Supporting Information

4-oxo-2-nonenal-induced α-synuclein oligomers interact with membranes in the cell, leading to mitochondrial fragmentation

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Preparation of recombinant α-syn. Recombinant human α-syn was produced in *E. coli* using a pET11-D expression vector as described previously (1). To produce N-terminally acetylated α-syn, a plasmid encoding the yeast N-acetyltransferase complex B (NatB) was co-overexpressed (2). To make uniformly ^{15}N-labelled αSN, *E. coli* was grown in minimal medium using $^{15}NH_4Cl$ as only nitrogen source. Cultures were induced at OD_{600} 0.8 with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG, Roth) for ~4 h at 37 °C. We used an established purification protocol (3) modified with two additional steps, which is acid precipitation and filtration through 0.45 µm filter before anion-exchange as described (1,4) to obtain high purity monomeric α-syn.

αSO preparation and purification: ONE-modified αSO was prepared as described in (5) by mixing 3 mM ONE in PBS with α-syn in PBS buffer to a final concentration of 200 µM αSN and 0.8 mM ONE. To avoid the significant losses of αSO caused by absorption onto the filter, we adopted the alternative approach of dialyzing αSOs against 5 L mQ water for 1 h followed by 5 L mQ water O/N to remove PBS. The protein was then lyophilized before dissolving it in electroporation buffer (EPB) (100 mM sodium phosphate, 5 mM KCl, 15 mM MgCl2, 15 mM HEPES, 2 mM ATP (Thermo Fisher), 2 mM reduced glutathione (Sigma) at pH 7.0).

Cell line and media: SHSY5Y (kindly provided by Poul Henning Jensen, Department of Biomedicine, University of Aarhus) were grown at 37 °C in humidified 5 % (v/v) CO_2 incubators in DMEM media with 4.5 g/L D-glucose and L-glutamine (Sigma) supplemented with 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were split at 70-80 % confluence and not passaged more than 6-12 times prior to NMR experiments. All cell lines were routinely tested for mycoplasma contaminations and are deemed mycoplasma free.

Cell lysate preparation: Around 50 x 10^6 cells were trypsinized and then subjected to lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 1% troton x-100, 2 mM EDTA, 1x protease inhibitor cocktail (cOmplete, EDTA-free), by pipetting until cells where lyzed (at room temperature). To prepare a full cell lysate, 300 µl lysed cells were spun 10 min at 1000xg to pellet remaining intact cells. To prepare a cell lysate without cell debris, nuclei and mitochondria, 300 µl lysed cells were spun for 10 min at 12.300xg to remove the membrane fraction.

Electroporation of recombinant α-syn into mammalian cells: For in-cell NMR samples, around 100 x 10^6 cells were used as the starting material. Cells were detached from culture flasks with trypsin/EDTA (0.05 %/ 0.02 %), centrifuged at 250 x g for 4 min at 37 °C, washed once in PBS and counted. ^{15}N-labelled α-syn was added to freshly prepared and sterile PEB buffer to final concentrations of 385 µM for monomer sample and 316 µM (monomer equivalent) for αSO sample.

Cells were pelleted again and mixed with α-syn in EPB at 30-60 x 10^6 cells per mL. 100 µL aliquots (3-6 x 10^6 cells) were then transferred into 2 mm cuvettes and electroporated on an Amaxa 4D-Nucleofector (Lonza). The CA-137 pulse program for the SHSY5Y cell line was used. For control experiments, cells were mock electroporated with EPB alone. Directly after electroporation, 1 mL of pre-warmed (37 °C) CO_2-adjusted and growth medium was added to each cuvette and samples were transferred to cell culture flasks. For immunofluorescence microscopy, aliquots of 300.000 cells were transferred to separate wells of a 24 well plate with 1 ml media and 10 mm coverslips with poly-L-lysine coating. For Western blotting, aliquots of 600.000 cells were added to separate wells of a 6-well plate (3.5 cm diameter), filled with 5 mL/well of pre-warmed growth medium and incubated 20 h. For in-cell NMR, aliquots of 50-100 x 10^6 cells were added to T175 flasks containing 25 mL of pre-warmed culture medium. Cells were returned to the incubator and allowed to recover for 5 h. Flasks were washed 2 times with pre-warmed media and once with PBS and cells were harvested by trypsinization. Cells were washed with pre-warmed PBS and counted. For in-cell NMR experiments, cells were taken up in 500 µL of growth media supplemented with 10 % D2O and 2 % melted agarose IX (sigma) at 37 °C. Agarose was added to ensure that the sample height and homogeneity remained the same during NMR experiment, otherwise the cells tend to sediment during the NMR experiment which leads to decreasing sample height during the experiment. This in turn gives lower quality NMR-spectra and less access to media for the cells in the bottom of the NMR-tube, increasing cell stress.

Western Blot: Proteins were separated on 12% SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked for 1 h in 0.5 % (w/v) BSA in TBST (0.1 % (v/v) Tween-20 in TBS), membranes were probed with anti-αSyn antibody sc-69977 (Santa Cruz, 1:100 dilution) and anti-actin IgM (Merck Millipore, JLA20, 1:5,000 dilution). Secondary antibodies were HRP-conjugated anti-mouse (Jackson, 1:10,000 dilutions). Membranes were developed using TMB Blotting PLUS (Kem-En-Tec). ImageJ 32 was used for densitometric scanning of bands. The total cell volume was calculated as the product of the number of cells and the average cell volume, approximated to 1 pL (10 µm cubed).

Immunofluorescence Microscopy for electroporation validation: For immunofluorescence imaging of α-syn in fixed specimens, cells were recovered in the incubator on poly-L-lysine-coated 10 mm cover slips as described in the electroporation protocol. Cells were washed with PBS, then fixed in PBS containing 4 % (w/v) PFA for 15 min and washed with PBS. Subsequently the cells were permeabilized with 0.1 % (v/v) Triton-X in PBS for 3 min. After washing for 3 x 10 min with

PBS, samples were blocked with 0.5 % (v/v) BSA (Sigma) in PBS for 1 h. Cells were incubated for 2 h with anti-α-syn sc-69977 (Santa Cruz, 1:200 dilution) in blocking buffer. After washing 3 x 10 min with PBS, specimens were incubated with anti-mouse IgG Alexa-647, (Sigma, 1:1,000 dilution) and fluorescein isothiocyanate (FITC)-labeled phalloidin (Millipore, 2 μg/mL) for 1 h in blocking buffer in the dark. Slides were washed for 3 x 10 min with PBS and nuclei stained with 1 μg/mL 4',6-diamidino-2-phenylindole (DAPI, Invitrogen) in PBS for 5 min. After washing thrice in PBS, samples were mounted with Immu-Mount (Thermo Scientific). Confocal images were taken at 20-60 x magnification using excitation wavelengths of 405, 488, and 633 nm on a Zeiss LSM510.

NMR: For in-cell NMR the cells prepared as described above were transferred to a Shigemi tube without plunger. The cells were sedimented in the tube using a hand-cranked centrifuge, and the height of the cells was measured. When cooled to 10°C in the NMR spectrometer, the agarose formed a gel, halting further cell sedimentation. Both in-cell NMR and reference experiments were recorded in a 950 MHz Bruker Avance spectrometer equipped with a cryogenically cooled triple resonance TCI probe. NMR spectra were acquired at 10 °C. ^{1}H-^{15}N-HSQC pulse sequence hsqcetfpf3gpsi2 was used (6). Experiments were recorded with interscan delay of 1 sec and with 128 scans and 2048 complex points. The spectra were processed using NMRPipe and analyzed with Sparky (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco) (7). Experiments of α-syn in buffer were acquired with 30-50 μM of N-acetylated α-syn in PBS supplemented with 10 % D_2O. In all cases, reference NMR spectra were recorded under identical conditions as the respective in-cell NMR experiments. Chemical shift perturbation of shifts in ^{1}H-^{15}N-HSQC spectra of in-cell compared to buffer was calculated using $\Delta\delta_{comp}=[\Delta\delta_{HN}^{2}+(\Delta\delta_{N}/R_{scale})^{2}]^{½}$, where $R_{scale}=6.5$ (8).

The concentration of α-syn in the cell was calculated by finding the number of cells and the total concentration of α-syn in the NMR sample. The number of cells was the fraction of cells within the lowest 18 mm of the NMR tube. For pellets with height < 18 mm, the total number of cells was used. The total concentration of α-syn in the sample was obtained by integrating the amide region (6-9ppm of the 1D projection of ^{1}H-^{15}N-HSQC) for α-syn in buffer (with known concentration) and the same was done for α-syn in cell. $[\alpha\text{-syn}]_{cell}=I_{buffer}/I_{in\text{-}cell}*[\alpha\text{-syn}]_{buffer}*V_{sample\text{-}in\text{-}coil}/(N_{cell}*V_{cell})$.

Immunofluorescence microscopy for fragmentation analysis and co-localization: Sample preparation was done as described above except that we used 1 mg/ml αSO in the electroporation for mitochondria fragmentation analysis, while 0.1 mg/ml αSO was added to media without electroporation for co-localization. The samples were fixed with 3.0% PFA/0.1% glutaraldehyde for 20 min at room temperature. Then the samples were reduced with 200 µl 0.1 % $NaBH_4$ (prepared

immediately before use) for 7 min at room temperature while shaking and washed 5x 5 min with PBS, after which the cells were permeabilized for 15 min 0.2 % Triton in PBS. Then the samples were blocked for 90 min with 500 µl 10 % NGS/0.05%Triton/PBS. The samples were then incubated with primary antibody at 4ºC overnight. For fragmentation analysis, the following antibodies was used: 1:300 anti-α-syn (sc-69977), 1:500 anti-ATP5A (ab 176569) in 5% NGS/0.05%triton/PBS. For co-localization analysis additionally 1:250 anti-VPS35 antibody [EPR11501(B)] (ab157220) and 1:250 anti-RAB7 antibody [EPR7588(B)] - Late Endosome Marker (ab126712) were used. Samples were washed 5 times with 1% NGS/0.05%triton/PBS (15 min pr wash while shaking), then incubated with secondary antibody (Goat-anti-rabbit with Alexa Flour® 488 (ab50077): 1:1000, Goat-anti-mouse with Alexa Flour® 647 (ab150115): 1:500) in 5% NGS/0.05%Triton/PBS in 30 min while shaking. This was followed by 5 times washing with 1% NGS/0.05% Triton/PBS (15 min pr wash while shaking), one wash with PBS 5 min (shaking), post fixing with 3.0% PFA/ 0.1% glutaraldehyde in 10 min (no shaking) and finally 3 washes with PBS of 5 min each while shaking. Samples were imaged on a Nikon A1 confocal microscope (Nikon, Tokyo, Japan) with Plan Apo λ 100x Oil objective.

Live cell imaging with Mitotracker: 30.000 cells pr well were seeded in an 8 well chamber slide with #1.5H glass bottom (Ibidi). The next day media was removed, and αSO was added in 0.4 mg/ml, 0.8 mg/ml and 1.6 mg/ml concentration in 100 µl Opti-MEM media. MitoTracker^{TM} Green FM (Invitrogen) was used according to manufactures procedure. A dilution of 200 mM Mitotracker in warm Opti-MEM was prepared. 200 µL of warm MitoTracker/OptiMEM was added to the cells 15-30 min before microscopy to a final concentration of 133 µM MitoTracker. Samples were imaged on a Nikon A1 confocal microscope (Nikon, Tokyo, Japan) with Plan Apo λ 100x Oil objective, using a Okolab Uno Stage Top Incubator to keep the cells at 37°C in 5 % (v/v) CO_2 and well humidified during experiment. A video with 1000 frames was recorded using 3 % 488 nm laser intensity, Scan size 512 pixel, 16x Line Average, 8x Line Integrate. The cells were kept in the stage incubator between measurements and in the dark.

Image analysis: *Fragmentation analysis:* To quantify the fraction of cells with fragmented mitochondria, cells were manually categorized either with predominantly fragmented mitochondria or with predominantly tubular mitochondria. To bin cells to either high or low in α-syn, the intensity was measured in the cell in a ROI of 40x40 pixels. A threshold of 900,000 relative fluorescence intensity units was used to divide cells into low and high α-syn signal.

Co-localization analysis: Background was subtracted from the images before analysis. To calculate Pearson's correlation coefficient, the ImageJ plugin BIOP JACoP was used on an ROI of the cytoplasm of the cell.

Live cell imaging: Mitochondria were measured using the Microsoft programme Visio in a selected ROI in the cell.

STED imaging: Sample preparation were done as described above where 1 mg/ml αSO is electroporated into SHSY5Y cells and fixed after 5 hours. Staining was done as described previously except here we used Abberior secondary antibodies: goat anti-mouse IgG Abberior STAR ORANGE and goat anti-rabbit STAR RED. Dual-color stimulated emission depletion (STED) image acquisition was carried out on an Abberior Facility Line STED microscope using a 100x magnification UplanSApo 1.4 NA oil immersion objective lens. Imaging of Abberior STAR ORANGE and Abberior STAR RED was done using a pulsed excitation laser of 561 nm or 640 nm, respectively, and a pulsed 775 nm depletion laser for both dyes Huygens software was used to make a deconvolution of the images.

Figure S1

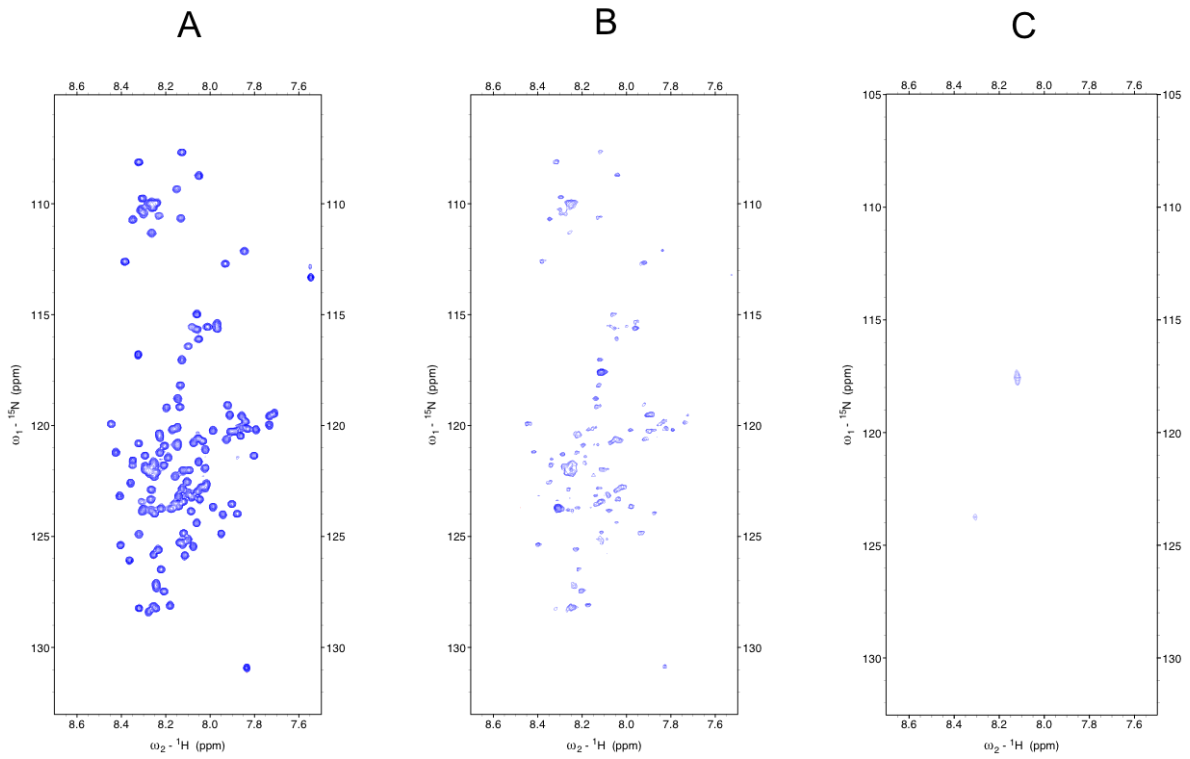

Figure S1: ^{15}N-^{1}H HSQC of (A) monomeric ^{15}N-α-syn in buffer, (B) monomeric ^{15}N-α-syn in cell and (C) SHSY5Y cells without ^{15}N-α-syn. The two peaks in panel C correspond to cellular metabolites arising from the 0.4 % natural abundance of ^{15}N.

Figure S2

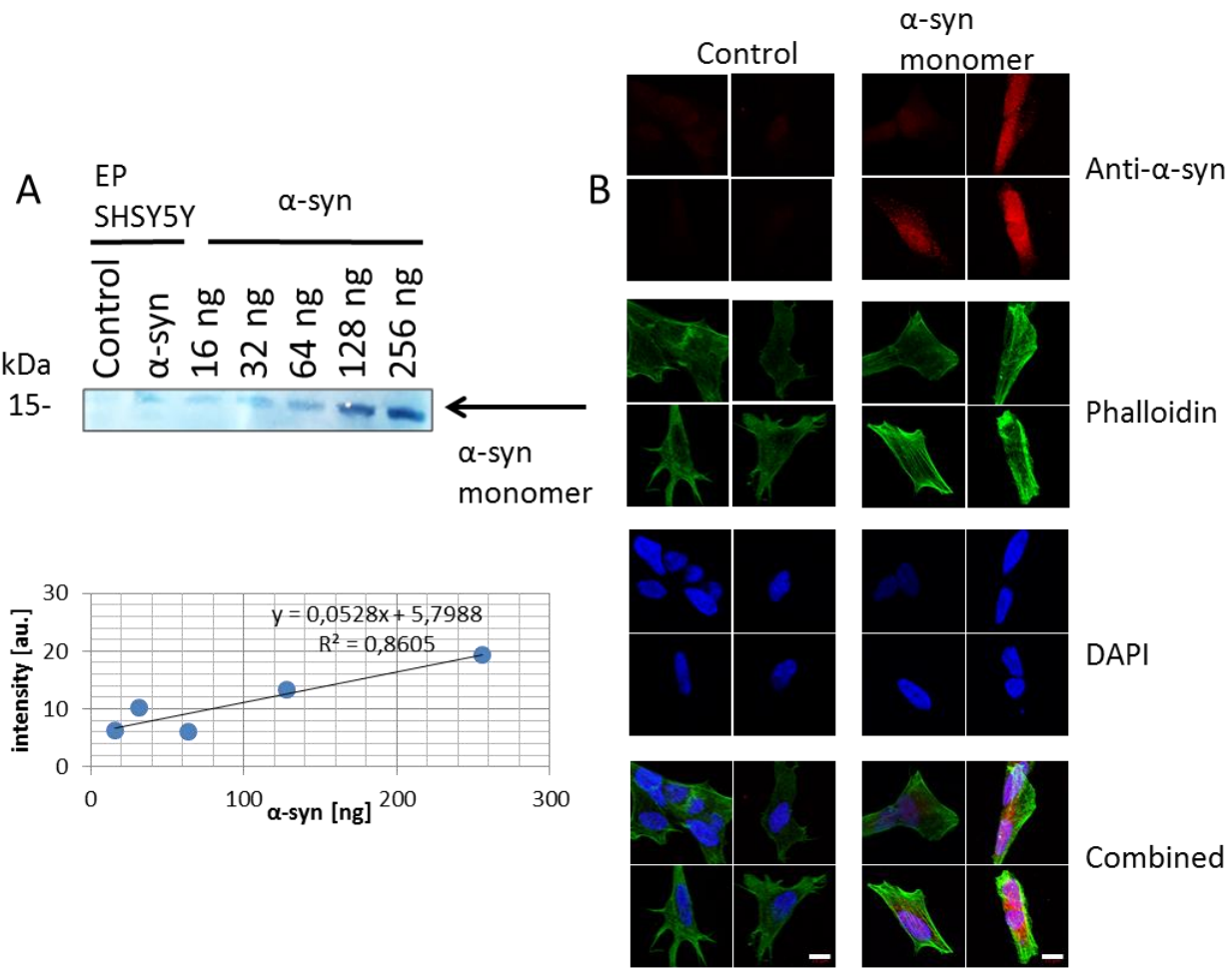

Figure S2: Western blot and confocal microscopy of SHSY5Y cells electroporated α-syn monomer. A: WB of cell electroporated (EP) with PBS and with α-syn monomer together with a dilution series of α-syn from 16-256 ng pr. well for concentration determination. Cells were washed before SDS-PAGE to limit analysis to intracellular proteins. Linear fitting used for concentration determination is showed below. B: Confocal microscopy of SHSY5Y cells electroporated with α-syn monomer or control buffer. Red: α-syn (anti-α-syn), green: actin (Phalloidin), blue: nuclei (DAPI). Scalebar is 10 µm.

Figure S3

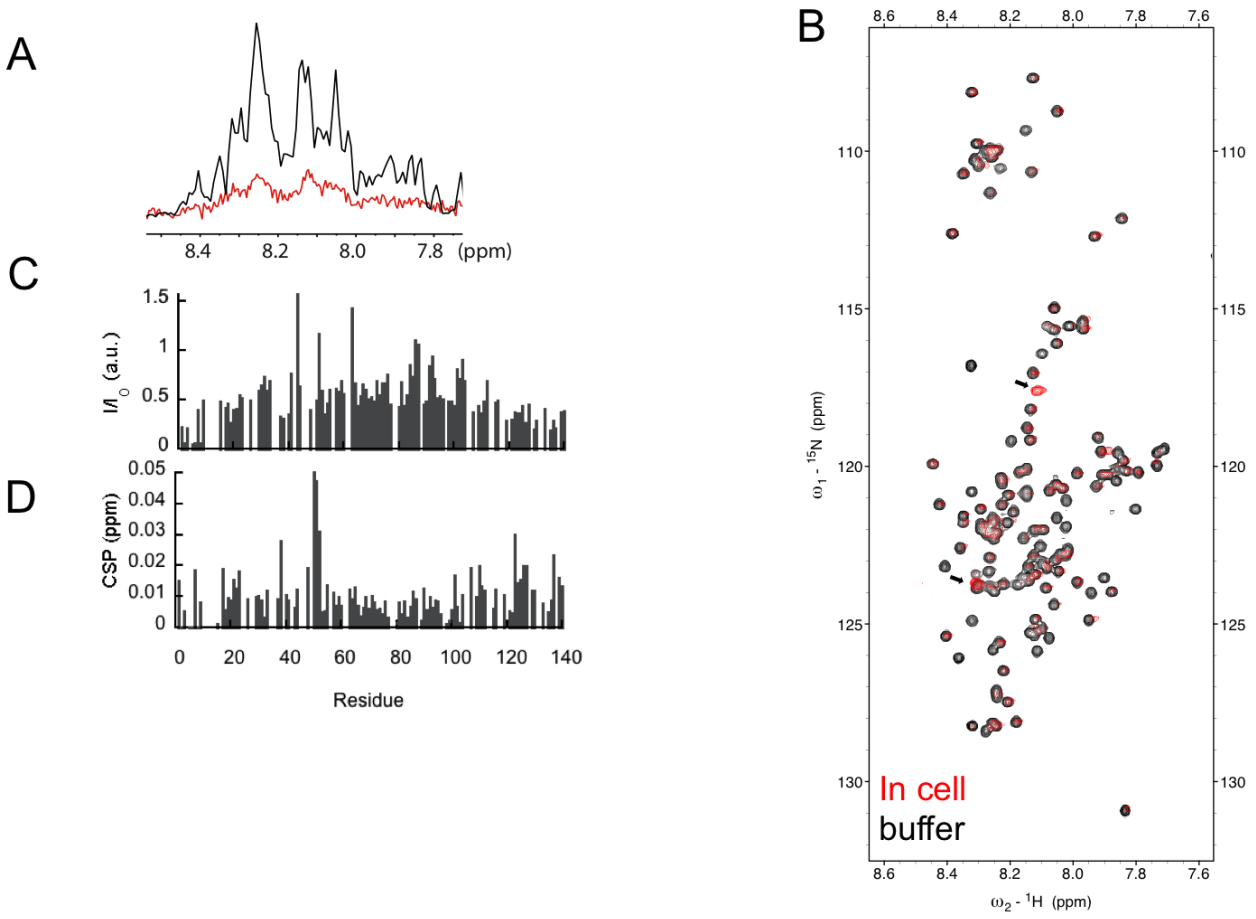

Figure S3: **in-cell NMR of α-syn monomer electroporated into SHSY5Y cells.** A: ^{15}N-edited 1D ^{1}H-NMR of 50 µM α-syn in PBS (in buffer) (black) and of α-syn in SHSY5H cells (red). B: ^{1}H-^{15}N-HSQC of α-syn in cell (red) and α-syn in buffer (black). Arrows denote growth medium specific metabolite signals (background). C: relative intensity of peaks in ^{1}H-^{15}N-HSQC (in B) of α-syn monomer in cell compared to α-syn in buffer. D: chemical shift perturbation of peaks in ^{1}H-^{15}N-HSQC of α-syn monomer in cell compared to α-syn in buffer.

Figure S4

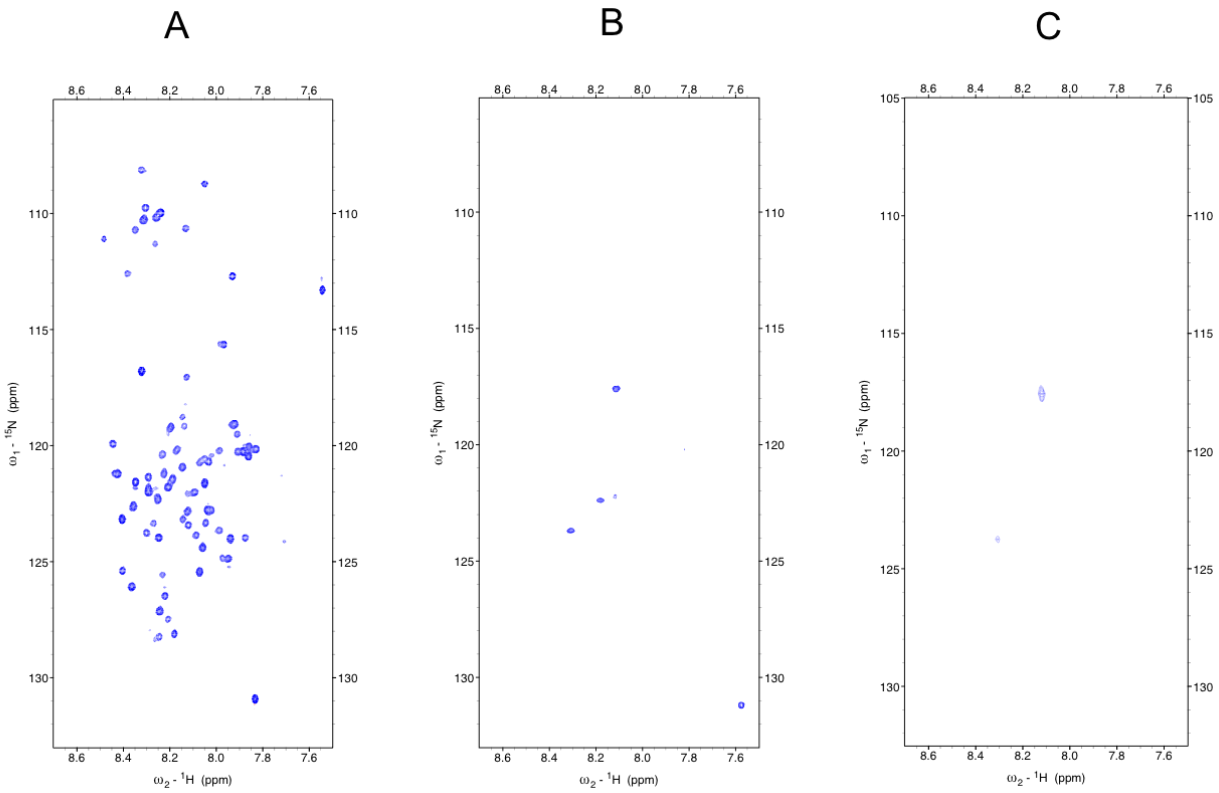

Figure S4: ^{15}N-^{1}H HSQC spectra of (A) ONE-oligomer in buffer, (B) ONE-oligomer in SHSY5Y cell and (C) SHSY5Y cells without ONE-oligomer (reproduced from Fig. S1C for comparison).

Figure S5

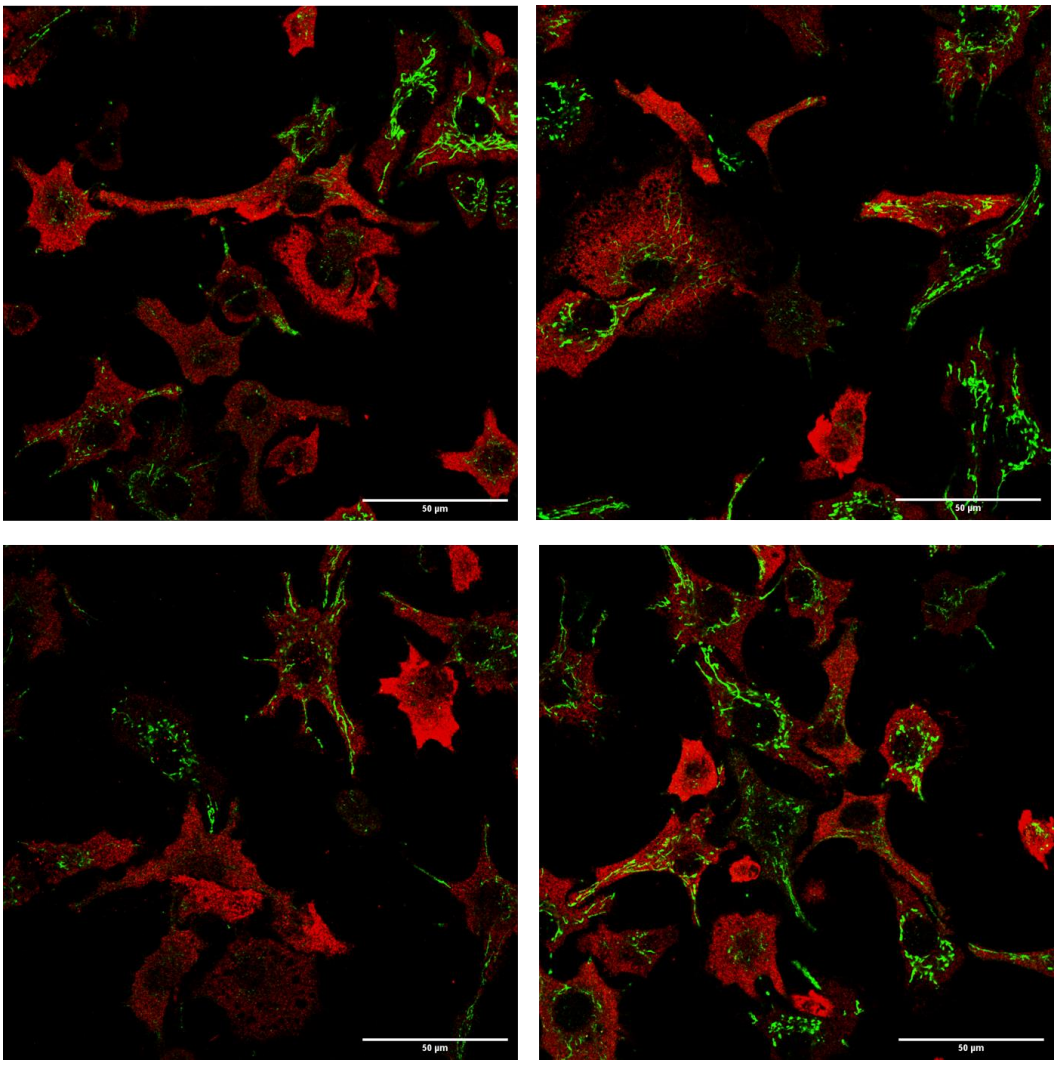

Figure S5: Confocal images of SHSY5Y cells electroporated with α-syn oligomer and stained with anti-α-syn (red) and anti-ATP5A (green, mitochondria).

Figure S6

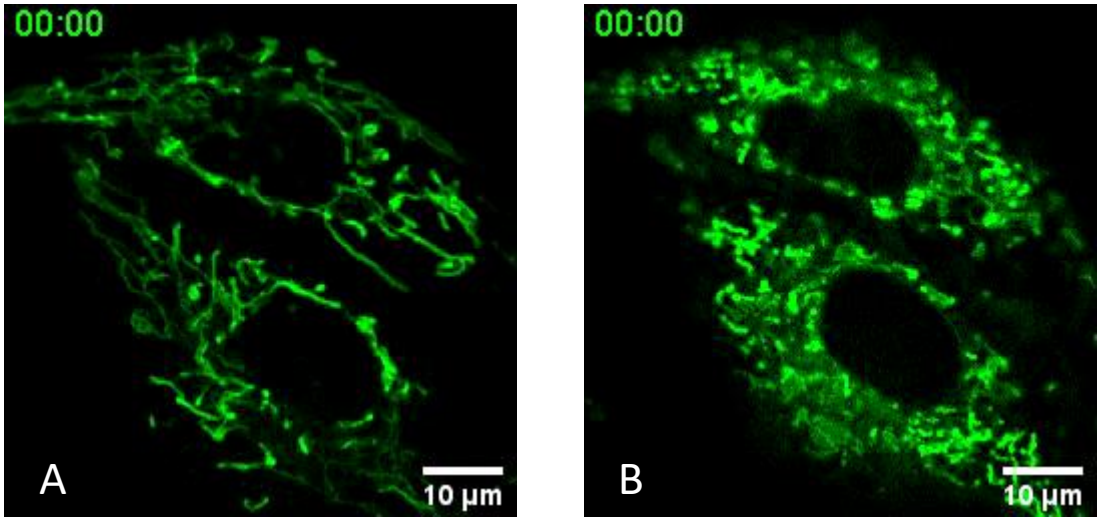

Figure S6: Live imaging of SHSY5Y cells with mitotracker: Samples with 1.6 mg/ml αSO after (A) 1 h and (B) 2 h after addition of αSO. Stained with Mitotracker Green.

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