European Review for Medical and Pharmacological Sciences 2013; 17: 143-151

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 MATERIALS: 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 model of Gaucher disease (GBAD409H/[illegible]) to detect the gene types of GBA.

RESULTS: The results showed functional knockdown (KD) of GBA in neuroblastoma cell culture causes a significant accumulation of alpha-synuclein and alpha-synuclein mediated neurotoxicity. Furthermore, [illegible] rat primary neurons expressing [illegible]A53T [illegible]ation of alpha-synuclein, decreased cell viability [illegible]tion, we observed that overexpression of GBA mutants (N370S, [illegible]44P, [illegible]D409V) significantly raised human alpha-synuclein levels of vector control. Glucosylceramide (substrate), the GCase substrate, enhanced formation of purified a-syn by [illegible]ilizing 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 a-syn and western blot analysis revealed that two forms of alpha-syn oligomers were present in brain homogenates of the hippocampus D409H mice.

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

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

Introduction

Gaucher disease is the most common lysosomal storage disease, is an inherited recessive autosomal metabolic defect due to 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 human cells, originating ceramide and glucose. The human GBA gene is located on chromosome 1q21 and comprises 11 exons and 10 exons spanning over 7kb[4].

Clinical, genetic and pathological studies demonstrate that mutations in GBA, are risk factors for Parkinson disease and related disorders[5]. 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 compacta[6]. 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 PD[7]. While the identification of these loci has been important, mutation of these genes is responsible for a relatively small proportion of PD cases[8]. 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 affecting 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

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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 controls[11]. 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 essential[5].

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 Dulb's Modified Eagle's Medium (DMEM) me containing 10% fetal bovine serum (FBS), streptomycin and penicillin. The ells were di ferentiated in neurobasal me emented with B-27 and 40 mM reti acid 7 days. All maintained in a 37°C CO_2, fu midified incubator, passed tw bric prepared for experi al p es when in log-phase growth.

RT-PCR

RNA was racted human neuroblastoma cell HSY5Y), Stat 60 (Testest, Friends , TX, USA) acc g to manufacturer' tructi . The cDNAs were prepared by se tra ption (Superscript III; Invitrogen Ca ad, CA SA). PCR was performed the A RISM 7000 Sequence tion ata Rosa, CA, USA) and pri . The e a-syn gene was calibrated aga that of the reference gene HPRT (hypoxan ribosyltransferase 1).

eparation of Primary Mesencephalic res

ry midbrain cultures were prepared via dissection of day 17 embryos obtained from pregnant Sprague-Dawley rats (Harlan, Indianapolis, IN, USA). The cells were pl d on poly-L-lysine-treated 48-well plates of 163,500 cells per well. Five day r platin the cells were treated with cytos arabinofuranoside (20 mM, 48 h) to inhib growth of glial cells. At this stage (i.e. 7 days o), the glial cells accounted for of the el population, and the neu appeared di ated with extended pr es.

Lentiviral and den ansd ons

Primary cult (7 da *vitro*) e transduced with l virus and/o us in the presence o ne (6 mg/m e transductions we rie for 72 h at a multiplicity of infection (MOI) of e case of A53T adenovir GBA shR hort hairpin RNA) le as. The cells were en treated with fresh a for an additional 24 h and analyzed by imnocytochem Control samples consisted ntransduced nary rat midbrain cultures.

Imm cence Assay

Treate ells were fixed in freshly prepared 4% paraformaldehyde in phosphate buffered BS) for 30 min at room temperature and en p meabilized with 0.2% Triton X-100 for 10 min. After blocking with 4% bovine serum albumin (BSA), slides were incubated with IKKα antibody (dilution, 1:100) for overnight at 4°C, washed three times in PBST and reacted with fluoresceinisothiocyanate (FITC)-conjugated anti-rabbit IgG (dilution, 1:100) for 1 h and counterstained for nuclei with 10 μg/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 speciesspecific 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 ± 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 Levels in SH-SY5Y Cells

To test the hypothesis that inhi n of GCase would elicit changes in cellular lein level, we evaluated the protein by using rn blot analysis in SH-SY5Y cel fferentia th neuronal phenotype. We ormed siRNA down of GBA in diff iated SY5Y c , GCase shRNA-me d KD ockdo n) by lentiviral infectio res 0% re on in GCase protein s com to co l, and a accumulatio glucosylce e) (Figure 1A). We hy d that endog s α-synucle- in protei ac te in neurons infected with GCase shRNA. Nota ockdown of GBA was

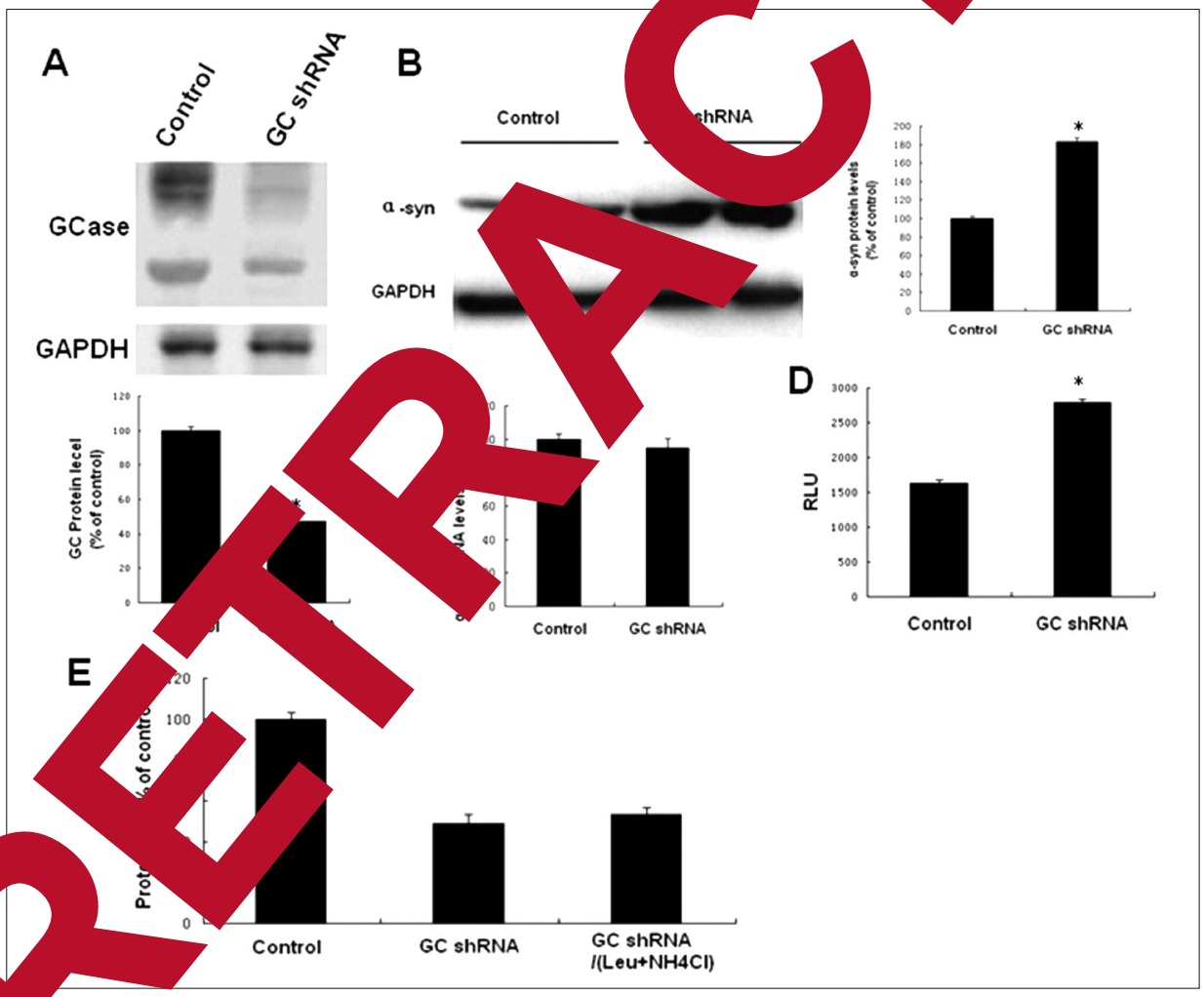


Figure 1. Depletion of GCase increases α-syn levels and compromises protein degradation. ***A,*** KD of GCase protein in corti- c ns by GCase shRNA is shown by western blot. ***B,*** α-syn expression analysis in GCase shRNA cells. Quantification is sho ht (n = 4, *$p < 0.01$). ***C,*** Expression of 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.

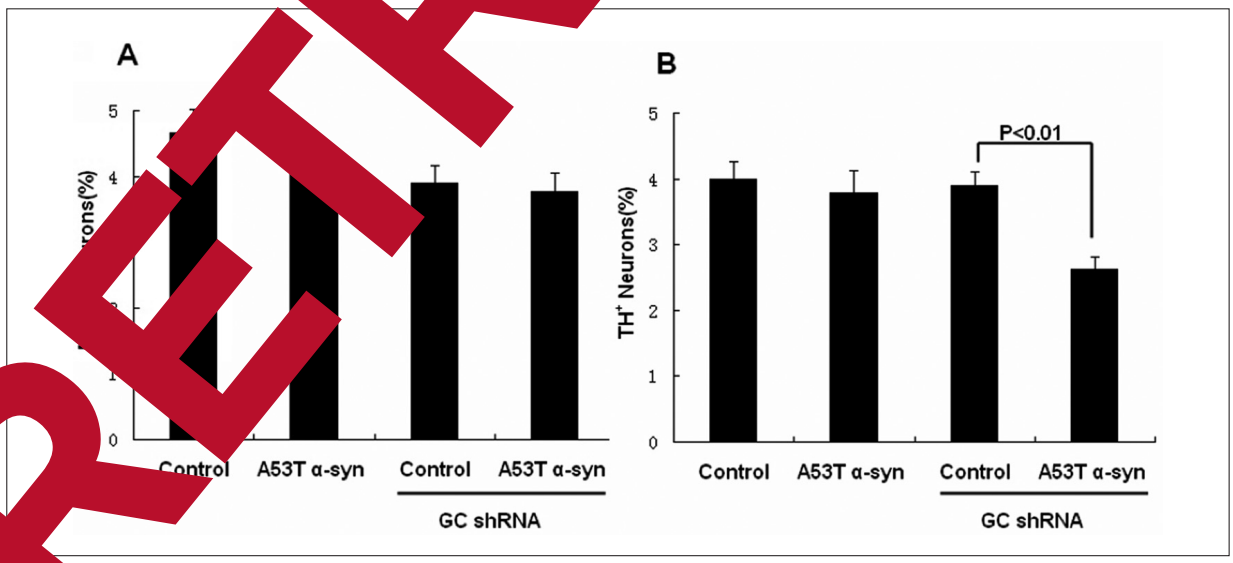
accompanied by a significant increase in α-synuclein protein levels (Figure 1B). Indeed, GCase KD increased the steady-state levels of α-synuclein by 1.8-fold relative to controls. In order to determine whether increased levels of the protein were due to enhanced transcription, RT-PCR was performed to 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 combine with a decrease in GBA results in increased cyto

GBA Reduction Increases Neurotoxici in Rat Midbrain Neurons Overexpresse A53T-α-synuclein

We also investigated whethe action o α-synuclein and GBA was ent fo e A53T mutation in α-synuclein. In this experiment, we examined how the reduction in endogenou GBA influences α-synuclein-mediated toxi tured primary rat midbrain neurons essing t A53T form of α-synuclein, an *i vo* model of PD. Primary rat midbrain neuro es consist of 4-5% tyrosine hydroxylase po (TH+) cells, which have been des d as be is itely sensitive to α-syn in levels in hours after transduc , we a sed rela e dopaminergic cell v by unocyt chemistry for TH, a m ke inergi rons. Transduction v the A -syn n adenovirus or wit er GAK sh alone did not affect ergic cell vi y compared with unt duce ures. However, simultaneous exposure to the α-synuclein adenovirus and of the GBA A dramatically reduced the number of TH neurons relative to P2+ (microtubule-associated protein 2) neurons, suggesting α-synuclein neurotoxicity is ered or enha d by the combination of overe ion of m t SNCA (synuclein alpha) and redu (glucosidase beta acid) (Figure 2A, B). These results indicating that GBA reduction plays an important role in cell toxicity when tant-α-synuclein is overexpressed.

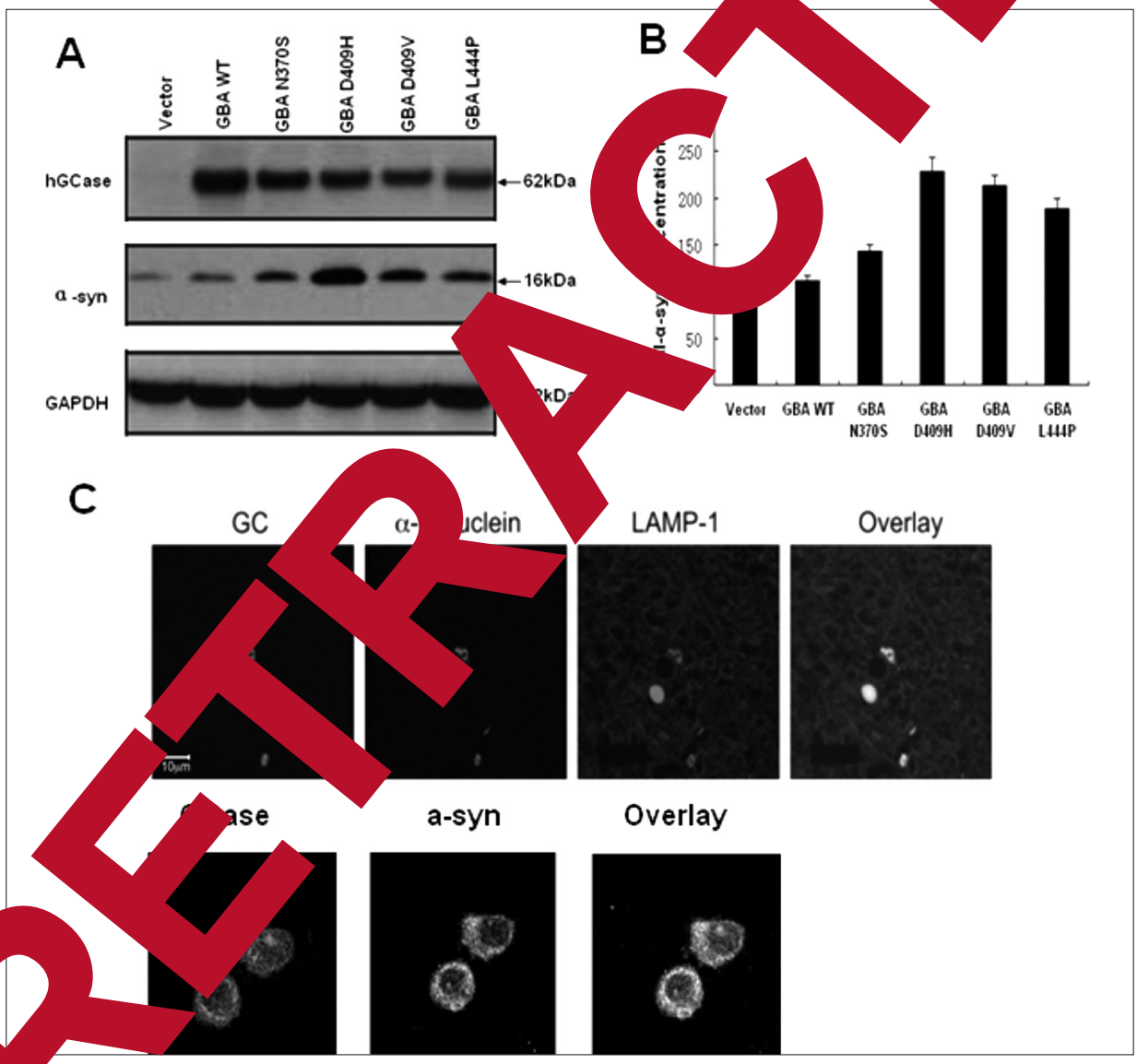
Mutant GBA Proteins Contribute to α-synuclein Aggregation

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



F 2. The effect of GCase KD in rat midbrain neurons overexpressed A53T-α-syn. Primary midbrain cultures were transduc h 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 protein[13], this could contribute to the enhanced aggregation of α-synuclein by a direct or indirect interaction between GCase 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 al[14] reported α- positive Lewy bodies are found in brains patients with GBA mutations an, we decided to investigate the associaiton ular level. Immunofluorescence analysis d that GCase was colocalized wi e lysoso er, LAMP-1 (lysosoma sociated me protein 1). Double-l immu uoresce showing GCase (g and nuclei (red), GCase and α-sy co as sho n the overlay panels ure 3 resu pported



re 3. Effects of mutant GCase expression on α-syn levels. ***A,*** GCase and α-syn analysis by western blot in cells tran- nsfected with GBA-encoding cDNA. ***B,*** Cell lysates were corrected for equal total protein content and subjected to quan tion 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 a-syn-mediated [illegible]-toxicity (Figure 4A). We next assess[illegible] ern blotting the level of the microtu[illegible]-associa[illegible] ed protein 1 light chain 3 (LC3), [illegible]hly specific autophagosomal marker. During [illegible]hagosome formation, the LC3-I cytosolic form [illegible]verted into lipid conjugated LC3-[illegible]rm, who[illegible]un correlates with the num[illegible] of autophago[illegible] Western blot analysis [illegible]cated [illegible]mparable [illegible]-crease in the levels [illegible]C3-II[illegible] by le[illegible]peptin treatment or GCa[illegible] K[illegible] 4B). [illegible]hemical analysis re[illegible]d an[illegible]se of [illegible]soluble α-syn in leu[illegible]n-treated ce[illegible] change in the amount[illegible]uble α-syn ([illegible]e 4C). Thus, despite [illegible]ar [illegible] on the total α-syn levels

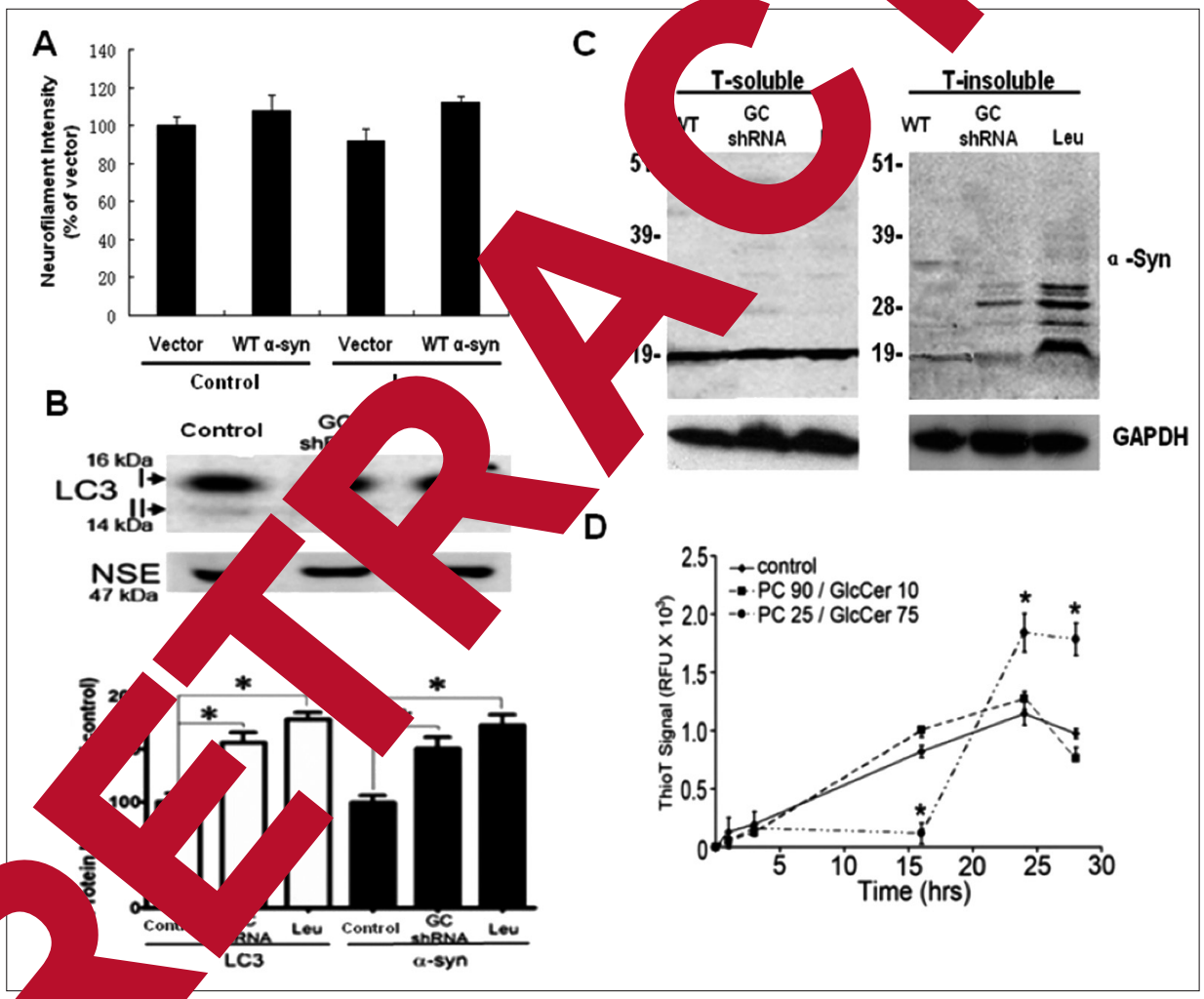


Figure 4. ***A,*** Neurotoxicity assessment by neurofilament immunostaining in either empty vector (vect) or WT α-syn infected [illegible] with or without leupeptin treatment (n = 4). ***B,*** Western blot of LC3-II upon GCase knockdown or leupeptin treatment. [illegible] used as a loading control. ***C,*** Western blot of T-soluble and T-insoluble α-syn in GCase knockdown or leupeptin treatment[illegible]s. ***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, *$p < 0.01$).

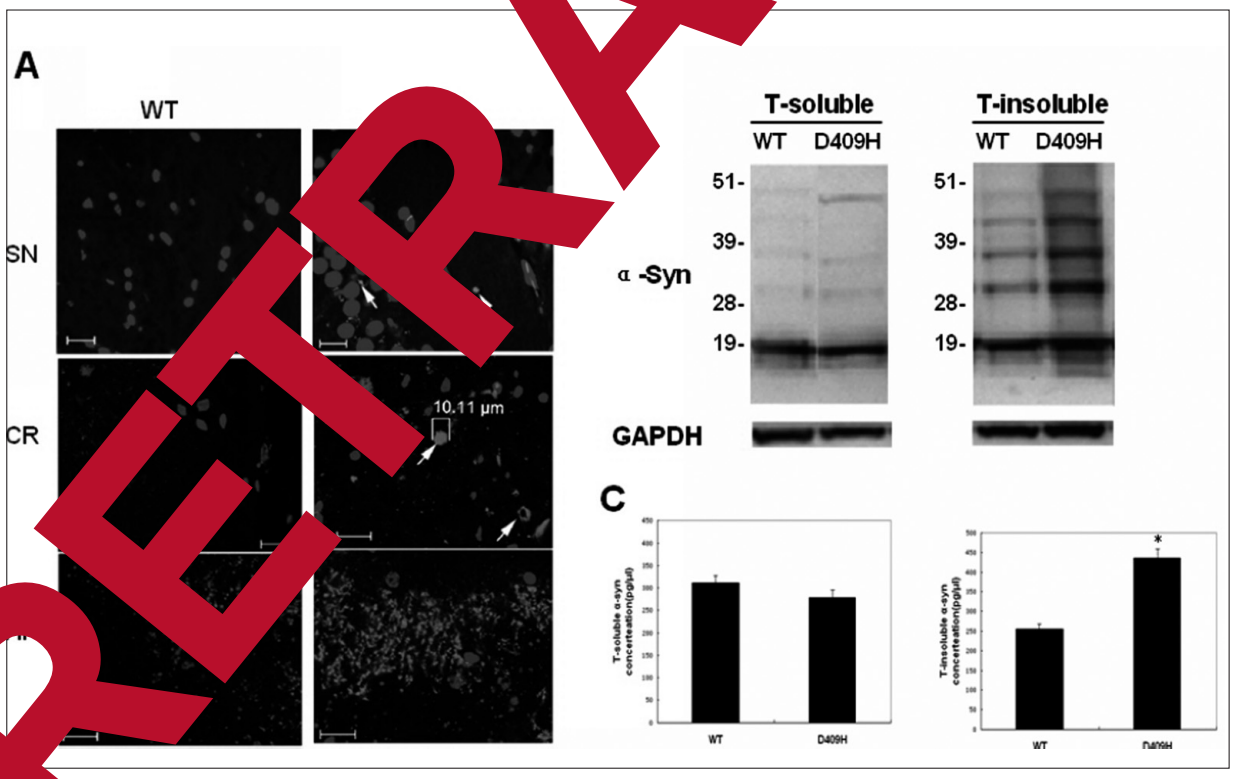
by leupeptin or GCase KD, only GCase KD increased the steady-state levels of soluble α–σψν.

We next examined whether GlcCer directly influences the *in vitro* aggregation of recombinant α–σψν. Lipid dispersions made of mixtures of purified GlcCer and brain phosphatidylcholines (PCs) were incubated with α–σψν 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 a-syn compared to control reactions containing a-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-σψν fibril formation by delaying the formation of insoluble thioT-positive a-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 ne[illegible] amined whether GCase depletion and GlcCer accumulation affect 12-mo-old levels and sol[illegible]ity *in vivo*. For this, brain tissues from [illegible]09H/D40[illegible] described GD mouse model (G[illegible] were analyzed to determine w[illegible]er endogenously expressed α-syn protein [illegible] were elevated. Serial sagittal brain sections [illegible]12-mo-old D409H mice were exa[illegible]d by im[illegible]fluorescence microscopy to [illegible]vide an overv[illegible] brain α-syn distribut[illegible] D409[illegible]ice show[illegible]d positive α-syn im[illegible]reac[illegible]y in m[illegible]ltiple brain regions incl[illegible]din[illegible]antia ni[illegible](SN), cortex (Ctx), [illegible]ell as [illegible]cam[illegible](Figure 5A). Immun[illegible]rescence a[illegible]ealed the presence o[illegible]ccumulation[illegible]the form of punctate [illegible]uctu[illegible]hereas WT mice showed a normal ne[illegible]ropil sta[illegible]attern expected for α-syn [illegible] α-syn part[illegible](≥ 5 μm) were obse[illegible]n cerebral cortex [illegible]bstantia nigra and of [illegible]no-old D409H mice, in comparison to the [illegible]pocampal reg[illegible]

[illegible]e next focu[illegible] on hippocampus, a region c[illegible]teristicall[illegible]ffected in humans with DLB or a[illegible]nd in Gaucher disease patients with Parkinsonism. Brain homogenates from the



[illegible]5. α-syn in mouse brain. ***A,*** Dual-label immunohistochemistry was performed in coronal brain sections detect α-syn. ***B,*** Wes[illegible]lot of T-soluble and T-insoluble α-syn in 12-mo-old D409H mice. GADPH 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 fr[om] archetypical nongenetic disease to one [with] clear genetic basis in a percentage of pat[ients][15]. Past work has evidenced the link between Ga[u]er disease and the synucleinopathies Parkin[son's] disease and dementia with Lewy b[odi]es, that di[s]play abnormal fibrillation and [accu]lation of proteinaceous, insoluble c[ytop]asm in n[eur]ons and glia, indicating a shared c[ommon] pathol[ogy] for the handling and clearance of [them][16].

α-syn is a small, [natu]rally unfolded cytoplasmic protein wh[ich] is highly [expr]essed in the central nervous [syst]em and con[cen]trated in presynaptic te[rmi]nals, representing 0.5[%] of the total cytosolic [pro]tein [co]ntent[17]. Previous studies of brain s[amp]les from subj[ects] with GBA-associated syn[u]cleinopathies demo[nstr]ated that glucocereb[ro]sidase is a component of α-syn positive intr[ane]uronal [incl]usions[14]. So we evaluated the rela[tion]ship between G[CBA] and α-synuclein by manipu[lating] GBA e[xpr]ession affects α-syn protein levels [and] synuclein-mediated toxicity, respectively in SY5Y cells, which are dopaminergic neuroblastoma cells used as a m[odel] and primary rat neuronal cells t[rans]duced with the virus expressing α-synuclein with the A53T mutation. In both models, knockdown of GBA expression resulted in dramatic increase in cell death. Specifically, in the SH-SY5Y cells, siRNA knockdown of GBA increased toxicity and overall α-synuclein protein levels. In the primary neuronal cultures, [over]expression of shRNA for GBA reduced the [num]ber of TH+ dopaminergic neurons. From [these] data we infer that down-regulation of GB[A] enhances the preferential toxicity of A53T [α]-synuclein to dopaminergic neurons.

Here, we demonstrate [in] cellular experiments that mutations in the GCase in[hibit] SNCA processing a[nd] its steady state lev[els]. Interestingly, GCase co[-loc]alized with the lysosomal marker LAMP1. There is considerable evidence indicating effects in the lysosome-autophagy pathway in neurodegenerative disorders. Furthermore, α-synuclein can be selectively transported into the lysosomes for degradation by chaperone-mediated autophagy and aggregated forms of 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 contribute to aggregation of α-synuclein through a gain-offunction 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 hadonly 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 inmost patients with synucleinopathies with or without GBA1 mutations[19].

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.

Acknowledgements

This work was supported by a grant from the National Natural Science Foundation of China (No. 81071037).

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