Dermal fibroblasts from patients with Parkinson’s disease have normal GCase activity and autophagy compared to patients with PD and GBA mutations [version 2; peer review: 2 approved]

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Abstract

Background: Recently, the development of Parkinson’s disease (PD) has been linked to a number of genetic risk factors, of which the most common is glucocerebrosidase (GBA) mutations.

Methods: We investigated PD and Gaucher Disease (GD) patient derived skin fibroblasts using biochemistry assays.

Results: PD patient derived skin fibroblasts have normal glucocerebrosidase (GCase) activity, whilst patients with PD and GBA mutations have a selective deficit in GCase enzyme activity and impaired autophagic flux.

Conclusions: This data suggests that only PD patients with a GBA mutation have altered GCase activity and autophagy, which may explain their more rapid clinical progression.

Keywords

GBA mutations, Parkinson’s disease, Gaucher disease, fibroblasts, lysosome, autophagy
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Introduction

Gaucher disease (GD) is an autosomal recessive condition caused by deficient glucocerebrosidase (GCase) enzyme. GCase (GBA gene) normally functions to breakdown lipids in the lysosomes and mutations in GCase cause accumulation of its substrate, glucoceramide (GlcCer). These mutations result in characteristic engorged macrophages, known as Gaucher macrophages (GMs) or ‘Gaucher cells’. The GlcCer buildup in macrophages manifests as a multi-systems disorder, with signs such as hepatosplenomegaly, anaemia, thrombocytopenia, and bony impairments, reviewed by 3. GD is common in Ashkenazi Jewish populations, although the disorder is pan-ethnic. GD has been historically divided into three types, based on the severity of clinical features and neurological involvement. Type I GD is classically defined as non-neuropathic, although neurological deficits have been described in some of these patients including Parkinsonism. Type II disease usually begins in infancy, with severe neurological involvement. Type III GD is an extended form of type II, also with neurodepathic problems but patients live into adolescence and adulthood largely due to the development of enzyme replacement therapy (ERT).

It is now apparent that there is a strong genetic connection between Gaucher and Parkinson’s disease (PD). Studies have shown that in large multi-center patient cohorts, patients with PD have an increased incidence of carrying GBA mutations1-4, including 3% of sporadic PD patients and up to 15% in Ashkenazi Jewish populations with PD5. Thus, we now know that heterozygote GBA mutations are the single commonest genetic risk factor for familial and sporadic PD; leading to a more rapid progression of PD with an early onset dementia6-8. The cellular pathology in lysosomal storage disorders (LSD) is centered around the misfolding of GCase and lysosomal dysfunction. Lysosomes have also been implicated in PD and several other neurodegenerative disorders including Alzheimer’s disease and Huntington’s disease9-11, suggesting that similar underlying defects in autophagy and lysosomal dysfunction may link the pathophysiology of PD to GD12. The accumulation of substrates in the lysosome impacts on their function and other intracellular pathways, resulting in secondary changes such as an impairment of autophagy13. Lysosomal and autophagy impairments are apparent in cell line models, mouse models and in post-mortem tissue from PD patients. In PD mice models, components of lysosomal function are affected, causing a reduction in lysosomal number and accumulation of autophagosomes14. Genetic mutations causing PD have also implicated the lysosome; these include ATPase Type 13A2 (ATP13A), which encodes a lysosomal ATPase, maintains lysosomal pH, and inhibits α-synuclein aggregation in the lysosome, as seen in PD and LSD, may in turn accelerate its own aggregation.

In this study, we sought to investigate autophagic function and GCase enzyme function using cells derived from patients. We tested patients with PD with and without GBA mutations as well as individuals with GD. We sought to define the extent of reduced GCase enzyme activity in all cases, and how this relates to autophagic flux. The activity of three other housekeeping enzymes was measured, with the expectation that reduced GCase enzyme activity in all cases, and how this relates to autophagic flux. The activity of three other housekeeping enzymes was measured, with the expectation that reduced GCase enzyme activity in all cases, and how this relates to autophagic flux. The activity of three other housekeeping enzymes was measured, with the expectation that reduced GCase enzyme activity in all cases, and how this relates to autophagic flux.

Methods

Patients and fibroblast cell lines

Fibroblasts were derived from dermal skin biopsies. In Table 1 the characteristics of the 13 patient derived and 4 healthy control fibroblast cell lines are summarised. Written informed consent was taken from each participant, and the ethics for this study was approved by the Cambridge Central Research Ethics Committee (REC09/ H0311/88). HFL1 (ATCC-CCL-153) cells were obtained from the American Type Culture Collection (ATCC), expanded in standard fibroblast medium and used as controls. Skin biopsies were taken from patients with:

(i) Both GD and PD (homozygous, PD/GD), n=3
(ii) PD with one GBA mutation (heterozygous, PD GBA), n=3
(iii) PD with no GBA mutations, idiopathic PD (iPD), n=3
(iv) Healthy controls, n=4
(v) GD only (homozygous), with no PD (GD), n=4

Western blot experiments

To detect protein expression, samples obtained from the lysed cells were loaded on to a 4–12% SDS-PAGE gel (Invitrogen). Cells were harvested using either Radioimmunoprecipitation assay buffer (RIPA), containing 1.0% sodium dodecyl sulfate (SDS), 0.5% Sodium Deoxycholate, 0.1% Triton X 100, 150 mM NaCl, 50 mM Tris-HCl pH 8.0 and protease inhibitors, or NP40 (Nonidet P40) buffer containing 150 mM NaCl, 50 mM Tris-HCl pH 8.0, NP-40 1.0% and protease inhibitors. The samples were sonicated at 1 second intervals for 10 secs, centrifuged at 20,000 g for 20 minutes at 4°C and the supernatant...
was snap frozen and stored at -80°C. The total protein concentration was quantified by a BCA assay (Thermo Scientific). The gel was run in 3-(N-morpholino) propanesulfonic acid (MOPS) buffer at 100 V, 40mA for 1 hour. The proteins on the SDS gel were transferred to a polyvinylidene fluoride (PVDF) membrane at 30 V, 100 mA for 2 hours. Then the membrane was incubated with Ponceau red to detect the proteins before being blocked (room temperature (RT), 1 hr) with 5% w/v semi skimmed milk, 1% bovine serum albumin (BSA) PBS with tween (PBS-T 0.3% v/v) and incubated with primary antibodies GBA(GCase) rabbit polyclonal, sigma (G4046, rabbit polyclonal 1:50) and Lamp1 (Abcam, ab25630, mouse monoclonal 1:50). Fibroblasts were grown to 50,000 cell/well confluency on 0.1% gelatin coated covers-slips in 24-well plates overnight in DMEM containing 10% FBS, and 1% penicillin/streptomycin (pen/strep) and fixed in 4% PFA for 30 minutes. Cells were washed twice with PBS, blocked in PBS, 10% FBS and 0.2% Triton and incubated for 1 hour at room temperature. After this the cells were then incubated overnight at 4°C with the relevant primary antibody (GBA (Sigma, G4046, rabbit polyclonal 1:50) and Lamp1 (Abcam, ab25630, mouse monoclonal 1:50)) in PBS, 0.2% Triton and 2% FBS. The cells were washed three times with PBS the following morning and incubated with the secondary antibodies (goat anti rabbit (green) and goat anti mouse (red) antibodies (Bio-Rad) were applied at 1:10,000 dilution in PBS, 0.2% Triton and 2% FBS in the dark for 2 hours at room temperature and washed with PBS three times for 5 minutes each and stained with Hoescht 1mg/ml in PBS for 15 minutes at room temperature in the dark. The cells were then washed with PBS 3 times for 5 minutes and imaged on a confocal microscope. In each well 10 fields of view were captured and this was repeated in triplicate.

### Gaucher disease (GD)

| Patient (ID, gender/age) | GBA Genotype | Clinical features | PD Family History |
|--------------------------|--------------|------------------|-------------------|
| GD002 F/58               | N370S?       | GD Type I        | Yes               |
| GD006 M/66               | N370S/N370S | GD Type I        | Yes               |
| GD008 M/68               | N370S/L444P  | GD Type I        | No                |
| GD009 M/69               | N370S?       | GD Type I        | No                |

### Parkinson’s disease normal GBA (IPD)

| Patient (ID, gender/age) | GBA Genotype | Clinical features | PD Family History |
|--------------------------|--------------|------------------|-------------------|
| PD005 F/68               | WT/WT        | PD               | No                |
| PD006 F/66               | WT/WT        | PD               | Yes               |
| PD007 M/65               | WT/WT        | PD               | No                |

### Healthy controls (Controls)

| Patient (ID, gender/age) | GBA Genotype | Clinical features | PD Family History |
|--------------------------|--------------|------------------|-------------------|
| C001 F/54                | WT/WT        | No neurological disease | No               |
| C002 M/57                | WT/WT        | No neurological disease | No               |
| C003 M/72                | WT/WT        | No neurological disease | No               |
| C004 M/69                | WT/WT        | No neurological disease | No               |

**Immunocytochemistry for localisation of intracellular GBA proteins**

The cellular localisation of the GBA protein in dermal fibroblasts was investigated using antibodies against GBA (Sigma, G4046, rabbit polyclonal 1:50) and Lamp1 (Abcam, ab25630, mouse monoclonal 1:50). Fibroblasts were grown to 50,000 cell/well confluency on 0.1% gelatin coated coverslips in 24-well plates overnight in DMEM containing 10% FBS, and 1% penicillin/streptomycin (pen/strep) and fixed in 4% PFA for 30 minutes. Cells were washed twice with PBS, blocked in PBS, 10% FBS and 0.2% Triton and incubated for 1 hour at room temperature. After this the cells were then incubated overnight at 4°C with the relevant primary antibody (GBA (Sigma, G4046, rabbit polyclonal 1:50) and Lamp1 (Abcam, ab25630, mouse monoclonal 1:50)) in PBS, 0.2% Triton and 2% FBS. The cells were washed three times with PBS the following morning and incubated with the secondary antibodies (goat anti rabbit (green) and goat anti mouse (red) antibodies (Bio-Rad) were applied at 1:10,000 dilution in PBS, 0.2% Triton and 2% FBS in the dark for 2 hours at room temperature and washed with PBS three times for 5 minutes each and stained with Hoescht 1mg/ml in PBS for 15 minutes at room temperature in the dark. The cells were then washed with PBS 3 times for 5 minutes and imaged on a confocal microscope. In each well 10 fields of view were captured and this was repeated in triplicate.

**Bafilomycin assay**

The dermal fibroblasts were seeded and grown at a density of 72,000 cells per well in 24-well plates overnight in DMEM with 10% FBS and 1% pen/strep. Bafilomycin A1 from Sigma-Aldrich (B1793) was used at a concentration of 0.1 mM and added to the cells for 2 hours, this concentration was derived from titrations of Bafilomycin A1 and 0.1 mM and found to be the concentration best tolerated by the cells. The cells were either treated with 1) Bafilomycin A1 alone, 2) Bafilomycin A1 and starvation medium (Hanks’ Balanced Salt Solution (HBBS) with 5% Sodium Bicarbonate) 3) starvation medium alone and 4) untreated cells as a control. The cells were incubated for 2 hours at 37°C and then washed twice with ice cold PBS. To harvest, the cells were incubated with prechilled NP-40 lysis buffer for 30 minutes with shaking at 4°C. The cells were then collected and spun at 20,000 g for 15 minutes, the supernatant was snap frozen and stored at -80°C until processed for western blotting analysis.

**Lysosomal activity assays**

**Cell preparation.** The enzyme activity assays were performed on fibroblasts harvested from 24-well plates. Cells were seeded at a density of 100,000 cells per well in DMEM containing 10% FBS and 1% pen/strep. After 24 hours the cells were harvested in ice-cold lysis buffer specific to each reaction described below. The lysate was spun for 20 minutes at 20,000 g at 4°C. The cell pellet was then resuspended in lysis buffer and sonicated for 1 minute, while keeping the sample on ice. A BCA assay (Thermo Fisher) was performed to quantify protein concentration.
Glucocerebrosidase activity assay
For the GCase activity assay, cells were harvested in lysis buffer containing 30 mM Citrate Phosphate buffer pH 5.5, 0.65% Sodium Taurocholate and 0.65% triton-X 100. Enzyme activity was measured using 4-Methylumbelliferyl (4MU) β-D-glucopyranoside, Sigma (M3633), as the substrate in running buffer containing 50 μl of 0.2M Citrate Phosphate buffer pH 4.4 (to inhibit GCase 2 and 3). Each well in a 96 well black microplate, contained 20 μg (for each sample ~20 μl of protein and 30 μl of buffer) of sample protein, 75 μl running buffer and 25 μl of 15 mM substrate (which was made up immediately before the assay at 59.15 mg/ml in Dimethyl sulfoxide (DMSO) and diluted 1/10 in running buffer and kept in the dark). Each assay was repeated in triplicate for each cell sample. The sample alone was added in triplicate as a blank and also, the buffer with the substrate alone was added as a blank. The samples were incubated for 1 hour at 37°C and the reaction was stopped using 100 μl of 0.2 M Glycine pH 10.5. The fluorescence was read on the FLUOstar Omega plate reader at excitation 355 nm and emission 460 nm.

Hexosaminidase activity assay
Total hexosaminidase activity was measured using fibroblasts harvested with lysis buffer containing 0.01 M Citrate phosphate buffer, pH 4.4, and 0.2 M Na₃HPO₄. A solution of 4 μg cell lystate (for each sample of ~10 μl of protein), 95 μl of 0.01 M Citrate Phosphate buffer and 20 μl of 2.5 mM 4-MU α-N-acetyl-b-D-glucosaminide, Sigma (M2133), in 0.01 M Citrate phosphate buffer was added to each well, the assay was performed in triplicate and blanks were included in lystate alone and a separate blank (buffer and substrate alone). The reaction ran for 20 minutes at 37°C and was stopped by adding 100 μl of 0.17 M Glycine pH 9.8 (made up from 2.5 g glycine and 3.6 g Na₂CO₃). The activity was measured on the FLUOstar Omega plate reader at excitation 360 nm and emission 415 nm.

Galactosidase activity assay
To measure α-galactosidase activity, 15 μg of cell lystate (each sample contained ~20 μl of protein and 30 μl of buffer), was incubated with 75 μl 0.2 M Citrate Phosphate buffer pH 4.4 and 20 μl of 10 mM 4-MU α-D-galactopyranoside, Sigma (M7633). This cell lystate solution was added to each well, the assay was performed in triplicate and blanks included lystate alone and a separate blank (buffer and substrate alone). The reaction was incubated for 30 minutes at 37°C and stopped by adding 100 μl of 0.17 M Glycine pH 9.8. The activity was measured on the FLUOstar Omega plate reader at excitation 360 nm and emission 415 nm.

Mannosidase activity assay
To measure mannosidase activity, 15 μg of cell lystate (for each sample ~20 μl of protein and 40 μl of buffer), was incubated with 50 μl of 0.2M Citrate Phosphate buffer pH 4.4 and 15 μl of 5 mM 4-MU α-D-mannopyranoside, Sigma (M3657). This cell lystate solution was added to each well, the assay was performed in triplicate and blanks included lystate alone and a separate blank (buffer and substrate alone). The reaction was incubated for 20 minutes at 37°C and stopped by adding 100 μl of 0.17M Glycine pH 9.8. The activity was measured on the FLUOstar Omega plate reader at excitation 360 nm and emission 415 nm.

Gold nanoparticle analysis
The fibroblasts were plated at a density of 50,000 cells/well on 0.1% gelatin coated coverslips and placed in a 24-well plate. The cells were grown over night in DMEM with 10% FBS and 1% pen/strep. The following day Gold nanoparticles were added to the cells (AuNPS). The cells were incubated with the AuNPS for 24 hours and then washed with cell culture medium to remove the AuNPS. After 16 hours the cells were fixed using 4% formaldehyde and imaged on a brightfield microscope.

Statistical analysis
All analysis was performed using SPSS version 20.1 (IBM). Normality for all the variables was tested using a one-sample Kolmogorov–Smirnov tests. Variables which did not follow a normal distribution, were log transformed and retested for normality. Non-parametric variables were compared with a Mann-Whitney U-test or Kruskal Wallis test. Parametric variables were compared with a t-test or ANOVA. Bonferroni post analysis was applied. Each experiment was repeated a minimum of three times.

Results
GCase activity is specifically decreased in patient fibroblasts carrying a GBA mutation
We started by assessing enzyme activity as an indication of overall lysosomal health and the extent to which this is restricted to GCase activity. A panel of lysosomal enzymes were screened for activity levels and included GCase, α-galactosidase, hexosaminidase and mannosidase. The enzyme activity was assessed in all the patient and healthy control fibroblast lines (n=17), and then grouped in to their respective disease phenotypes, based on GBA genotype for the analysis. GCase activity was found not surprisingly to be significantly lower in the PD GBA, GD and PD/GD compared to controls. In contrast, the iPD group was not significantly different compared to any other groups (Figure 1A and Dataset 1). There was no significant difference seen in the other lysosomal enzymes that were looked at (Figure 1B–D) and Dataset 1).

Endogenous GCase protein expression was assessed by western blot to check if there were differences in the protein concentration between the lines (n=17). The PD/GD skin fibroblasts had significantly lower GCase protein compared to control, and iPD. There was no significant difference seen between GD, PD GBA and the HFL1 (Figure 1E and Dataset 1). Fluorescence immunostaining revealed that the endogenous GCase and Lamp1 protein co-localised in the control lines but this was not the case in the PD/GD lines (Figure 1F and Dataset 1).

We then measured lysosomal function by looking at the uptake of gold nanoparticles (AuNP), and did not find any significant difference when comparing all cell lines (Figure 1G Dataset 1). Taken together, our result show a decrease in
**Figure 1. Lysosomal enzyme activity assays and Gold nanoparticle uptake assays.**

(A) GCase activity was significantly lower in the PD GBA (83.76 ± 8.16) (p=0.023), GD (55.33 ± 11.88) (p=0.006) and PD/GD (45.56 ± 2.99) (p=0.013) compared to controls (229.67 ± 79.96). The iPD group was not significantly different compared to any other groups (134.20 ± 33.94) (p>0.05).  

(B) α-galactosidase activity for PD (212.42 ± 22.30), PD GBA (189.81 ± 12.51), GD (188.10 ± 10.02), control (257.63 ± 17.13) and PD/GD (179.00 ± 16.25) (p>0.05).  

(C) Hexosaminidase activity for PD GBA (540.10 ± 178.66), iPD (539.61 ± 203.33), GD (485.08 ± 153.99), control (931.33 ± 120.93) and PD/GD (350.65 ± 111.99) (p>0.05).  

(D) Mannosidase activity for iPD (245.33 ± 28.76), PD GBA (222.67 ± 24.81), GD (234.83 ± 26.06), control (304.00 ± 14.00) and PD/GD (210.44 ± 19.92) (p>0.05). Enzyme activity measured by the FLUOstar Omega plate reader (Ex max = 360nm, Em max = 415nm).  

(E) Western blot of GCase protein levels. No reduction in GCase expression in GD (0.74 ± 0.08), PD GBA (1.41 ± 0.60) and the HLF (1.96 ± 0.48) (all p>0.05). PD/GD (0.33 ± 0.16) was significantly lower compared to control (1.95 ± 0.15) (p=0.042) and compared to the iPD (2.05 ± 0.55) (p=0.044). Ratio of GCase protein over total actin expression as a loading control.  

(F) Fibroblasts were stained for GCase (green), Lamp1 (red) and DAPI (blue). Representative images of Control and PD/GD lines. Density was measured in Image J.  

(G) Brightfield images of cell lines with AuNP uptake. No significant difference was found when measuring gold nanoparticle density in control (1.91 x 10^6 ± 5.89 x 10^5), PD/GD (1.31 x 10^6 ± 1.79 x 10^5), GD (1.24 x 10^6 ± 3.41 x 10^5), PD GBA (1.19 x 10^6 ± 9.04 x 10^5) and iPD (1.17 x 10^6 ± 2.11 x 10^5) (all p>0.05) (A.U arbitrary units). Density was measured in Image J. For each cell line 30 fields of view were analysed from three separate repeats. The AuNP uptake data was parametric and analysed by ANOVA and Bonferroni post hoc test. Analysis was repeated in three independent experiments for each cell line. Data are presented as mean ± s.e.m. Scale bar = 100μm.
Autophagy is affected in GD and PD GBA patient fibroblasts

Autophagy has a role in quality control in the cell, and to examine this we looked at autophagosome maturation as a measure of autophagic flux in the patient fibroblasts (n=17). We first investigated the autophagosome content by measuring the ratio of LC3IIb over total actin, in cells with or without Bafilomycin A1 treatment followed by starvation. Bafilomycin A1 is a specific inhibitor of vacuolar (H+)-ATPases, and a blocker of autophagosome-lysosome fusion. If the cell has a well-regulated autophagic flux, Bafilomycin A1 should increase LC3IIb without any starvation. If the cell has normal basal flux, starvation should increase LC3IIb, due to activation of autophagy. Using a combined starvation and Bafilomycin A1 treatment should produce the maximum amount of autophagosomes possible under these starvation conditions.

In light of this, autophagic flux was found to be elevated following treatment with Bafilomycin A1 and starvation in control, iPd and PD/GD fibroblasts (Figure 2 A–C and Dataset 2). In the GD and PD GBA lines, Bafilomycin A1 treatment had no effect on LC3IIb expression levels, indicating that autophagic flux was impaired in these cells (Figure 2 and Dataset 2).
Discussion

Our results show, for the first time in a large variety of PD/GD patient fibroblasts that the only lysosomal enzyme affected by GBA mutations is GCase. This finding is consistent with other’s work, who have shown GCase to be impaired in fibroblasts from GD and PD patients although in smaller patient numbers\textsuperscript{12–20}. However we have now extended these findings to show that GCase is also abnormal in fibroblasts from patients with PD/GD and normal in iPd fibroblasts. We also found that the impairments are not due to low protein amounts in line with other studies\textsuperscript{21}. This is in contrast with findings in brain post mortem tissue, where GCase amounts have been reported to be decreased in both PD GBA and iPd cases\textsuperscript{27}. In the case of PD/GD and GD the GCase protein and activity was reduced which was expected, however, in the PD GBA the protein amount was not reduced but the activity was reduced in this case, this leads us to believe in the case of PD GBA there is an additional factor, outside the GCase protein, causing pathology in these cells which could lead to dysfunction. In contrast to other studies, we did not find that the iPd line had significantly lower GCase activity compared to controls, although we were looking at fibroblasts rather than specific neuronal cells.

The screening of such a large number of GBA carriers with PD and GD, using a panel of lysosomal enzymes has not been previously undertaken. During this screening we found that there were no impairments in any other lysosomal enzymes measured besides GCase. These findings contradict published work which have reported that Hexosaminidase is elevated in PD GBA, GD and healthy GBA carriers fibroblasts\textsuperscript{24}. However, in this paper the enzyme activity was not directly measured, and the amount was determined by western blot, thus the protein amount may be elevated, but the specific activity may not be impaired.

In terms of looking at autophagic function, we used both Bafilomycin A1 and starvation treatments and observed a normal LC3IIb level in the PD/GD, iPd and control lines. However, in the case of the PD GBA and GD lines there was no increase in LC3IIb flux upon treatment. Most of the GD lines had N370S mutations and the PD GBA lines were carriers of N370S and E326K. Our results are in agreement with recent publications that found impaired autophagic flux was apparent in fibroblasts from a GD patient with homozygous L444P mutations\textsuperscript{29} and in cases with compound heterozygous (N370S/L444P) mutations\textsuperscript{30}. However, other studies have found no impairment in autophagy, but these GD patients had Saposin C, not GBA, mutations\textsuperscript{32}.

All of the PD/GD lines in our study were compound heterozygous and two out of the three cases included L444P. These cases had normal autophagic flux, and this was the first study to assess autophagy in patients with both diseases. It may be that having both diseases together also impacts on autophagy, but autophagic function differs depending on the GBA mutation or cell type studied. There are mixed findings in relation to autophagy in PD depending on the model being tested. Autophagy has been found to be increased in fibroblasts from patients with LRRK2 mutations\textsuperscript{31}, but in cell lines it is decreased when α-synuclein is overexpressed\textsuperscript{32}. Autophagy as also been found to be impaired in iPd derived DA neurons from a PD patient with GBA mutations\textsuperscript{33,34}. In our study, autophagic flux was normal in iPd patients but abnormal in the PD GBA lines. This could be further assessed by correcting the GBA mutation using CRISPR/Cas9 gene editing to see if the impairment in autophagy is dependent on GBA gene status. In addition it may be that GCase activity and autophagy are affected by mitochondrial function, which has been implicated in PD GBA previously\textsuperscript{25}, but as of yet, not extensively studied. Another factor to consider is that the affected GCase in GBA related PD, may be in the wrong location such as trapped in the ER, which has previously been observed to occur\textsuperscript{26}.

In summary, the GBA mutated patient fibroblast lines had impairments of 20–60% GCase activity compared to controls. These diseased cells had normal lysosomal function, with independent lysosomal uptake. However, PD GBA and GD lines display normal autophagy. Our results from these genetically relevant patient cell models provide evidence that GBA mutations could lead to impaired GCase and autophagic function, which may translate to CNS neurons and thus clinical expression and progression. If so, this cell model could be used in novel drug development.

Data availability

Dataset 1: Raw data underlying the results presented in Figure 1.

- Figure 1A. GCase assay raw data and statistics.
- Figure 1B. α-galactosidase assay raw data and statistics.
- Figure 1C. Hexominidase assay raw data and statistics.
- Figure 1D. Mannosidase assay raw data and statistics.
- Figure 1E. Western blot GCase raw data, blots and statistics.
- Figure 1F. Immunocytochemistry fibroblasts were stained for GCase (green), Lamp1 (red) and DAPI (blue). Representative images of control and PD/GD lines.
- Figure 1G. Gold nanoparticles, images, raw data and statistics.

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Dataset 2: Raw data underlying the results presented in Figure 2.

- Figure 2A. Western blots for autophagy assay with iPd, PD GBA, PD/GD and control.
- Figure 2B. Western blots for autophagy assay with GD.
- Figure 2C. Autophagy assay statistics.

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Competing interests

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Open Peer Review

Current Peer Review Status: ✔ ✔

Version 2

Reviewer Report 16 February 2018
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✔ Dario Besusso
Laboratory of Stem Cell Biology and Pharmacology of Neurodegenerative Diseases, Department of Biosciences, University of Milan, Milan, Italy

I read through the new version and I am happy with the amendments.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 14 November 2017
https://doi.org/10.5256/f1000research.13081.r26388

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✔ Mia Horowitz
Department of Cell Research and Immunology, Tel Aviv University, Tel Aviv-Yafo, Israel

In the present manuscript, the authors used dermal fibroblasts to assess possible changes in acid-b-glucocerebrosidase (GCase) levels and changes in autophagy markers between three Gaucher disease (GD) patients with Parkinson disease, three carriers of GD mutations with PD, three idiopathic PD patients, four healthy controls and three GD patients with no known PD signs. They also had one human fetal lung fibroblast line (HFL1).
The authors concluded that only PD patients with a GBA mutation have altered GCase activity and autophagy, which may explain their most rapid clinical progression. The authors mentioned that there was no decrease in GCase activity in iPD patients. However, the results in Figure 1A indicate reduced GCase activity in iPD patients. It may not be significant due to the small sample used in the study, but it is obvious and cannot be overlooked. Likewise, there is a notable decrease in the level of hexosaminidase and mannosidase activity.

The authors state that: "the impairments in (GCase) activity are not due to low protein amounts, in line with other studies". However, several reports have already documented decreased GCase amounts in GD derived cells. McNeill et al. (Reference 24 in the present manuscript) showed that: “Glucosylceramidase protein levels, assessed by western blot, were significantly reduced in fibroblasts from Gaucher disease (median glucosylceramidase levels 42% of control, P < 0.001) and heterozygous mutation carriers with (median 59% of control, P < 0.001) and without (median 68% of control, P < 0.001) Parkinson's disease". Other studies described a decrease in the amount of mutant GCase in cells that derived from GD patients and carriers of GD mutations. (Jonsson et al.¹; Ron et al.²; Lu, et al.³; Bendikov-Bar et al.⁴).

The authors mention in their discussion that: “Another factor to consider is that the affected GCase in GBA related PD may be in the wrong localization such as trapped in the ER”. It has already been shown that mutant GCase is recognized as misfolded in the ER, undergoes ER retention and, depending on the ability of the ER chaperones to refold it, it will either undergo ERAD or traffic to the lysosomes (Ron et al.²).

Typographical errors:
1. The authors mention HFL1 or HLF cells and I guess they are the same.

2. GBA (or better GBA1, since there are three GBA genes: GBA1, GBA2 and GBA3) is the gene encoding acid-b-glucocerebrosidase (GCase). It is the protein (GCase) that localizes in the cell and not the GBA protein. It could also be referred to as the GBA1 encoded protein.

3. In Materials and Methods: bafilomycin assay: ...."this concentration was derived from titrations of Bafilomysin A1 and 0.1mM and found ..." The second and should be replaced by was. In the same paragraph the authors mention that after NP-40 treatment “the cells were collected”. NP40 is a detergent that disrupts the plasma membranes so following its treatment there are lysates, not cells.

4. In the galactosidase assay the authors mention: “4-MUI a-D-galactopyranosidase”, should be: 4-MU a-D-galactopyranoside.

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Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Lysosomal storage diseases, Gaucher disease

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 11 Jan 2018
Lucy Collins, Cambridge University Hospitals NHS Foundation Trust, UK

I have made these changes, thank you

Competing Interests: No competing interests were disclosed.

Reviewer Report 13 October 2017
https://doi.org/10.5256/f1000research.13081.r26387

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Dario Besusso
Laboratory of Stem Cell Biology and Pharmacology of Neurodegenerative Diseases, Department of Biosciences, University of Milan, Milan, Italy
In the manuscript by Collins L.M. et al., the authors sought to investigate glucocerebrosidase (GCase) activity and autophagic flux in fibroblast derived from Parkinson's (PD) and Gaucher disease patients (GD). GD is specifically caused by mutations in the GCase gene that drive defective enzymatic activity. Since GCase mutation is the most single commonest genetic risk factor in PD and is associated with poorer prognosis, the authors set to compare PD, GD and PD-GD patient-derived fibroblast for enzymatic activity and autophagic flux.

The major point of concern is the physiological relevance of the cell models studied. Stating the obvious, since both PD and GD have neurological components, it would have been more appropriated to reprogram the fibroblasts in iPSCs and then convert these in the relevant cell type to assess GCase activity and autophagy. Unfortunately, this requires a good year of work.

Interestingly, the authors show that mutations to the GCase gene affect both enzymatic activity (Figure 1A) and amount of protein in PD/GD (Figure 1E - Maybe also in other GBA context, but the statistical power is too low to discern this aspect). Since the first can be a consequence of the latter, as a mere suggestion, it would be of interest to investigate this aspect in more detail maybe looking at protein stability, although also this goes beyond the aim of the present manuscript.

To this point, the authors state in the discussion about the GCase activity in PD/GD “We also found that the impairments are not due to low protein amounts in line with other studies”. As far as this reviewer understand, there is a significant difference between PD/GD and controls as indicated in Figure 1E. Authors may want to clarify this point.

Major comments:
1. The most obscure point to this reviewer is the fact that PD/GD fibroblasts carrying homozygous mutation in DBA show reduced enzymatic activity but normal autophagic flux when PD GBA and GD (carrying similar mutations) have abnormal flux. Could the authors elaborate better their considerations to this point in the discussion?
2. The patient-derived cell lines are actually 13 and not 17 since 4 are derived from healthy donor. Please, amend the entire text on this regard.
3. In Figure 1A, multiple bands are visible in the WB. It is not clear which band is accounted for quantification. Please, highlight the quantified band in the figure, if possible.
4. Figure 1F will benefit from a colocalisation plot and quantification. It is not clear how many cells have been recorder for this phenotype.

Minor comments:
1. The second column of Table 1 should specify “GBA genotype” in the title, for clarity.
2. Acronyms are first presented in the method session. Since many readers do not take the time to go through the methods, I kindly suggest repeating the acronyms in the main text.
3. Figure 2C is missing legend for the color-coding. Although, is the same as previous figure, a reminder would improve reading.
4. Legend of Figure 1F it may contain text that actually belong to the panel 1E.
5. In Figure 2A, please specify the subtype of LC3 in the legend.
6. The antibody used for LC3 WB usually recognizes both the LC3II and LC3IIb form of the protein. I wonder whether the authors have an explanation for not detecting the LC3II.

Is the work clearly and accurately presented and does it cite the current literature?
Yes
Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 11 Jan 2018

Lucy Collins, Cambridge University Hospitals NHS Foundation Trust, UK

Reviewer’s comments:

The major point of concern is the physiological relevance of the cell models studied. Stating the obvious, since both PD and GD have neurological components, it would have been more appropriated to reprogram the fibroblasts in iPSCs and then convert these in the relevant cell type to assess GCase activity and autophagy. Unfortunately, this requires a good year of work.

Thank you kindly for your comments. We really appreciate your review of our manuscript. As you point out it would have been more valuable as a module of neurodegeneration to convert these cells to iPS and neurons relevant to the disease. Janelle is now pursuing this through iN models with success in different starting from different cell types.

Interestingly, the authors show that mutations to the GCase gene affect both enzymatic activity (Figure 1A) and amount of protein in PD/GD (Figure 1E - Maybe also in other GBA context, but the statistical power is too low to discern this aspect). Since the first can be a consequence of the latter, as a mere suggestion, it would be of interest to investigate this aspect in more detail maybe looking at protein stability, although also this goes beyond the aim of the present manuscript.

Thank you for the suggestion. Indeed it would have been really useful to assess the protein stability and we will consider this for future work, in the lab.

To this point, the authors state in the discussion about the GCase activity in PD/GD “We also found that the impairments are not due to low protein amounts in line with other studies”. As far as this
To clarify this point in the case of PD/GD and GD the GCase protein and activity was reduced which was expected, however, in the PD GBA the protein amount was not reduced but the activity was reduced in this case, this leads us to believe in the case of PD GBA there is an additional factor, outside the GCase protein, causing pathology in these cells which could lead to dysfunction. We have clarified this point in the text.

**Major comments:**

1. The most obscure point to this reviewer is the fact that PD/GD fibroblasts carrying homozygous mutation in DBA show reduced enzymatic activity but normal autophagic flux when PD GBA and GD (carrying similar mutations) have abnormal flux. Could the authors elaborate better their considerations to this point in the discussion?

In the PD GBA and GD lines there was no increase in LC3II flux upon treatment, and this impairment could be due to the GBA mutations in these cases. Most of the GD lines had N370S mutations and the PD GBA lines were carriers of N370S and E326K. Autophagy may vary between different patients and indeed different GBA mutations/combinations of GBA mutations. However, our results from the GD line are in agreement with a recent publication where it was found that impaired autophagic flux was apparent in fibroblasts from a GD patient with homozygous L444P mutations (de la Mata et al., 2015). Also in agreement with our study, it was shown that three GD fibroblasts lines with compound heterozygous N370S/L444P mutations had decreased autophagic flux (Lay et al., 2012). Other studies have 144 lysosomal function in fibroblasts from Gaucher and Parkinson's Disease found no impairment in autophagy (Pacheco et al., 2007; Tatti et al., 2012), but this was seen in GD patients with Saposin C mutations (Vaccaro et al., 2010).

All of the PD/GD lines in our study were compound heterozygous and two out of the three cases included L444P. These cases had normal autophagic flux, and this was the first study to assess autophagy in patients with both diseases. It may be that having both diseases together also impacts on autophagy, but autophagic function differs depending on the GBA mutation or cell type studied. It is very rare to have both PD and GD so it would also be interesting to assess autophagic function in iN cells with a-syn in these cases. Indeed recently it has been shown that loss of GCase caused impairment in autophagy that was associated with PP2A (protein phosphatase 2A), further leading to a-syn accumulation, albeit in a neuroblastoma cell line (Du et al., 2015). It would be interesting to test the function of PP2A in future experiments in the patient fibroblasts.

1. The patient-derived cell lines are actually 13 and not 17 since 4 are derived from healthy donor. Please, amend the entire text on this regard.

Text has been amended:

Fibroblasts were derived from dermal skin biopsies. In Table 1 the characteristics of the 13 patient derived and 4 healthy control fibroblast cell lines are summarised.

Autophagy has a role in quality control in the cell, and to examine this we looked at autophagosome maturation as a measure of autophagic flux in the patient and healthy control fibroblasts (n=17).
The enzyme activity was assessed in all the patient and healthy control fibroblast lines (n=17), and then grouped

1. *In Figure 1A, multiple bands are visible in the WB. It is not clear which band is accounted for quantification. Please, highlight the quantified band in the figure, if possible.*

The whole band was taken for the quantification as there are multiple forms of GBA depending on cellular location and trafficking. Similar western blots of GBA have been seen by others (McNeill et al., 2014).

1. *Figure 1F will benefit from a colocalisation plot and quantification. It is not clear how many cells have been recorder for this phenotype.*

This is a good suggestion. However, the images were captured during my PhD study and I no longer have access to a microscope or image analysis to complete this. For this study the images were included as qualitative data to display the distribution of GCase. Others prior to this study have already shown that GCase is indeed sequestered in the ER. (McNeill et al., 2014). For this qualitative study 10 fields of view were taken for each fibroblast line in three separate wells.

**Minor comments:**

1. *The second column of Table 1 should specify “GBA genotype” in the title, for clarity.*

This has been updated on page 23

1. *Acronyms are first presented in the method session. Since many readers do not take the time to go through the methods, I kindly suggest repeating the acronyms in the main text.*

I have added main acronyms to the main text.

1. *Figure 2C is missing legend for the color-coding. Although, is the same as previous figure, a reminder would improve reading.*

This has been updated and included.

1. *Legend of Figure 1F it may contain text that actually belong to the panel 1E.*

The text has been updated to correct this.

1. *In Figure 2A, please specify the subtype of LC3 in the legend.*

The subtype used was LC3IIb, this has been added to the legend

1. *The antibody used for LC3 WB usually recognizes both the LC3II and LC3IIb form of the protein. I wonder whether the authors have an explanation for not detecting the LC3II.*

The LC3II band was quite faint in these experiments. This could be due to the pathological affect of GBA in the disease states on the lysosome, as in the control lines the bands were more detectable. This could also be due to experimental technique as part of the bafilomycin assay requires
washing and harvesting the cells and LC3 quite a small protein. It is possible that LC3 levels in the cell lines are very low, on the limit of detection. Also in these patient and healthy control lines there could be a rapid turnover of LC3.

**Competing Interests:** No competing interests were disclosed.

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