Glucocerebrosidase deficiency accelerates the accumulation of proteinase K-resistant α-synuclein and aggravates neurodegeneration in a Drosophila model of Parkinson’s disease

Mari Suzuki¹, Nobuhiro Fujikake¹, Toshihide Takeuchi¹, Ayako Kohyama-Koganeya², Kazuki Nakajima², Yoshio Hirabayashi², Keiji Wada¹, Yoshitaka Nagai¹,*

¹Department of Degenerative Neurological Diseases, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira 187-8502, Japan
²Laboratory for Molecular Membrane Neuroscience, RIKEN Brain Science Institute, Wako, Saitama 351-0198, Japan

*Corresponding author: Yoshitaka Nagai, Department of Degenerative Neurological Diseases, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira 187-8502, Japan. Tel.: 81-42-341-1715, Fax: 81-42-346-1745, E-mail: nagai@ncnp.go.jp
Abstract

Alpha-synuclein (αSyn) plays a central role in the pathogenesis of Parkinson’s disease (PD) and dementia with Lewy bodies (DLB). Recent multicenter genetic studies have revealed that mutations in the glucocerebrosidase 1 (GBA1) gene, which are responsible for Gaucher’s disease, are strong risk factors for PD and DLB. However, the mechanistic link between the functional loss of glucocerebrosidase (GCase) and the toxicity of αSyn in vivo is not fully understood. In this study, we employed Drosophila models to examine the effect of GCase deficiency on the neurotoxicity of αSyn and its molecular mechanism. Behavioral and histological analyses showed that knockdown of the Drosophila homologue of GBA1 (dGBA1) exacerbates the locomotor dysfunction, loss of dopaminergic neurons, and retinal degeneration of αSyn-expressing flies. This phenotypic aggravation was associated with the accumulation of proteinase K (PK)-resistant αSyn, rather than with changes in the total amount of αSyn, raising the possibility that glucosylceramide (GlcCer), a substrate of GCase, accelerates the misfolding of αSyn. Indeed, in vitro experiments revealed that GlcCer directly promotes the conversion of recombinant αSyn into the PK-resistant form, representing a toxic conformational change. Similarly to dGBA1 knockdown, knockdown of the Drosophila homologue of β-galactosidase (β-Gal) also aggravated locomotor dysfunction of the αSyn flies, and its substrate GM1 ganglioside accelerated the formation of PK-resistant αSyn. Our findings suggest that the functional loss of GCase or β-Gal promotes the toxic conversion of αSyn via aberrant interactions between αSyn and their substrate glycolipids, leading to the aggravation of αSyn-mediated neurodegeneration.
Introduction

Parkinson’s disease (PD) is one of the most common neurodegenerative diseases, characterized by motor dysfunction, such as resting tremor, bradykinesia, and rigidity. Dopamine (DA) replacement therapy is widely used to improve these motor symptoms, but does not attenuate disease progression. Pathological features of PD include a loss of dopaminergic neurons and the deposition of Lewy bodies (LBs), which are mainly composed of α-synuclein (αSyn), in the substantia nigra. LBs are also abundantly found in the brains of patients with dementia with Lewy bodies (DLB), suggesting a pathological overlap between PD and DLB (1).

Missense mutations in the SNCA gene encoding αSyn, as well as genomic multiplications in the region containing the SNCA gene, have been identified as causes of dominantly inherited PD (2). Moreover, genome-wide association studies identified sequence variations in the SNCA gene as major risk factors for sporadic PD (3, 4), suggesting that αSyn plays a central role in the pathogenesis of both familial and sporadic cases of PD. Missense mutations in αSyn, such as A30P, A53T, and E46K, have been shown to modulate αSyn fibril formation, implicating that the misfolding and subsequent aggregation of αSyn play key roles in its acquisition of neurotoxicity (5). However, in sporadic PD and DLB, normally harmless αSyn proteins without any mutations might gain toxic functions by unknown mechanisms. Thus, it is important to elucidate the factors promoting the toxic conversion of αSyn, towards understanding the pathogenesis of and developing disease-modifying therapies for PD and DLB.

Recently, multicenter genetic studies revealed that mutations in the glucocerebrosidase 1 (GBA1) gene are strong risk factors for PD (odds ratio: 5.31) (6) and DLB (odds ratio: 8.28)
Glucocerebrosidase (GCase) is a lysosomal enzyme, which hydrolyzes the glycolipid glucosylceramide (GlcCer). Homozygous mutations in the GBA1 gene, which result in decreased enzyme activities, cause Gaucher’s disease (GD), one of the lysosomal storage diseases. GD patients have a 21-fold increased life-time risk of developing parkinsonism (8), and GD patients who exhibit parkinsonism were found to have αSyn-positive LBs in their brains (9). In addition, PD patients with a heterozygous GBA1 mutation more frequently develop diffuse LB pathology, which is associated with earlier disease onset, compared with those without GBA1 mutations (10). These facts strongly suggest a tight link between αSyn toxicity and GBA1 mutations. However, the molecular mechanisms as to how GBA1 mutations affect αSyn toxicity in vivo are not fully understood.

In this study, we employed Drosophila models to examine the in vivo role of GCase deficiency in αSyn-mediated neurotoxicity, as well as the molecular mechanisms involved. We found that knockdown of the Drosophila homologue of GBA1 (dGBA1) accelerates αSyn neurotoxicity without any alterations in total αSyn levels. Instead, the accumulation of proteinase K (PK)-resistant αSyn was accelerated by dGBA1 knockdown, which correlated with the phenotypic aggravation. Furthermore, GlcCer, which is a substrate of GCase, directly promoted the formation of PK-resistant αSyn. We therefore conclude that the aberrant interaction of αSyn with GlcCer, which accumulates upon the loss of GCase activity, is a key event in the acquisition of toxic properties of αSyn.

Results

GCase deficiency increases αSyn toxicity in Drosophila

To investigate the effect of GCase deficiency on the neurotoxicity of αSyn, we used transgenic RNAi flies that express an inverted repeat RNA (IR) targeting the Drosophila homologues of the
GBA1 gene, namely, *CG31148 (dGBA1a)* or *CG31414 (dGBA1b)*, which show 49% or 31% amino acid similarity to the human gene, respectively, under the control of the GAL4-UAS system. We first analyzed the effect of GCase deficiency in the brain, by using the flies expressing *dGBA1a-IR* (dGBA1a-RNAi flies) or *dGBA1b-IR* (dGBA1b-RNAi flies) under the pan-neuronal *elav-GAL4* driver. The *elav-GAL4* driver was chosen because the effect of the *GBA1* mutation might not be limited to the DA neurons in the case of DLB (7), in which cholinergic neurons in the neocortex are also affected (11). Compared with the flies expressing *Ctr-IR* (Ctr-RNAi flies), the brain lysates of dGBA1a-RNAi-a and dGBA1a-RNAi-b flies exhibited a 92% and 81% decrease in GCase activity, whereas those of dGBA1b-RNAi-a, dGBA1b-RNAi-b, and dGBA1b-RNAi-c flies exhibited a 28%-51% decrease (Fig. 1A). We also confirmed that *dGBA1a* mRNA levels were decreased in the brains of the dGBA1a-RNAi flies to approximately 20% compared with Ctr-RNAi flies (Fig. 1B). Therefore, we decided to use dGBA1a-RNAi flies in the subsequent experiments.

To examine whether GCase deficiency affects the toxicity of αSyn in vivo, we crossed human wild-type αSyn-expressing flies (αSyn flies), which is a well-established PD model (12), with the dGBA1a-RNAi flies to obtain flies co-expressing both αSyn and *dGBA1a-IR* (αSyn/dGBA1a-RNAi flies) in the nervous system. We performed the climbing assay to evaluate the effect of *dGBA1* knockdown on the locomotor dysfunction of the αSyn flies. αSyn flies expressing *Ctr-IR* (αSyn/Ctr-RNAi flies) exhibited a mild decline in climbing ability from 4 weeks after eclosion, compared with the control flies (EGFP/Ctr-RNAi flies) (Fig. 1C). In contrast, the climbing ability of αSyn/dGBA1a-RNAi-a and αSyn/dGBA1a-RNAi-b flies significantly declined from 2-3 weeks after eclosion and continued to decline rapidly thereafter (Fig. 1C), exhibiting a statistically significant difference compared with αSyn/Ctr-RNAi flies. We next assessed whether *dGBA1* knockdown also accelerates the degeneration of DA neurons.
We performed immunohistochemical staining with an antibody against tyrosine hydroxylase (TH), which is an enzyme required for the biosynthesis of DA. We found that αSyn/Ctr-RNAi flies have fewer DA neurons than EGFP/Ctr-RNAi flies at 6 weeks after eclosion (Fig. 1D and E, EGFP/Ctr-RNAi vs αSyn/Ctr-RNAi flies). Moreover, the loss of DA neurons in αSyn flies was significantly exacerbated by dGBA1 knockdown in the protocerebral posterior lateral (PPL) cluster (Fig. 1E, αSyn/Ctr-RNAi vs αSyn/dGBA1a-RNAi-a or αSyn/dGBA1a-RNAi-b flies), and this tendency of exacerbation was also seen in the protocerebral posterior medial (PPM) clusters (Fig. 1E). Furthermore, the exacerbation of αSyn toxicity by GCase deficiency was also observed with the retinal degeneration phenotype under the eye-specific GMR-GAL4 driver. αSyn/dGBA1a-RNAi flies, but not the other flies, exhibited thinning and vacuolization of the retinal tissue resulting from photoreceptor degeneration (Fig. 1F, middle and lower panels, and Fig. 1G), although external eye appearances were almost the same in all the flies analyzed (Fig. 1F, upper panels). These behavioral and histological results indicate that GCase deficiency exacerbates the neurotoxicity of αSyn in vivo.

**GCase deficiency accelerates the accumulation of proteinase K-resistant αSyn**

Since αSyn has been reported to be degraded in lysosomes (13-15), we hypothesized that the loss of GCase activity compromises the αSyn-degrading function of lysosomes, resulting in αSyn accumulation. To test whether the aggravation of αSyn toxicity by GCase deficiency is caused by the accelerated accumulation of αSyn proteins, we performed immunoblotting analysis. We found that the amounts of αSyn in both the Triton X-100-soluble and -insoluble fractions are not significantly increased, but rather are almost unchanged by dGBA1 knockdown (Fig. 2A-C). Upon long-exposure of the membrane, we identified SDS-resistant bands (approximately 35 kDa), probably corresponding to dimers, in the Triton X-100-soluble fraction...
(arrow in Fig. 2A), and the amount of this dimer was decreased by \textit{dGBA1} knockdown. These results suggest that GCase deficiency does not result in the accumulation of \(\alpha\)Syn, and thus the impaired degradation of \(\alpha\)Syn in lysosomes is unlikely to be the mechanism responsible for the phenotypic aggravation of \(\alpha\)Syn-expressing flies upon \textit{dGBA1} knockdown.

From these results, we considered the possibility that not simply the total amount of \(\alpha\)Syn, but alterations in the toxic properties of \(\alpha\)Syn may contribute to the phenotypic aggravation of \(\alpha\)Syn/d\textit{GBA1a-RNAi} flies. Prion-like conformational changes of \(\alpha\)Syn have been shown to be associated with the pathogenesis of PD (16, 17), and these different \(\alpha\)Syn conformers can be distinguished biochemically by their susceptibility to PK digestion (18). Indeed, PK-resistant \(\alpha\)Syn accumulates in the brains of PD patients and animal models of PD, including mice and \textit{Drosophila}, suggesting a significant role of PK-resistant \(\alpha\)Syn in disease pathogenesis (19-21). We therefore treated fly head lysates with serial concentrations of PK and performed immunoblotting to assess the PK resistance of \(\alpha\)Syn. In \(\alpha\)Syn/Ctr-RNAi flies the immunoreactivity of \(\alpha\)Syn was considerably decreased by treatment with 1 \(\mu\)g/ml PK, and was almost completely abolished by treatment with more than 5 \(\mu\)g/ml PK (Fig. 2D). In contrast, \(\alpha\)Syn/d\textit{GBA1a-RNAi-b} flies showed residual \(\alpha\)Syn bands even upon treatment with 5 or 10 \(\mu\)g/ml PK. Quantification analyses revealed that the amounts of full-length \(\alpha\)Syn that remained after 1 \(\mu\)g/ml PK treatment are significantly increased in the \(\alpha\)Syn/d\textit{GBA1a-RNAi-b} flies compared with those in the \(\alpha\)Syn/Ctr-RNAi flies (1.68-fold, \(p < 0.05\), Fig. 2D). Furthermore, the band patterns of the \(\alpha\)Syn fragments after PK digestion were altered upon \textit{dGBA1} knockdown, suggesting that GCase deficiency may promote conformational changes in \(\alpha\)Syn (16).

Immunohistochemical analyses of retinal sections also demonstrated that the immunoreactivity of PK-resistant \(\alpha\)Syn is increased in the \(\alpha\)Syn/d\textit{GBA1a-RNAi} flies compared with \(\alpha\)Syn/Ctr-RNAi flies (Fig. 2E). Interestingly, the accumulation of PK-resistant \(\alpha\)Syn increased
with age in both the αSyn/dGBA1a-RNAi and αSyn/Ctr-RNAi flies (Fig. 2E), which is consistent with the progressive decline in locomotor dysfunction (Fig. 1C). Taken together, these results suggest that the accumulation of PK-resistant αSyn may be responsible for the phenotypic aggravation seen in αSyn/dGBA1a-RNAi flies.

**Glucosylceramide promotes the formation of PK-resistant αSyn**

Our results showing the accelerated accumulation of PK-resistant αSyn in the αSyn/dGBA1a-RNAi flies raised the possibility that GlcCer, a substrate of GCase, affects the conversion process of αSyn into the PK-resistant form. We first examined whether GlcCer accumulation occurs upon dGBA1 knockdown. Thin-layer chromatography (TLC) analysis showed that the amounts of GlcCer was significantly increased in the heads of the dGBA1a-RNAi flies compared with those of the Ctr-RNAi flies, when co-expressed with either EGFP or αSyn (Fig. 3A and B). Moreover, immunohistochemical staining using a GlcCer antibody also showed the accumulation of GlcCer in the dGBA1a-RNAi flies (Fig. 3C and D), confirming that GCase deficiency leads to GlcCer accumulation in our fly model.

We next tested whether GlcCer directly accelerates the formation of PK-resistant αSyn, using an in vitro assay. Recombinant αSyn was incubated with lipid dispersions consisting of phosphatidylcholine (PC) with or without GlcCer for 14 days, and after PK digestion, αSyn was subjected to immunoblotting analyses. On day 1 (24 hr after incubation), the relative amounts of undigested αSyn were almost equal between αSyn incubated with GlcCer-containing PC and that incubated with PC only (Fig. 4A). In contrast, on days 7 and 14, the relative amounts of undigested αSyn incubated with GlcCer were significantly greater than that incubated with PC only (Fig. 4B and C), indicating resistance to PK digestion. In addition, we noted that the amounts of the approximately 35 kDa bands, probably corresponding to SDS-resistant αSyn
dimers (arrows in Fig. 4), were also increased at 7 and 14 days after incubation with GlcCer (Fig. 4B-D), although the amounts of the αSyn dimer were not increased, but rather decreased, in the αSyn/dGBA1a-RNAi flies (Fig. 2A). These data strongly indicate that GlcCer directly promotes the conversion of αSyn into the PK-resistant form.

β-galactosidase deficiency increases αSyn toxicity in flies and GM1 ganglioside promotes the formation of PK-resistant αSyn

GD is one of lysosomal storage diseases (LSDs), in which the deficiency of specific lysosomal enzymes causes the accumulation of certain lipids and glycoproteins in lysosomes, resulting in various neurological and peripheral symptoms. Interestingly, αSyn accumulation is also found in the brains of patients of several LSDs, including GM1 gangliosidosis, GM2 gangliosidosis, and Sanfilippo syndrome (22, 23), as well as GD (24). Moreover, it was shown that induced pluripotent stem cells (iPSC)-derived neurons from GBA1-associated PD patients exhibit decreased GCase activity, as well as β-galactosidase (β-Gal) activity compared with the isogenic gene-corrected controls (25). These facts led us to examine whether knockdown of β-Gal accelerates αSyn toxicity as in the case of dGBA1 knockdown.

We obtained fly lines carrying IR transgenes targeted to the Drosophila homologue of the β-galactosidase (dβ-Gal, approximately 41% amino acid similarity to human β-Gal) gene, which is responsible for GM1 gangliosidosis. We confirmed that the amounts of dβ-Gal mRNA in dβ-Gal-RNAi fly brains were decreased to less than 10% of that in Ctr-RNAi fly brains when expressed under the elav-GAL4 driver (Fig. 5A). To assess the effect of dβ-Gal knockdown on αSyn toxicity, we crossed the dβ-Gal-RNAi flies with αSyn flies, and found that knockdown of dβ-Gal significantly exacerbates the locomotor dysfunction of αSyn flies (αSyn/Ctr-RNAi vs αSyn/dβ-Gal-RNAi-a or αSyn/dβ-Gal-RNAi-b flies, Fig. 5B). Moreover, the aggravation in
climbing abilities of the αSyn/dβ-Gal-RNAi flies was associated with an accumulation of PK-resistant αSyn, as revealed by immunoblotting analysis (Fig. 5C). To observe the direct effect of GM1 ganglioside (GM1), a substrate of β-Gal, on the formation of PK-resistant αSyn, we performed an in vitro assay and found that GM1-containing liposomes promoted the conversion of αSyn into the PK-resistant form, similarly to GlcCer-containing liposomes (Fig. 5D-F). In contrast to GlcCer, dimer formation was not affected by GM1-containing liposomes (Fig. 5G). These findings suggest that an increase in αSyn neurotoxicity by β-Gal deficiency is also mediated by an increase in the PK resistance of αSyn, supporting our hypothesis that aberrant interactions of αSyn with glycolipids trigger the toxic conversion of αSyn, resulting in an increase in neurotoxicity in vivo.

Discussion

In the present study, we investigated the molecular mechanisms underlying the effect of GCase deficiency on αSyn toxicity. We demonstrated that loss of GCase function exacerbates αSyn neurotoxicity in vivo and that this aggravation is associated with the accelerated accumulation of PK-resistant αSyn. In addition, GlcCer, which was accumulated in the brain of dGBA1 knockdown flies, directly promoted the formation of PK-resistant αSyn, suggesting that the accumulation of GlcCer by GCase deficiency promotes the toxic conversion of αSyn, leading to exacerbation of its neurotoxicity.

The phenotypic aggravation by GCase deficiency in αSyn flies was associated with the accumulation of PK-resistant αSyn, rather than with changes in the total amount of αSyn, suggesting that the production of this PK-resistant αSyn species might play a key role in the neurotoxicity. Although we did not directly demonstrate the toxicity of PK-resistant αSyn, there was a tight association between the neurotoxicity of αSyn and its PK resistance.
αSyn oligomers that are formed as an intermediate conformer in the course of in vitro αSyn fibrillization have been shown to cause oxidative stress in primary neurons at much higher levels than non-PK-resistant oligomers (26). Guo et al. found that two kinds of αSyn fibrils exhibiting different vulnerabilities to PK digestion can be isolated from repetitive seeded fibrillization, and the αSyn strain more resistant to PK digestion was more toxic to neurons (17). In addition, αSyn fibril strains produced using different buffers showed different vulnerabilities to PK digestion, and their toxicities were associated with their resistance to PK digestion (16). Interestingly, it was also demonstrated that αSyn fibrils with different levels of PK resistance have different structures, cross-seeding abilities, and propagation properties both in vitro and in vivo, all of which are reminiscent of the properties of prions (16, 17). Therefore, it is possible that the accelerated formation of PK-resistant αSyn that we observed in the GCase-deficient flies represents the “prion-like conversion” of αSyn, and that this toxic species leads to phenotypic aggravation by promoting the prion-like seeding and propagation of αSyn.

The idea that αSyn is degraded in lysosomes (13-15) has led to several studies based on the hypothesis that loss of GCase activity compromises the αSyn-degrading function of lysosomes, resulting in αSyn accumulation. Several groups demonstrated that decreased GCase activity results in increased amounts of αSyn, using cultured neurons, human iPSC-derived neurons from GBA1 mutation carriers, and mice treated with a GCase inhibitor (25, 27, 28). On the other hand, two other groups reported that GCase activity does not correlate with the amount of αSyn in neuronal cells (29), whereas the expression of a mutant GCase that maintains its enzyme activity increases the amount of αSyn (30), favoring a gain-of-function mechanism in the pathogenesis of GBA1-associated PD. In our fly model, the amount of total αSyn was not significantly increased by GCase deficiency, despite the phenotypic aggravation (Fig. 1C-G and Fig. 2A-C). However, a recent study using PD model mice with a GBA1 mutation showed that
the total amount of αSyn in the brain lysates was not increased, but the rate of αSyn degradation assessed by pulse-chase experiments was decreased in primary neurons from the same mice (31). Thus, we could not completely exclude the possibility that αSyn degradation is compromised by lysosomal dysfunction, even though changes in the total amount of αSyn were not detected.

In addition to the fly model experiments, we further demonstrated by *in vitro* experiments that GlcCer directly promotes the formation of PK-resistant αSyn (Fig. 4), as a mechanism for the increased accumulation of PK-resistant αSyn in the dGBA1a-RNAi flies. These results are consistent with a previous report showing a direct effect of GlcCer on the stability of αSyn oligomers (28). Moreover, we also found a significant increase in αSyn dimers by the incubation of αSyn with GlcCer-containing liposomes (Fig. 4B-D), which is consistent with the finding that the amount of αSyn dimers was significantly increased in GD patients (32). We also showed that β-Gal knockdown exacerbates the locomotor dysfunction of αSyn flies, and GM1 directly promotes the PK resistance of αSyn (Fig. 5), supporting our hypothesis that aberrant interactions of αSyn with glycolipids trigger the toxic conversion of αSyn, resulting in increased neurotoxicity *in vivo*. It has been demonstrated that GM1 specifically binds to αSyn and induces its oligomerization, thereby inhibiting its fibrillation (33). Interestingly, a recent report showed that iPSC-derived neurons from *GBA1*-associated PD patients exhibit not only decreased GCase activity, but also decreased β-Gal activity, which can be rescued by zinc-finger nuclease-mediated gene correction, implying a crosstalk between GCase and β-Gal activity (25). Taken together, it is possible that a loss of β-Gal activity also contributes to the acceleration of αSyn toxicity in *GBA1*-associated PD. It is noted that the direct binding of GM1 to the amyloid β protein also triggers its toxic conversion (34), implying a common or similar role of glycolipids in the conversion of neurodegenerative disease-related proteins from their non-toxic to toxic forms.
Then, where in a cell does the accumulated GlcCer interact with αSyn to convert it into a PK-resistant form? One possibility is that αSyn is transported into lysosomes via macroautophagy or chaperone-mediated autophagy (13-15), where it interacts with accumulated GlcCer. Then, GlcCer-associated αSyn is secreted from the cells, taken up by itself or by the surrounding cells, and accumulates in the cytosol (35, 36). The other possibility is that the accumulated GlcCer in the lysosome leaks into the cytosol, and interacts with αSyn in the cytosol, as the leakage of GlcCer into the cytosol has been reported in both GD patients and GD model mice (37, 38). There have been no reports to date of the level of GlcCer in the brain of PD patients with a GBA1 mutation. However, in iPSC-derived neurons from two PD patients with a heterozygous GBA1 mutation (RecNcil/wt and N370S/wt), which causes an approximately 50% decrease in GCase activity, the amount of GlcCer has been reported to be about 2-fold higher than isogenic gene-corrected iPSC-derived neurons (25). Furthermore, GlcCer was reported to be accumulated in the brains of GD patients (37) in which GCase activity was decreased (by 80%–90%) (39, 40). GCase activity was also found to be moderately decreased in the brains of GBA1 mutant carrier PD patients (58% decrease in the substantia nigra) (41, 42). Collectively, these data suggest that GlcCer accumulates in the brains of GBA1 mutation carrier PD patients.

Our present study focused on the loss-of-function aspect of GBA1 mutations, but there is another possibility arguing the gain-of-function toxicity of mutant GCase, because most mutant GCases are prone to misfold in the ER. Human skin fibroblasts derived from GD patients and carriers are reported to induce the unfolded protein response, which is also observed in Drosophila models of GD expressing human mutant GCase (43). Ambroxol, a potential pharmacological chaperone for mutant GCase has been shown to ameliorate both ER stress and the phenotypes of these Drosophila models (44). Interestingly, ambroxol treatment
also suppresses the misfolding of mutant GCase, subsequently resulting in an enhancement of cellular GCase activity (45). Therefore, chemical chaperone therapy can be expected to exert beneficial effects against GD, via the amelioration of both the gain-of-function aspect through ER stress and the loss-of-function aspect through decreased GCase activity. Since ER stress is suggested to be involved in the neurodegeneration that occurs in PD (46-48), the synergistic effects of chemical chaperone therapy would also be effective for GBA1-associated PD patients, through the suppression of both ER stress and the toxic conversion of αSyn by GlcCer accumulation.

In summary, our study revealed that the loss of function of GCase accelerates the toxic conversion of αSyn via an interaction with its substrate glycolipid GlcCer. Therefore, prevention of the conversion of αSyn to PK-resistant forms by altering GlcCer metabolism might be a promising approach as a disease-modifying therapy for PD and DLB.

Materials and Methods

Fly stocks.

Flies were grown on standard cornmeal medium at 25 °C. Transgenic fly lines bearing UAS-h[WT]αSyn (#8146) (12), UAS-EGFP (#6658), elav-GAL4\(^{155}\) (#458), and UAS-GFP-IR (Ctr-IR, #9330) were obtained from the Bloomington Stock Center. Transgenic RNAi fly lines bearing UAS-CG31148-IR-a (UAS-dGBA1a-IR-a, #14697), UAS-CG31148-IR-b (UAS-dGBA1a-IR-b, #14698), UAS-CG31414-IR-a (UAS-dGBA1b-IR-a, #21337), UAS-CG31414-IR-b (UAS-dGBA1b-IR-b, #101212), UAS-CG31414-IR-c (UAS-dGBA1b-IR-c, #21336), UAS-CG9092-IR-a (UAS-dβ-Gal-IR-a, #51445), and UAS-CG9092-IR-b (UAS-dβ-Gal-IR-b, #51446) were obtained from the Vienna Drosophila Research Center. Transgenic fly lines bearing GMR-GAL4 have been described previously (49). Male flies were
used in all the experiments.

*GCase activity assay.*

Ten fly brains were homogenized in 60 μl lysis buffer (0.25% Tauroso-deoxycholic acid and 1 mM ethylenediaminetetraacetic acid (EDTA) in citrate-phosphate buffer, pH 5.4) with a protease inhibitor mixture (cOmplete, EDTA-free, Roche Applied Science), and centrifuged at 20,000 × g for 20 min at 4 °C. The supernatants were collected and used for the assay. Lysates containing 2.5 μg protein were assayed for GCase activity in 100 μl of lysis buffer containing 1 mM 4-methyl-umbeliferyl-glucopyranoside (MUGlc, Sigma-Aldrich #M3633) for 60 min at 37 °C. The reaction was stopped by the addition of an equal volume of 1 M glycine, pH 12.5, and the amount of the product 4-methyl-umbeliferone (4-MU) was determined by measuring the fluorescence of the sample (excitation 355 nm, emission 460 nm) in a Wallac Victor plate reader (Perkin Elmer), using 4-MU (Sigma-Aldrich #M1381) standards.

*qRT-PCR.*

Total RNA was isolated from the brains of 1-week-old flies (5 brains per sample). Reverse transcription and real-time PCR were performed as described previously (50). The sequences of the forward and reverse primers were as follows:

* dGBA1a forward, 5′-CTGCTGGGAAGCTGGGAGCG-3′
* dGBA1a reverse 5′-TGGGGCATCCACCGTGTTGT-3′
* Rpl32 forward, 5′-AGCGACCAAGCACTTCATCCGCA-3′
* Rpl32 reverse, 5′-GCACCTTGTGCAACCAGGAACCTTC-3′
* dβ-Gal forward, 5′-TGCTGAGCGGAGATGGTG-3′
* dβ-Gal reverse, 5′-ATACCACCTGCACCTCTG-3′
**Climbing assay.**

The climbing assay was performed according to a published protocol (51) with slight modifications. Ten to twenty male flies were placed in a conical glass tube (length, 15 cm; diameter, 2.5 cm) without anesthesia. Ten seconds after tapping the flies to the bottom of the tube, the numbers of flies in each vertical area were counted and scored as follows: score 0 (0-2 cm), 1 (2-3.9 cm), 2 (4-5.9 cm), 3 (6-7.9 cm), 4 (8-9.9 cm), 5 (10-15 cm). Five trials were performed on each group at 20 sec intervals, and the climbing index was calculated as follows: each score multiplied by the number of flies was divided by the total number of flies, and the mean score of the 5 trials was calculated. Results are presented as the mean ± S.E. of the scores obtained in 4-9 independent experiments.

**Histological and immunohistochemical analyses.**

Fly heads were fixed in Carnoy’s fixative solution and embedded in paraffin. To analyze retinal tissues, serial 3 μm sections were stained with haematoxylin and eosin, or antibodies against αSyn (1:500, syn211, Life Technologies). To quantify retinal thicknesses, sections containing the same brain region were selected for each fly, and lines were drawn from the oesophagus to the retina across the center of the medulla using Image J 1.44p software (http://imagej.nih.gov/ij/). The retinal thickness was obtained for each fly by measuring the length of the line on the retinal tissue. PK treatment was performed as described previously (21).

For whole-mount staining, adult fly brains were dissected, fixed in 4% formalin in phosphate-buffered saline (PBS) and stained using an antibody against TH (1:100, Millipore) or GlcCer (RAS_0011, 1:50, Glycobiotech). The numbers of TH-positive neurons were scored under confocal microscopy (Olympus FV1000). The numbers of TH-positive cells in the PPM1
cluster per brain and those in the PPM2, PPM3, and PPL clusters per hemisphere were calculated. For quantification of anti-GlcCer staining, we selected the outer cellular cortex layer as a representative region because many neuronal cell bodies are detected in this region. Signal intensities were quantified using Image J 1.44p software. Thirty to sixty cells were analyzed in each brain and the mean intensity was calculated for each fly line.

**Immunoblotting.**

Fly heads were homogenized in Triton lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, and 1 mM EDTA with the protease inhibitor mixture (cOmplete, EDTA-free), centrifuged at 15,000 × g for 20 min at 4 °C, and the supernatants were collected as the Triton-soluble fractions. For the preparation of Triton-insoluble fractions, the remaining pellets were washed twice with Triton lysis buffer and lysed in sodium dodecyl sulfate (SDS) buffer (2% SDS, 50 mM Tris-HCl, pH 6.8, 150 mM NaCl). The proteins were separated by 5%-20% polyacrylamide gels, transferred to polyvinylidene fluoride membranes (Millipore) and incubated with PBS containing 0.4% paraformaldehyde for 30 min at room temperature (52) before blocking with 3% bovine serum albumin in PBS containing 0.1% Tween 20. The antibodies used in this study were as follows: anti-αSyn (1:1,000, BD Transduction Laboratories) and anti-β-tubulin (1:2,000, Developmental Hybridoma Bank). To assess the PK resistance of αSyn, fly heads were homogenized in PBS and centrifuged at 1,000 × g for 5 min at 4 °C. The supernatants were collected and the aliquots were treated with PK (final concentration: 0, 1, 5, 10, or 50 μg/ml) for 30 min at 37 °C. The reaction was stopped by the addition of SDS sample buffer. The samples were incubated for 10 min at 95 °C, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and subjected to immunoblotting. Signal intensities were quantified by densitometry using Multi Gauge software.
Glycosphingolipid extraction and TLC analysis were performed as described previously (53, 54) with slight modifications. Total lipid was extracted from 10 heads of 1-week-old flies, according to the method of Bligh and Dyer. Total lipids were hydrolyzed for 2 hr at room temperature in 2 ml of a 0.1 M KOH solution in chloroform/methanol (2:1, v/v) and then neutralized with acetic acid. The lower solution layer obtained after Folch’s partition of the digests were dried and resuspended in chloroform/methanol (2:1, v/v). The lipid extracts corresponding to 5 heads, and GlcCer (1 nmol) were applied on a TLC plate (HPTLC Silica gel 60, Merck), and the plate was developed with a solvent system of chloroform/methanol/water (65:20:2, v/v/v). The lipids were visualized with orcinol/H$_2$SO$_4$ reagent. The signal intensities were quantified using Image J 1.44p software.

**In vitro assessment of the PK resistance of αSyn in the presence of lipid dispersions.**

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (16:0, PC, #850355), Glucosyl (β) ceramide (d18:1/18:0, GlcCer, #860547), and GM1 ganglioside (ovine brain, GM1, #860065) were purchased from Avanti Polar Lipids. PC and GlcCer or GM1 dissolved in high performance liquid chromatography grade chloroform at 10 mg/ml were mixed thoroughly (molar ratio 1:1) and dried under N$_2$ gas, forming a thin film on the wall of the vial. The thin film was hydrated in 100 mM NaCl and 10 mM Tris buffer, pH 7.4, with gentle mixing. Hydrated samples were sonicated in a bath sonicator (Bioruptor, Cosmo Bio) for 10 min at 25 °C. The sonication program was set to a 10 sec sonication period followed by a 10 sec pause, to prevent overheating. The samples were then subjected to 2 freeze/thaw cycles, followed by 3 cycles of
10 min sonication with water replacement until the solution was nearly clear. The lipid dispersions were made fresh for each individual experiment.

Recombinant αSyn (#S-1001, rPeptide) was freshly dissolved at 1 mg/ml in 200 mM NaCl and 20 mM Tris, pH 7.4, and the solution was centrifuged at 15,000 × g for 15 min to remove insoluble material. Lipids (20 mg/ml) were added to αSyn to final concentrations of 0.4 mg/ml (28 μM) αSyn and 8 mg/ml liposomes (1:20 mass ratio), and incubated at 37 ºC. Aliquots were collected at the indicated time points and incubated with various concentrations of PK (final concentration: 0, 0.01, 0.1, 1, or 10 μg/ml) for 20 min at 37 ºC. Reactions were stopped by the addition of SDS buffer, and the samples were separated by SDS-PAGE (25 ng αSyn per lane) for subsequent immunoblotting, as described above.

**Statistical analyses.**

Quantitative data are expressed as the mean ± S.E. The statistical differences between 2 groups were analyzed by the two-tailed Student’s t-test. For comparison between more than 2 groups, one-way or two-way ANOVA was followed by the Bonferroni test. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software).
Funding

This work was supported in part by a Health Labour Sciences Research Grant for Research on Development of New Drugs to Y.N. from the Ministry of Health, Labour and Welfare, Japan; by Grants-in-Aid for Scientific Research on Innovative Areas (Synapse and Neurocircuit Pathology and Comprehensive Brain Science Network to Y.N.) and for Young Scientists (B) to M.S., by a Comprehensive Brain Science Network Award for Young Scientists to M.S. from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan; by a grant for Practical Research Project for Rare/Intractable Diseases to Y.N. from Japan Agency for Medical Research and Development; by a grant from Core Research for Evolutional Science and Technology (CREST) of the Japan Science and Technology Agency to Y.N., by Intramural Research Grants (grant number 24-5, 27-7, and 27-9) for Neurological and Psychiatric Disorders to Y.N. from the National Center of Neurology and Psychiatry. M.S. has received fellowships from the Japan Intractable Disease Research Foundation.

Acknowledgements

We thank Drs. H. Akiko Popiel, Morio Ueyama, Yuji Saitoh, Eiko Minakawa (National Center of Neurology and Psychiatry), and Tadashi Uemura (Kyoto University) for helpful discussions. We thank Hisae Kikuchi, Tomoko Okada, and Hiroko Bonkohara for their technical assistance.

Conflict of Interest Statement

The authors declare no competing financial interests.
References


Legends to Figures

Figure 1. Glucocerebrosidase (GCase) deficiency accelerates \(\alpha\)-synuclein (\(\alpha\)Syn) toxicity in \(Drosophila\). (A) GCase activity of 1-week-old fly brains expressing an inverted repeat (IR) RNA targeting the \(Drosophila\) homologues of the \(GBA1\) gene, namely, \(CG31148\) (\(dGBA1a\)) or \(CG31414\) (\(dGBA1b\)), showing 49\% or 31\% amino acid similarity to the human gene, respectively. An IR targeting \(GFP\) (Ctr-IR) was used as a control, and transgene expression was induced by the pan-neuronal \(elav\)-GAL4 driver. Data are expressed as the mean \(\pm\) S.E. (n = 3). **\(p < 0.01\) (one-way ANOVA with Bonferroni test). (B) qRT-PCR analysis of \(dGBA1a\) mRNA. Total RNA was extracted from 1-week-old fly brains, and relative amounts of \(dGBA1a\) mRNA were analyzed by qRT-PCR. Each value was normalized to the amount of a ribosomal protein gene \(RpL32\) mRNA. Data are expressed as the mean \(\pm\) S.E (n = 4–5). ***\(p < 0.001\) (one-way ANOVA with Bonferroni test). (C) \(dGBA1\) knockdown accelerates the locomotor dysfunction of \(\alpha\)Syn flies. Human wild-type \(\alpha\)Syn was co-expressed with \(Ctr-IR\) or \(dGBA1a\)-IR in the fly neurons. Locomotor function was evaluated by the climbing assay. Data are expressed as the mean \(\pm\) S.E. (n = 4–9). **\(p < 0.01\), ***\(p < 0.001\) EGFP/Ctr-RNAi vs \(\alpha\)Syn/dGBA1a-RNAi-b flies, \(^\dagger\)\(p < 0.05\) \(\alpha\)Syn/Ctr-RNAi vs \(\alpha\)Syn/dGBA1a-RNAi-a flies (two-way ANOVA with Bonferroni test). (D) Immunohistochemical staining of dopaminergic (DA) neurons in the fly brain. Representative images of protocerebral posterior lateral (PPM) 2 clusters in 6-week-old fly brains stained with an anti-tyrosine hydroxylase (TH) antibody. The bottom image indicates the location of the major DA neuron clusters in the fly brain. PPL: protocerebral posterior lateral. Scale bar, 10 \(\mu\)m. (E) Average numbers of TH-positive neurons per DA cluster in 6-week-old flies. Data are expressed as the mean \(\pm\) S.E. (n = 6-10 flies). **\(p < 0.01\), ***\(p < 0.001\) vs EGFP/Ctr-RNAi flies, \(^\dagger\)\(p < 0.05\) EGFP/Ctr-RNAi vs \(\alpha\)Syn/dGBA1a-RNAi-b flies (two-way
ANOVA with Bonferroni test). (F) dGBA1 knockdown exacerbates the compound eye degeneration of αSyn flies. Transgene expression was induced by the eye-specific GMR-GAL4 driver. Light microscopic images of the external eye morphologies (upper panels) and haematoxylin and eosin staining images of retinal paraffin sections (middle and lower panels) of 3-week-old flies. Fly eyes co-expressing αSyn and dGBA1a-IR (αSyn/dGBA1a-RNAi) showed photoreceptor degeneration resulting in the thinning and vacuolization (arrowheads) of retinal tissue. The black lines indicate the retinal thicknesses quantified in G. Upper scale bar, 10 μm; lower scale bar, 100 μm. (G) Quantification of retinal thicknesses. Data are expressed as the mean ± S.E. (n = 9–11 flies). ***p < 0.001 (two-way ANOVA with Bonferroni test). Fly genotypes in C-E: EGFP/Ctr-RNAi, elav-GAL4;UAS-EGFP/+;UAS-GFP-IR/+;
EGFP/dGBA1a-RNAi-a, elav-GAL4;UAS-EGFP/+;UAS-dGBA1a-IR-a/+;
EGFP/dGBA1a-RNAi-b, elav-GAL4;UAS-EGFP/UAS-dGBA1a-IR-b; αSyn/Ctr-RNAi,
elav-GAL4;UAS-h[WT]αSyn/UAS-GFP-IR; αSyn/dGBA1a-RNAi-a,
elav-GAL4;UAS-h[WT]αSyn/UAS-dGBA1a-IR-a; αSyn/dGBA1a-RNAi-b,
elav-GAL4;UAS-dGBA1a-IR-b+/UAS-h[WT]αSyn/. Fly genotypes in F-G were the same as those in C-E, except that the driver was GMR-GAL4.

Figure 2. GCase deficiency accelerates the accumulation of proteinase K (PK)-resistant αSyn without alterations in the total amount of αSyn. (A-C) Immunoblotting analysis of αSyn. Triton X-100-soluble and -insoluble head lysates of 6-week-old flies were subjected to immunoblotting. Arrowheads indicate αSyn monomers, and the arrow indicates SDS-resistant αSyn dimers. Asterisks indicate non-specific bands. The elav-GAL4 driver was used for transgene expression. B and C show quantifications of the amounts of Triton X-100-soluble (B) and -insoluble (C) αSyn. Data are the mean ± S.E. (n = 3-4). n.s., not significant (Student t-test). (D) PK resistance
of αSyn. Head lysates were treated with PK at the indicated concentrations for 30 min at 37 °C, followed by immunoblotting analysis. The graph shows relative amounts of undigested full-length αSyn upon treatment with 1 μg/ml PK. Data are the mean ± S.E. (n = 3). *p < 0.05 (Student t-test). (E) Immunohistochemical detection of PK-resistant αSyn. Retinal tissue sections were either untreated or treated with 25 μg/ml PK for 30 min at 37 °C before probing with an anti-αSyn antibody. The GMR-GAL4 driver was used for transgene expression. Bar, 50 μm. Fly genotypes used in A-D and E are the same as Fig. 1C-E and F-G, respectively.

**Figure 3.** Glucosylceramide (GlcCer) accumulates in the dGBA1a-RNAi fly brain. (A) Thin-layer chromatography (TLC) analysis of GlcCer using 3-week-old fly head lipid extracts demonstrated that the amount of GlcCer is increased by dGBA1 knockdown. The elav-GAL4 driver was used for transgene expression. Arrowheads indicate GlcCer bearing different ceramide conformation. The standard is GlcCer (d18:1/18:0). (B) Quantification of the TLC analysis. Data are shown as the mean ± S.E. (n = 4). *p < 0.05 (one-way ANOVA with Bonferroni test). (C) Immunostaining of GlcCer. The brains of 2-week-old flies were stained with an anti-GlcCer antibody. Photomicrographs show the cells in the outer cellular cortex layer (arrowheads). The elav-GAL4 driver was used for transgene expression. Upper scale bar, 100 μm; lower scale bar, 5 μm. (D) Quantification of the GlcCer staining (n = 7-10 flies). *p < 0.05 (one-way ANOVA with Bonferroni test).

**Figure 4.** GlcCer promotes the formation of PK-resistant αSyn in vitro. Recombinant αSyn was mixed with lipid dispersions consisting of phosphatidylcholine (PC) with or without GlcCer and incubated for 1 (A), 7 (B), or 14 (C) days. After PK digestion, αSyn was detected by immunoblotting. Arrowheads indicate full-length αSyn, and arrows indicate the SDS-resistant
\(\alpha\)Syn dimer quantified in D. The graphs under each of the immunoblotting images show the relative amounts of residual full-length \(\alpha\)Syn. Data are expressed as the mean ± S.E. (n = 3-4). *\(p < 0.05\), **\(p < 0.01\) (two-way ANOVA with Bonferroni test). (D) Quantification of the \(\alpha\)Syn dimer. Ratio of \(\alpha\)Syn dimer to monomer were quantified and expressed as the mean ± S.E. (n = 3-4). *\(p < 0.05\), ***\(p < 0.001\) (two-way ANOVA with Bonferroni test).

**Figure 5.** Deficiency of \(\beta\)-galactosidase (\(\beta\)-Gal) accelerates \(\alpha\)Syn toxicity in flies and GM1 ganglioside (GM1) promotes the formation of PK-resistant \(\alpha\)Syn *in vitro*. (A) qRT-PCR analysis of \(d\beta\)-Gal mRNA. The amounts of \(d\beta\)-Gal mRNA were normalized to the amount of \(Rpl32\) mRNA. The elav-GAL4 driver was used for transgene expression. Data are expressed as the mean ± S.E (n = 4). ***\(p < 0.001\) (one-way ANOVA with Bonferroni test). (B) \(d\beta\)-Gal knockdown exacerbates the locomotor dysfunction of \(\alpha\)Syn flies. The climbing scores of 5-week-old flies are shown as the mean ± S.E. (n = 3-9). *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\) (two-way ANOVA with Bonferroni test). The elav-GAL4 driver was used for transgene expression. (C) PK resistance of \(\alpha\)Syn. Head lysates of \(\alpha\)Syn flies co-expressing Ctr-IR (Ctr-RNAi) or \(d\beta\)-Gal-IR (\(d\beta\)-Gal-RNAi) under the elav-GAL4 driver were treated with PK at the indicated concentrations for 30 min at 37°C, followed by immunoblotting analysis. The graph shows relative amounts of undigested full-length \(\alpha\)Syn treated with 0.5 \(\mu\)g/ml PK. Data are expressed as the mean ± S.E. (n = 3). *\(p < 0.05\), **\(p < 0.01\) (one-way ANOVA with Bonferroni test). (D-F) *In vitro* assay for the formation of PK-resistant \(\alpha\)Syn by GM1-containing liposomes. Recombinant \(\alpha\)Syn was mixed with lipid dispersions consisting of PC with or without GM1 or GlcCer, and incubated for 1 (D), 7 (E), or 14 (F) days. The graphs under each of the immunoblotting images show the relative amounts of residual full-length \(\alpha\)Syn. Data are expressed as the mean ± S.E. (n = 4). *\(p > 0.05\), **\(p < 0.01\), ***\(p < 0.01\), PC vs
PC + GM1 (two-way ANOVA with Bonferroni test). (G) Quantification of the αSyn dimer.

Ratios of αSyn dimer to monomer are expressed as the mean ± S.E. (n = 4). *p < 0.05, **p < 0.001 (two-way ANOVA with Bonferroni test).
Figure 3

A

B

C

D

GlcCer (standard)

GlcCer

Ctr-RNAi
dGBA1a-RNAi-a
dGBA1a-RNAi-b

Legend

Related amount of GlcCer

Ctr-RNAi
dGBA1a-RNAi-a
dGBA1a-RNAi-b

Legend

Legend

Ctr-RNAi
dGBA1a-RNAi-a
dGBA1a-RNAi-b

Legend

Legend

Ctr-RNAi
dGBA1a-RNAi-a
dGBA1a-RNAi-b

Legend

Legend

GlcCer intensity

Ctr-RNAi
dGBA1a-RNAi-a
dGBA1a-RNAi-b
Figure 4

A  Day 1

B  Day 7

C  Day 14

D

PK (µg/ml)

Undigested αSyn (%)  PK (µg/ml)

Undigested αSyn (%)  PK (µg/ml)

Undigested αSyn (%)  PK (µg/ml)

PK (µg/ml)

αSyn/αSynmonomer ratio (a.u.)

Incubation time (days)