Peroxidase mechanism of lipid dependent cross-linking of synuclein with cytochrome c: protection against apoptosis versus delayed oxidative stress in Parkinson’s disease.

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Running head: Scavenging of cytochrome c by alpha-synuclein

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Damage of pre-synaptic mitochondria could result in release of pro-apoptotic factors that threaten integrity of the entire neuron. We discovered that alpha-synuclein (Syn) forms a triple complex with anionic lipids (such as cardiolipin) and cytochrome c, which exerts a peroxidase activity. The latter catalyzes covalent hetero-oligomerization of Syn with cytochrome c into high molecular weight aggregates. Syn is a preferred substrate of this reaction and is oxidized more readily than cardiolipin, dopamine and other phenolic substrates. Co-localization of Syn with cytochrome c was detected in aggregates formed upon proapoptotic stimulation of SH-SY5Y and HeLa cells, and in dopaminergic substantia nigra neurons of rotenone-treated rats. Syn/cardiolipin exerted protection against cytochrome c induced caspase 3 activation in a cell free system, particularly in the presence of H2O2. Direct delivery of Syn into mouse embryonic cells conferred resistance to pro-apoptotic caspase 3 activation. Conversely, siRNA depletion of Syn in HeLa cells made them more sensitive to dopamine induced apoptosis. In human Parkinson's disease substantia nigra neurons, two thirds of co-localized Syn/cytochrome c complexes occurred in Lewy neurites. Taken together, these results indicate that Syn may prevent execution of apoptosis in neurons through covalent hetero-oligomerization of cytochrome c. This immediate protective function of Syn is associated with the formation of the peroxidase complex representing a source of oxidative stress and postponed damage.

Lewy bodies (LBs), mitochondrial impairment, and oxidative stress are cardinal features of Parkinson’s disease (PD) and several related neurodegenerative disorders (1,2). Aggregation of alpha-synuclein (Syn), an abundant protein in synaptic terminals, plays a major role in the formation of LBs (3,4). Neither the mechanisms of LB production nor their pathogenic or protective roles in neurodegeneration are well understood.

In nigrostriatal dopaminergic synaptic terminals, mitochondria - harboring a host of death-initiating factors - are at a peril of damage by reactive oxygen species generated by disrupted electron transport and/or oxidative metabolism of dopamine (DA). Because cytochrome c (cyt c) dependent formation of apoptosomes and activation of caspases designates a point of no return in the apoptotic program, release of pro-apoptotic factors from...
synaptic mitochondria could threaten the integrity of the entire neuron. How neurons protect themselves against inadvertent release of death-signals from damaged synaptic mitochondria is not known.

The N-terminal fragment of Syn contains six variants of an 11-aminoacid consensus motif that comprise an apolipoprotein-like-class-A2 helix participating in binding of different lipids, particularly anionic phospholipids (5). This domain is believed to be important for Syn functions in regulation of neuronal lipid metabolism, particularly turnover of a mitochondria-specific phospholipid, cardiolipin (CL) (6). However, the relevance of the Syn lipid binding capacity in regulating neuronal injury (apoptotic) responses has not been established.

It is believed that oxidative stress participates in the accumulation of LB and Lewy neurites (LN) through yet to be identified pathways (7). Reportedly, Syn is co-localized with cyt c in LBs (8), indicating a potential interaction between the two proteins. Because cyt c is a redox-active heme-protein (9,10), its presence in the LBs in conjunction with Syn may also provide a mechanistic link of LBs with oxidative stress. We have recently reported that cyt c interacts with CL in mitochondria early in apoptosis and with phosphatidylserine (PS) in plasma membrane after its release into the cytosol (11,12). In both cases, this results in redox activation of cyt c and the production of complexes with high peroxidase activity that effectively catalyze peroxidation of the respective phospholipids (13).

Based on these facts, we hypothesize and provide experimental evidence that Syn acts as a sacrificial scavenger of cytosolic cyt c inadvertently released from synaptic mitochondria to prevent its migration into the soma, i.e., spread of the pro-apoptotic signal and cell death. This vital function is realized through the emergence of a peroxidase activity of cyt c/Syn/phospholipid complex that cross-links its components and yields covalently-conjugated protein-lipid hetero-oligomers. The latter maintain lingering peroxidase activity. Thus protection against acute apoptotic cell death comes with a penalty of Syn/cyt c aggregation into a peroxidase complex capable of inducing protracted oxidative stress. Our results present a novel biochemical mechanism likely involved in Lewy body formation and explain a known paradox of a dual protective and deleterious role that Syn plays in neuronal cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and treatment.** HeLa, HL-60 and SH-SY5Y cells were purchased from American Type Culture Collection and cultured in 1:1 mixture of Eagle's Minimum Essential Medium (EMEM) and Ham's F12 Medium supplemented with 10% of fetal bovine serum (FBS), 1.5 g/L sodium bicarbonate, 2 mM of L-glutamine, 0.5 mM sodium pyruvate and 0.05 mM nonessential amino acids. For apoptosis induction, HeLa cells were incubated with tert-butyl hydroperoxide (t-BuOOH) (400 µM) or ActD (200 ng/mL) for 16h; SH-SY5Y cells were incubated with t-BuOOH (10 µM for 16h) or ActD (10 µg/mL for 18 h). At the end of incubation, the attached cells were harvested by trypsinization and pooled with the detached cells from supernatant for PS externalization analysis and immunostaining. Cyt c deficient HeLa cells were generated using siRNA expressing plasmid (pSEC hygro vector, Ambion) as previously described (12) and cultured in DMEM medium supplemented with 15% FBS, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 50 mg/L uridine, 110 mg/L pyruvate, 2 mM glutamine, 1×nonessential amino acids, 0.05 mM 2'-mercaptoethanol. Syn knock-down HeLa cells were generated using siRNA expressing plasmid (pSEC hygro vector, Ambion). Target sequence (AAGAGGGTGTTCTCTATGTAG) was cloned into pSEC hygro vector and the resultant plasmid was transfected into HeLa cells. Positive clones were selected by hygromycin.

**Delivery of Syn into MECs.** Mouse embryonic cells (MECs) (courtesy of X. Wang, University of Texas, Dallas) were derived from 8- and 9-days old mouse embryos by Li et al (14). MECs were cultured in DMEM supplemented with 15% FBS, 25 mM HEPES, 50 mg/L uridine, 110 mg/L pyruvate, 2 mM glutamine, 1× nonessential amino acids, 0.05 mM 2'-mercaptoethanol, 0.5 × 10^6 U/L mouse leukemia inhibitory factor. Syn protein was delivered into cells using Chariot (Activemotif, Carlsbad, CA).
according to the manufacturer’s instructions. Briefly, cells were seeded at a density of 0.03x10^6/well in 24-well plate and allowed to attach overnight. Chariot: Syn complex (2 µl: 0.5 µg) was incubated with cells for 3 h for integration. After that, cells were treated with 50 ng/ml Actinomycin D (ActD) for 18 h. At the end of incubation, attached cells were harvested by trypsinization and pooled with detached cells from supernatant. Caspase-3/7 activity was determined using a caspase-3/7 Glo kit (Promega, San Luis Obispo, CA).

**Preparation of Liposomes.** Liposomes containing dioleoyl-phosphatidylcholine (DOPC) and tetraoleoyl CL (TOCL) (or other anionic lipids) (lipid/DOPC ratio 1:1), were prepared in 20 mM HEPES (pH 7.4) by sonication under N2 and used immediately after preparation. To prevent redox cycling with free metals diethylenetriaminepenta–acetic acid (DTPA) (100 µM) was added to all solutions used.

**Preparation of fibrillated (aged) Syn.** Fibrillated (aged) Syn was prepared by incubation of wt Syn and its mutants (200 µM) in 20 mM HEPES, 100 µM DTPA (pH 7.4) with shaking at 200 rpm for 6 days at 37°C. Isolation of mitochondria. Mitochondria were isolated as described previously (12). Briefly, harvested cells were resuspended in isolation buffer containing 300 mM mannitol, 10 mM HEPES-KOH (pH 7.4), 0.2 mM ethylenediamine-tetra-acetic acid (EDTA), 0.1% bovine serum albumin (BSA) and protease inhibitor cocktail (Roche) homogenized on ice with glass homogenizer, and then centrifuged at 1000 g for 10 min at 4°C. The resulting supernatants were centrifuged at 14,000 g for 15 minutes at 4°C. The resulting pellet was collected as the mitochondrial fraction. Protein concentration was determined using Bio-Rad assay (Bio-Rad).

**Conditions for model biochemical experiments.** Recombinant Syn was purchased from Chemicon International Inc. (Temecula, CA). Synuclein was diluted in water (to a final concentration of 1 mg/ml), divided into aliquots and stored at -20°C until use. In all model experiments in Figs 1B, C, D and 2A, the following conditions were utilized: Syn (1.5 µM) was incubated with 0.5 µM cyt c and TOCL/DOPC liposomes (TOCL/Syn ratio 25:1) in 20 mM HEPES (pH 7.4) for 60 min at 37°C. Incubation volume was 50 µl. 50 µM H2O2 was added to incubation mixture every 15 min. The reaction was stopped by addition of 5 µl of catalase (0.1mg/ml).

**Western blot analysis.** Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) run in Mini-Protein 3 system (Bio-Rad) in tris-glycine buffer. 0.8% agarose gel electrophoresis was run in horizontal gel system “Mupid-21” (Cosmo Bio Co., Ltd) in non-denaturing buffer (43 mM imidazole, 35 mM HEPES (pH 7.4)). In experiments with the HeLa cells, proteins were extracted with 1% SDS after cells were washed with PBS. The separated proteins were electrotransferred to nitrocellulose membrane. After blocking with 5% non-fat milk dissolved in phosphate buffered saline-tween-20 (PBS-T, 0.05%) or tris buffered saline-tween-20 (TBS-T) for 1 h, membrane was incubated with primary antibodies (anti-synuclein, anti-cyt c, anti-dityrosine or anti-5,5-dimethyl-1-pyrroline-N-oxide (DMPO) antibodies) overnight at 4°C. The membranes were washed 3-4 times followed by incubation with horseradish peroxidase (HRP)- or alkaline phosphatase (AP)-conjugated goat anti-rabbit or goat anti-mouse antibodies for 60 min at RT. The protein bands were visualized by SuperSignal West Pico Chemiluminescent Substrate (Pierce) for HRP-conjugated secondary antibody or Lumi-Phos WB (Pierce) for AP-conjugated secondary antibody as described by the manufacturer. The density of bands were determined by scanning with Epi Chemi II Darkroom (UVP BioImaging Systems, Upland, CA, USA).
Peroxidase activity in gel was determined after native electrophoresis in 0.8% agarose. Gels were incubated in solution containing SuperSignal West Pico Chemiluminescent Substrate (Pierce) and chemiluminescence was determined by scanning with Epi Chemi II Darkroom (UVP Biolimaging Systems, Upland, CA, USA). (iii) EPR spectroscopy of etoposide phenoxyl radicals produced by oxidation of etoposide was performed at 25 ºC under the following conditions: 3,350 G center field; 50 G sweep width, 0.5 G field modulation; 10 mW microwave power; 0.1 s time constant; 2,000 receiver gain; and 4 min time scan. Different amounts of Syn were incubated with 5 µM cyt c, liposomes (TOCL/DOPC ratio 1:1; TOCL/ cyt c ratio 25/1), 100 µM etoposide, and 100 µM H2O2. Low-temperature EPR measurements of protein-derived (tyrosyl) radicals. Cyt c (80 µM) was incubated with DOPC-TOCL liposomes (DOPC:TOCL=1:1; 4 mM total lipid) and Syn for 5 min at room temperature in 25 mM HEPES-Na buffer, pH 7.4, 100 µM DTPA (N2-conditions); then H2O2 (800 µM) was added. The reaction was stopped after 5 s by freezing the samples in liquid nitrogen. The EPR spectra were recorded at 77K under the following conditions: 3230 G, centered field; 100 G, sweep width; 5 G, field modulation; 5 mW, microwave power; 0.1 s, time constant; and 2 min, time scan. Dependence of relative magnitude (percentage of the maximal magnitude) of EPR signals of tyrosyl radicals on square root from microwave power (in milliwatts) was presented as saturation curves. The spin-lattice relaxation time was determined by fitting the experimental curve of radical signal saturation to the theoretical one as described previously (15).

Fluorescence spectroscopy analysis of dityrosine adducts. Samples were digested with proteinase K (20 µg/ml) at 37 ºC overnight. Digested samples were precipitated with cold perchloric acid (final concentration 0.53 N). Samples were allowed to stand 10 min on ice bath and centrifuged for 15 min at 3000g. After neutralization with 2 N KOH, precipitate of potassium perchlorate crystals was discarded by centrifugation (15 min at 3000g). Supernatants were diluted with 0.2 M HEPES (pH 9.0) and fluorescence was measured (λ exc 315, λ emission 420 nm).

Assessment of Syn binding to CL/cyt c using NBD-CL fluorescence. CL/cyt c complexes were formed by incubation of DOPC/CL liposomes (5 µM total lipids, including 1 µM CL and 0.05 µM 1,1',2-triroleoyl-2'-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-cardiolipin (NBD-CL, custom-synthesized by Avanti Polar Lipids, Alabaster, AL) and cyt c (0.05 µM) for 2min. NBD-CL fluorescence spectra were recorded in the range of 500-650 nm (excitation wavelength of 480 nm, slits 10 and 10 nm) using a Shimadzu F5301-PC spectrofluorometer. NBD-fluorescence was monitored 2 min after addition of Syn aliquots (0.25 µM each).

Mass spectrometry(MS) analysis of Syn/cyt c covalent cross-links. After SDS gel electrophoresis, proteins were visualized by Coomassie staining and bands corresponding to high molecular weight Syn/cyt c complexes were excised. Destaining was achieved by several washes with 25mM NH4CO3/50% CH3CN. Gel pieces were reduced in the presence of dithiothreitol (DTT) followed by alkylation with iodoacetamide. Gel pieces were washed, dehydrated and dried. The complexes were subjected first to overnight in-gel digestion with trypsin (25ng/ul in 25 mM ammonium bicarbonate buffer, pH 7.8) followed by overnight digestion with endoproteinase-Glu-C (25 ng/ul in the same buffer). Peptides were extracted with 5% formic acid/50% acetonitrile and evaporated to near dryness. Matrix Assisted Laser Desorption/Ionization Time-of -Flight (MALDI-TOF) mass spectrometry was performed on a on a Bruker Ultraflex mass spectrometer in positive reflector mode (20kV) with a matrix of alpha-cyano-4-hydroxycinnamic acid (CHCA, Sigma). At least 500 laser shots were averaged to get each spectrum. Masses were calibrated to known peptide standards (purchased from Applied Biosystems) on the same day of analysis. 30ul aliquots of each of the digests were acidified with 1.5 ul of 5% trifluoroacetic acid (TFA, Sigma), and then taken-up into a C18 ZipTip (Millipore) that had been prepared as per manufacturer’s instructions. The bound peptides were desalted with two 15ul washes of
0.1%TFA and then eluted with 2ul of aqueous, acidic acetonitrile (67%CH₃CN, 0.1%TFA). The elutant was mixed with 1ul freshly prepared CHCA stock solution (20mg/ml CHCA in aqueous acetonitrile as above), and 1ul portions of this mixture were spotted onto a MALDI sample plate for air drying. Potential cross-linked candidates identified by the MALDI run were subjected to sequence analysis by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS). LC-ESI-MS was performed on a Micromass triple quadrupole mass spectrometer (Waters, Inc.). A microcapillary column (10cm X 75 micron i.d.) was packed in-house using 5 micron C18 particles (PerSeptive Biosystems, Inc.). Flow rates were generated with a Rainin high performance liquid chromatography (HPLC) system equipped with an LC-Packings microflow processor and maintained at 180 nl/min. The Syn/cyt c tryptic/Glu-C digest fragments were loaded onto the microcapillary column, washed in 0.1% acetic acid in water (buffer A) and eluted with a linear gradient of acetonitrile containing 0.1% acetic acid (buffer B) over 30 minutes. Fragmentation of potential cross-linked species identified by LC-ESI-MS was performed using a collision energy ramp and Ar as the collision gas.

**CL/Syn binding constant assessments.**

DOPC/CL-liposomes (5 nmol total lipids, 1:1) were incubated with Syn (100 pmol) for 15 min. The mixture was then incubated in the presence of acridine 10-nonyl bromide (NAO, 2-10 nmol) in 20 mM HEPES buffer (pH=7.4) for 30 min at room temperature. Samples (10 µl) were applied to 7.5% PAGE and electrophoresis was performed (1 hr 120 V). Unbound Syn was stained with SilverSNAP Stain Kit from Pierce (Rockford, IL) and quantified by optical density. CL/Syn binding constants were calculated using the following equation:

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\frac{[\text{NAO}]}{[\alpha-\text{synuclein}]}_{\text{free}} = \frac{K_{\text{Lipid--α-synuclein}}}{K_{\text{Lipid--NAO}}},
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where \(K_{\text{Lipid--NAO}} = 2 \times 10^6 \text{ M}^{-1}\) for CL (16).

**Quantification and mass spectrometric analysis of CL hydroperoxides.** TLCL and its oxidized species were extracted from the incubation medium by Folch procedure (17) and evaporated under N₂. DOPC/TLCL liposomes (250 µM at a ratio of 1:1) were incubated with cyt c (5 µM) and H₂O₂ (100 µM were added every 15 min during incubation) in phosphate buffered saline (PBS) (pH 7.4 + 100 µM DTPA)) in the absence and presence of Syn (15 µM). TLCL hydroperoxides were determined by fluorescence HPLC of products formed in microperoxidase 11 (MP)-catalyzed reaction with a fluorogenic substrate, Amplex Red, N-acetyl-3,7-dihydroxyphenoxazone (Molecular Probes, Eugene, OR) as described previously (12,18). For ESI-MS analysis, TLCL and its oxidation products were re-suspended in chloroform:methanol 1:2 v/v (20 pmol/µl) and analyzed by direct infusion (flow rate of 5 µl/min) into a quadrupole linear ion trap mass spectrometer LXQ (Termo Electron, San Jose, CA) The electrospray probe was operated at a voltage differential of 5 kV in the negative ion mode. Source temperature was maintained at 150°C.

**Cell free apoptosis system (S-100 system).**

Human HL-60 cells were grown in RPMI-1640 supplemented with 15% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin sulfate. The cytosol extracts (S-100) was obtained as previously described (19) with minor modification. Briefly, the cells were washed twice in cold phosphate-buffered saline, pH 7.4, the resulting pellet was resuspended in buffer containing 25mM HEPES-KOH, pH 7.0, 10 mM KCl, 1.5mM MgCl₂, 1 mM EDTA, 1mM ethylene glycol tetraacetic acid (EGTA), 1mM DTT, 0.1mM phenylmethylsulfonyl fluoride, 0.05% digitonin and 1% protease inhibitor cocktail (Sigma) for 2 min at 4°C. Cells were centrifuged at 4°C for 10 min at 10,000 × g. The resulting supernatant was further centrifuged at 4°C for 50 min at 100,000 × g. The supernatant was collected as S-100 and kept at -80°C till further use. For caspase-3 activation, S-100 (5 µg/µL) were incubated with 1 mM deoxyadenosine triphosphate (dATP) and 1 µM cyt c for 90 min at 37°C, and caspase-3 activity was normalized as 100%. Syn (13 µM) was added alone or in complex with TOCL (at a ratio of 3:1). The caspase 3 activity was evaluated as described in the manufacturer’s manual (Invitrogen, Enzchek Caspase-3 assay kit).

**Rat rotenone model of Parkinson’s disease.** All animal use was in accordance with National...
Institutes of Health guidelines and was approved by the Pittsburgh University Institutional Animal Care and Use Committee. Surgeries were performed as described previously (20). Briefly, male Lewis rats (300–350 g) received 3.0 mg/kg/day rotenone for up to 4 weeks through subcutaneous osmotic mini-pumps (Alzet Corporation, Palo Alto, CA). Control rats received vehicle (dimethyl sulfoxide (DMSO): polyethylene glycol (PEG), 1:1). Rotenone-infused rats were euthanized at the time of severe systemic illness characterized by rigidity and akinesia that prevented adequate feeding and grooming. Control rats were euthanized at similar time intervals. Rats were euthanized using isoflurane (Abbott Laboratories, Chicago, IL). The brains were removed without prior perfusion and dissected midsagitally into 2 hemispheres. One hemisphere was immersion fixed in 4% paraformaldehyde in phosphate buffer and used for immunochemistry. From the other hemisphere, specific brain regions were dissected and immediately frozen in dry ice and stored at -80°C.

**Fluorescence Resonance Energy Transfer (FRET) protocol.** Co-localization (molecular proximity) of cyt c and Syn in histological preparations was assessed by analysis of FRET between fluorescently-labeled specific antibodies. Because FRET efficiency strongly depends on the distance between the fluorophores, increased FRET levels reflect the close proximity of the antibodies and, therefore, of the proteins of interest (reviewed in (21)). Primary antibodies were directly labeled using the DyLight 549 and DyLight 649 antibody labeling kits (ThermoFisher, Rockford, IL). Lyophilized primary antibodies were resuspended in 0.05 M borate buffer, pH 8.5, to a final concentration of 1 mg/ml; 100 µg were used for labeling. The dyes were reconstituted in 20 µL of dimethylformamide; 8 µL of DyLight 549 or 5 µL of DyLight 649 was added to the antibody solution and the mixture was incubated for 45 minutes at room temperature. The labeled antibodies were purified and the excess dye was removed by processing the solution with a Micro Bio-Spin chromatography column 6 (Biorad, Hercules, CA) according to the manufacturer’s instructions. Thus modified antibody (in 10 mM TrisHCl buffer, pH 7.4) was used for treatment of tissue sections obtained according to standard histological procedures. Images were acquired using a laser scanning confocal microscope (Fluoview1000, Olympus) equipped with spectral detector technology, which provides accurate wavelength separation of the emitted light. FRET was detected by reading the acceptor emission (DyLight 649-conjugated primary antibody) at 674 nm while exciting the donor (DyLight 549-conjugated primary antibody) with the 543 nm laser.

**Immunostaining of Syn and cyt c in cells.** Paraformaldehyde (4%)-fixed cytospin preparations were permeabilized with 0.2% Triton X-100. Cyt c was stained with mouse anti-cyt c antibody (Pharmingen, 1:100) followed by FITC conjugated goat anti-mouse IgG (Pharmingen, 1:100), which exhibits a green fluorescence. Syn was stained with rabbit anti-Syn antibody (Chemicon International, 1:200) followed by Texas Red conjugated sheep anti-rabbit IgG (GeneTex, 1:200), which shows red fluorescence.

**Immunostaining of Syn and cyt c in human and rat brains.** Double immunofluorescence analysis was performed as previously described (22). In brief, de-waxed substantia nigra sections from PD/LB disease (LBD) cases were heated in target retrieval solution at 95°C for 30min, blocked with Immunon protein blocking agent (Shandon, Pittsburgh, PA), and incubated at 4°C overnight with rabbit anti-cyt c (1:50, Cell signaling Technology, Beverly, MA) and mouse anti-α-Syn (1:1500, Zymed Laboratories). The sections were then incubated with Alexa Fluoro 488 donkey anti-mouse and Cy3- conjugated donkey anti-rabbit IgG (1:500, Jackson ImmunoResearch, West Grove, PA). Nuclei were counterstained with DAPI. To minimize bleed through from the green into the red channel, images are always collected sequentially. For negative controls, primary antibody was replaced with nonimmune rabbit or mouse IgG. Sections were visualized using Olympus IX 71 fluorescent microscope.

**Statistical analysis.** The results are presented as either mean ± SD or mean ± SEM values from at least three experiments, and statistical analyses was performed by paired Student’s t-test unless specified otherwise. The statistical significance of differences was set at P< 0.05.
RESULTS

Syn cross-links cyt $c$ in a peroxidase process requiring anionic phospholipids.

To explore the possibility of peroxidase catalyzed cross-linking of Syn with cyt $c$, we utilized a model system that included Syn, cyt $c$ and an anionic phospholipid. Given the high affinity of cyt $c$ for CL, we initially used CL as a prototypic anionic phospholipid. Because Syn can also bind lipids, we performed estimates of binding capacity of Syn towards CL. Migration of Syn as a single band was changed to a smeared profile in the presence of CL (Fig 1a).

Addition of NAO, as a competitive complexer of CL, reinstated the single band of Syn at NAO:CL ratios >1:2. Based on these estimates, the binding constant for Syn with TOCL is on the order of $8.5 \pm 1.0 \times 10^6$ M$^{-1}$ for non-fibrillated Syn. Previous studies demonstrated that, upon binding with acidic phospholipids, Syn undergoes structural rearrangements toward alpha-helical conformation (22). For PS, the high affinity binding with Syn was stimulated by its homo-oligomerization and the formation of (proto)fibrils (23,24). Therefore, we assessed binding constant for CL with fibrillated (aged) Syn that has been prepared according to Smith, et al (23). We found that aged Syn had a significantly higher affinity for CL than non-fibrillated Syn ($1.8 \pm 0.5 \times 10^7$ M$^{-1}$, $p<0.02$ vs non-fibrillated Syn). These results correspond with previous reports showing that protofibrils and fibrils of Syn interact with lipid membranes more effectively than the soluble monomeric protein (23-25). These data indicate that Syn oligomers with bound CL may act as substrates for peroxidase activity of cyt $c$ (see below).

To test whether PD-associated mutants of Syn – A30P and A53T – were also able to form hetero-oligomeric complexes with cyt $c$, we performed native PAGE with subsequent silver staining. We utilized conditions that are compatible with the expression of sufficiently high peroxidase activity of cyt $c$/TOCL complexes (1:20 mol/mol). We found that at Syn:cyt $c$ ratio of 1:1, the affinity of wild-type Syn for binding with cyt $c$/TOCL was markedly higher than for both Syn mutants (A30P and A53T). This was documented by almost complete disappearance of Syn monomer due to its oligomerization with cyt $c$/TOCL for the wild-type protein and similar but only partial decrease of the Syn monomeric forms for both mutants (Fig. 2a left panel). In order to quantitatively assess the formation of heterooligomeric complexes of cyt $c$/TOCL/Syn across different Syn concentrations, we used fluorescently labeled CL, NBD-CL. The assay is based on the ability of cyt $c$ to quench fluorescence of NBD-CL (Fig. 2a right panel, insert), likely due to the proximity of the NBD-chromophore to the heme moiety of cyt $c$ (26).

Competition between cyt $c$ and Syn for CL binding – upon addition of Syn - diminishes the quenching effect resulting in the increased fluorescence intensity (Fig. 2a right panel, insert). We found that wild-type Syn was more effective in reconstituting the NBD-fluorescence than both of the Syn mutants (A53T, A30P) (Fig. 2 insert, spectra 2 and 3, respectively). When the fluorescence intensity differences were plotted against several concentrations of added Syn (Fig 2a, right panel), one can see that both mutant forms of Syn were less potent as fluorescence enhancers than the wild-type protein.

We then studied peroxidase reactions and cross-linking of Syn/CL/cyt $c$ complexes. SDS-PAGE analyses and staining with anti-cyt $c$ and anti-Syn antibodies showed that different hetero-oligomers of Syn and cyt $c$, as well as very high molecular weight aggregates were produced by the H$_2$O$_2$-mediated cyt $c$-catalyzed cross-linking of proteins (Fig. 1b). Reducing conditions (treatment with 20 mM DTT) did not affect the accumulation of oligomerized protein aggregates (data not shown). Because SDS can mimic and interfere with the interactions of anionic phospholipids with Syn and cyt $c$, we performed native agarose gel electrophoresis. This revealed that the covalent hetero-oligomerization of Syn and cyt $c$ was induced by H$_2$O$_2$ in the presence of CL (Fig. 1c). All four components – Syn, cyt $c$, CL and H$_2$O$_2$ – were required for the formation of cross-linked polymers. Even in the presence of large excess of Syn (Syn:cyt $c$ ratio of 10:1), oligomerization did not occur without TOCL and H$_2$O$_2$.

In the presence of H$_2$O$_2$ and TOCL, wild-type Syn, A30P and A53T mutants
underwent hetero-oligomeric covalent cross-linking with cyt c (at different Syn: cyt c ratios, from 1:5 to 40:1) in a similar way as assessed by Western blot analysis and staining with anti-Syn antibodies (Fig 2b). Moreover, we observed that in the presence of cyt c, TOCL and H2O2, both intact and fibrillated Syn underwent covalent cross-linking in a similar way (Fig. 2c). The lack of significant difference in covalent conjugation with cyt c and CL between fibrillated and non-fibrillated Syn was observed not only with wild-type Syn but also with its mutants, A53T and A30P.

While the presence of an anionic phospholipid was critical for the hetero-oligomerization of Syn and cyt c, the requirement in CL was not absolute as other negatively charged phospholipids such as phosphatidic acid (PA), phosphatidylinositol (PI), and PS were effective in supporting the hetero-oligomerization as well (Fig. 1d). However, a non-charged PC was insufficient for the stimulation of protein hetero-oligimerization (Fig. 1d).

**Syn is a preferred substrate for reactive intermediates of the peroxidase complex.**

To test whether reactive intermediates of peroxidase activity of cyt c in its complexes with Syn and CL are essential for protein oxidative hetero-oligomerization, we employed immunospin trapping(27) and EPR techniques. A spin trap, DMPO, has been shown to form spin adducts with protein radicals generated during the peroxidase activation of cyt c by H2O2 (28). Utilizing anti-DMPO antibodies, we confirmed the H2O2-dependent production of protein-derived radicals by both non-oligomerized as well as by oligomerized complexes of Syn/CL/cyt c (Fig. 3a). Importantly, interaction of DMPO with protein radicals effectively inhibited hetero-oligomerization in a concentration-dependent manner (Fig. 3a) corroborating the involvement of the peroxidase mechanism in hetero-oligomerization.

EPR spectroscopy revealed a characteristic broad (half-width 15.5 G) signal of protein immobilized radicals (with g factor of 2.005) that have been tentatively ascribed to Tyr• radicals generated by cyt c/CL complexes treated with H2O2 (12,29). The signal was also detectable in the presence of Syn (Fig. 3b, left panel). As a participant of the peroxidase reaction leading to hetero-oligimerization, Syn is expected to interact with the cyt c protein radicals, likely resulting in quenching of these radicals due to the formation of Tyr-Tyr bonds. Indeed, increasing the ratio of Syn to cyt c/CL caused a significant quenching of the signal such that half-maximal magnitude of the signal was achieved at a ratio of 0.4:1. This is compatible with the expected central role of cyt c in generating the radical intermediates and relatively “passive” substrate participation of Syn leading to Tyr-Tyr hetero-oligomerization of the latter in a triple complex of cyt c/TOCL/Syn. More detailed studies of power saturation dependence of the signal demonstrated its relatively fast saturability in the cyt c/TOCL system (Fig 3b, right panel). Based on the comparable data in the literature, one can assume that the saturation course is indicative of a spin-lattice relaxation time of tyrosyl radical $T_1=(1.3\pm0.3)\times10^{-5}$s (30,31). The proximity of Tyr radicals to the heme iron moiety was unaffected by Syn based on a very similar saturation pattern of the radical in the triple system cyt c/TOCL/Syn exposed to H2O2.

To further characterize formation of dityrosine cross-links in Syn/cyt c/TOCL hetero-oligomeric complexes in the presence of H2O2, we performed i) fluorescence spectroscopy, ii) Western blotting with anti-dityrosine antibody, and iii) MS analysis. Measurements of characteristic dityrosine fluorescence demonstrated a 1.6-fold increase in dityrosine adducts upon addition of H2O2 to cyt c/TOCL (Fig. 3c). When Syn was present in this incubation system along with H2O2, the fluorescence intensity was further increased by 3.2-fold.

Western blot analysis with anti-dityrosine antibody showed a strong positive signal in cross-linked high molecular weight aggregates (non-dissociable in SDS) which were weaker in the absence of Syn or H2O2 (Fig. 3d). Syn enhanced the formation of dityrosine-based covalent cross-links – in line with measurements of di-tyrosine fluorescence (Fig. 3c). These results are also in agreement with above data showing significant quenching of the characteristic EPR signal of H2O2-induced Tyr• radicals of cyt c/CL by Syn.
Finally, MS-analysis of covalent aggregates further confirmed the formation of cyt c/Syn cross-links as a result of recombination of protein Tyr• radicals. After SDS gel electrophoresis, bands corresponding to potential high molecular weight Syn/cyt c aggregates were cut from the Coomassie-stained gel and subjected to an in-gel digest with trypsin at 37 °C for 24h followed by digestion with edoproteinase Glu-C for an additional 24h. The MALDI-TOF profile of the multi-enzyme in-gel digest included fragments from both Syn as well as cyt c which included one species of m/z 1450 whose m/z matched that of a potential Syn/cyt c cross-link (not shown). This species was further analyzed by LC-ESI-MS. Fragmentation of a proteolytic fragment with m/z 725.8 documented the cross-link involving Y133 of Syn (GYQDYE) and Y74 of cyt c (YIPGTK). This cross-link preferred to run as the doubly-charged species (m/z 725.8) as opposed to the parent molecular ion [(M+H)+, m/z 1450] under our instrumental conditions (not shown). Fragmentation of the doubly-charged species revealed ions with m/z 468, 495, 625, 730 and 896 corresponding to GY/YI (without CO), GY/YI, GYQD/Y, YQDY/Y and GY/YIPGTK, respectively (Fig. 3e). Since these fragments are all singly-charged species, it is not unusual to detect ions with masses above that of the doubly-charged parent ions.

To further prove the involvement of the peroxidase mechanism in protein aggregation, we assessed the effects of three prototypical peroxidase phenolic substrates – DA, Amplex Red and etoposide. Consistently, all three compounds caused concentration-dependent inhibition of protein hetero-oligomerization (Fig. 4a and b). Conversely, Syn displayed a concentration-dependent inhibition of the peroxidase activity of the complexes with the phenolic peroxidase substrates (Fig. 4b). Very low micromolar concentrations of Syn were sufficient to inhibit the peroxidase activity of cyt c/CL complexes. With Amplex Red, the IC50 for inhibition by Syn was 0.2 µM in the presence of two orders of magnitude higher concentrations of Amplex Red (50 µM). Low micromolar Syn concentrations were also effective in inhibiting the peroxidase activity in the presence of two orders of magnitude higher concentrations of DA or etoposide (Fig. 4b). Staining of native agarose gels for the peroxidase activity - using West Pico chemiluminescence reagent as a substrate - demonstrated that the high activity detectable in cyt c/CL complexes, was markedly diminished in the presence of Syn (Fig. 4c). Further, PD-associated Syn mutants - A30P, A53T and A53T/A30P – were tested for their peroxidase activity. We found that in the presence of Syn mutants (A30P and A53T/A30P), the peroxidase activity of cyt c/TOCL complexes was higher than in the presence of wild type Syn. Combined, these data suggests that Syn acted as a preferred substrate for the peroxidase activity of cyt c/CL complexes.

To determine whether the important peroxidase role of Syn is realized with another physiologically relevant substrate, we monitored oxidation of polyunsaturated TLCL by cyt c in the presence and absence of Syn. Using MS-analysis and fluorescence-HPLC we found that Syn was able to protect TLCL against oxidation (Fig. 4d). Expectedly, oxidation of Syn by cyt c/TCL was accompanied by accumulation of characteristic covalent aggregates detectable on SDS-PAGE gels (Fig. 4d, inset).

**Syn and Syn/CL complex inhibits apoptotic caspase activation.**

We reasoned that the covalent binding of cyt c through its Syn/CL-mediated peroxidase cross-linking should effectively decrease the amounts of cyt c required for its participation in apoptosome formation and caspase activation. To experimentally address this, we employed a well characterized pro-apoptotic cell free system composed of S100 fraction (obtained from human leukemia HL-60 cells lacking endogenous Syn) and activated apoptosis by cyt c/dATP (19). We demonstrated that, in the presence of CL, exogenously added Syn indeed was able to significantly suppress activation of caspase 3/7. Pre-incubation of cyt c/Syn/CL complexes with H2O2 completely abolished the pro-apoptotic effects of cyt c towards caspase-3 activation in S100 fraction (Fig. 5a). We found that wild-type Syn and two mutants, A53T and A30P, displayed equal inhibitory effects against caspase-3 activation after pre-incubation with cyt c/TOCL/H2O2. This suggests that covalent hetero-oligomeric cross-links produced by
peroxidase activity of cyt c with wild-type Syn as well as A30P and A53T mutants under conditions of oxidative stress result in equally effective inhibition of cyt c’s interactions with Apaf-1 and caspase activation. These results correspond with our data obtained by PAGE demonstrating similar effectiveness of wild-type Syn and its A30P and A53T mutants in H2O2-dependent formation of covalent cross-links shown in Fig.2b.

To further test whether Syn is able to protect cells against apoptosis we used HeLa cells containing Syn and knocked down its expression to less than 10% of its content in wild-type cells by siRNA protocol to generate clones HeLa A5 and A7 (Fig 5b). We then compared cell viability and apoptotic responses of HeLa cells and HeLa A5 and A7 cells to DA (Fig. 5c). Cell viability was decreased and two biomarkers of apoptosis - PS externalization and caspase 3/7 activation - were increased in Syn-deficient HeLa cells incubated for 24 h in the presence of DA (400 µM). Furthermore, the resistance of cells to apoptosis induced by DA was proportional to the levels of Syn expression. These results indicate that Syn acts as a protector against apoptosis and its deficiency is associated with increased sensitivity to DA-induced apoptosis.

In a separate series of experiments, we used a reverse approach and delivered Syn into MECs, naturally expressing very low levels of Syn. The efficiency of Syn delivery was confirmed by immunohistochemistry. Western blot analysis demonstrated a three-fold increase in Syn content compared to controls. In MEC, standard challenge with a pro-apoptotic agent, ActD, caused a robust activation of caspases 3/7 (Fig. 5d). Significantly lower caspase activation was found in MECs with delivered Syn. Delivery of a non-specific protein, green fluorescent protein (GFP), exerted no effect on ActD-induced caspase 3/7 activation in MECs. Thus both in a cell free system and in two types of cells, Syn afforded protection against cell death induced by different pro-apoptotic stimuli. Notably, Machida et al., (32) have demonstrated that increasing expression levels of Syn enhanced resistance to death promoting stimuli in SH-SY5Y cells.

**Syn and cyt c co-localize after pro-apoptotic stimulation in cells.**

In LBs, Syn has been reported to be co-localized with cyt c (8). If cyt c co-aggregation with Syn is essential for its anti-apoptotic action, the production of aggregates with co-localized Syn and cyt c is expected to occur in cells triggered to apoptosis. We utilized two different pro-apoptotic stimuli, a non-oxidant ActD and a pro-oxidant, t-BuOOH, to initiate apoptosis in Syn-containing HeLa cells. Pro-apoptotic stimulation with ActD resulted in the appearance of cells containing inclusions with co-localized cyt c and Syn (Fig. 6a). These inclusions were not detectable in cyt c deficient HeLa cells (HeLa 1.2) engineered (using siRNA protocol) to express 14% of their constitutive levels of cyt c (Fig 6a). Importantly, we were able to detect t-BuOOH-induced formation of Syn/cyt c aggregates in HeLa cells using Western blot analyses after electrophoresis in native agarose gel or SDS-PAGE (Fig. 6b). The presence and co-localization of Syn/cyt c hetero-oligomers in tBuOOH-treated cells was confirmed by immuno-precipitation with anti-Syn antibodies (Fig. 6c). Recent reports indicate that Syn can translocate to and form aggregates in mitochondria (33). Given that cyt c is one of the abundant proteins in the inter-membrane space of mitochondria, we reasoned that the peroxidase activity of cyt c/Syn/CL complexes could initiate the hetero-oligomerization. Indeed, we detected the accumulation of co-localized Syn/cyt c hetero-oligomers in mitochondria isolated from HeLa cells pre-treated with t-BuOOH (Fig. 6d).

Dopaminergic SH-SY5Y cells triggered to apoptosis by either ActD or t-BuOOH (as evidenced by 28.1 ± 7.4% and 36.9 ± 9.7% of cells with externalized PS by Annexin V binding) elicited inclusions in which Syn and cyt c were co-localized (Fig. 6e). Thus, pro-oxidant stimulation or pro-apoptotic stimulation accompanied by the production of reactive oxygen species (ROS) resulted in the formation of Syn/cyt c covalent aggregates.

**Syn co-localizes with cyt c in LB and LN in vivo.**

We used a well-established rotenone rat model of PD *in vivo* and followed cyt c and Syn
distribution in the midbrain. After the rotenone treatment, cyt c distribution changed from punctate (presumably mitochondrial) to apparent aggregates that co-localized with Syn in dopaminergic substantia nigra neurons (Fig. 7). Moreover, using FRET protocol we were able to prove that Syn and cyt c are within molecular proximity to each other in LNs (Fig. 7b). This strongly supports our hypothesis on the closeness and molecular availability of Syn and cyt c for peroxidase-catalyzed cross-linking.

To determine the extent to which Syn in synaptic terminals is capable of preventing retrograde expansion of apoptotic events to soma of neuronal cells in humans, we analyzed co-localization of Syn and cyt c in midbrain sections from Parkinson's/Lewy body disease patients. Demographic characteristics of patients are presented in Supplementary Table 1. By definition, control patient brains lacked Syn aggregates and were not used further for co-localization scoring. Case 1 and Case 2 showed classic PD presentation clinically, with late development of dementia prior to death (PDD). Case 3 had symptoms of "incipient dementia" within a year of clinical diagnosis of Parkinson's disease, and is classified as "pure" diffuse LB disease (DLB) in accord with the recommendations of the Third Consortium on Diffuse Lewy Body Disease. The neuropathology and clinical features of PDD and DLB are indistinguishable aside from the temporal sequence of symptoms (34). Case 4 represented a DLB patient with concurrent Alzheimer's type neuropathology. Using these samples, we confirmed the co-localization of Syn and cyt c (in approximately half of Syn immunoreactive structures) and found that it was 2-fold more frequent in LNs than in the soma (Fig. 8). This suggests that Syn acts as an anti-apoptotic scavenger of cyt c likely preventing undesirable spread of apoptotic signals from neuritic processes and terminals to the soma (35).

**DISCUSSION**

Oxidative stress has long been associated with the development of PD and accompanying LB formation (36). In addition to non-covalent aggregation of partially unfolded Syn molecules, covalent cross-linking and accumulation of granular material is typical of (dopaminergic) neurons of PD patients (37). While DA oxidation products and their adducts with Syn have been suggested to contribute to fibril formation, specific mechanisms translating oxidative stress into covalent cross-links and their role in pathogenesis of the disease, have not been well understood (38). Hashimoto et al. (8), has suggested that cyt c may be involved in H2O2-dependent Syn aggregation implying contribution of a peroxidase-type mechanism. However, cyt c is a poor peroxidase (39). Therefore, aggregation of Syn by cyt c required long-term incubations and high concentrations of cyt c/H2O2 to cause the aggregation (8). Thus specific catalytic mechanisms of cyt c-driven oxidative cross-linking of Syn have not been identified.

Mitochondrial impairment plays a key role in PD and its models and several mutations that cause PD are associated with mitochondrial abnormalities (40,41). Systemic deficits in mitochondrial complex I activity have been reported in PD patients (42). Evidence of mitochondrial oxidative stress and cyt c release are also observed in animal and cell culture models of PD (20,43-46). Yet, despite abundant evidence of mitochondrial alterations (47-49), classic morphologic changes of apoptosis are disproportionately rare (50), suggesting the possibility of additional mechanisms in dopaminergic neurons that serve to limit pro-apoptotic effects of mitochondrial damage.

Our results on anionic lipid dependent conversion of cyt c into a peroxidase (12) along with the data on anionic phospholipid binding capacity of Syn (5) prompted us to hypothesize that a triple complex of Syn/cyt c/anionic phospholipid can act as a peroxidase. Previous work has demonstrated that oxidative stress-mediated damage of mitochondria can trigger pro-apoptotic events in synaptic terminals likely associated with synaptic remodeling and plasticity (35). Here we present evidence that the peroxidase activity of this complex utilizes Syn as a peroxidase substrate resulting in oxidative catalytic aggregation of cyt c and Syn. In this way, Syn acts as a scavenger of the key-pro-apoptotic factor, cyt c, resulting in its covalent association with Syn.
There could be at least two possible ways through which phospholipids induce Syn/cyt c interactions. Tightly bound to the mitochondrial membranes, CL undergoes cyt c-catalyzed oxidation during apoptosis (12); oxidized CL, particularly when two or more of its fatty acid residues are oxidatively modified, has a significantly lower hydrophobicity, potentially resulting in the departure of cyt c/CL complexes from mitochondrial membranes. In this scenario, Syn scavenging of cyt c/CL complexes requires the presence of CL to mediate Syn/cyt c interaction. Alternatively, cytosolic Syn can be associated with one of anionic phospholipids (PI, PS, PA, free fatty acids) and is primed by this interaction to promptly bind cyt c. Clearly, both of these mechanisms may be realized in some proportions simultaneously. Previous reports showed that protofibrils and fibrils of Syn interact with lipid membranes more effectively than the soluble monomeric protein (23-25). In agreement with this, our data demonstrate that aged Syn has a significantly higher affinity for CL than intact Syn indicating that Syn oligomers with bound CL may act as substrates for peroxidase activity of cyt c. The presence of beta sheets in fibrillated Syn may facilitate hydrophobic interactions with cyt c. It has been shown that, after interaction with CL, cyt c forms fibrils containing beta-sheets (51).

Oxidative stress-dependent production of H$_2$O$_2$ acts as a source of oxidizing equivalents for the activation of the peroxidase complex resulting in cross-linking of Syn and cyt c and the formation of their high molecular weight aggregates. The peroxidase activity of triple complexes formed in the presence of cyt c, TOCL and Syn mutants - A30P and A53T/A30P – was higher than in the presence of wild type Syn. The significance of this for the PD process is that peroxidase activity of cyt c/CL/Syn complexes may act as a persistent source of oxidative stress. The low level of this activity compared to cyt c/CL alone defines a slow oxidative stress “regimen” and is compatible with the known pro-disease role of synuclein.

In separate experiments, we utilized three other proteins - lactoglobulin (not present in LBs), 14-3-3 (known to be in included in LBs) and β-amyloid-peptide (Abeta 1-42) - and tested their ability to undergo heterooligomerization catalyzed by cyt c/CL complex. We found that, similarly to Syn, the peroxidase activity of the complex effectively cross-linked 14-3-3 (data not shown) and Abeta-peptide (see Supplementary Fig. 1). In contrast, lactoglobulin incubated with cyt c/CL in the presence of H$_2$O$_2$ was not polymerized (data not shown). Heterooligomerization of cyt c with anionic lipid-binding proteins and formation of triple peroxidase complexes may not be uniquely selective towards Syn. Other proteins, particularly those with significant binding affinity towards CL may get involved in cyt c-driven covalent cross-linking. The likelihood of the cross-linking should be to a large extent dependent on the concentration and affinity of a given protein and its molecular proximity to CL and cyt c. While Syn is the major component of LBs, Abeta is the main structural component of senile plaques of Alzheimer’s disease (52). However, it is also possible that some specificity can be observed (eg, due to the lack of reactive and accessible Tyr residues in a protein). Notably, the presence of Tyr residues is essential for aggregation of Syn and LB formation as well as for its involvement in cross-linking through a catalytic peroxidase process (53), in line with our findings on protein-derived (Tyr•) radicals in oligomers and high molecular weight aggregates.

As one of the most abundant proteins in pre-synaptic terminals (54), Syn, in the presence of anionic phospholipids, can scavenge cyt c, and hence, prevent high affinity binding of the latter with Apaf-1 (55). This suggests a mechanism by which cyt c can be eliminated from its potential involvement in the execution of apoptotic caspase activation. Syn has been reported to play a beneficial role in preventing neuronal death (56). For example, forced expression of Syn diminished apoptotic response in dopaminergic cells (32). We demonstrated that siRNA knock-down of cyt c in HeLa cells with high endogenous levels of Syn essentially eliminated protein oligomerization. Reportedly, anti-apoptotic effects of Syn can also be realized through its interactions with other pro-apoptotic proteins - PKCdelta and BAD - as has been demonstrated by co-immunoprecipitation studies in mesencephalic dopaminergic neuronal cells.
challenged with 1-methyl-4-phenylpyridinium (MPP+) (57).

There is a significant body of literature supporting the notion that expression of normal Syn is essential for survival of neuronal cells. A recent communication (58) states, based on antisense oligonucleotide Syn silencing, that the protein has an essential pro-survival role towards primary neurons. Previous work has demonstrated that wild-type, but not mutated Syn, delayed death of neuronal cells after serum withdrawal (59), and protected cells by preventing activation of cell death-inducing proteins (caspases, p53, Bad) (60,61). An anticonvulsant drug, valproic acid, protected cerebellar granule cells from glutamatergic excitotoxicity via induction of Syn (62). However, this hypothesis may be in disagreement with several other important reports demonstrating that elevated levels of Syn lead to increased likelihood of PD (63-65), while experimental deletion of Syn is protective against standard models such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (66,67).

We believe that a beneficial role for protein expressed at normal levels does not translate into the assumption that more must be better. Indeed, there are many precedents for overactivation of adaptive responses proving to be harmful. Many biological phenomena show bell shaped curves, such that either too little or too much is harmful. Our proposal that a normal role for Syn includes sequestration of cyt c in LB-like aggregates does not at all contradict with observations that mutation or overexpression due to gene multiplication leads to human disease. Indeed, the data in our paper offer an explanation as one of the possible mechanisms for this type of trade-off - while apoptosis is prevented, increased peroxidase activity and/or excessive aggregation may interfere with cellular health if not effectively cleared. In agreement with this interpretation, wild-type Syn was shown to protect neurons from apoptosis by inhibiting caspase-3 (68). Aggregation of wild-type and PD-related mutated Syn was reported to be associated with enhanced cell death (69). This process could be caused by the loss of an anti-apoptotic function. The latter possibility is supported by the evidence that Syn lowers p53-dependent apoptotic response of neurons (68) and activates MAPK (mitogen-activated protein kinase) survival pathways (70). Although a number of studies have focused on toxic gain-of-function effects of Syn in PD, such as its aggregation, other dominantly inherited diseases such as spinocerebellar ataxia 1 are caused by concomitant gain-of-function and loss-of-function mechanisms (71). Most misfolding diseases involve both toxic gain of function and loss of normal protective functions (72).

In conclusion, our results demonstrate that the covalent conjugation of Syn with cyt c in LB-like aggregates prevents signaling effects of the cyt c as a death signal in the cytosol but contributes to the formation of LB and Lewy neurites (LN). Thus Syn acts as a sacrificial scavenger of cyt c and prevents acute cell death. However, this protection from acute neuronal cell death does not come without a price. Because the peroxidase activity is retained in aggregates of cyt c with Syn and anionic lipids, they could act as an additional source of oxidative stress in affected neurons, contributing to chronic neurodegeneration. This explains, at least in part, the conflicting protective and damaging roles of Syn and LB reported to date in PD. Manipulation of components of the Syn/cyt c/anionic phospholipid complexes and/or their peroxidase activity might lead to development of mechanism based therapies for neurodegenerative diseases associated with LB/LN formation.
REFERENCES

FOOTNOTES

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The abbreviations used are:

- Syn, synuclein; cyt c, cytochrome c; LBs, Lewy bodies; LN, Lewy neuritis; PD, Parkinson’s disease; DA, dopamine; CL, cardiolipin; TOCL, tetraoleoyl CL; TLCL, tetralinoleyl CL; PI, phosphatidylinositol; PA, phosphatidic acid; PC, phosphatidylcholine; PS, phosphatidylserine; NAO, 10-N-nonyl acridine orange; DMOO, 5,5-dimethyl-1-pyrroline-N-oxide; MEC, mouse embryonic cells; ActD, actinomycin D; t-BuO2H, tert-butyl hydroperoxide; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, HEPES; mouse embryonic cells, (MECs); dioleoyl-phosphatidylcholine, (DOPC); tetraoleoyl CL, TOCL; diethylenetriaminepenta–acetic acid, DTPA; ethylene-diamine-tetra-acetic acid, EDTA; bovine serum albumin, BSA; phosphate buffered saline-tween-20, (PBS-T, 0.05%); tris buffered saline-tween-20, TBS-T; 5,5-dimethyl-1-pyrroline-N-oxide, DMPO; horseradish peroxidase, HRP; alkaline phosphatase AP; electron paramagnetic resonance, EPR; dithiothreitol, DTT; Matrix Assisted Laser Desorption/Ionization Time-of –Flight, MALDI-TOF; alpha-cyano-4-hydroxycinnamic acid, CHCA; trifluoroacetic acid, TFA; liquid chromatography electrospray ionization tandem mass spectrometry, LC-ESI-MS; high performance liquid chromatography, HPLC; ethylene glycol tetraacetic acid, EGTA; deoxyadenosine triphosphate, dATP; dimethyl sulfoxide DMSO, polyethylene glycol, PEG; 1-methyl-4-phenylpyridinium, MPP+; 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, MPTP.

FIGURE LEGENDS

**Figure 1.** Electrophoretic evidence for H2O2-induced hetero-oligomerization of Syn with cyt c in the presence of TOCL (a, b) and other anionic phospholipids (c, d).

- **a** - PAGE-based assessment of competitive binding of CL with Syn. A typical PAGE gel stained for Syn with SilverStain Snap Kit. Note that monomeric form of Syn migrating to cathode in the absence of TOCL, changes its migration behavior upon binding with CL. Titration with different amounts of nonyl acridine orange (NAO), which has a known high affinity for CL(16), re-constitutes the Syn migration profile, due to competitive binding of TOCL with NAO. The amount of free Syn was quantified by densitometry using BioRad MultiImager and Multi-Analyst software.

- **b** - SDS-PAGE electrophoresis of aggregates formed after incubation of Syn with cyt c/CL/H2O2. Staining with anti-Syn (left panel) and anti-cyt c antibodies (right panel) reveals that all for components - Syn, cyt c, TOCL, and H2O2 - were required for the formation of hetero-oligomers (containing both Syn...
and cyt c, detectable on both panels, arrows). A weak H$_2$O$_2$-induced aggregation of Syn with cyt c was observed in the absence of TOCL (arrowheads) in line with the known induction of peroxidase activity of cyt c during incubation with H$_2$O$_2$(8). Expectedly, significant aggregation of cyt c occurs (asterisk) in the absence of Syn as cyt c/TOCL complexes exert significant peroxidase activity causing H$_2$O$_2$-dependent aggregation(12). However, the aggregates are formed due to homo-oligomerization of cyt c and they do not contain Syn (compare with the lack of staining for Syn on the left panel). Typical gels representative of 5 experiments are shown.

c - Native electrophoresis in agarose gel and western blotting of complexes formed after incubation of Syn with cyt c and TOCL. Staining with anti-Syn (left panel) and anti-cyt c antibodies (middle panel). Note that Syn and cyt c did not interact with each other in the absence of TOCL. In the absence of H$_2$O$_2$, Syn and cyt c migrated mostly as single monomeric proteins. TOCL changed migration profile of Syn and cyt c. TOCL plus H$_2$O$_2$ caused oligomerization of Syn/cyt (asterisks) as evidenced by the appearance of aggregates at the origin of the gel and dense smeared pattern on the bottom of the gel. Thus, all four components – Syn, cyt c, CL and H$_2$O$_2$ – were required for the catalytic formation of cross-linked polymers. Note that no non-specific reactivity was displayed by anti-Syn or anti-cyt c anti-bodies to either monomeric or aggregated forms of cyt c and Syn, respectively.

d - SDS-electrophoresis of aggregates formed after incubation of Syn with cyt c and H$_2$O$_2$ in the presence of different anionic phospholipids: CL, phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidic acid (PA). A non-anionic phospholipid phosphatidylcholine (PC) was used as a control (shown for Syn). Staining with anti-Syn antibody (left panel) and anti-cyt c antibody (right panel) revealed that all four components - Syn, cyt c, H$_2$O$_2$ and anionic phospholipids - were required (arrows) for the formation of hetero-oligomers (containing both Syn and cyt c and detectable on both panels). Note that all four tested phospholipids were active in inducing H$_2$O$_2$-dependent hetero-oligomerization of Syn with cyt c; PI was significantly less effective than other phospholipids. In the absence of cyt c, none of the phospholipids tested caused oligomerization of Syn (data not shown).

Figure 2. Formation of hetero-oligomeric complexes and covalent cross-linked aggregates of cyt c/TOCL with wild-type Syn (intact and aged) and its mutants A53T and A30P.

a - Native PAGE of Syn/cyt c/TOCL hetero-oligomeric complexes (staining with SilverSNAP stain kit, ThermoFisher Scientific) (left panel). Syn (8 µM) was incubated with TOCL/DOPC liposomes (TOCL/Syn ratio 20:1) and cyt c (8µM) for 60 min at 37ºC. Assessment of Syn binding to CL/cyt c using NBD-CL fluorescence (right panel). Insert: typical fluorescence spectra obtained from: CL/NBD-CL liposomes (uppermost curve), CL/NBD-CL/cyt c complexes (lowest curve) and CL/NBD-CL/cyt c after titration with wild-type Syn (1) and mutant forms of Syn A53T (2) and A30P (3) (1.75 µM).

b - SDS-PAGE (12%) with subsequent Western blot-analysis (using anti-Syn antibody) of cross-links formed after incubation of wild-type (wt) Syn, A53T Syn and A30P Syn with cyt c/TOCL in the presence of H$_2$O$_2$. Wild-type Syn, A30P and A53T mutants underwent hetero-oligomeric covalent cross-linking with cyt c in a similar way.

c - SDS-PAGE (7.5%) with subsequent Western blot-analysis (using anti-Syn antibody) of Syn/cyt c/CL covalent cross-links formed by incubation of non-fibrillated and fibrillated (aged) Syn with cyt c/TOCL in the presence of H$_2$O$_2$. Comparable covalent cross-linking of non-fibrillated and fibrillated Syn was observed in the presence of cyt c, TOCL and H$_2$O$_2$. 


**Figure 3.** Evidence for the involvement of peroxidase reactive intermediates in hetero-oligomerization of Syn/cyt c/TOCL in the presence of H₂O₂.

**a** - Immuno-spin trapping of protein-derived radicals during H₂O₂-dependent hetero-oligomerization of Syn with cyt c in the presence of TOCL. Staining of PAGE gels with anti-DMPO antibody (left panel) demonstrates the production of protein-derived radicals. Increasing concentrations of DMPO weakened the response from high molecular weight aggregates and enhanced staining in lower molecular weight oligomers as well as in non-oligomerized complexes of Syn/cyt c/TOCL. Staining with anti-Syn (middle panel) and anti-cyt c (right panel) antibodies. Note that the production of hetero-oligomeric forms of Syn/cyt c/TOCL aggregates is progressively inhibited by increasing concentrations of DMPO indicating that the formation of DMPO adducts competitively inhibited oligomerization. No oligomerization of Syn with cyt c and TOCL occurred in the absence of H₂O₂ (data not shown). Typical gels representative of 3 experiments are shown.

**b** - Low-temperature EPR measurements of H₂O₂-induced protein-derived (tyrosyl) radicals of cyt c/CL complexes is shown in the left panel. Syn quenched the formation of tyrosyl radicals of cyt c/CL complexes in a concentration-dependent manner. The insert shows a typical EPR spectra of protein-derived (tyrosyl) radicals of cyt c/CL complexes in the absence (1) and presence of Syn (2), cyt c: α-synuclein=1:0.5. Right panel shows power-saturation curves of protein-derived tyrosyl radical EPR signals of cyt c/CL complexes (1) and cyt c/CL/Syn complexes (2) (cyt c: α-synuclein=1:0.5).

**c** - Assessments of dityrosine cross-links formed by incubation of Syn/cyt c/TOCL with H₂O₂ by fluorescence intensity (λ exc 315 nm, λ em 420 nm). Formation of dityrosine adducts increased by 1.6-fold upon addition of H₂O₂ to cyt c/TOCL. When Syn was present in this incubation system along with H₂O₂, the fluorescence intensity was further increased by 3.2-fold (Mean ± SD, *p< 0.05 vs cyt c/TOCL/H₂O₂).

**d** - SDS-PAGE with subsequent Western blot analysis of covalent conjugates with dityrosine cross-links formed from syn/cyt c/TOCL hetero-oligomeric complexes in the presence of H₂O₂. Staining was performed using anti-dityrosine antibody (panel A), anti-Syn antibody (panel B) and anti-cyt c antibody (panel C). A strong positive signal was observed with anti-dityrosine antibody in cross-linked high molecular weight aggregates (non-dissociable in SDS).

**e** - MS/MS spectrum of m/z 725.8 (M+2H)²⁺ species. This species was obtained from the digestion of high molecular weight aggregates of Syn/cyt c/CL formed in the presence of H₂O₂. Note the presence of ions at m/z 468, 495, 625, 730 and 896 corresponding to various daughter ions of the Syn-(132-137) and cyt c-(74-79) crosslink.

**Figure 4.** Syn is preferred substrate for reactive intermediates of the peroxidase complex

**a** - Effects of two prototypical peroxidase phenolic substrates – Amplex Red (left panel) and DA (right panel) on oligomerization of Syn/cyt c/TOCL in the presence of H₂O₂. Staining with anti-Syn antibody. Note that both compounds caused concentration-dependent inhibition of protein hetero-oligomerization. Typical gels representative of 3 experiments are shown.

**b** - Inhibition of peroxidase activity of Syn/cyt c/TOCL complexes with Amplex Red, DA, and etoposide. Left panel - Syn inhibits the peroxidase activity of Syn/cyt c/TOCL complexes, with Amplex Red as a substrate, in a concentration-dependent manner. Note that the IC₅₀ for Syn was 0.2 µM in the presence of...
50 µM Amplex Red. The results of 3 independent experiments are shown. Middle panel - Syn inhibits the peroxidase activity of Syn/cyt c/TOCL complexes, with etoposide as a substrate, in a concentration-dependent manner. Note that the IC50 for Syn was 0.5 µM in the presence of 100 µM etoposide. A typical ESR spectrum of etoposide phenoxyl radical generated by the H2O2-dependent peroxidase activity is shown (insert). The results of 3 independent experiments are shown. Right panel - Syn inhibits the peroxidase activity of Syn/cyt c/TOCL complexes, with DA as a substrate (200 µM). Data are presented as mean ± s.d., (n=3).

c - Native agarose gels of Syn/cyt c/CL and cyt c/CL complexes stained for peroxidase activity with SuperSignal West Pico Chemiluminescence substrate (left panel). Note that the peroxidase activity of cyt c/TOCL complexes (single arrow) towards the chemiluminescence substrate is inhibited when Syn is included in the preformed complex (double arrows) and then exposed to H2O2/chemiluminescence substrate. Typical gels representative of 3 experiments are shown. Quantification of residual peroxidase activity of wild type (wt) and mutated forms of Syn (A53T, A30P, A53T/A30P) /cyt c/TOCL complexes (right panel). The peroxidase activity of cyt c/TOCL complexes was higher in the presence of Syn mutants (A30P and A53T/A30P) compared with wt Syn (Mean ± SD, *p< 0.05 vs wt Syn).

d - Syn protects polyunsaturated TLCL against oxidation induced by cyt c/H2O2. Cyt c-induced accumulation of oxidized TLCL [as assessed by fluorescence HPLC (left panel) and MS analysis of doubly-charged ions [M-2H]2- (right panel)] was markedly suppressed by Syn. This was accompanied by the formation of high molecular weight aggregates of Syn/cyt c/TLCL (see Western blot with Syn antibody (left panel, inset). Data are presented as Mean ± SE; * p< 0.05 vs. TLCL+cyt c+H2O2. Accumulation of monohydroperoxy/monohydroxy derivatives of TLCL molecular species was observed after incubation of liposomes with cyt c and H2O2 (M/z 724.2 + [O] = 732.2; 724.2 + [OO] = 739.7; 724.2 + [O]+[OO] = 748.2; 724.2 + [2OO]= 755.9; 724.2 + [O]+[2OO] = 763.8; 724.2 + [3OO] = 771.8; 724.2 + [O]+[3OO] = 779.7; 724.2 + [4OO] = 787.9; and 724 + [O]+[4OO] = 795.7). This effect was markedly reduced by Syn. Representative mass spectra from 3 independent experiments are presented.

Figure 5. Syn and Syn/CL complex inhibits apoptotic caspase activation.

a - Syn/TOCL complexes inhibit caspase-3 activated by cyt c/dATP in a cell-free pro-apoptotic system (S-100 fraction isolated from HL-60 cells). Note that Syn in combination with TOCL was most effective in preventing cyt c-dependent caspase-3 activation. Wild-type Syn and two mutants, A53T and A30P, displayed equal inhibitory effects against caspase-3 activation after pre-incubation with cyt c/TOCL/H2O2. S-100 (5 µg of protein/µL) fractions were incubated with 1 µM cyt c for 90 min at 37°C, in the absence and in the presence of complex of Syn/TOCL, (1) S-100; (2) 1 + cyt c; (3) 2 + TOCL/Syn; (4) complex of wild-type Syn/TOCL/cyt c was pre-incubated with H2O2 and then added into 1; (5) complex of A53T Syn/TOCL/cyt c was pre-incubated with H2O2 and then added into 1; (6) complex of A30P Syn/TOCL/cyt c was pre-incubated with H2O2 and then added into 1. All the data are presented as % of caspase-3 activation in complete system (2) taken as 100%. Data presented are means ± SD (n=6). The statistical significance was calculated using analysis of variance (ANOVA), followed by Tukey's procedure with the family-wise error rate of P < 0.05 to perform our pair wise comparisons of selected group means. Asterisk indicates a statistically significant difference resulting from the Tukey comparisons.

b - Densitometric and Western blot analysis (inset) with anti-Syn antibody in wild type (WT), non-targeting siRNA (C), two different clones (A5 and A7) of HeLa Syn knock-down. Data are presented as mean ± SD (n=3/condition). *p<0.05 vs WT, #p<0.05, A7 vs A5.
c - Biomarkers of apoptosis --PS externalization (left panel) and caspase 3/7 activation (middle panel) -- increase and cell viability (right panel) decrease in Syn deficient HeLa A5 and A7 cells after exposure to dopamine (DA). Data are presented as mean ± SD (n=3/condition). *p<0.05, 400µM vs 0 µM, , #p<0.05, A7 vs A5.

d - Delivery of Syn into mouse embryonic cells (MECs) confers resistance to pro-apoptotic caspase 3/7 activation. Note that robust activation of caspase 3/7 MECs stimulated with ActD was markedly attenuated in Syn-containing MECs (delivered by the direct introduction of Syn into cells). Delivery of a nonspecific protein, green fluorescent protein (GFP), exerted no effect on ActD-induced caspase 3/7 activation in MECs. Data are presented as mean ± SD (n=4/condition). *p<0.05, ActD vs ActD + Syn.

Figure 6. Detection of inclusions and co-localization of hetero-polymerized Syn and cyt c aggregates in HeLa and SH-SY5Y cells after exposure to ActD or t-BuOOH.

a - Immunostaining of cyt c (green) and Syn (red) in intact HeLa cells and cyt c-deficient HeLa 1.2 cells. In control cells, cyt c appears in a punctate, perinuclear pattern of staining with little overlap with cytosolic Syn staining. ActD induces cytoplasmic blebbing, nuclear fragmentation and transition from perinuclear to a diffuse staining pattern for cyt c. Focal regions of accentuated Syn staining, suggestive of aggregation, are observed and this frequently colocalize with focal regions of accentuated cyt c staining in cells with both preserved and fragmented nuclear contours. HeLa 1.2 clones engineered to express decreased levels of cyt c show a similar distribution of cyt c and Syn, although there is less cyt c immunoreactivity. HeLa 1.2 cells show preserved perinuclear cyt c and reduced propensity for clumped or aggregated Syn in response to the same doses of ActD, and there is no evidence of Syn and cyt c colocalization in these cells.

b - Western blot analysis of Syn/cyt c aggregates after native agarose gel electrophoresis (left panel) and SDS-PAGE (right panel) in HeLa cells exposed to t-BuOOH.

Left panel - Staining with anti-Syn (Syn) and anti-cyt c antibodies (cyt c) reveals the appearance of co-localized hetero-oligomers after treatment of cells with t-BuOOH.

Right panel - Staining with anti-Syn (Syn) and anti-cyt c antibodies (cyt c) demonstrates disappearance of monomeric form of Syn and accumulation of very high molecular weight Syn aggregates partially overlapping with aggregated forms of cyt c. Typical gels representative of 3 experiments are shown.

c - Detection of Syn/cyt c hetero-oligomers in HeLa cells exposed to t-BuOOH using immunoprecipitation with anti-Syn antibodies and Western blot analysis with anti-Syn and anti-cyt c antibodies after SDS-PAGE. Note that proteins immunoprecipitated with anti-Syn antibody contained high molecular weight aggregates positive for both Syn (asterisk) and cyt c (arrowhead) after treatment with t-BuOOH. Bands around 50kDa likely correspond to heavy chains of immunoglobulins remaining after immuno-precipitation. Arrow indicates Syn monomer. Typical gels representative of 3 experiments are shown.

d - Western blot analysis of Syn/cyt c aggregates after SDS-PAGE in mitochondria isolated from HeLa cells pre-treated with t-BuOOH. Staining with anti-Syn (Syn) and anti-cyt c antibodies (cyt c) demonstrates accumulation of very high molecular weight Syn aggregates partially overlapping with aggregated forms of cyt c. Note that mitochondria from control cells contain significant amounts of monomeric cyt c but hardly detectable levels of monomeric Syn. After treatment with tBuOOH, polymerized forms of Syn and cyt c are readily detectable and monomeric form of cyt c is decreased. Typical gels representative of 3 experiments are shown.
e- Immunostaining of cyt c (green) and Syn (red) in SH-SY5Y cells. Note that both pro-apoptotic agents, t-BuOOH and ActD – caused the appearance of LB-like inclusions revealed as yellow color in merged images (arrows). In normal SH-SY5Y cells, cyt c had a punctuate mitochondrial distribution, and Syn was localized in the cytoplasm and peri-nuclear area. Following ActD or t-BuOOH treatment, Syn displayed aggregated pattern of distribution and overlapped with anti-cyt c staining (yellow spots on merged images).

Figure 7. Syn distribution and colocalization with cyt c in substantia nigra dopaminergic neurons after rotenone. a - Brain sections through substantia nigra were triple-labeled for tyrosine hydroxylase (TH, blue) cyt c (red) and Syn (green) and imaged by laser scanning confocal microscopy. Note the increased fluorescence and altered distribution of Syn after rotenone. Additionally, there is FRET (fluorescence resonance energy transfer) between cyt c and Syn, strongly suggesting direct protein-protein interactions. b - Fluorescence intensity profiles of Syn immuno-reactivity in single dopaminergic neuron from control and rotenone-treated rats. Each fluorescence intensity profile is from an identical rectangular region of interest encompassing part of a single dopaminergic neuron. After rotenone, the neuronal levels of Syn increase and its distribution changes from relatively diffuse to markedly punctate. c - Quantification of the colocalization of Syn with cyt c in substantia nigra from a control animal and a rotenone-treated animal. After rotenone treatment there is marked increase in the Pearson correlation coefficient (R) and in the colocalization coefficients for both channel 1 (cyt c) and channel 2 (Syn). Data are from confocal images (60x) that each contain approximately 20 dopaminergic neurons and surrounding neuropil.

Figure 8. Colocalization of Syn and cyt c in human PD/Lewy body disease substantia nigra. Midbrain sections from patients with PD/Lewy body disease were immunolabeled for cyt c (red) and Syn (green). DAPI (blue) was used to stain nuclei. Normal nigral neurons exhibit a granular cytoplasmic staining pattern for cyt c, consistent with its mitochondrial localization (a, lower neuron). In contrast, the neuron containing a LB (arrow) shows light, diffuse cytoplasmic cyt c staining and colocalization of cyt c and Syn (yellow) in the LB (a, upper neuron). Many Lewy neurites also showed colocalization of cyt c and Syn (b, c), although cyt c negative Lewy neurites (asterisk) were also observed (inset c). Note the absence of red staining in the LB containing neuron (arrowhead) when non-immune rabbit antiserum was substituted for antibody to cyt c as a negative staining control (d). More than 1,200 consecutive Syn immunoreactive structures from four cases were scored by location in the neuronal soma (Lewy body/pale body - LB/PB) or in neuritic processes (Lewy neurites). These were analyzed for presence or absence of cyt c colocalization. The pie chart (e) shows Lewy neurites without cyt c (light green-blue), LB/PB without cyt c (dark blue), LB/PB colocalizing with cyt c (magenta), and Lewy neurites colocalizing with cyt c (yellow). Note that nearly half of all Syn immunoreactive structures show colocalization with cyt c (yellow & red combined). Moreover, more than two-thirds of Syn-cyt c colocalizing structures involved neurites (yellow).
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 8

**A**

Merge  
Cyt c (red)  
α-Syn (green)

**B**

**C**

**D**

**E**

α-Synuclein Immunoreactive Structures

- 33% Lewy neurites
- 20% No cyt c
- 15% Colocalized with cyt c
- 32% LB/PB

Figure 8
Peroxidase mechanism of lipid dependent cross-linking of synuclein with cytochrome c: protection against apoptosis versus delayed oxidative stress in Parkinson’s disease


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