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Defining the Oligomerization State of γ-Synuclein in Solution and in Cells

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Abstract

Gamma synuclein is expressed at high levels in neuronal cells and in multiple invasive cancers. Like its family member α-synuclein, γ-synuclein is thought to be natively unfolded but does not readily form fibrils. The function of γ-synuclein is unknown but we have found that it interacts strongly with the enzyme phospholipase Cβ (PLCβ) altering its interaction with G proteins. As a first step in determining its role, we have characterized its oligomerization using fluorescence homotransfer, photon-counting histogram analysis and native gel electrophoresis. We find that when expressed in *E. coli* and purified, γ-synuclein appears monomeric on chromatographs under denaturing conditions, but under native conditions it appears as oligomers of varying sizes. We followed the monomer to tetramer association by labeling the protein with fluorescein and following the concentration-dependent loss in fluorescence anisotropy due to fluorescence homotransfer. We also performed photon counting histogram analysis at increasing concentrations of fluorescein labeled γ-synuclein and found concentration dependent oligomerization. Addition of PLCβ2, a strong γ-synuclein binding partner whose cellular expression is correlated with γ-synuclein, results in disruption of γ-synuclein oligomers. Similarly, its binding to lipid membranes promotes the monomer form. When we exogenously express γ-synuclein or microinject purified protein into cells, the protein appears monomeric. Our studies show that even though purified γ-synuclein form oligomers, when binding partners are present, as in cells, it dissociates to a monomer to bind these partners which in turn may modify protein function and integrity.

Keywords

γ-synuclein; phospholipase Cβ2; oligomerization; fluorescence spectroscopy; photon counting histogram

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Supporting Information

To test whether γ-synuclein incorporates uniformly into the cytoplasm we compared the pixel intensity throughout the cell. Figure S1 shows the pixel intensity distribution, the intensity values vary by factor of two and one can conclude that the concentration difference between various regions is not more than two fold. We performed PCH measurements in several spots in one cell and did not detect differences in molecular brightness. PCH relies on mobile molecules. Thus any mobile aggregates would have been detected as they would diffuse in and out of the confocal volume. We performed measurements in multiple cells and in various concentration areas. Figure S2 shows the graph representing the PCH measurements for different number of molecules in the confocal volume. Tenfold increase in the number of particles did not result in increase in molecular brightness indicating that γ-synuclein is not aggregating. "This material is available free of charge via the Internet at http://pubs.acs.org.

Introduction

γ-Synuclein is a small, cytosolic protein comprising 127 amino acids. It is abundant in spinal cord, sensory ganglia, and retina, but also in metastatic breast cancer and other cancer tissue[1-4]. It was first identified in the invasive breast cancer tissue and termed breast cancer specific gene product 1[5]. Over-expression of γ-synuclein in cells leads to proliferation, motility and metastasis[6]. γ-Synuclein belongs to the synuclein family composed of three members: α-synuclein , β-synuclein , and γ-synuclein that are conserved throughout vertebrates. Synucleins are considered to be natively unfolded[7]. All family members share a common highly conserved α-helical lipid-binding motif on the N-terminal[4, 8, 9]. α-Synuclein is the best characterized member of the family. It has propensity to form oligomers that play a role in pathologies[10, 11]. γ-Synuclein is not expected to form fibrils but can form small stable oligomers[12].

Despite intense investigation, the exact biological function of both α- and γ- synuclein remains unknown. We have found that both proteins are cellular binding partners of PLCβ2[13, 14]. PLCβ2 is a member of a larger mammalian PLC family that catalyzes the hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP_2). Cleavage of PIP_2 generates the second messengers, diacylglycerol and 1,4,5 inositol trisphosphate, which activate protein kinase C and cause the release of Ca^{2+} from intracellular stores, respectively. PLCβ2, similar to γ-synuclein, is overexpressed in invasive breast cancer tissue[15] and when overexpressed in cells, PLCβ2 increases migration and proliferation[16, 17]. We have found that binding of either α- or γ-synuclein inhibits PLCβ2 activity and occludes binding of PLCβ2's activator Gαq which will disrupt cell signaling mediated by receptors coupled to Gq such as angiotensin, acetylcholine and dopamine. However, binding of γ-synuclein to PLCβ does not affect its binding to Gβγ subunits or small G proteins but instead will enhance these signals due to the relief of enzyme inhibition by γ-synuclein synergistic with activation by Gβγ [13, 14]. Additionally, synucleins bind to the same site as the TRAX subunit of the RNA-induced silencing complex and may interfere with PLCβ's role in gene regulation[18]. We have also observed that γ-synuclein protects PLCβ2 from digestion by the Ca^{2+} activated protease calpain[19] suggesting that γ-synuclein may contribute to the over-expression of PLCβ2 in breast cancer.

While α-synulcein aggregates are hallmarks of neurodegenerative diseases such as Parkinson's, over-expression of γ-synuclein is the hallmark of several cancers, including breast cancer. In addition to our lack of understanding of its cellular function, the role that it plays in promoting cancer is unknown. To begin to understand the potential function of γ-synuclein, we have characterized its oligomerization state in solution and in cells. We find that even though γ-synuclein purifies as a tetramer or a smaller oligomer, in cells it tends to be monomeric where it is most likely bound to lipids and protein partners such as PLCβ2. This finding leads to a model suggesting that γ-synuclein may act as a cofactor to modulate the function of other proteins rather than having an independent function.

Materials and Methods

Cell culture

MDA MB 231, HEK293 and HeLa cells were purchased from American Type Culture Collection (ATCC) and were cultured in Dulbecco's Minimum Essential Media (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 50 units/mL of penicillin and 50 μg/mL of streptomycin at 37°C and 5% CO_2. The transient transfections of 0.2 μg of DNA in MDA MB 231 and HeLa cells was performed on 35 mm glass bottom MaTek dishes at a cell density of 50–80% using Lipofectamine 2000™ and according to the manufacture's protocol.

Protein expression and purification

Human γ-synuclein was expressed in *E. coli* and purified using 15Q column as described for α-synuclein [20], to aid in labeling the serin 4 of γ-synuclein was replaced with cysteine. His_6-PLCβ2 was expressed in Sf9 cells using a baculovirus system with minor modifications. The purity of proteins was assessed by SDS-PAGE electrophoresis and western blotting. Concentrations of proteins were determined by a Bradford assay (Biorad).

To produce the mCherry-γ-synuclein construct we amplified the γ-synuclein DNA from bacterial plasmid using polymerase chain reaction and the following primers: forward: CAC AGA TCT ATG GAT GTC TTC AAG AAG GGC and reverse: ATC GGT ACC TCA CTA GTC TCC CCC ACT. It was then inserted into the mCherry-C1 vector between Bgl II and Kpn I sites.

Native Page

8% acrylamide/bis-acrylomide gel was prepared omitting SDS and replacing it with water. The samples were not boiled and diluted with sample buffer containing bromophenol blue, glycerol and Tris-HCl in water, pH 6.8.

Fluorescence labeling

γ-synuclein was labeled on ice for at least one hour. The protein was mixed with the probe at 1:4 protein:probe molar ratio. Prior to labeling with thiol-reactive CPM (7-diethylamino-3-(4'-maleimidylphenyl)-4 methylcoumarin), and modified with maleimide Alexa488, Alexa 546, and Oregon Green the protein was dialyzed to remove DTT present in the storage buffer. The reaction was stopped by adding 10 mM DTT. Prior to labeling with N-terminal reactive fluorescein and Alexa488 the pH of the solution containing the protein was raised to approximately pH=8 using phosphate buffer. The pH was 8.0, pKa of lysins is 10. In order for their pK to drop 2 units, their local environment would have to be highly anionic, which is not the case. Thus, the probability that any would be deprotonated is low. We note, though, that while it is remotely possible that a small fraction of the lysine residues also got labeled, it would not drastically change the interpretation of our results. The unreacted probe was removed using spin trap PD-25 column or desalting PD-10 column (GE Healthcare, Backinghamshire, UK).

Fluorescence binding studies

Fluorescence measurements were performed on an ISS spectrofluorometer (Champaign, IL) using 3 mm quartz cuvettes. Samples were diluted in buffer solution containing160 mM NaCl, 20 mM Hepes, 1 mM DTT, pH 7.2. CPM was excited with 380 nm wavelength and the emission spectrum was recorded from 415 to 530 nm. The background spectra of unlabeled protein were subtracted from each spectrum along the titration curve. All of the spectra were corrected for the 10–12% dilution that occurred during the titration. For anisotropy measurements fluorescein was excited with 480 nm and fluorescence emission was observed at 520 nm using an Oriel bandpass filter (Stratford, CT). Prior to the measurements, we added DMF to the protein solution and subjected it to low energy sonication to promote dissociation of oligomers as detected by fluorescence anisotropy and fluorescence correlation spectroscopy.

Preparation of Giant Unilammelar Vesicles

We used a rapid evaporation method [21-23] to form 1:1 phosphatidyl choline: phosphatidyl serine (PC/PS) giant unilamellar vesicles (GUVs) for photon counting histogram (PCH) measurements. The Alexa488-labeled γ-synuclein was either added to the solution prior to the organic solvent evaporation or after formation of GUVs.

Microinjections

For the microinjection experiments, cells were plated in 35 mm glass bottom dishes from MatTek (Ashland, MA) to achieve 50-70% confluency. To produce needles, thin-wall single-barrel standard borosilicate glass tubes (with filament), outer diameter 1.0 mm and inner diameter 0.75 mm (World Precision Instruments, Sarasota, FL) were pulled on a Flaming Brown micropipette puller (model P.80/PC, Sutter Instrument Co (Novato, CA) using the following settings Heat = 780, Pull = 15, Velocity = 13, Time = 20.

Microinjections were performed on Axiovert200M from Zeiss (Jena, Germany) equipped with 40 × long distance phase 2 objective. For microinjections, we used InjectMan NI2 with FemtoJet pump from Eppendorf. Cells were microinjected in the cytoplasm. We set the injection pressure Pi to 17 – 25 hPa and kept the compensation pressure Pc at 10-15 hPa. After microinjections we replaced media bathing the cells with phenol-free Liebovitz's 15.

Photon Counting Histograms

is a method based on the probability distribution of photon counts in a confocal volume (about one femtoliter). The probability of the photon count distribution stems from fluctuations of the particle number and the photon detection statistics. Both display Poisson statistics and when convoluted, result in a broader distribution that is further broadened by the optical properties of the instrument and the position of the fluorophore in the light beam resulting in the final super-Poisson shape. The super-Poisson shape is fit to a theoretical model to resolve the average number of fluorescence particles and their molecular brightness [24-26]. The data were collected using Zeiss LSM 510/confocor 2 microscope (Zeiss, Jena, Germany). We used a 40 × NA 1.2 C-Apochromat water immersion objective and adjusted pinholes at least daily using rhodamine 6G and rhodamine B dyes. Fluorescein, Alexa488 and Oregon Green were excited with 488 nm wavelength of Argon ion laser and the emission was recorded using 505 nm long pass filter. The laser power was set to 1% using AOTF. Alexa 546 and mCherry were excited with 543 nm HeNe laser and the emission of collected through 560 nm long pass filter. The laser power was set to 10% using AOTF. The data were saved in the photon arrival mode (raw format) that was converted to the time mode using SIMS FCS from the Laboratory of Fluorescence Dynamics (Champaign, IL). The Photon Counting Histograms were binned to achieve 2MHz sampling rate and the PCH model functions were fit using also SIMS FCS.

Results

Purified γ -synuclein is primarily a tetramer

It has been reported that γ -synuclein can form small oligomers[12]. Thus, we examined the migration of purified protein under native conditions. γ-Synuclein purified from *E. coli* migrates on an SDS-PAGE gel as monomer with a molecular weight of 14 kDa (**Figure 1A**). On the native gel, we noticed several bands corresponding to different oligomerization states including trimer and tetramer forms, (**Figure 1B**). This result indicates that γ-synuclein forms weakly associating oligomers that dissociate under denaturing conditions.

Monomeric γ-synuclein self-associates to higher order oligomers

We investigated whether purified γ-synuclein can self-associate using fluorescence homotransfer. Homotransfer is resonance energy transfer between chemically identical molecules. Because the donor and acceptor molecules are the same, homotransfer is most easily measured by the loss in polarization, or anisotropy, of the emitted light. For these studies, we labeled γ-synuclein on its N-terminal with fluorescein. After removal of the unreacted probe, we added 5-10% DMF and sonicated the labeled protein to insure that the

prevalent population was monomeric as suggested by a low value of the anisotropy that remained constant with increasing amounts of DMF up to 80% v/v or 10% SDS. A monomer free in solution is not expected to have high anisotropy because its rotational motion is unrestricted and the monomer molecular weight is low resulting in a small rotational volume. This free motion causes light to be emitted with all possible polarization[27]. We diluted the protein to about 1 nM, recorded its anisotropy and subsequently measured the anisotropy at increasing concentrations of fluorescein-labeled-γ-synuclein. The results are shown in **Figure 2**. As the concentration of γ-synuclein increases, the anisotropy decreases indicating that the γ-synuclein molecules are associating to dimers, tetramers and higher order oligomers [28]. A change in anisotropy was not observed when buffer replaced γ-synuclein titration. Our results indicate that the γ-synuclein self-associates to oligomers with an apparent dissociation constant of 8 ± 3 nM assuming a bimolecular association.

We corroborated these studies with fluorescence titrations in which we labeled γ-synuclein on its N-terminal with CPM and measured increase in intensity as unlabeled γ-synuclein is added to the solution (data not shown). We find that the apparent dissociation constant is ~5 nM which is consistent with the anisotropy measurements.

Photon counting histogram (PCH) analysis indicates that γ-synuclein forms oligomers in solution that can be broken by PLCβ2

We also investigated the oligomerization state of γ-synuclein using photon counting histogram (PCH) analysis. The PCH uses the intensity fluctuations from fluorescence correlation measurements to quantify the number of molecules in the observed confocal volume and the brightness associated with them [24-26].

We performed PCH measurements of γ-synuclein labeled with fluorescein, Alexa488, Oregon Green, and Alexa 546 either on N-terminal amine, or on cysteine introduced close to the N-terminal. These variations were done to ensure that the results are independent on the nature of the probe. We obtained similar results for all the probes tested. **Figure 3** shows data indicating that fluorescein-labeled γ-synuclein is 3-4 fold brighter than free dye which is consistent with the size of the aggregates seen in native gels. Raising the concentration of γ-synuclein increases the molecular brightness suggesting an increase in self-association. This increase is consistent with our homotransfer experiments. Addition of PLCβ2 reduces the molecular brightness indicating a reduction in oligomerization.

In **Figure 4** we show the change in brightness when we incubated Alexa 488 labeled γ-synuclein at 4°C for a week. We find a significant increase in the molecular brightness of Alexa488- γ-synuclein as compared with Alexa488 or freshly labeled Alexa488- γ-synuclein suggesting increased oligomerization. Addition of PLCβ2 resulted in decrease in molecular brightness suggesting that PLCβ2 is breaking oligomers formed by Alexa488- γ-synuclein.

We also induced oligomerization of γ-synuclein by incubating it at 37 °C for 24-72 hours. We observed appearance of larger (about 20-mer) oligomers. PLCβ2 was not able to break the larger oligomers (data not shown). The diffusion coefficient of the larger aggregates was not altered by addition of PLCβ2 which is ten-fold heavier than synuclein (MW~140,000 kDa) suggesting that the larger aggregates are no able to bind to it.

Binding to the lipid membranes reduces the oligomerization state of γ-synuclein

Since PLCβ2 resides predominantly on the plasma membrane, we tested whether γ-synuclein has intrinsic membrane affinity. To test this we measured the binding of γ-synuclein to PC/PS 1:1 lipid vesicles and found a strong molar partition coefficient 3×10^4

M^{-1} (**Figure 5A**). To test whether membrane binding affects the oligomerization state of γ-synuclein, we performed PCH analysis of Alexa488 labeled γ-synuclein bound to PC/PS GUVs. The data in **Figure 5B** shows that γ-synuclein bound to giant, unilamellar vesicles (GUVs) has a significantly reduced brightness indicating that upon binding to lipid membranes, the oligomer size of γ-synuclein is reduced.

γ Synuclein is not oligomerized in cells

To determine whether the native state of γ-synuclein in cells is also in the form of a homo-oligomer, we microinjected γ-synuclein labeled with Alexa 488 into HEK 293 cells and performed PCH measurements. After labeling and purification using PD10 columns, we verified that Alexa488-γ-synuclein was in monomeric state prior to microinjection (as assessed by PCH and FCS). Microinjected γ-synuclein incorporated uniformly throughout the cytoplasm of the cells (**Figure 6A**). From the pixel intensity distribution, the concentration difference between various regions is not more than two fold. We note that although gamma-synuclein is not incorporating into cellular vesicles, it does seem to interact with many partners that giving a grainy appearance distribution. We performed PCH measurements in several spots in one cell and did not detect differences in molecular brightness. The pixel intensity varied by a factor of two (see **Figure S1** for the intensity pixel distribution in a microinjected cell). **Figure 6B** shows the distribution of molecular brightness. The molecular brightness of Alexa488- γ-synuclein microinjected into HEK293 cells is not significantly different from the molecular brightness of the free Alexa488 probe microinjected into the HEK293 cells. The similarity of the brightness of free Alexa488 and the labeled protein suggests that γ-synuclein is monomeric under these cellular conditions. We performed PCH measurements in multiple cells with different concentration of Alexa488-γ-synuclein. It is important to note that there was no observable self-association of γ-synuclein with increasing concentration (see Figure S2 for the relation between number of particles and molecular brightness). The measurements were performed in cells with concentration of γ-synuclein ranging from 10 nM to 100 nM comparable to the concentration of the protein in the native gel (~100 nM).

We expressed mCherry-γ-synuclein in MDA MB 231 (**Figure 7A**) and HeLa cells (**Figure 7B**). In both cases, the molecular brightness of the protein is not significantly different from the molecular brightness of the mCherry protein expressed alone. This result indicates that γ-synuclein fused with fluorescent protein is monomeric when expressed in mammalian cells.

Discussion

The synucleins represent an important family of proteins because of their critical role in disease. While α -synuclein aggregates are hallmarks of many neurodegenerative disorders, γ - synuclein expression has been shown to promote cancer phenotypes most notably in breast and retinal cancers. Despite their prominent roles in promoting disease states, their high expression levels in neuronal tissue and their high homologies through vertebrates, the cellular function of these proteins remains unknown [10, 11, 29, 30]. γ -Synuclein differs from α-synuclein in that it lacks the acidic C-terminal tail and the region that is important for α-synuclein aggregation. In solution, γ-synuclein forms fibrils at much slower rate than α-synuclein [12, 31] although large γ-synuclein aggregates have been observed *in vivo* [32, 33]. It was shown that γ-synuclein, particularly after oxidation, can also form larger aggregates and serve as a seed for α-synuclein aggregation [32]. Moreover, transgenic mice over-expressing γ-synuclein showed neuropathological changes caused apparently by aggregation of this protein [33].

Here, we show that γ-synuclein is prone to oligomerization in its native state and in solution (see **Figure 8** for our working model). Specifically, using native-PAGE gels we show that γ-synuclein is forming small oligomers, and we can follow the self-association of γ-synuclein from monomer to tetramer by fluorescence methods. This tetrameric form is consistent with previous observations showing that γ-synuclein can form small soluble oligomers [12, 34]. Like α-synuclein, γ-synuclein tends to aggregate in solution in a time-dependent manner, and we find that its ability to bind to PLCβ2 as well as lipid membranes is critically dependent on its oligomeric state. Specifically, we show that lipid membranes and PLCβ2 disrupt these small oligomers suggesting that γ-synuclein oligomers dissociate into monomers or dimers in order to binds these species (see cartoon in **Figure 8**). Not surprisingly, PLCβ2 could not break the large aggregates of γ-synuclein formed in solution over time suggesting that the molecules are kinetically trapped in the aggregate or oxidized. The time, temperature and concentration dependence of γ-synuclein aggregation most likely is responsible for differences seen in the lipid binding properties here and in previous work [35] as well as those reported for α-synuclein. We note that even the membrane binding constant measured here is stronger than in previous studies, the trend of increasing strength of binding to membranes with increasing percent of negatively charged lipids is the same (data not shown).

Previous studies have shown that in cells, PLCβ1 is protected by α-synuclein from degradation by calpain, and in turn PLCβ1 ameliorates the aggregation of α-synuclein [3619] presumably by preserving the monomeric state. It is possible that similar mechanisms govern the interaction between PLCβ2 and γ-synuclein. γ-Synuclein may similarly protect PLCβ2 from protease degradation [19] to increase its cellular levels and in turn PLCβ2 can prevent γ-synuclein from forming oligomers. Lipid membranes also might preserve the monomeric state of γ-synuclein in cells. To determine the aggregation state of γ-synuclein in cells we viewed fluorescently-tagged γ-synuclein in live cells using fluorescence fluctuation methods. The expression of mCherry fusion protein results in mainly monomeric form, as assessed by PCH analysis, but the distribution of brightness has a long tail towards higher values indicating some degree of oligomerization in small populations of cells. mCherry is a 28.8 kDa protein and it is possible that it may interfere with normal behavior of γ-synuclein that is only 14 kDa. To eliminate this issue we microinjected γ-synuclein labeled with a small fluorophore, Alexa488, into HEK293 cells. Under these conditions, γ-synuclein appears to be monomeric based on the fluorescence fluctuation studies. We expect that it is bound to multiple binding partners as well as to the membranes. Some studies suggest that post-translational modification of γ-synuclein increases its aggregation propensity[32].

The results of this study suggest a model in which γ -synuclein monomers are bound to various cellular partners, such as PLCβ. This model implies that the isolated tetrameric form of γ -synuclein is not its native functional state but rather, γ -synuclein monomers function to modify or modulate the function of other cellular proteins. Although this idea is very speculative, it correlates well with the many cell culture and whole organism studies that have yet to uncover a clear function for this protein. This model also implies that neuropathologies as well as pathologies associated with invasive cancer attributed to γ-synuclein may be promoted by formation of small oligomers and γ-synuclein aggregates when it is released from its binding partners.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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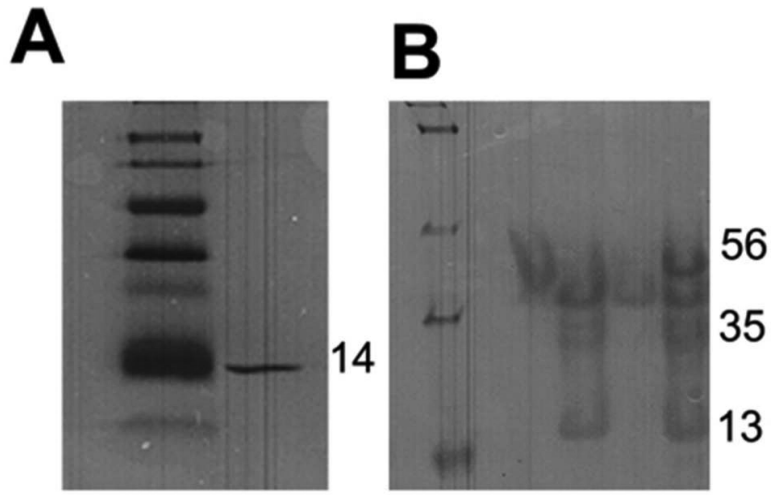

Figure 1. γ-Synuclein migrates as a tetramer on native gel

A) An SDS gel showing moleculear weight markers (lane 1) and γ-synuclein expressed in *E. coli* (lane 2) where the protein is seen to migrate as14 kDa monomer. B) Native gel showing multiple oligomeric states of γ-synuclein. Where lane1 is a marker and lanes 2-5 correspond to different protein preparations from *E. coli*.

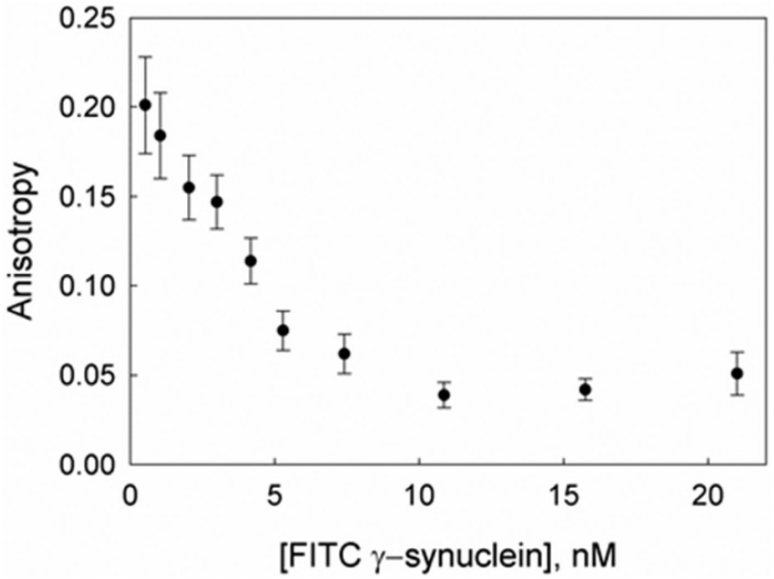

Figure 2. Fluorescence homotransfer indicates that FITC-γ-synuclein forms oligomers

The graph shows decrease in anisotropy (see Methods) when concentration of FITC-γ-synuclein is increasing consistent with oligomerization.

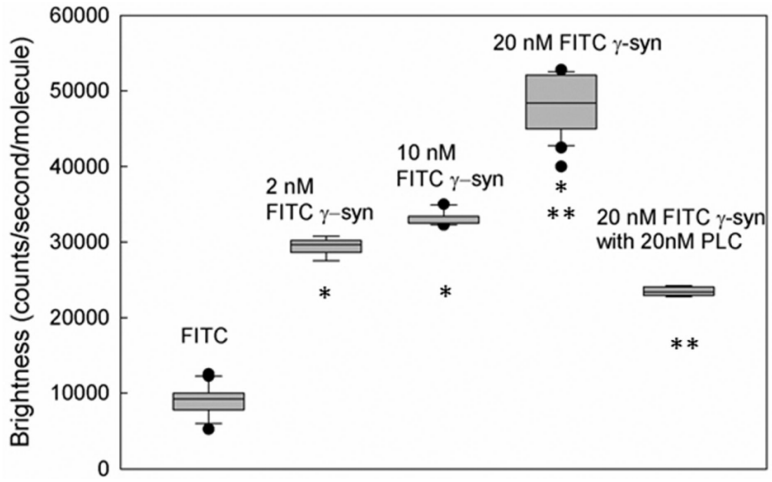

Figure 3. PCH analysis of FITC-γ-synuclein shows concentration dependent oligomerization

The bars show the distribution of brightness of FITC-γ-synuclein when the protein concentration protein is increased. Each bar represents distribution of at least 10 independent measurements. The line in the box represents median. The lower boundary of the box represents the 25th percentile and the top line represents the 75th percentile. Whiskers below and above indicate 90th and 10th percentiles respectively. The points represent outliers. Addition of PLCβ2 causes a decrease in molecular brightness indicating disruption of oligomers.

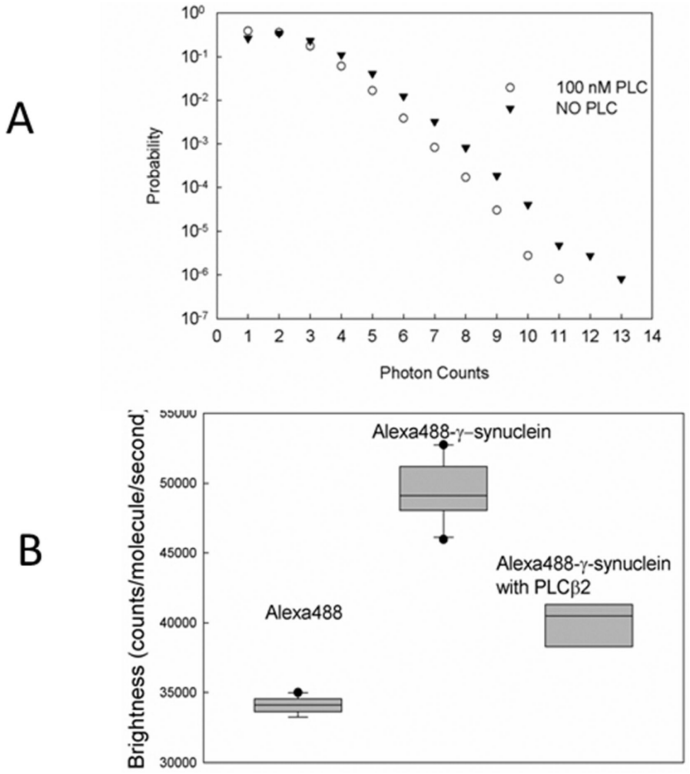

Figure 4. PCH analysis indicates that PLCβ2 disrupts γ-synuclein oligomers

Alexa488-γ-synuclein was left at 4°C for one week. A) Shows an example of PCH curves with 100 nM PLCβ2 (○) and without PLCβ2 (▼). B) Compilation of data showing that aggregation of the protein that is reduced by addition of PLCβ2. * indicates significant difference from the FITC alone ($P<0.05$) and ** indicates significant difference from the 2 nM FITC-γ-synuclein.

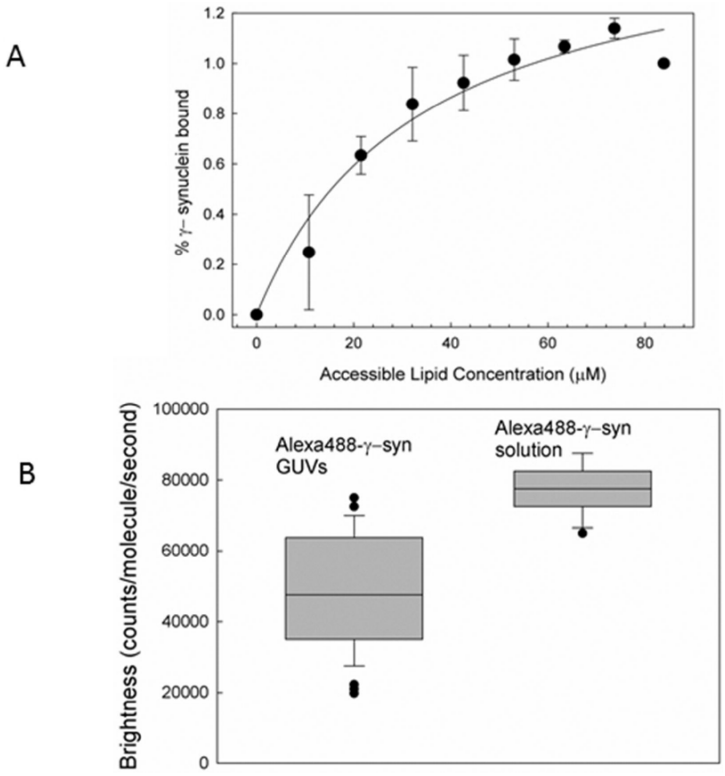

Figure 5. Binding to lipid membranes reduces the oligomerization of γ-synuclein

A) Binding of CPM- γ-synuclein to 1:1 POPC/POPS large unilammelar vesicles. The percent of γ-synuclein bound was determined as a function of accessible lipid concentration, [lipid], measuring the increase in CPM fluorescence upon membrane binding. The data was corrected for background and dilution. The points represent average of three independent experiments. The curve represents the least-squares fit of ligand binding with one site saturation to the data. The K_d = 33.6 μM. B) Graph shows the distribution of brightness of Alexa488-γ-synuclein in solution (N=18) and bound to GUVs (N=36). The brightness of γ-synuclein bound to GUVs is significantly lower than γ-synuclein in solution indicating that oligomers are dissociating upon membrane binding.

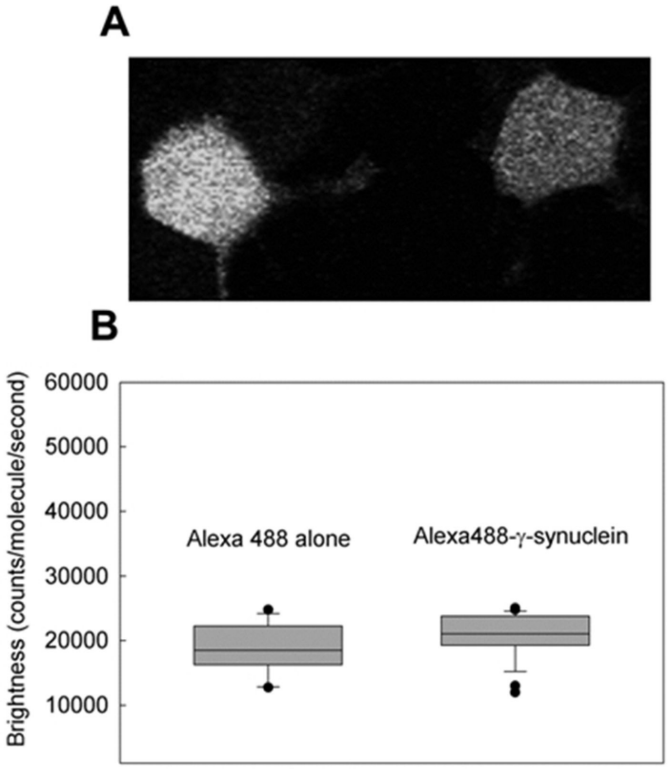

Figure 6. γ-Synuclein injected into HEK293 cells is monomeric

A) Examples of HEK293 cells after microinjection of Alexa488-γ-synuclein. The protein is uniformly distributed thought the cells. B) The graph shows distribution of molecular brightness of Alexa488 (N=13) and Alexa488-γ-synuclein (N=27) injected into HEK293 cells. There is no significant difference between the two groups. The brightness of Alexa488-γ-synuclein in solution was also not significantly different (*data not shown*).

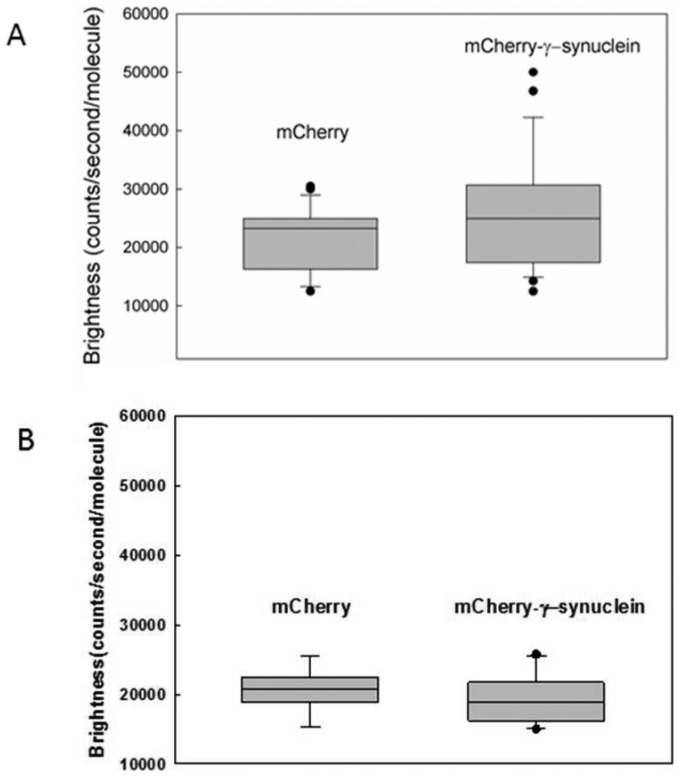

Figure 7. mCherry γ-synuclein expressed in MDA MB 231 and HeLa cells is monomeric

A) The graph shows distribution of molecular brightness of mCherry protein (N=23) and mCherry-γ-synuclein fusion protein (N=28) in MDA MB 231 cells. There is no significant difference between the two groups but the long tail in mCherry-γ-synuclein indicates small degree of oligomerization in some cells. B) The graph shows distribution of molecular brightness of mCherry protein (N=9) and mCherry-γ-synuclein fusion protein (N=10) in HeLa cells. There is no significant difference between the two groups.

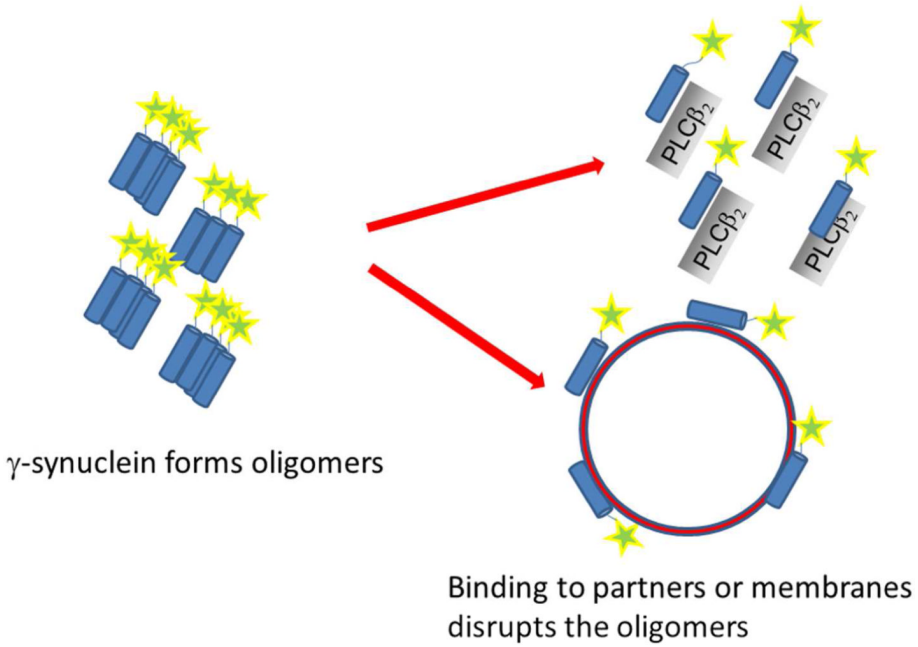

Figure 8. Cartoon of a working model

γ-Synuclein forms oligomers that can be disrupted by binding to protein partners, for example PLCβ2 or to lipid membranes.