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Role of α -synuclein penetration into the membrane in the mechanisms of oligomer pore formation

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Abstract

Parkinson's disease (PD) and Dementia with Lewy bodies are common disorders of the aging population characterized by the progressive accumulation of α -synuclein (α -syn) in the CNS. Aggregation of α -syn into oligomers with a ring-like appearance has been proposed a role in toxicity. However, the molecular mechanisms and the potential sequence of events involved in the formation of pore-like structures are unclear. We utilized computer modeling and cell-based studies to investigate the process of α -syn (wild type and A53T) oligomerization in membranes. The studies suggest that α -syn rapidly penetrates the membrane, changing its conformation from α -helical toward a coiled structure. This penetration facilitate the incorporation of additional α -syn monomers to the complex, and subsequent displacement of phospholipids, and formation of oligomers in the membrane. This process occurred more rapidly, and with more favorable energy of interaction for mutant A53T compared with wild type α -syn. After 4 ns of simulation for the protein-membrane model α -syn penetrated through two thirds of the membrane. By 9 ns, the penetration of the annular α -syn oligomers can result in the formation of pore-like structures that fully perforate the lipid bilayer. Experimental incubation of recombinant α -syn in synthetic membranes resulted in the formation of similar pore-like complexes. Moreover, mutant (A53T) α -syn had a greater tendency to accumulate in neuronal membrane fractions in cell cultures, resulting in greater neuronal permeability with the calcein efflux assay. These studies provide a sequential molecular explanation for the process of α -syn oligomerization in the membrane, and support the role of formation of pore-like structures in the pathogenesis of the neurodegenerative process in PD.

Introduction

α -synuclein (α -syn) is thought to be a natively unfolded synaptic protein [1, 2] that under physiological conditions plays a role in synaptic vesicle release via interactions with members of the SNARE family [3, 4]. Under pathological conditions, progressive accumulation of α -syn leading to the formation of oligomers has been proposed to play a critical role in the pathogenesis of Parkinson's Disease (PD), Dementia with Lewy bodies (DLB) and Multiple System Atrophy (MSA) jointly denominated synucleinopathies [5-7, 8-11].

In PD and DLB, which are common neurodegenerative disorders of the aging population, there is progressive accumulation of α -syn in cortical and subcortical regions of the brain leading to dementia and movements disorders [12-17]. Moreover, familial forms of parkinsonism have been associated with multiplication of the α -syn gene as well as with mutations in the N-terminus (E46K, A53T) of α -syn that enhance oligomerization [18-21]. The precise mechanisms through which accumulation of α -syn leads to neurodegeneration in PD are not completely understood, however some studies support the notion that α -syn oligomers rather than large polymers might be responsible [22]. Supporting this possibility, we showed that the most extensive in vivo neurotoxicity was observed in animals with the α -syn variants that form oligomers (i.e., E57K and E35K), whereas the α -syn variants that form fibrils very quickly are less toxic [23]. Alternative studies suggest that protofibrils and fibrils formed during the process of α -syn oligomerization contribute to cytotoxicity. For example oligomers with wreath-like shape appear to be consistent with an on-pathway role for the oligomer in the fibrillation process and suggests that oligomer-driven α -syn fibrillation has a cytotoxic potential via membrane permeabilization [24]. Previous molecular dynamics (MD) studies suggest that α -syn might undergo conformational changes resulting in the formation of propagating dimers that are more prone aggregate and form ring-like oligomers [25]. In addition, other MD simulation and biophysical studies support the view that α -syn binding to the membrane is important in this process [26-29]. Moreover, a number of studies have proposed that alternative splicing, post-translation modifications (eg: nitration, oxidation, C-terminal cleavage, phosphorylation), and interactions with phospholipids and PUFA in the membrane might play a role in the process of α -syn oligomerization [32-35].

Thus, interactions between α -syn and lipids in the neuronal cell membrane has been proposed to be an important step in the process of oligomerization and cytotoxicity [26-28, 32]. The oligomers might interfere with the normal function of cellular membranes and result in the formation of pore-like structures [37, 38]. It has been proposed that these oligomers might display channel-like activity resulting in abnormal calcium influx leading to neurodegeneration [25, 39-41]. However the molecular mechanisms and the potential sequence of events involved in the formation of these pore-like structures are unclear. To examine this process, we utilized computer modeling and simulations, as well as electron microscopy and cell-based studies to investigate the course of α -syn (wild type and A53T) oligomerization in membranes. Our studies support the possibility that α -syn penetration of the membrane plays an important role.

Results

Simulations of α -syn penetration into the membrane

To understand the molecular mechanisms of α -syn oligomerization and pore-like complex formation, MD simulations of α -syn in the membrane were performed. To facilitate the analysis of the simulations in the membrane, the initial studies were performed with a single wild type and A53T α -syn conformer as an example of how groups of α -syn molecules might behave in the membrane. We initiated the simulations by positioning the original, experimentally-defined, α -syn conformer (resolved by NMR, pdb ID 1XQ8) [42] on the surface of a 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) membrane (Figure 1A). This conformation was also used as the basis for the structure of the point mutation model of A53T α -syn. The membranes utilized for the simulations are organized into two interfacial regions (IFR) containing phospholipid polar head groups and an intrinsic hydrocarbon core (HC). To find the proper orientation of the α -syn conformer on the membrane, the MAPAS membrane-contact prediction program was used [43] (Figure 1A). Residues of the initial contact position are presented in Figure 1A. Consistent with previous studies [44], analysis of α -syn transmembrane scores using the dense alignment surface (DAS) method [45]

predicted that the region including residues 64-79 resembles a transmembrane helix (Figure 1B). This region contains a significant number of hydrophobic residues that play a critical role during the process of membrane penetration. Following the initial contact of the N-terminus with the surface of the membrane, the α -syn conformer penetrates the membrane, undergoing changes in secondary structure with portions of the α -helices converting into π -helices that eventually would extend into the β -sheet regions (Figure 1C, D).

After 2 ns of simulation of the α -syn-membrane complex (wild-type and A53T α -syn), a new coiled and turn region appeared, interrupting the α -helix around aa 70-80 (Figure 1B). During the first 3 ns of MD simulation, the region to the right of residue 38 (not shown) transforms initially to a π -helical formation (Figures 1 and 2A, B), then to a mixture of coils, 3-10 helical, and bended regions continuously evolving toward a β -strand structure (Figure 2A, B, D, E). Note that such conformations with different content of α -helical secondary structure has been recently shown in experiments [46]. Compared to wild type α -syn (Figure 2A-C), in the A53T α -syn-membrane complex (Figure 2D-F), the α -helices converted into π -helices more rapidly, and with a greater proportion of residues being involved in the π -helical formation (Figure 1C, D). During membrane penetration, the length of the α -helical region closest to the C-terminus decreased over time, evolving toward a β -strand structure (Figure 2A, B, D, E). This transformation produced an elongated structure that directly participates in the penetration of the molecule through the membrane (Figure 2).

During the process of membrane penetration, the hydrophilic groups of α -syn (wild-type protein shown, Figure 3) contact the polar lipid groups on the surface of the membrane bilayer. At the initial stage, GLN62 and ASN65 contact the oxygen atoms (hydrogen-bond acceptors) situated on the surface of the lipid structure (Figure 3A). In subsequent stages of the process of α -syn penetration (3ns and 5ns), hydrophobic residues such as VAL63 and VAL66 begin to play a role, leading to interactions with the hydrophobic parts of the membrane lipids (Figure 3B and C). During the final stage (7ns), most of the α -syn N-terminus has been transformed to an extended β -strand-like structure (Figure 3D) that facilitates penetration to the full depth of the membrane. Moreover, hydrophobic interactions of the valines with the membrane phospholipids continue to play a role at this stage. In addition, the leading hydrophilic groups (particularly ASN65) of the moving α -syn molecule interact with polar groups on the opposing side of the membrane, and with water molecules on the membrane (Figure 3D).

Further analysis of the course of α -syn penetration was performed utilizing a lateral projection in the membrane for the wild type (Figure 4A, C, E) and the A53T mutant α -syn conformers (Figure 4B, D, F). These studies showed that the leading penetrating residues in both wild-type and A53T α -syn are residues 59-72, with the first penetrating residue being ASN65 (Figure 4A and B), as shown in detail in Figure 3. Both the wild-type and A53T mutant α -syn molecules displayed greater penetration of the N-terminus domain after approximately 4 ns of MD simulation (Figure 4C and D), followed by extension of the leading edge region of the molecule at 7-8 ns (Figure 4E and F). When compared with the wild-type α -syn, penetration of the A53T α -syn molecule across the membrane occurred approximately 20% faster (Figure 4G). Consistent with the rate of penetration, levels of van-der-Waals energy diminished more rapidly for A53T than for the wild type α -syn during MD simulations (Figure 4H).

To further confirm the specificity of the results for the α -syn MD simulation studies in membranes, similar analysis was performed with β -syn, a member of the synuclein family that lacks the hydrophobic NAC domain [47]. Compared to wild-type α -syn (Figure 4), β -syn did not penetrate the membrane even after 7 ns of simulation (Figure 5A). Next studies were performed with conformers of the α -syn bearing mutations on the amino-acid residues

(E57A, E61A, K58A, Q62A, NS65A, V63A, V66A) predicted by the MD simulations to be critical for the process of membrane penetration (Figure 3). Similarly, these studies showed that compared to wild-type α -syn (Figure 4), the mutated α -syn was unable to penetrate the membrane even after 7 ns of simulation (Figure 5B).

Dynamics of the process of α -syn oligomer embedding into the membrane

We have shown that α -syn molecules might penetrate into the membrane after approximately 7ns of unrestrained MD simulation, thus next we examined whether the conformational changes of α -syn molecule during the process of membrane penetration might allow the generation of stable annular multimers on each stage of penetration. In order for consistent docking to proceed and annular structures to develop, the α -syn monomers on each stage of membrane penetration should have attractive energies of intermolecular contact. To obtain the energy-per-molecule value, the total energy was calculated for each complete oligomeric structure, and this value was divided by the number of individual α -syn molecules in the oligomer. This allowed for the comparison of oligomers containing different total numbers of α -syn molecules. We found that annular oligomers with an 8-mer array had a highly favorable energy of interaction between neighboring molecules of around -60 kcal/mol. The 9-mer and 11-mer had energies of interaction between approximately -30 and -50 kcal/mol respectively. All other possible oligomers had less favorable energy values. For the next step, we evaluated the oligomers for the energies of membrane-oligomer interactions. The structure of octamer α -syn ring presented in Figures 6, 7 has significant negative electrostatic energy. Note that this energy is decreased during the possible growing of the multimer from the single molecule to eight molecules within the multimer (Figure 7D). This fact supports the possibility of generation of such annular oligomers on all stages of transmembrane penetration.

Using the conformers of wild-type α -syn taken at different times of MD simulation, the program constructed a set of octamers embedded in the membrane on each time step of MD. Figure 6 shows the possible α -syn octamer immersion starting from initial state (Figure 6A-C), then at 4 ns (Figure 6D-F) and 9 ns (Figure 6G-I) of MD simulation. This reveals that at each stage of simulation, an oligomer can be generated that can penetrate into the membrane. The 9 ns position is shown in Figure 7A, B as a Corey-Pauling-Koltun (CPK) space-filling model, with intermolecular and multimer-membrane interactions characterized in the image inset (Figure 7C).

Next, we examined the organization of the wild-type and α -syn octameric structures and intermolecular interactions within the multimeric complex. This assembly possesses an intrinsic diameter of the opening in the middle of the oligomer of around $35\text{\AA}$. The external diameter of the 9 ns oligomer is approximately $130\text{\AA}$. In this conformation, the C-terminal tail of each α -syn molecule in the octamer, comprising the residues 94-140, are extended beyond the oligomer structure and are equally distributed in a concentric manner surrounding the interacting N-termini of the molecules. The intermolecular interactions of a single α -syn from the octamer with the membrane are described above. We analyzed the interactions between the neighboring molecules of α -syn in the octamer that are involved in organizing the oligomeric structure. We observed that several critical interactions support the close contact of single molecules of α -syn, and their binding within the oligomer. Table 1 shows the possible interactions that support the organization of the octamer after 9 ns of MD simulation. Hydrophobic interaction were counted when the distance between the side-chain heavy atoms of the neighboring residues were less than $4\text{\AA}$. Hydrogen bonds were counted when the distance between the corresponding side-chains donor and acceptor atoms of the neighboring residues were less than $2.9\text{\AA}$. Pi-cation interactions were counted between that center of the plain of the benzene ring of PHE and the positive charge of the LYS residues less than $4\text{\AA}$.

These residues participate in the intermolecular interactions depicted in the “node of interactions” that appears at the upper part of the oligomer in the octamer structure (Figure 7C). The overall organization of the oligomer shows the C-terminal tails of α -syn to be equally distributed in a radial configuration around the center of the structure; this arrangement prevents significant repulsive electrostatic interactions between these negatively charged tails. Next we evaluated the predictive value of MD simulations by comparing the dimensions of α -syn generated during the MD simulations with membrane bound recombinant α -syn aggregates analyzed by electron microscopy. For this purpose we selected annular oligomers that displayed the best energy of interaction and had an octamer array and overlay them into the electron micrographs of the α -syn aggregates (Figure 7E). Annular oligomers obtained by MD simulations or by incubation in synthetic membranes had similar characteristics ranging in diameter between 120 and 150 Å (Figure 7E).

Effects of α -syn membrane distribution in oligomerization in a neuronal cell model

To investigate the consequences of α -syn oligomerization in a cell-based system, neuronal cultures were infected with lentiviral vectors expressing wild-type or mutant A53T α -syn. By immunoblot analysis, the monomeric forms of wild type and mutant α -syn were found in equal amounts in cytosolic fractions (Figure 8A and B). In contrast, in the membrane fraction, mutant A53T α -syn monomer and oligomers were more abundant compared to cells expressing the wild-type construct (Figure 8A-B). Similarly, in native gels more abundant α -syn aggregates were detected in the membrane fraction of the neuronal cells infected with the LV-mutant A53T α -syn (Figure 8C). Immunocytochemical analysis and examination by laser scanning confocal microscopy showed that compared to control cells, the lentivirus-expressed wild-type α -syn was distributed as punctae throughout the cytoplasm of the neurons, and along the neurites (Figure 8D-E). In comparison, the A53T α -syn was more diffusely distributed in the cell bodies and tended to concentrate around the periphery of the neurons (Figure 8F).

To investigate whether the accumulation of α -syn in the neuronal membranes altered cellular function, cells expressing α -syn or empty vector were tested with the calcein permeability assay, and by Fluo-4 calcium imaging to measure intracellular calcium levels. With the calcein assay, neuronal cells expressing both wild-type or A53T α -syn displayed increased permeability, however this effect was more apparent in the cells expressing A53T α -syn (Figure 8G). Consistent with this increased permeability, both neuronal cell cultures expressing wild type or A53T α -syn displayed increased levels of intracellular calcium (Figure 8H). To further investigate if the α -syn immunoreactive signal distributed around the neurons was localized to the membrane ultrastructural immunogold analysis was performed. In neuronal cells infected with LV-control no gold particles were detected in the membrane in contrast neuronal cells infected with LV-wildtype (not shown) and mutant A53T α -syn displayed the presence of gold particles in close proximity to the plasma membrane as well as in the cytosol in association with other organelles (Figure 8J).

Discussion

Previous MD simulation and biophysical studies have shown that α -syn binding to the membrane is important in the process of oligomerization [26-29]. For the present study we utilized molecular modeling and molecular dynamics simulations of the protein during penetration to the membrane in combination with biochemical and ultrastructural analysis, to show that α -syn might be able to penetrate the cell membrane. The theoretical studies predict that α -syn molecules initially dock to the membrane surface and begin to penetrate the membrane, resulting in changes to their secondary and tertiary structures. In the initial stages of the molecule's structural changes over time, compared with the wild-type α -syn, the mutant A53T molecule possessed a greater percentage of aa residues that were involved

in α -helical conformations. After transformation of regions within the α -syn molecule from α - to π -helices, the N-terminal region of each α -syn molecule begins to shift towards a coiled structure and into a β -strand conformation. Later in the simulation times, a greater percentage of aa residues in the mutant α -syn than in the wild-type molecule were involved in π -helical structures. This is followed by eventual perforation of the membrane by the leading hydrophobic edge formed by two helices and a linker region of each α -syn molecule.

Interestingly, and in support of our observations that the polar lipid heads play an important role in α -syn-membrane interactions, previous studies have shown that α -syn binds preferentially to membranes composed of lipids with small polar head groups (e.g. phosphatidic acids), where inherent membrane instabilities may be present, and may contribute to the binding of α -syn with the membrane [48]. Similarly, a recent study demonstrated that binding of α -syn to negatively charged lipid membranes is much stronger than α -syn interactions with neutral lipid bilayers, and this interaction is less sensitive to other membrane alterations [49]. Intrinsic surface disruptions present in negatively-charged membranes could play a role in promoting α -syn membrane penetration, as bilayer charge and accessibility of the hydrocarbon core modulates oligomer-membrane interaction [48]. Consistent with these observations, the present study reveals that interactions between hydrophobic valine residues of α -syn and the membrane hydrocarbon core play a pivotal role in the process of membrane penetration of α -syn molecules. The MD simulations propose that α -syn penetration in to the membrane facilitates oligomerization and embedding that could eventually result in the formation of pore-like structures. In support of this possibility, we found similar ring-like structures when comparing the α -syn model with oligomers obtained by incubating α -syn with a synthetic lipid membrane. The characteristics and dimensions (120 and 150 Å) of our α -syn model and oligomers is very similar to those reported by Lashuel et al. [50] and Quist et al. [51].

For the present study we performed the MD simulations with full length α -syn in a neutral POPC membrane and showed a fast speed of penetration. Previous MD studies have used segments of α -syn including residues 1-41 [52], residues 35-43 [26] and 1-99 [27]. In the latter study it was shown the penetration of this fragment into the membrane during MD on the length around 5 Å. For this study negatively charged DOPS membrane was utilized. The N-terminal part of α -syn has an overall positive charge (and the C-terminal part is negative). This suggests that the truncated N-terminal part having the positive charge most probably was attracted by the negative phospholipid heads and penetrated slightly beyond their level. The position of the truncated α -syn immediately below the charged heads of the lipids can be explained by the sharp decrease of dielectric constant when moving from water to the place inside the lipid layer. The constant changes from around 80 to around 5 would cause the sharp (around 16 folds) increase of electrostatic energy of lipid- α -syn interactions.

While the present study proposes that the process of membrane penetration was energetically favorable for both wild-type and mutant A53T forms of α -syn, we have observed that the immersion of the mutant A53T monomer into the membrane occurs more rapidly than the wild-type molecule, and with more favorable Van der Waals energy of interaction. This observation provides a potential molecular mechanism that may explain previous findings that neural cells expressing mutant alpha-synuclein (A30P and A53T) have higher plasma membrane ion permeability than control cells [40]. In the present study we demonstrated using MD simulations that the mutant A53T form of α -syn possesses a greater proportion of residues involved in a π -helical formation, and over time the length of the α -helical region closest to the C-terminus decreased, evolving toward an elongated, β -strand structure that facilitates the penetration of the molecule into the membrane. Enhanced membrane penetration and distinct changes in the secondary and tertiary structure of mutant

A53T α -syn may also contribute to altered aggregation kinetics compared to the wild-type molecule. In support of this possibility, previous studies reveal that mutant A53T α -syn displays accelerated aggregation compared to wild-type α -syn [53, 54], which may be related to an increase in β -strand character of the molecule [43].

In summary, these results suggest a model where penetration of α -syn into membranes gives rise to the formation of annular pore-like oligomer structures with the ability to increase cell permeability and calcium influx. We focused as an example on α -syn octamers because they displayed favorable negative energies of interaction (around -60 kcal/mol), however other multimeric arrays are also possible in particular 9-mer and 11-mers. The MD simulations suggest three stages in this process (Figure 9). In the first, hydrophilic residues around aa 59-72 of α -syn interact with the polar heads on the surface of the membrane (Figure 9A). Next, as α -syn could penetrate into the membrane, hydrophobic valine residues interact with the non-polar tails of the lipids (Figure 9B). Finally, the hydrophilic aa residues—particularly ASN65 at the leading edge of the molecule—could participate in hydrogen bonding interactions with polar heads of the lipids on the opposing side of the membrane bilayer, and allow the penetration of the molecule through the membrane (Figure 9C). Understanding the precise mechanisms, and specific amino acid residues, involved in α -syn interactions with the membrane and with other α -syn monomers will aid in the development of molecular structure-based therapeutic approaches for disorders of α -syn aggregation.

Materials and Methods

Molecular dynamics simulations and modeling of the α -syn-membrane system

The modeling and simulations were based on the previously reported NMR structure of α -syn (PDB index 1xq8 [42]). Molecular dynamics simulations of α -syn molecule were conducted as previously described [25, 56] using periodic boundary conditions at constant pressure (1 atm) and temperature (310 K) initially with the α -syn molecule in the water box in which the shortest distance between the protein molecule and the box walls was $30\text{\AA}$. For the α -syn-membrane model, the NMR structure α -syn was located on the surface of the membrane according to the predicted membrane-contacting surface (prediction was performed using the MAPAS program [43]). This program was applied to the initial α -syn conformer and the top scoring predicted membrane attractive surface was used to dock the protein molecule to the membrane. For modeling α -syn-membrane interactions, a rectangular POPC polar lipid bilayer membrane with fixed edges, and dimensions of $100\times 180\text{\AA}$, was used.

The NAMD molecular dynamics program (version 2.5 [57]) was used with the CHARMM27 force-field parameters [58] to simulate the behavior of α -syn molecules in water under normal conditions, and the interaction of the POPC membrane with the α -syn aggregates. The temperature was maintained at 310 K by means of Langevin dynamics using a collision frequency of 1/ps. A fully flexible cell at constant pressure (1 atm) was employed using the Nosé-Hoover Langevin Piston algorithm [59, 60] as used in the NAMD software package. Initial coordinates were taken from a previously equilibrated 500ps system. The van der Waals interactions were switched smoothly to zero over the region $10\text{\AA}$ and electrostatic interactions were included via the smooth particle-mesh Ewald summation [61]. The impulse-based Verlet-I/r-RESPA method [62, 63] was used for multiple time-stepping: 4fs for the long-range electrostatic forces, 2fs for short-range nonbonded forces, and 1fs for bonded forces.

The simulation was performed in four steps. Initially the systems were minimized for 10,000 iterations. Then the systems were heated in 0.1° increments and equilibrated for 10ps; then

the molecular dynamics simulation was conducted. Data for analysis were taken between 50ps and 5ns of the simulation.

Cell culture of neuronal cells expressing wild-type or mutant A53T α -syn

B103 rat neuroblastoma cells were grown in 10 cm dishes at 50% confluence in DMEM (Mediatech) containing 10% fetal calf serum (FCS) at 37°C, 5% CO₂. Cells were infected with lentiviruses expressing α -syn (wild-type or mutant A53T) or GFP (each at 1.0×10^7 TU) prepared as previously described by transient transfection in 293T cells [64-66]. After incubation for 48 hrs, the efficiency of transduction of lenti-GFP was more than 90%. Cells were then plated onto 12-mm coverslips, or into 6-well or 96-well plates, and then processed for subsequent biochemical and functional assays.

Subcellular fractionation and immunoblot analysis of α -syn distribution in lentivirus-infected neuronal cells

To analyze the distribution and levels of α -syn in B103 cells expressing α -syn, briefly as previously described [66], lentivirus-infected cells grown on 6-well plates were homogenized and fractionated into cytosolic and membrane fractions by ultracentrifugation at 100,000 rpm for 1 hr in a TL-100 rotor (Beckman-Coulter). Approximately 20 μ g of protein per lane were loaded into 4-12% Bis-Tris gels with 3-[N-morpholino] propanesulfonic acid (MOPS)/SDS buffer and blotted onto polyvinylidene fluoride (PVDF) membranes. Blots were incubated with antibodies against α -syn (1:1000, Chemicon, Temecula, CA or BD Transduction Laboratories), followed by secondary antibodies tagged with horseradish peroxidase (HRP, 1:5000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunoreactive signals were visualized by enhanced chemiluminescence and analyzed with a Versadoc XL imaging machine using the QuantityOne program (BioRad, Hercules, CA). Analysis of actin levels was used as a loading control.

Double-labeling immunocytochemical analysis of patterns α -syn immunoreactivity in B103 cells infected with α -syn lentiviruses

For double-labeling immunocytochemical analysis, cells were infected with lentiviruses as described above and cultured on coverslips. Coverslips were then fixed in 4% paraformaldehyde for 20 min, and blocked overnight at 4°C in 10% FCS and 5% bovine serum albumin. Cells on coverslips were then incubated overnight at 4°C with the antibody against α -syn in PBST (1:2500, Chemicon or BD Transduction Laboratories). The following day, the antibody was detected with the Tyramide Signal Amplification-Direct (Red) system (NEN Life Sciences). Coverslips were then double-labeled with an antibody against microtubule-associated protein-2 (MAP2, marker of dendritic integrity, 1:250) detected with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:75, Vector Laboratories, Burlingame, CA). Coverslips were air dried overnight, then mounted under glass coverslips with ProLong Gold antifade reagent with DAPI (Invitrogen) and imaged with a Zeiss 63X (N.A. 1.4) objective on an Axiovert 35 microscope (Zeiss, Germany) with an attached MRC1024 laser scanning confocal microscope system (BioRad) [67]. All sections were processed simultaneously under the same conditions and the experiments were performed twice to assess reproducibility.

Electron microscopy and immunogold

Briefly, as previously described [56] recombinant α -syn was incubated in synthetic lipidic membranes and then aggregates were analyzed by electron microscopy 24 hrs later. The synthetic lipid monolayer was generated as described by Ford M et al [68]. For studies of α -syn distribution, cells were fixed in 0.25% glutaraldehyde and 3% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) and then pre-embedded with epoxy resin, sectioned with an

ultra microtome (Reichert Ultracut E) at 60 nm thickness and collected in nickel grids for immunogold labeling. The grids were treated with sodium periodate saturated in water and then incubated with the anti-synuclein antibody (1:100) overnight. Next day, the grids incubated with the secondary antibody IgG-anti-rabbit/ 10 nm gold particles (AURION Immunogold reagents) for 2 hrs. at room temp. The immunostained grids were post stained using saturated Uranyl Acetate and analyzed with a Zeiss EM10 electron microscope and electron micrographs obtained at a magnification of 50,000.

Dye efflux (calcein) assay

Assessment of membrane permeabilization was carried out as previously described [48] using calcein dye as an indicator (Invitrogen, Carlsbad, CA). Briefly, lentivirus-infected cells were plated at a density of 25,000 cells/well on Costar 96 well-black plates with flat clear bottom (Corning, Corning, NY). After an additional 24 hrs of incubation, complete media was replaced by 200 μ L of phenol red-free media containing 1 μ M calcein dye (Invitrogen). Cells were kept in the incubator at 37°C for 1 hr before measuring fluorescence with an excitation/emission filter at 470-495/515-575 nm on a DTX 880 Multimode Detector (Beckman Coulter, Fullerton, CA). As a positive control of dye efflux, detergent was added to control wells to lyse the cells.

Calcium mobilization FLUO-4 assay

Assessment of calcium influx was carried out as previously described [56] using a modified protocol of the FLIPR 4 calcium assay (Molecular Devices, Sunnyvale, CA). Briefly, B103 cells were infected with lentiviral constructs. Cells were infected at a MOI of 30, and two days after infection, cells were plated at a density of 30,000 cells/well on Costar 96 well-black plates with flat clear bottom (Corning). After an additional 24hrs of incubation, media was replaced by 100 μ l of HBSS buffer, and 100 μ l of calcium dye (FLUO-4 NW, Invitrogen) was added to each well. Cells were kept in the incubator at 37°C for 30 mins, followed by incubation in the dark at room temperature for an additional 30 mins before measuring fluorescence with an excitation/emission filter at 470-495/515-575 nm on a DTX 880 Multimode Detector (Beckman Coulter). As a positive control of calcium influx, 0.6 μ g of ionomycin (Sigma, St. Louis, MO) was added to control wells.

Statistical analysis

The data are expressed as mean values $\pm$ standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by post hoc Dunnett's or Tukey-Kramer tests (Prism Graph Pad Software, San Diego, CA, USA). Differences were considered significant at p values of < 0.05 .

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Figure 1. Simulated penetration of α -syn into the membrane

(A) Initial position of α -syn before membrane penetration. Highlighted residues interact with the membrane upon the initial contact. This position was obtained using our program MAPAS. (B) Prediction of the possible transmembrane character of the α -syn primary sequence. Score of the possible transmembrane character of the initial α -syn helix. The existing transmembrane helices typically have a score above 500. A score of approximately 400 suggests that this helix has a tendency to be transmembrane and may insert into the membrane. (C) Evolution of α -syn α -helices during membrane penetration. (D) Evolution Rate of formation of α -syn π -helices during membrane penetration.

Figure 2. Time course and structural analysis of α -syn penetration into the membrane

(A) Snapshots of wild type α -syn at different time points during membrane penetration (no membrane in image), (B) Snapshot of wild type α -syn at different time points during membrane penetration (membrane present). (C) Close-up of the membrane penetrating region of wild type α -syn. (D) Snapshots of A53T mutant α -syn at different time points during membrane penetration (no membrane in image), (E) Snapshot of A53T mutant α -syn at different time points during membrane penetration (membrane present). (F) Close-up of the membrane penetrating region of the A53T mutant α -syn. In images A, B, D and E: 0 ns-cyan; 2 ns-violet; 4 ns-pink; 6 ns-yellow; 8 ns-red; 9 ns-magenta. For A53T the deepest penetration is achieved at 7 ns therefore the snapshot 9 ns is not shown. Arrows point to the A53 residues (CPK, red) and T53 (CPK, yellow).

Figure 3. Characterization of the amino acid residues interacting with lipids in the membrane in the membrane-penetrating region of wild type α -syn

(A) Initial position, (B) 3 ns, (C) 5 ns and (D) 7 ns snapshots of membrane penetration by wild type α -syn. The stick-like structures represent the residues that interact with the phospholipids and the insets represent an overview of the position of the α -syn interaction with the membrane.

Figure 4. Analysis of α -syn membrane penetration utilizing lateral projection for the wild type and A53T mutant α -syn

(A, B) Initial position on the membrane of wild type (yellow) and A53T (red) mutant α -syn monomers respectively, (C, D) 4 ns position of wild type and A53T mutant α -syn respectively and (E, F) 7 ns position of wild type and A53T mutant α -syn respectively. (G) Distance between the front penetrating residue of α -syn to the median of the membrane (red – wild type, blue – A53T mutant). (H) Van der Waals energies of α -syn during membrane penetration (red – wild type, blue – A53T mutant). Arrows point to the A53 residues (CPK, red) and T53 (CPK, yellow).

Figure 5. Analysis of β -synuclein and mutated α -syn embedding into the membrane utilizing lateral projection

(A) β -synuclein initial position on the membrane and progression after 4 and 7 ns. (B) Docking and MD simulations with mutated (E57A, E61A, K58A, Q62A, N65A, V63A, V66A) α -syn. These aa substitutions are predicted to reduce interactions with lipids in the membrane. Initial position on the membrane and progression after 4 and 7 ns

Figure 6. Model of α -syn octamer penetration into the membrane

(A, B, C) Initial position of α -syn octomer on the membrane in semi-transparent membrane view, side view and view from beneath respectively, (D, E, F) 4 ns snapshot of α -syn octomer on the membrane in semi-transparent membrane view, side view and view from beneath respectively and (G, H, I) 9 ns snapshot of α -syn octomer on the membrane in semi-transparent membrane view, side view and view from beneath respectively.

Figure 7. Structure of possible octamer constructed from the 9 ns conformers of α -syn
 (A) The α -syn octamer viewed from above and (B) the side. The region highlighted in the yellow box is depicted in (C) which shows in greater detail the contact residues of neighboring α -syn molecules in the octamer. (D) Electrostatic energy changes during possible growth from the single wt α -syn molecule to octamer for the conformers taken at different times of MD of the single α -syn in the membrane. (E) Overlay of the model for the α -syn octamer with that obtained by incubating α -syn with synthetic lipid membrane and analysis by electron microscopy, merged image is on the right side.

Figure 8. Characterization of the effects of α -syn oligomers in a neuronal cell line
 (A) Immunoblot analysis of α -syn species in the soluble (cytoplasmic) and membrane-bound (membrane) fractions from B103 cells infected with LV-wild type α -syn or LV-A53T α -syn, uninfected cells were used as a control. Analysis of α -syn monomers in the soluble and membrane-bound fractions from B103 cells infected with LV-wild type α -syn or LV-A53T α -syn. (B) Analysis of α -syn oligomers in the soluble and membrane-bound fractions from B103 cells infected with LV-wild type α -syn or LV-A53T α -syn. Co-localization of α -syn aggregates and the dendritic marker MAP2 in (C-F) control cells, (C) Native gel analysis of membrane fractions from control B103 cells and neurons infected with LV-wild type α -syn or LV-A53T α -syn (D-F) cells infected with LV-wild type α -syn and (J-L) cells infected with LV-A53T α -syn. (M, N) Calcein and calcium assays respectively in control cells or cells infected with LV-wild type α -syn or LV-A53T α -syn.

Figure 9. Schematic hypothesis of α -syn annular oligomer penetration into the membrane
 (A) Hydrophilic residues of α -syn interact with polar heads on the surface of the membrane.
 (B) As α -syn begins to penetrate into the membrane, the hydrophobic residues interact with the non-polar tails of the lipids. (C) Finally, the hydrophilic residues participate in hydrogen bonding interactions with polar heads of lipids on the opposing side of the membrane bilayer allowing the molecule through the membrane.

Table 1

α -Syn amino acid residues involved in intermolecular interactions during oligomerization.

Type of interaction	Residue of the “left” α -syn	Residue of the “right” α -syn
Hydrophobic	VAL66	VAL71
Hydrogen Bond	GLN62	THR75
Hydrogen Bond	Backbone Nitrogen of VAL55	THR81
Hydrophobic	VAL55	VAL82
Hydrophobic	VAL55	ALA85
Hydrogen Bond	GLN24	THR92
Hydrophobic	LYS23 (hydrophobic region of side chain)	VAL95
Pi-cation	LYS23	PHE94