Several lines of evidence indicate that mutations in the GBA gene coding for glucocerebrosidase (GCase) contribute to the development of Parkinson’s disease (PD) and dementia with Lewy bodies (DLB); however, the underlying mechanism is still to be established. Whilst there is a variable strength of association in certain populations, a large meta-analysis has demonstrated the odds ratio for any GBA mutation in patients versus controls to be 5.43 across centres, providing validation of GBA as a risk gene for PD (Sidransky et al. 2009). GBA-associated parkinsonism seems to be characterized by an earlier onset and an increased likelihood of cognitive decline and dementia, compared with typical PD (Neumann et al. 2009; Sidransky et al. 2009; Seto-Salvia et al. 2012). Features typical of DLB also cluster with GBA-associated phenotypes (Goker-Alpan et al. 2008) and population studies have shown an increased frequency of GBA mutations in DLB as in PD (Goker-Alpan et al. 2006; Mata et al. 2008). DLB, PD and PD with dementia (PDD) belong to the common spectrum of Lewy body disease.
(LBD), as they are characterized by the overlapping clinical symptoms reflecting the common pathogenesis, i.e. the dismetabolism of α-synuclein and formation of Lewy bodies in the brain (Lippa et al. 2007). Mutant GCase, therefore, may be a contributory factor to the development of Lewy body disease in general.

Homzygous mutations in the GBA gene cause Gaucher’s disease (GD), the most prevalent lysosomal storage disorder, due to deficiency of glucocerebrosidase activity, and the accumulation of its main substrate glucocerebroside (α-glucosyl-N-acylsphingosine, glucosylceramide) in lysosomes in macrophages (Barranger and Ginns 1989). GD patients present a wide spectrum of clinical phenotypes with three subtypes of GD: type 1–non-neuronopathic (MIM:230800), type 2–acute neuronopathic (MIM:230900) and type 3–subacute neuronopathic (MIM:2301000) with involvement of the nervous system in type 2 and 3, in contrast to type 1 (Barranger and Ginns 1989). Early-onset parkinsonism has also been associated with GD. Initial reports described mild late-onset GD patients with typical PD that included tremor, bradykinesia, rigidity and often cognitive decline, with a poor response to conventional anti-parkinsonian therapy (Neudorfer et al. 1996), with typical neuropathological changes (Wong et al. 2004). A family history of parkinsonism in GD probands has also been reported, demonstrating the potential predisposition of GBA heterozygotes to parkinsonism (Goker-Alpan et al. 2004). The presence of glucocerebrosidase in Lewy bodies and Lewy neurites in patients carrying GBA mutations suggests that glucocerebroside can contribute to the aggregation of α-synuclein and Lewy body formation (Goker-Alpan et al. 2010). Increased α-synuclein immunoreactivity has been observed in cells treated with conductubol B epoxide (CBE), an inhibitor of GCase activity, along with accumulation of α-synuclein within the substantia nigra in CBE-treated mice (Manning-Bog et al. 2009). This indicates that reduced glucocerebrosidase activity may promote alterations in α-synuclein biology and these changes are also present in the brains of mice and in neuronal cells carrying GBA mutations (Cullen et al. 2011; Mazzulli et al. 2011; Sardi et al. 2011). It is plausible that mutant glucocerebrosidase interferes with cellular clearance of α-synuclein via lysosomal pathway and stimulates protein aggregation (Cuervo et al. 2004; Mazzulli et al. 2011), thus predisposing to the development of synucleinopathies.

To gain an insight into the effects of glucocerebrosidase on neuronal function, we aimed to evaluate the effects of mutant GBA gene in human frontal cortex tissue by examining glucocerebrosidase protein levels and activities in GBA mutation carriers and establishing whether lysosomal changes were evident. Furthermore, a hypothesis, that the presence of misfolded proteins, perhaps glucocerebrosidase, in the ER induces ER stress and contributes to the development of LBD, was also explored.

Materials and methods

Case control analysis

All procedures were approved by the Local Research Ethics Committee. A prospective clinicopathological series of patients with DLB (n = 105) or PD (n = 79) fulfilling clinical and neuropathological criteria (Gibb and Lees 1988; McKeith et al. 1996; McKeith 2006) was screened for mutations in GBA. PD and DLB cases were grouped together as LBD since pathological changes were similar, clinical presentation differing only in the timing of any dementia in relation to PD. A cohort of elderly normal individuals (n = 164 pathologically confirmed) without a history of neurological or psychiatric impairment was also genotyped and used as a control population. DNA was extracted from frozen post-mortem brain tissue using standard methods. Seven specific mutations in GBA were identified and screened using PCR-RFLP-based assays and cycle sequencing (see Table 1 for case details and Table S1).

Tissue analysis

Post-mortem frontal cortex (Brodmann Area 8) tissue was used as an area relatively spared from major LBD pathology and cell loss, which may confound studies in regions such as substantia nigra. GBA mutation being present in all cells would be expected to have effects in most cell types. Tissue from individuals identified from the cohort as carrying GBA mutations (4 DLB, 3 PD and 5 controls) and non-carriers (7 DLB, 8 PD and 11 controls) was rapidly thawed and approximately 250 mg of grey matter homogenized over ice using a rotor stator-type homogenizer in 10 volumes of 0.32 M sucrose, 1 mM EDTA, 50 mM Tris, pH 7.4 (Sigma, Gillingham, Dorset, UK), protease inhibitor cocktail tablets EDTA-free (Roche, Burgess Hill, West Sussex, UK) containing buffer. After addition of Triton X-100 (Sigma) to a final concentration of 1%, samples were sonicated for 20 min on ice in a sonicating bath. Another subset of four human spleen and four frontal cortex tissue samples (30–180 mg wet tissue) were homogenized as previously. Triton X-100 was then added to a final concentration of 1%, followed by incubation on ice for 30 min and centrifugation at 25 200 g for 60 min. Supernatants were subjected to the further analysis as Triton X-100-soluble fractions. Protein concentration was determined using Bradford assay.

GCase enzymatic activity

To investigate the effects of GBA mutation on brain enzyme activity, a standard glucocerebrosidase activity assay at acidic pH was employed. Residual enzyme activity was determined as the production of fluorescent 4-methylumbelliferone (4-MU) from 4-methylumbelliferyl-β-D-glucopyranoside (4-MUG) substrate as described previously (Takagi et al. 1999). Sample triplicates, each containing 50 μg protein (brain homogenates) or 10 μg protein (cell lysates) were diluted in assay buffer of 50 mM citrate phosphate buffer, pH 5.0 and 0.25% sodium taurocholate (Sigma) containing 0.5 mM 4-MUG in 96-well black polystyrene plates. The reactions were incubated at 37°C for 1–2 h and terminated by addition of 200 μL of 200 mM carbonate-bicarbonate buffer, pH 10.5. Fluorescence was measured (Ex 360 nm, Em 460 nm) and a 4-MU standard solution (0–4000 nM) was used to generate a standard curve. Control of glucocerebrosidase inhibition was carried out with the addition of

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analysed by Western blot.

Treated samples and non-treated controls were resolved using 4–10% sodium dodecyl sulphate–polyacrylamide gels (Invitrogen) and treated with either PNGase F or Endo-H (New England Biolabs, Hitchin, Herts., UK) according to the manufacturer's instructions.

The residual Cathepsin D activity was measured in the human brain tissue samples using akinetic assay (Yasuda et al. 1999). Cathepsin D enzymatic activity was measured on glucocerebrosidase inhibitor CBE (Sigma), which reduced activity to approximately 15% of total values.

**Western blot analysis**

Samples of brain tissue homogenates and cell lysates containing 10 μg of protein were resolved by electrophoresis on 10% or 4–12% sodium dodecyl sulphate–polyacrylamide gels (Invitrogen, Paisley, UK), and transferred to nitrocellulose membranes using iBlot Gel Transfer Stacks (Invitrogen). The membranes were probed with primary and secondary antibodies (see Table S2) using standard methods and developed using Amersham ECL detection reagents (GE Healthcare, Buckinghamshire, UK) and exposed to autoradiography film (Kodak, Sigma, Gillingham, Dorset, UK). The blots were scanned and band intensities were analysed using ImageJ software. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to normalize the results and the ratios of mean protein/GAPDH were calculated. The results are presented as mean ± SD.

**Endo-H and PNGase F treatment**

To determine the effects of GBA mutation on glucocerebrosidase glycosylation and oligosaccharide chain development, either Endoglycosidase H, to strip proteins of high mannose oligosaccharides, or PNGase F, to remove all types of asparagine N-linked oligosaccharides, was used. Frontal cortex tissue homogenates or spleen and frontal cortex Triton X-100-soluble fractions, containing 20 μg of protein, were treated with either PNGase F or Endo-H (New England Biolabs, Hitchin, Herts., UK) according to the manufacturer’s instructions. Treated samples and non-treated controls were resolved using 4–12% sodium dodecyl sulphate–polyacrylamide gels (Invitrogen) and analysed by Western blot.

**Cathepsin D enzymatic activity**

The residual Cathepsin D activity was measured in the human brain tissue samples using a kinetic assay (Yasuda et al. 1999). Cathepsin D substrate solution (Enzo Life Sciences, Exeter, UK) was used at a final concentration of 20 μM. Pepstatin A (Enzo Life Sciences) solution at a final concentration of 0.2 mg/mL was used for the selective inhibition of Cathepsin D. Briefly, 5 μg of protein samples (prepared in native lysis buffer containing 0.32 M Sucrose, 1 mM EDTA, 50 mM Tris, pH 7.4 (all Sigma) and EDTA-free protease inhibitor cocktail tablets (Roche)) were diluted in an assay buffer (50 mM sodium acetate buffer, pH 4.0 (Sigma)) in a final volume of 10 μL. In 96-well black polystyrene plates, 40 μL of the assay buffer or for the control samples with inhibitor, 40 μL of Pepstatin A solution in the assay buffer were added to the protein samples and pre-incubated at 37°C for 10 min and subsequently, 50 μL of the substrate solution in the assay buffer pre-heated to 37°C were added to all samples. The increase in fluorescence was measured (Ex 320 nm, Em 400 nm, sensitivity 100,37°C). For 30-min incubation, the readings were taken at intervals of 5 min. 7-methoxycoumarin-4-acetic acid (Sigma) in the assay buffer (0–1.5 nmol) was used to generate a standard curve. Pepstatin A caused a complete inhibition of enzyme activity.

### Table 1: Case details of mutation-carrying donor samples

<table>
<thead>
<tr>
<th>Patient</th>
<th>GBA mutation</th>
<th>Clinical diagnosis</th>
<th>Gender</th>
<th>AAO-Dem (years)</th>
<th>AAO-PD (years)</th>
<th>Age-Death (years)</th>
</tr>
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<tbody>
<tr>
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<td>IVS2 + 1</td>
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<td>57</td>
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<tr>
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<td>DLB</td>
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<td>79</td>
<td>82</td>
</tr>
<tr>
<td>DLB_4</td>
<td>L444P</td>
<td>DLB</td>
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<td>75</td>
<td>75</td>
<td>77</td>
</tr>
<tr>
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<td>73</td>
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<td>74</td>
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<tr>
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<td>L105R</td>
<td>PDD</td>
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<td>76</td>
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<td>78</td>
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<td>51</td>
</tr>
</tbody>
</table>

*For case DLB_1, no clinical details indicating parkinsonism could be identified on the case note review. Pathology showed widespread substantia nigra neuron loss and cortical Lewy bodies. AAO-Dem, Age at onset of dementia where this could be accurately ascertained; AAO-PD, Age at onset of parkinsonism movement disorder where this could be accurately determined; NA, not applicable.*

Human neural stem cell cultures

Human neural precursor stem cell lines were grown as neurospheres according to previously described methods (Burnstein et al. 2004) at 37°C in a 95% air/5% CO₂, humidified incubator. Proliferation medium was replenished at 2–3-day intervals by replacing 60–70% of the medium with fresh medium. For assay, cells were extensively triturated to small neurospheres and seeded into 25 cm flasks coated with 0.5% gelatine (Sigma) and grown for 10 days. Growth medium was replaced with differentiating medium containing Dulbecco’s modified Eagle’s medium /F12 supplemented with 10% heat-inactivated Foetal Bovine Serum (Sigma), N-1, B27 and N-2. Conversion of neurospheres into cells with neural morphology took 2–3 days. Cells were allowed to grow for a further 14 days to develop neuritic networks after which they were treated for 72 h with 100 μM CBE or dimethyl sulfoxide (0.02%) vehicle. Cells were lysed using ice-cold native lysis buffer [Tris-buffered saline containing 0.32 M Sucrose, 1% Triton X-100 (all Sigma), 1 x protease/phosphatase inhibitor cocktail (Roche)] and sonicated.
for 20 min on ice in a sonicating bath. Subsequently, lysates were centrifuged at 1000 g for 10 min and the supernatants subjected to Western blotting. Protein concentration was determined using Bradford assay. Data are the mean of three independent replicates.

**Statistical analysis**

F-test two-sample for variances and t-test: two-sample assuming equal or unequal variances were used to assess statistical significance of the enzyme activity and Western blot analysis data. For the stem cell culture studies, the data consist of the mean of all three independent replicates and the paired sample statistics of the three independent experiments was performed using spss Statistics 17.0 (SPSS, Chicago, IL, USA). Statistical significance was considered when $p$ two-tail $\leq 0.05$. Graphs were generated using GraphPad Prism 4 software (GraphPad, San Diego, CA, USA). The results are presented as mean ± SD. Fold changes (FC) represent: average of ratios of mean (protein/GAPDH) for treated (mutant) samples/average of ratios of mean (protein/GAPDH) for untreated (control) samples.

**Results**

**GCase activity**

Brain tissue from the frontal cortex of LBD cases and controls, both with and without GBA mutation, were screened for the relative levels of glucocerebrosidase protein by Western blotting (Fig 1). Analysis of relative

**Fig. 1** Peptide N-glycosidase F (PNGase F)-treated glucocerebrosidase in normal and GBA mutation carriers: Brain homogenates were treated with PNGase F to show protein molecular weight and Western blotting for glucocerebrosidase used to detect glucocerebrosidase protein (a). The major isoform of glucocerebrosidase was reduced in GBA-mutation heterozygotes (GBA mutations: L444P, RecNci, IVS2 + 1 G>A, N370S and L105R) (LBDmt, n = 5; Cmt, n = 5) compared to wild-type GBA individuals (LBDwt, n = 5; Cwt, n = 5) (b). Reductions in the major isoform were seen irrespective of the presence of Lewy body disease. Changes were seen in the levels of the short isoform of glucocerebrosidase in controls with GBA mutations, although this was not significant in LBD cases and GBA mutation carriers; overall, the short isoform was significantly reduced (c). Figures are Mean GBA/GAPDH ratio ± SD. Numbering of mutation bearing cases is according to Table 1.
levels of glucocerebrosidase protein (following PNGase F treatment) showed a reduction in the longer isoform of glucocerebrosidase of approximately 20% in mutation carriers \( (p < 0.05, \text{mutant v. wild type}, \text{LBD}^{\text{mt}} n = 5, \text{LBD}^{\text{wt}} n = 5, \text{Cmt} n = 5, \text{Cwt} n = 5) \), irrespective of disease state, with the shorter isoform showing a reduction in levels in the control samples carrying mutation \( (p < 0.05, \text{mutant v. wild type}, \text{LBD}^{\text{mt}} n = 5, \text{Cmt} n = 5, \text{Cwt} n = 5) \), although this was not significantly reduced in LBD cases \( (p > 0.05, \text{mutant v. wild type}, \text{Fig} 1) \). Alterations in GCase were not because of either neuronal loss or gliosis as we found no significant change in numbers of cases studied. This was confirmed by analysis of glucocerebrosidase enzyme activity, which again showed an approximately 25% reduction in enzyme activity in GBA mutation carriers irrespective of disease state \( (\text{Fig} 2) \), with no reduction in activity because of the presence of LBD. Individual analysis of specific mutations did not show any consistent pattern of enzyme activity reduction \( (\text{not shown}) \), although we cannot rule out a genotype–phenotype relationship, given the reduced numbers of cases studied.

**Lysosomal markers**

As glucocerebrosidase is a major lysosomal enzyme, we tested whether the presence of reduced glucocerebrosidase levels as a result of GBA mutation causes any change in lysosomal composition. Using the major lysosomal membrane protein \( (\text{LAMP1}) \) as a marker of lysosome mass \( (\text{Eskelinen} 2006) \), an elevation of LAMP1 associated with the presence of GBA mutation was seen in both normal individuals \( (p < 0.05, \text{Cmt} n = 5 \text{ vs. Cwt} n = 10) \), and in individuals with LBD \( (p < 0.05, \text{LBD}^{\text{mt}} n = 7 \text{ vs. LBD}^{\text{wt}} n = 15) \), Fig 3). Similarly, LAMP2 relative protein levels were also found to be elevated by approximately 10–15% in GBA mutation carriers \( (p < 0.05, \text{LBD}^{\text{mt}} n = 5 \text{ vs. LBD}^{\text{wt}} n = 14; p < 0.05 \text{Cmt} n = 5 \text{ vs. Cwt} n = 10) \). As glucocerebrosidase uses LIMP-2/SCARB2 for import and sequestration by lysosomes \( (\text{Reczek et al.} 2007) \) the effects of GBA mutation on expression were determined. Control cases carrying GBA mutation showed significantly increased LIMP-2 expression \( (p < 0.05, \text{Cmt} n = 5 \text{ vs. Cwt} n = 11) \), and whilst increased, this was not significant in LBD cases with GBA mutation \( (0.05 < p < 0.1, \text{LBD}^{\text{mt}} n = 7 \text{ vs. LBD}^{\text{wt}} n = 15) \), Fig 3). Whilst the elevation of these major lysosomal proteins pointed towards a general increase in lysosomal mass, not all lysosomal proteins were increased.

Specifically, the Parkinsonism-related protein ATP13A2, involved with Kufor–Rake syndrome, was reduced in LBD GBA mutation carriers \( (p < 0.05, \text{LBD}^{\text{mt}} n = 7 \text{ vs. LBD}^{\text{wt}} n = 14) \), see Fig 4), as was the major lysosomal protease Cathepsin D \( (\text{Fig} 4) \) in LBD cases \( (p < 0.05, \text{LBD}^{\text{mt}} n = 7 \text{ vs. LBD}^{\text{wt}} n = 14) \), but not in controls carrying GBA mutation \( (p > 0.05, \text{Cmt} n = 5 \text{ vs. Cwt} n = 11) \). Consistently, LBD cases with GBA mutations showed significantly decreased activities of Cathepsin D enzyme compared with non-carriers \( (15\% \text{ depletion}, p < 0.05, \text{LBD}^{\text{mt}} n = 7, \text{LBD}^{\text{wt}} n = 15) \) \( (\text{Figure} \text{ S1}) \). This was, however, not characteristic for control individuals with GBA mutations \( (p > 0.05, \text{not significant, Cmt} n = 5, \text{Cwt} n = 12) \). For the individuals with no alterations in GBA, LBD patients had significantly lower Cathepsin D activities compared with controls \( (16\% \text{ depletion}, p < 0.05) \), indicating deficiency may be more associated with LBD.

As the majority of lysosomal proteins show extensive post-translational modification, and in particular the incorporation of major carbohydrate chains which aid in protein folding and resistance to lysosomal hydrolases, we sought to determine how glucocerebrosidase is glycosylated and the status of the endoplasmic reticulum activity in GBA mutation carriers. The glycosylation state of glucocerebrosidase was investigated using Endo-H and PNGase F treatment to remove high-mannose carbohydrate chains and all asparagine N-linked oligosaccharides respectively. PNGase F treatment of glucocerebrosidase showed the presence of two molecular weight species of glucocerebrosidase \( (\text{see} \text{ Fig} \text{ 1}) \) with evidence of glycosylation of approximately 10–15 kDa. Frontal cortex tissue samples from GBA mutation carriers showed significantly less cross-reacting material and little evidence of resistance to Endo H treatment compared with samples of human spleen, suggesting the presence of less extensive glycosylation pattern on brain glucocerebrosidase \( (\text{see} \text{ Figure} \text{ S2}) \).

**Endoplasmic reticulum markers**

As there is little Endo-H-resistant glucocerebrosidase fraction in brain which may indicate ER retention and reduced

---

Fig. 2 Brain glucocerebrosidase enzyme activity is decreased in GBA mutation carriers: Samples of grey matter \( (\text{LBD}^{\text{mt}} n = 7, \text{LBD}^{\text{wt}} n = 15, \text{Cmt} n = 5, \text{Cwt} n = 10) \) were assayed for glucocerebrosidase enzyme activity using the specific substrate and levels of 4-methylumbelliflone \( (4\text{-MU}) \) production were determined. Comparison with tissue from individuals with wild-type GBA were used as control and showed reduced glucocerebrosidase enzyme activity in GBA mutation carriers. Glucocerebrosidase activity was reduced in both normal and Lewy body disease \( (\text{LBD}) \) cases to a similar extent. Note that there is an overlap in glucocerebrosidase activity between control and mutant samples.
processing of glucocerebrosidase, it is possible that ER
marker proteins are altered by the presence of GBA mutation.
Western blot analysis of the major UPR protein BiP/GRP78
showed a marked reduction in LBD which was independent
of GBA mutation status, suggesting that LBD shows an
abnormal UPR ($p < 0.05$, LBD vs. Control, LBDmt $n = 7$,
LBDwt $n = 15$, Cwt $n = 10$) (a, c). Elevated levels of LAMP1 and LAMP2 were seen in Lewy
body disease (LBD) and normal individuals in the presence of GBA mutation (b). The
major protein involved in import of glucocerebrosidase into lysosomes LIMP-2
(SCARB2) was also seen to be elevated in control mutation carriers (c) but was not
significantly elevated in LBD mutation carriers. Error bars are ± SD.

Fig. 3 Major lysosomal membrane proteins are elevated in GBA mutation carriers:
Western blotting was used to determine the relative levels of LAMP1, LAMP2 and LIMP-2 protein in brain homogenates from individuals with and without GBA mutations (LBDmt $n = 5–7$, LBDwt $n = 14–15$, Cmt $n = 5$, Cwt $n = 10–11$) (a, c). Elevated levels of LAMP1 and LAMP2 were seen in Lewy
body disease (LBD) and normal individuals in the presence of GBA mutation (b). The
major protein involved in import of glucocerebrosidase into lysosomes LIMP-2
(SCARB2) was also seen to be elevated in control mutation carriers (c) but was not
significantly elevated in LBD mutation carriers. Error bars are ± SD.
tissue homogenates showed that the monomeric form of the protein was elevated in LBD cases carrying GBA mutations compared with wild-type LBD cases (p < 0.05; LBDmt n = 6 vs. LBDwt n = 11; Fig 7), whilst no alterations were observed within controls (Cmt n = 5, Cwt n = 11).

Cell culture analysis of GBA inhibition
To determine if changes identified in the brains of GBA mutation carriers were attributable directly to reduced glucocerebrosidase activity, differentiated human cortical neural stem cells were treated with the glucocerebrosidase inhibitor, CBE. Cell extracts from treated cells showed a slight but significant increase in glucocerebrosidase protein (p < 0.05; Fig 8), although glucocerebrosidase activity was almost completely inhibited (1.8% ± 0.9 of control). Acute reduction of glucocerebrosidase activity caused a small, however significant reduction of BiP/GRP78 protein (p < 0.05; Fig 8), indicating similar reductions in the UPR response to that found in tissue samples, although significant lysosomal protein changes were not seen and no alteration in α-synuclein was observed (data not shown).

Discussion
Previous studies in several populations have demonstrated the association between GBA mutations and Lewy body disease (Sidransky et al. 2009). The results of this study indicate that GBA heterozygotes show reduced activities of glucocerebrosidase enzyme in the frontal cortex tissue irrespective of disease state, consistent with previous reports (Choi et al. 2011; Mazzulli et al. 2011). This reduction in GCase enzyme activity is relatively mild, approximately 25% in both cases and controls carrying GBA mutation, and would suggest that if glucocerebrosidase activity does influence development of LBD, it does so relatively efficiently or shows a subtle effect in concert with other mechanisms. Furthermore, the results show a slight depletion of GCase protein in mutation carriers, with the main isoform significantly reduced in controls and LBD cases with GBA mutation; however, the significant reduction of the shorter isoform was detected only in control individuals with GBA mutation. Importantly, in neural tissue from LBD cases and control subjects, glucocerebrosidase appears to show little in the way of extensive glycosylation, as evidenced by almost complete sensitivity to Endo-H treatment in contrast to tissues such as spleen. This finding of different patterns of glucocerebrosidase activity and processing is not new (Pentchev et al. 1978; Ginns et al. 1982), but the finding of a reduced glycosylation in the brain may have important implications for enzyme function. Normally, glycosylation has the effect of enhancing the folding and solubility of a protein (Helenius and Aebi 2001) and in the context of lysosomally directed proteins, of enhancing protein half-life by protecting it from lysosomal proteases (Kundra and Kornfeld 1999). An intriguing possibility is that the preferential effects of GBA mutation on the CNS in GD on the CNS (Sidransky 2004) may be a combination of enhanced GCase sensitivity to lysosomal degradation because of reduced glycosylation along with a reduced

Fig. 4 Selective alterations in lysosomal proteins in GBA mutation carriers with Lewy body disease: Brain tissue from GBA mutation carriers was screened for levels of the Kufor–Rakeb Parkinsonism protein ATP13A2 and the lysosomal protease Cathepsin D by Western blotting (LBDmt n = 7, LBDwt n = 14, Cmt n = 5, Cwt n = 11) (a) and relative levels determined against GAPDH levels. Reduced levels of ATP13A2 were seen in LBD cases carrying GBA mutations but not in normal individuals with GBA mutation (b) and similar reductions in Cathepsin D heavy chain (28 kDa) were seen associated selectively with LBD patients. Error bars are ± SD.
enzyme activity because of mutation. Whilst enzyme activity shows a relatively small change, it suggests that restoration of activity may be a therapeutic option, particularly if blood-brain barrier-penetrating small molecules affecting glucocerebrosidase and also glucosylceramide synthase, to reduce substrate accumulation, could be used (Mu et al. 2008; Khanna et al. 2010; Marshall et al. 2010; Marugan et al. 2011).

Whilst the enzyme defect in glucocerebrosidase in heterozygous carriers is modest, there appear to be additional changes within the endoplasmic reticulum and lysosomes. The enhanced expression of LAMP1 and LAMP2 along with the glucocerebrosidase carrier protein LIMP-2 indicates an abnormal cellular lysosomal activity. The presence of abnormal lysosomal processing and evidence of autophagy associated with synucleinopathies is a relatively common finding (Cuervo et al. 2004; Chu et al. 2009; Dehay et al. 2010). Whilst the enhanced expression of LAMP1 and LAMP2 might be a response to remove aggregated proteins and damaged organelles (because of for example α-synuclein accumulation), this appears to be inefficient as evidenced by the reduced
Fig. 8 Effects of glucocerebrosidase inhibition in human neural cultures using CBE: Differentiated human neural stem cells were exposed to 100 µM of the specific glucocerebrosidase inhibitor conduritol Beta epoxide (CBE) for 72 h and protein extracts prepared for western blotting. CBE caused a slight elevation of steady-state levels of glucocerebrosidase (a) but caused an acute change of ER stress and the UPR as evidenced by reduced levels of BiP (b). Overall, no significant change was seen in the major lysosomal protein LAMP1 (c). Example blots are representative of three independent experiments. The data presented consist of the mean of all three independent replicates. Error bars are ± SD.

Cathpsin D levels and activities. The loss of lysosomal protease activity measured as reduced Cathpsin D and ATP13A2 protein selectively in LBD cases carrying mutation may indicate why only some mutation carriers develop LBD. Several studies have shown that reduced lysosomal Cathpsin D activity can lead to α-synuclein accumulation as can ATP13A2 reduction (Qiao et al. 2008; Chu et al. 2009; Cullen et al. 2009; Gitler et al. 2009) and it is possible therefore that lysosomal changes contribute to LBD development. Given that elevated lysosome expression is evident in normal individuals heterozygous for GBA mutations, it is possible that the lysosomal defect predisposes individuals to disordered protein metabolism associated with aging, such as α-synuclein processing. The development of LBD in individuals with heterozygous GBA mutations may be because of additional factors such as enhanced α-synuclein expression (Chu and Kordower 2007; Satake et al. 2009; Simon-Sanchez et al. 2009) and/ or inefficiency of protein degradation machinery (Collier et al. 2011). The changes identified in the endoplasmic reticulum markers of the UPR in GBA mutation carriers and in LBD may be an indication of abnormal protein processing associated with α-synuclein dismetabolism (Cooper et al. 2006; Hoozemans et al. 2007). The abnormal UPR in GBA mutation carriers, a finding previously noted in animal and cell models of GD (Wei et al. 2008), indicates that GBA mutation predisposes cells to abnormal protein folding, exacerbated perhaps by α-synuclein accumulation (Cullen et al. 2011; Sardi et al. 2011). The reduced levels of BiP/GRP78 seen in mutation carriers and in CBE-treated cells may predispose to neuronal cell loss as selective knockout of BiP/GRP78 in Purkinje cells causes neuronal cell death (Wang et al. 2010). Similarly, the failure to mount an adequate UPR in GBA carriers evidenced by GRP78 and GRP94 reductions may indicate a predisposition to abnormal protein folding in GBA mutation carriers. The current cell studies show that acute inhibition of glucocerebrosidase function leads to ER stress and reduced levels of BiP, although no changes in monomeric α-synuclein levels were detected. The present data corresponds with the previous report by Cullen et al., where similar concentrations of CBE were used to inhibit GCase activity in PC12-SNCA cell lines, with no corresponding α-synuclein deposition (Cullen 2011). In contrast, the initial findings by Manning-Bog et al. demonstrated elevated levels of α-synuclein following CBE exposure in SH-SY5Y cells differentiated to the neuronal phenotype and in the substantia nigra of CBE-treated mice (Manning-Bog et al. 2009). The predisposition of cells carrying GCase defect to synucleinopathy might be therefore a combined effect of the presence of mutant GCase protein and the loss of its enzymatic activity. It is possible that reduced GCase activity causes ER stress and UPR induction through abnormal lysosomal activity, which then
exacerbates α-synuclein accumulation (Mazzulli et al. 2011). Alternatively, the abnormal UPR in GBA mutation carriers identified in this study might be caused by the enduring production of mutant GCase protein, enhanced by age-related impairment of the ER chaperone systems (Nuss et al. 2008), which may impede protein folding and degradation (Ron et al. 2010). Prolonged activation of the UPR leads to cell death (Tabas and Ron 2011), with nitric oxide-mediated protein damage and polyubiquitination of neuronal components being contributory factors (Uehara et al. 2006), all hallmark features of LBD.

In summary, the findings of this study indicate that the presence of glucocerebrosidase mutation rather than reduced GCase activity may predispose neurons to the accumulation of misfolded proteins through lysosomal abnormalities and defects in the endoplasmic reticulum. Whilst on their own, these changes do not appear to be associated with LBD, such abnormalities may underlie the vulnerability of neurons to α-synuclein dismetabolism. The small number of GBA mutation-bearing LBD patients and controls analysed in this study should be acknowledged and it may represent a limitation factor, although various approaches were used to investigate the potential biochemical defect in GBA heterozygotes. It becomes apparent that LBD may be associated with a wider spectrum of lysosomal storage disorders than GCase pathology alone (Shachar et al. 2011; Winder-Rhodes et al. 2012), implying that more general cellular mechanisms, as presented herein, may be involved.

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Supporting information

Additional supporting information may be found in the online version of this article:

Figure S1. Residual Cathepsin D activities in GBA mutation carriers.

Figure S2. Endoglycosidase-H sensitivity of glucocerebrosidase species in GBA mutation Carriers.

Table S1. Patient demographics of post mortem cohort.

Table S2. Primary antibodies used in analysis.

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References


Weil H., Kim S. J., Zhang Z., Tsai P. C., Wisniewski K. E. and Munkherjee A. B. (2008) ER and oxidative stresses are common medi-
ators of apoptosis in both neurodegenerative and non-neurodegenerative lysosomal storage disorders and are alleviated by chemical chaperones. *Hum. Mol. Genet.* **17**, 469–477.

