Glucocerebrosidase gene therapy prevents α-synucleinopathy of midbrain dopamine neurons

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β-glucocerebrosidase (GCase) and clinical and neuropathological evidence links GBA1 to PD and related α-synucleinopathies (Eban et al., 2006; Gegg et al., 2012; Neumann et al., 2009; Rocha et al., 2015a; Sidransky et al., 2009; Velayati et al., 2010). Approximately 4–7% of PD-patients are carriers of a heterozygous GBA1 mutation, resulting in 30–40% diminished GCase activity (Neumann et al., 2009; Sidransky et al., 2009). PD-patients who harbor a GBA1 mutation that results in a reduction in GCase activity usually develop more severe symptoms, are often diagnosed earlier and are associated with increased α-synuclein accumulation than non-GBA1 mutation carrying PD-patients (Bras et al., 2009; Murphy et al., 2014; Sidransky et al., 2009). As a consequence of this genetic link, attention is being focused on uncovering the relationship between GCase and sporadic PD, which should give insight into the pathophysiology of the disease. Several studies, including our own, have demonstrated a reduction of GCase activity in the brain of sporadic PD patients (Gegg et al., 2012; Rocha et al., 2015a), and our data also showed that reduced GCase activity is paralleled by increased levels of the glycolipid substrate glucosylsphingosine. Moreover, we have shown that there is a progressive age-dependent reduction in GCase activity in healthy subjects in brain regions most affected in PD (Rocha et al., 2015a).

Diminished GCase activity can promote α-synuclein aggregation in vitro and in vivo (Mazzulli et al., 2011; Sardi et al., 2011) and systemic administration of the GCase inhibitor, conduritol β-epoxide, CBE, induces α-synuclein aggregation in the substantia nigra of wildtype mice (Rocha et al., 2015b). Overexpression of GBA1 has been shown to...
reduce α-synucleinopathy (Sardi et al., 2011; Sardi et al., 2013) and therefore would presumably protect vulnerable nigral dopamine neurons from PD-like degeneration. To investigate the therapeutic potential of GCase for PD, we overexpressed GBA1 by means of adenoviral-associated virus (AAV) in two rodent models of α-synucleinopathy. To determine whether overexpression of GBA1 can reduce α-synuclein inclusions in the striatum and substantia nigra we used a transgenic mouse model causing widespread α-synucleinopathy (Fleming et al., 2004; Hallett et al., 2012). Second, to measure the direct neuroprotective effects of increased GCase activity on midbrain dopamine (DA) neurons, we utilized a well-established rat model of α-synucleinopathy that causes progressive loss of DA neurons (Chung et al., 2009).

2. Materials and methods

2.1. Animals

Transgenic mice that overexpress human wildtype α-synuclein (AS0) on the Thy-1 promoter (Rockenstein et al., 2002; Fleming et al., 2004; Hallett et al., 2012) were bred in house. Male mice were used for experimental procedures. Female Sprague–Dawley rats approxi mately 250–300 g (Charles River Laboratories) were used for the neuro-protection experiment. Animals were housed in standard conditions in a dark/light cycle of 12 h, with ad libitum access to food and water. All animal procedures were performed in accordance with National Institute of Health guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) at McLean Hospital, Harvard Medical School.

Two- to three-month old AS0 mice were randomly selected for one of two treatment groups: either AAV2/2-synapsin GFP (n = 10) or AAV2/5-synapsin GBA1 (n = 13). AAV viruses containing each of the transgenes were delivered to mice by intra-cerebral injections and mice were returned to their homecages after the procedure. A cohort of these mice was euthanized 3-months post-gene delivery for biochemical analysis (AAV2/2-synapsin GFP, n = 6; AAV2/5-synapsin GBA1, n = 8), and the remaining mice were euthanized 8-months post-gene delivery for histological analysis (AAV2/2-synapsin GFP, n = 4; AAV2/5-synapsin GBA1, n = 5).

Female rats (250–300 g) were randomly selected for one of three treatment groups: either AAV2/2-synapsin GFP (n = 8) or AAV2/2-synapsin A53T α-synuclein + AAV2/2-synapsin GFP (n = 14) or AAV2/2-synapsin A53T α-synuclein + AAV2/2-synapsin GBA1 (n = 15). AAV viruses containing each of the transgenes were delivered to rats by intra-cerebral injections and rats were returned to their homecages after the procedure. A cohort of these rats was euthanized 8-weeks post-gene delivery for biochemical analysis (AAV2/2-synapsin GFP, n = 4; AAV2/2-synapsin A53T α-synuclein + AAV2/2-synapsin GFP, n = 5; AAV2/2-synapsin A53T α-synuclein + AAV2/2-synapsin GBA1, n = 5), and the remaining rats were euthanized 24-weeks post-gene delivery for histological analysis (AAV2/2-synapsin GFP, n = 4; AAV2/2-synapsin A53T α-synuclein + AAV2/2-synapsin GFP, n = 9; AAV2/2-synapsin A53T α-synuclein + AAV2/2-synapsin GBA1, n = 10).

2.2. Recombinant AAV preparation

For the WT and AS0 mouse surgeries, AAV2/5-GBA1 vector containing the coding sequence for the human GBA1 gene and under the synapsin promoter was injected into various brain regions. The final titers for the vectors encoding human GBA1 and GFP were 2.0 × 1012 genome copies/mL. For the rat surgeries, AAV2/2-GBA1 and AAV2/2-A53T-α-synuclein vectors containing the coding sequences for the human GBA1 gene and for the human α-synuclein gene, respectively, were co-injected into the substantia nigra in the same injectate, at a final titer of 2.0 × 1012 genome copies/mL. Vectors were under the control of the synapsin promoter. The GBA1 plasmid was obtained from Origene, and Virovek completed virus production. Details on the virus production for mutant A53T-α-synuclein have been previously described (Chung et al., 2009).

2.3. Stereotaxic surgeries

Stereotaxic coordinates for WT and AS0 mice surgeries were obtained using a mouse atlas by Paxinos and Watson (1986). Before surgery, mice were anesthetized using Nembutal sodium (50 mg/kg). The mice were placed in a stereotaxic frame (Stoelting), where a 10 μL Hamilton syringe and 31 gauge needle were used as a delivery system. Two injection sites into the striatum were achieved using the following coordinates: anteroposterior (AP): +1.4, +0.1 mm; mediolateral (ML): −1.5, −2.0 mm; and dorsoventral (DV): −2.5, −3.5 and −2.2 mm, all relative to bregma. Two injections into the hippocampus were achieved using the following coordinates: AP: −2.0, −3.2 mm; ML: −1.5, −3 mm; and DV: −1.5, −3.2 mm, all relative to bregma. Two microliters of either AAV-GFP or AAV-GBA1 was injected unilaterally at a rate of 1 μL/min using a microinfusion pump (Stoelting), with a wait time of 2 min between injections.

Stereotaxic coordinates for rat surgeries were obtained using a rat atlas by Paxinos and Watson (1986). Before surgery, rats were anesthetized using xylazine and ketamine (3 mg/kg and 60 mg/kg, respectively). The mice were placed in a stereotaxic frame (Kopf), where a 10 μL Hamilton syringe and 31 gauge needle were used as a delivery system. Two injections into the substantia nigra were achieved using the following coordinates: AP: −4.8, −5.5 mm; ML: −2.1, −1.9 mm; and DV: −7.1, −7.0 mm, all relative to bregma. Toothbar was set at −3.3 mm. Two microliters of AAV-GFP, AAV-A53T α-synuclein + AAV-GFP, or A53T α-synuclein + AAV-GBA1 was injected unilaterally at a rate of 0.5 μL/min using a microinfusion pump (Stoelting) with a wait time of 5 min between injections.

2.4. Immunohistochemistry and antibodies

Animals were terminally anesthetized with sodium pentobarbital and perfused transcardially with heparinized saline (0.1% heparin in 0.9% saline) followed by 4% paraformaldehyde in phosphate buffer. Brains were removed and post-fixed in 4% paraformaldehyde for 4 hours (h) before placing them 30% sucrose. Forty-micrometer thick coronal sections were then collected, using a sledge microtome and stored in antifreeze at −20 °C until use. Free-floating mouse brain sections were collected in a 1 in 6 series and free-floating rat sections were collected in a 1 in 12 series.

The primary antibodies used included chicken anti-GFP (Aves, 1:1000), mouse anti-GBA (Abcam, 1:1000), rabbit anti-LC3 (Millipore, 1:500), mouse anti-α-synuclein (BD Transduction, 1:1000), rabbit anti-LAMP-2A (Invitrogen, 1:200), rabbit anti-p62 (Cell Signaling, 1:200), chicken anti-GAPDH (Millipore, 1:5000), anti-rabbit TH (Pelfreeze, 1:300), and anti-mouse NeuN (Millipore, 1:1000). Horseradish peroxidase-conjugated goat anti-chicken, anti-rabbit, and anti-mouse (all Jackson ImmunoResearch, 1:10000) were used for Western blot analysis. Donkey biotin-conjugated anti-rabbit (Jackson ImmunoResearch, 1:200) was used for brightfield microscopy.

Transgene expression was assessed at 24-weeks post-gene delivery in every rat using immunofluorescence based histology; co-stains were carried out using a 1 in 12 series of sections. Brain sections were rinsed 3 x for 5 min using PBS to remove antifreeze and were blocked with 10% normal serum using PBS-0.3% Triton-X for 1 h at room temperature. Sections were incubated with primary antibodies at 4 °C overnight using blocking solution. Sections were then washed 3 x using PBS and incubated with secondary antibodies diluted in PBS for 1 h at room temperature. Sections were then submersed in Hoechst (1 mg/200 mL) for 4–5 min and washed 3 x using PBS. Tissue sections were mounted using
Mowiol mounting media (Sigma) and visualized using a Zeiss LSM 510 confocal microscope using Zen 2009 software.

2.5. Glucocerebrosidase activity

GCase activity was measured in the striatum (n = 5–6), hippocampus (n = 3–6) and substantia nigra (n = 6–8) of mice treated with either AAV-GFP or AAV-GBA1 3-months post-gene delivery and in rats treated with either AAV-GFP, AAV-α-synuclein + AAV-GFP or AAV-α-synuclein + AAV-GBA 8 weeks post-gene delivery, using a previously described protocol with some modifications (Sellos-Moura et al., 2011); brain tissues (~5 mg) were homogenized in 300 μL of water. Samples were diluted in a 2 mg/mL BSA, citric acid sodium phosphate buffer (pH 5). Ten microliter of sample was added to 75 μL of 10 mM 4-methylumbelliferyl-β-D-glucopyranoside (Sigma, USA) substrate. After incubation with the substrate for 60 min at 37 °C, the reaction was terminated using 200 μL of stop solution (0.3 M glycine/0.2 M sodium carbonate, pH 10.7). Plates were read (Ex 360/Em 460) in a Molecular Devices SPECTRAMax plate reader using Softmax Pro software. Enzymatic activity was assessed from a 4-methylumbelliferyl (Sigma, USA) standard curve and normalized to protein content in each sample as determined using a BCA kit (Thermo Scientific Pierce, USA).

2.6. Staining and quantification of insoluble α-synuclein aggregates

To visualize insoluble α-synuclein aggregates, free-floating tissue sections were collected using 1 in 6 series from ASO mice injected with AAV2/5-synapsin GFP (n = 4) or AAV2/5-synapsin GBA1 (n = 5) and pre-mounted on gelatin-coated slides. Tissue sections were incubated with proteinase-K solution (10 μg/mL; Promega) for 20–30 min at 37 °C. Endogenous peroxidases were quenched in 3% hydrogen peroxide for 7 min and placed in a blocking solution (Vectashield MOM kit) for 1 h at room temperature. Tissue sections were incubated with anti-α-synuclein (1:1000; BD Transduction) overnight at 4 °C using primary antibody diluent (Vectashield MOM kit). Sections were incubated with anti-mouse biotinylated secondary antibody (1:200) for 1 h at room temperature and visualized using a standard peroxidase-based method (Vectastain Elite, ABC kit, Vector Laboratories, Burlingame CA, USA) and the chromogen, 3,3′-diaminobenzadine (Sigma). A semi-quantitative score for the number of insoluble α-synuclein aggregates was quantified using the threshold function in Image J software (Version 1.46r). Photomicrographs were taken using 25× and 60× magnification in defined regions of the substantia nigra and striatum, respectively. Photomicrographs were duplicated and converted to grayscale. The threshold of the grayscale was adjusted until only the dark α-synuclein inclusions remained visible on the image, removing all background staining. The image was then inverted and the number of α-synuclein inclusions was quantified for each image using the analyze particles function. Parameters of this function were set to measure parameters at the size (pixels²) of 1–infinity and the circularity of 0–1.

2.7. Stereology

To quantify the number of surviving dopamine neurons in the substantia nigra pars compacta, every 12th section of the substantia nigra was collected from rats injected with AAV2/2-synapsin GFP (n = 4) or AAV2/2-synapsin A53T α-synuclein + AAV2/2-synapsin GFP (n = 9) or AAV2/2-synapsin A53T α-synuclein + AAV2/2-synapsin GBA1 (n = 10). Free-floating tissue sections were rinsed in PBS before endogenous peroxidases were quenched in 3% hydrogen peroxide for 7 min. After rinsing, the sections were incubated in 0.1% Triton X-100 in PBS containing 10% normal serum. Tissue sections were then incubated sequentially with anti-TH and anti-NeuN antibodies overnight at 4 °C. After each primary antibody, sections were washed in PBS, incubated in biotinylated secondary antibody (1:200), and visualized using a standard peroxidase-based method (Vectastain Elite, ABC kit, Vector Laboratories, Burlingame CA, USA) and the chromogen, 3,3′-diaminobenzadine (Sigma). An investigator blinded to animal treatment groups counted the number of neurons colabeled for TH and NeuN using nonbiased stereology using the optical fractionator method at 20× magnification from MBF Bioscience (Stereoinvestigator 7) and the following parameters: a 150 μm × 150 μm counting frame and 200 × 200 μm grid. One-way ANOVA statistical analyses were performed followed by Bonferroni post-hoc test.

2.8. Western blotting

Transgene expression and changes in autophagy were measured in the substantia nigra, striatum, and hippocampus by Western blot in mice treated with either AAV2/5-synapsin GFP (n = 3–8) or AAV2/5-synapsin GBA1 (n = 5–6) 3-months post-gene delivery and in the substantia nigra and striatum of rats treated with either AAV2/2-synapsin GFP (n = 4) or AAV2/2-synapsin A53T α-synuclein + AAV2/2-synapsin GFP (n = 5) or AAV2/2-synapsin A53T α-synuclein + AAV2/2-synapsin GBA1 (n = 5) 8-weeks post-gene delivery. Mice and rats were terminally anesthetized and perfused transcardially with heparinized saline (0.1% heparin in 0.9% saline) and proteinase-K solution (10 μg/mL; Promega) for 20–30 min at 37 °C. Endogenous peroxidases were quenched in 3% hydrogen peroxide for 7 min and placed in a blocking solution (Vectashield MOM kit) for 1 h at room temperature. Tissue sections were incubated with anti-α-synuclein (1:1000; BD Transduction) overnight at 4 °C using primary antibody diluent (Vectashield MOM kit). Sections were incubated with anti-mouse biotinylated secondary antibody (1:200) for 1 h at room temperature and visualized using a standard peroxidase-based method (Vectastain Elite, ABC kit, Vector Laboratories, Burlingame CA, USA) and the chromogen, 3,3′-diaminobenzadine (Sigma). Semi-quantitative scores for the number of insoluble α-synuclein aggregates were quantified using the threshold function in Image J software (Version 1.46r). Photomicrographs were taken using 25× and 60× magnification in defined regions of the substantia nigra and striatum, respectively. Photomicrographs were duplicated and converted to grayscale. The threshold of the grayscale was adjusted until only the dark α-synuclein inclusions remained visible on the image, removing all background staining. The image was then inverted and the number of α-synuclein inclusions was quantified for each image using the analyze particles function. Parameters of this function were set to measure particles at the size (pixels²) of 1–infinity and the circularity of 0–1.

2.9. Statistical analysis

All analyses were conducted using GraphPad Prism (Version 5.0) (GraphPad Software, Inc). Statistical significance was determined at the alpha level of 0.05.

3. Results

3.1. Overexpression of GCase reduced the number and size of insoluble α-synuclein aggregates in mice overexpressing human wildtype α-synuclein

The mechanism(s) by which α-synuclein oligomeric species increase neuronal vulnerability are not fully understood, but likely involve disruption of proteostasis (Kayed et al., 2004; Manning-Bog et al., 2009). Therefore, we wanted to address whether overexpression of GCase activity could decrease levels of the oligomeric and aggregated forms of α-synuclein. Unilateral injections of either AAV-GBA1 or AAV-GFP control were delivered to 2–3-month old ASO mice in the striatum, substantia nigra and hippocampus (Fig. 1A). GCase activity increased in the striatum (T1,7 = 2.602, p < 0.05), substantia nigra (T1,7 = 2.496, p < 0.05), and hippocampus (T1,7 = 4.033, p < 0.05) 3 months post-AAV injection (Fig. 1B). This corresponded to an increase in the protein levels of GCase in the substantia nigra (T1,5 = 4.033, p < 0.05), striatum (T1,7 = 2.60, p < 0.05), and hippocampus (T1,7 = 2.496, p < 0.05) (Fig. 1C).
At 5 months of age ASO mice display a modest amount of α-synuclein pathology (Fleming et al., 2004; Hallett et al., 2012). Overexpression of GBA1 in ASO mice increased LC3-II levels in the substantia nigra (T2,7 = 5.06, p < 0.05) and striatum (T2,7 = 6.65, p < 0.05), in comparison to AAV-GFP injected ASO mice (Fig. 2A). This increase in LC3-II coincided with a 40% reduction in high molecular weight (HMW) α-synuclein oligomeric species in the SN (T2,7 = 3.18, p < 0.05) and in the striatum (T2,7 = 4.67, p < 0.05) in comparison to AAV-GFP injected mice (Fig. 2B). In contrast, levels of LC3-II and HMW α-synuclein remained unchanged in the hippocampus in AAV-GBA1 treated ASO mice in comparison to ASO mice treated with AAV-GFP (Fig. 2A and B).

In addition to reducing soluble α-synuclein oligomeric intermediates, we wanted to assess whether GBA1 overexpression could decrease the number of insoluble α-synuclein aggregates when ASO mice were 10–11 months of age (corresponding to 8 months post-AAV injection), a timepoint when α-synuclein pathology is extensive (Chesselet et al., 2012). Transgene expression of GBA1 was first confirmed at 8 months post-gene delivery in the substantia nigra (Fig. 3A), striatum (Fig. 3D) and hippocampus (data not shown) by immunofluorescence. We used a well-established histology protocol to visualize aggregates of insoluble α-synuclein (Fernagut et al., 2007). The number of protease-K resistant insoluble α-synuclein aggregates was quantified and normalized to the contra-lateral (non-injected) side. Overexpression of GBA1 reduced the number and size of the protease-K resistant insoluble α-synuclein aggregates in the substantia nigra (T2,7 = 2.733, p < 0.05) and striatum (T2,8 = 2.863, p < 0.05), 8-months post-gene delivery. To determine if AAV-GBA1 prevented the accumulation of α-synuclein aggregates of different sizes or was able to prevent accumulation of aggregates of a specified size, we arbitrarily grouped the α-synuclein aggregates into groups and used the same protocol to quantify the reduction in the number of aggregates. AAV-GBA1 caused a reduction in the number of α-synuclein aggregates in the substantia nigra between 0 and 10 μm² (T2,7 = 2.73, p < 0.05), 11–20 μm² (T2,7 = 2.59, p < 0.05), 21–50 μm² (T2,7 = 2.61, p < 0.05) and >100 μm² (T2,7 = 2.73, p < 0.05) compared to AAV-GFP injected littermate control ASO mice (Fig. 3B–C). AAV-GBA1 also reduced the number of α-synuclein aggregates in the striatum between 0 and 0.5 μm² (T2,8 = 5.05, p < 0.05), 0.6–1 μm² (T2,8 = 4.34, p < 0.05), 2–5 μm² (T2,8 = 2.85, p < 0.05) and 6–10 μm² (T2,7 = 5.06, p < 0.05) compared to AAV-GFP injected littermate control ASO mice (Fig. 3E–F). Therefore, AAV-GBA1 prevented the accumulation of aggregates of all sizes except those between 51 and 100 μm². There was a trend towards a reduction in number of aggregates between 51 and 100 μm², however, due to the variability this was statistically insignificant.

3.2. Overexpression of GCase protects nigrostriatal dopaminergic neurons from degeneration

The therapeutic potential of GBA1 was tested using a rat model of α-synucleinopathy, which causes a 50% reduction in TH-positive neurons and accumulation of α-synuclein inclusions, 24-weeks post-AAV injection (Chung et al., 2009). This is in contrast to our first model of α-synucleinopathy, which exhibits a progressive and widespread accumulation of insoluble α-synuclein but does not cause a loss of the TH-positive dopamine neurons in the substantia nigra.

Naive rats received an intra-nigral injection of either (1) AAV-GFP (control), (2) AAV-GFP + AAV-mutant A53T α-synuclein, or (3) AAV-GBA1 + AAV-mutant A53T α-synuclein (Fig. 4A). AAV-GBA1 caused an increase in GCase activity (F2,11 = 32.34, p < 0.05) in the substantia nigra, 8-weeks post-AAV injection (Fig. 4B), which was paralleled by an increase GCase protein levels (F2,11 = 9.994, p < 0.05) (Fig. 4C). At 24-weeks post-AAV injection, the transgene expressions of the viruses remained elevated in the dopamine neurons of the substantia nigra (Fig. 4D). Consistent with previous reports (Chung et al., 2009), we found that intra-nigral administration of AAV-mutant A53T α-synuclein caused a significant (50%; F2,22 = 7.89, p < 0.05) decrease in the number of dopamine neurons compared to control rats injected with AAV-GFP (Fig. 5). Co-administration of AAV-GBA1 with AAV-mutant A53T α-synuclein prevented mutant A53T α-synuclein induced degeneration of the dopamine neurons of the substantia nigra pars compacta and dopamine neuron number was comparable to that in AAV-GFP injected control rats (Fig. 5). Therefore, this data demonstrates that intra-nigral administration of AAV-GBA1 can rescue dopaminergic neurons from A53T α-synuclein-induced degeneration.
Mutations in GBA1 are frequently observed in sporadic PD patients. These mutations are associated with a reduction in GCase activity and an increase in Lewy body inclusions (Neumann et al., 2009; Sidransky et al., 2009). Genetic studies have also implicated other lysosomal-associated genes with sporadic PD. A loss-of-function mutation in the lysosomal cation-transporting ATPase, Adenosine-3-phosphate 13A2 (ATP13A2) will result in abnormal lysosomal acidification in Kufer-Rakeb syndrome as well as some juvenile and young-onset forms of PD (Di Fonzo et al., 2007; Park et al., 2011; Ramirez et al., 2006). Mutations in the lysosomal proton transporting ATPase, ATP6AP2 causes X-linked parkinsonism with spasticity (Kovatska et al., 2013), and a mutation in VPS35, an endosomal–lysosomal trafficking gene, causes autosomal dominant forms of PD (Vilarino-Guell et al., 2011; Zimpich et al., 2011). More recently, a loss-of-function mutation in SMPD1 (p.L302P), a gene linked with Niemann–Pick diseases Type A and Type B, and which leads to an accumulation of sphingomyelin, has been shown to be strongly associated with PD (Gan-Or et al., 2013), and the presence of phosphorylated α-synuclein overexpression was associated with diminished levels of α-synuclein in the striatum of A53T α-synuclein transgenic mice (Neumann et al., 2009; Sidransky et al., 2013). Collectively, these lysosomal gene mutations suggest that impairments in lysosomal degradation systems are implicated in the disease process and may even be causative for PD.

This report demonstrates that overexpression of GBA1 is neuroprotective and can modulate α-synuclein accumulation using two rodent models of PD. First, we demonstrate that overexpression of GBA1 increases levels of LC3-II and prevents accumulation of insoluble α-synuclein in a transgenic mouse model of α-synucleinopathy. Second, using a rat model of selective midbrain dopamine neuron degeneration, we show for the first time that overexpression of GBA1 protects against mutant A53T α-synuclein induced neurodegeneration. In support of our data, AAV-mediated overexpression of GBA1 has also been shown to prevent both α-synuclein and Tau pathology in the hippocampus in a mouse model of Gaucher’s disease and modestly diminish Tris soluble α-synuclein in the striatum of A53T α-synuclein transgenic mice (Sardi et al., 2011; Sardi et al., 2013). Collectively, these data suggest that increasing GCase activity may have therapeutic potential to treat PD.

5. Overexpression of GCase protected dopaminergic neurons in the substantia nigra against mutant A53T α-synuclein induced neurodegeneration

We have previously demonstrated that an intra-nigral injection of AAV-A53T α-synuclein induces a progressive retrograde degeneration of nigrostriatal dopamine neurons over a 6-month period, which is preceded by the development of dystrophic dopamine axons in the striatum as early as 4–8 weeks after AAV injection (Chung et al., 2009).

4. Discussion

Mutations in GBA1 are frequently observed in sporadic PD patients. These mutations are associated with a reduction in GCase activity and an increase in Lewy body inclusions (Neumann et al., 2009; Sidransky et al., 2009). Genetic studies have also implicated other lysosomal-associated genes with sporadic PD. A loss-of-function mutation in the lysosomal cation-transporting ATPase, Adenosine-3-phosphate 13A2 (ATP13A2) will result in abnormal lysosomal acidification in Kufer-Rakeb syndrome as well as some juvenile and young-onset forms of PD (Di Fonzo et al., 2007; Park et al., 2011; Ramirez et al., 2006). Mutations in the lysosomal proton transporting ATPase, ATP6AP2 causes X-linked parkinsonism with spasticity (Kovatska et al., 2013), and a mutation in VPS35, an endosomal–lysosomal trafficking gene, causes autosomal dominant forms of PD (Vilarino-Guell et al., 2011; Zimpich et al., 2011). More recently, a loss-of-function mutation in SMPD1 (p.L302P), a gene linked with Niemann–Pick diseases Type A and Type B, and which leads to an accumulation of sphingomyelin, has been shown to be strongly associated with PD (Gan-Or et al., 2013), and the presence of phosphorylated α-synuclein in the striatum of A53T α-synuclein transgenic mice (Neumann et al., 2009; Sidransky et al., 2013). Collectively, these data suggest that impairments in lysosomal degradation systems are implicated in the disease process and may even be causative for PD.

This report demonstrates that overexpression of GBA1 is neuroprotective and can modulate α-synuclein accumulation using two rodent models of PD. First, we demonstrate that overexpression of GBA1 increases levels of LC3-II and prevents accumulation of insoluble α-synuclein in a transgenic mouse model of α-synucleinopathy. Second, using a rat model of selective midbrain dopamine neuron degeneration, we show for the first time that overexpression of GBA1 protects against mutant A53T α-synuclein induced neurodegeneration. In support of our data, AAV-mediated overexpression of GBA1 has also been shown to prevent both α-synuclein and Tau pathology in the hippocampus in a mouse model of Gaucher’s disease and modestly diminish Tris soluble α-synuclein in the striatum of A53T α-synuclein transgenic mice (Sardi et al., 2011; Sardi et al., 2013). Collectively, these data suggest that increasing GCase activity may have therapeutic potential to treat PD.

3.3. Overexpression of GBA1 prevents altered expression of autophagy-related proteins

To determine whether overexpression of GBA1 altered levels of proteins associated with either macroautophagy and/or chaperone mediated autophagy (CMA), key proteins involved in both pathways were explored at a pre-degenerative time point, 8-weeks post-AAV gene delivery. Specifically, Western blots were performed using total lysate from the SN and the striatum of naive rats that received intra-nigral injections of (1) AAV-GFP (control), (2) AAV-GFP + AAV-mutant A53T α-synuclein or (3) AAV-GBA1 + AAV-mutant A53T α-synuclein. At 8-weeks post-gene delivery, the lipidated form of LC3 (LC3-II) remained unchanged in the substantia nigra at 8 weeks after AAV injection, possibly indicating a change in p62 in the striatum of rats; however, when co-administered with AAV-GBA1, p62 was significantly increased ($F_{2,12} = 5.98, p < 0.05$) in the striatum (Fig. 6E). The CMA receptor LAMP-2A remained unchanged in the substantia nigra and striatum following a co-injection of AAV-mutant A53T α-synuclein and AAV-GBA1 (Fig. 6C,F).

Fig. 2. Intra-cerebral injection of AAV-GBA1 in ASO mice increases LC3-II and reduces levels of high molecular weight (HMW) α-synuclein, 3-months post-gene therapy delivery. Gene delivery of AAV-GBA1 increased protein levels of LC3-II in the substantia nigra and striatum in comparison to AAV-GFP control mice (A). In contrast, levels of LC3-II remained unchanged in the hippocampus (A). AAV-GBA1 caused an increase in LC3-II, which corresponded to a reduction in the number of HMW α-synuclein oligomeric species in the substantia nigra and striatum in comparison to AAV-GFP control mice (B). Consistent with protein levels of LC3-II in the hippocampus, the number of HMW α-synuclein oligomeric species remained unchanged in the hippocampus (B). N = 5 animals per group. Data are analyzed using an unpaired two-tailed T-test, *p < 0.05, graphs expressed as mean ± SEM. Western blots show two representative samples from each group.
in the striatum was prevented by co-administration of AAV-GBA1 with AAV-A53T α-synuclein. Our data did not show alterations in the protein expression of LAMP-2A, which is required for CMA, following AAV-A53T α-synuclein overexpression. There are several reports linking α-synuclein turnover with LAMP-2A and CMA (reviewed in Xilouri and Stefanis (2015)) and further in-depth studies that examine multiple time points during the degenerative period in the AAV-A53T α-synuclein model, as well as the use of lysosomal enriched fractions, may reveal further insights into LAMP-2A-mediated mechanisms.

Importantly, at 6 months after AAV injection, GBA1 overexpression protected against mutant A53T α-synuclein-induced degeneration of dopaminergic cell bodies. This is the first evidence to our knowledge demonstrating that increased GCase can prevent dopamine neuron loss in a rodent model of α-synucleinopathy. Intra-nigral injection of AAV-GBA1 was associated with increased GCase activity, and overexpression of GCase in dopaminergic neurons was maintained even up to 6-months post-gene delivery. Future studies will determine the proportion of GCase localized in lysosomes in this GBA1 overexpression paradigm—although it is possible that excess GCase may accumulate in the ER and disrupt trafficking of GCase from the ER to the lysosome, it is unlikely that in such a scenario the protective effect of AAV-GBA1 against α-synucleinopathy-induced dopamine neuron degeneration would have been observed.

The hypothesized link between GCase and lysosomal function suggested by our current findings is supported by recent data showing that a loss of GCase activity early in sporadic PD corresponds to reduced protein levels of beclin-1 (regulator of autophagy), LAMP-2A (CMA receptor), and increased oligomeric α-synuclein levels (Murphy et al., 2014). Therefore, up-regulation of specific genes in the autophagy–lysosomal degradation pathway early in the disease process may delay or even prevent the progression of PD.

6. Overexpression of GCase enhanced lysosomal activity and improved α-synuclein clearance in mice overexpressing human wildtype α-synuclein

Using a mouse model of α-synucleinopathy, which causes widespread α-synuclein inclusions in the absence of overt neurodegeneration,
overexpression of GBA1 prevented the accumulation of α-synuclein inclusions in the substantia nigra and striatum and not in the hippocampus. GBA1 overexpression has previously been shown to reduce levels of soluble α-synuclein in the striatum in a different mouse model of α-synucleinopathy that overexpresses mutant A53T α-synuclein (Sardi et al., 2013). However, to our knowledge, ours is the first report demonstrating that GBA1 gene therapy can initiate the clearance of insoluble α-synuclein aggregates within the substantia nigra. In the current study, the

Fig. 4. Intra-nigral administration of AAV-GBA1 causes an increase in GCase protein levels and activity. Naive rats received an intra-nigral injection of AAV-GFP control transgene or a coinjection of either AAV-GFP + AAV-mutant A53T α-synuclein or AAV-GBA1 + AAV-mutant A53T α-synuclein (A). At 8-weeks post-AAV injection, this resulted in an increase in GCase activity in the substantia nigra (B, C). N = 5 animals per group. At 24-weeks post-AAV injection, the expression of transgenes in the substantia nigra dopamine neurons was confirmed using immunofluorescence (D) labeling for GFP, human α-synuclein and TH and GBA1, human α-synuclein and TH. N = 4–10 animals per group. Western blots show two representative samples from each group. Data are analyzed using a 1-way ANOVA followed by Bonferroni post-hoc test, *p < 0.05, graphs are expressed as mean ± SEM.

Fig. 5. Intra-nigral administration of AAV-GBA1 is neuroprotective against the A53T α-synuclein induced neurodegeneration. Immunolabeling for TH (brown) and NeuN (black) (A, B, C), and subsequent stereological analysis of the total number of dopamine neurons (TH/NeuN colabeled cells) in the substantia nigra pars compacta, indicated that an intra-nigral administration of AAV-mutant A53T α-synuclein caused ~50% decrease in the dopamine neurons compared to control rats injected with AAV-GFP (A, B, D). Intra-nigral administration of AAV-GBA1 rescued dopamine neurons from the mutant A53T α-synuclein induced degeneration (B, C, D). N = 4–10 animals per group. Data are analyzed using a 1-way ANOVA followed by Bonferroni post-hoc test. *p < 0.05, graphs are expressed as mean ± SEM.
observed reduction in α-synuclein inclusions in the substantia nigra and striatum of ASO mice treated with AAV-GBA1 coincided with an increase in levels of LC3-II protein. Despite an increase in GCase activity in the hippocampus, levels of LC3-II were not elevated in this region. The observed increase in LC3-II in the substantia nigra and striatum through overexpression of GBA1 could indicate either an induction of macroautophagy or defective clearance of autophagosomes. Further experiments will be required to elucidate the role of LC3-II in the AAV-GBA1-mediated reduction of α-synucleinopathy. Overall, our data showing a therapeutic effect of GBA1 gene therapy on α-synucleinopathy are not surprising given that reduced levels of GCase results in the accumulation of oligomeric and aggregated forms of α-synuclein (Mazzulli et al., 2011; Osellame et al., 2013; Rocha et al., 2015b; Sardi et al., 2011; Sardi et al., 2013). Moreover, overexpression of beclin-1 causes an increase in LC3-II, which is accompanied by a reduction of cortical α-synuclein levels (Spencer et al., 2009), and overexpression of TFEB (master regulator of autophagy–lysosomal pathways) up-regulates many autophagy-related proteins and prevents the loss of nigral DA neurons caused by accumulation of toxic α-synuclein oligomers (Decressac et al., 2013). Lastly, GBA1-PD patient derived DA neurons are associated with defects in autophagic–lysosomal degradation pathways, including dysregulated LC3-II levels, reduced lysosomal GCase levels and elevated α-synuclein. These pathological changes are not found in corresponding gene corrected iPSC-derived DA neurons (Schondorf et al., 2014). Further studies will examine in more detail how lysosomal function is affected in the α-synucleinopathy models used in the current study, including markers of lysosomal activity and autophagic flux, and determine which aspects of lysosomal function are altered by GBA overexpression.

In addition to these data, in vivo and in vitro models of PD further support the role of dysfunctional autophagy–lysosomal degradation pathways in PD. The autophagy-related protein Atg7 is required for the conversion of LC3-I to its lipidated form LC3-II, and conditional knockdown of atg7 results in age-dependent neuropathological and motor deficits reminiscent of PD, including loss of dopaminergic neurons, accumulation of α-synuclein, and the accumulation of ubiquitinated protein aggregates (Ahmed et al., 2012). Moreover, overexpression of beclin-1 causes an increase in LC3-II, which is accompanied by a reduction of cortical α-synuclein levels (Spencer et al., 2009), and overexpression of TFEB (master regulator of autophagy–lysosomal pathways) up-regulates many autophagy-related proteins and prevents the loss of nigral DA neurons caused by accumulation of toxic α-synuclein oligomers (Decressac et al., 2013). Lastly, GBA1-PD patient derived DA neurons are associated with defects in autophagic–lysosomal degradation pathways, including dysregulated LC3-II levels, reduced lysosomal GCase levels and elevated α-synuclein. These pathological changes are not found in corresponding gene corrected iPSC-derived DA neurons (Schondorf et al., 2014). Further studies will examine in more detail how lysosomal function is affected in the α-synucleinopathy models used in the current study, including markers of lysosomal activity and autophagic flux, and determine which aspects of lysosomal function are altered by GBA overexpression.
Lysosomal degradation systems, as well as GCase are known to gradually decline with aging (Martinez-Vicente et al., 2005; Rocha et al., 2015a). Moreover, there is an age-dependent reduction in GCase activity in healthy subjects in the brain regions most affected in PD (Rocha et al., 2015a). It is unclear if this age-dependent reduction in GCase activity is pathological by contributing to age-related lysosomal impairments that result in deficits in autophagy degradation pathways. The only known regulator of GCase expression is the TFEB, which regulates lysosomal biogenesis, and lysosomal clearance of organelles, lipids and proteins (Settembre et al., 2011). Histological examination of postmortem PD midbrains reveals a significant reduction of nuclear TFE3 expression, and accumulation of cytoplasmic TFE3 colocalizes with α-synuclein in Lewy body containing nigral neurons (Decressac et al., 2013). Several other autophagy–lysosome related proteins, including ATP13A2 and LAMP-2A, are downregulated in dopaminergic neurons in sporadic PD patients (Decressac et al., 2013; Dehay et al., 2012; Gegg et al., 2012; Murphy et al., 2014). Disruption in autophagy–lysosomal protein levels during the early stages of PD will likely alter lysosomal biogenesis, and lysosomal clearance of organelles, lipids and proteins (Settembre et al., 2011). Histological examination of postmortem PD midbrains reveals a significant reduction of nuclear TFE3 expression, and accumulation of cytoplasmic TFE3 colocalizes with α-synuclein in Lewy body containing nigral neurons (Decressac et al., 2013). Several other autophagy–lysosome related proteins, including ATP13A2 and LAMP-2A, are downregulated in dopaminergic neurons in sporadic PD patients (Decressac et al., 2013; Dehay et al., 2012; Gegg et al., 2012; Murphy et al., 2014). Disruption in autophagy–lysosomal protein levels during the early stages of PD will likely alter lysosomal membrane properties and impair proper protein degradation, thus exacerbating any age-related lysosomal dysfunction. Diminished GCase activity levels have been reported in the amygdala, striatum and substantia nigra of non-GBA1 PD patients (Gegg et al., 2012; Rocha et al., 2015a). This reduction in GCase activity is associated with an accumulation of GluSph, high levels of α-synuclein and alterations in LAMP-2A early during the disease progression (Murphy et al., 2014; Rocha et al., 2015a).

In summary, using two models of α-synucleinopathy, we have demonstrated that GBA gene delivery to the substantia nigra and striatum can facilitate the clearance of insoluble α-synuclein aggregates and protect against mutant A53T α-synuclein induced neurodegeneration. These data validate lysosomal genes as new potential targets to reduce levels of misfolded α-synuclein in PD.

Author contributions

P.J.H. and O.I. designed research; E.M.R., G.A.S., M.A.H., J.B., J.R.M., S.C.I., E.P-T., and P.J.H. performed research; E.P., H.C., and E.B. performed data interpretation; and E.M.R., G.A.S., P.J.H. and O.I. wrote the paper.

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