocated into the lysosomes for degradation by chaperone-mediated autophagy and aggregated forms of α -synuclein may be degraded by lysosomes. It is possible that mutant GC may contribute to impaired α -synuclein clearance in the lysosome, and thus may enhance the formation of the α -synuclein inclusions. The pathology exhibited in GBA homozygotes, as well as heterozygotes, encompasses the spectrum of synucleinopathies, including DLB, suggesting that glucocerebrosidase may contribute to aggregation of α -synuclein through a gain-of-function mechanism whereby mutated glucocerebrosidase enhances the quantity of aggregates.

Our studies in mice clearly support the contention that there is a link between mutations in GBA and the development of synucleinopathies. We examined a well-characterized, hypomorphic mouse model of Gaucher disease. In mice that express 2 D409H gba knockin alleles, GCase activity is reduced to > 20% compared with WT littermates (depending on their age)¹⁸. D409H mice showed positive α -syn immunoreactivity in multiple brain regions including the substantia nigra (SN), cortex (Ctx), and hippocampus as well as higher levels of soluble oligomers and insoluble α -syn species. In clinic, brains from patients with GBA1-associated parkinsonism also showed that most patients with GBA1 mutations and synucleinopathies exhibited oligomeric forms of α -syn in the SDS-soluble fraction, while controls and patients with GD without synucleinopathies had only the monomeric form of α -synuclein in the same fraction. Insoluble α -syn oligomers, appearing as a ladder in the SDS- and urea-soluble fractions, were seen in most patients with synucleinopathies with or without GBA1 mutations¹⁹.

Conclusions

Despite the great progress made in the last two decades, the precise mechanisms underlying the genesis and progression of Parkinson's disease and are Gaucher disease still not fully understood. Further studies of the association between them will stimulate new insights into the pathophysiology of the two disorders, and will prove crucial for both genetic counseling of patients and family members and the design of relevant therapeutic strategies for specific patients with parkinsonism.

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