

# Reduced glucocerebrosidase is associated with increased $\alpha$ -synuclein in sporadic Parkinson's disease

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Heterozygous mutations in *GBA1*, the gene encoding lysosomal glucocerebrosidase, are the most frequent known genetic risk factor for Parkinson's disease. Reduced glucocerebrosidase and  $\alpha$ -synuclein accumulation are directly related in cell models of Parkinson's disease. We investigated relationships between Parkinson's disease-specific glucocerebrosidase deficits, glucocerebrosidase-related pathways, and  $\alpha$ -synuclein levels in brain tissue from subjects with sporadic Parkinson's disease without *GBA1* mutations. Brain regions with and without a Parkinson's disease-related increase in  $\alpha$ -synuclein levels were assessed in autopsy samples from subjects with sporadic Parkinson's disease ( $n = 19$ ) and age- and post-mortem delay-matched controls ( $n = 10$ ). Levels of glucocerebrosidase,  $\alpha$ -synuclein and related lysosomal and autophagic proteins were assessed by western blotting. Glucocerebrosidase enzyme activity was measured using a fluorimetric assay, and glucocerebrosidase and  $\alpha$ -synuclein messenger RNA expression determined by quantitative polymerase chain reaction. Related sphingolipids were analysed by mass spectrometry. Multivariate statistical analyses were performed to identify differences between disease groups and regions, with non-parametric correlations used to identify relationships between variables. Glucocerebrosidase protein levels and enzyme activity were selectively reduced in the early stages of Parkinson's disease in regions with increased  $\alpha$ -synuclein levels although limited inclusion formation, whereas *GBA1* messenger RNA expression was non-selectively reduced in Parkinson's disease. The selective loss of lysosomal glucocerebrosidase was directly related to reduced lysosomal chaperone-mediated autophagy, increased  $\alpha$ -synuclein and decreased ceramide. Glucocerebrosidase deficits in sporadic Parkinson's disease are related to the abnormal accumulation of  $\alpha$ -synuclein and are associated with substantial alterations in lysosomal chaperone-mediated autophagy pathways and lipid metabolism. Our data suggest that the early selective Parkinson's disease changes are likely a result of the redistribution of cellular membrane proteins leading to a chronic reduction in lysosome function in brain regions vulnerable to Parkinson's disease pathology.

**Keywords:** Parkinson's disease; glucocerebrosidase;  $\alpha$ -synuclein; chaperone-mediated autophagy; ceramide

## Introduction

Homozygous mutations in the glucocerebrosidase gene (*GBA1*) cause Gaucher disease, a lysosomal storage disorder characterized by the accumulation of the lipid glucocerebroside in cells as a result of the functional loss of the lysosomal enzyme glucocerebrosidase (Hruska *et al.*, 2008). Although considered non-neuropathic, patients with type 1, the mildest form of Gaucher disease, have a significantly increased risk for developing clinical Parkinson's disease (Bultron *et al.*, 2010), with pathological Lewy bodies at autopsy (DePaolo *et al.*, 2009). Parkinson's disease and associated Lewy body disorders are also observed in heterozygous *GBA1* mutation carriers (Sidransky *et al.*, 2009; Nalls *et al.*, 2013), with similar levels of pathological Lewy bodies compared with sporadic Parkinson's disease (Parkkinen *et al.*, 2011). In large cohorts of patients with Parkinson's disease, 4–7% were found to carry heterozygous *GBA1* mutations (Neumann *et al.*, 2009; Sidransky *et al.*, 2009), rendering mutations in this gene the most common genetic risk factor for Parkinson's disease (Leverenz *et al.*, 2009).

All cases of sporadic Parkinson's disease are characterized by the abnormal accumulation and aggregation of the neuronal protein  $\alpha$ -synuclein in inclusions called Lewy bodies (Dickson *et al.*, 2009). Normal  $\alpha$ -synuclein is degraded primarily through lysosomal autophagic mechanisms (Cuervo *et al.*, 2004; Vogiatzi *et al.*, 2008), and the insoluble accumulation of this protein indicates defective lysosomal function in Parkinson's disease (Boya and Kroemer, 2008; Martinez-Vicente *et al.*, 2008; Alvarez-Erviti *et al.*, 2010; Dehay *et al.*, 2010). In cell and animal studies, decreased wild-type glucocerebrosidase leads to  $\alpha$ -synuclein accumulation, and increased  $\alpha$ -synuclein inhibits normal glucocerebrosidase function (Manning-Bog *et al.*, 2009; Mazzulli *et al.*, 2011; Yap *et al.*, 2011). In post-mortem brain tissue from patients with Parkinson's disease and *GBA1* mutations, glucocerebrosidase and  $\alpha$ -synuclein colocalize in Lewy bodies (Goker-Alpan *et al.*, 2010) and aggregated forms of  $\alpha$ -synuclein are present (Choi *et al.*, 2011). Recent reports show reduced glucocerebrosidase protein levels and enzyme activity in a range of affected brain regions in patients with Parkinson's disease (Gegg *et al.*, 2012), although whether this reduction relates to the amount of accumulated  $\alpha$ -synuclein has not been determined. The reason for reduced glucocerebrosidase in patients with Parkinson's disease without *GBA1* mutations remains unknown.

This study assessed Parkinson's disease-specific changes in glucocerebrosidase expression and function in two brain regions, one with increased  $\alpha$ -synuclein levels in Parkinson's disease (anterior cingulate cortex) and one without (occipital cortex). Proteins and sphingolipids in related lysosomal, autophagic and sphingolipid pathways were assessed to identify the cellular mechanisms most disrupted. Our aim was to determine whether deficient glucocerebrosidase, changes in glucocerebrosidase-related pathways, and increased  $\alpha$ -synuclein levels were related in patients with sporadic Parkinson's disease without *GBA1* mutations to

identify potential therapeutic targets and early disease biomarkers.

## Materials and methods

### Cases

Brain samples from longitudinally followed, autopsy-confirmed subjects with Parkinson's disease ( $n = 19$ ) and age- and post-mortem delay-matched neurological and neuropathological controls ( $n = 10$ ; Table 1) were obtained from the Sydney Brain Bank and New South Wales Tissue Resource Centre after study approval and with appropriate institutional ethics approval. All cases with Parkinson's disease were levodopa-responsive, had no other neurodegenerative conditions, and met the UK Brain Bank Clinical Criteria for diagnosis of Parkinson's disease (Gibb and Lees, 1988). Parkinson's disease cases with few concurrent non-Parkinson's disease-related pathologies were selected (Montine *et al.*, 2012) as well as a group with later stages of Parkinson's disease, many of whom had dementia based on assessments within 2 years of their death using the clinical dementia rating (Morris, 1997) (Table 1). All cases and controls were of non-Jewish ancestry.

### DNA extraction

Genomic DNA was extracted from 50–100 mg of fresh-frozen occipital cortex tissue from each of the 19 cases with Parkinson's disease using a proteinase K and phenol method for the isolation of high molecular weight mammalian DNA (Sambrook and Russell, 2006), modified for tissue samples. Briefly, minced tissue samples were suspended in lysis buffer (0.32 M sucrose, 10 mM Tris-HCl pH 7.5, 5 mM MgCl<sub>2</sub>, 1% Triton<sup>TM</sup> X-100), centrifuged at 3000 rpm for 10 min at 4°C, and the resulting pellet repeatedly washed in lysis buffer before overnight incubation at 55°C in digestion buffer (50 mM Tris-HCl pH 8.5, 1 mM EDTA, 0.16 M NaCl, 20 µg/ml proteinase K and 0.4% SDS). Repeated addition of phenol:chloroform:isoamyl alcohol (25:24:1; Sigma P2069) and centrifugation at 3000 rpm for 20 min at 4°C resulted in the isolation of DNA in the upper aqueous phase. DNA was precipitated by the addition of 100% ethanol and pelleted by centrifugation at 13 000 rpm for 5 min at room temperature. The final DNA pellet was air-dried before resuspension in TE solution (10 mM Tris pH 8.0, 1 mM EDTA). DNA was extracted from 25 mg of frozen occipital cortex from each of the 10 control samples using the DNeasy<sup>®</sup> Blood and Tissue Kit (Qiagen) according to manufacturer instructions. DNA concentration was measured using a NanoDrop<sup>TM</sup> spectrophotometer.

### GBA1 screening

*GBA1* mutation status was assessed in the Parkinson's disease cases by performing complete sequencing of the 11 exons and flanking intronic regions of *GBA1*, amplified from genomic DNA in three fragments encompassing exons 1–5, 5–7 and 8–11, as previously described (Koprivica *et al.*, 2000; Stone *et al.*, 2000). Amplified fragments were purified using a QIAquick<sup>®</sup> PCR Purification Kit (Qiagen) and cycle sequencing was performed using the Dye Terminator Cycle Sequencing kit (Applied Biosystems) with both forward and reverse

**Table 1** Demographic details for early and later stage Parkinson's disease and control cohorts

| Diagnosis        | Sex (M:F) <sup>a</sup> | Age at death (years) <sup>b</sup> | Post-mortem delay (hours) <sup>c</sup> | Disease duration (years) <sup>d</sup> | Braak Lewy stage (/6) | CERAD plaque score (/3) <sup>e</sup> | Braak neuritic stage (/6) <sup>e</sup> | Clinical dementia rating (/3) <sup>f</sup> |
|------------------|------------------------|-----------------------------------|--|---------------------------------------|-----------------------|--------------------------------------|--|--|
| Early PD (n = 7) | 6M:1F                  | 78.3 ± 2.4 (71–88)                | 13.7 ± 3.0 (3–24)                      | 14.9 ± 1.6 (8–20)                     | 7IV                   | 0.6 ± 0.3 (0–2)                      | 1.1 ± 0.3 (0–2)                        | 0  |
| Late PD (n = 12) | 8M:4F                  | 77.8 ± 1.3 (69–85)                | 16.2 ± 3.6 (3–42)                      | 15.2 ± 2.2 (7–36)                     | 7V:5VI                | 1.0 ± 0.3 (0–3)                      | 1.2 ± 0.4 (0–4)                        | 1.9 ± 0.3 (1–3)                            |
| Control (n = 10) | 5M:5F                  | 74.7 ± 2.9 (60–88)                | 18.0 ± 3.2 (7–35)                      | –                                     | –                     | 0.3 ± 0.2 (0–2)                      | 0.6 ± 0.3 (0–2)                        | 0  |

<sup>a</sup>Not significantly different between groups (Pearson chi-square,  $P = 0.31$ ).

<sup>b</sup>Not significantly different between groups (one-way ANOVA,  $P = 0.48$ ).

<sup>c</sup>Not significantly different between groups (one-way ANOVA,  $P = 0.72$ ).

<sup>d</sup>Not significantly different between groups (independent samples  $t$ -test,  $P = 0.91$ ).

<sup>e</sup>Parkinson's disease cases and controls do not meet diagnostic criteria for Alzheimer's disease (Montine *et al.*, 2012), with CERAD scores and Braak neuritic stages not significantly different between groups (one-way ANOVA,  $P = 0.16$  and  $P = 0.44$ , respectively).

<sup>f</sup>Later stage Parkinson's disease cases were significantly demented compared to both early stage Parkinson's disease cases and controls (one-way ANOVA with *post hoc* Bonferroni comparisons,  $P < 0.0001$ ), with early stage Parkinson's disease cases and controls not significantly different ( $P = 1.0$ ).

CERAD = Consortium to Establish a Registry for Alzheimer's Disease; PD = Parkinson's disease.

Values are given as mean ± standard error and range for age at death, post-mortem delay, disease duration, Parkinson's disease severity (Braak Lewy stage, Braak *et al.*, 2003), Alzheimer's disease severity (CERAD plaque score and Braak neuritic stage), and clinical dementia rating.

primers (Stone *et al.*, 2000), using an ABI Prism 3730XL DNA Analyzer. The sequencing data were analysed by SeqScape v2.6 using GeneBank NG\_009783.1 as the reference sequence.

The control cases were screened for six common and two recombinant *GBA1* mutations that account for 70% of causative alleles for type one Gaucher disease in non-Jewish populations (Beutler *et al.*, 1991; Koprivica *et al.*, 2000). Specifically, sequence analysis of exons 9 and 10 was used to test for N370S (c.1226A>G), L444P (c.1448T>C), R463C (c.1504C>T) and D409H (c.1342G>C) mutations, whereas testing for the IVS2 + 1 (c.115 + 1G>A) mutation used restriction enzyme analysis and the 84GG (c.84dupG) mutation used amplification-refractory mutation system analysis. The recombinant alleles RecNcil and RecTL were excluded based on the absence of the L444P mutation and other sequence variants in exon 10.

## Protein extraction and lysosomal-enriched fraction preparation

Soluble and insoluble proteins were serially extracted from 250 mg of fresh-frozen brain from the anterior cingulate (with increased  $\alpha$ -synuclein levels) and occipital (no increased  $\alpha$ -synuclein) cortices, as previously described (Zhou *et al.*, 2011). Briefly, tissue was homogenized in TBS homogenization buffer [50 mM Tris, 125 mM NaCl, pH 7.4, 5 mM EDTA, 0.02% sodium azide containing protease inhibitor cocktail (Complete, EDTA-free; Roche 04693132001)], centrifuged at 120 000g for 2 h at 4°C and the supernatant collected as the TBS-soluble fraction containing cytosolic proteins. The pellet was resuspended in TBS homogenization buffer containing 5% SDS, centrifuged at 100 000g for 30 min at 25°C, and the supernatant collected as the SDS-soluble fraction containing membrane-associated proteins.

Lysosomal membrane-enriched fractions were isolated from 300 mg fresh-frozen tissue from each region of interest. Tissue was thawed on ice, minced with a scalpel blade and homogenized in 10 × volume of homogenization medium [0.32 M sucrose, 1 mM EDTA, 10 mM Tris-HCl pH 7.4, containing protease inhibitor cocktail (Complete, EDTA-free; Roche)] using 20 strokes of a Potter homogenizer rotating at 600 rpm. A small aliquot of total homogenate (whole tissue extract) was reserved for later analysis. Total homogenate was centrifuged at 1000 g for 10 min at 4°C to sediment the nuclear pellet and cellular debris. The pellet was washed twice, and the resulting supernatant centrifuged at 17 000g for 15 min at 4°C to obtain the lysosome-enriched pellet. The supernatant was centrifuged at 100 000g to obtain a pure cytosolic fraction (supernatant) and a microsomal pellet. The lysosomal-enriched pellets were resuspended in homogenization medium. The three fractions of interest (whole tissue extract, lysosomal-enriched and cytosolic fractions) were assessed by western immunoblotting with appropriate cellular markers to confirm the enrichment of lysosomal membranes in the 17 000g pellet and their absence in the cytosolic 100 000g supernatant fraction (Supplementary Fig. 1).

Protein concentration of all fractions was measured using the BCA assay (Pierce BCA Protein Assay Kit; Thermo Scientific 23225), according to manufacturer instructions. Samples were stored at –80°C.

## Western immunoblotting

TBS-soluble, SDS-soluble and lysosomal-enriched protein fractions were assessed by routine western immunoblotting for levels of glucocerebrosidase,  $\alpha$ -synuclein, and lysosomal proteins. Briefly, 25 µg of protein samples were added to loading buffer (2% SDS, 20% glycerol, 2.5% bromophenol blue, 12.5 mM Tris-HCl, pH 6.8, 5% 2-mercaptoethanol), separated by reducing SDS-PAGE and transferred to

nitrocellulose membranes (BioRad 162-0112). Membranes were blocked in 5% skimmed milk dissolved in TBS-T (0.87% NaCl, 0.01 M Tris, pH 7.4, 0.05% Triton<sup>TM</sup> X-100 or 0.1% Tween-20) and incubated overnight in primary antibodies, before detection with enhanced chemiluminescence (Amersham ECL Plus Western Blot Detection System; GE Healthcare RPN2133) using horseradish peroxidase-conjugated secondary antibodies. The relative levels of each protein of interest were analysed using ImageJ software (National Institutes of Health). The intensity of each protein band was quantified and expressed as arbitrary units standardized to  $\beta$ -actin protein levels.

Primary antibodies used for western immunoblotting were mouse monoclonal NeuN (Millipore MAB377),  $\beta$ -actin (Abcam ab6276), glucocerebrosidase (Abnova H00002629-M01),  $\alpha$ -synuclein (BD Biosciences 610787), LAMP2 (Developmental Studies Hybridoma Bank H4B4) and LC3 (NanoTools 0231-100/LC3-5F10), rabbit polyclonal LAMP1 (Santa Cruz Biotechnology sc5570), LAMP3 (Aviva Biosystems ARP41598-P050), Beclin1 (Novus Biologicals NB500-249) and cathepsin K (Abcam ab19027), and goat polyclonal LIMP2 (Everest Biotech EB09295), cathepsin D (Santa Cruz Biotechnology sc6486) and cathepsin A (Santa Cruz Biotechnology sc26049).

## Glucocerebrosidase enzyme activity assay

Glucocerebrosidase enzyme activity was assessed in lysosomal-enriched protein fractions using the standard fluorimetric assay (Peters *et al.*, 1976). Thirty microlitres of the lysosomal-enriched protein fraction was incubated at 37°C for 40 min with 40  $\mu$ l of 1 mM 4-methylumbelliferyl  $\beta$ -glucopyranoside (4MU $\beta$ Glu; Sigma M3633) in 0.2 M citrate buffer (pH 4.5) containing 1% bovine serum albumin, 0.25% sodium taurocholate and 0.25% Triton<sup>TM</sup> X-100. The reaction was stopped by addition of 70  $\mu$ l of 1 M glycine-NaOH (pH 10.4), and 4-methylumbelliferone fluorescence measured at excitation 355 nm and emission 460 nm using a POLARstar plate reader (BMG 415-201). One unit of enzyme activity is the amount of enzyme that hydrolyses 1 nmol of substrate per minute at 37°C. Values are expressed as  $\mu$ mol normalized to the 4MU $\beta$ Glu standard curve, with enzyme activity normalized to the total protein amount in each sample.

## RNA extraction and quantitative polymerase chain reaction

Total RNA was extracted from 50 mg of fresh-frozen brain tissue from each region of interest using TRIzol<sup>®</sup> Reagent (Sigma 15596-026), according to manufacturer instructions. Five micrograms of RNA was reverse transcribed into complementary DNA using Moloney-murine leukaemia virus reverse transcriptase and random primers in a 20  $\mu$ l reaction volume. Relative expression of *GBA1*,  $\alpha$ -synuclein and  $\beta$ -actin messenger RNA was measured by quantitative PCR using a Mastercycler ep realplex S (Eppendorf) with the fluorescent dye SsoAdvanced<sup>TM</sup> SYBR<sup>®</sup> Green Supermix (BioRad 172-5264), according to manufacturer instructions. Briefly, each 20  $\mu$ l reaction contained SYBR<sup>®</sup> Green Supermix, 5 pmol primers, and 1  $\mu$ l complementary DNA template. Amplification consisted of 40 cycles of denaturation (94°C, 15 s), annealing and extension (60°C, 1 min). A complementary DNA template-free control was included for each PCR amplification. Primer sequences are provided in Supplementary Table 1. Gene expression was normalized to  $\beta$ -actin messenger RNA levels and relative gene expression calculated using the comparative threshold cycle ( $\Delta\Delta C_T$ ) method.

## Sphingolipid analyses

Sphingolipid species were extracted from 10 mg of fresh-frozen brain tissue from each region using a recently developed and validated mechanical bead homogenization (Precellys<sup>®</sup>24, Bertin Technologies) and methyl-*tert*-butyl ether lipid extraction protocol, as described (Abbott *et al.*, 2013). The sphingolipid extracts were analysed using electrospray ionization mass spectrometry and sphingolipid species quantified in nmol/g of tissue wet weight using AB SCIEX Lipidview software as described (Abbott *et al.*, 2013).

## Statistical analyses

All statistical analyses were performed using SPSS Statistics software (IBM) and statistical significance set at  $P < 0.05$ . A series of analyses were performed to identify clinical and diagnostic structural changes between the cohort groups. Differences between group demographics (Table 1) were assessed using Pearson chi-square test (sex), ANOVA with *post hoc* Bonferroni comparisons (age at death, post-mortem delay, CERAD plaque score, Braak neuritic staging, and clinical dementia rating), or independent samples *t*-test (disease duration). A univariate ANOVA was performed to determine if there was any loss of neurons in the different cohort groups and regions, and a multivariate statistical analysis was performed to determine if there were any neuronal changes in the levels of  $\alpha$ -synuclein protein and messenger RNA between cohort groups and brain regions. Two multivariate analyses were performed to determine any changes in glucocerebrosidase protein, enzyme, messenger RNA, lysosomal protein, and sphingolipid levels that related to Parkinson's disease-specific increases in  $\alpha$ -synuclein protein: one with control and early stage Parkinson's disease cases to determine if there were early changes in these variables, and one with early and late stage Parkinson's disease cases to determine if there were later changes in these variables in the anterior cingulate cortex when  $\alpha$ -synuclein aggregates occur. Full details of these analyses are given in the relevant results sections. Non-parametric Spearman's correlations and step-wise multiple linear regressions were used to identify related variables.

## Results

### Confirmation of changes typical of sporadic Parkinson's disease

All Parkinson's disease cases had typical pathological changes (Dickson *et al.*, 2009) with limited other pathologies, seven reaching Braak Parkinson's disease stage IV, seven reaching Braak Parkinson's disease stage V and five reaching Braak Parkinson's disease stage VI (Braak *et al.*, 2003). For all analyses, the Parkinson's disease cases were grouped into early stage Parkinson's disease (Braak stage IV) and later stage Parkinson's disease (Braak stages V and VI; Table 1). The absence of *GBA1* mutations was confirmed in the 19 cases with sporadic Parkinson's disease by sequencing all exons of *GBA1*, and in the 10 control cases by screening for eight common mutations (Beutler *et al.*, 1991; Koprivica *et al.*, 2000). To determine if there was any loss of neurons in the different cohort groups and regions, a two-way ANOVA was performed with the relative levels of NeuN protein as the dependent variable and cohort groups and brain regions as



factors, covarying for the clinical dementia rating differences between the groups (other variables did not differ between groups). Among the early stage Parkinson's disease cases, neither the anterior cingulate nor occipital cortex had substantial neuron loss using NeuN as a marker of neuron number in protein extracts ( $P > 0.92$ ; Fig. 1A and B), as expected for early stage disease; however, NeuN was reduced in the cases with later stages of Parkinson's disease ( $74 \pm 15\%$  reduction from control levels,  $P = 0.014$ , Fig. 1A and B).

Recent studies demonstrate that in regions vulnerable to Parkinson's disease-specific aggregations of  $\alpha$ -synuclein, the soluble form of  $\alpha$ -synuclein is either unchanged or progressively reduced in sporadic Parkinson's disease, whereas the SDS-soluble or membrane-associated fraction of  $\alpha$ -synuclein increases significantly as pathological Lewy bodies form (Tong *et al.*, 2010; Zhou *et al.*, 2011; Lue *et al.*, 2012; Murphy *et al.*, 2013). To determine if there were any neuronal changes in the levels of  $\alpha$ -synuclein protein and messenger RNA between cohort groups and brain regions, a multivariate statistical analysis was performed with the relative levels of  $\alpha$ -synuclein protein and messenger RNA as the dependent variable and cohort groups and brain regions as factors, covarying for the clinical dementia rating and NeuN differences identified between groups. As expected (Zhou *et al.*, 2011), we identified a significant increase in SDS-soluble  $\alpha$ -synuclein in both early and late stage Parkinson's disease anterior cingulate cortex (Fig. 1C and D, Table 2) but not in Parkinson's disease occipital cortex at any disease stage. No change in TBS-soluble  $\alpha$ -synuclein protein was observed (Fig. 1C and Table 2). This analysis was performed covarying for the NeuN identified neuronal loss in the later stages of Parkinson's disease to ensure that the cellular comparisons were similar within the structurally changed anterior cingulate cortex, as there was a significant negative correlation between Braak stage and NeuN in all Parkinson's disease samples (non-parametric Spearman's  $\rho = -0.47$ ,  $P = 0.003$ ; Fig. 1E). This confirms the association of lower NeuN levels with the higher Braak stages of late Parkinson's disease. In addition, there was a significant negative correlation between NeuN and SDS-soluble  $\alpha$ -synuclein levels in Parkinson's disease anterior cingulate (non-parametric Spearman's  $\rho = -0.69$ ,  $P = 0.001$ ; Fig. 1F), indicating that neuron loss is associated with greater accumulation of membrane-associated  $\alpha$ -synuclein. The additional increase observed in SDS-soluble  $\alpha$ -synuclein in late stage Parkinson's disease anterior cingulate cortex (Table 2) was confirmed by the significant positive correlation between SDS-soluble  $\alpha$ -synuclein and Braak staging in all Parkinson's disease cases (non-parametric Spearman's  $\rho = 0.54$ ,  $P = 0.018$ ). The multivariate analysis also revealed  $\alpha$ -synuclein messenger RNA to be significantly increased to a similar extent in both early and late stage Parkinson's disease anterior cingulate cortex (Table 2), but not in Parkinson's disease occipital cortex at any disease stage (Table 2 and Fig. 1D).

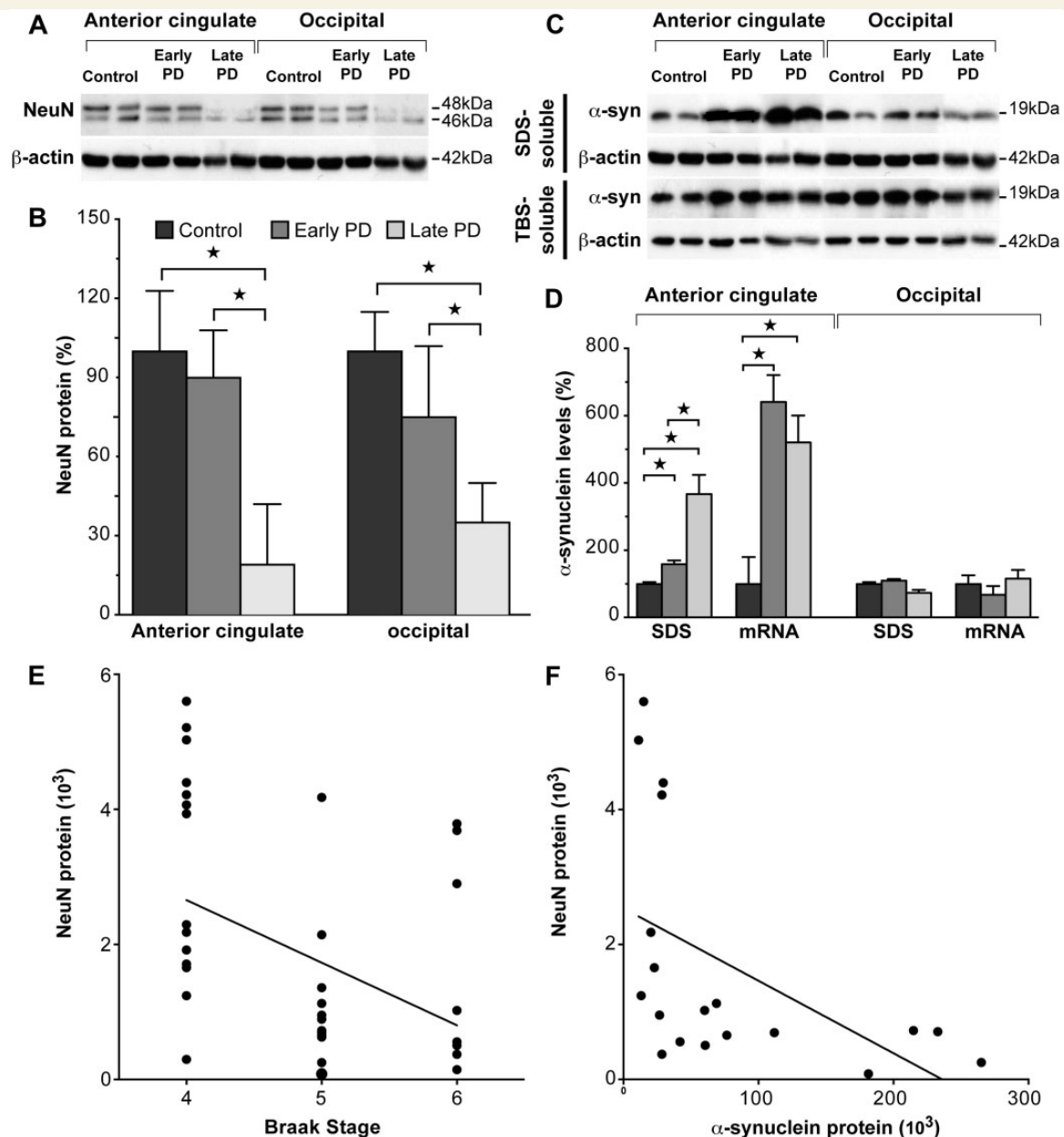
Collectively, these data provide biochemical confirmation of the regional selectivity of increased  $\alpha$ -synuclein levels in early stage Parkinson's disease because of increased production of  $\alpha$ -synuclein before substantial inclusion formation and cortical neuronal loss (Halliday and McCann, 2010). To identify the earliest cellular changes associated with increased  $\alpha$ -synuclein before structural

tissue changes, we first compared tissue changes between early stage cases with Parkinson's disease and control subjects, then comparing the early and later stage Parkinson's disease groups to identify any factors accompanying the later stage neuronal loss that might be associated with further increases in  $\alpha$ -synuclein.

## Decreased glucocerebrosidase protein levels and enzyme activity in regions with $\alpha$ -synuclein accumulation in early stages of Parkinson's disease

Glucocerebrosidase is predominantly a lysosomal membrane-associated protein under normal conditions (Qi and Grabowski, 1998). In controls, the majority of glucocerebrosidase protein was seen in the SDS-soluble fraction (99% is SDS-soluble in the anterior cingulate cortex). To determine if there were early changes in glucocerebrosidase protein, enzyme, messenger RNA, lysosomal protein and sphingolipid levels that related to Parkinson's disease-specific increases in  $\alpha$ -synuclein protein, a multivariate statistical analysis was performed with control and early stage Parkinson's disease cases and the relative levels of  $\alpha$ -synuclein protein and messenger RNA; glucocerebrosidase protein, enzyme activity and messenger RNA; lysosomal proteins; and sphingolipid levels as dependent variables and cohort groups and regions as factors, covarying for NeuN. SDS-soluble glucocerebrosidase was selectively reduced in anterior cingulate cortex samples from subjects with early stage Parkinson's disease (Fig. 2A and B, and Table 2). The selective reduction of glucocerebrosidase protein in early stage Parkinson's disease anterior cingulate, but not occipital cortex (Table 2), was confirmed in the lysosomal fraction using western blotting (Fig. 2A and B, Table 2). Glucocerebrosidase enzyme activity, assessed in the lysosomal-enriched protein fraction using standard methods (Peters *et al.*, 1976), was also selectively reduced in early stage Parkinson's disease anterior cingulate (Fig. 2B and Table 2) but not occipital cortex (Table 2). Non-parametric Spearman's correlation analysis showed that the amount of increased  $\alpha$ -synuclein observed in early stage Parkinson's disease was related to the reduction in SDS-soluble glucocerebrosidase protein ( $\rho = -0.66$ ,  $P = 0.01$ ; Fig. 2C) and step-wise multiple regression analysis determined that the amount of increased  $\alpha$ -synuclein observed in early stage Parkinson's disease corresponded to the reduction in lysosomal glucocerebrosidase enzyme activity in the same cases ( $\beta = 0.3$ ,  $P = 0.001$ ). These data indicate that diminished glucocerebrosidase activity and protein levels occur selectively in brain regions with increased levels of  $\alpha$ -synuclein in the early stages of Parkinson's disease.

In contrast with the selective reduction in glucocerebrosidase in regions accumulating  $\alpha$ -synuclein, *GBA1* messenger RNA expression was not significantly altered in early stage Parkinson's disease anterior cingulate cortex (Table 2), although there was a strong trend for a non-selective reduction in both brain regions (Fig. 2D and Table 2). This non-selective *GBA1* messenger RNA reduction did not differ between early and later stage Parkinson's disease (Table 2). This suggests that the reduction and redistribution of glucocerebrosidase in the anterior cingulate cortex is related to the



**Figure 1** Characterization of neuron loss and  $\alpha$ -synuclein changes in early and later stage sporadic Parkinson's disease (PD).

(A) Representative western blots of NeuN protein in control, early stage Parkinson's disease and later stage Parkinson's disease anterior cingulate and occipital cortices. (B) Quantitative western blot data of NeuN protein levels in control ( $n = 10$ ), early stage Parkinson's disease ( $n = 7$ ) and later stage Parkinson's disease ( $n = 12$ ) cases. In early stage Parkinson's disease anterior cingulate and occipital cortices, NeuN was not significantly different ( $P > 0.92$ ), whereas NeuN was reduced in later stage Parkinson's disease ( $P = 0.014$ ). This confirms the lack of substantial cortical neuron loss in early stage Parkinson's disease. \* $P < 0.05$ . (C) Representative western blots of SDS- and TBS-soluble  $\alpha$ -synuclein protein in control, early stage Parkinson's disease and later stage Parkinson's disease anterior cingulate and occipital cortices. (D) Quantitative data of SDS-soluble  $\alpha$ -synuclein protein and  $\alpha$ -synuclein messenger RNA levels in control ( $n = 10$ ), early stage Parkinson's disease ( $n = 7$ ) and later stage Parkinson's disease ( $n = 12$ ) cases. SDS-soluble  $\alpha$ -synuclein was selectively increased in early stage Parkinson's disease ( $P < 0.0001$ ), and further increased in later stage Parkinson's disease ( $P < 0.0001$ ) anterior cingulate cortex, with unchanged levels in the occipital cortex.  $\alpha$ -Synuclein messenger RNA was selectively increased to a similar extent in early stage Parkinson's disease ( $P = 0.004$ ) and later stage Parkinson's disease ( $P = 0.004$ ), with unchanged levels in the occipital cortex. These results confirm the regional selectivity of abnormal  $\alpha$ -synuclein accumulation in early stage Parkinson's disease. \* $P < 0.05$ . (E) Braak stage was significantly correlated with NeuN protein levels in all Parkinson's disease samples (non-parametric Spearman's  $\rho = -0.47$ ,  $P = 0.003$ ), confirming the association of lower NeuN levels with the higher Braak stages of later stage Parkinson's disease. (F) SDS-soluble  $\alpha$ -synuclein protein levels were significantly correlated with NeuN levels (non-parametric Spearman's  $\rho = -0.69$ ,  $P = 0.001$ ) in Parkinson's disease anterior cingulate cortex. This indicates that neuron loss associates with greater accumulation of membrane-associated  $\alpha$ -synuclein in Parkinson's disease anterior cingulate cortex.

**Table 2** Results from the two main multivariate statistical analyses

| Variable                               | % control<br>AC standard<br>deviation | % change<br>from control<br>in early PD AC | % control<br>OCC standard<br>deviation | % change<br>from control<br>in early PD OCC | P-value <sup>a</sup> | % early PD<br>AC standard<br>deviation | % change<br>from early PD<br>in late PD AC | P-value <sup>b</sup> |
|--|---------------------------------------|--|--|---|----------------------|--|--|----------------------|
| SDS-soluble $\alpha$ -synuclein        | 78                                    | 59 $\pm$ 11 $\uparrow$                     | 45                                     | 10  | <0.0001              | 36                                     | 130 $\pm$ 36% $\uparrow$                   | <0.0001              |
| TBS-soluble $\alpha$ -synuclein        | 71                                    | 55   | 64                                     | 3   | 0.963                | 94                                     | 151  | 0.215                |
| $\alpha$ -Synuclein messenger RNA      | 73                                    | 540 $\pm$ 80 $\uparrow$                    | 101                                    | 32  | 0.004                | 52                                     | 19   | 0.215                |
| SDS-soluble glucocerebrosidase         | 79                                    | 85 $\pm$ 15 $\downarrow$                   | 53                                     | 39  | 0.006                | 40                                     | 73   | 0.215                |
| Lysosomal glucocerebrosidase           | 46                                    | 38 $\pm$ 11 $\downarrow$                   | 49                                     | 28  | 0.013                | 35                                     | 24   | 0.215                |
| Glucocerebrosidase enzyme activity     | 9                                     | 27 $\pm$ 4 $\downarrow$                    | 9                                      | 18  | 0.003                | 23                                     | 32   | 0.215                |
| <i>GBA1</i> messenger RNA <sup>c</sup> | 88                                    | 70   | 81                                     | 27  | 0.252                | 122                                    | 72   | 0.215                |
| SDS-soluble LAMP1                      | 44                                    | 26   | 47                                     | 36  | 0.123                | 32                                     | 33   | 0.215                |
| SDS-soluble LAMP2                      | 72                                    | 71 $\pm$ 20 $\downarrow$                   | 56                                     | 139   | 0.005                | 40                                     | 22   | 0.215                |
| SDS-soluble LAMP3                      | 65                                    | 23   | 54                                     | 10  | 0.761                | 34                                     | 16   | 0.215                |
| SDS-soluble LIMP2                      | 24                                    | 3  | 33                                     | 14  | 0.681                | 18                                     | 31   | 0.215                |
| TBS-soluble cathepsin A                | 90                                    | 216 $\pm$ 61 $\uparrow$                    | 60                                     | 59  | 0.004                | 88                                     | 217  | 0.215                |
| TBS-soluble cathepsin D                | 12                                    | 312 $\pm$ 61 $\uparrow$                    | 22                                     | 57  | 0.001                | 89                                     | 63   | 0.215                |
| TBS-soluble cathepsin K                | 30                                    | 17   | 68                                     | 38  | 0.410                | 48                                     | 19   | 0.215                |
| SDS-soluble beclin 1                   | 57                                    | 59 $\pm$ 13 $\downarrow$                   | 13                                     | 113   | <0.0001              | 19                                     | 5  | 0.215                |
| SDS-soluble LC3-II                     | 62                                    | 2  | 80                                     | 5   | 0.909                | 58                                     | 0  | 0.215                |
| Ceramide                               | 17                                    | 59 $\pm$ 9% $\downarrow$                   | 50                                     | 39  | <b>0.059</b>         | 56                                     | 12   | 0.215                |
| Sphingomyelin                          | 23                                    | 48   | 58                                     | 40  | 0.142                | 35                                     | 22   | 0.215                |

AC = anterior cingulate cortex; OCC = occipital cortex.

<sup>a</sup>Dependent variables were the relative levels of  $\alpha$ -synuclein protein,  $\alpha$ -synuclein messenger RNA, glucocerebrosidase protein, enzyme, messenger RNA, lysosomal proteins, and sphingolipid levels, factors were cohort groups and regions, covariates were NeuN protein levels.

<sup>b</sup>Dependent variables were the relative levels of  $\alpha$ -synuclein protein,  $\alpha$ -synuclein messenger RNA, glucocerebrosidase protein, enzyme, messenger RNA, lysosomal proteins, and sphingolipid levels, factors were cohort groups and regions, covariates were Clinical Dementia Rating and NeuN protein levels.

<sup>c</sup>As described in the results, *GBA1* messenger RNA levels were not significantly altered in early stage PD anterior cingulate cortex ( $P = 0.252$ ), but there was a strong trend for a non-selective reduction in both brain regions (54  $\pm$  21% reduction from controls,  $P = 0.060$ ), with no difference between early and later stage Parkinson's disease anterior cingulate cortex ( $P > 0.215$ ). The above data and Fig. 2D show that this reduction in *GBA1* messenger RNA was greater in the anterior cingulate cortex than in the occipital cortex.

To determine any early changes in Parkinson's disease, the first<sup>a</sup> analysis compared early stage Parkinson's disease cases with controls. To determine whether any early changes in Parkinson's disease were enhanced with increasing disease stage, or if new changes occurred, the second<sup>b</sup> analysis compared early stage Parkinson's disease cases to later stage Parkinson's disease cases. Data are given as a percentage of control values or early Parkinson's disease values (i.e. percentage change  $\pm$  SEM). Significant differences (italicized and direction of change arrowed), trends toward significance (bold and direction of change arrowed) or no change (plain text) are indicated.

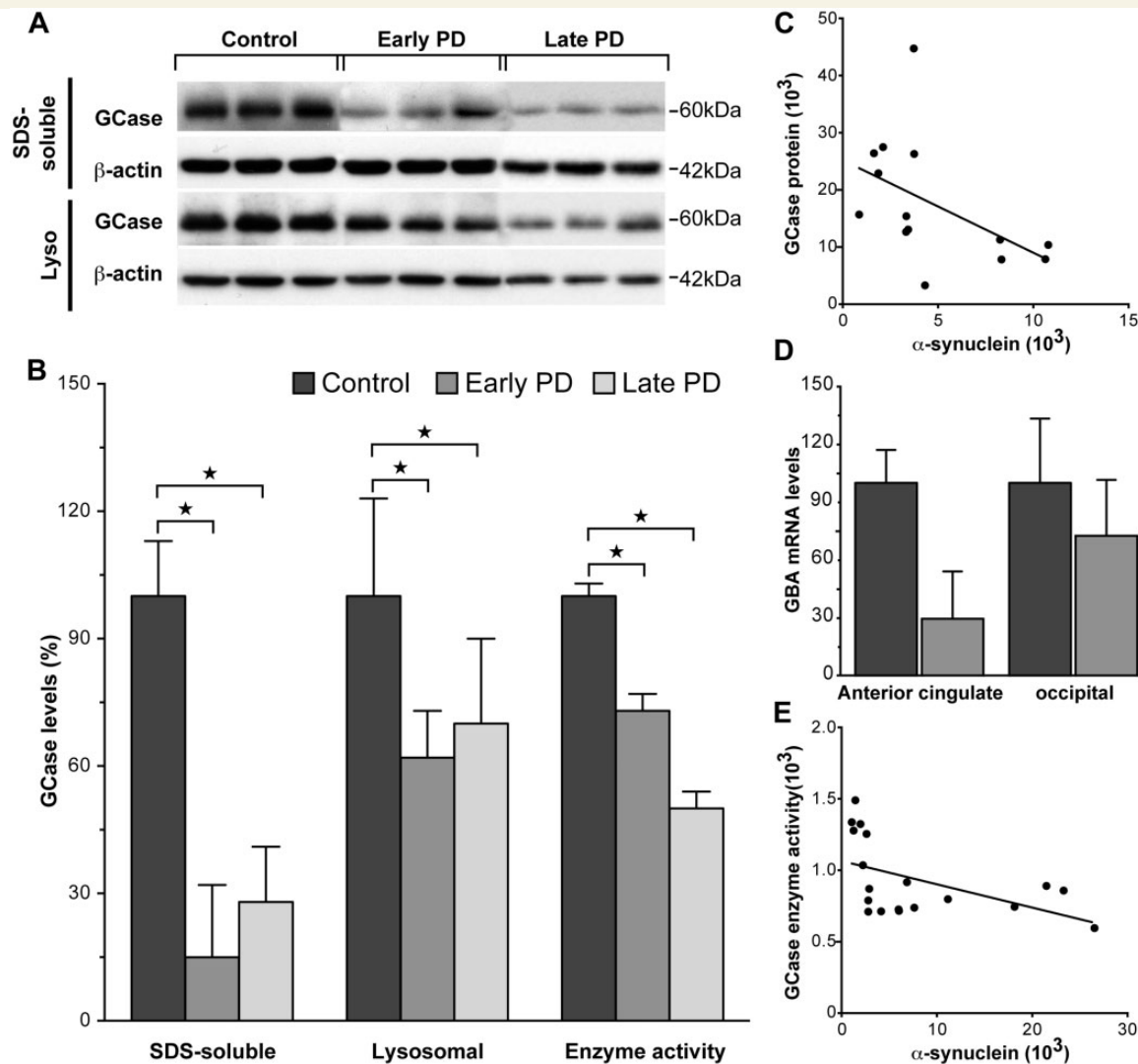
increase and pathological redistribution of  $\alpha$ -synuclein in this region.

To determine if there were later changes in glucocerebrosidase protein, enzyme, messenger RNA, lysosomal protein, and sphingolipid levels that related to Parkinson's disease-specific increases in  $\alpha$ -synuclein protein in the anterior cingulate cortex when  $\alpha$ -synuclein aggregates occur, a multivariate statistical analysis was performed with early and late stage Parkinson's disease cases and the relative levels of  $\alpha$ -synuclein protein and messenger RNA; glucocerebrosidase protein, enzyme activity and messenger RNA; lysosomal protein; and sphingolipid levels as dependent variables and cohort group as the factor, covarying for the clinical dementia rating and NeuN differences identified between groups. Covarying for the neuronal loss in late stage Parkinson's disease anterior cingulate cortex, the multivariate analysis showed that glucocerebrosidase protein levels and enzyme activity were not significantly different from the levels measured in early stage Parkinson's disease (Fig. 2A and B, Table 2). NeuN protein levels had a significant negative correlation with lysosomal glucocerebrosidase protein levels (non-parametric Spearman's  $\rho = -0.56$ ,  $P = 0.013$ ) as well as glucocerebrosidase enzyme activity (non-parametric Spearman's  $\rho = 0.80$ ,  $P < 0.0001$ ). SDS-soluble  $\alpha$ -synuclein levels in all Parkinson's disease anterior cingulate

cortex negatively correlated with glucocerebrosidase enzyme activity (non-parametric Spearman's  $\rho = -0.62$ ,  $P = 0.005$ ; Fig. 2E). This suggests that while glucocerebrosidase protein and enzyme activity deficits are an early pathological event associated with abnormal  $\alpha$ -synuclein accumulation in Parkinson's disease, glucocerebrosidase enzyme activity continues to decline in the absence of additional glucocerebrosidase protein deficits.

## Glucocerebrosidase deficits in early stage Parkinson's disease are not because of fewer lysosomes but are associated with lysosomal dysfunction and reduced ceramide levels

At the early Parkinson's disease stage examined, many constituent lysosomal membrane proteins were not affected in regions accumulating  $\alpha$ -synuclein, with no change in SDS-soluble levels of LAMP1, LAMP3 or LIMP2 observed in the anterior cingulate cortex (Fig. 3A and B, Table 2). Factoring in neuronal loss, these lysosomal membrane proteins were also unchanged in late stage Parkinson's disease anterior cingulate cortex (Table 2). This suggests no overt loss of lysosomes with  $\alpha$ -synuclein accumulation



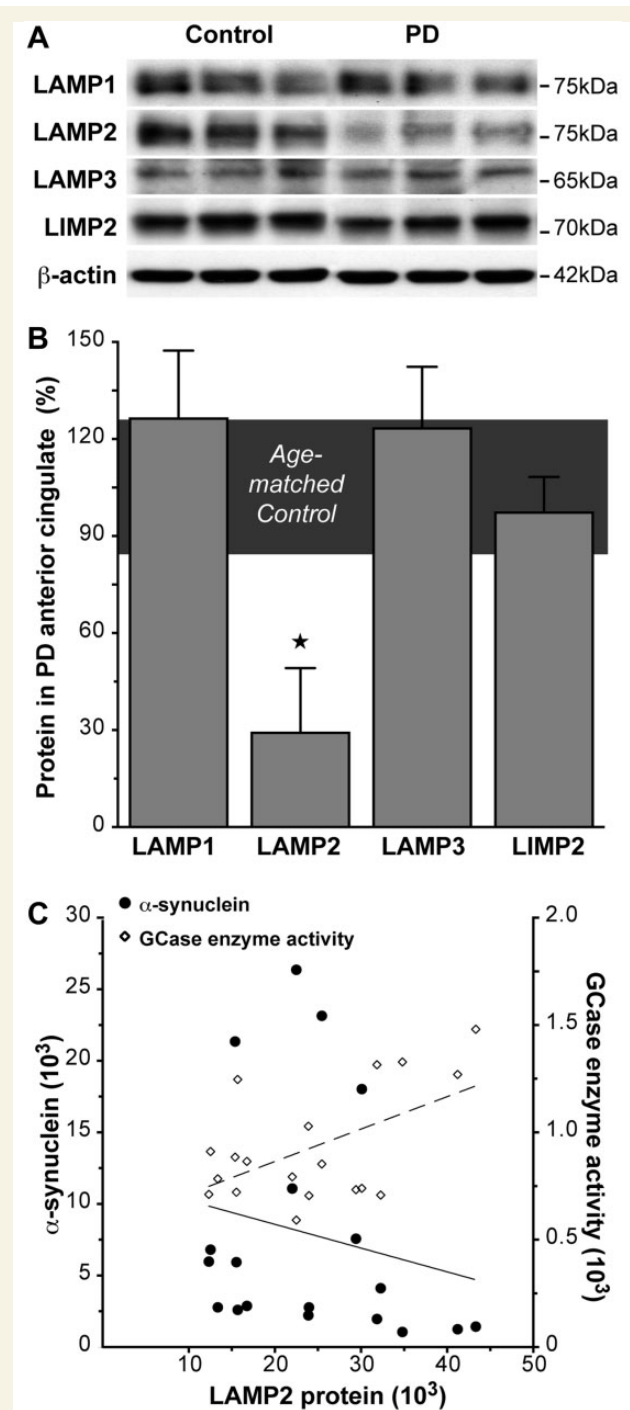
**Figure 2** Glucocerebrosidase protein levels and enzyme activity were selectively reduced in regions that accumulate abnormal  $\alpha$ -synuclein in sporadic Parkinson's disease. (A) Representative western blots of SDS-soluble and lysosomal glucocerebrosidase (GCase) protein in control, early stage Parkinson's disease and later stage Parkinson's disease anterior cingulate cortex. (B) Quantitative data of SDS-soluble and lysosomal glucocerebrosidase protein and glucocerebrosidase enzyme activity in control ( $n = 10$ ), early stage Parkinson's disease ( $n = 7$ ) and later stage Parkinson's disease ( $n = 12$ ) cases. There were selective reductions in SDS-soluble glucocerebrosidase ( $P = 0.006$ ), lysosomal glucocerebrosidase ( $P = 0.013$ ), and glucocerebrosidase enzyme activity ( $P = 0.003$ ) in early stage Parkinson's disease anterior cingulate cortex. There was no difference between early and later stage Parkinson's disease anterior cingulate cortex for SDS-soluble glucocerebrosidase ( $P > 0.21$ ), lysosomal glucocerebrosidase ( $P > 0.21$ ), or glucocerebrosidase enzyme activity ( $P > 0.21$ ). \* $P < 0.05$ . (C) The amount of increased SDS-soluble  $\alpha$ -synuclein protein observed in early stage Parkinson's disease anterior cingulate cortex was related to the reduction in SDS-soluble glucocerebrosidase protein (non-parametric Spearman's  $\rho = -0.66$ ,  $P = 0.01$ ). (D) Although *GBA1* messenger RNA expression was not significantly altered in early stage Parkinson's disease anterior cingulate or occipital cortex ( $P > 0.25$ ), the anterior cingulate cortex seemed to have less *GBA1* messenger RNA. (E) The reduction in lysosomal glucocerebrosidase enzyme activity was significantly correlated with the increase in SDS-soluble  $\alpha$ -synuclein protein in early and later stage Parkinson's disease anterior cingulate cortex (non-parametric Spearman's  $\rho = -0.63$ ,  $P = 0.005$ ).

before neuronal loss in Parkinson's disease. Therefore, a loss of lysosomes does not appear to be the underlying cause of the glucocerebrosidase reductions observed.

Lysosome dysfunction in early stage Parkinson's disease is indicated by changes in selected lysosomal proteins. In early stage Parkinson's disease anterior cingulate cortex, there was a selective reduction in the lysosomal membrane protein LAMP2 (Fig. 3A and

B, Table 2). LAMP2A, an isoform of LAMP2, is important for chaperone-mediated autophagic degradation of  $\alpha$ -synuclein (Cuervo and Dice, 2000; Cuervo *et al.*, 2004). Non-parametric Spearman's correlation analysis showed that in early stage Parkinson's disease the reduction in SDS-soluble LAMP2 was related to increased SDS-soluble  $\alpha$ -synuclein protein ( $\rho = -0.82$ ,  $P = 0.023$ ) and decreased glucocerebrosidase





**Figure 3** SDS-soluble lysosomal membrane protein LAMP2 levels were selectively reduced in association with reduced glucocerebrosidase (GCase) in regions that accumulate abnormal  $\alpha$ -synuclein in early stage Parkinson's disease (PD). (A) Representative western blots of SDS-soluble lysosomal membrane proteins LAMP1, LAMP2, LAMP3 and LIMP2 in control and early stage Parkinson's disease anterior cingulate cortex. (B) Quantitative data of SDS-soluble lysosomal membrane proteins in control ( $n = 10$ ; horizontal bar at  $100 \pm 15\%$ ) and early stage Parkinson's disease ( $n = 7$ ) anterior cingulate cortex showed no significant changes in LAMP1 ( $P = 0.12$ ), LAMP3 ( $P = 0.76$ ) and LIMP2 ( $P = 0.68$ ), but a selective reduction of LAMP2 in early stage Parkinson's disease ( $P = 0.005$ ). \* $P < 0.05$ . (C) The

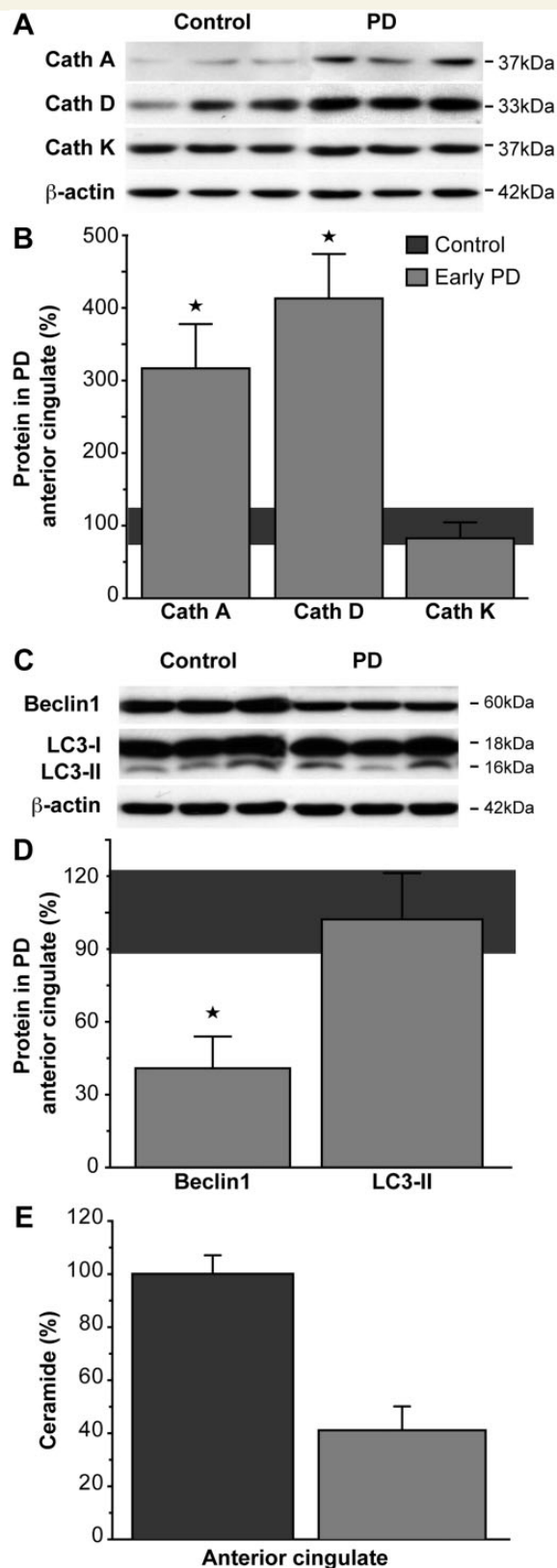
enzyme activity ( $\rho = 0.89$ ,  $P = 0.007$ ) (Fig. 3C). Accounting for neuronal loss, LAMP2 protein levels in late stage Parkinson's disease anterior cingulate cortex did not differ from early stage Parkinson's disease anterior cingulate cortex (Table 2). These data implicate that chaperone-mediated autophagy pathways contribute to the glucocerebrosidase deficits observed in the early stages of Parkinson's disease.

Lysosomes degrade chaperone-mediated autophagy-delivered cytosolic proteins using a range of soluble hydrolases called cathepsins. Two cathepsins associated with chaperone-mediated autophagic degradation of  $\alpha$ -synuclein include cathepsin D, the primary lysosomal enzyme that degrades  $\alpha$ -synuclein (Sevlever *et al.*, 2008); and cathepsin A, involved in the regulation of the chaperone-mediated autophagy membrane receptor and rate-limiting factor LAMP2A (Cuervo *et al.*, 2003). Cathepsin K is involved in bone matrix destruction and its gene expression is upregulated in Gaucher disease (Moran *et al.*, 2000). In early stage Parkinson's disease anterior cingulate cortex, there was a selective increase in the chaperone-mediated autophagy-related cathepsins A and D, but not the bone-related cathepsin (Fig. 4A and B, Table 2). However, there were no correlations between the levels of these affected chaperone-mediated autophagy-related cathepsins and the levels of glucocerebrosidase or  $\alpha$ -synuclein in early stage Parkinson's disease. Furthermore, the multivariate analysis showed that the levels of cathepsins A, D and K in the anterior cingulate cortex were similar in early and late stage Parkinson's disease (Table 2). This indicates that although significant early dysfunction is likely in the lysosomal chaperone-mediated autophagy system, the glucocerebrosidase deficits observed in Parkinson's disease may be more related to membrane interactions or factors external to the lysosome than to lysosomal hydrolase dysregulation.

Chronic reductions in chaperone-mediated autophagy activity can result in constitutive activation of macroautophagy (Massey *et al.*, 2006; Kaushik *et al.*, 2008), where autophagosomes are delivered to the lysosome for degradation. To determine if changes in this pathway are relevant, we assessed two proteins involved in the induction of macroautophagy, LC3-II is required for autophagosome formation and beclin 1 is involved in the early formation and maturation of autophagosomes (Tanida, 2011). In early stage Parkinson's disease anterior cingulate cortex, there was a selective reduction in levels of beclin 1 but not LC3-II (Fig. 4C and D, Table 2). The multivariate analysis revealed that the levels of beclin 1 and LC3-II in late stage Parkinson's disease anterior cingulate cortex were not different from early stage Parkinson's disease anterior cingulate cortex (Table 2). Step-wise multiple

#### Figure 3 Continued

reduction in SDS-soluble LAMP2 protein was negatively correlated with increased SDS-soluble  $\alpha$ -synuclein (circles with unbroken line; non-parametric Spearman's  $\rho = -0.82$ ,  $P = 0.023$ ), and positively correlated with decreased glucocerebrosidase enzyme activity (diamonds with dashed line; non-parametric Spearman's  $\rho = 0.89$ ,  $P = 0.007$ ) in early stage Parkinson's disease.



**Figure 4** Chaperone-mediated autophagy- and macroautophagy-related proteins and total ceramide were selectively altered in regions that accumulate abnormal  $\alpha$ -synuclein in early stage Parkinson's disease. (A) Representative western blots of TBS-soluble lysosomal cathepsins (Cath) in control and early

regression analysis showed that the reduction in SDS-soluble beclin 1 related to the reduction in SDS-soluble glucocerebrosidase ( $\beta = 0.32$ ,  $P < 0.0001$ ), but not to increasing levels of  $\alpha$ -synuclein in early stage Parkinson's disease. The LC3-II results indicate no change in steady-state levels of autophagosome number in Parkinson's disease; however, they do not rule out an alteration in the rate of autophagosome formation (Mizushima and Yoshimori, 2007).

Lipid accumulation within the lysosome is the major defect observed in Gaucher disease (Schueler *et al.*, 2004). We analysed the relevant sphingolipids using mass spectrometry. In early stage Parkinson's disease anterior cingulate cortex, there was a strong trend for a selective reduction in total ceramide (Fig. 4E) but not sphingomyelin (Table 2). Total ceramide and sphingomyelin in late stage Parkinson's disease anterior cingulate cortex were not different from early Parkinson's disease anterior cingulate cortex in the multivariate analysis (Table 2). These data indicate that ceramide is selectively reduced in brain regions that accumulate  $\alpha$ -synuclein and show glucocerebrosidase deficiency in sporadic Parkinson's disease.

## Discussion

The present study provides a comprehensive analysis of the relationship between wild-type glucocerebrosidase,  $\alpha$ -synuclein, and the consequent lysosomal changes in a region with biochemical but not structural changes early in sporadic Parkinson's disease. The loss of lysosomal glucocerebrosidase early in sporadic Parkinson's disease corresponds to reduced lysosomal chaperone-mediated autophagy (but not macroautophagy), increased  $\alpha$ -synuclein levels, and a decrease in ceramide. Although a deficit in glucocerebrosidase is expected in Parkinson's disease cases harbouring *GBA1* mutations, determining why glucocerebrosidase is

### Figure 4 Continued

stage Parkinson's disease anterior cingulate cortex. (B) Quantitative data of TBS-soluble lysosomal cathepsins in control ( $n = 10$ ; horizontal bar at  $100 \pm 15\%$ ) and early stage Parkinson's disease ( $n = 7$ ) anterior cingulate cortex showed that the cathepsins related to the chaperone-mediated autophagic degradation of  $\alpha$ -synuclein were significantly increased in early stage Parkinson's disease (cathepsin A  $P = 0.004$ ; cathepsin D  $P = 0.001$ ), with no change to the *GBA1*-related cathepsin K levels ( $P = 0.41$ ).  $*P < 0.05$ . (C) Representative western blots of SDS-soluble autophagosome marker proteins in control and early stage Parkinson's disease anterior cingulate cortex. (D) Quantitative data of SDS-soluble autophagosome marker proteins in control ( $n = 10$ ; horizontal bar at  $100 \pm 15\%$ ) and early stage Parkinson's disease ( $n = 7$ ) anterior cingulate cortex showed that beclin1 was significantly reduced in early stage Parkinson's disease ( $P < 0.0001$ ), while LC3-II levels remained within normal levels ( $P = 0.91$ ).  $*P < 0.05$ . (E) Ceramide trended toward a selective reduction in early stage Parkinson's disease anterior cingulate ( $P = 0.059$ ). Data are presented as percentage changes in early stage Parkinson's disease ( $n = 7$ ) compared to controls ( $n = 10$ ).

deficient in sporadic Parkinson's disease without *GBA1* mutations is important. In cell and mouse models of Gaucher disease there are direct and reciprocal interactions between glucocerebrosidase and  $\alpha$ -synuclein, where decreased glucocerebrosidase activity causes abnormal  $\alpha$ -synuclein accumulation and elevated  $\alpha$ -synuclein inhibits normal glucocerebrosidase function (Manning-Bog *et al.*, 2009; Cullen *et al.*, 2011; Mazzulli *et al.*, 2011; Sardi *et al.*, 2011, 2013; Xu *et al.*, 2011; Yap *et al.*, 2011, 2013). Reduced glucocerebrosidase protein levels and enzyme activity have been previously reported in sporadic Parkinson's disease cases, with greatest reductions seen in the vulnerable substantia nigra, and no significant loss of glucocerebrosidase in frontal cortex (Gegg *et al.*, 2012). This contrasts with the results of the present study and other reports of glucocerebrosidase reductions in frontal cortex in sporadic and *GBA1*-mutant Parkinson's disease (Mazzulli *et al.*, 2011; Kurzawa-Akanbi *et al.*, 2012), although our data would suggest that such variation is most likely because of the degree of  $\alpha$ -synuclein accumulation and neuronal loss in different frontal cortical regions at different stages of Parkinson's disease (Braak *et al.*, 2003). Overall, these studies confirm diminished glucocerebrosidase in Parkinson's disease.

Although there was some evidence for reduced *GBA1* messenger RNA expression in the same early stage Parkinson's disease cases, this reduction did not reach significance, did not occur selectively in the region accumulating  $\alpha$ -synuclein and was not associated with reduced glucocerebrosidase activity in the occipital cortex. These data are consistent with other studies showing relatively normal levels of *GBA1* messenger RNA in sporadic Parkinson's disease putamen, despite a 23–48% reduction in glucocerebrosidase protein and enzyme activity in this region (Gegg *et al.*, 2012), and differs from data obtained in patients with Gaucher disease. In patients with Gaucher disease with different types of *GBA1* mutations and a reduction in normal messenger RNA, greater residual glucocerebrosidase enzyme activity tends to be associated with milder Gaucher disease symptoms (Montfort *et al.*, 2004; Goker-Alpan *et al.*, 2005), and *GBA1* mutation carriers remain asymptomatic despite a 50% reduction in glucocerebrosidase activity (Choy, 1988; Aerts *et al.*, 2011). The reduced glucocerebrosidase activity levels in asymptomatic *GBA1* mutation carriers are presumably sufficient for normal lysosomal function. It would be of interest to know the level to which normal *GBA1* expression can be reduced before significant deficits in glucocerebrosidase levels and activity occur. It should be noted that therapeutic enzyme replacement therapies have significant efficacy for symptomatic patients with Gaucher disease (Aerts *et al.*, 2011).

Importantly, there have been no previous studies assessing the relationships between glucocerebrosidase protein levels and enzyme activity and  $\alpha$ -synuclein accumulation, or other relevant lysosomal proteins and lipids in Parkinson's disease brains. Our data identifies important relationships between levels and activity of glucocerebrosidase and the early, selective increase in membrane-associated  $\alpha$ -synuclein before substantial neuronal loss and Lewy body formation in sporadic Parkinson's disease. Glucocerebrosidase is transported from the endoplasmic reticulum to the lysosome by the trafficking receptor LIMP2 (Reczek *et al.*, 2007). Cells over-expressing  $\alpha$ -synuclein show less glucocerebrosidase delivered to the lysosome as a result of less binding to

LIMP2 (Gegg *et al.*, 2012). We confirm that the glucocerebrosidase deficiency seen in early and late Parkinson's disease is not because of reduced LIMP2 protein levels, consistent with previous studies (Gegg *et al.*, 2012; Kurzawa-Akanbi *et al.*, 2012), and although the deficit is associated with increased SDS-soluble  $\alpha$ -synuclein, it is not associated with increased  $\alpha$ -synuclein messenger RNA, suggesting that the glucocerebrosidase deficits in Parkinson's disease may be associated with the cellular redistribution of both  $\alpha$ -synuclein and glucocerebrosidase proteins. Some retention of glucocerebrosidase in the endoplasmic reticulum has been observed in Parkinson's disease (Mazzulli *et al.*, 2011). We also observe that lysosomes are not overtly lost in Parkinson's disease, the formation of autophagosomes is not reduced, and that changes in the levels of *GBA1* messenger RNA do not account for this relationship. We show that the reduction in glucocerebrosidase is not simply because of a loss of neurons in early stage Parkinson's disease, consistent with known regional selectivity of neurodegeneration in Parkinson's disease (Halliday and McCann, 2010). Finally, we show associated dysregulation in chaperone-mediated autophagy-associated proteins and enzymes, and a decrease in cellular ceramide in both early and later stages of Parkinson's disease.

Notably, these abnormalities are not significantly different between the early and later stages of Parkinson's disease, although our analysis included covarying to account for the degree of neuronal loss. The data suggest that the pathological event/s underlying such lysosomal dysfunction occur early within surviving neurons in Parkinson's disease and may contribute to the increase in  $\alpha$ -synuclein aggregation observed over time. Of interest, the degree of lysosomal dysfunction appears insufficient to immediately kill the neurons, but was of a substantial level and, in the neurons remaining, was not exacerbated with disease progression. Perhaps this indicates that any increasing level of deficit would have more detrimental consequences. Of course, ongoing and/or more substantial neuronal lysosomal dysfunction is likely to contribute to the loss of neurons we and others have observed in regions with  $\alpha$ -synuclein pathology. The identification of such relationships enhances our understanding of the lysosomal pathways involved in Parkinson's disease.

Glucocerebrosidase is a membrane-associated lysosomal enzyme, and a portion of  $\alpha$ -synuclein is normally delivered to the lysosome via chaperone-mediated autophagy (Cuervo *et al.*, 2004) and degraded primarily by the lysosomal enzyme cathepsin D resident in the lysosome lumen (Sevlever *et al.*, 2008). Our data in early stage Parkinson's disease show a selective increase in cathepsin D, consistent with some (Kurzawa-Akanbi *et al.*, 2012) but not all (Chu *et al.*, 2009; Gegg *et al.*, 2012) previous studies in Parkinson's disease and Alzheimer's disease (Cataldo *et al.*, 1991; Mackay *et al.*, 1997). Again, the variation in Parkinson's disease studies may relate to the amount of regional  $\alpha$ -synuclein accumulation and neuronal loss at different disease stages. Although cathepsin D-deficient mice display abnormal  $\alpha$ -synuclein accumulation that can be rescued by over-expression of cathepsin D (Qiao *et al.*, 2008; Cullen *et al.*, 2009), our data show the opposite effect in sporadic Parkinson's disease, with an increase rather than a decrease in this enzyme. However, we also found a novel increase in the levels of cathepsin A in sporadic



Parkinson's disease, an enzyme that contributes to multienzyme complexes to protect glycosidases against proteolytic inactivation (Hiraiwa, 1999). The proteolytic activity of cathepsin A also triggers the degradation of LAMP2A, with cathepsin A-deficient cells displaying reduced rates of LAMP2A degradation, increased LAMP2A levels and chaperone-mediated autophagy rates (Cuervo *et al.*, 2003). Our data do not support the elevation of cathepsin A levels in Parkinson's disease brain tissue as a protective response to the reduction in glucocerebrosidase, as although reduced glucocerebrosidase protein levels stabilized in late Parkinson's disease, glucocerebrosidase enzyme activity continued to decline, indicating that the remaining glucocerebrosidase was not protected against inactivation. Rather, our data suggest that increased cathepsin A would subsequently lead to increased LAMP2A degradation and reduced chaperone-mediated autophagy. This would result in the increased accumulation of chaperone-mediated autophagy substrates like  $\alpha$ -synuclein.

Chaperone-mediated autophagy and macroautophagy exhibit crosstalk that results in the constitutive activation of one pathway when the other is impaired (Massey *et al.*, 2006; Kaushik *et al.*, 2008). In Parkinson's disease,  $\alpha$ -synuclein accumulates and neuronal dysfunction occurs over a long progressive disease course, and hence upregulated macroautophagy might be expected due to chronic impairment of chaperone-mediated autophagy (Massey *et al.*, 2008). However, we saw no change in the autophagosome marker LC3-II, whereas others have found increased levels of this marker in Parkinson's disease (Yu *et al.*, 2009; Alvarez-Erviti *et al.*, 2010; Gegg *et al.*, 2012). Again, this may be because of the assessment of tissues at different disease stages. As stated in the results, LC3-II levels in post-mortem human tissue only represent autophagosome number, and do not identify whether an LC3-II increase is because of greater induction of autophagosome formation (upregulation of macroautophagy) or decreased fusion of autophagosomes with lysosomes. We also identified a decrease in beclin 1, also required for autophagosome formation in macroautophagy, which was significantly correlated with the reduced glucocerebrosidase in the same cases. This differs from previous studies showing no change (Crews *et al.*, 2010) or an increase (Yu *et al.*, 2009) in beclin 1 in Parkinson's disease (variation could be related to disease stage), with our data being more consistent with cell and mouse models of synucleinopathy (representing early disease stages) where over-expression of beclin1 reduces abnormal  $\alpha$ -synuclein accumulation by inducing autophagy (Spencer *et al.*, 2009). Interestingly, beclin 1 has been implicated in the regulation of retrograde endosomal transport as well as macroautophagy (Ruck *et al.*, 2011; Wirawan *et al.*, 2012), suggesting that the beclin 1 deficits we observed may be related to endosomal trafficking. Recent work has identified dysfunction of endosome to Golgi trafficking by the retromer complex as important in Parkinson's disease, with three Parkinson's disease susceptibility genes involved in this pathway, *LRRK2*, *PARK16/RAB7L1* and *VPS35* (MacLeod *et al.*, 2013). The association of beclin1 and glucocerebrosidase deficits in early stages of Parkinson's disease may also link *GBA1* to this Parkinson's disease-related pathway rather than to deficits in macroautophagy.

We provide novel data on relevant sphingolipid changes that occur selectively in brain regions with glucocerebrosidase deficits

and increased  $\alpha$ -synuclein levels in early stages of sporadic Parkinson's disease, identifying reductions in total ceramide. Ceramide may be generated through three main pathways: (i) *de novo* synthesis beginning with serine and palmitoyl-CoA; (ii) through the breakdown of sphingomyelin by sphingomyelinase; and (iii) by the breakdown of glucosylceramide by glucocerebrosidase (Kitatani *et al.*, 2008). The latter route for ceramide production, also known as the salvage pathway, has been proposed as the most energy efficient mechanism for ceramide generation in non-dividing cells, and could account for 50% to 90% of sphingolipid production, depending on the lipid molecular species and the cell type (Gillard *et al.*, 1998; Tettamanti *et al.*, 2003). The decreased levels of ceramide detected in the Parkinson's disease cases suggest that an impairment in the salvage pathway, which is critically dependent on lysosomal glucocerebrosidase, is not compensated for by an upregulation of *de novo* synthesis, and this may eventually have an impact on cellular membrane structure and signalling pathways (Soreghan *et al.*, 2003; Fortin *et al.*, 2004; Jana *et al.*, 2009). This could suggest significant changes in neuronal membrane properties in patients with Parkinson's disease (Fabelo *et al.*, 2011). It should be noted that ceramide is not reduced in patients with Gaucher disease, even though they have significantly reduced glucocerebrosidase activity (Almeida Mdo, 2012), supporting the concept of alternate production pathways for this lipid, at least in the peripheral macrophages that are most abnormal in Gaucher disease. This suggests that neurons rely more on the ceramide-producing salvage pathway compared with other cell types, a concept that may increase their vulnerability to certain lysosomal perturbations. Interestingly, mutations affecting enzymes involved in alternate ceramide synthesis pathways (*PANK2* in the *de novo* pathway and *PLA2G6* in the sphingomyelin breakdown pathway) also present with parkinsonian symptoms and display  $\alpha$ -synuclein neuronal pathology (Bras *et al.*, 2008), supporting a relationship between the dysregulation of ceramide production and Parkinson's disease.

This study assessed post-mortem Parkinson's disease cases and cannot address early cellular changes occurring before, or at, symptom onset in Parkinson's disease. However, the early stage Parkinson's disease cases examined were the earliest disease stage with largely biochemical rather than structural  $\alpha$ -synuclein changes in frontal cortical regions (Zhou *et al.*, 2011; Lue *et al.*, 2012; Murphy *et al.*, 2013) and limited cortical pathology compared with later disease stages (Braak stages V/VI) (Braak *et al.*, 2003). We analysed two cortical regions that do not undergo substantial neuron loss at this early Parkinson's disease stage, to assess the earliest cellular changes associated with increases in  $\alpha$ -synuclein. We then assessed later stage Parkinson's disease cases (Braak stages V/VI) to determine what effect increased  $\alpha$ -synuclein accumulation and aggregation have on these early cellular changes. We determined that there are reduced NeuN levels in the anterior cingulate cortex in these later disease stages indicative of neuron loss. The neuronal loss was associated with increased  $\alpha$ -synuclein accumulation and continued, but not exacerbated, lysosomal dysregulation. Chronic lysosomal dysregulation in Parkinson's disease is consistent with suggestions of lysosomal membrane destabilization and permeabilization as potential cell



death mechanisms in neurodegenerative disorders (Ditaranto-Desimone *et al.*, 2003; Boya and Kroemer, 2008).

This study suggests that glucocerebrosidase is reduced in association with the early abnormal accumulation of  $\alpha$ -synuclein in sporadic Parkinson's disease, leading to substantial alterations in lysosomal chaperone-mediated autophagy pathways and altered lipid metabolism. Although reduced glucocerebrosidase levels alone are not sufficient to cause Parkinson's disease, as only a small percentage of *GBA1* mutation carriers develop Parkinson's disease (Westbroek *et al.*, 2011), reduced glucocerebrosidase levels are present even in sporadic Parkinson's disease. Our data show that the redistribution of cellular membrane proteins may be key to the selective Parkinson's disease changes observed, and that such protein redistribution may occur through endosomal membrane trafficking. The reduced glucocerebrosidase is likely to contribute to lysosomal dysfunction by altering lysosomal contents and membrane properties, exacerbating any age-related diminished lysosomal capacity. Further elucidation of the nature and timing of dysregulation in these affected pathways in sporadic Parkinson's disease will aid in identifying the catalytic event(s) that initiate the pathogenic process, as well as potential targets for disease-modifying therapies and early disease biomarkers (Balducci *et al.*, 2007; Parnetti *et al.*, 2009).

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## Supplementary material

Supplementary material is available at *Brain* online.

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