Quantitative biochemical profiling of GCase activity and α-synuclein proteoforms in post-mortem human brains from GBA-related and idiopathic Parkinson's disease

--- Supplementary Material ---

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Detailed methods

Post-mortem human frozen tissue cohorts

Post-mortem human brain tissue was obtained from either the Netherlands Brain Bank (NBB; www.brainbank.nl) or Normal Aging Brain Collection Amsterdam biobank (NABCA; www.nabca.eu) from neuropathologically verified donors. Informed consent for brain autopsy, use of brain tissue, and sharing of clinical information for research was obtained from either the donors or their families, adhering to all local ethical and legal guidelines. Brain dissections followed standard operating protocols established by the NBB, with neuropathological assessments conducted by a qualified neuropathologist [1, 2].

For the *GBA1 genotyping cohort*, we identified cases with a neuropathologically confirmed clinical diagnosis of PD, with or without dementia (PDD), who exhibited Lewy body disease (LBD) pathology and of which frozen brainstem and GTM tissue was available in the brain banks archives. We excluded cases with severe Alzheimer's disease pathology (Braak NFT stage >3 and Thal phase >3; n = 114) [3, 4]. Cases with severe cerebral amyloid angiopathy (CAA) type 1 pathology or microinfarcts were also excluded. Control cases had no detectable αSyn pathology (Braak αSyn stage 0) and were required to have "none" or "low" AD pathology (n = 21). Control cases with Braak αSyn stage >0 were included as incidental LBD (iLBD) cases (n = 25). The demographic information of this cohort (*genotyping cohort*) and details on the *GBA1* risk variants identified are presented in Table 1 and Table 2. Fresh-frozen cerebellum blocks from the selected cases were obtained for DNA extraction and *GBA1* genotyping.

Based on tissue availability, 86 cases from the *genotyping cohort* were selected for biochemical analyses (10 controls, 14 iLBD, 40 IPD, 22 GBA-PD). To improve statistical power and ensure that sufficient tissue was available from every brain region, we established a *biochemistry cohort* by including additional cases from our brain bank for which genotyping information of *GBA1* was already available from earlier studies [5, 6]. The same inclusion criteria used for the *genotyping cohort* were applied to these additional cases. 52 cases met these criteria: 16 controls, 16 iLBD and 20 PD cases. 4 PD brains (3 IPD and 1 GBA-PD) originated from [5] and had been Sanger-sequenced for *GBA*, whereas the remaining 48 cases (16 controls, 16 iLBD, 7 IPD and 9 GBA-PD) came from [6], where *GBA1* status had been determined with the Infinium NeuroChip Consortium array [7] and supplemented with the imputation of additional variants. These additional cases included one control and one iLBD brain carrying the E326K variant (see Suppl. Tab. 2), which were not included in the presented biochemistry analysis, except for the analysis presented in Figure 7-8. The resulting cohort (*biochemistry cohort*) consisted of 26 control cases (CTRL), 30 iLBD cases and 50 PD cases without a *GBA1* missense variant (CTRL), and 32 PD cases with a *GBA1* missense variants (GBA-PD). For details on the demographics of the *biochemistry cohort*, see Table 1, Supplementary Table 2, and Supplementary File 1. Additional

clinical information, such as presence of dementia, age at death, age at disease onset (age at onset), disease duration, time interval from motor symptoms to dementia development, and presence of visual hallucinations were retrieved from clinical files and are reported in Supplementary File 3.

DNA purification

To isolate purified DNA for the sequencing of *GBA1* from fresh-frozen cerebellum blocks, about 30 mG of tissue were obtained from the blocks by cryosectioning using a cryostat (CryoStar NX50, Epredia). The DNA was purified using NucleoSpin DNA Lipid Tissue kit (#740471.50; Macherey-Nagel) following the protocol of the producer. Briefly, tissue was disrupted in MN Bead Tubes (Type D) using the kit lysis buffer (Buffer LT) with Proteinase K, and the clarified lysate was applied to silica-membrane spin columns. Columns were washed according to the manufacturer's protocol and genomic DNA was eluted in Elution Buffer BE. To minimize RNA carryover prior to quantification, an RNase A treatment step was included according to protocol. Initial quality and concentration of the obtained genomic DNA (gDNA) was assessed by measuring absorbance (A) at 230 nm (A230), 260 nm (A260) and 280 nm (A280) wavelength with a Nanodrop spectrophotometer (NanoDrop Lite Plus, Thermo Fisher Scientific Inc.). An A260/A280 ratio of >1.7, an A260/A230 ratio of >1.8 and a concentration of at least (5 nG/µL) were used as cutoff to proceed with sample analysis.

GBA1 genotyping

-*GBA1* sequencing

GBA1 gene in the *genotyping cohort* was performed at GenomeScan B.V. (Leiden, The Netherlands) in accordance with a previously established protocol [8]. Quality check of the gDNA samples was performed using a fragment analyser (Fragment Analyzer System, Agilent Technologies Inc.). Precise DNA concentration was determined using a Quant-IT measurement (Quant-iT dsDNA Assay Kit, Thermo Fisher Scientific Inc). A long-range PCR using TaKaRa LA Taq DNA Polymerase Hot-Start (#RR042B, Takara Bio USA Inc.) and target-specific primers were used to amplify *GBA1* [8]. After library preparation (NEBNext Ultra II Ligation Module, #E7595, New England Biolabs), amplicons were fragmented with Bioruptor Pico (Diagenode Inc.). A-tailing and ligation of sequencing adapters of the resulting product was performed according to the NEBNext Ultra II Ligation Module Instruction Manual (New England Biolabs). Quality check was performed with a fragment analyser (Fragment Analyzer System, Agilent Technologies Inc.). Finally, sequencing was performed on an Illumina Novaseq 6000 System (Illumina Inc.).

-Sequencing data analysis

Quality of raw data was inspected using FastQC v.0.11.9 [9]. Next, sequencing adapters were removed using Trimmomatic v.0.39 [10], allowing a maximum of 2 mismatches and a minimum alignment score 12. Sickle v.1.33 [11] was used to trim and filter the paired reads. Bases were required to have a minimum PHRED score of Q30, and the splitted reads to have a minimum length of 36bp. QC-passing reads were mapped against *GBAP1*-masked GRChg37/hg19 reference genome using the Burrows-Wheeler algorithm (BWA-mem v. 0.7.17b [12]). After duplicates marking, the GATK v.4.2.4.1 [13] guidelines were followed to identify the variants. Coverage was calculated over the genomic interval chr1:155204151-155211199 using GATK DeptOfCoverage. Samples with a median read depth below 30x were excluded from downstream analyses, with 4 samples being filtered out. The number of variants in the region chr1:155204151-155211199 per sample were inspected to identify outliers. No sample had a number of variants bigger than the mean plus 3 standard deviations. Variants were deemed of interest if: 1 – In any of the samples passing both the "Sample read depth" and "Number of variants called" quality criteria as described above; 2 – Falling in the transcript NM_000157.3; 3 – Exonic or within ± 5 bp from canonical splice site. Variants were further annotated in silico using CADD [14] and GERP [15] scores from WGSA v0.85 [16] and the allele frequency from public databases including gnomAD [17] and GoNL [18]. Splicing effect was predicted using ADA, RF [19], SpliceAI [20] from WGSA v0.85 and SQUIRLS v.1.0.0 [21]. The identified variants are presented in Table 2 and Supplementary Table 1. *GBA1* variant severity was determined according to their association with Gaucher disease (GD) type II or III (severe), type I (intermediate) or with Parkinson's disease (mild), corresponding to Parlar et al. using the *GBA1-PD browser*, when available [22]. Frameshift and null variants were considered as severe (see Table 2 and Suppl. Table 1).

Imputation of GBA1 variants from microarray genotyping

For the samples genotyped on the Infinium NeuroChip Consortium Array (Illumina, San Diego, CA USA), initial quality checks and filtering were performed as described in a previous publication [23]. Imputation was performed using the Michigan Imputation Server [24] with European ancestry reference data from the Haplotype Reference Consortium [25].

Tissue sectioning

Fresh-frozen tissue blocks were manually dissected to isolate the areas of interest of the LC, SN and GTM blocks (Suppl. Fig. 7). After incision, the tissue was cut in 60 µM sections using a cryostat (CryoStar NX50, Epredia). Several tubes of about 10 mG of tissue (wet weight) were produced for the different analysis by collecting sections from the area of interest per each block and kept frozen. The tissue was anonymized during cutting. Blocks of the brainstem at the level of the pons and containing the locus

coeruleus (LC) were incised to separate the pons from the tegmentum, containing the LC (referred as LC; Suppl. Fig. 7a). Blocks of the midbrain were incised to isolate the SN area (referred as SN; Suppl. Fig. 7b). Blocks of the GTM were incised to isolate grey matter and discard the white matter (referred as GTM; Suppl. Fig. 7c). The tissue collected during cryo-sectioning and used for further biochemical analysis.

Tissue fractionation

Tissue processing was randomized and anonymized to exclude technical biases and performed separately per brain region. For the quantification of total GCase enzyme activity and GCase protein levels by ELISA, the tissue was extracted in GCase Lysis Solution (McIlvaine buffer (100mM di-Sodium Hydrogen Phosphate Dihydrate, 50mM Citric Acid Monohydrate, pH 5.2) containing 0.25% (v/v) Triton X-100 (#108603, Millipore), cOmplete Protease Inhibitor Cocktail (#04693116001, Roche; according to manufacturer instruction) and PhosSTOP (#4906837001, Roche; according to manufacturer instruction)). 250 µL of GCase lysis solution at room temperature (RT) was added to each tube of 10 mG of frozen tissue sections, which was then inverted 10 times and vortexed (5 seconds, maximum speed). The sample was subsequently homogenized using a tissue dissociator (TissueLyser LT, Qiagen) with a single 5 mm stainless steel bead (#69989, Qiagen) per tube (50 Hz for 2 minutes at RT). The tube was then incubated in ice for 30 minutes and the homogenate was then centrifuged at 15,000 × G for 15 minutes at 4 °C to separate the pellet and supernatant. The supernatant was divided into single-use aliquots and stored at -80 °C until further analysis.

For the quantification of αSyn protein levels by AlphaLISA, the tissue was subjected to sequential protein extraction (Suppl. Fig. 3) with a procedure adapted from published protocols [26-28]. First, 10 mG of tissue was thawed on ice and immediately incubated in 250 µL of SDS-free RIPA (RIPA Buffer (10X), #9806, Cell Signaling Technologies) containing 2% octyl-b-D-glucoside (w/v; #850511, Avanti Polar Lipids LLC.), 1 mM phenylmethylsulfonyl fluoride (PMSF), cOmplete Protease Inhibitor Cocktail (according to manufacturer instruction) and PhosSTOP (according to manufacturer instruction) (OG-RIPA). The tissue was then homogenized using a tissue dissociator (TissueLyser LT, Qiagen) with a single stainless-steel bead per tube (50 Hz for 2 min at RT) and then incubated in ice for 20 minutes. 200 µL of the homogenate was then transferred in an ultracentrifuge tube (0.2 mL Open-Top Thickwall Polycarbonate Tube, #343775, Beckman Coulter Inc.) and centrifuged at 100’000g at 4 °C for 1 hour in an ultracentrifuge (Optima MAX-TL, Beckman Coulter Inc.). After ultracentrifugation, the supernatant was collected and stored at - 80 °C in single-use aliquots. This fraction contains OG-RIPA-soluble, non-aggregated and membrane associated proteins (“Soluble” fraction). Subsequently, the resulting pellet (Pellet 1) was resuspended in 200 µL and centrifuged again at 100’000g at 4 °C for 30 minutes to remove all remaining OG-RIPA-soluble protein. The resulting pellet was then solubilized in

volume of UTC buffer (7M Urea, 2M Thiourea, 4% CHAPS, 30mM Tris/HCl) equal to 100 µL per mG of total protein in the Soluble fraction measured by BCA assay (#A55864, Thermo Fisher Scientific Inc.). The solution was then sonicated with a probe sonicator (Ultrasonics Sonifier 250, Branson) for 100 seconds (3 seconds on, 7 seconds off) and incubated at 100 °C for 10 minutes. The sample was then centrifuged at 100'000g at 4 °C for 30 minutes to yield an insoluble pellet (Pellet 2) and a supernatant containing OG-RIPA-insoluble and UTC-soluble proteins ("Insoluble" fraction). The Insoluble fraction was stored in single-use aliquots at - 80 °C for further analysis.

GCase enzymatic activity quantification

The total GCase enzyme activity assay was based on the conversion of the artificial GCase substrate Resorufin-β-D-glucopyranoside and was adapted from previously-published protocols for the high-throughput use in 384-wells plates in post-mortem human brain lysate [5, 29-31]. One single-use aliquots from samples extracted with GCase Lysis Solution were initially thawed on ice and their protein concentration was measured by BCA in a 384-well format. In order to run all the samples at the same time and in the same plate, a second aliquot per each sample was thawed in ice and transferred to a 384-wells pre-dilution plate (Polypropylene Storage microplates, # 3657, Corning Inc.) by diluting it to 0.5 mG/mL in GCase Lysis Buffer. From the pre-dilution plate, an aliquot was taken to run a second BCA assay in a 384-well format for normalization on total protein. From the same pre-dilution plate, 5 µL of each sample and a blank (GCase Lysis Buffer) were transferred in triplicate to a black 384-well plate (#732-3724, VWR International LLC) for GCase enzyme activity measurement. Similarly, 5 µL of a dilution series of recombinant human GCase enzyme (Recombinant Human Glucosylceramidase, #7410-GHB-020, Bio-Techne) was prepared in GCase Lysis Solution (concentration in plate 0-2000 nG/mL) and added to the plate. 25 µL of GCase Assay Buffer (McIlvaine Buffer (Citrate/Phosphate buffer, 0.15M, pH 5.2) with 0.25% (w/v) of Taurocholic acid (#T4009, Sigma-Aldrich)) and 30 µL of Res-β-Glc-Substrate solution (Resorufin-β-D-glucopyranoside, #R4758, Sigma-Aldrich; 40µM Resorufin-β-glucopyranoside in GCase Assay Buffer) per each well (final substrate concentration 20 µM). All wells were then brought to 60 µL with GCase Assay Buffer. In parallel, a standard curve with free Resorufin (#73144, Sigma-Aldrich) was prepared in GCase Assay Buffer and diluted 1:2 with GCase Assay Buffer to a final volume of 60 µL per each well (final concentration: 0-20 µM) and added to the plate. Both the plate and all solutions were pre-warmed at 37 °C. The plate was then sealed with a transparent plastic sealer (TopSeal-A PLUS, #6050185, Revvity), spun down (2000g x 10 seconds) a shaken for 1 min in an orbital vibrating shaker. The plate was then incubated at 37 °C in a spectrophotometer (SpectraMax iD3, Molecular Devices LLC.) and the fluorescence of free resorufin was read (excitation: 535 nm, emission: 595nm) every 1 hour for 12 hours.

The enzymatic activity was calculated using R (R version 4.3.2, 2023-10-31) [32] by fitting a 4-parameter logistic curve to the free Resorufin standard curve to compute the moles or free resorufin product formed at each time point. The rate of reaction was then calculated per each time point (nmol/hour), normalized on total protein concentration (previously measured by BCA assay) and reported as median nmol of Resorufin product per hour per mG of total protein (nmol/hour/mG). Representative standard curves for the assay are presented in Supplementary Figure 8 (linear range 30 – 2000 nG/mL of hrGCase; Lower limit of detection (LLOD)= 49.5 nG/mL hrGCase; Lower limit of quantification (LLOQ) = 150.0 nG/mL hrGCase).

GCase protein levels quantification by ELISA

The ELISA assay to measure GCase protein levels in post-mortem human brain lysate was developed using recombinant rabbit monoclonal anti-GCase antibody EPR26755-29 (Abcam ab309228; Capture antibody) and recombinant rabbit monoclonal anti-GCase antibody EPR26755-42 (Abcam ab309229; Detection antibody) [33]. The Detection antibody was biotinylated with a biotinylation kit (Biotin Conjugation Kit (Fast, Type A), #ab201795, Abcam) following manufacturer instructions. The first day, 30 µL of a 2 µG/µL Capture antibody solution in Coating Buffer (0.1 M Sodium Carbonate/Bicarbonate, pH 9.4) was added to each well of a high binding 384-well black plate (Immuno Plates, #460518, Thermo Fisher Scientific Inc.) and incubated overnight at 4 °C. On the second day, each well was washed four times with 60 µL of Base Buffer (TBS (0.15 M NaCl, 0.050 M Tris-HCl, pH 7.2) + 0.05% Tween-20) and incubated for 1,5 hours at RT with 90 µL of Blocking Buffer (2% w/v BSA (Bovine Serum Albumin Fraction V, #03117332001, Roche) + 5% v/v Normal Rabbit Serum (#011-000-120, Jackson ImmunoResearch LTD.). Then, one single-use aliquot from the samples extracted with GCase Lysis Solution were initially thawed on ice and their protein concentration was measured by BCA in a 384-well format. In order to run all the samples at the same time and in the same plate, a second aliquot per each sample was thawed in ice and transferred to a 384-wells pre-dilution plate (Polypropylene Storage microplates, # 3657, Corning Inc.) and diluted to 1 mG/mL in GCase Lysis Buffer. From the pre-dilution plate, an aliquot was taken to run a BCA assay in a 384-well format for normalization on total protein. A second aliquot was taken from the pre-dilution plate to produce a second pre-dilution plate where the samples were diluted 1:10 in Sample Diluent (2% w/v BSA in Base Buffer). A standard curve with a serial dilution (0 – 10’000 pG/mL) of recombinant human GCase (Recombinant Human Glucosylceramidase, #7410-GHB-020, Bio-Techne) was prepared in Sample Diluent containing the same amount of GCase Lysis buffer as the samples (1:10) and added to the plate. Then, the Blocking Buffer was removed from the ELISA plate and 30 µL of diluted samples and standards were added and incubated for 2 hours at RT shaking (500 RPM). After, the wells were washed three times for 2 minutes with 60 µL of Washing Buffer and incubated with 30 µL of biotinylated Detection antibody solution (2 µG/mL in Sample Diluent) for 1 hour at RT shaking (500 RPM). The wells were then

washed 6 times with 60 µL of Washing Buffer for 3 minutes and incubated with 30 µL of Sreptavidin HRP solution (50 nG/ml Streptavidin-HRP (#N100, Thermo Fisher Inc.) in Sample diluent) for 1 hour at RT. After, the wells were washed 6 times with 60 µL of Washing Buffer for 3 minutes and 30 µL of LumiPhos solution (LumiPhos-HRP, #PSA-100, Lumigen; according to manufacturer indication) was added to each well. After 5 minutes at RT, luminescence was read at a spectrophotometer (SpectraMax iD3, Molecular Devices LLC.). A representative standard curve for the assay is presented in Supplementary Figure 9. The assay was linear between 30 and 4000 pG/mL. GCase protein concentration in the samples was calculated based on the standard curve by interpolating a 5-parameter logistic curve using the software GraphPad Prism (10.2.0, GraphPad Software) and by normalizing the values on the total protein concentration as measured by BCA.

α-synuclein proteoforms quantification by AlphaLISA

Quantification of αSyn proteoforms was performed with an AlphaLISA assay (Revvity), adapting the protocol from Moors et al. [27] and following the manufacturer's recommendations to target three αSyn species: phosphorylated Ser129 (pSer129), truncated at αSyn122 (CTT122), and a C-terminal region encompassing residues 118–123 (referred as "Total" αSyn). A biotinylated antibody directed against the NAC domain of αSyn (Biotin anti-α-Synuclein, Clone A15115A, #848306, BioLegend; epitope aa80–96) served as the universal detection antibody. Specific acceptor antibodies — Syn-142 (gift Roche, [27]) for pSer129, MJFR1 (#ab209420, Abcam) for the "Total", and A15127A (#848402, BioLegend) for CTT122 — were conjugated to AlphaLISA Acceptor Beads (Unconjugated AlphaLISA Acceptor Beads, #6772002, Revvity) at a 10:1 beads-to-antibody weight ratio. No cross-reactivity was observed between the assays. For the Total αSyn assay, MJFR1 conjugate detected 76% of pSer129 αSyn and did not recognize CTT122 αSyn (see Suppl. Fig. 10g). Each pair of antibodies underwent concentration optimization to achieve robust signal-to-noise ratios without reaching the hook point.

To run all the samples at the same time and in the same plate, sample aliquots were thawed in ice and total protein amounts were measured by either BCA assay (Soluble samples) or Pierce 660nm assay (Insoluble samples). A master plate (Polypropylene Storage microplates, # 3657, Corning Inc.) was then prepared from a second sample aliquot as to equalize the concentration of all samples to either 3 mG/mL (Soluble samples) or 1.2 mG/mL (Insoluble samples) by diluting them in Assay Buffer (25 mM HEPES (#H3375, Sigma-Aldrich), 0.5% (v/v) Triton X-100 (#8603, Merck Millipore), 0.1% (w/v) Casein (# C0376, Sigma-Aldrich), and 0.1% (w/v) Dextran (Dextran-500, #9219.3, Carl Roth)). From the master plate, an aliquot was taken to run a second BCA assay (Soluble samples) or Pierce 660nm Assay (Insoluble samples) in a 384-well format for the normalization of the results on total protein. For the Soluble fractions, aliquots from the master plate were further diluted in Assay Buffer to 1:100 and 1:600

in pre-dilution plates. These diluted samples were used to run the pSer129, CTT122 (Dilution 1:100) and the Total (dilution 1:600) αSyn AlphaLISA assays. For the Insoluble fractions, aliquots from the master plate were further diluted in Assay Buffer to 1:90 and 1:270 in pre-dilution plates. These diluted samples were used to run the pSer129, CTT122 (Dilution 1:90) and Total (dilution 1:270) αSyn AlphaLISA assays.

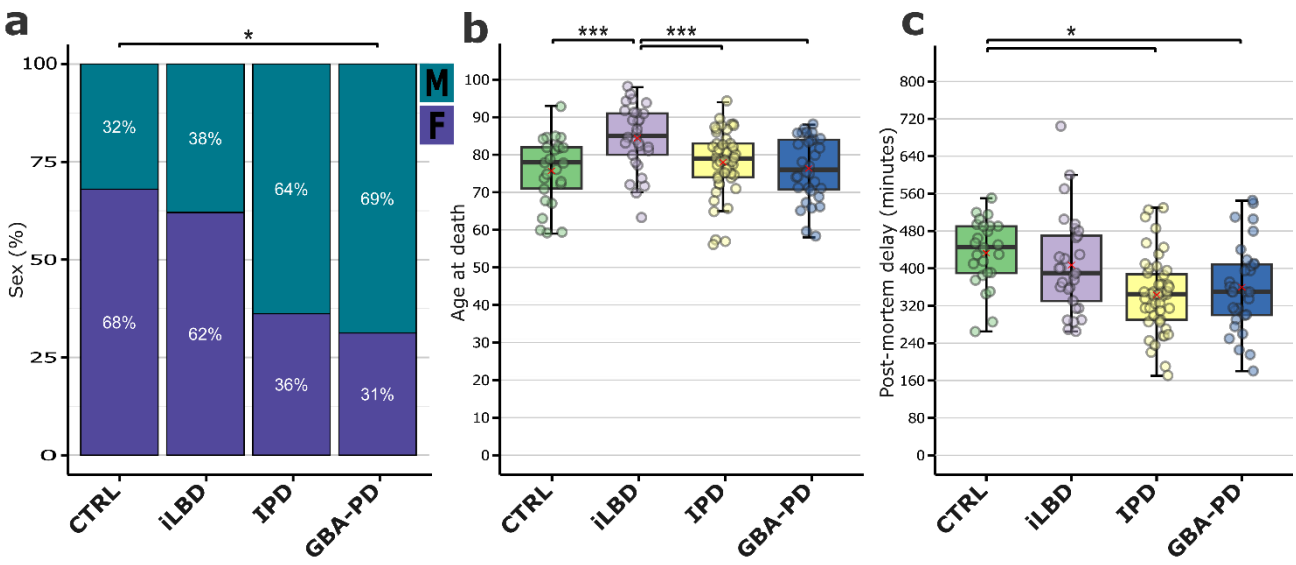
Assays were conducted in 384-well AlphaPlates (#6005350, Revvity) with a 50 µL total volume per well. Standard curves were generated using recombinant wild-type αSyn (#S-1001-2, rPeptide), pSer129 (#RP-004, Proteos), and CTT122 (gift Roche, [27]) in the same buffer conditions (same OG-RIPA or UTC buffer dilution) as for the lysate samples (see Suppl. Fig. 10). For each well, 5 µL of either diluted sample or recombinant standard was combined with 10 µL of Acceptor Beads conjugated to the respective Acceptor Antibody (75 µG/mL in the Total and CTT122 αSyn assays, 50 µG/mL in the pSer129 assay). The plate was then shaken for 1 minute and incubated at room temperature (RT) in the dark for 2 hours. Next, 10 µL of the biotinylated detection antibody (5 nM in Assay Buffer) was added, followed by another 1-minute shake and a 1-hour incubation under the same conditions. Then, 25 µL of Streptavidin Donor Beads (AlphaScreen Streptavidin Donor Beads, Revvity, Cat. 6760002) at 80 µG/mL was added per well. After shaking for 1 minute, the plate was incubated for an additional 30 minutes at RT in the dark. All samples were then measured on a VICTOR Nivo reader (PerkinElmer). All samples were run in triplicate. Data was graphed and analysed using R (R version 4.3.2, 2023-10-31) by fitting a 4-parameter logistic model to the standard curves to quantify αSyn levels in the samples (see Suppl. Fig. 10). The lower limit of detection (LLOD) and lower limit of quantification (LLOQ) were computed calculated as the concentration at $\beta * 3.3\sigma$ (for LLOD) and $\beta * 10\sigma$ (for LLOQ) where σ is the standard deviation of the blank signal and β is the signal of the blank. Sample signal lower than the signal of the LLOD were considered as negative (undetectable) and reported as 0 pG/mL. A list of the antibodies used, their concentration and LLOD and LLOQ values per each assay are presented in Supplementary Table 2. All cases were measured in triplicate and mean values calculated per each case. The assays had a 6.6-10.0% coefficient of variance (CoV) in the Soluble assays (Total: 6.6%; pSer129: 8.2%; CTT122: 10.0%) and of 5.3-7.0% CoV in the Insoluble assays (Total: 7.0%; pSer129: 5.3%; CTT122: 5.6%) across all measurements.

Statistics and computing

The data were analysed and graphed using R (R version 4.3.2, 2023-10-31) and R Studio [34]. Graphs were created using the *ggplot2* R package [35] and graphical methods were created with BioRender (BioRender.com, 2025). Figures were composed using Inkscape (Inkscape 1.3, Inkscape.org). All statistical comparisons were adjusted using age, sex, and post-mortem delay (PMD) as covariates (See Suppl. Fig. 1). Outliers were handled by capping values above 1.5× the interquartile range (IQR) beyond the third quartile (Q3) within each group, and zero values (if present)

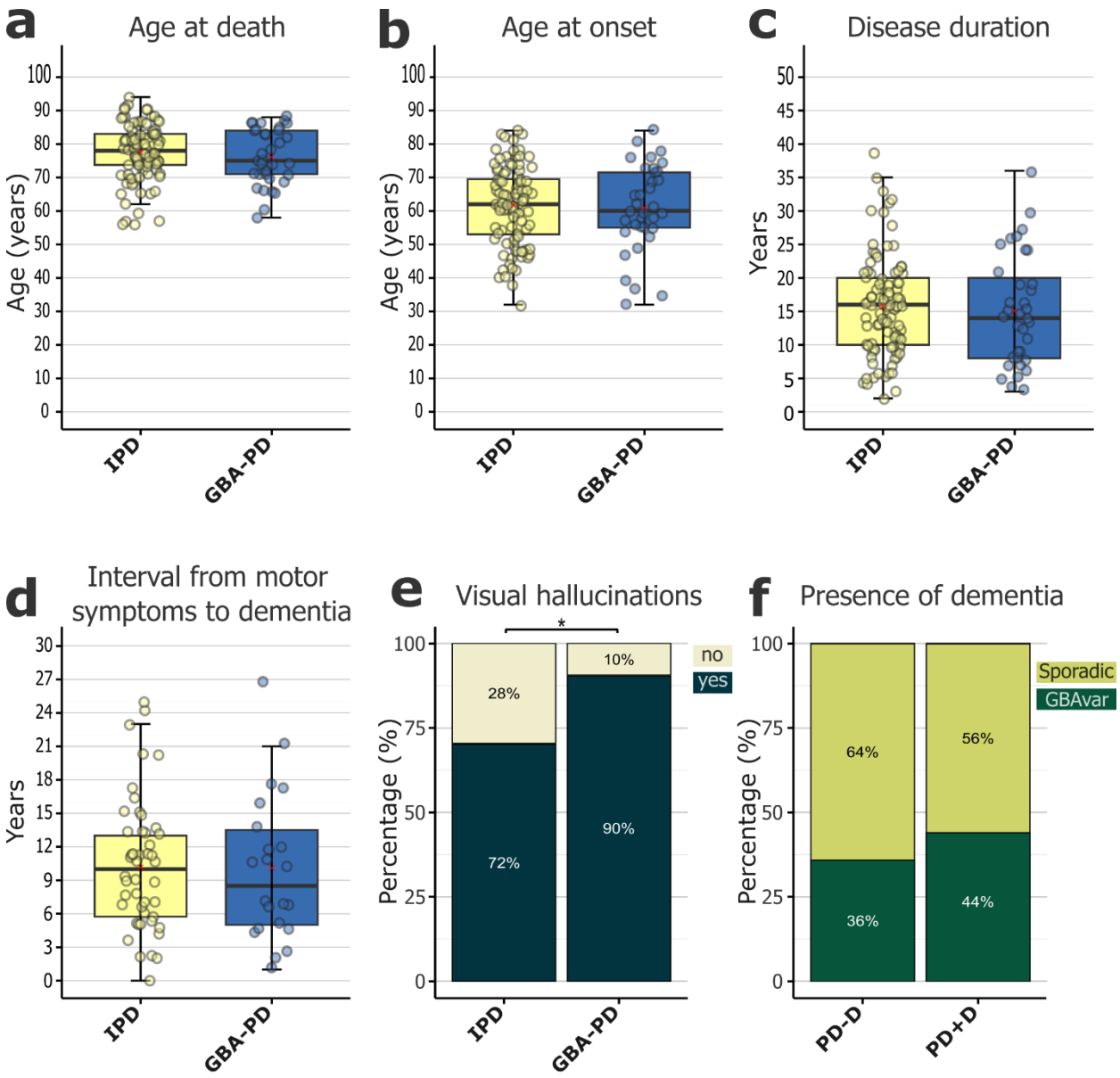
were offset by a small constant (+0.03). In all tests, significance threshold was set at $p \leq 0.05$. When comparing multiple groups for a given outcome, a generalized linear model (GLM) with a Gamma distribution and identity link was fitted. As data from the CTT122 AlphaLISA assay followed a non-normal Tweedie distribution, here we used a GLM model with Tweedie family to examine group differences. When comparing the disease groups irrespective of the anatomical area, a gamma generalized linear mixed-effects model with log link was used with disease group as the primary fixed effect, anatomical region, age at death, sex and PMD as covariates, and a random intercept for each case. For subpopulations described by a covariate, the corresponding covariate was removed from the model. When significant, pairwise group comparisons were performed using the package *emmeans* (version 1.10.6) to generate estimated marginal means with a Tukey's p value adjustment for multiple comparisons. Values and percentage difference for each comparison reported in the text are based on median values. When comparing two groups for a continuous variable, a one-way ANCOVA was performed using group as the main factor and including covariates (age, sex, PMD) in the model. When comparing two groups for binary outcomes, we used a GLM with a binomial distribution and logit link was fitted, with group and relevant covariates (age, sex, PMD) included as predictors. Receiver operating characteristic (ROC) analysis was performed using logistic regression, and the area under the curve (AUC) with 95% confidence intervals was calculated. The optimal threshold for each variable was determined using Youden's J statistic [36], defined as $sensitivity + specificity - 1$. Correlation analysis was performed using a Pearson's correlation test (continuous variables) or a Spearman's correlation test (ordinal variables). Correlation coefficients and the associated p-values are reported in the figures. Multiple correlation was performed by computing pairwise Pearson's correlations among all the variables of interest. The statistical test used in each comparison is indicated in the figure legend.

Supplementary figures and tables



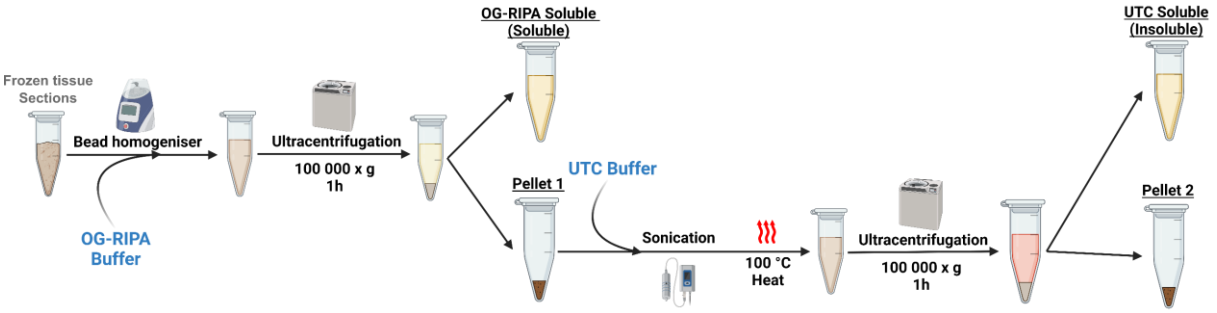
Supplementary Figure 1: Graphs of sex, age at death and post-mortem delay of the *biochemistry cohort*.

Graph of the variables included as covariates in the statistics analysis performed in the *biochemistry cohort*. **a**: Percentage of male (M) and female (F) cases in each disease group. **b**: Comparison of age at death between disease groups. **c**: Comparison of post-mortem delay between disease groups. a: Bonferroni-corrected pairwise Fisher test. b,c: generalized linear model + pairwise Tukey-adjusted estimated marginal means). * $p < 0.05$; *** $p < 0.005$.



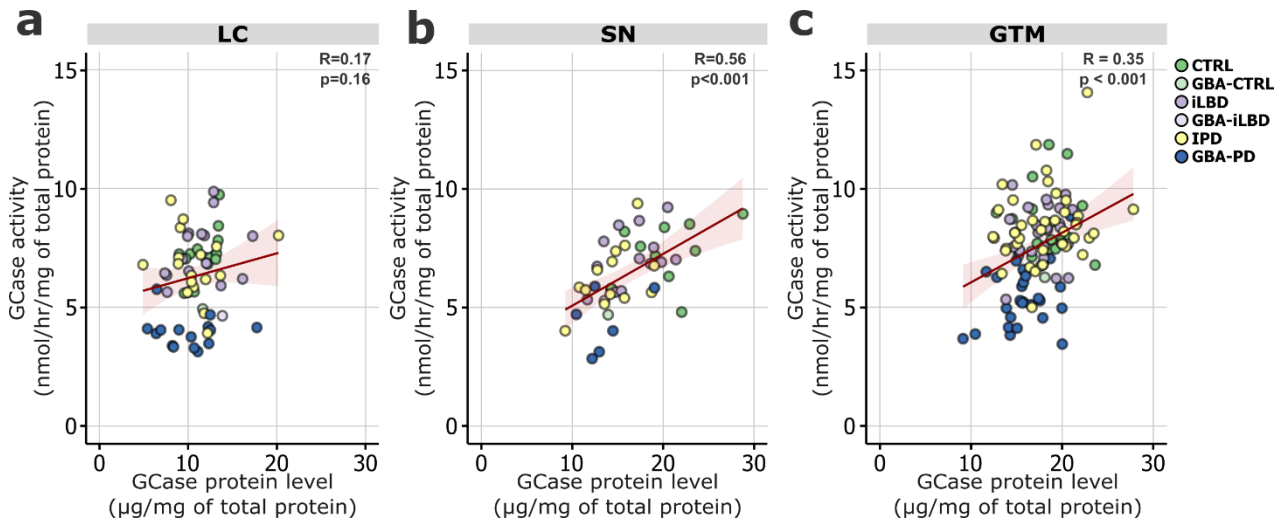
Supplementary Figure 2: Comparison of clinical characteristics between idiopathic and GBA-related PD cases in the *biochemistry cohort*.

a-c: Comparison of age at death (**a**), age of disease onset (**b**) and disease duration (**c**) between idiopathic (IPD) and GBA-related (GBA-PD) PD cases. **d**: Time interval between motor symptoms and the dementia appearance IPD and GBA-PD cases with dementia (IPD+D and GBA-PD+D, respectively). **e**: Percentage of cases with the occurrence of visual hallucinations in IPD and GBA-PD groups. **f:** Percentage of cases with a *GBA1* risk variant (GBAvar) in PD cases with (PD+D) and without (PD−D) dementia. No differences were observed between groups in age at death, age of disease onset, disease duration, time interval between motor symptoms and dementia appearance or % of cases with dementia. A higher prevalence of visual hallucinations was observed in GBA-PD versus IPD (a-d: ANCOVA analysis; e-f: binomial generalized linear model. *$p < 0.05$).



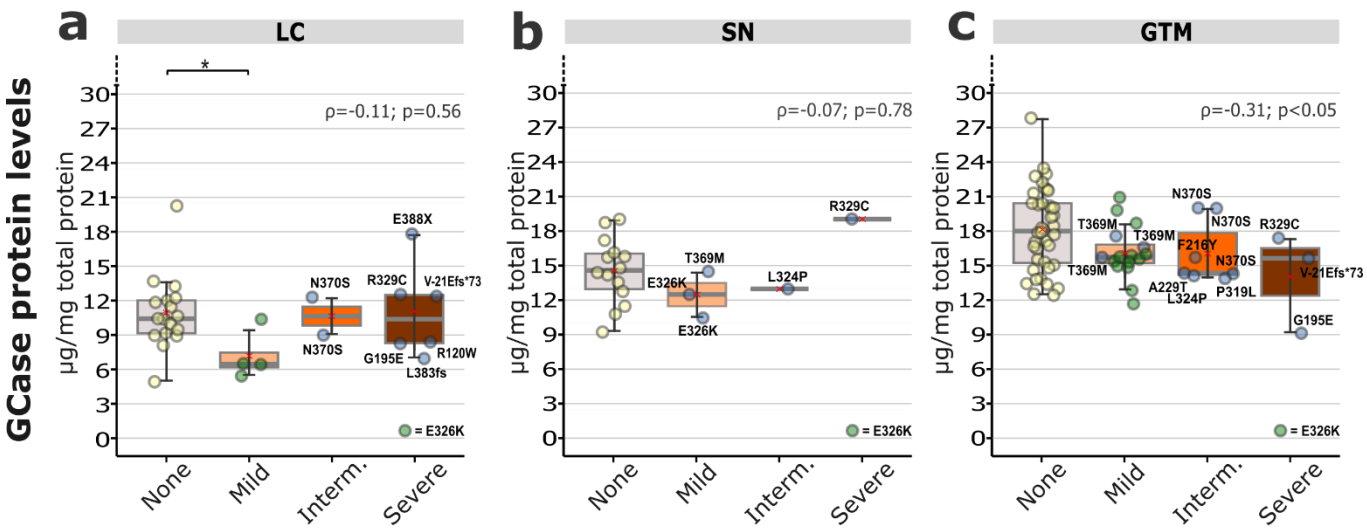
Supplementary Figure 3: Schematic representation of the sequential protein extraction performed in this study.

Representation of the sequential protein extraction procedure performed on the post-mortem human tissue sections. The frozen tissue was first incubated in SDS-free OG-RIPA buffer and homogenized using a bead homogenizer. OG-RIPA-insoluble material was then precipitated (Pellet 1) by ultracentrifugation to obtain the OG-RIPA-soluble proteins (Soluble fraction), primarily containing any non-aggregated cytosolic and membrane-associated αSyn protein. Pellet 1 was then subjected to a second extraction in UTC buffer by sonication and subsequent boiling. A second ultracentrifugation was performed to obtain the UTC-insoluble material (Pellet 2) and the OG-RIPA-insoluble UTC-soluble proteins (Insoluble fraction).



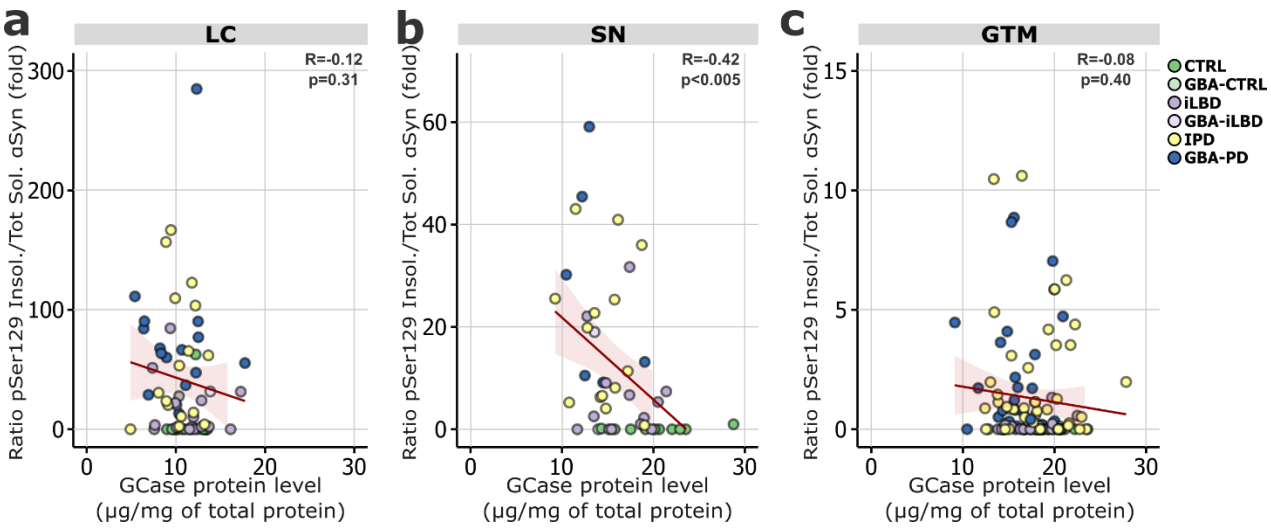
Supplementary Figure 4: Correlation between GCase activity and GCase protein levels in post-mortem human LBD brain.

a-c: Correlation between GCase activity and GCase protein levels in the *locus coeruleus* (LC; **a**), *substantia nigra* (SN; **b**), and *gyrus temporalis medius* (GTM; **c**). Pearson's correlation coefficient (R) and p values are reported within each panel.



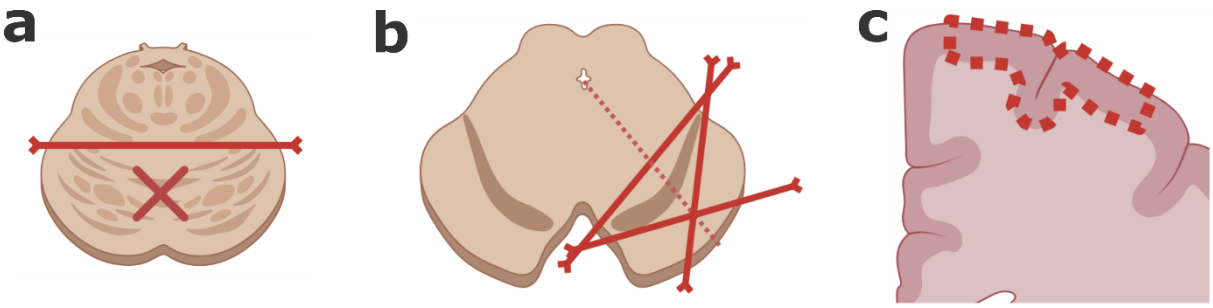
Supplementary Figure 5: Correlation between GCase protein levels and *GBA1* variant severity.

a-c: Comparison of total GCase protein levels measured by ELISA among PD cases carrying different *GBA1* variants classes based on variant severity (none, mild, intermediate, severe), across the three anatomical regions analyzed. *p < 0.05 (generalized linear model + pairwise Tukey-adjusted estimated marginal means). Spearman's correlation coefficient (ρ) and p value are reported per each panel.

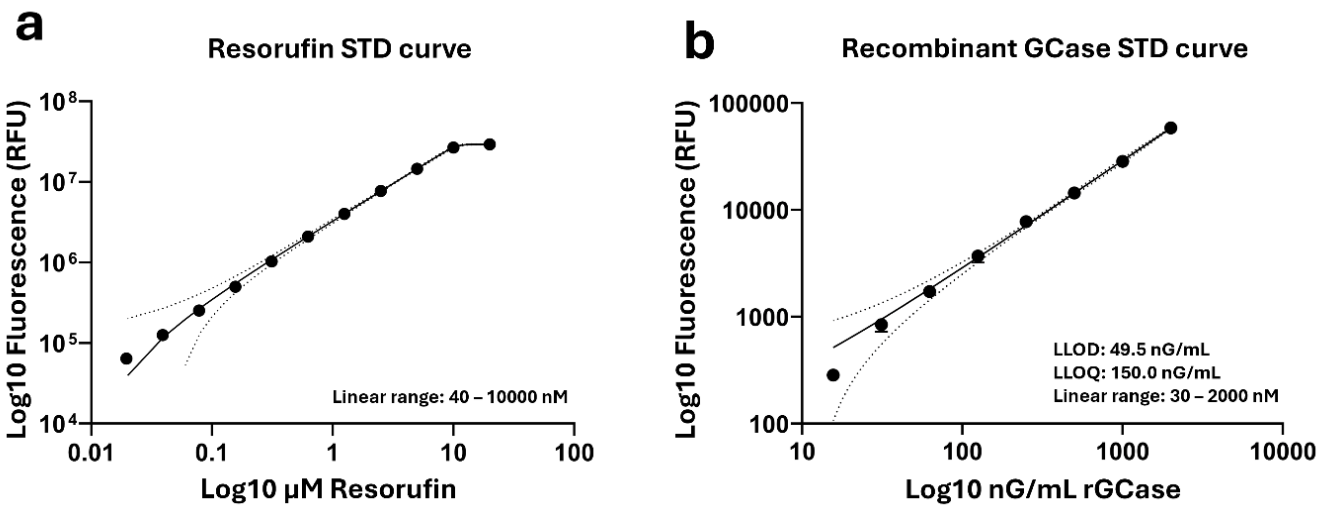


Supplementary Figure 6: Correlation between Insoluble pSer129 / Soluble Total α-synuclein ratio and GCase protein levels in post-mortem human LBD brain.

a-c: Insoluble pSer129 / Soluble Total α-synuclein (αSyn) ratio and total glucocerebrosidase (GCase) activity negatively correlate in the *locus coeruleus* (LC; **a**), *substantia nigra* (SN; **b**), and *gyrus temporalis medius* (GTM; **c**). αSyn levels are ratios of Insoluble pSer129 αSyn / Soluble Total αSyn expressed as fold change relative to the overall median. Pearson's correlation coefficient (R) and p values are reported within each panel.

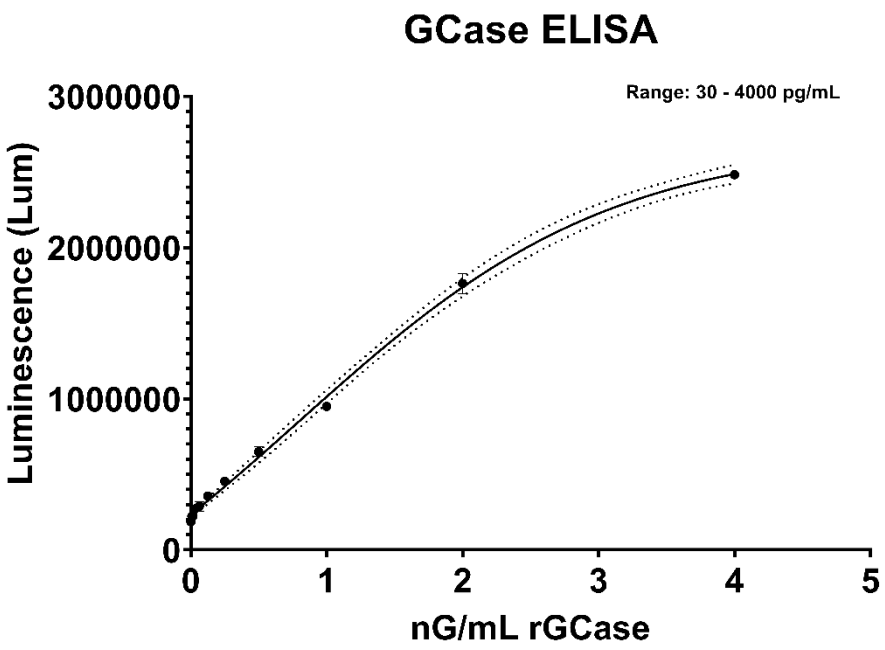


Supplementary Figure 7: Schematic representation of the tissue cutting performed prior to cryo-sectioning of the tissue.
Representation of the anatomical dissection performed on the post-mortem human tissue sections during the cryo-sectioning and collection of the tissue for biochemical characterization. **a**: Post-mortem fresh-frozen blocks of the brainstem at the level of the pons and containing the *locus coeruleus* (LC) were incised as indicated in the panel to separate the pons from the tegmentum, containing the LC. The tegmentum was collected during cryo-sectioning and used for further biochemical analysis. **b**: Post-mortem fresh-frozen blocks of the midbrain including *substantia nigra* (SN) were incised as indicated in the panel to isolate the SN area. **c**: Post-mortem fresh-frozen blocks of the *gyrus temporalis medius* (GTM) were incised as indicated in the panel to isolate gray matter and discard the white matter and processed for biochemical analysis.



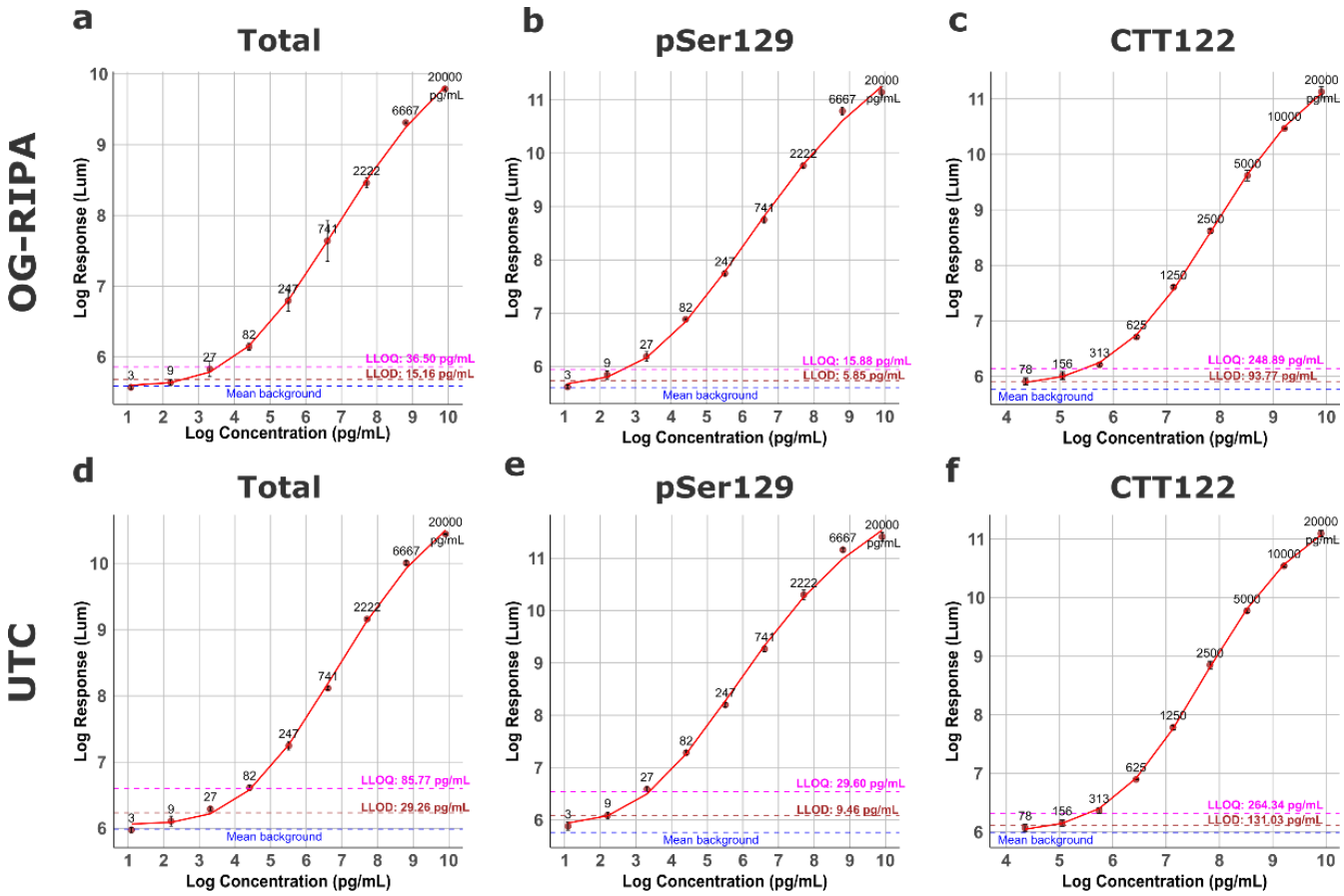
Supplementary Figure 8: Standard curves of the GCase activity assay for the quantification of GCase enzymatic activity.

a: Standard curve made with free resorufin for the quantification of resorufin produced by the enzymatic reaction. Mean relative fluorescence unit (RFU) and standard deviation are presented per each curve point. Linearity range is indicated in figure. **b:** Curve made with recombinant human GCase (rGCase) protein used to measure the sensitivity of the assay. Mean Luminescence (Lum) of the resorufin produced by the enzymatic reaction and standard deviation is presented per each curve point. Linearity range, lower limit of detection (LLOD) and lower limit of quantification (LLOQ) are indicated in figure.



Supplementary Figure 9: Standard curves of the ELISA assay used for the quantification of GCase protein levels.

Standard curve made with recombinant human GCase protein (rGCase) used for quantification of GCase protein levels in brain lysates. Mean Luminescence (Lum) and standard deviation (dotted line) are presented per each curve point. Linearity range is indicated in figure.



g

		Recombinant Prot			
		unmodified	pSer129	CTT119	CTT122
αSyn Assay	Total	100%	76%	0%	0%
	pSer129	0%	100%	0%	0%
	CTT122	0%	0%	0%	100%

Supplementary Figure 10: Standard curves of the AlphaLISA assays for α-synuclein proteoforms quantification.

a-f: Representative standard curves made with recombinant α-synuclein (αSyn) protein used for quantification in the Total (**a,d**), pSer129 (**b,e**) and CTT122 (**c,f**) αSyn AlphaLISA assays in the OG-RIPA fraction (Soluble fraction; **a-c**) and UTC fraction (Insoluble fraction; **d-f**). Mean value ± Standard Deviation are presented. Lower Limit of Detection (LLOD, brown line), Lower Limit of Quantification (LLOQ, magenta line) and Mean background (blue line) are indicated in each panel. **g:** Cross-reactivity of each αSyn AlphaLISA assay (αSyn Assay) with unmodified, Ser129-phosphorilated (pSer129), aa119-truncated (CTT119), aa122-truncated (CTT122) recombinant protein performed in standard assay buffer. Mean value of measurements at 20000, 2000 and 200 pG/mL compared to the corresponding recombinant protein standard.

Genotype Information																Cohort		
Position Chr 1 (hg19/GRCh37)	Ref	Alt	NM_000157.3	pNomen	Allelic Name	Zygosity	rsID	GERP RS	CADD	Splicing effect (ADA, RF, SpliceAI, SQUIRLS)	Label-den Heijer [37]	gnomAD v2.1.1	GoNL	Assigned Severity	Newly reported	CTRL nr. [Total = 26]	iLBD nr. [Total = 30]	PD nr. [Total = 82]
Missense																		
155205540	GGGACTGTCGACAAAGTTACGCACCCAATTGGGTCCTCCTTCGGGGTTCAGGGCAA	G	c.1265_1319del	p.(Leu422fs)	L383fs (RecΔ55) /	Het	rs80356768	-	.	SQUIRLS (0.021)	.	not found	not found	Severe	No	0	0	1
155205634	T	C	c.1226A>G	p.(Asn409Ser)	N370S /	Het	rs76763715	3,53	23,8	.	GD	632/282786	not found	Intermediate	No	0	0	3
155206037	G	A	c.1223C>T	p.(Thr408Met)	T369M /	Het	rs75548401	3,57	22,2	SQUIRLS (0.0137)	PD	1730/282484	11/998	Mild	No	0	0	3
155206158	G	A	c.1102C>T	p.(Arg368Cys)	R329C /	Het	rs374306700	3,67	27,6	.	GD	3/251484	not found	Intermediate	No	0	0	1
155206167	C	T	c.1093G>A	p.(Glu365Lys)	E326K /	Het	rs2230288	3,67	17,3	.	PD	3035/282824	23/998	Mild	No	1	1	13
155206172	A	G	c.1088T>C	p.(Leu363Pro)	L324P /	Het	-	3,67	25,5	.	GD	not found	not found	NA	No	0	0	1
155207329	C	T	c.802G>A	p.(Ala268Thr)	A229T /	Het	-	3,51	25,6	.	.	not found	not found	Intermediate	Yes	0	0	1
155207367	A	T	c.764T>A	p.(Phe255Tyr)	F216Y /	Het	rs74500255	3,51	24,3	SQUIRLS (0.028)	GD	5/281254	1/998	Intermediate	No	0	0	1
155207932	A	C	c.754T>G	p.(Phe252Val)	F213V /	Het	-	3,67	25	.	.	1/251488	not found	NA	No	0	0	1
155207985	C	T	c.701G>A	p.(Gly234Glu)	G195E /	Het	rs74462743	2,73	22,5	.	.	2/251472	not found	Severe	No	0	0	1
155208361 155206167	C A	G G	c.535G>C c.1093G>A	p.[(Asp179His;Glu365Lys)]	D140H + E326K /	Combined Het	rs2230288 rs147138516	2.62 3.67	19.06 17.3	.	GD PD	36/282226 3035/282824	1/998 23/998	Severe	No	0	0	2
155208421	G	A	c.475C>T	p.(Arg159Trp)	R120W	Het	rs439898	3,55	25,1	.	GD	6/282498	not found	Severe	No	0	0	1
155210482	TA	T	c.53delT	p.(Val18fs)	V-21fs	Het	-	-	-	.	.	not found	not found	Severe	Yes	0	0	1
155206167 155206167	C C	T T	c.1093G>A c.1093G>A	p.[(Glu365Lys)];[(Glu365Lys)]	E326K / E326K	Hom	rs2230288 rs2230288	3.67 3.67	17.3 17.3	.	PD PD	3035/282824 3035/282824	23/998 23/998	Mild	No	0	0	1
155235790	C	A	c.1279G>T	p.Glu427Ter	E388* /	Het	-	-	-	-	NA	not found	not found	Severe	No	0	0	1
155206147	G	A	c.1073C>T	p.Pro358Leu	P319L /	Het	-	-	-	-	NA	not found	not found	Intermediate	No	0	0	1
															Total:	**1**	**1**	**33**
Synonymous																		
155206117	A	G	c.1143T>C	p.(Cys381=)	C342= /	Het	rs121908306	-3,08	11,63	.	.	3/251456	not found	none	No	0	0	1
															Total:	**0**	**0**	**1**

Supplementary Table 1 Detailed information of the *GBA1* variants in the *biochemistry cohort*.

Protein coordinates (pNomen) are presented according to NP_000148.2. Common variant name historically used (Allelic name) is presented after

removing the 39 aa signal sequence. Protein coordinates of the variant according to NP_000148.2. rsID = Reference SNP ID assigned by dbSNP or EVA. Clinical significance of the variant (Label-denHeijer) is presented as previously reported in [37] (GD = Gaucher Disease, PD = Parkinson's Disease). GERP RS = Genomic evolutionary rate profiling (GERP) "rejected substitutions" (RS) score. CADD = Combined Annotation Dependent Depletion (CADD) score in PHRED scale. gnomADv2.1.1 = Allele Count/Allele number in gnomAD v.2.1.1. GoNL = Allele Count/Allele number in GoNL. PD variant browser = Allele frequency (Allele count/Allele number) reported in cases and controls from the PD variant browser.

Supplementary Table 2: Acceptor Antibodies used for the quantification of α-synuclein proteoforms by AlphaLISA.

				OG-RIPA		UTC	
Antibody Epitope	**Antibody Clone**	**Producer (Cat. #)**	**Bead-Ab Conjugate Final Concentration**	**Mean LLOD (pg/mL)**	**Mean LLOQ (pg/mL)**	**Mean LLOD (pg/mL)**	**Mean LLOQ (pg/mL)**
aa118-123 (Total)	MJFR1	Abcam (ab209420)	15 µg/mL	18.97	49.95	19.38	64.03
pSer129	Syn-142	Roche (N.A.)	10 ug/mL	4.31	11.91	6.46	19.86
CTT122	A15127A	BioLegend (848402)	15 µg/mL	130.55	301.58	115.33	231.96

LLOD = Lower Limit of Detection; LLOQ = Lower Limit of Quantification.

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