Published in final edited form as:

ACS Chem Neurosci. 2015 March 18; 6(3): 403–416. doi:10.1021/cn500332w.

Molecular determinants of α -synuclein mutants' oligomerization and membrane interactions

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Abstract

Parkinson's disease (PD) is associated with the formation of toxic α -synuclein oligomers and their penetration the cell membrane. Familial forms of PD are caused by the point mutations *A53T*, *A30P*, *E46K*, and *H50Q*. Artificial point mutations *E35K* and *E57K* also increase oligomerization and pore formation. We generated structural conformations of α -synuclein and the abovementioned mutants using molecular dynamics. We elucidated four main regions in these conformers contacting the membrane and found that the region including residues 37–45 (Zone2) may have maximum membrane penetration. *E57K* mutant had the highest rate of interaction with the membrane by Zone2, followed by *A53T*, *E46K*, *E35K* mutants, and wt α -synuclein. The mutant *A30P* had the smallest percentage of conformers that contact the membrane than all other mutants and wt α -synuclein. These results were confirmed by experiments. We identified the key amino acids that can interact with the membrane (Y38, E62, and N65 (1st hydrophilic layer); E104, E105, and D115 (2nd hydrophilic layer), and V15 and V26 (central hydrophobic layer)) and the residues that are involved in the interprotein contacts (L38, V48, V49, Q62, and T64). Understanding the molecular interactions of α -synuclein mutants is important for the design of compounds blocking the formation of toxic oligomers.

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Conflict of Interests

The authors of the manuscript do not have direct financial relations with the commercial identities mentioned in this manuscript that might lead to a conflict of interest for any of the authors.

Author contribution

IFT, YS, VLK and JPG developed the models and conducted MD, VLK performed image processing and quantitative analysis of experimental data. WW, CO, TG, MT, BS, KK and EM planned, provided and conducted experiments. All authors contributed to preparation of the manuscript.

Supporting Information

Scheme of studies, calculated data for wt and mutant α syn conformers and oligomers, and their membrane interactions. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Keywords

α -synuclein; α -synuclein mutants; oligomers; neurodegeneration; molecular dynamics; membrane interactions

INTRODUCTION

Oligomerization of misfolded neuronal proteins and peptides may play a primary role in the pathogenesis of neurodegenerative disorders, including Parkinson's disease (PD), Dementia with Lewy bodies and Multiple System Atrophy.^{1–3} α -synuclein (α syn, a protein with 140 amino acids, accumulates intracellularly in PD, forming oligomeric annular structures and amyloid fibrils, the main components of Lewy bodies). Familial early-onset forms of PD are caused by the point mutations *A53T*, *A30P*, *E46K*, *G51D*, and *H50Q*.^{4–7} Artificial point mutations *E35K* and *E57K* also show increased oligomerization and toxicity.⁸ Biophysical analysis demonstrated that *A30P* and a set of artificial mutants prepared with proline reduced α syn fibrillization propensity but could generate an increased amount of soluble oligomers.²

α syn oligomers interact with the membrane lipids and disrupt the membranes.^{2,9–13} Approximately 15% of α syn molecules are membrane-bound *in vivo*.¹⁴ Interaction with the membrane for α syn has been predicted on the basis of sequence analysis and assigned its amphipathic helices containing seven 11-residue repeats as main membrane-contacting regions.^{15–17}

There are a number of reports of molecular dynamics (MD) use simulating the behavior of wt α syn in solution and in a lipid-exposed environment; however, little attention has been paid to modeling mutant α syn. During past MD simulations of wt and *A30P* mutant α syn with the truncated C-terminal tail (residues 96–140),¹⁸ nonsignificant loss of helicity had been shown up to 90 ns. As demonstrated by the surface sensitive imaging technique, supercritical-angle fluorescence microscopy, and Förster resonance energy transfer,¹⁹ *A53T* and *E57K* mutants damaged the membrane at submicromolar concentrations. *E46K*, *A53T*, and *A30P*/*A53T* α syn peptides accelerated fibril formation compared with the wt α syn; on the other hand, *A30P* peptide decelerated fibril formation.²⁰ The role of *A30P* mutation is not completely clear, because another study with a set of experimental techniques showed the *A30P* mutant was in greater propensity to the membrane than wt α syn. It was interesting to note that in mouse wt α syn mutation *A53T* already exists, and this does not increase oligomerization.²¹ This is because other mutations in the wt mouse sequence, especially the mutation *S87N* preventing phosphorylation, compensate for the effect of *A53T* mutation.²² Extension of the human *A53T* mutant α syn tertiary structure (demonstrated by the greater radius of gyration) in comparison with wt α syn can correlate with the increased protein oligomerization.²²

Replica exchange molecular dynamics (REMD) studies have been performed on several α syn mutants. The α syn mutant *A30P*²³ showed a helical propensity for the region 18–31 reduction compared to wt, with an increase of the extension of the tertiary structure of *A30P* mutant vs wt. *E46K* mutant showed some decrease in the average gyration radius of the

protein, which agreed with the more compact structure of the protein.^{24,25} The α syn mutant *A53T* showed no significant changes in helicity in the first 100 residues, which is consistent with the NMR studies; however, the more abundant 3_{10} -helix formation was demonstrated for residues V16–A18, E20–T22, E28–E31, V74–V77, and E131–Q134, and the gyration radius for the *A53T* mutant in these MD simulations grew larger than that of wt α syn.²⁶ In addition to studies of these mutant α syn monomers, REMD analysis of 20 ns replicas of possible ensembles of α syn showed the potential for α syn to simultaneously form oligomers with high alpha-helices along with oligomers with high beta-strand content and unstructured monomers.²⁷

MD simulation studies of some of the α syn mutants have identified conformational changes leading to oligomerization that might be linked to interactions with the membrane; however, the general mechanism leading to increased oligomerization of these mutants is not completely clear. For this reason we generated by MD multiple structural conformations of α syn. We showed that there are four main regions that contact the membrane among all the mutants and that one of these regions—the region including residues 37–45 (Zone2)—has the maximum membrane penetration. We also showed that the maximum percentage of conformers contacting the membrane with Zone 2 had *E57K* mutant followed by *A53T* and *E46K*, *E35K* mutants, wt α syn, and the *A30P* mutant. We studied the possible ring oligomers formation for all mutants and analyzed their protein-lipids interaction. Based on these results, key amino acids were identified that stabilized the monomers, interacted with the membrane, and involved interprotein contacts within the rings.

RESULTS

Overview of approach

To study the structural diversity of α syn mutants and possible annular oligomers, as well as to identify aa residues involved in membrane interactions, we developed a new combined modeling approach (Figure S1). We generated different structural conformations of α syn using implicit molecular dynamics (MD) and then examined secondary and tertiary structural change conformers along their MD traces. Then we analyzed the membrane interaction of the conformers along their MD traces. We elucidated the main regions of the proteins that interacted with the membrane and calculated percentages for these regions of wt and each mutant α syn. The conformers of wt and mutant α syn were then used in attempts to estimate whether they would be able to form the propagating dimers and further annular oligomers using the consecutive docking procedure to evaluate the possibility of quaternary structural formation. As a result, we obtained the possible annular oligomers that were embedded to the membrane and then underwent MD equilibration in the membrane surrounded by explicit solvent to estimate the stability of the possible transmembrane channels created by wt α syn and its mutants. Then we analyzed the possible protein–lipid contacts for each ring structure and studied the intermolecular interactions between the neighboring α syn monomers within the annular oligomers. The general scheme of three-level sets of protein residue interactions made it possible to suggest the points of drug interactions.

α syn and its mutants' conformational changes during MD

α syn and its mutants, as the unstructured proteins had a number of different conformations (Figure 1). The secondary structure of the initial micelle-bound conformation of α syn (as in the initial PDB file) did not change significantly during 100 ns of MD in implicit solvent. No extensive unfolding was noticed during the simulation time. That is consistent with the results of 90 ns MD simulation of *A30P* α syn mutant.¹⁸ At the same time, the helical parts of the conformers underwent some changes during MD. Additional turns interrupting helices appear in the early stages of MD in *E35K* and *E57K* mutants. C-terminal tail acquired a compact form in the early stages of MD in wt, *E46K*, *H50Q*, *A53T*, and *E57K* mutants. A set of dimers leading to annular oligomers can be generated from these initial conformers. Interactions between N-terminal and the middle parts of the protein prevail in forming these dimers (Figure 2). C-terminal tails mostly do not participate in that formation. The alpha-helical content decreases toward 100 ns of MD, in the *A53T* mutant and increases in all other cases (Figure 3, Figure S2). DSSP analysis shows that in general the alpha-helical content does not change more than 20% in all cases, which is consistent with the results of Chatterjee and Sengupta.¹⁸ α syn alpha-helices are transformed into turns and loops only within specified regions. In wt, α syn are transformed to the turn-loop structures in the regions of residues 43–56, 83–87, and 96–99 (Figure 3A). Such a transformation also occurs in the mutants at the region around 39–55 residues, though this varies for different mutants. For *A30P* mutant (Figure 3B), and more profoundly for *E35K* and *H50Q* mutants (Figure 3C and E), the transformation region shifts toward the region 39–45. For *E46K* mutant, such shift occurs after around 50 ns MD point (Figure 3D). Interestingly, these regions correspond to the increased mobility regions found by Perlmutter and coauthors.²⁸ Moreover, the turn around the region 39–45 has been shown to be the most probable membrane-penetrating zone by Fantini and Yagi²⁹ for α syn. Note that during MD new alpha-helical regions start to appear in the C-terminal part of the protein (Figure 3). In wt α syn, they appear within the region 110–130 and in the later stage of MD at the region 130–140 (Figure 3A). In *A30P* mutant in the region 100–130 and also on the later stages of MD at the region 130–140 (Figure 3B). In *E35K* and *E46K* mutants the alpha-helices appear almost on the entire length of the C-terminal interrupted by small turns and coils (Figure 3C and D). In *H50Q* mutant alpha-helices appear only in the small sections 100–106 and 130–140 (Figure 3E). In *A53T* mutant most of the relatively long-term new helices appear in the section 130–140 (Figure 3F). In *E57K* mutant some new alpha-helices appear on the early stages of MD in the region 100–110 and then disappear, and on the latest stages of MD they appear in the region 130–140.

We conducted the cluster analysis of all MD conformers. This analysis defined the clans including the most represented conformational states during MD runs (Table S1). The most populated clans' centroids for wt and mutant α syn molecules were found in the MD times after 50 ns. Other small clans included one or two conformers. Only the centroids of the most populated clans were used for generation of dimers and further oligomers. Interesting to note that there were no more than four most populated clans of conformers in all mutants and wt protein. These clans' centroids are situated mostly in the second half of the 100 ns runs. For *H50Q* mutant only one clan has been determined. For each centroid we created the dimers and checked their possible propagation toward fibrils or rings. The percentage of

rings that can be generated from all dimers indirectly corresponds to the possibility of the ring to bind and further penetrate the membrane. Our analysis shows that this parameter in general corresponds to the experimental results (Figure 8E).

Elongation of conformers and their membranophilicity

Starting from the same initial conformation, the mutants and wt conformers had quite different tendencies of elongation. The *E57K* mutant and wt α syn, closer to 100 ns, had the smaller radii of gyration (R_{gyr}). Maximum R_{gyr} and corresponding elongation showed the *A30P* and *E35K* mutants, followed by *H50Q*, *A53T*, and *E46K* mutants (Figure 4A). The increase in the elongation of conformers during various MD experiments was previously demonstrated for *A30P*²⁵ and *A53T*²⁶ mutants; some decrease in elongation was also shown previously for *E46K* mutant conformers.²⁵ The gyration radii of α syn and its mutants decreased from around 40 Å at the initial state to between 14 Å and 30 Å for various mutants.

We calculated membrane-binding propensities of the mutant and wt α syn MD conformers with a 10 ns interval and elucidated the membrane interactions of these proteins with membranes using our program MAPAS.³⁰ We discovered that almost all interactions of these proteins with the membrane occur in the four zones of the α syn sequence (Figure 4B and C). These zones correspond to the regions 2–5, 39–45, 62–67, and 96–100 of the α syn sequence. We called these regions Zone1, Zone2, Zone3, and Zone4, respectively. MAPAS calculations revealed striking differences in the predicted depths of membrane penetration for different zones. When the predicted membrane penetration for Zones1, 3, and 4 calculated with the program Orientations of Proteins in Membranes (OPM)³¹ is around 5 Å (Figure 4D and Figure 5A–H); that means slight immersion to the upper level of the membrane; for Zone2 the program predicted a significantly greater immersion, up to 15 Å (Figure 4D and Figure 5I–N), indicating the possible start of membrane penetration.

Preference for Zone2 in membrane contacts of mutants' conformers

The program MAPAS³⁰ selects the best position of a protein on the membrane and calculates the membrane-binding propensity (that we call “membranophilicity”) parameters. When we explored each of the snapshot conformers of mutant and wt α syn, we found that for the wt protein the main membrane-interacting region is Zone1. For all mutants, we observed that the occurrences of the main-membrane-penetrating Zone2 increase from 40% in the cases of wt, *E35K*, and *E46K* mutants toward 60% for *A53T* and *E57K* mutant α syn (Figure 6). The mutant *A30P* shows the lowest percentage of occurrences of Zone2 contacts. Such a result can support a hypothesis that Zone2 can serve as a tip-of-the-membrane penetration process. This result is consistent with the results of Bortolus and coauthors.³² The increase in the number of conformers having the strongest interactions with the membrane in this region can definitely lead to greater membrane penetration and consistently higher mutant's toxicity. During MD, membranophilicity of all mutants varies, with wt α syn showing the lowest values. (Figure 6H). Only *A30P* has comparable membranophilicity at the last 20 ns of the MD run, which is consistent with the frequency of its Zone2-membrane contacts during MD.

Formation of membranophilic conformations in mutant and wt α syns

The conformers of the mutant and wt α syns that connect the membrane with the residues of Zone2 have the strongest tendency towards membrane penetration (Figure 6).

The wt α syn and *A30P*, *E35K*, and *E46K* mutants show the lowest percentage of the membrane contact by the Zone2. It increases in the *H50Q*, *A53T*, and *E57K* mutants. Note that such distribution correlates with the experimental gold staining results (Figure 8E).

Table 1 summarizes the contacts that stabilize these conformers. The most frequent contact residues that stabilize the two-helix bundle among all mutant and wt α syn conformers are valines 15, 26, and 37 from the N-terminal alpha-helical part and valines 63, 70, and 71 from the central alpha-helical part of the molecules. We analyzed the dynamic of the conformational changes in all of these proteins during MD, trying to understand how the mutations effected the conformational change that shifted the membranophilicity of the mutants.

1. *In wt α syn*, during MD, there are significantly fewer stabilizing hydrophobic nodes than in the majority of mutants. Note that there are only four such nodes: F4–I88, V15–V71, C29–V55, and the hydrophobic part of K45–V52 (Figure S3A). Such a limited stabilization network may cause less stable conformation with the membrane-penetrating set of residues at the tip and may permit more conformational changes that would lead to fewer propensities toward membrane penetration.
2. *In the A30P mutant α syn* (Figure S3B) after the first 5 ns the protein keeps its two-helices structure, with region 39–45 (Zone2) serving as a link/loop between the two helices. Then, at 10 ns, it transforms into the extended conformation that does not have any strong intramolecular contacts. At 20 ns, the mutant evolves into the conformation that shows interactions between the two helices initiated by the mutated P30. This proline became involved in the hydrophobic interaction, with the residue V37 creating a loop that included residues 31–37. Such structural reorganization moves region 26–30 toward region 46–54. The intermediate hydrophobic interaction node appears at 30 ns, between residues P30 and V55. In the next stage, the membranophilic conformation organizational role of P30 ends; finally, at 100 ns, the stabilizing hydrophobic interaction “nodes” appear in the α syn conformation (V37–V55, V26–V63, and the hydrophobic part of K21 with P70), and, farther from them, F4 interacts with A85 and I88 (Figure S3B). This stabilized conformation shows Zone2 in a membranophilic configuration that further leads to the possibility of deeper membrane penetration and intramembranous ring organization. The residues on the putative penetrating tip of *A30P* mutant are K45 (which has great membranophilicity), V49, and S42.
3. *In the E35K mutant α syn* (Figure S3C) the process of conformation stabilization works differently than in wt α syn. In wt α syn, negative E35 interacts with neighboring positive K34, keeping the helix in stable

conformation. When E35 is mutated into the positive residue *E35K*, a repulsion force appears between K35 and K34 that pushes K34 in another direction until it reaches the first step, stabilizing interaction with the second helix residue, E57. At this stage, the helix is corrupted in region 32–37. The transformation of the helix in this region into a loop-like structure leads to the further movement of residues, with stabilization caused by the intra-helix interaction E28–K34. Further, a set of hydrophobic nodes appears: F4–G84, L8–V82, V26–K60, V26–A56, V37–V49, and V40–V48 (Figure S3C). All these interactions lead to the relatively stable conformation that has a membranophilic loop at Zone2. At the putative membrane-penetrating tip reside the membranophilic residues Y39, S42, T44, K43, and K45.

4. *In the E46K mutant* α syn residues S42, K43, and V49 are situated on the tip of the penetrating conformation. During MD, the conformation-stabilizing hydrophobic interaction nodes F4–A85, F4–I88, K21–V70, V26–V63, and V37–V55 are introduced (Figure S3D). *In the H50Q mutant* α syn, the stabilizing hydrophobic nodes are located closer to N-terminal and the center of the molecule residues: F4–A85, Z11–A78, V15, V71, A18, V70, and only one node close to the putative penetrating tip, V37–V48. H50Q exposed V40, T44, K43, K45, and E46 after penetrating Zone2: (Figure S3E). *In the A53T mutant* α syn, we noticed the conformational changes that brought to the tip of the putative membrane-penetration region residue Y39²⁹ and also the membranophilic K43, H44, E46, and K45. The *A53T* mutant conformation and configuration of the two-helical bundle is heavily stabilized by a set of hydrophobic nodes: F4–V82, the hydrophobic part of K12–V70, V15–V70, V26–V55, V37–V48, V70, and possibly hydrogen bond T22–Q62 (Figure S3F).
5. *In the E57K mutant* α syn (Figure S3G) at 10 ns the *E57K* mutation starts to affect the protein conformational changes. Positive K57 interacts with negative E61, organizing an intermediate salt bridge. The presence of such a bridge strongly changes a possible conformational space. At the second stage (20 ns), a new electrostatic interaction node is formed between E61 and two positive residues, K57 and K58. This interaction helps to bring together the hydrophobic residues V26 and V63, creating the first hydrophobic node. Eventually a set of hydrophobic nodes appears. The first is formed by F4 interacting with A85 and I88; the other nodes are formed by A19 and V70, V26 and V63, the hydrophobic part of K32 and A56, V40 and V52, and V37 and V48 (Figure S3G). On the putative penetrating tip of the *E57K* mutant are membranophilic residues K45 and H50, along with the less membranophilic E46 (similar to the wt α syn).

Neutralization of Zone2 by additional mutants

One of the common features of all conformers presented in Figure S3 is the presence of K43 and K45 residues at the tip of Zone2. Positively charged lysine was shown to be one of the

residues with the greatest propensity to the cell membranes, which led us to hypothesize that we could exchange this residue with the residue of an opposite charge or hydrophobic residue. We conducted an *in-silico* experiment, preparing two pairs of additional mutations for all previously described wt and mutant α syns. The mutations, *K43V* and *K45V* or *K43D* and *K45D*, were added to the already mutated α syn. We selected *A53T* and *E57K* as the mutants to which these additional mutations (which could abolish Zone2 membrane binding) would have the most notable effect on their interaction with the membrane, since they had the greatest percentage of Zone2 contacts (Figure 7A and 7B). Additional K43 and K45 mutations strikingly changed the picture of the membrane contact. Actually, no Zone2 contacts remained in any of the K43 and K45 mutants (Figure 7C–F). These results could possibly lead to the design of drugs that would prevent these residues from contacting the membrane.

Generation of dimers and oligomers and selection and characterization of oligomeric rings that function as transmembrane channels

Comparative *in vitro* biophysical studies³³ demonstrated that acceleration of oligomerization rather than acceleration of fibrillization is a property of α syn mutants *A53T* and *A30P*. That is why elucidation of possible directions of oligomerization of α syn and its mutants to annular oligomeric structures is important for understanding of the mechanism of their membrane interactions.

Using the docking program Hex,³⁴ we constructed dimers for each 10 ns MD conformer for the mutant and wt α syns. The conformers were docked to their copies, resulting in the generation of distinct dimers. After docking the dimers underwent the 1.5 ns MD equilibration in the water box. All of the selected dimers showed a stable configuration after equilibration (Figure S4). These dimers were then expanded through consecutive docking (see *Materials and Methods* section). The obtained dimers undergone 1.5 ns equilibration in water box. Both members of the dimers were reasonably stable after the first ns of equilibration (Figure S5). Examples of such monomers, dimers, and rings are presented in Figure 2. The oligomers generated from α syn and its mutants were filtered to identify ring structures that could form transmembrane channels by embedding them to the membrane and MD. Most of the rings created from the most populated clans conformers with the maximum membrane-binding propensity at Zone2 generated stable rings, which were not distorted during the following MD runs (Figure S6). Energy of interaction of these rings with the membrane sharply stabilizes after the short MD equilibration (Figure S5).

We studied the membrane lipids' contacts with the protein rings embedded to the membrane (Table 2). If the prediction of the possible transmembrane statuses of such rings is reasonable, these contacts must have a specific pattern. The hydrophilic residues must connect the upper and bottom hydrophilic regions with the heads of lipids, and the hydrophobic residues must contact the hydrophobic lipids region in the middle of the membrane. In the case of *A53T* mutant, residues K80 and D121 contact the top heads-of-lipids region, V66 and V69 contact the middle hydrophobic part of the bilayer, and the group of T44, E46, and R61 interact with the bottom heads-of-lipids layer. Overall, we see the interacting hydrophobic contacts in the middle of the helices and hydrogen bond interactions

on both the top and bottom regions of these helices. The most frequent hydrophilic residues in the top level are Y38, E62, and N65 (1st hydrophilic layer); in the bottom level (2nd hydrophilic layer), E104, E105, and D115; and the most frequent hydrophobic residues interacting with the nonpolar parts of the lipids are V15 and V26 (central hydrophobic layer). In all cases the H-bond interactions can occur between the ring residues and that both the top and bottom hydrophilic layers of the membrane and hydrophobic residues interact with the central hydrophobic lipid parts at the central part of the membrane.

Interprotein contacts in the transmembrane rings

Table S2 shows the interprotein interactions in the membrane-embedded α syn rings. The neighboring monomers in all cases have quite a significant number of ring-stabilizing interprotein interactions. In all cases, these were the combinations of hydrophobic interactions, H-bond, and electrostatic interactions. When for wt α syn, *H50Q*, and *E57K* mutants interactions between the neighboring monomers in the rings are supported by the combination of hydrophobic, electrostatic forces, and hydrogen bonds, in the other mutants clearly is seen the bias toward either hydrophobic interactions (*A30P* and *E35K* mutants), or combinations of electrostatic interactions and hydrogen bonds (*E46K*, *A53T*). These results can be taken in consideration in development of strategies for removing of the membrane-embedded α syn rings.

Immunolocalization of α syn mutants to in-neuronal cell membranes

First, the relative levels of α syn expression between the wt and the mutants were analyzed by immunoblot (Figure 8A). The monomer α syn band was detected as expected at 14 kDa, with comparable levels of expression between the wt and mutant α syn. Higher molecular weight bands consistent with dimers, trimers, and higher order oligomers were observed in the wt and mutants, with the *A53T* and *E57K* mutants having the most abundant amount of protein (Figure 8A). Immunocytochemical analysis showed α syn immunoreactivity in the cytoplasm and around the edges of the neuronal cells infected with LV-control, LV- α syn wt, and α syn mutants (Figure 8B). The arrows indicate intensified staining at the edge for the wt, *E46K*, *H50Q*, and *E57K* infected cells, whereas *A30P*-infected cells showed more diffuse staining throughout the cells (Figure 8B). To further determine the subcellular localization of the wt and mutants of α syn, immunogold and electron microscopy was performed (Figure 8C). These studies showed that gold particles were often found in close proximity to the cell membrane. Gold particles close to the neuronal cell membrane were more abundant in the *H50Q*, *A53T*, and *E57K*- α syn infected cells and less abundant in the *E46K*, *E35K*, *A30P*, and wt- α syn infected cells (Figure 8C).

Consistent with our theoretical results, those mutants (*E57K*, *E53T*) that displayed a greater affinity to interact with membranes via Zone2 and therefore a higher propensity to form ring oligomers were the same that showed by electron microscopy a higher concentration of gold particles in the neuronal cell membranes and a greater number of tetrameric oligomers (demonstrated by western blot) (Figure 8E).

Discussion

Our study showed that increased oligomerization of α syn mutants might be associated with greater membranophilicity and a propensity to penetrate the membrane. We analyzed snapshots of all mutant and wt α syn molecules during MD for their propensity to interact with the membrane. Our studies showed that there are four domains within α syn that mostly contact the membrane, including aa 3–6, 39–45, 63–67, and 94–99, which we denominated Zone1 to Zone4, respectively. We demonstrated that, compared to other domains, the aa within Zone2 predicted increased membrane penetration. The presence of defined domains within α syns that interact with the membrane is consistent with the findings of previous studies³⁵ that used fluorescent probes and ESR to show that the N-terminal region was the most immersed in the membranes around residue 3 (corresponding to our Zone1), followed by the region around residue 90 (our Zone4), and that the C-terminal was the least immersed. The increased propensity of α syn Zone2 to penetrate the membrane is consistent with the findings of previous studies^{28,29,36} that used mutants *A30P*, *A53T*, and *E46K* in sodium dodecyl sulfate (SDS) detergent micelles and in 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (DOPS) lipid bilayer. MD simulations were performed with the explicit solvent method for a maximum of 65 ns for the 1–99 portion of the α syn²⁸. In all these cases, the highest level of protein motion occurs in two regions: the first around 39–45 residues (equivalent to Zone2) and the second around the residues 64–74 (equivalent to Zone3). Moreover, Fantini and Yaji²⁹ demonstrated, using monolayer experiments with short peptides from the α syn sequence and MD, that tyrosine 39 insertion through the membrane is a molecular basis for the glycosphingolipid-binding specificity of α syn and its membrane penetration. The same sphingolipid-binding domain is also common for beta-amyloid and prions.^{29,37} Di Pasquale and coauthors,³⁶ using MD and electrophysiological measurements, showed that the major effects of applying the *E46K* mutation were the alteration of the channel properties of α syn oligomers and the generation of nonstop activity. This mutation is located directly in the region 35–46 (Zone2), which is a bend/loop break between the helices in α syn and is a main candidate for initiation of the membrane penetration of the protein.

NMR studies^{38,39} showed that micelle-bound α syn contains two alpha-helical regions: V3–V37 and K45–T92 with unstructured C-terminal tail. The unstructured loop including residues 38–44 (Zone2) links these two helices. A break in the helical structure at region 36–45 (Zone2) of the vesicle-bound α syn has also been found using NMR.⁴⁰ Helix breaks at residues 35–43 (Zone2) were demonstrated using electron spin resonance and MD simulations.³² Recently, evidence has accumulated regarding the secretion of α syn in extracellular space and its interaction with specific lipids.^{36,41,42} Using the NMR technique, Waudby and coauthors demonstrated that, with the SDS micelles bound to α syn, the alpha-helical regions remained mostly stable.⁴³ Three regions underwent transformations into unstructured loops: 39–45 (Zone2), 63–67 (Zone3), and, to a lesser extent, 83–87 (Zone4). These results correspond to our findings that there are four main zones of contact of α syn conformers with the membrane, with the Zone2 contacts having significantly greater membrane propensity and penetration. Finally, our results agree with a recent study that used a combination of solid-state and solution NMR spectroscopy to characterize the

conformations of α syns bound to lipid membranes.⁴⁴ This study showed three α syn regions, including an N-terminal helical segment performing the role of membrane anchor, an unstructured C-terminal region that is weakly associated with the membrane, and a central region acting as a sensor of lipid properties and determining the affinity of α syn membrane binding.⁴⁴

Consistent with our previous studies using wt α syn,^{12,45} we found that α syn mutants have a greater propensity to interact with and penetrate the membrane through the domain of Zone2 in comparison with the other Zones, that is frequent in the generated conformers with the best energies that forms dimers and oligomers. These oligomers have significant interaction with the membrane as they form possibly pore-like structures able to lead to outer ions influx and eventual cell death. These oligomers were stable in the membrane because of specific membrane–protein interactions. The oligomer rings interact with the hydrophilic residues in the top of the membrane that most probably organize the H-bonds with the lipid heads. In the middle hydrophobic area of the membrane, the ring proteins interact using their hydrophobic residues, while, at the bottom of the membrane, proteins interact using their hydrophilic residues. Such an arrangement leads to stable embedding of the rings in the membrane.

Of the various mutations investigated, our study showed that the mutants *E57K* and *A53T* adopted conformations that most frequently favored possible Zone2 interactions with the membrane. *H50Q* is the next in this frequency, and *A30P* is the last. The results of the MD simulation studies are in agreement with our western blot and immunogold results and are consistent with recent studies¹⁹ showing that *E57K*, followed by *A53T* and wt, has the most interactions with the membrane; the results are also in agreement with those produced by Ono and coauthors showing that *A30P* has less propensity to interact with the membrane.²⁰ Analysis of the frontal region of Zone2, the zone that would lead in membrane penetration, showed that, in all cases, K43 and/or K45 were present. We added additional mutations of these two residues to the opposite charge aspartic acid and hydrophobic valine. These mutations led to completely eradicating Zone2 as a possible membrane-contacting region. Our predictions correlate with the experimental results⁴⁶ demonstrating that *K45E* mutation of α syn significantly delays its aggregation.

The general dimensions and number of monomers in the rings predicted by our method correspond to the experimental results. The pore-like annular oligomers with diameters 80–120 Å have been shown using electron microscopy for wt α syn⁴⁷ and its mutants *A53T* and *A30P*.⁴⁸ Analysis of the EM images shows possible numbers of protein monomers 5 to 7 in these oligomers. Recently cryo-EM images of wt α syn annular oligomers of with polygon structures including from 3 to 6 monomers and diameter around 100 Å were demonstrated by Zhang and colleagues.⁴⁹ Our annular oligomers obtained from α syn dimers have from 5 to 7 monomers of α syn. Interesting to note that a percentage of annular oligomers in relation to the total number of dimers for wt and mutant α syn (one dimer can generate only one oligomer, some of the dimers can generate rings, some - fibrils, and some do not generate any oligomeric structures) corresponds to our experimental data (Figure 8E). This percentage is higher for *E57K*, *A53T*, and *H50Q* mutants and lower for *E46K*, *E35K*, wt, and *A30P* mutants of α syn that correlates with the experimental gold staining results.

Further supporting our initial MD results is the fact that the tertiary structures changes reflected in the radii of gyration are consistent with the small-angle X-ray scattering (SAXS) results⁵⁰ demonstrated that the gyration radii for wt α syn and its mutants *A30P*, *E45K*, and *A53T* are between 20 and 42 Å. In our MD simulations for wt α syn and its mutants radii of gyration are in the range from 14 to 40 Å. In this study we used the single trajectories of MD for all mutant α syn. We conducted additional MD run for 80 ns for wt α syn. Comparison of conformers from two runs shows that the RMSD difference between the same heavy atoms of the same time snapshots of two runs is not more than 2 Å. We consider the presented MD simulations and other results as a proof of concept. We plan to continue these studies with simulating multiple trajectories for all mutants and wt α syn.

We therefore concluded that developing strategies that will prevent interactions of α syn with the membrane might reduce the formation of oligomers and cell toxicity. Our studies suggest four possible points for intervention to abolish the generation of ring-like α syn oligomers. First, residues that serve in the hydrophobic nodes stabilizing the monomer conformers with the part of Zone2 that interacts with the membrane—here, small molecules affecting the conformational evolution of α syn in solution leading to other, less toxic conformations. Second, our *in-silico* experiments showed that mutations of K43 and K45 may lead to completely eradicating Zone2 contacts with α syn mutants. Compounds that would bind to this region may prevent the toxic oligomers' formation in the membrane. Third, residues involved in intermolecular interaction in the rings can also serve as points of intervention—here, compounds that would have a stable binding to the specific protein regions and serve as the interface inhibitors. Fourth would be the residues of the ring oligomers, which interact with the membrane. This is most challenging point, because the residues can change their interaction regions depending on the environment. For example, lysines can interact with the hydrophobic regions of lipids with their hydrophobic stems, while the charged region would be stabilized by the neighboring residue of the same or a neighboring protein.

In summary, this study suggests that oligomer-prone α syn mutants favor conformations that result in the increased interaction of Zone2 with the membrane. Understanding the conformational diversity of α syn mutants might be important for the rational design of small molecules to block the formation of their toxic oligomers.

MATERIALS AND METHODS

Molecular dynamics simulations of α -synuclein and its mutants

The micelle-bound form of α syn (PDB ID 1xq8) was used as the initial structure.³⁸ The structure was mutated into *A53T*, *A30P*, *E46K*, *E35K*, *E57K*, and *H50Q* using the VMD program.⁵¹ We conducted implicit molecular dynamics (MD) for 100 ns using wt α syn and its mutants as the initial structures, along with the NAMD program (version 2.7).⁵² The CHARMM27 force-field parameters⁵³ were used for MD simulations. The temperature was maintained at 310K. We used the Generalized Born using Molecular Volume (GBMV) solvent model and Generalized Born Implicit Solvent (GBIS) method. A dielectric constant of water 78.5, intrinsic radius of atoms 0.09 Å, molar ion concentration 0.15 mol/dm³ was used. Solvent-accessible surface area (SASA) was calculated using the Linear Combination of Pairwise Overlap (LCPO) method.⁵⁴ Surface tension was maintained at 0.005

kcal/mol/Å². The alpha cutoff used for calculating Born radius was 15 Å, and nonbonded parameters were cut off at 17 Å. The simulation was performed with initial minimization for 10,000 iterations followed by 0.1° increments heating and equilibrated for 1 ns; then the molecular dynamics simulation was conducted. Data for analysis were taken every 100 ps throughout the simulation. For the water box MD simulations we used the NAMD 2.6 program with the CHARMM27 forcefield with CMAP correction at the temperature 310K stabilized using Langevin dynamics and with the fully flexible cell at constant pressure (1 atm) supported by Nosé–Hoover Langevin piston algorithm.^{55,56} The studied molecular system had at least 30 Å separation from the box walls. The TIP3P water model was used.

Analysis of mutant and wt α syn conformers

The secondary structures of all MD conformers have been analyzed using the program Define Secondary Structure of Proteins (DSSP).⁵⁷ The results were used for elucidation of the specific region in these proteins. Gyration radii were calculated as the parameters related to the protein elongation of all conformers. Membranophilicity parameters for all of them were also measured with our program, MAPAS.³⁰ The energies of the membrane interactions of these conformers and possible membrane-penetration depths were measured using the Positioning of Proteins in Membranes (PPM) server.³¹ Conformers undergone clustering by structural similarity using Shindyalov's algorithm⁵⁸ with the maximum cutoff Z-scores and minimal RMSD between selected members of the clan. The conformer having the best Z-score along with the smallest summary RMSDs of C- α atoms to the same atoms of other conformers was selected as the centroid and used as a representative of its clan.⁵⁹

Elucidation of the tertiary structure elements interacting with the membrane

To address a question of possibly common membrane-contacting regions in the α syn and mutants, we studied interaction with the membrane of the mutant and wt α syn conformers for each 10 ns step of their MD traces. Membrane propensities were studied with the programs PPM³¹ and MAPAS.³⁰

Dimer and oligomer construction

The conformers representing the most populated clusters of the 100 ns MD run were used for generation of possible annular oligomers. Using the docking program Hex based on the Fast Fourier Transform (FFT) method,³⁴ selected conformers were docked to the copies of themselves, and the best energy dimers were chosen. After that, we conducted equilibration on each of the obtained dimers in a water box. Such time was sufficient to obtain the stable dimer configurations (Figure S4). During the first 100 ps of the equilibration possible close contacts introduced by the rigid body docking are corrected and during further 1 ns most of the dimers stabilize their conformations and their RMSD do not change significantly after this time. Then, we consequently docked additional copies of the same conformer (one at a time) to each of these dimers, with docking rotation and translation parameters, as in the equilibrated dimer, to extend the dimers. This procedure used three-dimensional (3D) transformation matrices that were preliminarily calculated for each of the starting dimers. Such procedure has been implemented by Yu and Zheng.⁶⁰ This step-by-step extension of the original dimer led to three different possible scenarios. The first scenario unfolded when adding an additional α syn molecule to the dimer became impossible. We called it

“nonpropagating dimer.” In the second scenario, a dimer could propagate, resulting in the eventual formation of a fibril. In the third scenario, a dimer could be consistently enlarged to form an annular structure (“propagating dimer”). For further studies, we used only the conformers that formed propagating dimers, leading to annular oligomers.

Elucidation of rings' stability in the membrane

We selected the annular oligomers with the maximum membrane propensity, or “membranophilicity.” For this, we used the PPM server.³¹ This server uses the polarity profile of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) bilayer, determined by neutron and X-ray scattering, and distribution of water in DOPC bilayer, determined by spin-labeling experiments. Then, we embedded the selected rings to the combined membrane containing 30% 1,2-palmitoyl-oleoyl-*sn*-glycero-3-phosphoserine (POPS) and 70% 1,2-palmitoyl-oleoyl-*sn*-glycero-3-phosphocholine (POPC) lipids and conducted 0.8 ns MD equilibration for each ring in the explicit water environment to study their stability. We measured the electrostatic and van der Waals energy of interactions of the embedded oligomers with the membrane. All explored oligomers remained stable in the membrane after this procedure (Figure S5).

Study of intermolecular interactions between the neighboring molecules of α -synuclein in oligomers

We measured the distances between the heavy atoms of the neighboring monomers of α syn within the ring formations. We considered the possible hydrophobic interaction when the minimum distance between the centers of heavy atoms of the neighboring amino acid was less or equal to 4 Å. We considered the possible H-bond interaction if the distance between the centers of bond-creating atoms was equal or less than 3 Å. We considered the possible electrostatic interaction if the distance between the centers of charged heavy atoms was less or equal 4.5 Å.

Construction of lentiviral vectors

The LV-wt α syn was characterized in a previous study.⁶¹ LV- α syn mutant vectors *A30P*, *E35K*, *E46K*, *H50Q*, *A53T*, and *E57K* α syn were prepared by site-directed PCR mutagenesis (Agilent Technologies). Lentiviruses were packaged by the transient transfection of the three packaging plasmids and the vector plasmid in 293T cells, as previously described.^{62,63}

Neuronal cell line treatments, lentiviral vectors

The rat neuroblastoma cell line B103 was used for our *in vitro* experiments because this cell line displays neuronal features, and we have shown that the pathological features of synucleonopathies emerge when infected with LV- α syn.⁶² B103 cells were plated at 3.5×10^4 cells/well on poly-L-lysine-coated glass coverslips. Cells were allowed to attach for 6 h before being infected with LV-control, LV-wt α syn, or LV-mutant α syn for 48 h (MOI = 50).

Immunoblot

The levels of LV-wt and LV-mutant α syn were analyzed using B103 cell lysates that were extracted and fractioned into soluble and insoluble fractions by ultracentrifugation.⁶⁴ Protein (20 μ g/lane) was loaded onto 4-12% SDS/PAGE gels, blotted onto PVDF membranes, and incubated with anti- α syn (SYN211; 1:1,000; Millipore) followed by HRP-tagged secondary antibodies (1:5,000; Santa Cruz Biotechnology). Bands were visualized by enhanced chemiluminescence (ECL; PerkinElmer, Boston, MA). β -Actin (1:3,000) was the loading control.

Immunocytochemistry

B103 cells were fixed in 4% paraformaldehyde and then immunolabeled with antibodies against α syn (SYN211, Millipore). Briefly, coverslips were incubated overnight at 4°C with antibodies against total α syn (1:500, affinity purified rabbit polyclonal, Millipore),⁶⁵ followed by biotin-tagged anti-rabbit IgG1 (1:100, Vector Laboratories, Inc., Burlingame, CA) secondary antibodies, Avidin D-HRP (1:200, ABC Elite, Vector), and visualization with diaminobenzidine (DAB). Sections were scanned with a digital Olympus bright field digital microscope (BX41).

Electron microscopy and immunogold analysis

B103 cells infected for 48h with LV-control, LV-wt α syn, or LV-mutant α syn were post-fixed in 1% glutaraldehyde, treated with osmium tetroxide, embedded in epon araldite, and sectioned with an ultramicrotome (Leica, Germany).⁶⁵ Sections were mounted on nickel grids, etched and incubated with the α syn antibody (1:50), and labeled with 10 nm Aurion ImmunoGold particles (1:50, Electron Microscopy Sciences, Fort Washington, PA) with silver enhancement. Electron micrographs (25,000 $\times$ magnification) were obtained.

Image processing and analysis

Immunoblot image quantification—The immunoblot image was quantified using the program ImageJ, v. 1.46r (National Institute of Health, Bethesda, MD). The obtained image was 205 $\times$ 290 pixels with 100 dpi resolution. A rectangular mask with axes 30 $\times$ 24 pixels was created. The patches were marked with the mask. The *Set Measurements* menu was used to measure Min and Max Gray Value, Centroid, and Integrated Density. The brightness of each patch was measured and then inverted to correspond to expression.

Electron micrograph image quantification and particle analysis—The obtained electron micrographs are 372 $\times$ 600 pixels at 300 dpi. To process them, the program ImageJ, v. 1.46r (National Institute of Health, Bethesda, MD), was used. The algorithm includes the following steps: Convert RGB images to 32-bit gray-scale images; enhance the image contrast using the *Normalize* function, set to 0.6% of the *Saturated Pixels* value; *Find Edges*, using a Sobel edge detector with 3 $\times$ 3 convolution kernels for vertical and horizontal derivatives; set a threshold to increase the minimum threshold value; remove noise, using the *Despeckle* median filter; use the *Remove Outliers* filter; and use *Analyze Particles*, which created a list with a particles count and provided the area and location of each particle.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful for computational support from the San Diego Supercomputer Center (SDSC) at UCSD.

Funding

Funding was provided by NIH grant AG188440 and Neuropore Therapies Inc. grant to EM.

Acronyms and Abbreviations

3D	three-dimensional
aa	amino acid
DAB	diaminobenzidine
DOPC	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphocholine
DOPS	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphoserine
DSSP	Define Secondary Structure of Proteins (program)
ELC	enhanced chemiluminescence
EM	electron microscope
FFT	Fast Fourier Transform
GBIS	Generalized Born Implicit Solvent (method)
GBMV	Generalized Born using Molecular Volume
LV	lentivirus
LCPO	Linear Combination of Pairwise Overlap (method)
MAPAS	Membrane-Associated-Proteins Assessment
MD	molecular dynamics
MOI	multiplicity of infection
MSA	Multiple System Atrophy
OPM	Orientations of Proteins in Membranes
PD	Parkinson's disease
POPC	1,2-palmitoyl-oleoyl- <i>sn</i> -glycero-3-phosphocholine
POPS	1,2-palmitoyl-oleoyl- <i>sn</i> -glycero-3-phosphoserine

PPM	Positioning of Proteins in Membranes (WEB server)
REMD	Replica Exchange Molecular Dynamics
SASA	solvent-accessible surface area
SAXS	small-angle X-ray scattering
SDS	sodium dodecyl sulfate
wt	wild type
αsyn	α-synuclein

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Figure 1.

20 ns interval MD snapshots of mutants and wt α syn conformers. The secondary structure of the initial NMR conformation containing a significant percentage of alpha-helices changes during MD. Additional turns interrupting alpha-helices appeared on early stages of MD in *E35K* and *E57K* mutants. C-terminal tail became less extended on early stages of MD in wt α syn and *E46K*, *H50Q*, *A53T*, and *E57K* mutants.

Figure 2.

Examples of possible dimers propagating to annular oligomers. These dimers are generated from the initial conformers by selection of the best energy docking positions, consecutive elongation (see *Materials and Methods* section), and MD equilibration in water box. The annular oligomers have from 5 to 7 monomer proteins.

Figure 3.

Secondary structure evolution during MD of mutant- and wt- conformers. wt α syn (A) and mutants: A30P (B), E35K (C), E46K (D), H50Q (E), A53T (F), and E57K (G). Secondary-structure bar: T—turn, E—beta strand, B—beta-turn, H—alpha-helix, G—3₁₀-helix, I—pi-helix, C—coil. The turn-loop secondary structures appear in wt α syn at the regions of residues 43–56, 83–87, and 96–99. In E35K and H50Q these structures appear at the region 39–45. The new alpha-helical structures start to appear at the C-terminal of the wt and all mutants of α syn.

Figure 4.

α syn tertiary structure evolution and interactions with membrane during MD. (A) Evolution of gyration radii of these proteins during MD. Maximum gyration radii showed the *A30P* and *E35K* mutants, followed by *H50Q*, *A53T*, and *E46K*. (B) Membrane interactions of wt and mutant α syn. The majority of interaction with the membrane of all selected with the interval 10 ns wt α syn and mutant conformers occurred in the four regions of the wt α syn sequence. These regions are located at the residues 2–5, 39–45, 62–67, and 96–100. We called these regions Zone1, Zone2, Zone3, and Zone4. (C) Zones of the greatest membrane interactions for all mutants and wt α syn. (D) Depth of membrane penetration of proteins depending on the region of contact. Penetration to around 5 Å means slight immersion to the upper layer of the membrane, For Zone2 we predicted a significantly greater immersion, up to 15 Å indicating the possible start of the further membrane penetration.

Figure 5.

Membrane interaction of mutant and wt α syn conformers. (A) wt 80 ns conformer contacting the membrane within Zone1. (B) A30P mutant 60 ns conformer contacting the membrane within Zone1. (C) A53T mutant 20 ns conformer contacting the membrane within Zone3. (D) H50Q mutant 100 ns conformer contacting the membrane within Zone3. (E) wt α syn 50 ns conformer contacting the membrane within both Zone1 and Zone2. (F) A53T mutant 40 ns conformer contacting the membrane within Zone4. (G) E57K mutant 80 ns conformer contacting the membrane within Zone4. (H) H50Q mutant 80 ns conformer contacting the membrane within Zone4. (I) A30P mutant 100 ns conformer contacting the membrane within Zone2. (J) A53T mutant 100 ns conformer contacting the membrane within Zone2. (K) E57K mutant 100 ns conformer contacting the membrane within Zone2. (L) E35K mutant 80 ns conformer contacting the membrane within Zone2. (M) H50Q mutant 40 ns conformer contacting the membrane within Zone2. (N) E46K mutant 20 ns conformer contacting the membrane within Zone2.

Figure 6.

Membrane interactions of wt and mutant α syn MD conformers. As presented in Figure 4, the main predicted regions of interactions of all wt and mutants conformers with the membrane are Zones1–4. Zone1—residues 2–5, Zone2—residues 39–45, Zone3—residues 62–67, and Zone4—residues 96–100 of the α syn sequence. Interactions with the membrane differ for various mutants. For all mutants the occurrences of the main-membrane-penetrating Zone2 increase from 40% in the cases of wt and *E35K* and *E46K* mutants toward 60% for *A53T* and *E57K* mutant α syn. The mutant *A30P* shows the lowest percentage of occurrences of Zone2 contacts. Such a result can support a hypothesis that Zone2 can serve as a tip-of-the-membrane penetration process. Percentages of these specified regions of the proteins interacting with the membrane during 100 ns MD: (A) wt, (B) *A30P*, (C) *E35K*, (D) *E46K*, (E) *H50Q*, (F) *A53T*, and (G) *E57K*. (H) Membranophilicity of conformers during MD. Wt and *A30P* mutant α syn show the lowest values of the membrane-binding propensity at last 20 ns of MD which is consistent with the Zone2 contacts prediction of these proteins.

Figure 7.

Percentage of conformers contacting the membrane within Zone2 in *A53T* (A) and *E57K* (B) mutants, with the additional mutations *K43D* (C) and *K45D* (D) and *K43V* (E) and *K45V* (F) during MD. Both additional mutations completely eradicate the Zone2 contacts with the membrane. These results predict that substitution of the lysines 43 and 45 in α syn to either opposite charge residues (aspartic acid) or hydrophobic residue (valine) would completely eradicate the possible Zone 2 contacts with the membrane and consistently will significantly inhibit the α syn membrane penetration.

Figure 8.

In-vitro analysis of α syn wt and mutant peptides using western blot, immunocytochemistry, and electron microscopy. (A) Representative western blot showed the presence of α syn monomer as well as dimers and oligomers for wt and mutant α syn with the greatest accumulation of protein for *A53T* and *E57K* α syn mutations. (B) Immunocytochemistry of B103 cells infected with LC-control and LV- α syn wt and α syn mutations. Arrows indicate intensified staining at the edge for wt, *E46K*, *H50Q*, and *E57K* infected cells, whereas *A30P*, *E35K*, and *A53T* infected cells had more diffuse staining throughout the cells. Scale bar = 10 μ m. (C) Representative electron micrographs from cells infected with LV-control (Cntl), LV-wt, or LV- α syn mutants show immunogold deposits associated with the edge of the cells for all LV- α syn infected cells, which was somewhat increased in *E46K* infected cells, and (D) greatly increased in *H50Q*, *A53T*, and *E57K* α syn infected cells. Scale bar = 1 μ m. (E) Percentages of conformers contacting the membrane by Zone2 during MD (conformers), western-blot-defined tetramers, and immunogold deposits (Gold staining), percentages of ring theoretically formed from the dimers (Ring percentage) for mutant and wt α syn. The two greatest values for *E57K* and *A53T* are similar for both the theoretical and experimental methods.

Table 1

Contacts stabilizing monomers of wt and mutant α -synuclein at 100 ns.

Protein						
wt	H50Q	A30P	E35K	E57K	A53T	E46K
I88-F4	A85-F4	F4-A85	G84-F4	A85-F4	A78-F4	A85-F4
V71-V15	A78-A11	F4-I88	V82-L8	I88-F4,	V82-F4	I88-F4
V55-A29	V71-V15	V70-K21	A56-V26	V70-A19	V70-K12	V70-K21
Stabilizing contacts	V52-K34	V70-A18	V63-V26	V63-V26	V70-V15	V63-V26
		V55-V37	V49-V37	A56-K32	Q62-T22	V55-V37
			V48-V40	V52-V40	V55-V26	
				V46-V37	V48-V37	

Table 2

Contacts of the embedded rings with the membrane

Protein	Top hydrophilic region	Middle hydrophobic region	Bottom hydrophilic region
WT	GLU 104	hydrophobic part of LYS32	TYR39
	GLU 105	VAL 55	LYS43
	GLU 110		
A30P	GLU114	VAL15	LYS34
	ASP115	VAL26	TYR39
	GLU137	VAL63	
E35K	GLU29	VAL16	ASP2
	LYS35	hydrophobic part of LYS21	LYS6
	HIS50		
E46K	GLU105	ALA76	LYS54
	ASP115	VAL77	GLU61
		hydrophobic part of LYS50	ASN65
H50Q	ASP98	ALA19	GLU57
	GLN99	hydrophobic part of LYS23	LYS60
	GLU104	VAL71	GLU61
			ASN65
A53T	LYS80	ALA63	THR44
	ASP121	VAL66	GLU46
E57K	GLU104	ring of TYR39	HIS50
	GLU105	hydrophobic part of LYS43	
		VAL40	