Supplementary Information for

Pathological a-syn aggregation is mediated by glycosphingolipid chain length and the physiological state of a-synuclein

Authors: Kristina Fredriksen, Stefanos Aivazidis, Kevin J. Burbidge, Karan Sharma, Caleb Pitcairn, Friederike Zunke, Eilrayna Gelyana, and Joseph R. Mazzulli*

* Correspondence: jmazzulli@northwestern.edu

This PDF file includes:

Supplementary Information

I. Supplementary Materials and Methods

Mice

Wild-type C57BL/6 mice were obtained from Charles River (Strain code 027) and housed in accordance with the US National Institutes of Health Guide to the Care and Use of Laboratory Animals and Society for Neuroscience guidelines. Mice were housed with a 12h-12h light-dark cycle and provided free access to food and water. Up to 5 littermates were housed in each cage. Mice were age-matched for all experiments, and an equal number of males and females was used.

Administration of Conduritol beta epoxide

CBE (EMD Millipore / Sigma Aldrich #234599; 100mg) was dissolved fresh in 1X PBS at 25 mg/ml and sterile filtered. Body weight of each mouse was recorded before the start of each treatment, at the same time every day. Mice were injected intraperitoneally (i.p) daily for 7 days with CBE at 100 mg/kg body weight or PBS vehicle starting at postnatal day 8, or 3 months of age.

Co-administration of CBE and venglustat

Venglustat was dissolved at 7.5 mg/ml in 200 mM citrate buffer pH 5, containing 0.5M sodium citrate buffer (Boston Bioproducts, SKU: BB-88r) diluted in 0.9% sodium chloride saline solution, and sterile filtered. Adult mice at 3 months of age were co-injected by i.p daily with 100 mg/kg CBE + 60mg/kg venglustat dissolved in citrate buffer, or with citrate buffer alone as a vehicle control for 7 days. To determine if venglustat can reverse pre-existing a-syn pathology, mice were first injected with CBE dissolved in PBS or PBS vehicle for the first 7 days, followed by co-injection with CBE + venglustat in citrate buffer or citrate buffer vehicle for an additional 7 days. The site of injection alternated every day from the left or right abdomen.

Behavioral Analysis

Balance and motor behavior was determined at 4, 7 or 14 days of treatment using a Rotarod apparatus (Ugo Basile) with increasing acceleration from 4–40 rpm during a 300 second period. Latency to fall was recorded over 4 trials per mouse, with 5 minutes between each trial. Data are presented as the average of all mice analyzed for each of the 4 trials on the same day. Equal number of males and females were used. Treatment groups were not blinded.

Immunohistochemistry

Mice were anesthetized with isoflurane and perfused transcardially with 50 mL phosphate-buffered 0.9% saline (PBS). Right hemibrains were post-fixed in 1X PBS with 4% paraformaldehyde for 48 hours, while the left hemibrain was frozen for biochemical analysis. Post-fixed brains were then washed in PBS for 24 h, cryoprotected by immersion in 20% sucrose, then 30% sucrose, and stored at 4°C. Free-floating 40-μm serial coronal sections were collected with a freezing sliding microtome (SM2400; Leica Microsystems). Brain slices were stored in PBS containing 0.1% sodium azide at 4°C until use. Free-floating sections from each treatment group were pre-treated in 3% hydrogen peroxide for 30 minutes and washed 3 times for 10 minutes in PBS. Sections were blocked for 1 hour at room temperature in PBS containing 5% normal goat serum and 0.1% Triton X-100. Sections were incubated overnight at 4°C in blocking solution with anti- GFAP antibody (Z0344-Agilent/DAKO, 1:2000). The next day, slices were washed 3 times for 10 minutes with PBS and incubated with biotinylated Goat anti-Rabbit IgG antibody (AP132B, EMD Millipore) at room temperature for 1 hour, followed by incubation with the Vectastain Elite ABC HRP Kit (PK-6100, Vector Laboratories) for 30 minutes. Staining was revealed with a peroxidase substrate solution (SK-4100, Vector Laboratories) for 1-5 minutes and the reaction was quenched with PBS, followed by H20. Sections were mounted on microscopy slides and left to dry overnight. Nuclei were stained with hematoxylin-based blue counterstain (Trevigen). Slides were then dehydrated in increasing concentrations of ethanol, cleared 3 times for 5 minutes with Histoclear II (101412-882, National Diagnostics), and coverslipped with DPX mounting medium (06522-Sigma Aldrich). The experimental groups were blinded during the entire staining procedure. The slides were imaged in a ZEISS Axio Imager M2 microscope (20x magnification). GFAP staining in the cortex was determined using a semiquantitative scoring system. GFAP intensity was scored by 4 independent assessors on a scale of 1 (healthy/absence of GFAP staining) to 5 (severe astrogliosis/ extensive GFAP staining) according to a reference template of

representative images. 3-4 slices per mouse were reviewed, and assessors were blinded to the treatment groups corresponding to the images.

iPSc cell culture and neuronal differentiation

iPSCs were maintained on matrigel coated dishes with mTeSR1 media and were groomed and passaged weekly. Differentiation of iPSCs into midbrain dopamine neurons (iPSn) was performed according to an established protocol (1). For differentiation, iPSC colonies were enzymatically dissociated (accutase) and seeded onto matrigel-coated 6 well dishes to reach 80% confluency. When fully confluent, the differentiation protocol was initiated by adding Knockout Serum medium (KSR) media supplemented with dual SMAD inhibitors for 10-15 days, in combination with growth factors and small molecules in various concentrations (1). Confluent cell layers were mechanically dissociated between day 10 and 15 to be passaged en block to prevent neuralization variability. This was done by carving the cell layers into squares of ca. 2 mm2 and plating them on 10 cm poly-d-lysine (PDL, 33 mg/ml) / 5 mg/ ml laminin dishes. After 25 to 30 days, the cells were enzymatically dissociated (accutase), counted, and plated on poly-d-lysine (PDL) / laminin-coated culture dishes until analysis. The growth factors for neuralization were withdrawn at day 40 to 50. Neurons were maintained in neurobasal media (Thermo Fisher Scientific, #21103-049) containing NeuroCult SM1 supplement (StemCell Technologies #05711). iPSC lines were differentiated up to 270 days depending on the experiment, as indicated in the figure legends.

Sequential extraction of iPSC-derived neurons or mouse brain, and western blot

iPSc derived neurons (iPSn) were harvested in phosphate buffered saline (PBS), pH 7.4, and pelleted by centrifugation at 400x g for 5 minutes. Cell pellets were lysed in 1% Triton X-100 buffer (1% Triton X-100, 20 mM HEPES pH 7.4, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1.5 mM MgCl2, 1 mM phenylmethanesulfonyl fluoride (PMSF), 50 mM sodium fluoride (NaF), 2 mM Na orthovanadate, and a protease inhibitor cocktail (Roche Diagnostics, 11-836-170-001)) followed by pestle homogenization. Whole mouse brain or mouse cortex was dissected and lysed directly in 1% Triton X-100 buffer in 1:5 weight/volume ratio and homogenized. Following homogenization, the samples were incubated in ice-water slurry for 30 minutes, subjected to two freeze / thaw cycles using a 100% EtOH bath at -80^{o}C (2 min) then a 37^{o}C water bath (30 seconds), and ultracentrifuged at 100,000 x g, 4°C for 30 minutes. Supernatant (Triton-soluble fraction) was

used for MicroBCA protein assay (ThermoFisher Scientific, 23235). The Triton-insoluble pellets were dissolved in SDS-lysis buffer (2% SDS, 50 mM Tris, pH 7.4 and a protease inhibitor cocktail (Roche diagnostics, 11836170001). The samples were boiled for 10 minutes, sonicated for 10 minutes at 20mV in a sonic water bath, boiled again for 10 minutes and centrifuged at 100,000xg for 30 minutes at 22°C. Protein concentration was measured by MicroBCA assay. 30-70 µg of protein from each iPSn or mouse sample (Triton-soluble and insoluble) were loaded in a 12% gel, separated via SDS-PAGE and transferred onto PVDF membranes. Membranes were post-fixed in 0.4% paraformaldehyde (PFA) for 30 minutes followed by three washes (water) and blocked with a 1:1 mixture of TBS: Odyssey blocking buffer (Li-Cor Biosciences) for 1 hour. Primary antibodies were incubated overnight at 4°C and detection was carried out with fluorescent-conjugated anti-rabbit or anti-mouse antibodies with Alexa 680 (Thermo Life Technologies) or IRDye 800 (Li-Cor Biosciences) and scanned / quantified using the Odyssey Li-Cor infrared imaging system. Intensity levels were normalized to a loading control (CBB or GAPDH). Where noted in the figure legends, two different anti-a-syn antibodies from different species (mouse or rabbit) were simultaneously added on the same blots and detected on separate channels. In these cases, the same loading controls apply to both a-syn signals (eg, Figure 2B). C20 is a rabbit antibody and was detected with IRDye800 conjugated anti-rabbit, while and syn505 and syn303 are mouse antibodies that are detected with Alexa 680 conjugated anti-mouse antibodies.

In vitro cell treatments

iPSC-derived midbrain dopamine neurons were treated with 50 µM CBE or PBS every other day for 7 days starting at day 23 (immature neurons) or day 113 (mature neurons). Cells were harvested and sequentially extracted as described above.

Size exclusion chromatography and isolation of HMW and LMW a-syn forms

For size exclusion chromatography (SEC), mouse brain or iPSn samples were homogenized as described above in 1% Triton buffer and then centrifuged at 100,000 x g for 30 minutes at 4°C. 1-1.2 mg of triton-soluble lysate (supernatant) was injected on a Superdex 200 increase HR 10/300 gel filtration column (GE17-5175-01, GE Healthcare) using a mobile phase of phosphate buffered saline at pH 7.4 (PBS) (sample injection volume: 1-1.2 mg lysate into 250 ml, flow rate at 0.5 ml/min; 0.5 mL sized fractions), an Agilent HPLC 1200 series pumps, autoinjector, UV/vis

detector, and fraction collector. Fractions 2-7 (100 Å) were combined to obtain HMW forms of a-syn and fractions 13-19 (35 Å) for the LMW forms. Amicon Ultra centrifugal concentrators (10 K, Millipore #UFC501096) were used to concentrate the fractions. The entire sample was then mixed with 5X SDS sample buffer, boiled (5 minutes at 100°C), and loaded onto SDS-PAGE gels for western blot analysis as described above.

The blots were probed with a-syn antibodies C20 (pAb rabbit), syn303 (mAb mouse), or syn505 (mAb). Mouse and rabbit antibodies were added to the same blot and detected on separate channels with either Alexa 680 (for mAb) or IRDye 800 (for pAb) using a two-channel Li-cor odyssey scanner. HMW oligomeric and LMW monomeric fractions were normalized to total protein (CBB stain), added together, and the % oligomer was obtained by dividing the normalized oligomer intensity by the total, X 100. For GCSi studies, CBE + veh / CBE + I pairs were analyzed together on the same blot, but individual replicates were loaded on separate blots. CBE + Veh was assigned as 1 fold for each analysis in order to limit inter-blot variability of the integrated intensity.

Oligomer stability assay

Triton-soluble mouse brain lysates were incubated with 0.1% SDS for 30 minutes on ice to denature a-syn oligomers and determine oligomer stability. HMW and LMW species were then isolated by SEC-HPLC followed by western blot as described above.

Native Dot Blot analysis

HMW a-syn fractions from CBE or PBS-treated 3-month-old mice were applied onto nitrocellulose membranes, fixed in 0.4% PFA for 30 minutes, and blocked in TBS with Odyssey blocking buffer (1:1) for 30 minutes. Primary antibodies OC and A11 that detect in-register or out-of-register b-sheets respectively, were incubated overnight in 1:1 TBST: Odyssey blocking buffer. Primary antibodies were detected similar to western blot analysis as described above. Antibody reactivity was quantified and normalized to tubulin using Li-Cor Image Studio (V 3.1.4).

GCase activity assay

GCase activity was measured as described previously (2) from triton-soluble mouse brain lysates. 2-5 μg of protein was incubated for 1 hour at 37°C with 1% BSA, 1 mM 4-methylumbelliferyl β-D-glucopyranoside (4-MU-Gluc; Sigma-Aldrich) in GCase activity assay buffer containing:

0.25% (v/v) Triton X-100 (Sigma-Aldrich, #T-8787), 0.25% (w/v) Taurocholic acid (Sigma-Aldrich, # T9034), 1 mM EDTA, in citrate phosphate buffer, pH 5.4. The reaction was quenched by adding equal volume of 1 M glycine, pH 12.5. 4-MU-Gluc fluorescence (ex = 355 nm, em = 460 nm) was detected in a Molecular Devices i3 microplate reader. To control for substrate specificity, each lysate (including from mice that were injected with CBE) was also incubated with 150 nM CBE for 1 hour in activity assay buffer with 4-MU-Gluc. Duplicate samples were run to account for technical variability. Relative fluorescent units (RFU) were measured and the difference between GCase activity with or without CBE-treated lysates was normalized to total protein concentration. Activity from 4-12 mice was measured from each experiment, as indicated in figure legends.

Lipidomic analysis

Lipidomic analysis was performed by the lipidomics core facility at the Medical University of South Carolina on frozen cortical tissue from neonatal mice (day 15) or 3-month-old mice. Glucosylceramide, glucosylsphingosine, and Galactosylceramide were analyzed by supercritical fluid high-performance liquid chromatography/ mass spectrometry (SFC/LC-MS/MS) as described previously (2). The analyzer at the core facility was blinded to the treatment group of the samples. Lipids were normalized to inorganic phosphate levels and expressed as fold change. iPSC neurons were analyzed in the same way, using culture material from a confluent 12 well from cultures aged to day 120.

Formulation of lipid dispersions

Purified brain L-α-phosphatidylcholines (PC, # 840053P), C16 Glucosyl (β) Ceramide (16:0, C16 GluCer, #860539P), C24:1 Glucosyl (β) Ceramide (24:1, C24 GluCer, #860549P) and C11 TopFluor Glucosyl Ceramide (#810267P) were purchased from Avanti Polar Lipids (www.avantilipids.com). HPLC grade chloroform containing 1% ethanol stabilizer was used to dissolve PC at 10mg/ml while the different chain length GluCer and C11 TopFluor Glucosyl Ceramide were dissolved in chloroform:methanol:water (80:20:2, v:v) at 10mg/ml. All lipids were stored at -20^{o}C in glass Teflon capped vials with a nitrogen gas overlay. The lipids mixture (3) was made in a 25:75 molar ratio of PC:GluCer. A thin layer of lipid was formed after drying the lipid mixture under a stream of nitrogen gas. This layer of lipid was resuspended in PBS and transferred

to a thin-walled PCR tube (BrandTech #781305). The lipid mixture suspension was then sonicated using Qsonica (Q800R3) water bath sonicator at 20% amplitude for about 5-15 minutes until the solution was clear. The lipid dispersions were prepared fresh for every separate experiment. The lipid dispersions were added to 2135 iPSC-derived midbrain neurons (day 170) at 100µM final concentration and incubated for 3 days.

Immunofluorescence analysis of C11 TopFluor Glucosylceramide

The lipid dispersions were added to H4 cells grown on coverslips in a 24 well plate (Corning, #3524) at 100µM final concentration. After 24 hours of incubation, the cells were rinsed with PBS and fixed with 4% paraformaldehyde prepared in PBS solution for 15 minutes. After fixation, cells were blocked in 2% (w/v) bovine serum albumin (BSA) (Roche, #3117057001), 4% normal goat serum (Jackson Immunoresearch Laboratories, www.jacksonimmuno.com, #005-000-121) and 0.1% (v/v) Triton X-100 prepared in PBS for 1 hour at room temperature. The cells were then incubated overnight at 4°C with LAMP1 (H4A3) (1:100) primary antibody (Santa Cruz Biotechnology, #sc-20011) diluted in blocking solution. The cells were washed thoroughly with PBS and incubated with the goat anti-mouse conjugated Alexa 568 secondary antibody (1:400) for 1 hour followed by a thorough wash with 0.1% Tween-20 prepared in PBS. The coverslips were then mounted onto glass slides with 5µl of 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) containing Fluoromount G (Southern Biotech, www.southernbiotech.com, #0100-20) and then imaged using a confocal microscope.

Cathepsin B Assay Protocol

Cathepsin B activity fluorometric assay kit from BioVision (Catalog number: K140). Mouse cerebellum from neonates and 3mo old mice was lysed in 1:5 weight/volume ratio of the provided CB cell lysis buffer. Tissues were homogenized with a pestle and incubated in ice for 30 minutes. The samples were centrifuged at 14,000 g for 5 minutes, the supernatant was collected and protein quantity was measured by the MicroBCA protein assay. 80 µg from each sample was added to a black flat bottom 96 well plate and lysis buffer was added to reach 50µl volume. 50µl of CB reaction buffer and 2µl of Cathepsin B substrate (Ac-RR-AFC, 200uM final concentration) were added to each well and the plate was incubated for 90 minutes at 37°C. Samples treated with

Cathepsin B inhibitor (20 μM final concentration) were used as negative controls. The samples were read in a fluorometer with 400-nm excitation filter and 505-nm emission filter.

iPSn list and studies of clearance inhibition

Label	**Genotype**	**Citation / source**
Ctrl (2135)	wt / wt (Control)	Mazzulli et al, *Cell*, 2011 (3)
GDC	N370S / c.84dupG	Mazzulli et al, *Cell*, 2011 (3)
49-1	L444P/L444P	Schondorf et al, *Nat. Commun.*, 2014 (4)
Idiopathic PD	N/A	Mazzulli et al, *PNAS* 2016 (5)
GBA-PD	N370S / wt	Coriell Cell Respository, reprogrammed from fibroblast line ND34982

iPSC-derived midbrain neurons (aged at day 65-75) were treated with venglustat (50nM), Ca74Me (Cayman chemicals, Item No. 18469, 10μM), MDL 28170 (Cayman chemicals, Item No. 14283, 10uM), Leupeptin (Sigma Aldrich, L2884, 200 μM), 3-Methyladenine (Sigma Aldrich, M9281, 5mM) or Epoxomicin (Sigma Aldrich, E3652, added on day 13 of the treatment for 24hr only, 50nM) every other day for 14 days.

Generation of lentiviral particles and iPSC-midbrain culture transduction

Depletion of cathepsin B in control (2135) or GD iPSC-midbrain cultures was achieved by lentiviral transduction using the Mission® scramble shRNA (Sigma Aldrich, #SHC016V) or CTSB shRNA (Sigma Aldrich, #TRCN0000003655) lentiviral constructs. The *CTSB* shRNA sequence targets 3'- CCAACACGTCACCGGAGAGAT-5'. The production of lentiviral particles by 293FT cell (Invitrogen, R70007) transfection was performed as described in previous publications (5). In brief, transfection media was prepared by adding psPAX2, VSV-G, and either scramble or CTSB shRNA constructs totaling 16.66 ug of DNA and at a ratio of 3:1:4 to 2.25 ml of Optimem (ThermoScientific, #31985-070). 36 μl of Xtreme gene HP DNA transfection reagent

(Roche, #6366236001) was added to the Optimem, allowed to combine over 20 minutes, and then added dropwise to a 90% confluent 15 cm plate of 293FT cells. The transfection media was changed ~16 hours later to DMEM (ThermoFischer, #11995-073) supplemented with 10% BSA (GEminiBio, #900-108), Penn/Strep (ThermoFischer, #15140163), and Geneticin (Gibco, #10131-027). The cultured media was collected 48 hours later and was centrifuged at 500 g for 10 minutes at 4oC. The supernatant was added to Lenti-X Concentrator (Clontech, #631232) at a ratio of 3:1, incubated overnight at 4°C, and the lentiviral particles were pelleted by centrifugation at 1,500 g for 45 minutes at 4°C. The pellet was resuspended in SM1 culture media and viral particle concentration was determined by p24 ELISA (ZeptoMetrix, #801111) as per the manufacturer's protocol. The viral particle concentration and the plated iPSC-midbrain cell culture number was used to calculate the multiplicity of infection (MOI). A MOI of 5 was used to facilitate efficient transduction. Control (2135) iPSC-midbrain cultures were collected 5 days post-transduction. By contrast, 48 hours post-transduction, GD iPSC-midbrain cultures were treated by supplementing media with either vehicle (0.1% DMSO) or 50 nM venglustat for 14 days with media changes occurring every 48 hours. Lysates were then collected and sequential extracted as previous described and evaluated by western blot.

Cathepsin B-MYC over-expression by transfection of H4 a-syn over-expressing cells

H4 cells over-expressing a-syn were transfected with either empty vector or cathepsin B-MYC (CTSB-MYC) pcDNA 3.1 constructs. The CTSB-MYC construct was generated using polymerase chain reaction and restriction enzymes to modify the hCathepsin B construct (Addgene plasmid #11249; http://n2t.net/addgene:11249 ; RRID:Addgene_11249). Transfection of H4 cells was performed using a modified version of the Lipofectamine 3000 manufacturer protocol (Invitrogen, #L3000015). 5.5 µl of lipofectamine 3000 or 4 µg of construct DNA with 8 µl of p3000 reagent was added to 125 µl of Optimem, allowed to incubate for 10 minutes, and combined at a 1:1 ratio. Following a second 10-minute incubation, Optimem was added to the transfection mixture to a total of 2 ml and then added to a 90% confluent well of a 6-well plate. 16 hours later, the cells were split at equal concentrations into two wells of a 12-well plate, and cultured in Optimem supplemented with 5% BSA, Penn/Strep, Geneticin, and Hygromycin B (Invitrogen, #10687010) as well as either vehicle (0.1% PBS) or 50 µM CBE treatment conditions. Cells were cultured for

five days post re-plating, during which the media was refreshed every 48 hours. Lysates were then collected, underwent sequential extraction, and the fractions were analyzed by western blot.

Quantification and Statistical Analysis

Statistical tests were performed using GraphPad Prism software V6 (https://www.graphpad.com/scientific-software/prism/). ANOVA with Tukey's or Dunnett's post-hoc test was used when comparing more than 2 groups. Two-tailed Student t-test was used when comparing two groups. All values are expressed as the mean +/- SEM. Graphpad Prism software was used to perform correlation analysis between GluCer species and insoluble a-syn (syn303 reactivity) for each individual mouse.

II. Supplemental Figures and Figure Legends

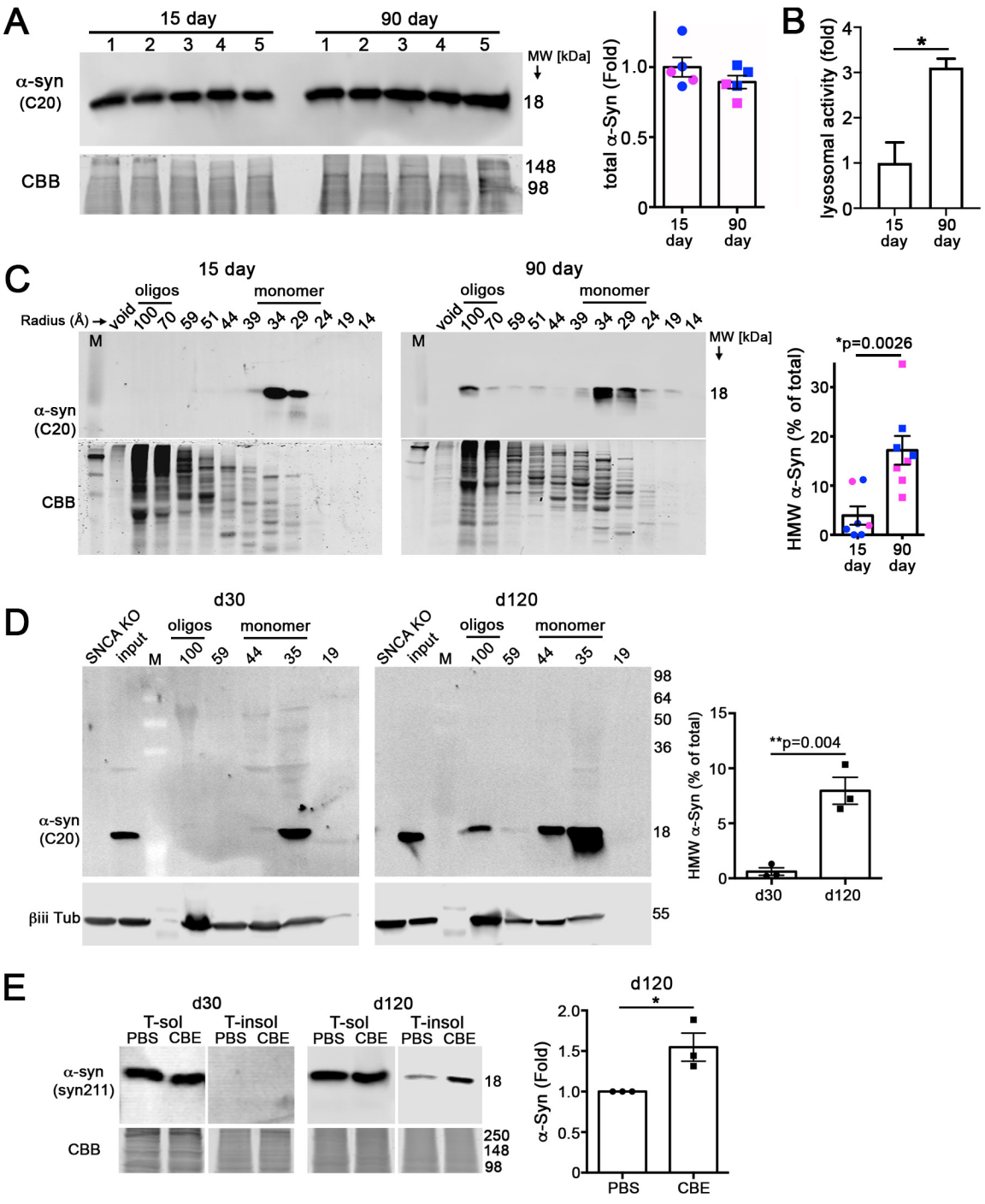
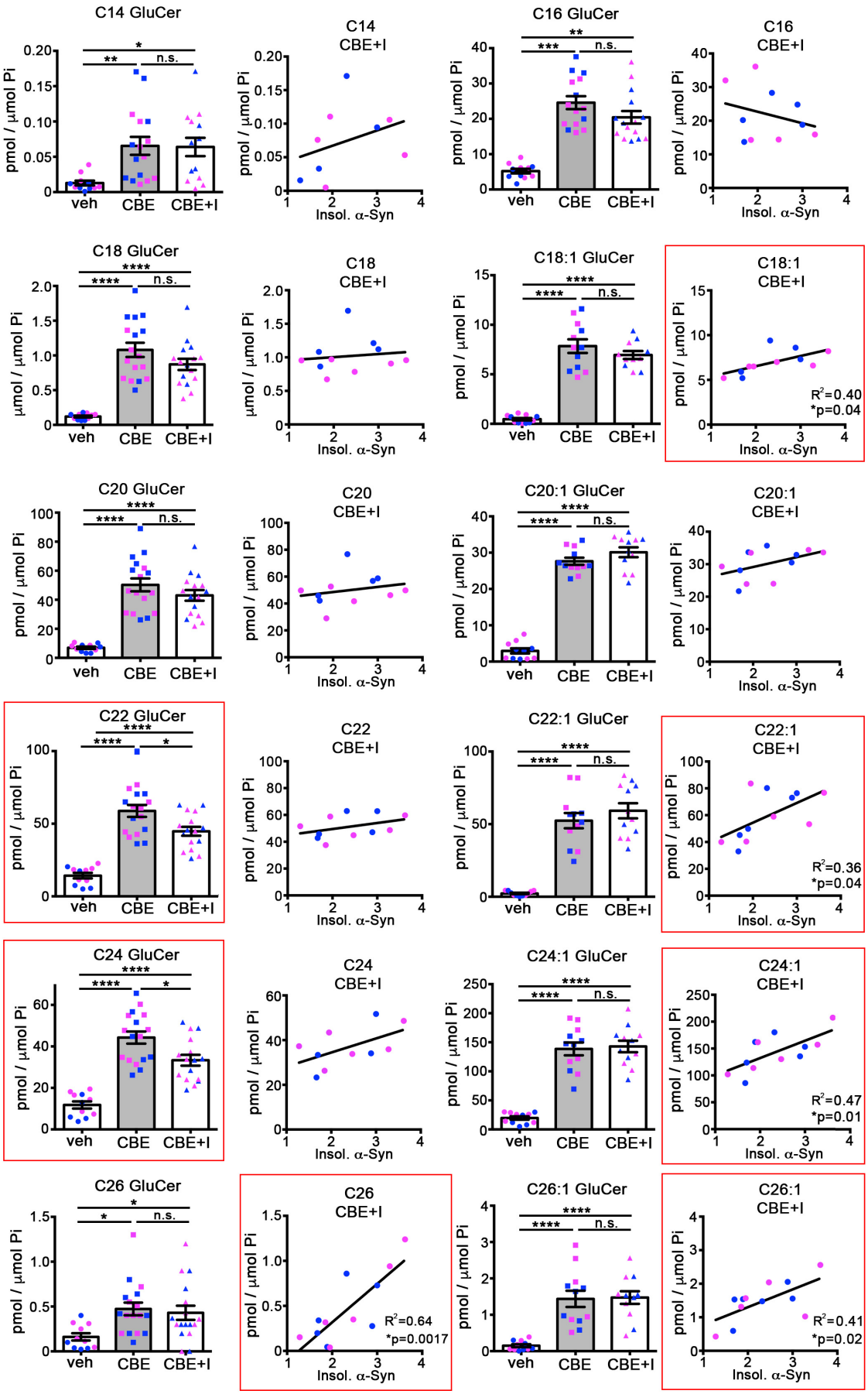


Figure S1 related to Figures 1 and 2. High molecular weight physiological oligomers of a-synuclein increase with age in mice and human iPSC-derived midbrain neurons. A) Western blot analysis of cortical extracts from healthy 15 day or 90-day old mice. A-syn levels were normalized to Coomassie brilliant blue (CBB). Each plot represents a measure from an individual mouse (n=5). **B**) Lysosomal activity was assessed in cerebellum brain extracts by measuring cathepsin B activity and normalized to total protein (n=10 mice) **C**) Size exclusion chromatography / western blot

analysis of mouse cortical extracts reveals an elevation of oligomer a-syn that elutes as a 100Å-sized species. Monomeric a-syn elutes as a ca. 34Å-sized particle. Right, quantification of high molecular weight (HMW) a-syn oligomers normalized to monomeric a-syn. n=7-8, Blue = male; Pink = female. **D**) Size exclusion chromatography / western blot analysis of wt iPSC -derived neurons shows that HMW physiological oligomers are only present in d120 cultures. SNCA knock-out (KO) lysate was used as a negative control. Equal amounts of a-syn were injected on the column, as indicated by the input lane. Values are the mean +/- SEM, student's t-test. **E**) WT iPSCs were differentiated into midbrain dopamine neurons, matured for 30 or 120 days, and treated with 50μM CBE for 7 days. Sequential extraction / western blot indicates the accumulation of insoluble a-synuclein only in neurons matured to 120 days (n=3).


C14 GluCer
pmol / µmol Pi
veh
CBE
CBE+I
C14
CBE+I
Insol. α-Syn
C16 GluCer
C16
CBE+I
C18 GluCer
µmol / µmol Pi
C18
CBE+I
C18:1 GluCer
C18:1
CBE+I
R²=0.40
*p=0.04
C20 GluCer
C20
CBE+I
C20:1 GluCer
C20:1
CBE+I
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CBE+I
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C24
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C26
CBE+I
R²=0.64
*p=0.0017
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C26:1
CBE+I
R²=0.41
*p=0.02
n.s.

Figure S2 related to Figure 3. Lipidomic analysis of venglustat-treated CBE mice and the correlation with pathogenic a-synuclein. Mice were treated as described in figure 3, and cortical lysates were analyzed. GluCers were quantified and separated based on their fatty acyl chain length, and normalized to inorganic phosphate (Pi). For the CBE + I group, correlations of GluCer species with fold-change of insoluble a-synuclein (syn303 reactivity) are plotted to the right of each quantification. Statistically significant changes are highlighted by red boxes. Values are the mean +/- SEM, student's t-test, *p<0.05; **p<0.01, ***p<0.001, ****p<0.0001. Each plot shown represents an individual mouse (pink, female; blue, male). The same graphs of the strongest correlations (C24:1, and C26 GluCer) are duplicated in main figure 3 as representative examples, and shown here again for convenience.

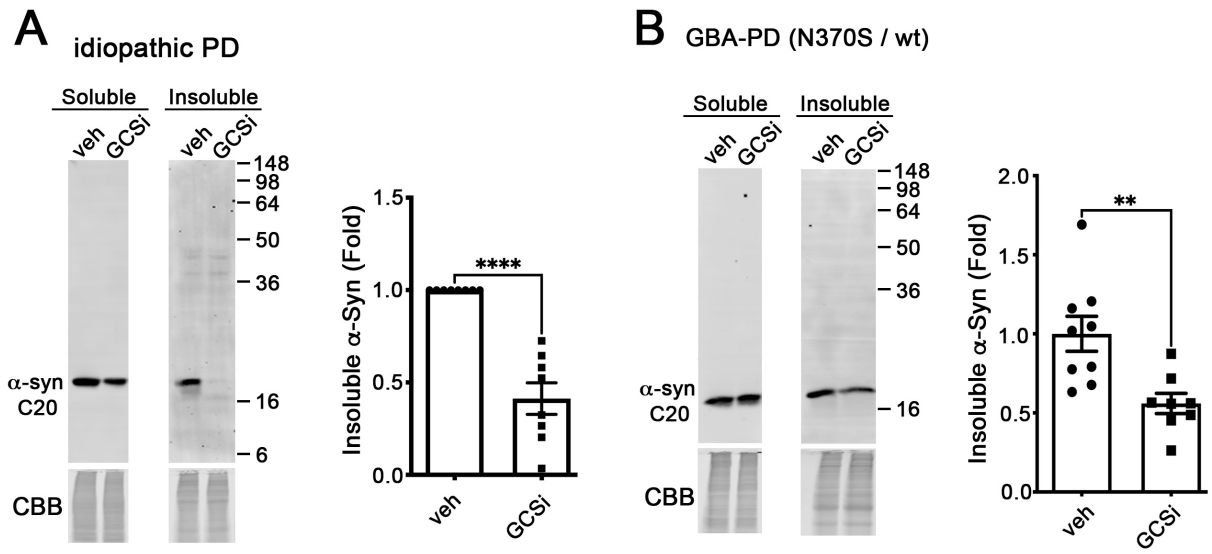

Figure S3. Treatment of iPSC midbrain neurons from PD patients with GCSi reduces insoluble a-syn. A) Idiopathic PD cultures at 9 months of age were treated with 50nM GCSi (eliglustat) for 4 weeks, followed by sequential extraction / western blot for a-syn (C20 antibody). Quantification of insoluble a-syn is shown on the right, normalized to CBB. **B)** GBA-PD cultures expressing N370S / wt GCase at 9 months of age were treated and analyzed as in panel A. Values are the mean +/- SEM, **p<0.01, ****p<0.0001, Student's t-test.

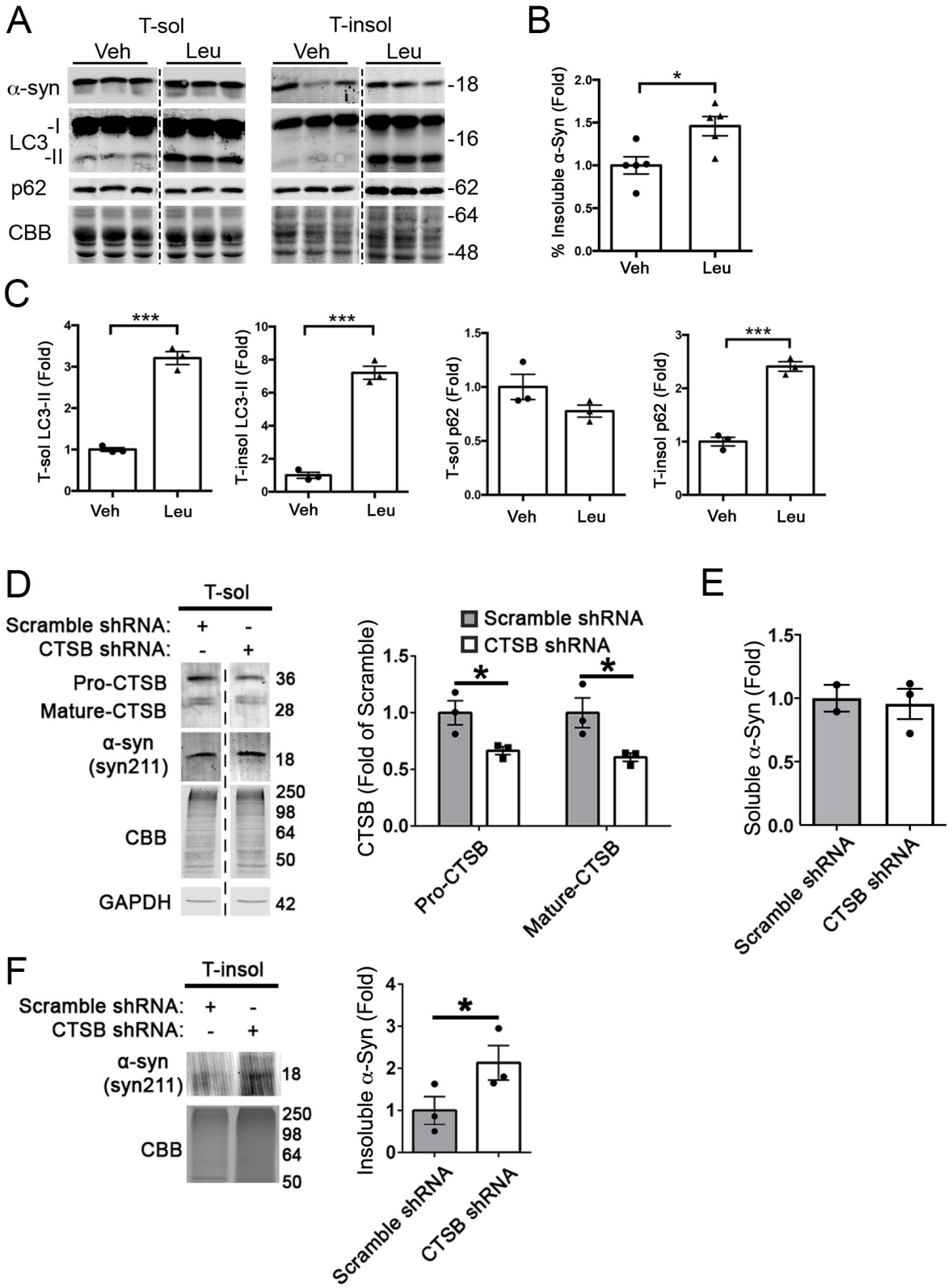

Figure S4. Insoluble a-Synuclein increases upon lysosomal inhibition. A) Wild-type midbrain neurons from a healthy control were treated with 3-methyladenine (3-MA), (10mM, 24 hr) or

leupeptin (Leu) (100μM,24 hr) then analyzed by sequential extraction / western blot of a-synuclein. LC3 and p62 are autophagy proteins that are lysosomal substrates, and used to determine effective inhibition of the autophagic-lysosomal pathway. Coomassie blue (CBB) was used as a loading control. **B)** Quantification of % insoluble a-syn (n=5). A-Syn levels from each fraction were normalized to CBB, and % insoluble a-syn was calculated (T-insol / (T-sol+T-insol)*100) then expressed as fold change of vehicle (veh) treated cultures. **C)** Quantification of P62 and LC3 in each fraction normalized to CBB (n=3). **D**) Wild-type midbrain cultures were infected with lentiviral particles to express either scrambled or CTSB shRNA constructs. CTSB and a-syn levels were measured five days after lentiviral transduction by western blot from (MOI = 5; n = 3 culture wells). **E)** Quantification of soluble a-syn normalized to CBB. **F)** Quantification of insoluble a-syn normalized to CBB (n=2-3). Values are the mean +/- SEM, *p<0.05, ***p<0.001, ANOVA with Tukey's post hoc test (Panels B, C) or unpaired two-tailed student's t-test (panels E, F).

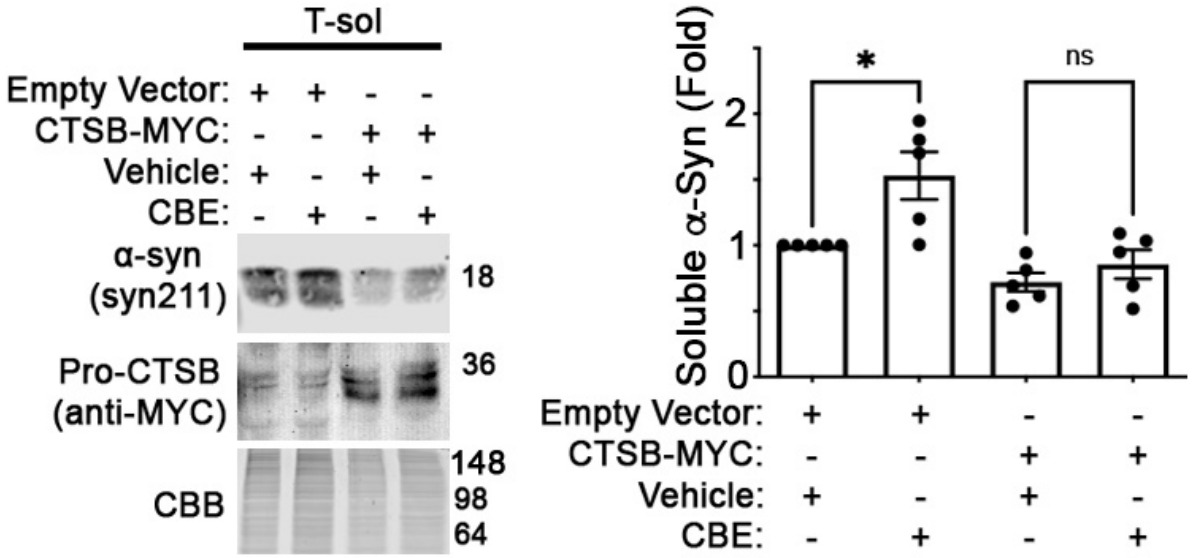

Figure S5. Overexpression of CTSB in human H4 cells blocks GSL-induced a-syn accumulation. H4 cells overexpressing a-syn (described previously (3) were transfected with empty vector or cathepsin B-MYC (CTSB-MYC) pcDNA 3.1 and then treated with vehicle (PBS) or 50 μM CBE for five days. a-Syn levels were measured in the soluble fraction by western blot and CTSB-MYC expression was verified using anti-MYC antibodies (T-sol, *n* = 6). Values are the mean +/- SEM, *p<0.05, ns, not significant, ANOVA with Tukey's post-hoc test.

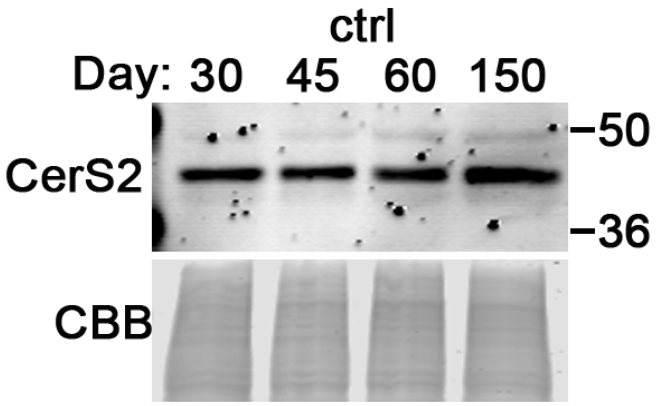

Figure S6. iPSC-derived midbrain cultures express CerS2. Western blot analysis demonstrates the expression of CerS2 within iPSC-derived midbrain cultures from wt controls aged to day 30, 45, 60, and 150.

SI References

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