SUPPLEMENTAL INFORMATION

Molecular mechanism of olesoxime-mediated neuroprotection through targeting α-synuclein interaction with mitochondrial VDAC

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Methods

Immunofluorescence

Cells grown on 8 well chamber slides were fixed in 2% paraformaldehyde for 10 min at room temperature and permeabilized with block solution (0.1% Triton™ X-100, 10% normal goat serum in PBS) for 1 h at room temperature (RT). Rabbit anti- αSyn (Cell Signaling Technology: 1:100) was diluted in block solution and incubated overnight at 4°C. Cells were then washed three times in 0.1% Triton™ X-100 in PBS solution and appropriate Alexa Fluor®594 IgG secondary antibody (Invitrogen) was applied for 1 h at room temperature, protected from light. Cells were mounted with VECTASHIELD® Antifade Mounting Medium with DAPI and imaged using a Zeiss LSM 710 confocal microscope at 63 × magnification.

Western blot analysis

Proteins were extracted in cell lysis buffer (Cell Signaling Technology) with additional phosphatase and protease inhibitors (Sigma). Protein fractions were separated by SDS-PAGE using 4-12% Bolt NuPage gel (Invitrogen) and electrotransferred to PVDF membranes for Western blot analysis. The following antibodies were used for immunoblotting: αSyn 2628 antibody (1:1000) (Cell Signaling Technology, Inc), VDAC1/porin antibody (1:1000) (Abcam) and β-actin (1:1000) (Santa Cruz).The membranes were washed in TBST (3×5 min) at RT followed by incubation for 1h at RT with fluorescently conjugated goat anti-mouse or rabbit IR Dye 680 or 800 antibodies (Licor). The blots were washed in TBST (3×5 min) at RT and scanned on an ODYSSEY® CLx (Licor). Quantitation of western blots was performed using Image Studio (Licor), the intensity of target proteins was standardized with the loading control. A relative quantification was performed by densitometry analysis using the NIH Image J software compared to the loading controls.

Proximity ligation assay (PLA)

Cells were grown, transfected and differentiated on Nunc™ Lab-Tek™ II CC2™ Chamber Slide System (ThermoFisher scientific). PLA was carried out with Duolink® In Situ Detection Reagents Orange (Sigma Aldrich) with primary antibodies for anti-αSyn (Cell Signaling Technology, 1:100, rabbit) and TOM20 (Santa Cruz,1:100, mouse). Slides were mounted using a minimal volume of Duolink in situ Mounting Medium containing DAPI and images were taken with a Zeiss LSM 710 confocal microscope at 63 × magnification. The microscope settings were kept constant for all images to enable direct comparison. Quantification of signals (number of dots per cell) was

obtained from thresholded images using the "analyze particles" feature of ImageJ, which detects isolated continuous objects in the image.

Gramicidin A channel measurements

Bilayer membranes were formed from DOPE/DOPC (4:1) (w/w) without or with 20% (w/w) of olesoxime. Membrane-bathing solutions contained 1 M KCl buffered with 5 mM HEPES at pH 7.4. Gramicidin A (gA) was added from 1 nM ethanol solutions to both sides of the membrane as described previously [1]. gA was a generous gift of O. S. Andersen, Cornell University Medical College.

Single-channel gA measurements were performed using an Axopatch 200B amplifier (Axon Instruments, Inc., Foster City, CA) in the voltage clamp mode at + 100 mV of applied voltage. For gA channels lifetime analysis the signal was filtered by a low-pass Bessel filter at 1 kHz and saved into the computer memory with a sampling frequency of 3 kHz. Then the records were digitally filtered at 500 Hz using Bessel algorithm and analyzed using Clampfit 10.3 software as described previously [1]. Channel lifetimes were calculated by fitting logarithmic exponentials to logarithmically binned histograms [2]. The mean number of events for each experiment was 250 to 2500. All lifetime histograms used 10 bins per decade.

Fluorescence Correlation Spectroscopy (FCS) measurements and data analysis

Liposome preparation. Large Unilamellar Vesicles (LUVs) were prepared from aliquots of 10 mg/ml stock lipid solutions in chloroform as described previously [3]. LUVs were made of DOPC/DOPE (1:1) mixture and either with 10% (w/w) of olesoxime or cholesterol. The dried lipid mixtures were re-hydrated in 150 mM KCl, 5 mM HEPES buffer at pH 7.4 to a final concentration of 1 mM lipid. The lipid-buffer solutions were then vortexed for 30 seconds to fully homogenize the sample and passed through lipid extruder (Avanti Polar Lipids, Inc.) using polycarbonate membrane filters (Millipore) with the pore sizes of 200 and 100 nm, sequentially. Liposomes' size and polydispersity were determined for each lipid composition and preparation by light scattering using Zetasizer Nano-ZS90 (Malvern). Homogeneous populations of LUVs of 152 ± 11 nm diameter with a polydispersity < 0.2 were used in FCS measurements. The liposome diameter measured for each lipid sample was used in analyzing the FCS data from that sample.

Sample preparation. FCS measurements were made in eight-well cover-glass slides (Grace Biolabs) pretreated with Sigmacoat (Sigma Aldrich) before each experiment to prevent liposome and αSyn adhesion to the surfaces. FCS measurements were carried out using a Hamamatsu Photonics K.K. C9413-01 spectrometer with a 473 nm excitation laser as described previously [4].

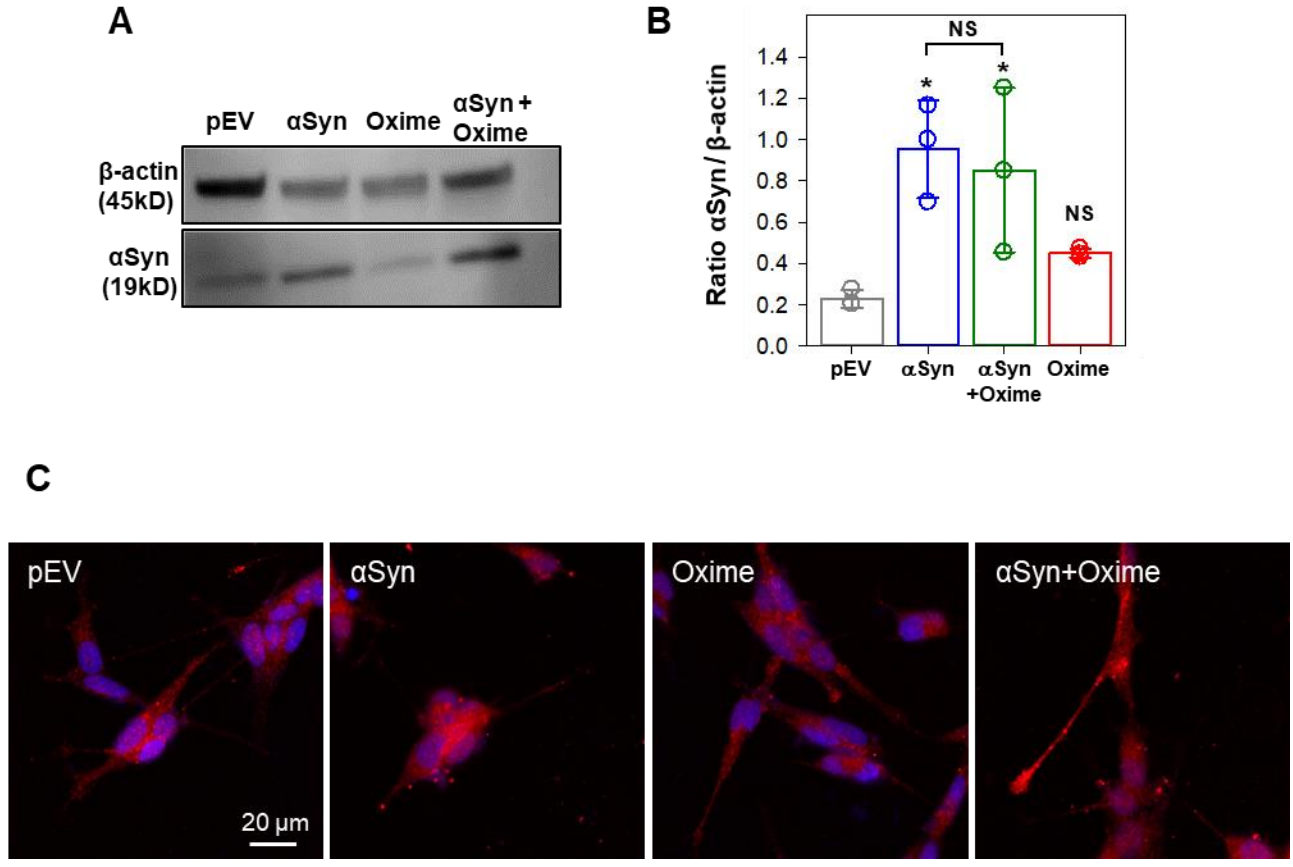
In order to minimize the potential influence of the fluorescent dye on αSyn-membrane binding, Alexa488 was placed in position Y136C in the C-terminus of αSyn. Considering that the N-terminal domain is generally accepted to be membrane-binding, the presence of Alexa488 at the C-terminus is unlikely to affect binding (see also [5]). Samples for FCS measurements contained

Alexa488-labeled αSyn alone or in liposome solution in 150 mM KCl, 5 mM HEPES buffer, pH 7.4. The concentrations of the Alexa488-labeled αSyn were calculated relatively to a calibration with free Alexa488 dye.

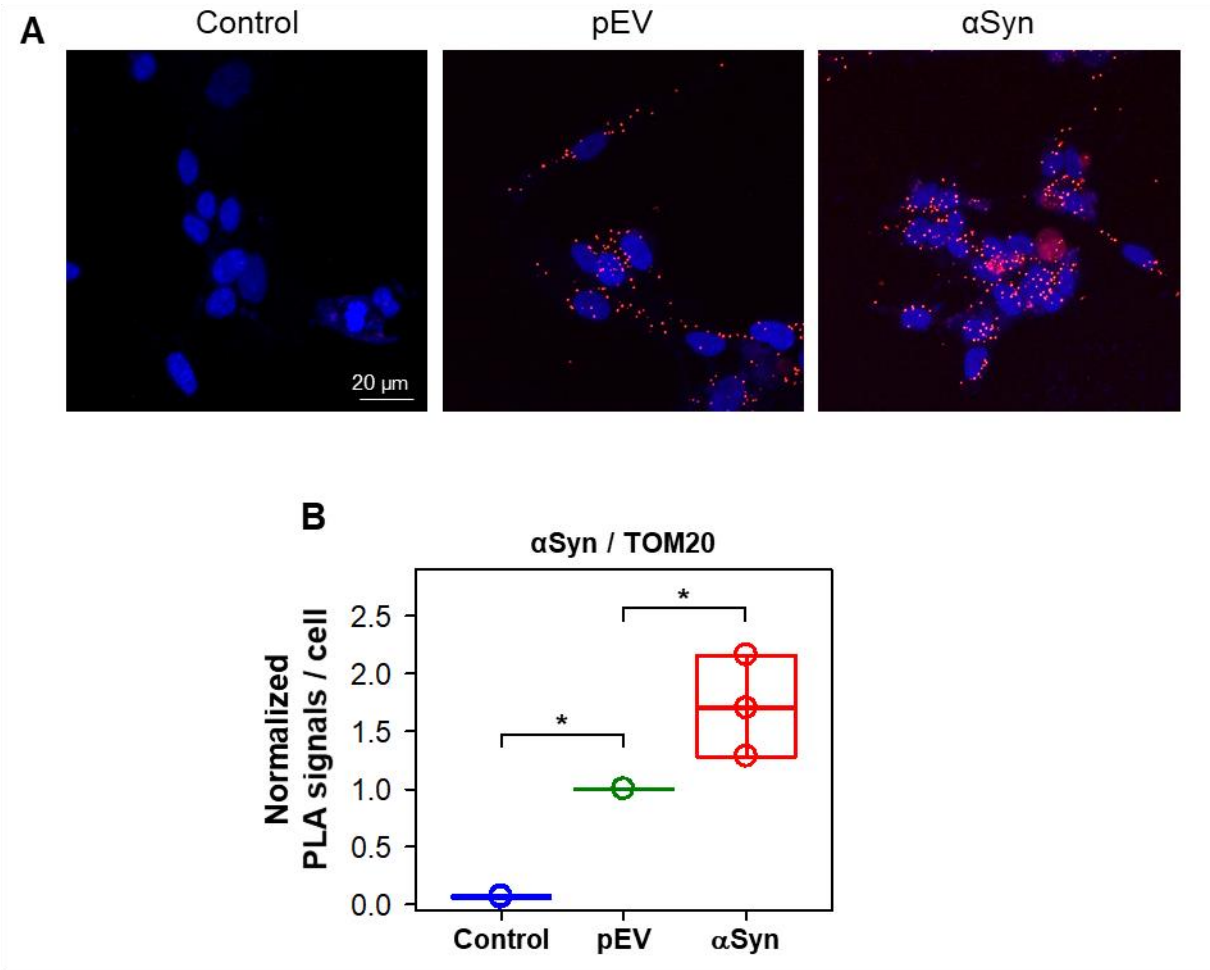
Analysis of correlation functions. The normalized FCS autocorrelation curve obtained with free Alexa488-labeled αSyn (Figure S5 A), shifted towards longer diffusion times with addition of non-labeled LUVs at constant concentration of Alexa488-labeled αSyn (30 nM, measured by comparison to an Alexa488 dye standard). The observed ~10 times increase of diffusion times is due to the binding of fluorescently labeled αSyn to the liposome membranes. As the diffusion time scales linearly with the hydrodynamic radius of the diffusing species, the diffusion time of the αSyn molecule is distinguishably smaller than that of much larger liposomes. The characteristic diffusion time of the free αSyn is ~ 0.21 ms (Figure S5 A) which is ~ 10 times faster than the diffusion time of ~ 120 nM diameter liposomes (~ 3 ms) measured using rhodamine-labeled LUVs. Therefore, the FCS functions obtained with αSyn and LUVs (Figure S5 A) are a sum of diffusion functions of free labeled αSyn and αSyn-liposome bound. A higher binding affinity of αSyn to lipid membrane corresponds to the higher portion of liposome-bound αSyn and, consequently to the lower content of free αSyn, resulting in a shift of correlation curves towards longer times characteristic for LUVs diffusion.

In order to quantify αSyn binding to the membranes with different lipid compositions we followed the approach described in [3]. This approach uses the amplitude of the non-normalized autocorrelation functions thus avoiding some uncertainty in fitting of autocorrelation functions with multiple parameters. It calculates the average number *n* of liposome-bound labeled αSyn per vesicle. The difference in αSyn-membrane binding to the LUVs of different lipid composition is best seen if the results are presented as the number of vesicle-bound proteins in the FCS focal volume, $n \cdot N_L$, where N_L is the number of lipid vesicles in the focal volume, plotted against the total accessible lipid concentration, $c_L/2$ (half the lipid concentration used to prepare the liposomes, assuming that αSyn binds to only the outer leaflet of liposome membrane [5]).

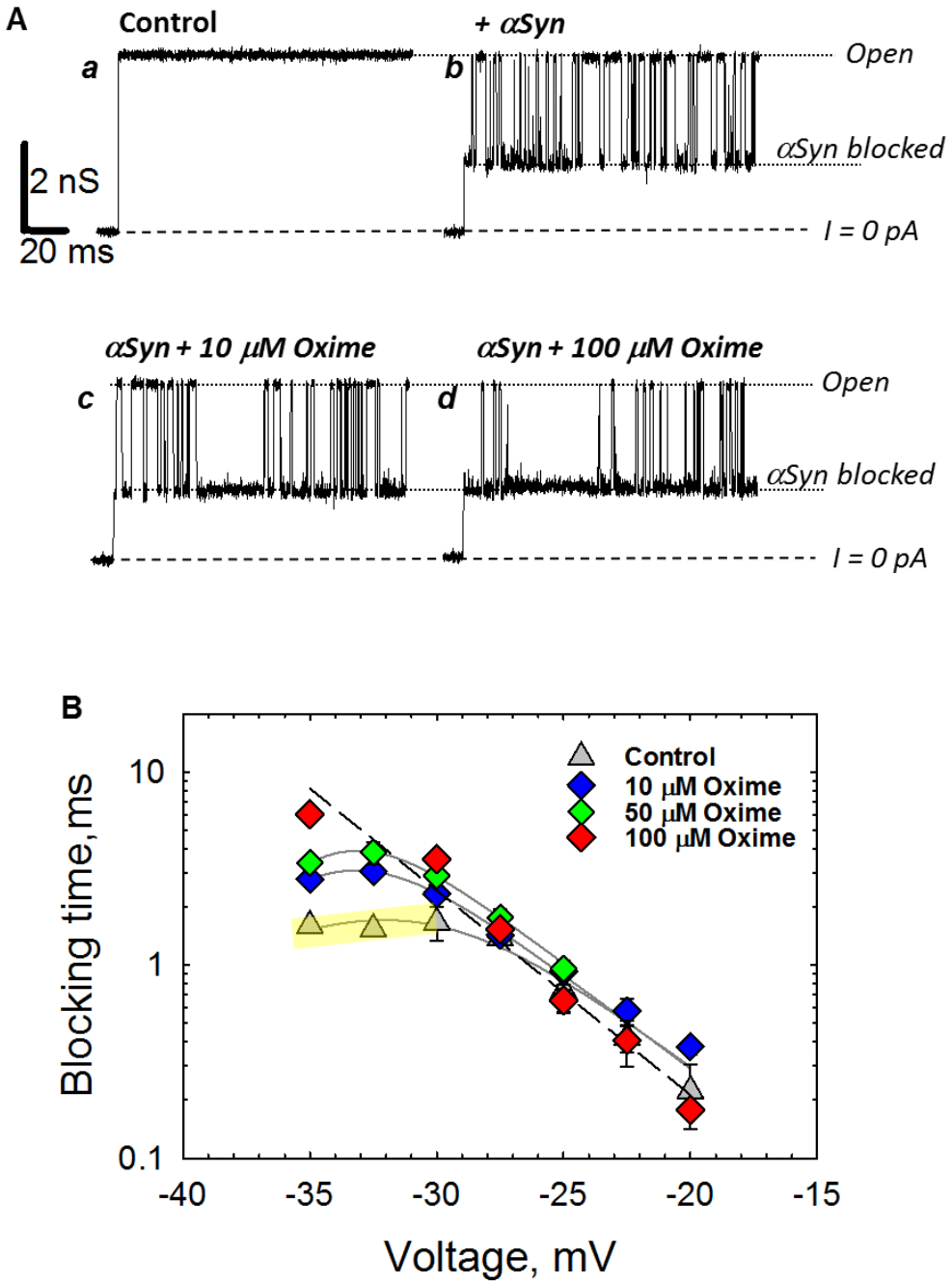
SUPPLEMENTAL FIGURES



Supplemental Figure S1. Effect of olesoxime on αSyn expression level in differentiated SH-SY5Y cells. Immunoblot for αSyn is shown in (A) and quantifications of changes in αSyn levels are shown in (B) (mean ± standard deviation; n = 3). Unless indicated by brackets, significance was tested against the corresponding pEV controls (NS (not significant): $p > 0.5$; *: $p < 0.05$; one-way ANOVA; β-actin served as an internal loading control). (C) Representative confocal images of αSyn immunostaining in differentiated SH-SY5Y cells following αSyn overexpression and in cells treated or not with olesoxime (x63 objective, scale bar 20 µm).

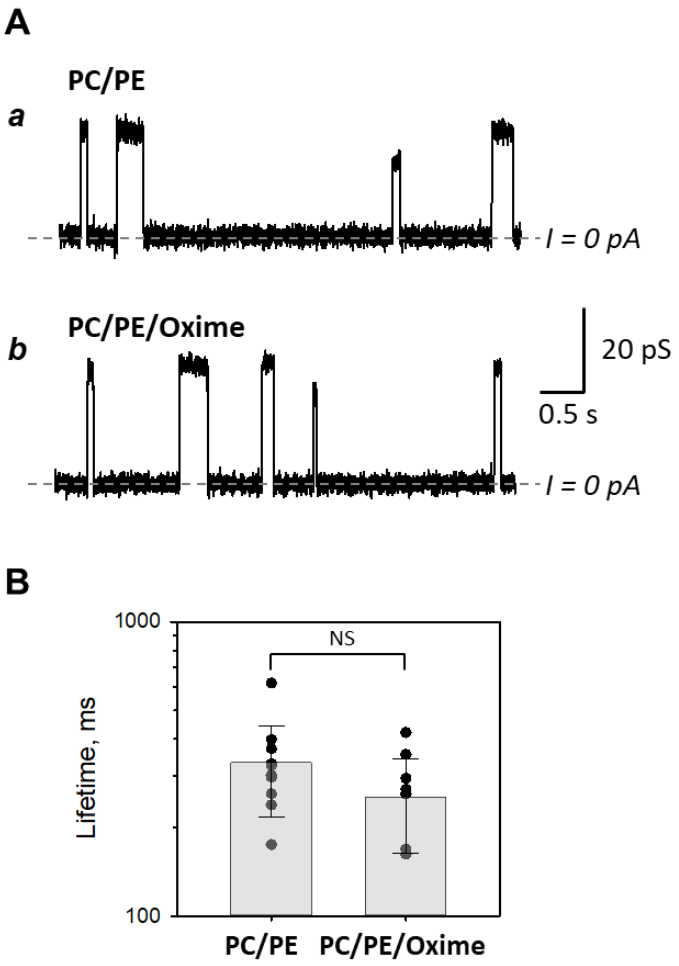


Supplemental Figure S2. Proximity ligation assay between αSyn and TOM20. (A) Representative confocal images of PLA showing αSyn in close proximity (~30 nm) to TOM20 at the MOM in cells overexpressing αSyn. Nuclei are visualized by DAPI. Negative control is obtained by omitting the primary antibodies (x63 objective, scale bar 20 µm). (B) Box plots represent the normalized distribution of signals per cell. The PLA signals were normalized to the average PLA values in pEV control cells of the corresponding experiment. Error bars indicate SD (*: $p<0.05$; one-way ANOVA).

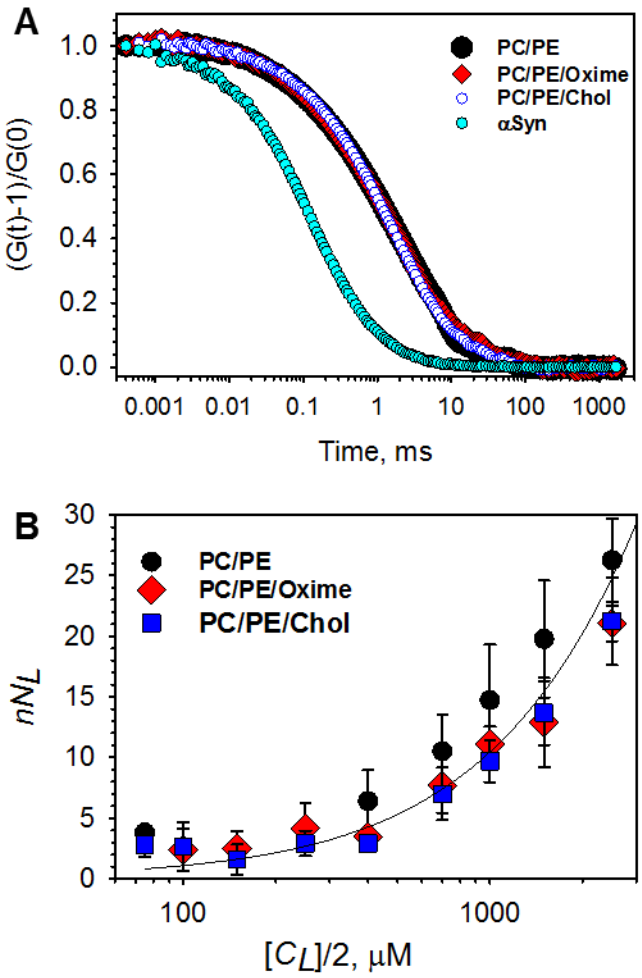


Supplemental Figure S3. Olesoxime added to the membrane-bathing solution prevents αSyn translocation through reconstituted VDAC. (A) Ion current records of the same single VDAC channel reconstituted into a planar lipid bilayer formed from a DOPC/DOPE (1:4 w/w) mixture with 5% (w/w) of cholesterol before (*trace a*) and after addition of 50 nM of αSyn to the *cis* compartment (*trace b*). Traces *c* and *d* were obtained after consequent additions of olesoxime in DMSO to the final concentrations of 10 and 100 μM in the membrane bathing solution. All records

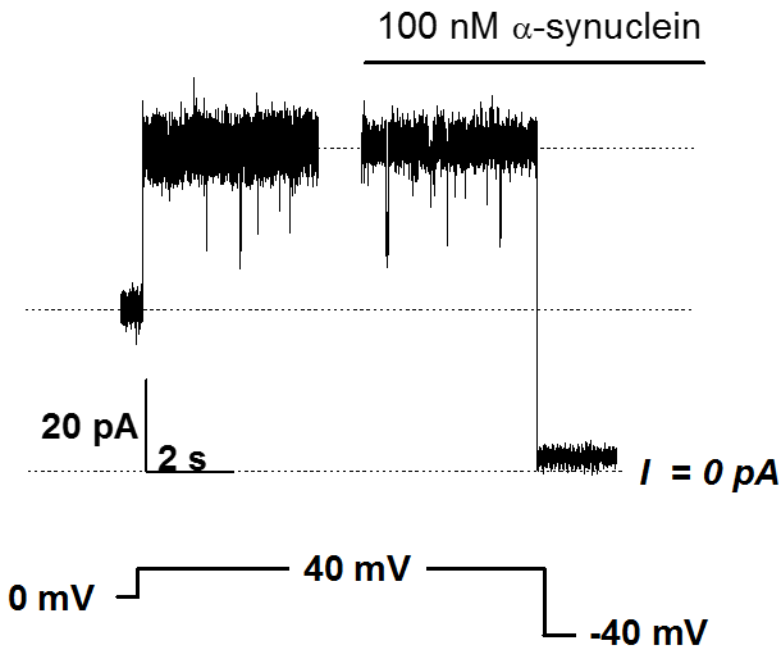
were taken at -30 mV applied voltage. The membrane-bathing solutions contained 1 M KCl buffered with 5 mM HEPES at pH 7.4. Dotted lines indicate VDAC open and αSyn-blocked states and dashed lines show zero current. All current records were smoothed with 5 kHz lowpass Bessel digital filter using pClamp 10.3. (B) The voltage dependences of mean blockage times of αSyn-induced blockages obtained at different olesoxime concentrations on the same membrane. The regime of αSyn translocation through VDAC, corresponding to a decrease of blockage time with voltage amplitude, is highlighted in yellow for data obtained in control conditions as in trace *b* in (A). At $|V| > 27.5$ mV the blockage time increases with olesoxime concentration. The voltage dependence of the mean blockage time starts deviating from the translocation regime, indicating inhibition of αSyn translocation through the channel. Data points and error bars represent the mean ±SD for 4 different fitting protocols of log-binned distributions of blockage times.



Supplemental Figure S4. Olesoxime does not affect gA channel lifetime. (A) Representative current traces of gA channels in planar lipid bilayers formed from DOPE/DOPC (4:1) (w/w) without (*a*) and with 20% (w/w) of olesoxime (*b*). The applied voltage is +100 mV. Dashed lines indicate zero current level. Current recordings were filtered with an 8-pole Bessel filter at 500 Hz cutoff frequency. (B) gA channel lifetimes in DOPE/DOPC (4:1) bilayers without and with 20% (w/w) of olesoxime. Each data point is calculated from at least 250 channel formation events. Error bars represent the mean ±SD for at least 7 independent experiments. NS (not significant): $p \geq 0.05$. The membrane-bathing solutions contained 1 M KCl buffered with 5 mM HEPES at pH 7.4.



Supplemental Figure S5. Olesoxime does not affect αSyn binding to the liposome membranes. (A) Normalized FCS correlation functions obtained on liposomes made of DOPC/DOPE (1:1) and with 10% (w/w) of olesoxime (PC/PE/Oxime) or cholesterol (PC/PE/Chol) in DOPC/DOPE mixtures in the presence of constant concentration of Alexa488-labeled αSyn (measured to be 30 nM). Liposomes were formed in 150 mM KCl buffered by 5 mM HEPES at pH 7.4. Total lipid concentration was 2 mM for all lipid compositions. Addition of liposomes to labeled αSyn causes a shift of correlation functions towards longer diffusion times due to the αSyn binding to the liposome membranes. (B) Quantification of labeled αSyn binding to the liposome membranes presented as number of labeled αSyn bound to one liposome, n, calculated at different lipid concentrations, c_L. Data presented as a product of $n \cdot N_L$, where N_L is a number of liposomes in the effective illuminated volume of FCS instrument, versus a half of the total lipid, $c_L/2$, to account for the liposome outer bilayer leaflet only. Solid line represents fit to the simple binding curve to guide the eye. Data points and error bars represent the mean ±SD for 3 - 4 independent experiments.



Supplemental Figure S6. Reconstituted pore-forming protein of the outer mitochondrial membrane TOM complex, TOM40, is insensitive to addition of αSyn to both sides of a membrane. Ion current shows no increase in fluctuation upon addition of 100 nM of αSyn. Experimental conditions: *cis*-side 250 mM KCl, 5 mM $CaCl_2$, *trans*-side: 20 mM KCl, both sides are buffered with 5 mM HEPES, pH 7.4, membrane is prepared from PLE. Purified and refolded TOM 40 from *Candida glabrata* (a gift from Susan Buchanan and Adam Kuszak, NIDDK, NIH) was added to the *cis*-side solution in proteoliposomes made from L-α-phosphatidylcholine. Recombinant TOM40 isolation and reconstitution were performed as previously described [6]. Dashed lines indicate Tom40 currents at different applied voltages shown in the bottom panel. Pore identity is manifested through its cationic selectivity (zero current corresponds to -40 mV applied), characterized by the reversal potential of ~40 mV (~80% cationic selectivity) in the current conditions.

Supplemental References

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