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Path mediation analysis reveals GBA impacts Lewy body disease status by increasing α-synuclein levels

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A B S T R A C T

Synucleinopathies including Parkinson's disease (PD) and Dementia with Lewy bodies (DLB) are characterized by the accumulation of abnormal α-synuclein in intraneuronal inclusions, named Lewy bodies. Mutations in *GBA1*, the gene encoding the lysosomal hydrolase glucocerebrosidase, have been identified as the most common genetic risk factor for PD and DLB. However, despite extensive research, the mechanism by which glucocerebrosidase dysfunction increases the risk for PD or DLB still remains elusive.

In our study we expand the toolbox for PD-DLB post-mortem studies by introducing new quantitative biochemical assays for glucocerebrosidase and α-synuclein. Applying causal modelling, we determine how these parameters are interrelated and ultimately impact disease manifestation.

We developed quantitative immuno-based assays for glucocerebrosidase and α-synuclein (total and phosphorylated at Serine 129) protein levels, as well as a liquid chromatography–mass spectrometry method for the detection of the glucocerebrosidase lipid substrate glucosylsphingosine. These assays were applied on tissue samples from frontal cortex, putamen and substantia nigra of PD (n = 15) and DLB (n = 15) patients and age-matched non-demented controls (n = 15).

Our results confirm elevated p-129 over total α-synuclein levels in the insoluble fraction of PD and DLB post-mortem brain tissue and we found significantly increased α-synuclein levels in the soluble fractions in PD and DLB. Furthermore, we identified an inverse correlation between reduced glucocerebrosidase enzyme activity and protein levels with increased glucosylsphingosine levels. In the substantia nigra, a brain region particularly vulnerable in Parkinson's disease, we found a significant correlation between glucocerebrosidase protein reduction and increased p129/total α-synuclein ratios.

We assessed the direction and strength of the interrelation between all measured parameters by confirmatory path analysis. Interestingly, we found that glucocerebrosidase dysfunction impacts the PD-DLB status by increasing α-synuclein ratios in the substantia nigra, which was partly mediated by increasing glucosylsphingosine levels.

In conclusion, we show that the introduced immuno-based assays enable the quantitative assessment of glucocerebrosidase and α-synuclein parameters in post-mortem brain. In the substantia nigra, reduced glucocerebrosidase levels contribute to the increase in α-synuclein levels and to PD-DLB disease manifestation partly by increasing its glycolipid substrate glucosylsphingosine. This interrelation between glucocerebrosidase, glucosylsphingosine and α-synuclein parameters supports the hypothesis that glucocerebrosidase acts as a modulator of PD-DLB.

1. Introduction

Parkinson's disease (PD) and Dementia with Lewy bodies (DLB) are synucleinopathies, characterized by the accumulation of Lewy bodies containing the aggregated protein α-synuclein (Spillantini et al., 1997; Mezey et al., 1998; Moore et al., 2005). Genetic, biochemical as well as animal studies have centred α-synuclein as a main pathological driver and target for therapeutic intervention in Parkinson's disease. (Polymeropoulos et al., 1997; Singleton et al., 2003; Miller et al., 2004; Luk et al., 2012; Mougenot et al., 2012).

Furthermore, it has been shown that α-synuclein within LBs is subjected to several post-translational modifications (Oueslati et al., 2010). One of these post-translational modifications is the phosphorylation at residue Ser129 of α-synuclein, which has been correlated with the degree of PD pathology (Lue et al., 2012; Walker et al., 2013). > 90% of α-synuclein within LBs is phosphorylated at Ser129. (Fujiwara et al., 2002; Anderson et al., 2006). Dysfunction of cellular degradation mechanisms, including lysosomal dysfunction, may directly contribute to the accumulation of pathological α-synuclein in PD and DLB (Migdalska-Richards and Schapira, 2016).

The *GBA*1 gene encodes the lysosomal enzyme glucocerebrosidase, which hydrolyses its glycolipid substrates glucosylceramide and glucosylsphingosine. Genetic deficiency of glucocerebrosidase leads to the accumulation of both glycolipid substrates and causes the lysosomal

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storage disorder Gaucher disease (Brady et al., 1965). Gaucher disease patients and their relatives, carrying a heterozygous GBA1 mutation, were shown to have an increased risk for developing PD and DLB (Tayebi et al., 2003; Goker-Alpan et al., 2004). Moreover, mutations in the glucocerebrosidase (*GBA*1) gene are the most common known genetic risk factor for PD and DLB (Sidransky et al., 2009; Bultron et al., 2010; Nalls et al., 2013).

The mechanism by which *GBA*1 mutations increase the risk for PD and DLB is still unknown. Hypotheses include a gain-of-function mechanism by which dysfunctional glucocerebrosidase directly interacts with α-synuclein, which then promotes α-synuclein accumulation and aggregation (Sidransky and Lopez, 2012). An alternative loss-of-function hypothesis proposes that glucocerebrosidase deficiency leads to an accumulation of its lipid substrates, which affects α-synuclein aggregation, trafficking and clearance (Mazzulli et al., 2011; Westbroek et al., 2011; Sidransky and Lopez, 2012; Taguchi et al., 2017). The third hypothesis represents a bi-directional feedback loop between glucocerebrosidase and α-synuclein, where oligomeric α-synuclein interferes with GCase trafficking, which further exacerbates α-synuclein pathology (Mazzulli et al., 2011).

Recent post-mortem studies aimed to address disease relevance of altered glucocerebrosidase in idiopathic PD by using immunohistochemical and biochemical methods (Gegg et al., 2012; Murphy et al., 2014; Chiasserini et al., 2015; Rocha et al., 2015; Moors et al., 2018). They find reduced glucocerebrosidase activity in idiopathic PD without *GBA* mutations, most evident but not exclusively in brain areas like the substantia nigra and anterior cingulate cortex (Chiasserini et al., 2015; Rocha et al., 2015; Moors et al., 2018). Biochemical analyses by semi-quantitative western blotting indicate a reciprocal relationship between reduced glucocerebrosidase and increased α-synuclein protein levels (Gegg et al., 2012; Murphy et al., 2014). However, given the limited number of studies and methodological restrictions, the interrelation between glucocerebrosidase, α-synuclein and potential synergistic effects in idiopathic PD is still poorly understood.

In this study, we aimed to characterize the interrelation between glucocerebrosidase and α-synuclein in human brain tissue to obtain more insight into the mechanism underlying GBA-associated PD. For this purpose, we combined quantitative measurements of glucocerebrosidase and α-synuclein protein levels with path analysis.

In order to do this, we developed tools to quantitatively assess glucocerebrosidase and α-synuclein parameters in brain tissue of PD and DLB patients and age-matched non-neurological controls ($n = 15$ per group). We measured these parameters in three different brain regions (frontal cortex, putamen and substantia nigra). By applying statistical path analysis we investigated the link between GBA carrier status, glucocerebrosidase activity and protein levels, its lipid substrate glucosylsphingosine and α-synuclein (total and p129-phosphorylated). We find that in the substantia nigra, glucocerebrosidase dysfunction correlates with an increase in soluble α-synuclein species partly mediated by increased glucosylsphingosine lipid levels.

2. Results

2.1. Biochemical assessment of PD and DLB post-mortem brain tissue

In the current study we analysed post-mortem tissue samples from three different brain regions (frontal cortex, putamen and substantia nigra) of 15 PD, 15 DLB cases and 15 non-neurological controls from the Netherlands Brain Bank (NBB, Amsterdam, The Netherlands) (Fig. 1). Results from genetic screening of the *GBA* gene in this cohort was reported in an earlier study, revealing the presence of different *GBA* variants (Moors et al., 2018) (Table S1). PD and DLB cases showed characteristic α-synuclein pathology with no significant differences in the distribution or load of β-amyloid or tau pathology (Table 1). All PD and DLB cases were at Braak Parkinson's disease stage IV to VI at autopsy whereas non-neurological controls did not show Lewy body pathology in the brain except for one case with Braak score I (Table 1) (Braak et al., 2003). Post-mortem tissue from the three brain regions was processed and fractionated into triton-soluble and triton-insoluble fractions for further biochemical assessments (Fig. 1).

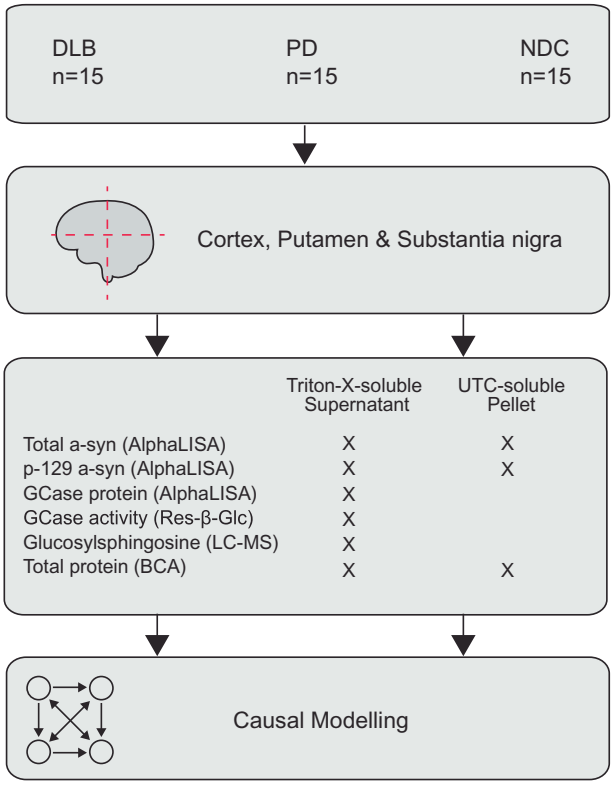


Fig. 1. Biochemical assessment of PD and DLB post-mortem brain tissue.

Table 1
Cohort demographics.

		NDC	DLB	PD	
	N	(N = 15)	(*N* = 15)	(N = 15)	P-value
Age (yrs)†	45	78 (75, 81)	78 (73, 81)	78 (74, 81)	0.942*
Sex (Males)‡	45	4 (27)	10 (67)	11 (73)	0.021**
Braak NFT (2–3)	45	7 (47)	6 (40)	4 (27)	0.516
Braak LB (0–2)	45	15 (100)	0 (0)	0 (0)	< 0.001
(3–4)		0 (0)	3 (20)	3 (20)	
(5–6)		0 (0)	12 (80)	12 (80)	
PMD (hrs)	45	6.2 (5.5, 7.1)	4.7 (4.4, 5.5)	5.8 (4.8, 6.5)	0.082
Disease duration (yr)	45	0	6 (4.0, 8.5)	13 (11.5, 18.0)	< 0.001
GBA variant carriers	43	2 (14)	4 (29)	6 (40)	0.304

† Median (Q1, Q3) for numerical variables
‡ n (%) for categorical variables
* Kruskal-Wallis test for numerical variables
** Pearson test for Categorical variables

We quantified glucocerebrosidase protein le id substrate glucosylsphingosine and both total as well as p129 phosphorylated α-synuclein in frontal cortex, putamen and substantia nigra samples (Fig. 1).

2.2. pSer129/ total α-synuclein ratios are increased in PD-DLB tissue and predict disease status

Previous post-mortem studies using semi-quantitative western blotting indicated that insoluble forms of α-synuclein are increased in

Parkinson's disease brains whereas soluble forms are not significantly changed or even reduced (Tong et al., 2010; Zhou et al., 2011; Murphy et al., 2014). α-Synuclein phosphorylation at serine 129 (pSer129 α-syn) is considered to contribute to α-synuclein pathology in LB disorders (Fujiwara et al., 2002; Anderson et al., 2006) and is selectively increased under pathological conditions as shown by immunohistochemistry (Obi et al., 2008; Beach et al., 2010). We aimed to advance biochemical assessment of α-synuclein in human post-mortem tissue by developing immuno-based assays for quantitative measurements of total and serine pSer129 phosphorylated α-synuclein in both soluble and insoluble fractions. Both AlphaLISA immunoassays, for total and pSer129 a-syn, were generated using two anti-α synuclein specific monoclonal antibodies and tested against recombinant total and PLK2 phosphorlyated synuclein (Fig. SM1A + B).

Our biochemical assessment of α-synuclein pathology did not reveal statistically significant differences between the DLB and PD cases, which prompted us to group PD and DLB cases into one cohort for further analysis (Fig. S2A + B).

We detected a pronounced increase of pSer129 α-syn/total α-syn ratios in the insoluble fraction for the PD-DLB group versus non-neurological controls in all analysed brain regions (Fig. 2A). In frontal cortex we find a 36 fold ($p < 0.001$), in putamen a 72 fold ($p < 0.001$) and in the substantia nigra a 20 fold ($p < 0.001$) increase of pSer129 α-syn/total α-syn ratios in the PD-DLB group compared to controls. Notably, we also found significantly increased ratios of pSer129 α-syn/total α-syn in the soluble fraction (Fig. 2B) of all three brain regions: cortex (20%, $p < 0.001$), putamen (50%, $p = 0.012$) and substantia nigra (50%, $p < 0.001$). Absolute amounts of pSer129 α-syn were also elevated in both insoluble and soluble fractions (S2C, D). Furthermore, pSer129 α-syn in the insoluble fraction reflects the Braak LB disease staging in all brain regions (Fig. 2C).

Applying both immuno-based α-synuclein assays on the soluble and insoluble fraction, we detected increased ratios of pSer129 α-syn/total α-syn in all three analysed brain regions in the PD-DLB group and were able to separate the PD-DLB group from the NDC group.

2.3. Reduced glucocerebrosidase protein and increased glucosylsphingosine levels in PD-DLB patient brain tissue

Reduced glucocerebrosidase protein levels and enzyme activity have previously been detected in PD brain tissue using semi-quantitative western blotting and a 4-methyl-umbellferyl β-glucopyranoside based enzyme activity assay (Gegg et al., 2012; Murphy et al., 2014; Chiasserini et al., 2015). In order to comprehensively analyse glucocerebrosidase status in PD-DLB brain tissue, we a) developed a sensitive and specific glucocerebrosidase protein assay b) measured glucocerebrosidase enzyme activity with the artificial fluorogenic substrate resorufin-β-D-glucopyranoside and c) quantified the endogenous glucocerebrosidase lipid substrate glucosylsphingosine by liquid chromatography coupled to mass spectrometry. The glucocerebrosidase AlphaLISA immunoassay was established with two newly generated mouse monoclonal antibodies and tested against human recombinant glucocerebrosidase (Fig. SM1C).

First, we assessed the overall correlation of the different readouts across all tissues and disease groups. We identified statistically significant correlations between all three glucocerebrosidase readouts (Fig. S3A). Glucocerebrosidase activity and protein levels were tightly correlated with each other (Spearman's rho = 0.9, $p < 0.001$) and were inversely correlated to glucosylsphingosine lipid levels (Spearman's rho = −0.44, $p < 0.001$).

Comparing the total levels of all three parameters between brain regions, we found a trend for glucocerebrosidase protein reduction in the PD-DLB group in all regions (CORTEX -20.1%, $p = 0.25$; PUTAMEN -10%, $p = 0.26$, SUBSTANTIA NIGRA -21% $p = 0.081$) (Fig. 3A). For the glucocerebrosidase enzyme activity we only find a trend for reduction in cortex and putamen (CORTEX 11,3%, $p = 0.346$; PUTAMEN

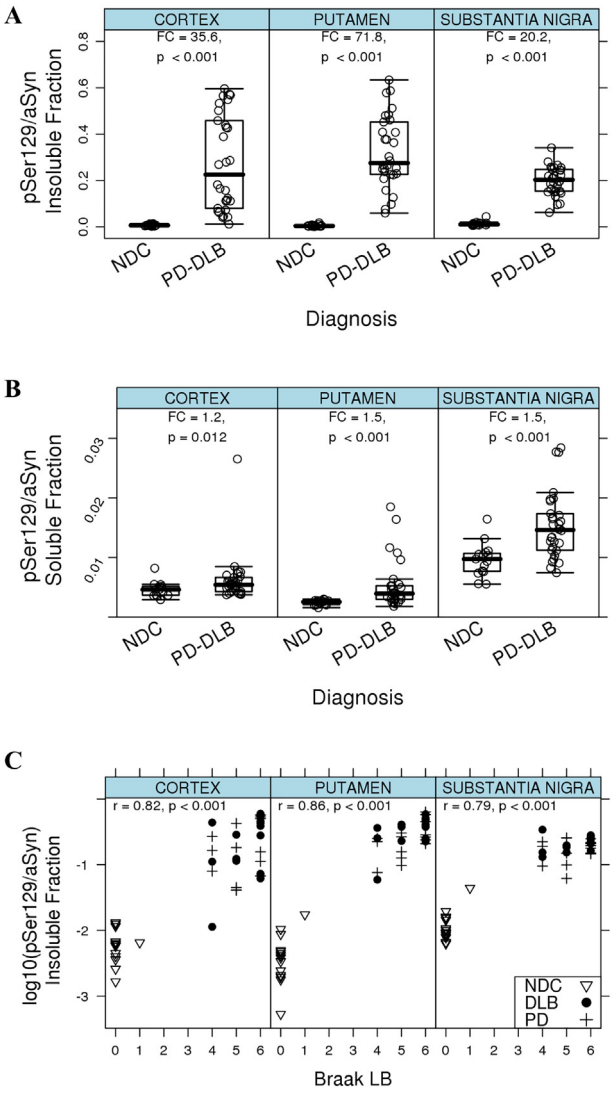


Fig. 2. pSer129/ total α-syn protein ratios are increased in PD-DLB brain tissue and predict disease status. Box plots showing the ratio of pSer129 and total α-synuclein protein in the insoluble (A) and soluble fraction (B) from brain tissue derived from NDC and PD-DLB subjects. FC = fold change, p = Wilcoxon test P-value. C pSer129 α-synuclein levels in the insoluble fraction with Braak Lewy body staging. NDC = non-demented controls. PD-DLB = Parkinson's disease and Dementia with Lewy bodies. r = Spearman correlation coefficient. p = p-value.

7,9%, $p = 0.31$; SN -0,6%, $p = 0.358$) (Fig. 3B). In line with a reduction in glucocerebrosidase protein levels, glucosylsphingosine levels were found to be increased in PD-DLB patients compared to controls (CORTEX 30%, $p = .015$; PUTAMEN 10%, $p = 0.25$; SN 10%, $p = 0.497$) (Fig. 3C).

When stratifying by mutant carrier status, a variable that substantially impacts glucocerebrosidase variance, we find association of glucocerebrosidase and PD/DLB disease status (Fig. S3). We also assessed the three glucocerebrosidase readouts comparing the NDC group to a subgroup of PD-DLB subjects carrying GBA variants (PD-DLB + GBA) (Fig. S3B-D). We found that the differences between PD-DLB + GBA and controls are most pronounced in the substantia nigra with a reduction in glucocerebrosidase activity of −24% ($p = 0.048$) and a reduction in protein levels of −39% ($p = 0.004$). In line with this, a trend for increased glucosylsphingosine levels (30%, $p = 0.129$) was observed in PD-DLB + GBA compared to controls (Fig. S3 B-D).

In summary, using the three glucocerebrosidase assays we identified

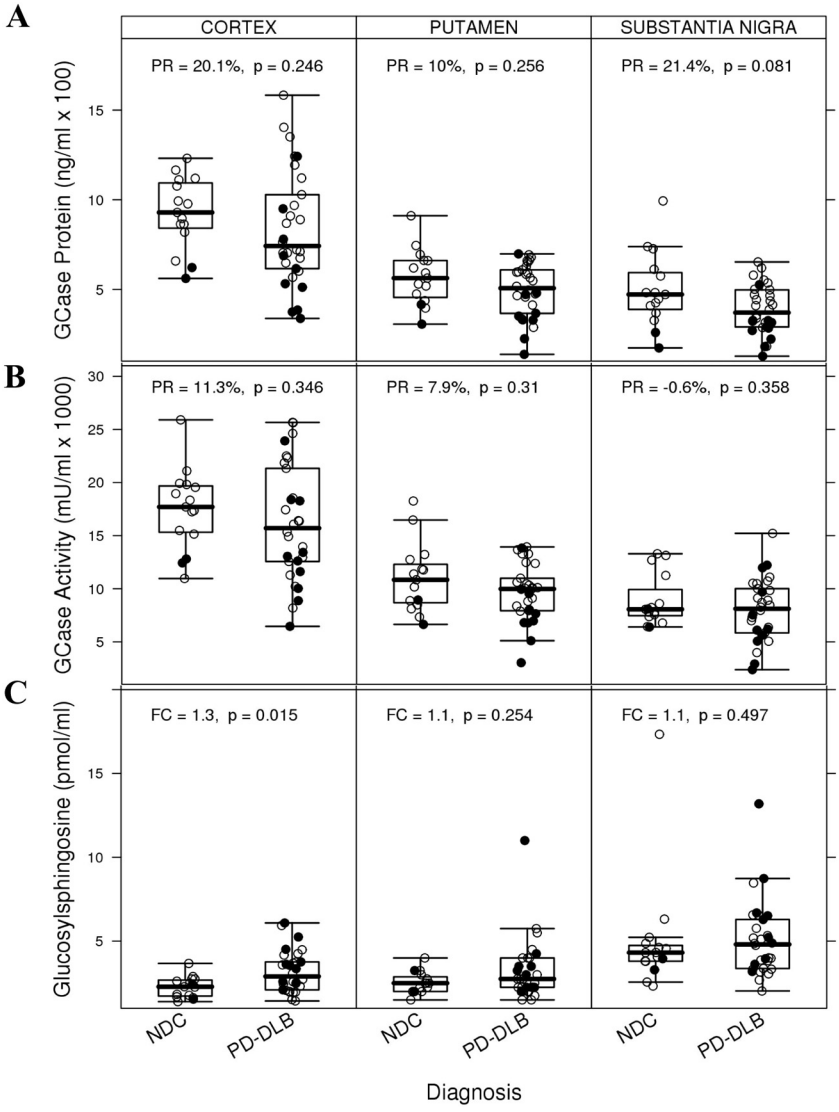


Fig. 3. Reduced glucocerebrosidase protein and increased glucosylsphingosine levels in PD-DLB patient tissue. Box plots showing protein levels (A), glucocerebrosidase activity (B) and the glucosylsphingosine lipid substrate levels (C) in the soluble fraction of brain tissue derived from NDC and PD-DLB subjects. GBA carriers are marked with black filled circles. PR = percent reduction, FC = fold change, p = Wilcoxon test P-value.

reduced glucocerebrosidase levels, which inversely correlated with elevated glucosylsphingosine levels in the DLB-LB group compared to the control group, which is most pronounced in the DLB-LB + GBA carrier subgroup.

2.4. Reduced glucocerebrosidase levels correlate with increased α-synuclein levels in the Substantia nigra

Our data demonstrate that we can quantitatively assess both glucocerebrosidase and p129/total α-synuclein protein levels in the soluble fraction of post-mortem brain tissue which enabled us to perform correlation analysis.

We found a strong association between reduced glucocerebrosidase protein levels with increased p129Ser/total α-synuclein ratios in the substantia nigra (Spearman's rho = −0.4, p = 0.0064) (Fig. 4). Furthermore, we find that all GBA variant carriers cluster in the region of reduced glucocerebrosidase levels and increased p129Ser/total α-synuclein.

2.5. The impact of glucocerebrosidase dysfunction on α-synuclein accumulation is partly mediated through increasing glucosylsphingosine levels in the Substantia nigra

Our analyses described so far have addressed the total effects of one measured parameter on another (e.g. association of glucocerebrosidase protein levels with PD-DLB disease status), omitting putative indirect effects through other parameters (e. g. through glucosylsphingosine and/or α-synuclein). In order to determine how different measured parameters are inter-related with each other, reflecting a more complex physiological environment, we integrated our data into a path analysis framework.

We defined six hypothetical path models describing how glucocerebrosidase and α-synuclein parameters could impact the PD-DLB disease status (Fig. 5). The GBA variant carrier status (yes/no), glucocerebrosidase protein levels, glucosylsphingosine levels, p129/total α-synuclein ratios and the disease status (NDC/PD-DLB) were included into the analysis. The competing models were selected to a) reflect plausible biological pathways (C, D, F), b) to test consistency and relevance of certain mediators (B, D, E) and c) to illustrate an statistical approach typically used for the analysis of such data (A, the

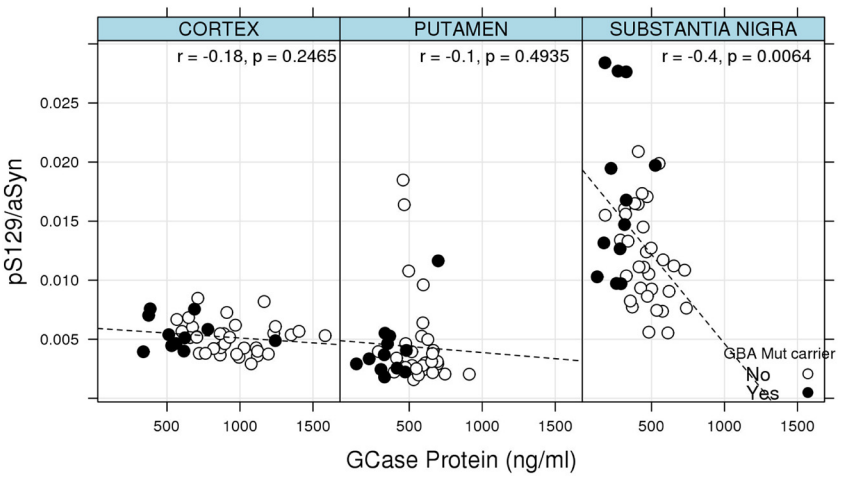


Fig. 4. Reduced glucocerebrosidase levels correlate with increased pSer129/ total α-syn protein ratios in the Substantia nigra. Correlation analysis between glucocerebrosidase protein levels and pSer129/ total α-synuclein ratios in the soluble fraction of NDC and PD-DLB brain tissue. GBA carriers are marked with black filled circles. r = Spearman correlation coefficient. P = *p* value.

multivariable regression). We evaluated the competing models against our empirical data by testing whether each model predictions of dependence and independence among variables are consistent with our data (Shipley, 2009, 2013) and estimated direct and indirect effects (MacKinnon et al., 2000; Lazic, 2012). We tested the hypothetical paths for the substantia nigra (Fig. 6). Fig. 6A shows the path coefficients estimates for all six models integrated in one path diagram. Corresponding posterior probabilities that the path coefficient β is away from

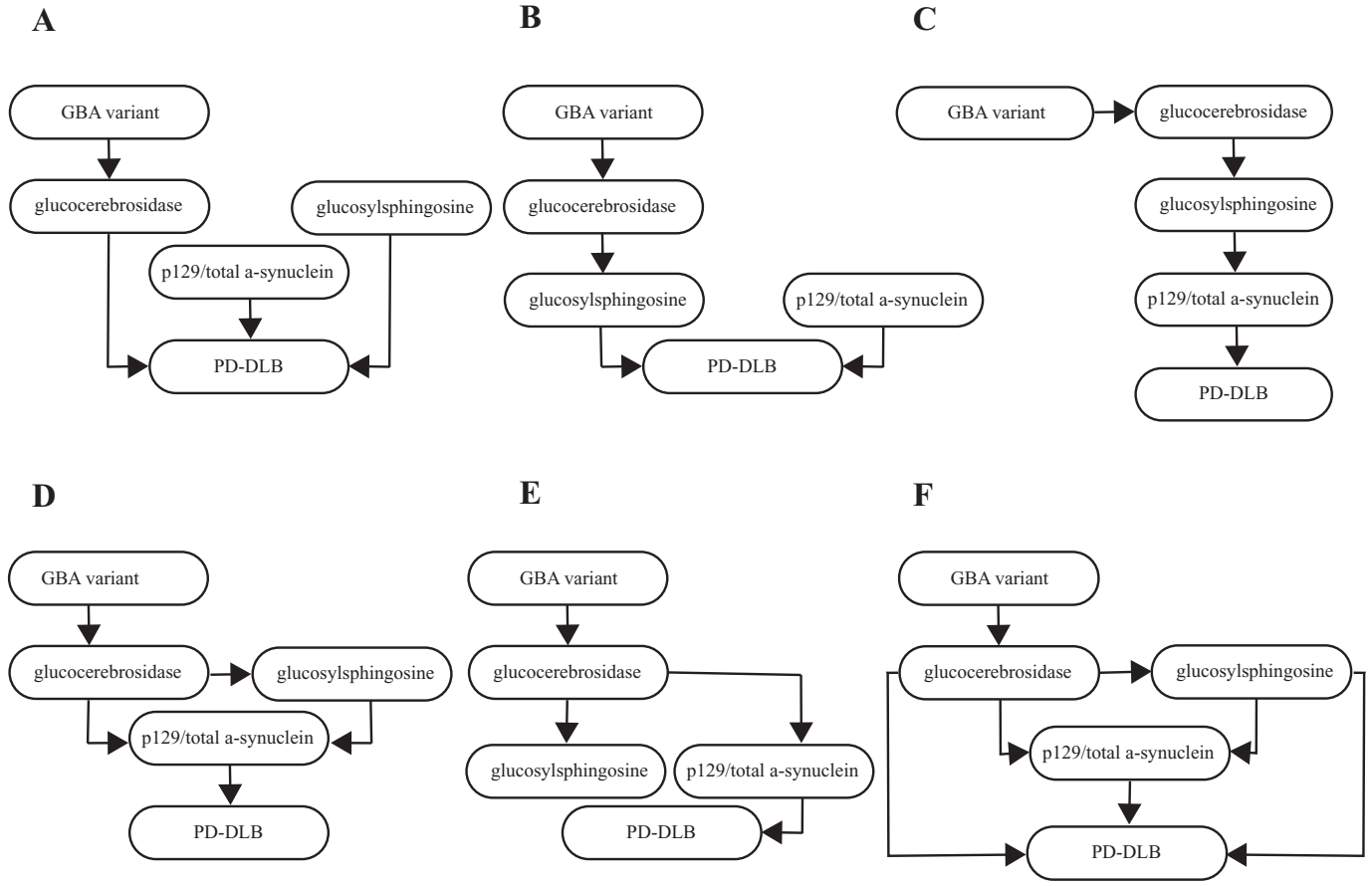
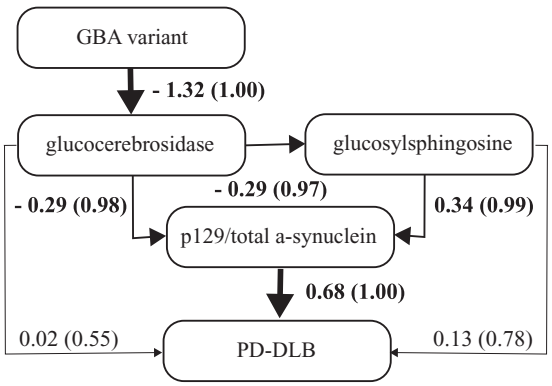


Fig. 5. Hypothetical networks linking glucocerebrosidase to PD-DLB. A-F Hypothetical models encompassing the GBA variant carrier status (yes/no), glucocerebrosidase protein levels, glucosylsphingosine levels, p129/total α-synuclein ratio and the disease status (NDC/PD-DLB). Nodes denote the variables and arrows the direction of the functional relationship. A In model A glucocerebrosidase levels depend on the GBA carrier status, but are linked to disease independently from glucosylsphingosine or pSer129/total α-synuclein ratios. B Model B postulates that GBA variants lead to a reduction in glucocerebrosidase and increased glucosylsphingosine levels, but pSer129/total α-synuclein ratios have an independent effect on the PD-DLB status. C Model C describes a cascade, where GBA variants lead to a reduction in glucocerebrosidase protein and increased glucosylsphingosine levels. Increased glucosylsphingosine levels cause an increase in pSer129/total α-synuclein ratios and ultimately lead to the PD-DLB disease manifestation. D Model D proposes that glucocerebrosidase levels impact p129/total α-synuclein ratios either directly or through glucosylsphingosine and subsequently impact the PD-DLB disease status. E In model E glucocerebrosidase protein levels influence the PD-DLB disease status only through direct effects on p129/total α-synuclein ratios not through changes in glucosylsphingosine levels F Model F encompasses all previously described models and allows independent effects for all measured parameters.

A



B

		Posterior Density Statistics			
	Effect	Lower†	Estimate††	Upper	P(|coef|>0)
Direct	Glucosylsphingosine -> Disease	-0.16	0.13	0.37	0.7807
	GCAse -> Disease	-0.25	0.02	0.27	0.5490
	pSyn Ratio -> Disease	**0.409**	**0.68**	**0.87**	**0.9997**
Indirect	GBA mut -> GCase -> Disease	-0.36	-0.02	0.33	0.5490
	GBA mut -> GCAse -> GlucSphin -> Disease	-0.06	0.04	0.19	0.7608
	GBA mut -> GCAse -> GlucSphin -> pSyn -> Disease	**0.002**	**0.07**	**0.22**	**0.9555**
	GBA mut -> GCAse -> pSyn -> Disease	**0.04**	**0.24**	**0.54**	**0.9758**

† Lower, Upper: 5% and 95% Posterior distribution percentiles

†† Estimate: Posterior Median of the Effect estimate of interest

Fig. 6. The impact of glucocerebrosidase dysfunction on a-synuclein accumulation is partly mediated through increasing glucosylsphingosine levels in the substantia nigra A Path diagram showing path coefficients (standardized regression weights:β) of all competing models. Within brackets, the posterior probability that β is greater than or smaller than the null (P|β| > 0| Data). B Summary statistics of the posterior densities of all direct and indirect effect estimates of models. Bold font highlights the effects that have a high certainty of being different from the null.

the null are presented within brackets.

We found that GBA variants are strongly related to reduced glucocerebrosidase levels ($\beta = -1.32$, $P(\beta < 0) = 1.00$). Furthermore, reduced glucocerebrosidase protein levels are linked to elevated glucosylsphingosine levels ($\beta = -0.29$, $P(\beta < 0) = 0.97$) and the p129/total α-synuclein ratio is strongly linked to the PD-DLB disease status ($\beta = 0.68$, $P(\beta > 0) = 1.00$).

Testing the six hypothetical models, we found strong evidence against models A and B (see d-separation tests and Akaike information criterion (AIC) results in Table S6A). Path coefficients for both models are low (Fig. 6A). Notably, both models propose that α-synuclein is not a component of the glucocerebrosidase path, i.e. glucocerebrosidase dysfunction would impact the PD-DLB disease status independent of pSer129/total α-synuclein ratios.

Among the remaining models (C—F), models C + D fit best to our data. Model D has the smallest AIC, none of the predicted claims of independence were rejected (Fig. S6A), all path coefficients of this model are elevated (Fig. 6A), and the two indirect effects that characterize this model have both > 95% probability of being different from null (Fig. 6B).

In contrast to models A + B, models C + D propose that α-synuclein is part of the glucocerebrosidase path to PD-DLB disease status.. In these models, changes in glucocerebrosidase and its downstream effects impact the disease status through increased pSer129/total α-synuclein ratios. Interestingly, model D proposes that glucosylsphingosine is not the only mediator of elevated α-synuclein, but that changes in glucocerebrosidase contribute to α-synuclein accumulation via alternative mechanisms ($\beta = -0.29$, $P(\beta < 0) = 0.98$) (Fig. 6A).

We replicated our analysis by replacing the PD-DLB disease status with p129/total α-synuclein ratios in the insoluble fraction as a quantitative proxy of disease status, and were able to confirm the above described findings (Fig. S6B + C).

Taken together, our path analyses suggest that in the substantia nigra reduced glucocerebrosidase levels lead to an increase in p129/total α-synuclein ratios, either by increasing its glycolipid substrate glucosylsphingosine and/or by yet unknown mechanisms, thereby ultimately impacting PD-DLB manifestation.

3. Discussion

Despite extensive research, the mechanism by which glucocerebrosidase dysfunction increases the risk of PD and DLB still remains elusive. Hypotheses include a toxic gain-of-function due to misfolded glucocerebrosidase; lipid substrate accumulation caused by glucocerebrosidase loss of function or a bi-directional feedback loop between glucocerebrosidase and α-synuclein (Manning-Bog et al., 2009; Cullen et al., 2011; Mazzulli et al., 2011).

In the present study we introduce a set of new biochemical tools that allowed us to quantitatively assess the status of the glucocerebrosidase as well as α-synuclein in PD-DLB post-mortem brain tissue. By combining our results in the substantia nigra with path analyses, we postulate a model in which glucocerebrosidase dysfunction increases α-synuclein levels, thereby contributing to the PD-DLB disease manifestation.

Immunohistochemical detection of pSer129 α-synuclein represents a specific and sensitive method to detect this pathological form of α-synuclein in human brain tissue (Fujiwara et al., 2002; Anderson et al., 2006; Beach et al., 2010). Histological analyses, the gold standard for post-mortem diagnosis of Lewy-body disorders, are used to classify the degree of pathology (Braak et al., 2003).

We have established α-synuclein immunoassays directed against total and pSer129 phosphorylated α-synuclein to allow for quantification of soluble and insoluble α-synuclein in different brain regions. Analysis of insoluble α-synuclein clearly discriminated PD-DLB from control samples in all brain regions analysed. We found that insoluble α-synuclein levels are increased in the frontal cortex of PD-DLB patients, a region affected at a late stage of the disease (Braak staging 5–6). Furthermore, levels of insoluble α-synuclein in the cortex were in correspondence with Braak staging. Thus, we conclude that our biochemical approach enables both quantitative and sensitive detection of

α-synuclein pathology.

Analysis of the soluble fraction revealed increased levels of pSer129 α-synuclein and pSer129/total α-synuclein ratio in PD-DLB cases compared to controls for all brain regions analysed. Basal levels of pSer129 α-synuclein are observed in controls, which are in line with previous findings and implies a role of pSer129 α-synuclein in normal cellular homeostasis (Muntane et al., 2012). As pSer129 α-synuclein is described to affect α-synuclein conformation and solubility, accumulation of soluble pSer129 α-synuclein could represent an early event in disease pathology leading to subsequent formation and aggregation of insoluble α-synuclein into Lewy bodies as previously proposed (Lue et al., 2012; Walker et al., 2013). While we are not able to address this in our human post-mortem study, our approach can help to assess the relationship between pSer129 phosphorylation and α-synuclein aggregation in future studies.

We used three independent readouts to assess glucocerebrosidase status in PD-DLB brain tissue. We introduced the first quantitative immuno-based assay to measure total glucocerebrosidase protein levels and found a good overall correlation between the newly established glucocerebrosidase protein assay, enzyme activity and glucosylsphingosine levels.

In all three analysed tissues we found a trend for reduced glucocerebrosidase protein level in relation to the disease status. For the substantia nigra, we detected a 21% reduction in glucocerebrosidase protein levels in the PD-DLB group and 39% reduction in the PD-DLB + *GBA* subgroup carrying GBA variants. These results are in line with previous reports, which identified reduced glucocerebrosidase protein levels and/or activity in brain tissue from idiopathic as well as PD patients carrying GBA variants (Gegg et al., 2012; Murphy et al., 2014; Chiasserini et al., 2015; Moors et al., 2018). They also describe the strongest reduction in glucocerebrosidase protein levels in the substantia nigra using semi-quantitative western blotting (Gegg et al., 2012; Chiasserini et al., 2015; Moors et al., 2018). Furthermore, they describe comparable results with 22% reduction in glucocerebrosidase protein levels for the sporadic PD group and 57% for the PD-*GBA* population. Unexpectedly, we only detected a 24% reduction of glucocerebrosidase activity in the PD-DLB + GBA carrier group and no reduction in the PD-DLB group. The reasons for these differing results are unclear, since a reduction in protein levels would be expected to lead to a reduction in enzyme activity. We anticipate that the resorufin-β-D-glucopyranoside based glucocerebrosidase activity assay used in our experiments might be less sensitive to assess glucocerebrosidase activity in post-mortem brain tissue than the 4-methylumbelliferyl-β-D-glucopyranoside based assay used in previous studies (Gegg et al., 2012; Murphy et al., 2014). In addition, we also found a trend for increased glucosylsphingosine levels in the PD-DLB group, which further suggests that glucocerebrosidase activity is reduced in the disease group.

To the best of our knowledge, we provide the first quantitative analysis of p129 α-synuclein, total α-synuclein and glucocerebrosidase in PD-DLB post-mortem brain tissue. We found an inverse correlation between glucocerebrosidase protein levels and α-synuclein levels specifically in the substantia nigra supporting in vitro and in vivo studies that propose an inverse relationship between the two proteins in Parkinson's disease and Dementia with Lewy Bodies (Aflaki et al., 2017). To gain a deeper understanding on how glucocerebrosidase and α-synuclein are interrelated with each other and impact the PD-DLB disease status, we included our data into path analyses. We found that, in the substantia nigra, reduced glucocerebrosidase protein levels induce an increase in p129/total α-synuclein ratio, either by increasing its glycolipid substrate glucosylsphingosine and/or by other mechanisms, thereby ultimately impacting the PD-DLB manifestation.

The increase in α-synuclein levels could be induced by different mechanisms. One possibility, which our data support, is that a reduction in glucocerebrosidase leads to an increase in its glycolipid substrate glucosylsphingosine. Glucosylsphingosine has been shown to accumulate to a high degree in the plasma and tissue of Gaucher disease patients with glucocerebrosidase dysfunction (Nilsson and Svennerholm, 1982; Orvisky 2002; Ferraz 2014). Furthermore, increased glucosylsphingosine levels have been found in the substantia nigra of sporadic PD patients (Rocha et al., 2015). A recent study by Taguchi et al. shows that glucosylsphingosine triggers the formation of oligomeric α-synuclein species in human cells and accumulates in homozygous GD/PD mouse brain. This supports the hypothesis that glucosylsphingosine promotes pathological α-synuclein accumulation, thereby increasing PD risk in GD patients and *GBA* mutation carriers (Taguchi et al., 2017). An alternative explanation could be that elevated glucosylsphingosine levels, which under normal conditions are extremely low, interfere with lysosomal homeostasis and lead to an altered lysosomal processing and degradation of α-synuclein.

A second scenario, which our data support, is that glucocerebrosidase dysfunction increases p129/total α-synuclein ratio independent of its glycolipid substrate glucosylsphingosine. Yap et al., have shown that α-synuclein and glucocerebrosidase can physically interact (Yap et al., 2011; Yap et al., 2013; Yap et al., 2015). They postulate that α-synuclein binding to glucocerebrosidase in the lysosome interferes with glucocerebrosidase membrane binding and enzyme activity and that glucocerebrosidase moves membrane-bound α-synuclein away from the lysosomal membrane and thereby perturbing its degradation.

Our path analyses in the substantia nigra support both scenarios with comparable probabilities; Glucocerebrosidase would impact α-synuclein levels either by increasing glucosylsphingosine levels, by independent mechanisms or even a combination of both.

These findings further underscore the complexity of the relationship between glucocerebrosidase and α-synuclein and suggest that glucocerebrosidase dysfunction could contribute through a combination of factors to the accumulation of toxic α-synuclein species and ultimately to Parkinson's disease and Dementia with Lewy Bodies. Because of the complexity of the relationship, the availability of sensitive and quantitative methods to assess the status of glucocerebrosidase and α-synuclein are an important prerequisite for understanding this relationship.

Our study introduces new quantitative glucocerebrosidase and α-synuclein assays in combination with path analysis as useful tools for modelling and understanding the pathological link between glucocerebrosidase and α-synuclein in PD and DLB. We anticipate that future studies applying these quantitative tools on larger cohorts will help to understand the role of glucocerebrosidase in Parkinson's disease and may eventually support the development of biomarkers and ultimately disease modifying therapies.

4. Material and methods

4.1. Post-mortem brain material

Human post-mortem brain tissue was obtained from clinically-diagnosed and neuropathologically confirmed PD patients with dementia, DLB patients, and age-matched control subjects (15/group) from the Netherlands Brain Bank (NBB, Amsterdam, The Netherlands). The details of the cohort demographics are specified in Table 1. In compliance with local ethical and legal guidelines, informed consent for brain autopsy and the use of brain tissue and clinical information for scientific research was given by either the donor or the next of kin. Brains were dissected in compliance with protocols of the Netherlands Brain Bank (www.brainbank.nl). Medial frontal gyrus was collected from 1 cm slice of the brain at the level of the tip of the anterior horn of lateral ventricle. Putamen was collected from 1 cm slices at the level of globus pallidus substantia nigra was collected from midbrain in 1 cm slice at the level of the oculomotor nerve.

4.2. Tissue processing

Frozen human tissue samples were pulverized under liquid nitrogen and aliquoted. For tissue lysis, tissue samples (250 mg) were solubilized in 1 ml lysis buffer (50 mM potassium phosphate dibasic, 50 mM citric acid monohydrate, 110 mM potassium chloride, 10 mM sodium chloride, 0.10% Triton X-100) + protease inhibitor cocktail (Roche) using the Precellys hard tissue homogenizing kit and a PreCellys24 homogenizer (both Bertin instruments). After 30 min incubation on ice, lysates were centrifuged at 14′000 x g/4 °C for 10 min and separated into pellet and supernatant fraction. The supernatant fraction (= Triton X-100-soluble fraction) was used for subsequent protein and enzyme activity analysis. The pellet was washed with lysis buffer to remove Triton X-100 soluble proteins, the remaining insoluble material was extracted with UTC buffer (30 mM Tris-HCl pH 7.5; 7 M Urea; 2 M Thiourea; 4% Chaps). The measurements in the soluble fraction were corrected for total protein. Additional analyses correcting for neuronal (neuron-specific enolase) and non-neuronal protein (S100b) content did not reveal significant differences between groups in any of the studied brain regions(Data not shown).

4.3. Protein determination

Protein determination for the Triton X-100 soluble fraction was performed using two different protein determination kits according to the manufacturer's protocols (μBCA Kit/ Cat. No. 23227 and 660 nm: Cat No. 22662, both Thermo Fisher). Correlation analysis revealed a high association of total protein content with different protein readouts in the Triton-X-100 soluble fraction, therefore soluble readouts were normalized to total protein content. No normalization was performed for glycosphingolipids analysis and analysis of the Triton-X-100 insoluble pellet.

4.4. Immuno-based assays

Immunoassays for different proteins have been developed in a 384 well plate format (AlphaLISA platform, Perkin Elmer) according to manufacturer's instructions. Detection was performed on EnVision Multilabel Plate Reader (Perkin Elmer) in AlphaScreen mode.

Glucocerebrosidase immunoassay was developed using two in house generated anti-human GCase specific mouse monoclonal antibodies (1/23, Biotin-labelled at 2.5 nM final concentration; 1/17, directly coupled to acceptor beads). Specificity of the immunoassay was confirmed using human GBA-KO H4 cell lines (Data not shown). Human recombinant glucocerebrosidase served as reference standard (Fig. SM1C). The Triton X-100-soluble fraction of human brain was diluted 1:10 to 1:20 for analysis.

Alpha synuclein immunoassay was developed using anti-alpha synuclein specific mouse monoclonal antibodies (syn 23E8, Biotin-labelled at 1 nM final concentration; clone 42 (BD), directly coupled to acceptor beads). E.coli derived recombinant human alpha synuclein served as reference standard (Fig. SM1A). The Triton X-100-soluble and Triton X-100-insoluble fractions of human brain were diluted 1:50 for analysis.

Alpha synuclein (phosphorylated at pSer 129) immunoassay was developed using two anti-alpha-synuclein specific mouse monoclonal antibodies (23E8, Biotin-labelled at 2 nM final concentration; a-syn p129 11A5; antibodies provided by Prothena, South San Francisco, CA, USA) directly coupled to acceptor beads. E.coli derived recombinant, PLK2 in-vitro phophorylated human pSer129 alpha-synulein served as reference standard (Fig. SM1B). The Triton X-100-soluble and Triton X-100-insoluble fractions of human brain were diluted 1:50 for analysis.

4.5. Glucocerebrosidase activity assay

The glucocerebrosidase enzyme activity was determined with the fluorogenic substrate resorufin β-D-glucopyranoside (Sigma). Samples from the Triton X-100-soluble fraction were diluted 1/8 in acidic GC assay buffer (50 mM citric acid, 50 mM KPi, 110 mM KCl, 10 mM NaCl, 1 mM MgCl2/ pH 6) and 50 μl transferred to 96 well assay plates (COSTAR 3904). 30 μl resorufin β-D-glucopyranoside solution (40 mM in GC assay buffer) was added per sample. Directly after addition of the substrate the fluorescence intensity (ex: 535 nm, em: 595 nm) was detected for baseline signal with a SpectraMax paradigm multi-mode microplate reader (Molecular Devices). After 2 h incubation in the dark on a plate shaker the fluorescence signal was detected, baseline values subtracted and GCase activity calculated.

4.6. Glucosylsphingosine analysis by mass spectrometry

100 mg frozen pulverized brain tissue was weighed, transferred into 2 ml hard tissue homogenizing vials prefilled with ceramic beads (Bertin, CatNo.03961–1-002.2) and homogenized with 1000 μl distilled water with a Precellys homogenizer (Bertin), operated in two cycles for 15 s at 5000 rpm. Calibration standards, quality controls and brain samples were cleaned up by supported liquid extraction (SLE). For calibration standards and quality controls D-glucosyl-β-1-1′-D-erythro-sphingosine and D-galactosyl-β-1-1′-D-erythro-sphingosine purchased by Avanti Polar Lipids were spiked into pooled brain homogenate. 100 μl aliquots of each brain homogenate were diluted with 300 μl methanol containing D-glucosyl-β-1-1′-D-erythro-sphingosine-d5 and D-galactosyl-β-1-1′-D-erythro-sphingosine-d5 as internal standards and loaded onto a Isolute 400 μl SLE + 96-well-plate from Biotage (Part No. 820–0400-P01). After applying a short pulse of pressure the samples were left for 5 min on the plate to complete the absorption process. Then two portions of 900 μl of tertiary butyl-methyl-ether (Sigma-Aldrich No.179787) were added for elution. The eluates were collected, evaporated to dryness (45 °C, 4 h) and reconstituted in 50 μl acetonitrile/ H_2O 90/10 containing 1% DMSO. 2 μl were injected. Compounds were glycospecifically separated on a BEH glycan amide column (100 × 2.1 mm, 1.7 μM particle size, purchased from Waters) using 100 mM ammonium acetate (Fluka No.09690) and acetonitrile (HPLC grade, Merck) as eluents and measured on a Waters Xevo-TQ-S mass spectrometer operating in positive ion electrospray mode and multiple reaction monitoring mode (MRM) recording the transition m/z 462.3 to 282.3 at a cone voltage of 30 V and a collision energy of 18 eV.

4.7. Statistical analyses

Descriptive statistics: Continuous variables were compared between disease groups by Wilcoxon/Kruskall-Wallis tests. Reported fold changes and relative percent changes are based on group medians. The association between continuous variables was tested by Spearman correlation and between categorical variables by the Fisher exact test. Significance level was set at 0.05. Statistics were implemented in R.

In addition, instead of assuming all measured parameters were independent from each other and finding those with stronger association with the disease, we postulated hypothetical models that describe the way the measured factors are interrelated and how glucocerebrosidase is linked to disease (Fig. 5). Agreement between such models and our empirical data was tested using Confirmatory Path Analysis. Such an approach allowed separating direct influences from mediated effects (e.g. if A affects B and B affects C, Does A affect C because of B?, i.e. A - > B - > C). Statistical parameters for direct and indirect effects were expressed in terms of path coefficients (standardized regression weights) and were derived from generalized linear model equations (dichotomous/continuous data as appropriate) structured according to the hypothesized causal paths. Parameters were estimated using both Markov Chain Monte Carlo simulation (Bayesian framework with uninformative priors) and likelihood-based methods (frequentist framework) using JAGS software in R (R2jags library) and base/stats tools in R respectively. Posterior median and posterior probabilities are

reported as summary statistics of direct and indirect effects. Models adequacy with our empirical data and goodness of fit were assessed by d-separation tests (Shipley, 2009) and information criteria indexes (AIC and BIC) as recommended (Shipley, 2013).

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Author contribution

T.K., S.Z., R.J., W.vdB., I.R., A.G. designed the protocols and the experiments; S.Z., T.M., T.K., I.R. and G.D. performed the experiments and analysed the data; GD performed the statistical analyses; T.K., S.Z., R.J., W.vdB., A.G., K.B., T.M. discussed the experiments; A.G., G.D. and T.K. wrote the manuscript; K.B., W.vdB., T.M. and R.J. gave critical input on the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2018.09.015.

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