

Cytosol-Exposed Lysine Residues Affect the Cytosol-Membrane Distribution and Serine-129 Phosphorylation of Alpha-Synuclein

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Abstract

Familial Parkinson's disease and other neurodegenerative diseases known as synucleinopathies are strongly associated with α -Synuclein (α S) missense mutations. One of these mutations, E46K, had been hypothesized to increase the electrostatic attraction of α S to neuronal vesicle membranes due to positively charged lysine that attracts negatively charged phospholipid head groups. Here, we confirm the biochemical mechanism of E46K through four compound mutants, each with three replacements with lysine on a cytosol-exposed position of the α S alpha helix. We show that the cytosolic α S to membrane-bound α S ratios are significantly lower, and that the phosphorylation rates of serine-129-a pathological marker-are pronouncedly higher for the mutants than for wild type. This experiment addresses the previous knowledge gap in the understanding of basic amino acid replacements in cytosol-exposed positions of α S. Importantly, the validated effect of cytosol-exposed lysine residues has implications for exploring the mechanism of pathogenesis of α S mutants in familial Parkinson's disease.

Keywords: α -Synuclein, Parkinson's disease, neurodegenerative disorder

1. Introduction

α -Synuclein (α S) is a neuronal protein that plays a central role in the pathological pathways of synucleinopathies, a group of neurodegenerative diseases comprising Parkinson's disease (PD), multiple system atrophy (MSA), dementia with Lewy bodies (DLB), and the Lewy body variant of Alzheimer's disease (AD) (Burré et al., 2017). In particular, several types of aggregates containing α S, including the accumulation of misfolded α S oligomers and Lewy bodies, are signature pathologies of PD (Vargas et al., 2014; Burré et al., 2017). Sporadic and familial PD (fPD) is strongly linked to the α S gene *SNCA*; specifically, fPD is associated with α S missense mutations (Krüger et al., 1998; Zarranz et al., 2004; Appel-Cresswell et al., 2013; Ciely et al., 2013; Lesage et al., 2013; Pasanen et al., 2014), locus triplication (Singleton et al., 2003), increased gene expression (Fuchs et al., 2008).

The α S protein consists of 140 amino acids, of which the N-terminus forms amphipathic alpha-helix with nine 11-residue repeats characterized by the consensus core motif KTKEGV (Bendor et al., 2013). This periodicity is also present in other amphipathic alpha-helical domains of apolipoproteins. Specifically, α S classifies as an A2 lipid-binding helix, which is characterized by basic residues, mostly lysine compared to arginine, between the polar and apolar sides, positioned $\pm 100^\circ$ from the apolar side's center, and several glutamates on the surface of the polar side (Zhu and Fink, 2003). Of the nine repeats of α S, repeats #6, #8, and #9 are poorly conserved, and repeats #3 and #4 fully conform to the core motif (**Figure 1A**). The alpha-helical structure of this domain has been represented as a helical wheel (Bendor et al., 2013; Dettmer, 2018); thus, this paper will also use this schematic representation (**Figure 1B**). Positions 11, 7, 3, 10, and 6 make up the helix's hydrophobic side and interact with the similarly hydrophobic fatty acid chains of membrane phospholipids. Positively charged lysine residue on positions 4 and 2 electrostatically interact with negatively charged phospholipid head groups. The remaining positions (8, 1, 5, and 9) are primarily hydrophilic or polar amino acids, and consequently have a higher affinity for the cytosol than the membrane. All known PD-associated point mutations, which include A53T (Polymeropoulos et al., 1997), A30P (Krüger et al., 1998), E46K (Zarranz et al., 2004), H50Q (Appel-Cresswell et al., 2013), A30P (Krüger et al., 1998), and G51D (Lesage et al., 2013) occur in the N-terminal domain. Along with this N-terminal domain, the highly hydrophobic non-amyloid-beta component (NAC) and the C-terminal domain make up the three major domains of this small protein (Piqué et al., 2016).

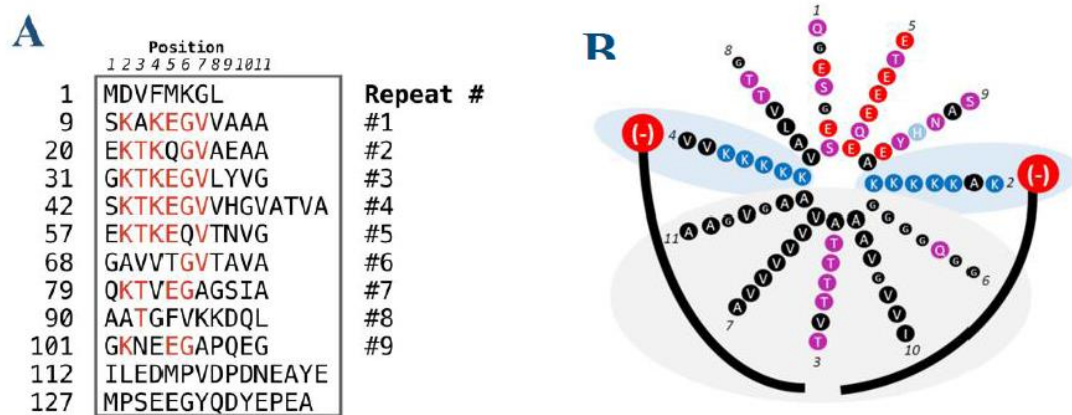


Figure 1. The structure of wild-type human α S

(A) The complete sequence of human α S aligned by position (top), highlighting the repetitive structure of α S. The amino acid counts are on the right and the repeat number on the left. Amino acids that conform to the core motif “KTKEGV” are shown in red. (B) The helical wheel diagram of α S depicting repeats 1-7, with the “ATVA” in repeat 4 omitted. The colors blue, light blue, red, purple, and black respectively indicate basic, weakly basic, acidic, uncharged polar, and nonpolar amino acids. The protein is shown embedded in the outer layer of the membrane of a curved vesicle, with negatively charged phospholipid head groups in red and fatty acid tails in black. The wheel diagram design is adapted from Bendor et al. (2013).

Purified recombinant α S *in vitro* is natively unfolded (Bertoncini et al., 2005) but forms an alpha-helix when bound to artificial membranes (Davidson et al., 1998). Recent studies have also shown that α S occur in monomeric and multimeric, predominantly tetrameric, forms inside neurons (Dettmer et al., 2015a) and that α S occurs physiologically in a dynamic equilibrium between unfolded monomers and helically folded tetramers (Glajch et al., 2021). All the known fPD-causing α S mutations A53T, A30P, E46K, H50Q, and A30P have decreased the physiologic tetramer-monomer ratio (Dettmer et al., 2015a). Thus, a low tetramer-monomer ratio may be seen as a pathological marker.

Another significant marker of α S pathology is the phosphorylation of serine-129 (S129). This post-translational modification has been reported to influence α S aggregation and toxicity considerably (Oueslati et al., 2010). A30P and A53T mutants have been reported to slightly affect S129 phosphorylation compared to WT, but the most notable increase in S129 phosphorylation was seen in the E46K mutant, both *in vitro* and *in vivo* (Mbefo et al., 2015).

In order to dissect the ways in which naturally occurring mutations of α S affect its conformational homeostasis, many studies have investigated how various designed α S point mutations affect the behavior of α S (Dettmer 2018). These rationally designed variants offer various biochemical insights for understanding the structural properties of α S *in vivo*.

One of these strategically designed variants is the “p5-3K” = E35K + E46K + E61K mutant, which extrapolates the effect of the mutation E46K (Rovere et al., 2019; Dettmer et al., 2015a). Specifically, this mutant changes the three negatively charged glutamates (E) on position 5 of repeats #3, #4, and #5 to positively charged lysines (K). It has been hypothesized that the E46K mutation increases the electrostatic attraction between α S and the vesicle phospholipid membrane due to the increased attraction of the positively charged lysine (K) to the negatively charged head groups of membrane phospholipids (Perlmutter et al., 2019). Indeed, the significantly increased ability of the E46K mutant to bind to phospholipid membranes was demonstrated as early as 2004 (Choi et al., 2004). This mutant has also been shown to increase α S oligomerization and aggregation more than other mutants, such as A30P (Lázaro et al., 2014).

Thus, the “p5-3K” variant confers an increased “dose” of the naturally occurring E46K mutation by changing more glutamates to lysines on position 5; the “dose-dependent” effects of the “p5-3K” mutant include significant increases in α S toxicity and membrane binding, vesicle cluster formation (Dettmer et al., 2015a; Dettmer et al., 2017), and the loss of α S curvature selectivity (Rovere et al., 2019).

As switching glutamate residues to lysine residues raises α S binding to phospholipid membranes, the

opposite-changing lysine residues to glutamate residues-lowers α S membrane binding. The two compound mutants K10E + K12E and K21E + K23E both reduced membrane binding compared to WT when determined by a quantitative phospholipid ELISA assay (Zarbiv et al., 2014). These mutations occur on positions 2 and 4, the highly basic positions that interact mainly with negatively charged phospholipid head groups.

While mutations on positions 11, 7, 3, 10, and 6 of the hydrophobic side of the α S amphipathic helix, as well as positions 4 and 2 which primarily interact with phospholipid head groups, have been extensively studied (Volles and Lansbury, 2007; Zarbiv et al., 2014; Burr *et al.*, 2012; Burr *et al.*, 2015; Dettmer et al., 2015b; Perrin et al., 2000; Wang et al., 2014; Kim et al., 2006), there have been relatively few studies exploring positions 8, 1, 5, and 9 of the hydrophilic side (Volles and Lansbury, 2007; Winner et al., 2011; Dettmer et al., 2015a). Notably, as lysine residue mutations are highly specific to different positions, it remains uncertain whether the extrapolation of lysine residue on less-known positions of the cytosolic side-namely, positions 8, 1, and 9-would result in the same increase in membrane binding as manifested by the “3K” compound mutation on position 5. Moreover, no studies so far have tested whether “3K” mutations on positions 8, 1, 5, and 9 would increase S129 phosphorylation. Therefore, this paper aims to address this gap in the current scientific knowledge of α S by determining the effect of replacing other amino acids on each of the positions 8, 1, 5, and 9 with three lysines (K).

2. Methods

2.1 Cell Lines and Transfection

Human neuroblastoma cells [BE (2)-M17 = M17D; ATCC number CRL-2267] were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% FBS, 50 U/mL penicillin, 50 μ g/mL streptomycin, and 2 mM L-glutamine. Cells were transfected using Lipofectamine 2000 following the manufacturer’s directions. In brief, cells in 48-well plates (pS129:total assay) were transfected with 0.5 μ g DNA, 1.2 μ L Lipofectamine-2000, and 60 μ L Optimem (Invitrogen) per well; cells in 12-well plates (sequential extraction assay) were transfected with 2 μ g DNA, 4.8 μ L Lipofectamine-2000 and 240 μ L Optimem (Invitrogen) per well.

2.2 DNA Constructs

Plasmid pcDNA4/ α S has been described by Dettmer et al. (2013); The new constructs pcDNA4/ α S-p1-3K, pcDNA4/ α S-p5-3K, pcDNA4/ α S-p8-3K, and pcDNA4/ α S-p9-3K were generated in the Dettmer lab. Inserts were synthesized as GeneArt Strings DNA fragments (GeneArt/Life Technologies) and inserted into pcDNA4-TO-mycHisA with the In-Fusion HD Cloning Kit (Clontech).

2.3 Cell Lysis and Sequential Protein Extraction

For the pS129:total assay, M17D cells were lysed directly in the plate; media was aspirated, attached cells were washed in PBS, and 100 μ L 1xLDS protein loading buffer was added to cells. Trituration lysates were transferred to 1.5 mL Eppendorf tubes and boiled for 10 minutes at 95 $^{\circ}$ C. Then, lysates were spun at room temperature, and the supernatant was subjected to immunoblotting. For sequential extraction, M17D cells in 12-well plates were washed in PBS and transferred to 1.5 mL Eppendorf tubes. Cells were first lysed by hypotonic shock (addition of H₂O/PI, followed by 10 minutes of incubation at RT while shaking), then 10x PBS was added for a final concentration of 1x. Lysates were spun at 4 $^{\circ}$ C (> 12,000g). The supernatant was collected (PBS fraction, cytosol). Then, pellets were lysed in PBS/1% TX-100/PI by sonication, followed by centrifugation at 4 $^{\circ}$ C (>12,000g) and the collection of the supernatant (TX-100 fraction, membranes). Protein concentrations were determined by BCA assay, and LDS sample buffer was added, followed by boiling.

2.4 Immunoblotting

Protein samples were electrophoresed on NuPAGE 4-12% Bis-Tris gels (Invitrogen) at 100 V and transferred in the iBlot 2 system (Invitrogen) to PVDF membranes (iBlot 2 PVDF regular stacks; IB24001). Membranes were fixed for 10 minutes in 0.4% paraformaldehyde in PBS. PVDF membranes were blocked in Odyssey Blocking Buffer, PBS (Li-Cor, ref. 927-40000) for 1 h. Membranes were incubated with primary antibodies in Odyssey Blocking Buffer, PBS/0.2% Tween 20, and washed 5 \times 5 minutes in PBS/0.2% Tween 20. Secondary antibodies were prepared in the same buffer containing 0.01% SDS and added for 1 h at RT, protected from light. Membranes were washed 5 \times 5 minutes in PBS/0.2% Tween 20 and scanned (Odyssey CLx, Li-Cor). Measurements were taken with Fiji ImageJ software with equal area and signals were subtracted from the background.

2.5 Antibodies

Rat monoclonal antibody 15G7 to α S was from a hybridoma supernatant (Kahle et al. 2000) kindly provided by N. Exner and C. Haass and used at 1:500 in WB. Rabbit polyclonal antibody to α S (DIR1R, Clontech) was used at 1:2000 in WB. Rabbit polyclonal antibody to GAPDH was purchased from Sigma (G9545) and utilized at 1:10,000 in WB. Rabbit polyclonal antibody to transferrin receptor (TfR; ab84036) was purchased from Abcam and used at 1:1000 in WB.

2.6 Statistical Analyses

One-way ANOVA tests, including Dunnett's post-hoc test comparing the mean of each set (p1-3K, p5-3K, p8-3K, p9-3K) with the WT set, were performed with GraphPad Prism Version 9 according to the program's guidelines. Normal distribution and similar variance were observed for all values. The criteria for significance, routinely determined relative to wt α S, were: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Sufficient experiments and replicates were analyzed to achieve statistical significance, and these judgments are consistent with earlier, similar work.

3. Results

3.1 DNA Sequencing

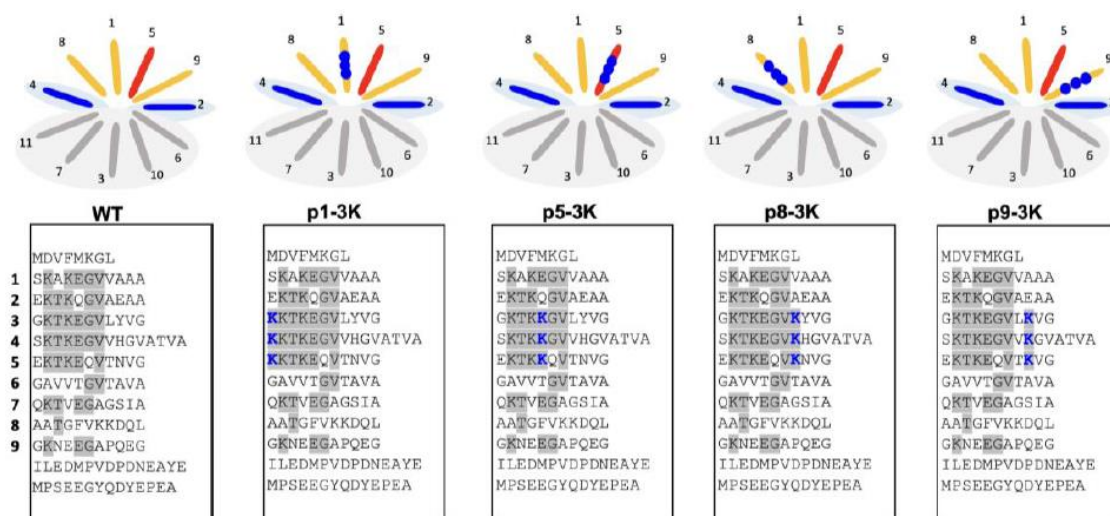


Figure 2. Schematic of WT and the compound mutants p1-3K, p5-3K, p8-3K, and p9-3K

In the boxes showing the amino acid sequences, changed amino acids are shown in blue and amino acids that conform to the core motif are highlighted in grey. Simplified helical wheel diagrams are shown on top with blue lines representing mostly basic positions, yellow as mixed hydrophilic positions, red as mostly acidic positions, grey as primarily uncharged positions, and blue dots as changed amino acids.

The compound mutants were designed to examine the effect of lysine residue on various positions, and DNA sequencing confirmed that the mutants were designed correctly. The four compound mutants that were designed in this study are “p1-3K” (G31K, S42K, E57K), “p5-3K” (E35K, E46K, E61K), “p8-3K” (L38K, V49K, T64K), and “p9-3K” (Y39K, H50K, N65K), which are shown, along with the wild type (WT) sequence, in **Figure 2**. Each mutant has three amino acids changed into lysine. Sequencing data confirms that the mutants were designed correctly (**Figure 3**), with the submitted WT α S amino acid sequence aligning perfectly with that of α S in the BLAST X database and the other sequences having exactly three deviations in the correct locations from the database wild-type α S sequence.

wt	235	MDVFMKGLSKAKEGVVAAAEEKTKQGVAAEAAKTKKEGVLYVGSKTKKEGVVHGVATVAEKTK	414
wt αS	1	MDVFMKGLSKAKEGVVAAAEEKTKQGVAAEAAKTKKEGVLYVGSKTKKEGVVHGVATVAEKTK	60
wt	415	EQVTNVGGAVVTGVTAVAQKTVEGAGSIAAATGFVKKDLGKNEEGAPQEGILEDMPVDP	594
wt αS	61	EQVTNVGGAVVTGVTAVAQKTVEGAGSIAAATGFVKKDLGKNEEGAPQEGILEDMPVDP	120
wt	595	DNEAYEMPSEEGYQDYEP EA 654	
wt αS	121	DNEAYEMPSEEGYQDYEP EA 140	
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p1-3K	237	MDVFMKGLSKAKEGVVAAAEEKTKQGVAAEAAKTKKEGVLYVGSKTKKEGVVHGVATVAEKTK	416
wt αS	1	MDVFMKGLSKAKEGVVAAAEEKTKQGVAAEAAKTKKEGVLYVGSKTKKEGVVHGVATVAEKTK	60
p1-3K	417	EQVTNVGGAVVTGVTAVAQKTVEGAGSIAAATGFVKKDLGKNEEGAPQEGILEDMPVDP	596
wt αS	61	EQVTNVGGAVVTGVTAVAQKTVEGAGSIAAATGFVKKDLGKNEEGAPQEGILEDMPVDP	120
p1-3K	597	DNEAYEMPSEEGYQDYEP EA 656	
wt αS	121	DNEAYEMPSEEGYQDYEP EA 140	
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p5-3K	236	MDVFMKGLSKAKEGVVAAAEEKTKQGVAAEAAKTKKEGVLYVGSKTKKEGVVHGVATVAEKTK	415
wt αS	1	MDVFMKGLSKAKEGVVAAAEEKTKQGVAAEAAKTKKEGVLYVGSKTKKEGVVHGVATVAEKTK	60
p5-3K	416	EQVTNVGGAVVTGVTAVAQKTVEGAGSIAAATGFVKKDLGKNEEGAPQEGILEDMPVDP	595
wt αS	61	EQVTNVGGAVVTGVTAVAQKTVEGAGSIAAATGFVKKDLGKNEEGAPQEGILEDMPVDP	120
p5-3K	596	DNEAYEMPSEEGYQDYEP EA 655	
wt αS	121	DNEAYEMPSEEGYQDYEP EA 140	
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p8-3K	237	MDVFMKGLSKAKEGVVAAAEEKTKQGVAAEAAKTKKEGVLYVGSKTKKEGVVHGVATVAEKTK	416
wt αS	1	MDVFMKGLSKAKEGVVAAAEEKTKQGVAAEAAKTKKEGVLYVGSKTKKEGVVHGVATVAEKTK	60
p8-3K	417	EQVTNVGGAVVTGVTAVAQKTVEGAGSIAAATGFVKKDLGKNEEGAPQEGILEDMPVDP	596
wt αS	61	EQVTNVGGAVVTGVTAVAQKTVEGAGSIAAATGFVKKDLGKNEEGAPQEGILEDMPVDP	120
p8-3K	597	DNEAYEMPSEEGYQDYEP EA 656	
wt αS	121	DNEAYEMPSEEGYQDYEP EA 140	
<hr/>			
p9-3K	238	MDVFMKGLSKAKEGVVAAAEEKTKQGVAAEAAKTKKEGVLYVGSKTKKEGVVHGVATVAEKTK	417
wt αS	1	MDVFMKGLSKAKEGVVAAAEEKTKQGVAAEAAKTKKEGVLYVGSKTKKEGVVHGVATVAEKTK	60
p9-3K	418	EQVTNVGGAVVTGVTAVAQKTVEGAGSIAAATGFVKKDLGKNEEGAPQEGILEDMPVDP	597
wt αS	61	EQVTNVGGAVVTGVTAVAQKTVEGAGSIAAATGFVKKDLGKNEEGAPQEGILEDMPVDP	120
p9-3K	598	DNEAYEMPSEEGYQDYEP EA 657	
wt αS	121	DNEAYEMPSEEGYQDYEP EA 140	

Figure 3. Full sequencing data of the αS amino acid constructs, compared with WT αS on the BLAST X database

The top rows labeled with “wt”, “p1-3K”, “p5-3K”, “p8-3K”, and “p9-3K” indicate the sequencing data from the constructs used in this experiment, the bottom rows labeled with “wt-αS” show the original sequence from the BLAST X database, and the middle rows show the matching between the two. Sequences before and after the αS constructs are omitted, with numbers on the right and left showing the location of amino acids in the sequencing data.

3.2 Cytosol-Membrane Distribution

The cytosol-membrane distribution ratios of the mutants were significantly lower than that of WT (Figure 4A). The Western Blot (WB) data were measured for cytosol-bound and membrane-bound α S pairs with excessively weak bands omitted, and cytosol-membrane ratios were calculated and normalized to WT. Due to the omission of weak WB signals, an ordinary one-way ANOVA test was conducted. Results showed one degree of significance with a p-value of 0.0113. Subsequently, Dunnett's multiple comparisons test showed that only the cytosol-membrane ratio of p1-3K significantly differed from that of WT, with two degrees of significance and an adjusted p-value of 0.0035. The non-significant results of multiple comparisons may have been caused by a lack of sufficient data (as in the case of p9-3K, where only one data point was conserved because other bands were two weak) and high variance.

3.3 Serine-129 Phosphorylation

The phosphorylation rate of serine-129 was consistently higher for all mutants, as compared to WT (Figure 4B). WB data for total α S and S129-phosphorylated α S (pS129 α S) were measured, and the ratios of pS129 α S to total α S were calculated. Because the experiments were conducted in several trials, the ratios were normalized to WT for each trial. The one-way ANOVA test with repeated measures and Geisser-Greenhouse correction indicated two degrees of significance with a p-value of 0.0094. A subsequent Dunnett's multiple comparisons test revealed that the pS129 to total α S ratios of all four mutants differed significantly from that of WT, with adjusted p-values of 0.0102, 0.0462, 0.0271, and 0.0020 for WT vs. p1-3K, p5-3K, p8-3K, and p9-3K, respectively. The mean differences of WT from the mutants were all negative, i.e., the pS129 to total α S ratios of mutants were higher than that of WT. The normalized mean pS129 to total α S ratio, or the mean phosphorylation rate, was highest for the mutant p8-3K at 8.492 times that of WT, followed by p5-3K (4.159 times) and p9-3K (3.329); p1-3K had the lowest normalized phosphorylation rate (2.786 times) but is still significantly higher than that of WT as indicated by the adjusted p-value. In none of the trials did the phosphorylation rate of any mutant dip below that of WT.

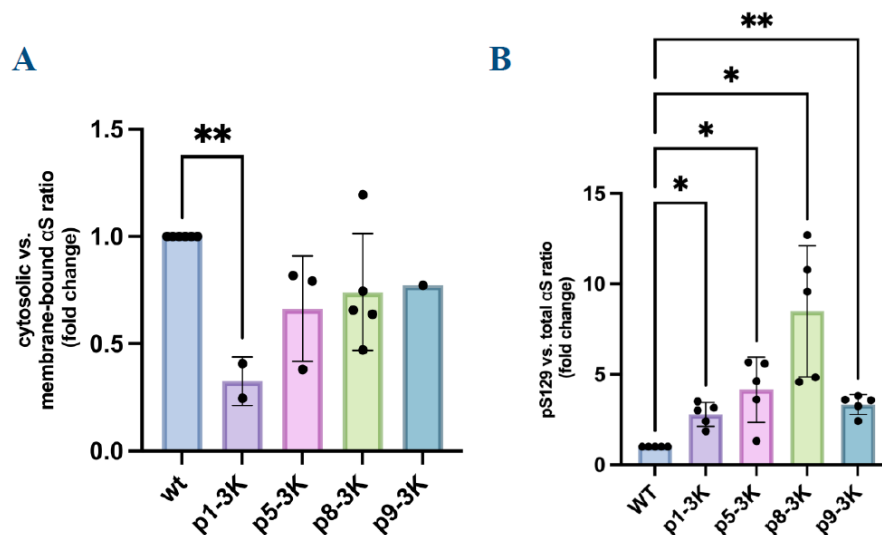


Figure 4. Bar graphs with means \pm standard deviation displaying the ANOVA and Dunnett's port-hoc test results

Data points are shown as black dots. (A) The cytosolic α S to membrane-bound α S ratio for different variants, normalized to WT. Only significant Dunnett's pairwise comparisons are shown for clarity. (B) The ratio of pS129 to total α S ratio, normalized to WT.

4. Discussion

The data from this experiment has shown that the cytosol-membrane distribution ratio was significantly lower (compared to WT) for various "3K" mutants occurring in the cytosol-exposed positions of the α S alpha helix-namely, 1, 5, 8, and 9. The lower ratio signifies that the α S mutants have more α S bound to the membrane than cytosolic α S, implying that the mutations confer a higher affinity for the membrane. This is consistent with the hypothesis that cytosol-exposed lysine residue would increase the electrostatic attraction between α S and the vesicle phospholipid membrane. Therefore, this experiment shows that the increased electrostatic attraction

between positively charged lysine (K) residue and negatively charged phospholipid head groups would lead to greater membrane binding. This result was previously confirmed for the p5-3K mutant (Dettmer et al., 2017). This experiment showed, furthermore, that the p1-3K mutant also displayed this property of a higher cytosol-membrane α S ratio. However, further trials are needed to verify this result for positions 8 and 9, which have displayed non-significance in Dunnett's multiple comparisons test.

Moreover, this experiment had indicated that the phosphorylation rate of S129 increased when cytosol-exposed lysine residues were introduced. The results displayed, with high significance, that the pS129 to total α S ratio is higher for all the mutants p1-3K, p5-3K, p8-3K, and p9-3K. Although the exact mechanism for this phenomenon is currently unknown, it has been hypothesized that α S phosphorylation depends on membrane binding—that when α S is bound to membranes, cellular mechanisms phosphorylate it.

Through investigating the effect of an increased “dose” of lysine residue on each of the four cytosol-exposed positions, three of which have not been explored before, this experiment highlights and confirms the mechanism of pathogenesis for the E46K mutant that causes familial Parkinson's disease. The substitution of non-basic amino acids for lysine on positions in the cytosolic side of the α S alpha helix results in higher vesicle-membrane affinity and S129 phosphorylation. This fills the previous knowledge gap in understanding basic amino acid changes in cytosol-exposed positions and elucidates an essential aspect of the biochemistry of α S, a significant step towards a fuller understanding of this crucial protein involved in Parkinson's disease and other synucleinopathic neurodegenerative diseases.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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