α-Synuclein-dependent Increases in PIP5K1γ Drive Inositol Signaling to Promote Neurotoxicity

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JONATHAN D. HORVATH
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DAVIS

Approved:

_____
Eamonn J. Dickson, Chair

_____
Manuel F. Navedo

_____
Yang K. Xiang

Committee in Charge

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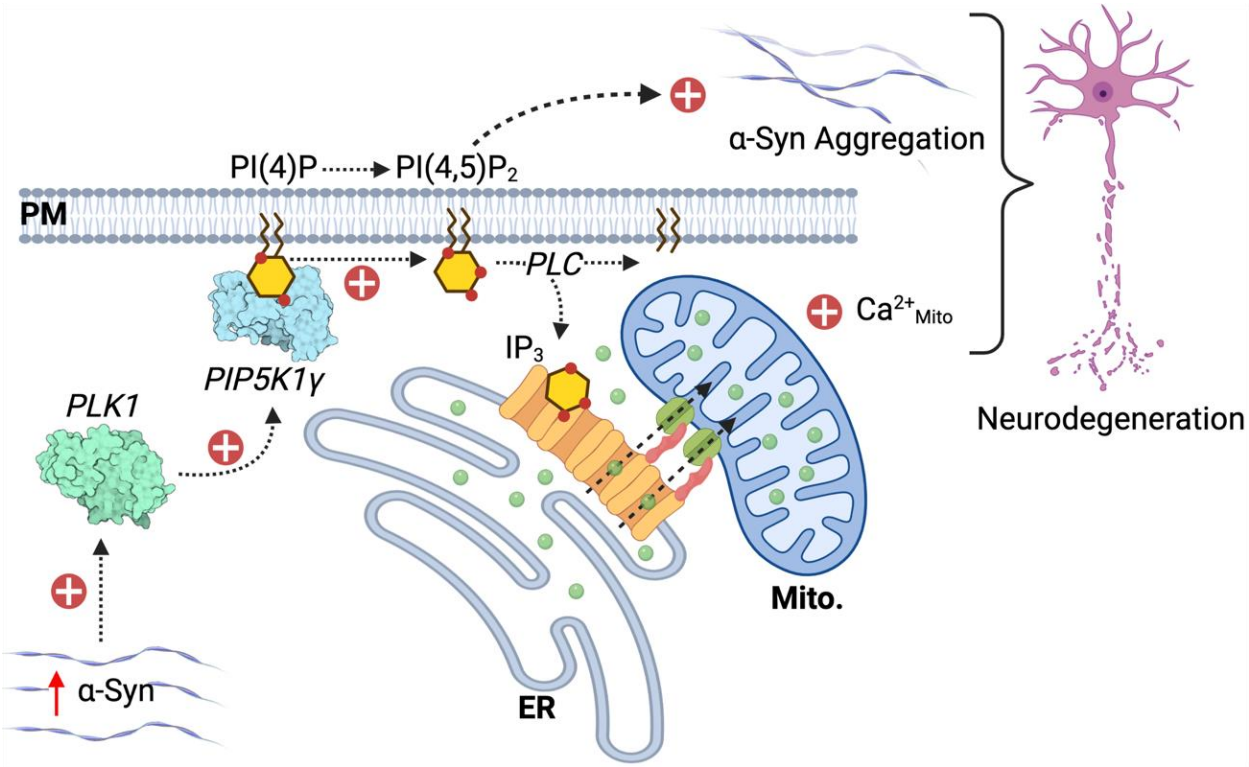
Abstract

Anomalous aggregation of the neuronal protein α-Synuclein (α-Syn) is a pathological hallmark of Parkinson's Disease (PD). Despite its strong link to PD and other synucleinopathies, the precise molecular mechanisms that facilitate α-Syn aggregation leading to neurodegeneration have yet to be elucidated. Here, we find that elevations in α-Syn lead to an increase in the plasma membrane (PM) phosphoinositide $PI(4,5)P_2$, which precipitates α-Syn aggregation and drives toxic increases in mitochondrial Ca^{2+} and reactive oxygen species leading to neuronal death in PD. Upstream of this deleterious signaling pathway is PIP5K1γ, whose abundance and localization is enhanced at the PM by α-Syn-dependent increases in PLK1 activity. Selectively inhibiting these upstream mediators prevents α-Syn aggregation and rescues cellular phenotypes of toxicity. Collectively, this data suggest that modulation of phosphoinositide metabolism may be a therapeutic target to slow neurodegeneration in PD and other related neurodegenerative disorders.

Graphical Abstract



Abbreviations

27-OHC- 27-hydroxycholesterol

AAV- Adeno-associated Virus

ABCA1- ATP binding cassette subfamily A member 1

ADP- Adenosine diphosphate

ALS- Amyotrophic lateral sclerosis

ANT- Adenine nucleotide translocator

ARF- ADP ribosylation factor

ATP- Adenosine triphosphate

Aβ- Amyloid beta

BAK- BCL2 homologous antagonist killer

BAPTA- 1,2-bis(o-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid

BAX- BCL2 associated X

BK- Bradykinin

Ca^{2+}- Calcium

CAMK- Ca^{2+}/calmodulin-dependent protein kinase

CDK- Cyclin-dependent kinase

CFP- Cyan fluorescent protein

CHO- Chinese hamster ovary-derived cell line

CNS- Central nervous system

DAG- Diacylglycerol

DJ1- Protein deglycase DJ-1

DLB- Lewy body dementia

DRP1- Dynamin-related protein 1

ER- Endoplasmic reticulum

GABA- Gamma-aminobutyric acid

GCase- Glucocerebrosidase

GDP- Guanosine diphosphate

GFP- Green fluorescent protein

GPCR- G-protein coupled receptor

GRP- Glucose-regulated protein

GTP- Guanosine triphosphate

H^+- Hydrogen

HDL- High-density lipoprotein

HEK- Human embryonic kidney-derived cell line

HeLa- Human cervical cancer-derived cell line from Henrietta Lacks

Hsc- Heat shock complex

HSP- Heat shock protein

HSPG- Heparan Sulfate Proteoglycan

INPP5E- Inositol Polyphosphate-5-Phosphatase E

IP_3- Inositol trisphosphate

IP_3R- Inositol trisphosphate receptor

IRE1- Inositol-requiring enzyme 1

K_{ATP}- ATP-sensitive potassium

LAG- Lymphocyte-activation gene

LAMP- Lysosomal associated membrane protein

LC-MS/MS- Liquid chromatography tandem mass spectrometry

LTCC- L-type calcium channel

LTP- Lipid transfer protein

LXR- Liver X receptor

MAP2- Microtubule-associated protein 2

MPP^+- 1-methyl-4-phenylpyridinium

MPTP- 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MSA- Multiple systems atrophy

NAC- Non-amyloid component

NFAT- Nuclear factor of activated T-cells

NLS- Nuclear localization sequence

NMDA- N-methyl-D-aspartate

NPC1- Niemann–Pick C1 protein

OSBP- Oxysterol-binding protein

Oxo-M- Oxotremorine

PD- Parkinson’s disease

PDD- Parkinson’s disease dementia

PH- Pleckstrin homolgy

PI- Phosphatidylinositol

PINK1- PTEN-induced kinase 1

PIP_2- Phosphatidylinositol 4,5-bisphosphate

PIP5K1γ- PI(4)P 5-kinase 1 γ

PKA- Protein kinase A

PKC- Protein kinase C

PLC- Phospholipase C

PLD- Phospholipase D

PLK- Serine/threonine-protein polo-like kinase

PM- Plasma membrane

PPI- Poly phosphoinositides

PS- Phosphatidylserine

PTEN- Phosphatase and tensin homolog

Rab- Ras-related protein

RNAi- RNA interference

Ro- Ro-3280

ROS- Reactive oxygen species

SERCA- Sarcoendoplasmic reticulum calcium transport ATPase

sgRNA- Single guide RNA

shRNA- Small hairpin RNA

SH-SY5Y- Human neuroblastoma-derived cell line

siRNA- Small interfering RNA

SMLM- Single-molecule localization microscopy

SN- Substantia nigra

SNARE- Soluble NSF attachment proteins receptor

SYNJ- Synaptojanin

TH- Tyrosine Hydroxylase

TIRF- Total internal fluorescence microscopy

TOM- Translocase of outer mitochondrial membrane

tsA-201- HEK cell line expressing an SV40 temperature-sensitive T antigen

UNC- UNC-3230

UTP- Uridine triphosphate

VAMP2- Vesicle-associated membrane protein 2; Synaptobrevin

VAP- VAMP-associated protein

VDAC- Voltage-dependent anion channel

VGCC- Voltage-gated calcium channel

VMAT- Vesicular monoamine transporter

VTA- Ventral tegmental area

WT- Wild-type

α-Syn- α-Synuclein

α-Syn fibrils- human pre-formed alpha-synuclein fibrils

$\alpha\text{-Syn}^{Dox}$- Doxycycline-inducible alpha-synuclein expressing SH-SY5Y cell line

1. Introduction

1.1 Parkinson's Disease

Parkinson's Disease (PD) is the second-most frequent neurodegenerative disorder (Obeso et al., 2010). Currently, there is no cure or therapy targeting the slowing of the progression of PD. The pathological hallmarks of the disease are dopaminergic neuron loss in the substantia nigra (SN) and the presence of cytotoxic inclusions composed predominantly of the protein α-Synuclein (α-Syn). These neuropathologies exist in other neurodegenerative disorders, but taken together, they provide a definitive diagnosis for PD (Poewe et al., 2017). The greatest risk factor for idiopathic PD is aging, but mutations of gene loci associated with familial PD can also be inherited, often leading to early-onset and autosomal-dominant forms of the disease. Mutations in over 40 gene loci have been identified for PD; however, the first and most studied is *SNCA* (PARK1), which encodes α-Syn (Chang et al., 2017). Although hereditary forms of PD make up the minority of all cases (< 10%), studying them may be critical for uncovering the molecular basis of neurodegeneration (Klein & Westenberger, 2012). Nevertheless, the precise pathogenesis and molecular mechanisms underlying both idiopathic and familial PD remain to be elucidated, illustrating an important need for future research.

1.2 α-Synuclein

1.2.1 Structure

α-Syn is a small, acidic protein found at synaptic nerve terminals in neurons throughout regions of the brain. The molecular structure of α-Syn is well studied. It is composed of 140 amino acid residues, making up three distinct regions: the N-terminal

domain, which acts as the lipid-binding region, the centrally located non-amyloid component (NAC) domain, and the C-terminal domain. A highly conserved sequence (*KTKEGV*) is repeated 6 times throughout the first 95 amino acid residues of the protein structure. This repeated sequence is required for the formation of the α-helix secondary structure of the protein. Upon forming a helical structure, α-Syn prefers to bind between the polar heads of two adjacent lipid molecules. Therefore, an increased lipid-to-protein ratio at biological membranes augments the propensity of α-Syn affinity (Galvagnion, 2017). It may exist physiologically as an unfolder monomer or a stable helical tetramer that doesn't aggregate, but due to mutation or misfolding, it can form larger oligomers which eventually produce protofibrils and ultimately beta-sheet-rich fibrillar structures (Bartels, Choi, & Selkoe, 2011). These fibrils are the molecular basis of Lewy body inclusions, the intracellular protein accumulations that cause neuronal death and lead to neurodegenerative disorders, such as PD and other synucleinopathies (Figure 1).

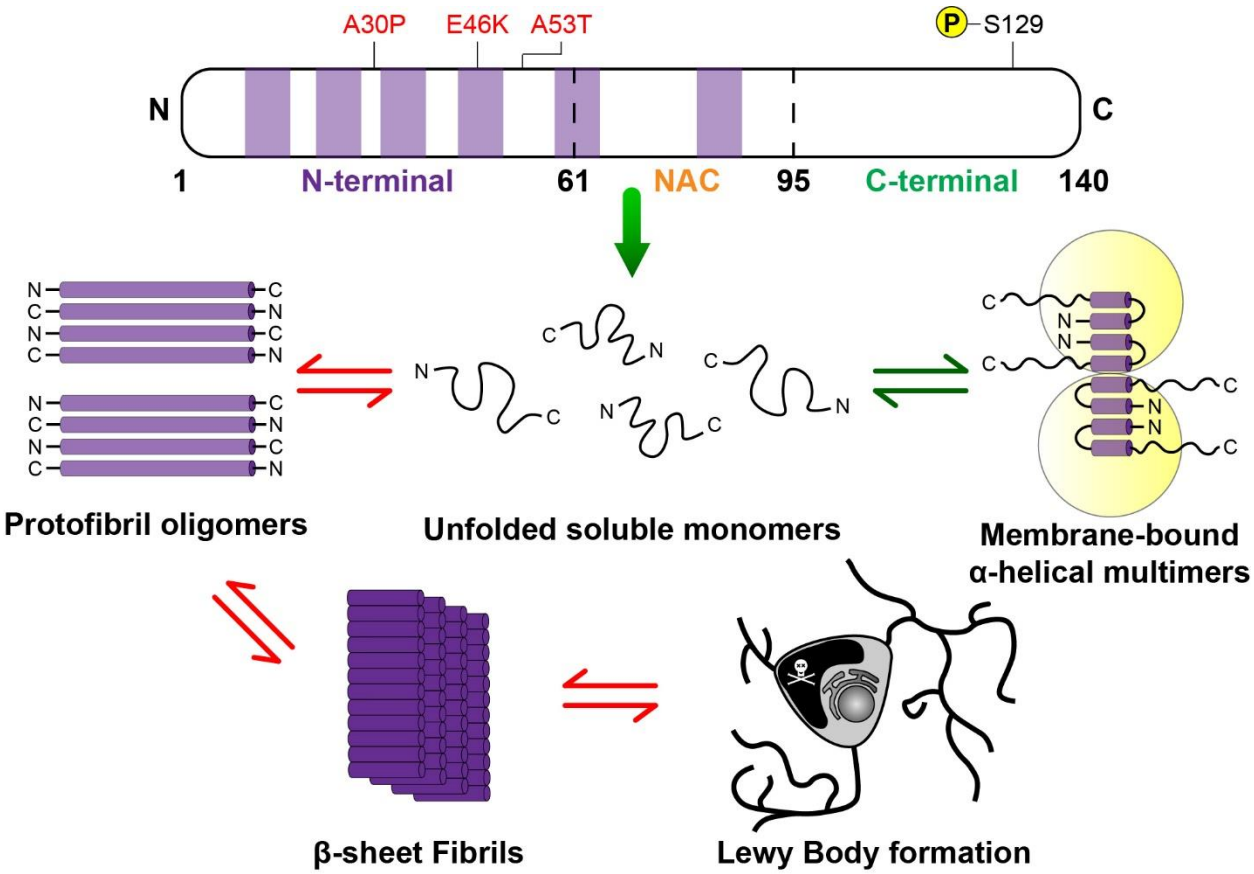


Figure 1. α-Syn structure and pathological aggregation. 14 kDa monomeric α-Syn consists of three distinct regions: N-terminal, NAC, C-terminal. Six *KTKEGV* repeats are found in the first 95 residues of the protein. The common familial PD mutations A30P, E46K, and A53T, are found in the N-terminal region. The predominant pathological post-translational phosphorylation of α-Syn occurs at Ser-129, as 90% of total α -Syn in Lewy bodies is phosphorylated at this residue. While physiological species of α-Syn associate with lipid membranes and aid in neuronal function, under deleterious conditions, natively unfolded monomers are prone to aggregation, forming protofibrils, and ultimately toxic β-sheet fibrils. These are the pathological components found in Lewy bodies and are thought to be the initiation of neurodegeneration.

1.2.2 Expression and localization

α-Syn is expressed to a varying degree in different brain regions such as the olfactory bulb, medulla oblongata, pons, midbrain, cerebrum, and cerebellum. It predominantly localizes to presynaptic terminals, but it is also found less abundantly in the soma in some brain regions, namely in the SN pars compacta, the region of the brain that degenerates during PD. In excitatory neurons, α-Syn colocalizes with vesicular glutamate transporter-1 (vGlut-1), while in inhibitory neurons it associates with glutamate decarboxylase (GAD), the enzyme that catalyzes the decarboxylation of glutamate to GABA and CO_2 (Taguchi, Watanabe, Tsujimura, & Tanaka, 2016). Non-neuronal glial cells, such as astrocytes and oligodendrocytes, may also display α-Syn-positive aggregates, as evident in some cases of PD, DLB, and MSA (Bruck, Wenning, Stefanova, & Fellner, 2016). Furthermore, α-Syn is highly expressed in other non-neuronal mammalian cells, such as erythrocytes and cardiac/skeletal myocytes, as well as HeLa and HEK-293 cell lines.

1.2.3 Post-translational modification

1.2.3.1 Implications in disease

Post-translational modifications are critical for regulating the cellular function of all proteins. α-Syn is modified in a number of manners, including phosphorylation, ubiquitination, truncation, nitration, and *O*-GlcNAcylation (J. Zhang, Li, & Li, 2019). However, the most common modification of α-Syn is phosphorylation of Serine-129 (Ser(P)-129). Its prominence is highlighted as the pathological form of α-Syn, as 90% of fibrils that comprise Lewy bodies exhibit phosphorylation at this specific residue. Yet,

several groups have shown only 4% of all soluble α-Syn is phosphorylated on this serine residue in healthy individuals (Anderson et al., 2006; Fujiwara et al., 2002). Interestingly, post-mortem studies of patients lacking Lewy body or other neurodegenerative pathology present relatively increased amounts of Ser(P)-129 in the SN, the most impacted brain region in PD, compared to other regions not subject to degradation by synucleinopathies. Notwithstanding, the expression of endogenous unphosphorylated α-Syn in those same control patients is lower than the compared brain regions (Muntane, Ferrer, & Martinez-Vicente, 2012). These data suggest that brain regions more vulnerable to synucleinopathies, such as the SN, may have a diminished basal amount of soluble α-Syn, yet a greater proportion of which is phosphorylated at Ser-129, implicating the importance of the SN in PD pathoprogression.

1.2.3.2 Kinases responsible for phosphorylation

Several groups have postulated the kinases responsible for this α-Syn Ser(P)-129. Historically, PKA or PKC were identified as enzymes responsible for phosphorylation due to homology of several substrate binding sequences. However, through co-immunoprecipitation studies, activating PKA and PKC in the presence or absence of certain phosphatase inhibitors had no effect on α-Syn Ser(P)-129. Instead, initially Casein Kinase-1 (CK1) and Casein Kinase-2 (CK2) were shown to be influential for the phosphorylation state of α-Syn (Okochi et al., 2000). Future biochemical studies demonstrated that an isoform of the polo-like kinase family, PLK2, has unique *in vivo* and *in vitro* phosphorylation capability of Ser-129. This data was confirmed with the use of inhibitors of PLK2, short hairpin RNA knockdown of PLK2, and complete knockout of the

PLK2 gene in mice (Inglis et al., 2009). Following this discovery, other members of the PLK family, such as PLK1, were identified as being capable of this particular α-Syn phosphorylation as well (Mbefo et al., 2010). Furthermore, although α-Syn Ser(P)-129 had no effect on the lipid-binding affinity (detailed in the subsequent portion) of wild-type (WT) α-Syn, α-Syn$_{A53T}$ demonstrated a decreased affinity when phosphorylated at Ser-129, while α-Syn$_{A30P}$ experienced an increased affinity (Samuel et al., 2016). This data seems to indicate that phosphorylation of certain mutant forms of α-Syn may alter the already high propensity for the protein to form fibrils, influencing pathological cellular lipid dynamics.

1.2.4 Function in health

Although the precise wild-type function of α-Syn still eludes investigators, several important roles of the protein impacting neurotransmission, synaptic plasticity, and neurodevelopment have been identified. Furthermore, α-Syn physically interacts with a variety of membrane-associated lipids and proteins in most organelle compartments (Figure 2).

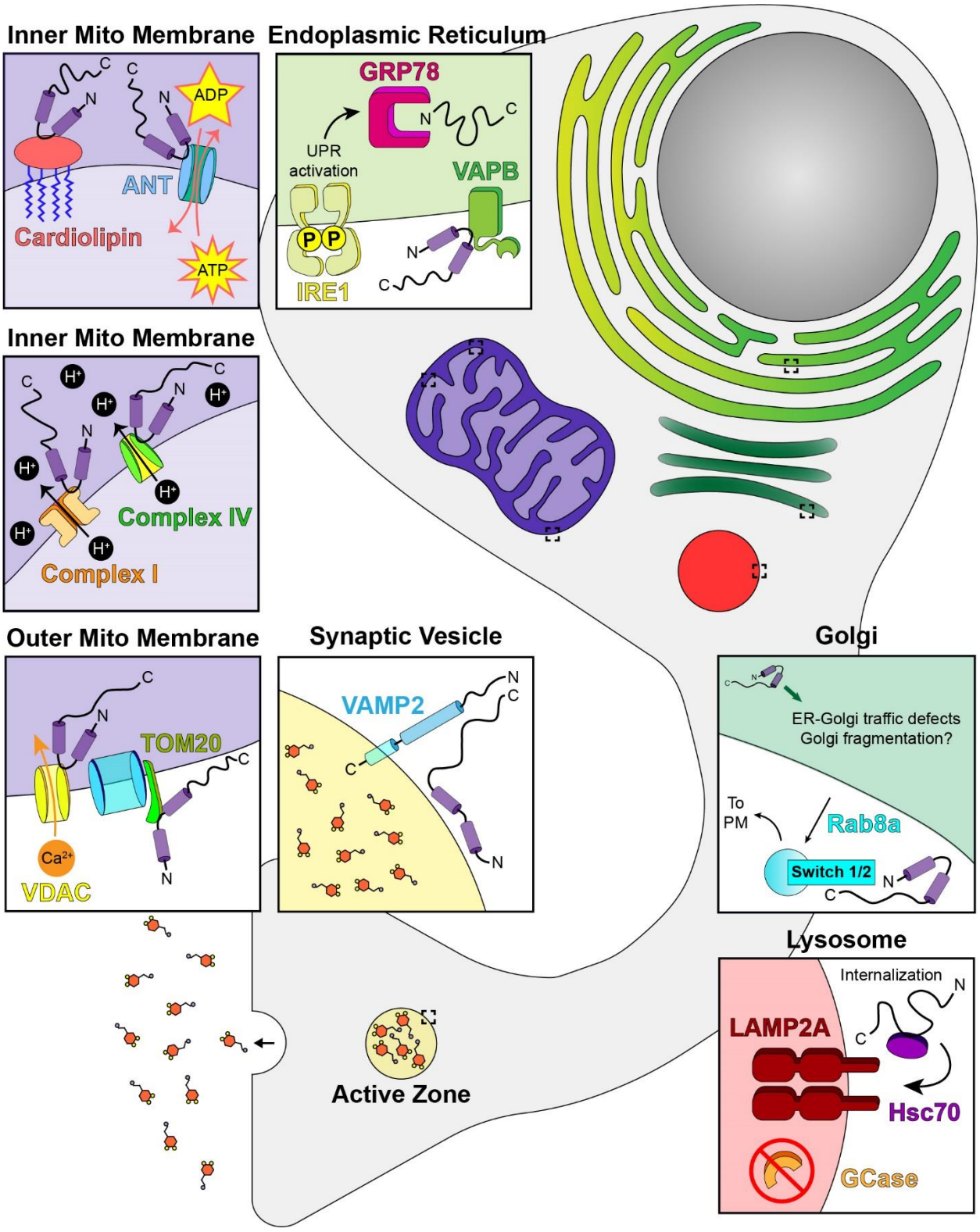

Inner Mito Membrane
ADP
ANT
Cardiolipin
ATP
Endoplasmic Reticulum
GRP78
UPR
activation
VAPB
P P
IRE1
Inner Mito Membrane
Complex IV
Complex I
Outer Mito Membrane
TOM20
Ca2+
VDAC
Synaptic Vesicle
VAMP2
Active Zone
Golgi
ER-Golgi traffic defects
Golgi fragmentation?
To
PM
Rab8a
Switch 1/2
Lysosome
Internalization
LAMP2A
Hsc70
GCase

Figure 2. α-Syn organellar interactions and pathophysiological functions. α-Syn has a high propensity to interact with lipid membranes and their associated proteins, including but not limited to those of mitochondria, endoplasmic reticula, synaptic vesicles, Golgi structures, and lysosomes. α-Syn is implicated in cellular functions such as bioenergetics, mitophagy, mitochondrial morphology, ER-mitochondrial contact site regulation, UPR activation, synaptic vesicle dynamics, neurotransmission regulation, Golgi trafficking, and lysosomal function.

1.2.4.1 Lipid associations

Due to the propensity of α-Syn to bind to lipid membranes, it has a strong effect on lipid transport, packing, and membrane biogenesis. When α-Syn is knocked-out in an *in vivo* murine model, a protein known to induce membrane curvature, endophilin A1, experiences upregulation of protein expression. Inversely, when α-Syn is overexpressed, endophilin A1 experiences a 20% reduction in protein expression compared to wild-type controls. By inserting its N-terminal helix into lipid membranes, α-Syn can tubulate liposomes in a similar manner as endophilin A1 *in vitro*, suggesting that expression of these two proteins is interdependent, likely due conserved physiological function (Westphal & Chandra, 2013). In addition to binding to lipid membranes, α-Syn has the ability to remodel membranes. The amphipathic helical characteristics of α-Syn can cause membrane thinning, relieve curvature due to a lipid packing defect, or potentiate membrane curvature, depending on the composition of the membrane (Ouberai et al., 2013). α-Syn also directly interacts with and inhibits phospholipase D1 (PLD1), an enzyme that catalyzes the hydrolysis of phosphatidylcholine to phosphatidic acid and choline. Since phosphatidylcholine proportionally constitutes roughly one-third of the lipids forming synaptic vesicles (Takamori et al., 2006), α-Syn thus plays an important role in the regulation of lipid cleavage and novel membrane formation related to neurotranmission (Ahn et al., 2005). The significance of α-Syn-lipid interactions in relation to disease is highlighted by altered lipid-binding properties of α-Syn mutants. For example, *in vitro* phospholipid vesicle studies, x-ray diffraction (Jo, McLaurin, Yip, St George-Hyslop, & Fraser, 2000), NMR spectroscopy (Bussell & Eliezer, 2004), and simulated predictive models (Perlmutter, Braun, & Sachs, 2009), revealed decreased lipid

affinity for the A30P mutation, increased lipid affinity for the E46K mutation, an no relative change in lipid affinity for the A53T mutation, in comparison to wild-type α-Syn. Clearly, common α-Syn pathological mutations display the ability to alter membrane structure and function.

1.2.4.2 Synaptic vesicle regulation

One protein that interacts with α-Syn is membrane-associated GTP-bound Rab3a. However, co-immunoprecipitation studies show that α-Syn doesn't associate with GDP-Rab3a, the soluble cytosolic form. GDP-dissociation inhibitors, heat shock protein inhibitors, and selective Rab 3a inhibitors, which all act to prevent Rab3a recycling from lipid membranes, cause an increase in α-Syn sequestration, suggesting Rab3a plays a critical role in stabilizing α-Syn at vesicles (R. H. C. Chen et al., 2013). Since this small GTPase protein plays an important role in trafficking synaptic vesicle to the active zone of presynaptic terminals and thus neurotransmitter exocytosis (Leenders, Lopes da Silva, Ghijsen, & Verhage, 2001), it is critical that α-Syn expression is finely regulated in neurons. In addition to synaptic vesicle trafficking, α-Syn also interacts with two proteins of high physiological relevance to dopamine synthesis and transport, the neurotransmitter whose release from the SN is diminished in PD.

Due to its similar homology to 14-3-3, a chaperone molecule which binds to and modulates activity of other protein substrates, α-Syn was proposed to alter dopamine production, given its interaction with the rate-limiting enzyme in dopamine synthesis, tyrosine hydroxylase (TH). While overexpression of wild-type and mutant α-Syn doesn't alter protein levels of TH, it reduces dopamine synthesis in a dose-dependent manner

(Perez et al., 2002). Furthermore, α-Syn seems to be involved the mechanism of dopamine transport into vesicles, as knockdown and overexpression of α-Syn, either increases or decreases the activity of Vesicular Monoamine Transporter-2 (VMAT2), respectively (J. T. Guo et al., 2008). Given that VMAT2 is a proton-dependent antiporter protein that packages synaptic vesicles with dopamine molecules, regulated physiological levels of α-Syn in healthy neurons are necessary to aid in the modulation of neurotransmitter release.

1.2.4.3 Neurotransmission and synaptic plasticity

Although α-Syn has been identified to interact with several proteins related to vesicle trafficking and neurotransmitter release, its exact role in modulating neurotransmission and synaptic plasticity remains unclear. α-Syn displays chaperone activity of SNARE-complex assembly, a group of proteins on separate lipid membranes that allow for the docking and fusion of vesicles to their target membrane. Coimmunoprecipitation experiments with full-length and truncated forms of α-Syn and synaptobrevin-2 (VAMP2), a vesicular protein in the SNARE-complex, demonstrated that the C-terminal region of α-Syn directly binds directly to the N-terminal region of VAMP2 (Burré et al., 2010). Furthermore, knockout of α-Syn in a murine model decreases SNARE-complex assembly activity and causes age-dependent neurodegeneration (Burré et al., 2010). Taken together, along with α-Syn's preference to localize to Rab3a-positive presynaptic terminals, these data suggest α-Syn has a strong role in neurotransmission. Nevertheless, α-Syn knockout and overexpression studies have shown inconsistent results in whether the protein exacerbates, diminishes, or has a negligible effect on

neurotransmitter release. In addition, several studies have shown that α-Syn affects synaptic plasticity via increasing GluNR1 NMDA receptor phosphorylation (J. Yang et al., 2016) and reducing synaptic localization of the GluN2A NMDA receptor subunit (Durante et al., 2019). The former of these interactions promotes ER exit and transit of the receptor to the plasma membrane enhancing NMDA-mediated inward current, while the latter hinders NMDA-mediated inward current, suggesting contradicting regulation of long term potentiation in neurons overexpressing α-Syn. Overall, the aforementioned data suggest that α-Syn may act physiologically to finely tune neurotransmission, as opposed to directly upregulating or downregulating its activity (Burre, Sharma, & Sudhof, 2018).

1.2.5 Cell-to-cell transmission in disease

One of the most frightening pathological characteristics of α-Syn aggregation is its ability to seemingly propagate from vulnerable neuronal networks to other brain regions with ease. In 2003, a landmark study established the staging pathology of PD (Braak et al., 2003). Interestingly, patients diagnosed with sporadic PD demonstrate little interindividual variation during the course of Lewy body spread. Although much PD-focused research remains on the SN, Braak postulated that synucleinopathy lesions initiate in the lower brainstem or olfactory bulb before progressing to affect the SN and inducing parkinsonian-like symptoms. This pathway indicates a clear role for α-Syn in cell-to-cell transmission in disease and raises many questions as to why some brain regions appear to be more susceptible to α-Syn-related pathology.

Although the mechanism of translocation into vesicular structures is unknown, α-Syn has the capacity to aggregate in the lumen of vesicles, leaving monomeric and

aggregated forms of the protein prone to exocytosis. Indeed, α-Syn is constitutively released by neurons into extracellular space as early as two hours after transient overexpression, independent of consequences of artifactual overexpression, membrane damage, or activation of cell death pathways. Additionally, extracellular α-Syn accumulation is correlated with the degree of intracellular aggregation, highlighting the capability of disease-affected neurons to inflict deleterious effects on adjacent cells (H. J. Lee, Patel, & Lee, 2005). It is not surprising then that α-Syn has been detected in both blood and cerebrospinal fluid of healthy and PD patients (El-Agnaf et al., 2003). Given these data, clearly, exogenous seeding of α-Syn aggregates in otherwise unaffected healthy neurons may potentiate the pathological progression of disease. Another disease-related mechanism of accelerated transcellular α-Syn transmission involves Glucocerebrosidase (GCase). Deficiency in *GBA1*, the gene that codes for GCase, leads to lysosomal dysfunction and is the predominant pathological component of Gaucher's Disease (GD). Deletion of *GBA1* impairs lysosomal clearance of α-Syn and potentiates cell-to-cell transmission. Furthermore, transduction of exogenous *GBA1* into cells lacking endogenous expression of the gene demonstrated a rescued effect of α-Syn transmission, while transduction of an activity-deficient mutant did not (Bae et al., 2014). Clearly, the pathological progression of α-Syn-related diseases is augmented due to lysosomal impairment. In addition to lysosomal impairment, various point mutations of α-Syn (discussed in more detail in the following section) also poentiate cell-to-cell transmission of the protein. Two of the eight common missense mutations of α-Syn highly associated with early-onset familial PD (H50Q and A53T) increase α-Syn secretion compared to wild-type α-Syn expression in differentiated SH-SY5Y cells (Guan et al.,

2020). Finally, α-Syn exocytosis appears to occur in a calcium-dependent manner. Treatment of SH-SY5Y cells with thapsigargin or ionomycin, which both elevate cytosolic calcium concentration, increases α-Syn secretion in cultured media, while BAPTA, a membrane-permeable Ca^{2+} chelator, downregulates α-Syn exocytosis (Emmanouilidou et al., 2010). Given that mitochondrial dysfunction and perturbed Ca^{2+} homeostasis are prominent phenotypes of α-Syn-related pathology, these data provoke an interesting perspective on a possible mechanism of α-Syn spread throughout the CNS in disease.

1.2.6 Function in PD

1.2.6.1 Familial variants of α-synuclein

α-Syn was first linked to PD through identification of a genetic defect in a large family lineage of Italian descent (Polymeropoulos et al., 1997). The original defect was a missense mutation in the *SNCA* gene, leading to a threonine substitution for alanine at amino acid residue position 53 of α-Syn (A53T). Subsequent studies have identified other PD-related missense mutations of α-Syn in other disparate familial lineages, including but not limited to A30P (Krüger et al., 1998), E46K (Zarranz et al., 2004), H50Q (Appel-Cresswell et al., 2013), G51D (Kiely et al., 2013), and A53E (Pasanen et al., 2014). Additionally, multiplication of the *SNCA* gene locus leads to autosomal-dominant inheritance of early-onset PD pathology, confirmed by augmentation of total α-Syn protein expression (Miller et al., 2004; Singleton et al., 2003). Similarly to familial cases of PD, the involvement of α-Syn in idiopathic origins of PD is not to be understated. *SNCA* is linked to both sporadic and familial PD through large-scale unbiased genome-wide association studies (Stefanis, 2012). Interestingly, a common feature of all pathological

α-Syn missense mutations is their occurrence in the N-terminal region of the protein. This apolipoprotein motif is the region that preferentially binds to lipid membranes and upon association forms alpha-helices (Fortin et al., 2004). This suggests that detrimental mutations of α-Syn potentiate deleterious interactions with lipid structures, quickening many of the pathological processes related to disease. Furthermore, PD-associated mutations of α-Syn, such as A53T, also cause accelerated fibrillar formation, potentially contributing to the progression of neurodegeneration (Conway, Harper, & Lansbury, 1998). Surely, future studies should continue to elucidate other novel α-Syn genetic risk factors and associations for PD.

1.2.6.2 Upregulation of expression

Although disease-related mutations potentiate aggregation and promote fibril formation, little is known about the molecular mechanisms that underlie elevated α-Syn expression at the transcriptional level in PD. One of the earliest discoveries of transcriptional regulation of *SNCA* was demonstrated in non-neuronal cells. Using erythroid cells, GATA1 was identified as a transcriptional regulator of *SNCA*. GATA2, which belongs to the same family and shares functional morphology with GATA1, is highly expressed in dopaminergic midbrain neurons and occupies intron 1 of *SNCA*, controlling expression of the endogenous neuronal pool of α-Syn (Scherzer et al., 2008). Soon after, an independent group identified another candidate transcriptional factor that associates with intron 1 of *SNCA*, ZCAN21 (Clough, Dermentzaki, & Stefanis, 2009), which is activated through the PI3K/ERK-dependent processes (Clough & Stefanis, 2007). Given the importance of a multitude of α-Syn protein-lipid interactions, including those involving

phosphoinositide (PI) kinases, perhaps agitation of phospholipid metabolism and phospholipid dyshomeostasis augment total α-Syn expression. Additionally, GATA2 was also described as capable of occupying a specific region within intron 2 (Brenner, Wersinger, & Gasser, 2015). Clearly, the transcriptional architecture of α-Syn is incredibly complex with multiple modulators acting within non-exclusive transcriptional sites. Future studies should be directed at the simplification of pathogenic α-Syn transcription pathways.

In addition to transcription factors, other molecules impact α-Syn expression and fibril induction. Continuing with the trend in α-Syn-lipid associations, 27-OHC, the predominant cholesterol metabolite in circulation, has the ability to penetrate the blood-brain barrier and activate the Liver X Receptors (LXR), which binds to the promoter region and upregulates *SNCA* gene expression (Marwarha, Rhen, Schommer, & Ghribi, 2011). Given that cholesterol homeostasis is disrupted during lysosomal dysfunction, as outlined earlier as a major pathological consequence of neurodegeneration, these data provide insightful knowledge for a detrimental positive feedback model in which α-Syn demonstrates greater propensity to aggregate, thus further impacting cholesterol metabolism pathways and intensifying cytotoxic gene expression. Indeed, this interaction promotes a compensatory mechanism by which α-Syn potently stimulates cholesterol efflux in a dose-dependent manner via the plasma membrane transporter protein ABCA1, further disrupting neuronal cholesterol homeostasis and metabolism which may exacerbate cellular dysfunction (Hsiao, Halliday, & Kim, 2017). Unrelated to transcriptional modulation but still highly impacting α-Syn expression, the oxidative properties of the neurotransmitter dopamine and other structurally related

catecholamines promote ligation to α-Syn, creating neurotoxic adducts. This association encourages protofibril formation, which is reversed by antioxidant activity, suggesting that dopamine or catecholamine oxidation is responsible. Since mitochondrial oxidative stress is a phenotype strongly correlated with PD-like pathology, this highlights one of the selective vulnerabilities of dopaminergic neurons for α-Syn-mediated neurodegeneration (Conway Kelly, Rochet, Bieganski Robert, & Lansbury Peter, 2001).

1.2.6.3 Ca^{2+} hypothesis of neurodegeneration

Another molecular regulator of α-Syn is the gregarious second messenger, Ca^{2+}. The calcium hypothesis of neurodegeneration was first postulated by Michael Berridge for Alzheimer's Disease, which investigated how neuronal Ca^{2+} signaling cascades were remodeled due to neurodegenerative consequences (Berridge, 2010). It stipulates that atrophic and degenerative processes are accompanied by alterations in calcium homeostasis. Ilya Bezprozvanny expanded this hypothesis to other neurodegenerative disorders, including PD, given that they also share the phenotype of aberrant Ca^{2+} signaling and dyshomeostasis (I. B. Bezprozvanny, 2010). This hypothesis is particularly relevant to PD, given that SN neurons manifest great impairment in endogenous calcium buffering capacity (Foehring, Zhang, Lee, & Callaway, 2009) and oligomeric α-Syn causes increases in basal intracellular calcium concentration due to amplification of extracellular influx and reduction in calcium efflux (Angelova et al., 2016). In fact, the SN relies primarily on L-type $Ca_V1.3$ channels for oscillatory pacemaking activity, which open at a relatively lower membrane potential compared to other voltage-gated calcium channels (VGCC) (Mercuri et al., 1994), causing greater amounts of calcium influx upon

depolarization. ER Ca^{2+} homeostasis is also dysregulated in PD-like pathology. Coimmunoprecipitation and proximity ligation assays elucidate a close physical proximity between α-Syn and SERCA, as well as functional modification of ATPase activity, calcium pumping, and rate of dephosphorylation. This interaction highly impacts ER Ca^{2+} refilling and maintenance of tightly regulated cytosolic calcium levels, both of which are augmented due to α-Syn oligomerization (Betzer et al., 2018). Clinically, increased $Ca_V1.3$ expression in the SN even precedes pathological symptoms of early-stage PD patients, supporting the viewpoint that dysregulation of calcium homeostasis is a pathological contributor to PD and not a compensatory response (Hurley, Brandon, Gentleman, & Dexter, 2013). Finally, these findings contribute to the potential explanation as to why dopaminergic neurons of the ventral tegmental area (VTA) remain relatively unaffected by the pathological progression of PD, as these neurons don't display the same calcium-related characteristics (William Dauer & Przedborski, 2003).

1.2.6.4 Ca^{2+}-induced neurotoxicity

Overall, the relative increase in SN calcium signaling provides compelling evidence for increases in α-Syn expression and aggregation ultimately leading to cell death in PD. One mechanism by which this may happen is via heightened mitochondrial calcium load, leading to oxidative processes and calcium cytotoxicity. Indeed, cytochrome c, an electron transfer protein that plays a major role in physiological function of the electron transport chain, was hypothesized to trigger α-Syn aggregation and colocalize with oligomers in Lewy bodies (Hashimoto, Takeda, Hsu, Takenouchi, & Masliah, 1999). Cytochrome c, which is released from the mitochondria under various forms of cellular

stress, requires an assembled mitochondrial-derived heme protein to ultimately activate the caspase-9-dependent apoptosis pathway (Green, 1998). H_2O_2/cytochrome c-induced oxidative stress was induced in populations of SN neurons to cause α-Syn aggregation, which was inhibited by application of the antioxidant *N*-acetyl-L-cysteine and iron chelator deferoxisamine, demonstrating the detrimental effect of mitochondrial oxidative stress on neurotoxicity (Hashimoto et al., 1999).

Perhaps the most compelling piece of evidence that disruption of calcium homeostasis is implicated in regulation of neuronal cell death is the fact that calcium binds the C-terminal domain of α-Syn and modulates its ligand interactions (Nielsen, Vorum, Lindersson, & Jensen, 2001). Furthermore, intracellular calcium concentration is dependent on α-Syn dosage (Caraveo et al., 2014). Consequently, one calcium-related target of its upregulation is calcineurin, a phosphatase highly enriched in neurons. This Ca^{2+}/calmodulin-dependent phosphatase is responsible for dephosphorylating the hyperphosphorylated cytosolic form of NFAT, which causes nuclear translocation to regulate gene transcription (Macian, 2005). Calcineurin/NFAT signaling pathways are activated upon transfection of either wild-type or A53T α-Syn, as calcineurin phosphatase activity and NFAT nuclear translocation are both upregulated in HEK cells and midbrain dopaminergic neurons. This α-Syn-dependent phenotype is ameliorated by cyclosporine A, a pharmacological inhibitor of calcineurin, which also rescued dopaminergic neuron viability, indicating that this pathway is critical in PD-associated neuron loss (Luo et al., 2014). Interestingly though, given NFAT nuclear translocation is correlated with promoting neuronal survival (Benedito et al., 2005), clearly more research is required to elucidate the precise mechanism by which calcineurin and NFAT mediate their α-Syn-dependent

effects. In fact, both deletion and overexpression of calcineurin induces cytotoxic effects, demonstrating that perhaps a regulated intermediate amount of calcineurin activation protects against neurotoxicity due to its interaction with multiple downstream substrates (Caraveo et al., 2014).

Finally, another Ca^{2+}-dependent enzyme implicated in PD is calpain. Under normal physiologic function, this protease regulates neuronal function and synaptic plasticity, but post-mortem PD patients express an increased amount of the overactivated form (Annick Mouatt-Prigent, Karlsson, Yelnik, Agid, & Hirsch, 2000). Calpain is responsible for cleavage of the C-terminal domain of α-Syn, which promotes oligomerization, and cleavage of the activator of CDK5, p35, into a pathologic form (Zaichick, McGrath, & Caraveo, 2017). Expression of the pathological activator, p25, increases CDK5 activity, leading to phosphorylation and subsequent inactivation of MEF2D, a neuronal survival factor. S444A mutagenesis of MEF2D, the CDK5-dependent phosphorylation site, protects dopaminergic neurons from apoptosis, indicating the pathological importance of the calpain/CDK5 signaling cascade in PD (Smith et al., 2006).

Not surprisingly, the role of α-Syn in disrupting calcium signaling cascades and initiating apoptotic signaling pathways is supported by its capacity to regulate mitochondrial morphology. Importantly, mitochondrial fragmentation occurs as a physiological response to senescence and due to physiological stressors (Herndon et al., 2002; Westermann, 2008). As previously mentioned, due to its various lipid-protein associations, α-Syn is able to mediate membrane curvature. Its high affinity for cardiolipin, a phospholipid expressed in mitochondrial membranes, makes α-Syn a prime candidate for remodeling mitochondrial morphology (Nakamura et al., 2008). Indeed, exogenous

overexpressed α-Syn impairs mitochondrial fusion *in vitro* in SH-SY5Y cells. This phenotype is rescued with wild-type overexpression of multiple PD-associated gene loci PINK1, DJ1, and Parkin, but not their PD-associated mutants (Kamp et al., 2010). Although mitochondrial fission does not always directly indicate apoptosis, subsequent cytochrome c release and caspase activation can occur due to colocalization of mitochondrial fission proteins DRP1 and BAX/BAK (Youle & Karbowski, 2005). However, α-Syn-mediated mitochondrial fission does not require DRP1, as fragmentation occurs due to direct interaction of α-Syn oligomers with cardiolipin. Overall, data suggest that deleterious PD-associated mutations may cause changes to mitochondrial morphology, leading to mitochondrial dysfunction and a bioenergetic reduction, which makes neurons expressing this morphology more susceptible to cell death (Nakamura et al., 2011).

1.2.7 Function in AD

Alzheimer's Disease (AD) is currently the most prevalent neurodegenerative disorder worldwide, characterized by pathological aggregation of amyloid beta (Aβ) and neurofibrillary tangles composed primarily of the hyperphosphorylated microtubule-associated protein tau (Twohig & Nielsen, 2019). Like PD, most AD cases are initiated idiopathically, with longevity (> 65 years) being one of the greatest risk factors. A small percentage of patients carry autosomal dominant mutations in a select number of genes, namely presenilin-1 (*PSEN1*), presenilin-2 (*PSEN2*), or Amyloid Beta Precursor Protein (*APP*), which predisposes them to early-onset AD (~45-65 years). Emerging evidence suggests that α-Syn also plays an important role in the pathogenesis of AD, as α-Syn pathology is striking in the amygdala of many AD patients carrying *PSEN1* mutations

(Leverenz et al., 2006). Furthermore, structural evidence of this pathophysiological interaction is encoded by the amino acid sequence of α-Syn. What is now known as the non-Aβ component (NAC) region of α-Syn, which comprises residues 61-95, was discovered to be enriched in Aβ plaques (Uéda et al., 1993). Although the overall secondary structure of α-Syn remains to be elucidated, the NAC region has been determined to interact with the N-terminal region to create α-helices critical to the membrane-bound state of α-Syn (Trexler & Rhoades, 2009). The NAC region is also necessary for anomalous aggregation and misfolding of α-Syn by producing β-sheet structures (H. T. Li, Du, Tang, Hu, & Hu, 2002). Interestingly, not only do exogenous α-Syn fibrils have the capability to seed PD-like Lewy body inclusions (Kelvin C. Luk et al., 2009), but α-Syn can also induce tau fibrillization in addition to promoting aggregation of itself (Giasson Benoit et al., 2003). This aberrant role of α-Syn demonstrates its potential importance in other neurodegenerative pathologies aside from PD.

1.2.8 Function in other synucleinopathies

Other pathologies caused by an atypical role of α-Syn include Lewy Body Dementia (DLB), Multiple Systems Atrophy (MSA), and Parkinson's Disease Dementia (PDD). Current diagnostic criteria for DLB is similar to that of AD, and is often clinically misdiagnosed as AD, since the two diseases share many pathologies. For example, DLB patients show pathological overlap of both α-Syn and Aβ deposition (Halliday, Holton, Revesz, & Dickson, 2011). However, what distinguishes DLB from PD is that whereas dopaminergic denervation of the SN characterizes PD, causing it to be primarily a movement disorder, Lewy bodies are most prevalent in non-limbic systems and the

neocortex associations in DLB, establishing it predominantly as a dementia disorder. PDD is a disorder where patients exhibit parkinsonian symptoms along with dementia. Like DLB, Lewy body pathology often exists in the least limbic systems of the brain, contributing to the phenotype of dementia (W. S. Kim, Kågedal, & Halliday, 2014). Lastly, although MSA also involves α-Syn aggregation and parkinsonian-like symptoms, the localization of Lewy bodies is distinct, and patients also exhibit cerebellar ataxia and autonomic dysfunction, indicating that other locations of the CNS are affected by α-Syn pathology (S. Gilman et al., 2008). Strikingly, along with neuronal cytoplasmic inclusions, which are common in other synucleinopathies, inclusions can be found in the cytoplasm or nucleus of oligodendrocytes (Papp, Kahn, & Lantos, 1989), and less commonly in nucleus or processes of neurons (W. L. Lin, DeLucia, & Dickson, 2004) in MSA patients. While post-translational modification of fibrillar α-Syn is the core component of inclusions, MSA patients with parkinsonian symptoms respond poorly to levodopa, unlike PD patients, indicating the complex nature of the pathogenesis of the disease (S. Gilman et al., 2008). Taken together, a multitude of neurodegenerative disorders related to α-Syn exist on a continuous spectrum with overlapping pathological characteristics. This makes it particularly difficult to accurately diagnose and clinically treat patients, in addition to the difficulty of investigating precise molecular mechanisms for each specific disease. Future studies should be directed at delineating commonalities and differences between α-Syn-related disorders.

1.3 Phosphoinositides

1.3.1 PI(4,5)P_2 structure and function

The phosphoinositide (PI) class of phospholipids are a physiologically important membrane-derived species of lipids containing an inositol ring linked to diacylglycerol (DAG) backbone via a phosphodiester bond. The hydroxyl positions 3, 4, and 5 on the inositol ring can be reversibly phosphorylated or dephosphorylated to create seven distinct species of polyphosphoinositides (PPIs). Each PI species has distinctive subcellular localization and physiological function, although they exist in a dynamic equilibrium with one another (Figure 3). Many PIs carry specific localization sequences, giving them a unique zip code for spatial distribution (Dickson & Hille, 2019). PI(4,5)P_2 (often referred to hereafter as PIP_2) is the signature PI of the plasma membrane. It acts in a myriad of cellular processes, including but not limited to endocytosis/exocytosis, cell motility, membrane protein anchoring, modulating ion channel activity, and second messenger signaling (Falkenburger, Jensen, Dickson, Suh, & Hille, 2010).

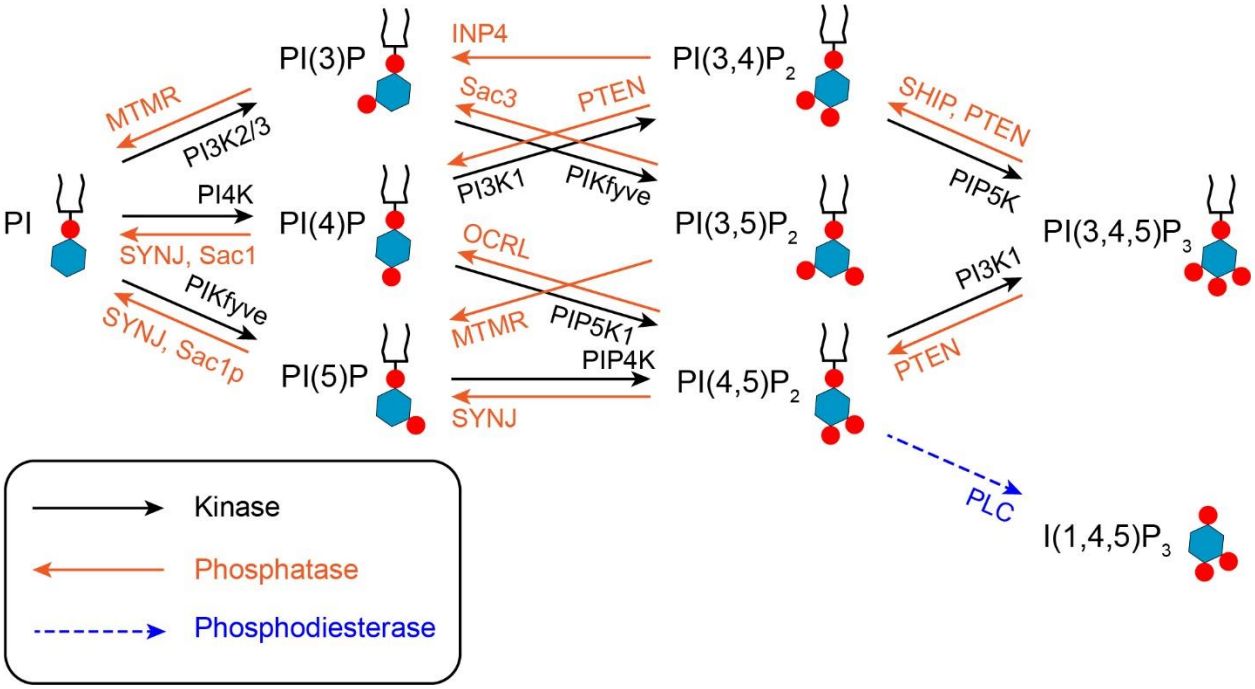


Figure 3. Phosphoinositide metabolism. Seven distinct PPI species are produced from the parent molecule PI. Species of PPI molecules may have varying fatty acyl chain lengths and double bond saturation. However, unique phosphorylation of the inositol head group determines the function and subcellular localization of each PPI. Phosphate groups can be added (black arrow; lipid kinases) or removed (orange arrow; lipid phosphatases) by the respective enzymes listed to the inositol head group to create a dynamic equilibrium of PPI species that play a precise role in various cellular processes. The signature PPI of the plasma membrane, $PI(4,5)P_2$ is also hydrolyzed by isoforms of PLC (dashed blue arrow; phosphodiesterase) to create DAGl (not pictured) and soluble IP_3.

1.3.2 PI(4,5)P_2 synthesis

PIP_2 can be produced either by adding a phosphate group to the 4 or 5 position on the inositol ring of PIP or removing a phosphate group from the 3 position of PI(3,4,5)P_3. The predominant enzyme enriched in neuronal tissue that catalyzes the production of PIP_2 is the lipid kinase PI(4)P 5-kinase 1 γ (PIP5K1γ), which phosphorylates PI(4)P (Wenk et al., 2001). Two other mammalian isoforms of the PIP5K family also exist, including PIP5K1α and PIP5K1β, albeit at lesser levels in the brain. The role of PIP5K1γ in neuronal function is underscored by its involvement in synaptic vesicle trafficking, long-term potentiation and depression, ER-mediated Ca^{2+} signaling, and neuronal development (Di Paolo et al., 2004; Unoki et al., 2012; Y. Wang, Lian, Golden, Morrisey, & Abrams, 2007; Y. J. Wang et al., 2004; Yu et al., 2011). Being a critical substrate for PIP_2 production, PI(4)P is found in two distinct subcellular pools, the PM and Golgi (Dickson, Jensen, & Hille, 2014). At least four isoforms of the PI 4-kinase (PI4K) exist, PI4K type II (α and β) and III (α and β). PI4K type II enzymes are localized solely at the plasma membrane due to palmitoylation at the catalytic domain (Barylko et al., 2001), while PI4K type III enzymes are found at either the plasma membrane (PI4KIIIα) (Nakatsu et al., 2012), Golgi (PI4KIIIα and PI4KIIIβ) (A. Balla, Tuymetova, Tsiomenko, Varnai, & Balla, 2005; Godi et al., 1999), or nucleus (PI4KIIIβ) (de Graaf et al., 2002). After being produced, PI(4)P translocates to the plasma membrane where it acts as the precursor for PIP_2 production. This process can occur in multiple ways. PI(4)P can traffic to the plasma membrane through fusion of standard ER-Golgi transport vesicles where it directly is added to the PM PI(4)P pool, or it can be phosphorylated by vesicle-associated PI5K enzymes as it is directly delivered to the plasma membrane PIP_2 pool (Dickson et al.,

2014). Several mechanisms and subcellular locations for PI(4)P production illustrate the cellular importance of PIP_2 production for biological processes.

1.3.3 PI(4,5)P_2 degradation

In addition to its production via PI(4)P synthesis, PM PIP_2 expression is also highly dependent on its turnover and degradation. While multiple lipid kinases contain 4-phosphatase and/or 5-phosphatase domains, the most relevant to synucleinopathies is synaptojanin-1 (SYNJ1). SYNJ1 has both 4-phosphatase and 5-phosphatase activity, making it capable of producing either PI(4)P or PI(5)P from the parent PI(4,5)P_2 molecule. SYNJ1 is present at the PM, endomembranes, and the nucleus, suggesting that SYNJ1-mediated 5-phosphatase activity of PIP_2 is physiologically more important, as the latter PIP is not as pertinent for maintaining precursor pools for PIP_2 production. However, contrary to this viewpoint, a homozygous missense mutation in the Sac1 domain of SYNJ1, which impedes 4-phosphatase activity, causes autosomal dominant early-onset PD, as observed in two independent familial lineages of Italian and Iranian descent (Krebs et al., 2013; Quadri et al., 2013). Moreover, this specific mutation impairs clathrin-mediated endocytic synaptic vesicle recycling, through which PI(5)P plays an important role (Cao et al., 2017). Indeed, this mutation has now been identified as the *PARK20* gene loci responsible for early-onset PD, demonstrating the significance PI, specifically PIP_2, potentially play in the pathogenesis of neurodegenerative disorders. In addition to SYNJ1, PIP_2 is also a substrate for phospholipase C (PLC), where it is hydrolyzed to produce soluble IP_3 and membrane-localized DAG (Figure 3, dashed blue arrow). While DAG activates PKC to carry out Ca^{2+}-dependent cellular processes, IP_3 diffuses to the

ER membrane where it binds ligand-gated IP_3R to release Ca^{2+} from its luminal stores (Kadamur & Ross, 2013). This potent second-messenger pathway is important for many cellular processes, including cytosolic Ca^{2+} elevation crucial for neuronal signaling, which will be discussed in detail later.

1.3.4 Phospholipid trafficking

Not only do PPIs undergo phosphorylation and dephosphorylation to modify and regulate their physiological function, a variety of lipid transfer proteins (LTPs) exist at closely linked organellar membrane junctions to move PIs to and from different subcellular locations to carry out particular roles. Specifically, oxysterol-binding proteins (OSBP) such as OSBP1 promote the transfer of cholesterol from the ER to the trans-Golgi in exchange for backwards PI(4)P transfer from the trans-Golgi to the ER (Mesmin et al., 2013). This critical step allows the ER-resident phosphatase Sac1 to hydrolyze PI(4)P into PI (S. Guo, Stolz, Lemrow, & York, 1999; Rohde et al., 2003), which is the major elimination pathway for this PPI (Tahirovic, Schorr, & Mayinger, 2005). Another LTP that aids in the transfer of PI(4)P to the ER is OSBP-related protein 5/8 (ORP5/8), which tethers ER-PM membrane junctions to replenish PM phosphatidylserine (PS) in exchange for PI(4)P targeted to the ER for Sac1-mediated degradation (Chung et al., 2015). PS is the most common negatively charged phospholipid enriched in eukaryotic lipid bilayers. It carries out various physiological functions across many cell types, such as clotting cascade activation in platelets, histamine release in mast cells, and differentiation in immature neurons (Leventis & Grinstein, 2010). Importantly, in all cell types, scramblase enzymes translocate PS to the outer leaflet of the plasma membrane as a recognition factor to

initiate macrophage-induced apoptosis (Balasubramanian, Mirnikjoo, & Schroit, 2007). Finally, PIP_2 is also capable of being transferred to the cell surface. This is accomplished by the PIP_2 floppase activity mediated by ABCA1 independent of the PS floppase activity, which is crucial for apolipoprotein A1 lipid binding and high-density lipoprotein (HDL) assembly (Gulshan et al., 2016). Altogether, due to the ability of PPIs to regulate diverse cellular processes, as well as their ability to influence their own dynamic metabolism, many mechanisms exist for synthesis, transfer, and degradation, which are all tightly monitored in order to maintain the delicate balance of PPI expression. Alterations in distribution have vast impacts on overall cell integrity and health, which underlies the pathology of many neurodegenerative diseases, including PD.

1.3.5 Implications in neurodegenerative diseases

One of the earliest documentations of PPIs being implicated in the pathological progression of PD involved the sequence homology of α-Syn and a molecular chaperone, 14-3-3, which stabilizes PKC in an inactive conformation (Ostrerova et al., 1999). Given that PKC requires DAG for activation, which is a product of PIP_2 hydrolysis mediated by phospholipase C (PLC), pathological consequences are imminent under the alteration of this biochemical pathway. Furthermore, α-Syn functions as a selective inhibitor of phospholipase D2 (PLD2), which is potently activated by PIP_2 (Jenco, Rawlingson, Daniels, & Morris, 1998). Evidently, due to these interactions, α-Syn is implicated in PPI metabolism regulation, hinting at areas of investigation for cellular and molecular mechanisms of neurodegeneration. Further studies were subsequently initiated, and additional findings included the increased propensity of α-Syn to oligomerize under the

presence of various lipid species. The N-terminal region of α-Syn was identified as a lipid-interacting domain capable of inducing self-aggregation in a surface-dependent interaction specific to negatively charged phosphate head groups (E. N. Lee, Lee, Lee, Kim, & Paik, 2003). In addition, metal-catalyzed oxidation of α-Syn is also potentiated by anionic phospholipids (E. N. Lee et al., 2003), specifically PI, potentially elucidating the etiology of neurodegeneration in regard to abnormal metal ion homeostasis (Paik, Shin, Lee, Chang, & Kim, 1999). This data is reinforced by the increase of PIP_2 in whole-tissue homogenate from the SN of PD patients as observed through dot blots (Sekar & Taghibiglou, 2018). This elevation underlies the pathophysiological importance of PIP_2 in PD, as it impacts α-Syn-dependent axon growth and endocytosis (Schechter, Atias, et al., 2020; Schechter, Grigoletto, et al., 2020). Finally, not surprisingly, given the previously present data, PIP_2 levels also correlate spatially with plasma membrane α-Syn localization. Overexpression of PIP5K1γ increases α-Syn intensity in A2780 cells while overexpression of 5-phosphatase INPP5E and 3-phosphatase PTEN decrease α-Syn intensity, suggesting PIP_2 acts as a rheostat for finely tuning α-Syn expression (Jacob, Eichmann, Dema, Mercadante, & Selenko, 2021).

Taken together, the involvement of PI metabolism in the molecular mechanisms of PD remains to be a lucrative point of investigation due to its action in many physiological processes. In this present dissertation, I outline the repercussions of aberrant PPI signaling in PD, which include G_q-mediated processes, mitochondrial dysfunction, and ultimately neurotoxicity.

1.4 G_q-protein-mediated signaling

1.4.1 Pathway overview

PIP_2 acts as a substrate for PLC in a ubiquitous intracellular second messenger pathway (Figure 4). Upon ligand activation of a wide variety of G_q protein-coupled receptors (GPCRs), the GDP from GDP-bound G_q alpha subunit is exchanged for a GTP to produce its active form, which dissociates from the beta and gamma subunits to activate PLC. PIP_2 is hydrolyzed by this enzyme, creating membrane-associated DAG and soluble inositol trisphosphate (IP_3). IP_3 freely diffuses through the cytosol, but upon binding the ER membrane-localized ligand-gated IP_3R, ER Ca^{2+} stores are mobilized, elevating intracellular calcium concentration for various cellular functions, which are highly dependent on cell type (A. G. Gilman, 1987). In neurons, due to the wide role the IP_3R plays in cell proliferation, neuronal development, learning, memory, and apoptosis, it is clear the phosphoinositol signaling cascade is critical for physiological processes involving elicitation of spatiotemporal Ca^{2+} release (Mikoshiba, 2015).

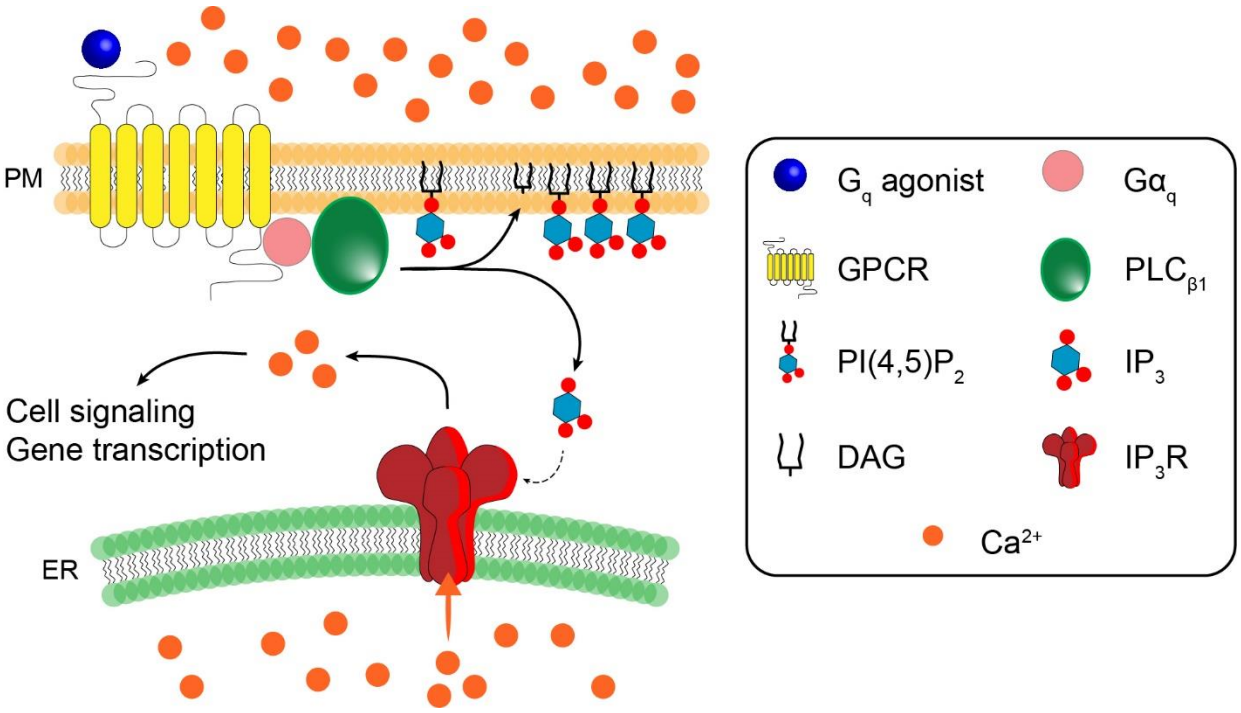


Figure 4. G_qPCR signaling cascade. Upon binding their appropriate neurotransmitters, hormones, peptides, or other ligands, G_qPCRs containing seven trans-membrane domains undergo a conformational change that is transmitted to the bound $G\alpha_q$ subunit of the G-protein complex, exchanging a bound-GTP for bound-GDP. The GTP-activated form of the $G\alpha_q$ subunit activates PLC to hydrolyze $PI(4,5)P_2$, producing membrane-associated DAG and soluble IP_3. IP_3 freely diffuses in the cytosol, and upon reaching the ER, it binds to its receptor, IP_3R. This ligand-gated Ca^{2+} channel opens and allows Ca^{2+} to travel down its concentration gradient, causing local increases in cytosolic Ca^{2+}. As a second messenger signal, Ca^{2+} may induce a plethora of cellular processes. However, specific signaling information is encoded with the total quantity, oscillatory nature, and further IP_3R activity modulation of Ca^{2+} release. Initiated neuronal signaling pathways include cell growth and differentiation, gene transcription, and cell death, among others.

1.4.2 Modulation of IP_3R activity

Not only can IP_3R activity be modified by modulation of components of the G_q-mediated pathway, it can be altered by cytosolic Ca^{2+} concentration, IP_3 activity, IP_3R clustering, kinase-mediated phosphorylation, and protein-protein interactions.

1.4.2.1 Ca^{2+}

Although IP_3 is necessary for ER Ca^{2+} release via IP_3R, it is not sufficient. Ca^{2+} also acts as a coagonist in IP_3-mediated Ca^{2+} release, via a specific Ca^{2+} sensor region of the IP_3R that includes glutamate residue 2100, which is critical in determining Ca^{2+} sensitivity (Finch Elizabeth, Turner Timothy, & Goldin Stanley, 1991; Miyakawa et al., 2001). However, prolonged periods of elevated cytosolic calcium concentration have a detrimental effect on cellular function due to induction of multiple and often conflicting physiological processes, frequently leading to cell death. IP_3Rs avoid this deleterious fate by releasing calcium in an oscillatory pattern, in which physiological instructions are encoded in the frequency and amplitude of Ca^{2+} release (Berridge, Lipp, & Bootman, 2000; S. Zhang, Fritz, Ibarra, & Uhlen, 2011). The ability to produce Ca^{2+} oscillations is also accomplished by the ability of cytosolic Ca^{2+} to terminate IP_3R release, creating a biphasic pattern of channel open probability (I. Bezprozvanny, Watras, & Ehrlich, 1991).

1.4.2.2 IP_3

IP_3Rs are able to spatially cluster to modulate ER Ca^{2+} release, which is mediated by IP_3 itself. An IP_3R can open to cause a single channel event, or Ca^{2+} “blip”, but low concentrations of IP_3 cause IP_3Rs to cluster so the opening of one channel can cause the

opening of its neighbors in close proximity in order to augment the effects of Ca^{2+} within a cluster, known as a Ca^{2+} “puff” (Rahman & Taylor, 2009).

1.4.2.3 IP_3R isotype

To further regulate ER Ca^{2+} release, different isoforms of the IP_3R exist, which have tissue-specific expression levels and varying affinities for IP_3 (Newton, Mignery, & Südhof, 1994). All three isoforms (IP_3R1, IP_3R2, IP_3R3) are expressed in the brain. However, only IP_3R1 and IP_3R3 are expressed in neurons, while IP_3R1 and IP_3R2 are the isoforms found in glial cells (Sharp et al., 1999). Intriguingly, all tissues and cell lines express a varying amount of IP_3R isoforms due each one's unique ability to dictate cell fate by activating cell death and/or cell survival pathways (Ivanova et al., 2014).

1.4.2.4 Protein interactions

Finally, many proteins form complexes with IP_3Rs or phosphorylate IP_3Rs to either enhance or inhibit their activity (Prole & Taylor, 2016). Not surprisingly, among these are proteins and kinases associated with Ca^{2+}, such as calmodulin and CaMKII which both inhibit IP_3R activity through their respective functions (Hirota, Michikawa, Natsume, Furuichi, & Mikoshiba, 1999; Toussaint, Charbel, Blanchette, & Ledoux, 2015). Taken together, all of these physiological features of the IP_3R allow cells to modulate Ca^{2+} specificity for activation of various signaling pathways.

1.5 ER-mitochondria junctions

The ER forms dynamic junctions with other organellar membranes, including but not limited to the plasma membrane, Golgi membrane, and mitochondrial membranes. The importance of these junction sites is underscored by the ability of ER to locally funnel Ca^{2+} into other organelles following IP_3R activation. Principally, mitochondria sequester Ca^{2+} to help homeostatically maintain low cytosolic levels, keeping a resting concentration of roughly 100 to 200 nM. However, upon activation of Ca^{2+} signaling pathways, mitochondrial calcium concentration can spike up to 500 μM, demonstrating the high capacity mitochondria have for handling Ca^{2+} (Xu, Zhang, He, Huang, & Shao, 2016). Furthermore, mitochondrial Ca^{2+} is required as a cofactor for certain enzymes, namely pyruvate dehydrogenase, isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase, involved in Krebs Cycle-dependent aerobic metabolism (Traaseth, Elfering, Solien, Haynes, & Giulivi, 2004; Wan, LaNoue, Cheung, & Scaduto, 1989), illustrating the critical cellular function of ER-mitochondria contact sites.

Various proteins are involved in the ER-mitochondrial Ca^{2+} shuttling, four of the most crucial being IP_3R, GRP75, DJ1, and VDAC1. VDAC1, a voltage dependent anion channel that controls mitochondrial metabolic flux, including that of calcium, is physically linked to the IP_3R by two molecular chaperone proteins: GRP75 and DJ1 (Y. Liu et al., 2019; Szabadkai et al., 2006). The latter is associated with the *PARK7* gene locus, in which mutations of DJ1 are a risk factor for early-onset PD (Bonifati et al., 2003). IP_3Rs are required for ER-mitochondrial contact sites to form. However, interestingly, each IP_3R isoform has distinct capabilities for regulating these junctions, with IP_3R2 being the most efficient in propagating Ca^{2+} signals to the mitochondria (Bartok et al., 2019). Together,

the close spatial proximity of these proteins mediates mitochondrial Ca^{2+} influx. Nevertheless, dysfunction of ER-mitochondria contact sites are implicated in several neurodegenerative diseases, including AD, ALS, and PD (E. L. Wilson & Metzakopian, 2021).

1.6 Mitochondrial dysfunction

Although Ca^{2+} is required for proper mitochondrial physiological function, overabundance of Ca^{2+} causes cytotoxic effects. However, the precise mechanism by which this occurs in neurodegenerative diseases requires clarification. For PD, several hypotheses have been postulated as to why dopaminergic neurons of the SN are particularly vulnerable to Ca^{2+}-induced mitochondrial dysfunction. First, SN neurons are highly arborized and unusually large, resulting in an elevated metabolism compared to other dopaminergic neurons, such as those in the ventral tegmental area which are relatively spared throughout the progression of PD (Pacelli et al., 2015). This metabolic requirement places stress on the mitochondria, utilizing greater amounts of Ca^{2+} to produce sufficient ATP. Metabolic stress also elicits higher activity from K_{ATP} channels, which are coupled to mitochondria in SN neurons (Liss et al., 2005), increasing the likelihood of NMDA-induced excitotoxicity and potentiating neuronal Ca^{2+} influx (Schiemann et al., 2012). Finally, SN neurons express a high amount of $Ca_V1.3$ channels, which activate at a more negative potential than their $Ca_V1.2$ counterparts and control their oscillatory pacemaking patterns (Chan et al., 2007; Philippart et al., 2016). All in all, it is apparent that dopaminergic neurons of the SN are selectively susceptible to elevations in calcium signaling cascades, placing a high load on the mitochondria. Given

that mitochondria are a key source of reactive oxygen species (ROS) production via oxidative properties of the electrons transport chain and subsequent superoxide leakage, upregulation of mitochondrial Ca^{2+} import and thus ATP production induces a neurotoxic effect (Yuanbin Liu, Fiskum, & Schubert, 2002). This pathological feature of oxidative stress is augmented by atypical α-Syn aggregation in dopaminergic neurons (Dryanovski et al., 2013). Generally speaking, aberrant regulation of the PLC-IP_3 signaling axis results in increased mitochondrial Ca^{2+} load and ATP synthesis dysfunction as demonstrated by decreased viability in glutamatergic neurons and shortened lifespan of *Drosophila* (Wong et al., 2021).

1.7 Introduction summary

Taken together, although there is a surfeit of data suggesting the pathological role of α-Syn in disease, there is a paucity of information regarding its physiological importance. Thus, it is difficult to discriminate whether the damaging effects of the protein outweigh those that are beneficial, and perhaps PD progression causes the function of α-Syn to reach a pathophysiological tipping point. Therefore, investigation of the tight regulation of α-Syn expression, aggregation, and clearance remain of the utmost importance for slowing progression of PD and other related synucleinopathies. The goal of this body of work is to outline a novel signaling axis, in which augmented $PI(4,5)P_2$ metabolism alters Ca^{2+} signaling platforms and shifts the cellular role of α-Syn from benign to deleterious. Alterations in this pathophysiological nexus provide opportunities for future investigation of therapeutic intervention, which would rescue detrimental consequences and reduce neurotoxicity.

2. Materials and Methods

Cell Culture

Embryonic hippocampal, cortical, and substantia nigra neurons were isolated from mice at day 18 of gestation and plated on 35 mm glass coverslips. Neuron cultures contained Neurobasal (21103-049; Gibco) supplemented with B27 (17504-044; Gibco), 5% FBS (26140-079; Gibco), Glutamax (35050-061; Gibco), and 0.2% penicillin/streptomycin. 50% neuronal media was exchanged every 3 days for fresh media. CHO, tsA-201, HeLa, and HEK293-Cas9-RFPcells (CRL-1573Cas9; ATCC) were cultured in DMEM (11995-065; Gibco) containing 10% FBS and 0.2% penicillin/streptomycin and passaged twice weekly at 1:20. Fibroblast cell lines from a healthy male patient (GM05659), a male patient with PD (AG20445), a healthy female patient (ND36091) and a female PD patient harboring α-Syn$_{A53T}$ (NDS00188) with were acquired from NINDS human cell repository and the Coriell Institute. Fibroblasts were passaged twice weekly and were cultured in MEME (M5650; Sigma) containing 15% FBS, 2 mM L-glutamine, and 0.2% penicillin/streptomycin. eGFP-IP$_3$R HeLa cells were a gift from Colin Taylor (Thillaiappan, Chavda, Tovey, Prole, & Taylor, 2017) and were cultured in the same media conditions as wild-type HeLa cells. IP$_3$R type-1 $^{-/-}$ HEK293 cells were purchased from Kerafast and were cultured in the same media as tsA-201 cells. Doxycycline- inducible α-Syn-expressing SH-SY5Y cells were a gift from Muralidhar Hegde (Vasquez, Mitra, Perry, Rao, & Hegde, 2018). Undifferentiated cells were treated with 3 μg/mL doxycycline hyclate (J60579; Alfa Aesar) for 72 hr to induce α-Syn overexpression. All cell lines were incubated in 5% CO_2 at 37 °C.

Transfections, Plasmids, and siRNA

Lipofectamine 2000 (11668-019; Invitrogen), LTX (15338-030; Invitrogen), and RNAiMax (13778-030; Invitrogen) were used for 24-hour cDNA transfections as per manufacturer's recommendations for cultured cells. Neurons were transfected between DIV 5-8 and culture media was replaced with a 2:1 ratio of old:fresh media following neuronal transfections. Neuronal media was exchanged once per week while cultured. The following cDNA plasmids were used in the present study: $PH_{PLC\delta1}$ –CFP (gift from Tamas Balla), α-$synuclein_{A53T}$-GFP (gift from Bjoern Falkenberger; (Opazo, Krenz, Heermann, Schulz, & Falkenburger, 2008)), 3xNLS-PH-GFP (gift from Michael Sheetz; (Y. H. Wang et al., 2017), GFP-PIP5KIgamma(90 kDa) (gift from Pietro Di Camilli; (Di Paolo et al., 2002)), pCS2-PLK1-mCherry (Addgene: 127154), pCAG mito-RCaMP1h (Addgene: 105013;), p3E-ARF6-DN (Addgene; 109592). DsiRNA Duplex targeting PIP5K1γ and sgRNA targeting SNCA were purchased from Integrated DNA Technologies and transfected as per manufacturer's recommendations.

Live cell AiryScan superresolution imaging

Coverslips containing transfected cells were imaged in 2 mM Ca^{2+} Ringer's solution (160 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 2.5 mM KCl, 10 mM Hepes, and 8 mM glucose) and were excited using 405 nm, 488 nm, or 594 nm lasers. Resulting light was collected using a Plan-Apochromat 63×/1.40 oil-immersion lens and a Zeiss 880 Airyscan microscope at room temperature (21 °C). Images were processed with Airyscan post-image processing using Zen software. For cells transfected with PH-CFP and PIP5KIγ-GFP, mean intensity values of in-focus plasma membrane was divided by the mean

intensity value of in-focus cytoplasm to obtain PM/Cyto ratio. For cells transfected with 3xNLS-PH-GFP, mean intensity values of nucleoli was divided by mean intensity value of nucleus without nucleoli to obtain normalized nuclear intensity.

Lipid Mass Spectrometry

Phosphoinositides were quantified as described previously (Traynor-Kaplan et al., 2017). Briefly, endogenous lipids from isolated neurons or cell lines were extracted with n-butanol and chloroform. After derivatization, samples were ran on a C4 column using an acetonitrile/formic acid gradient. Post-column eluate was infused with sodium formate and monitored using a Waters XEVO TQ-S MS/MS in multiple reaction monitoring (MRM), positive ion mode. Elution profiles were quantified by integrating areas under peaks using MassLynx software. Peak areas of individual phosphoinositide species from the biological sample were normalized to synthetic standards and corrected for tissue amount using total protein.

Immunocytochemistry

Cultured cells were initially washed with PBS and fixed in 4% PFA for 10 min. Neurons were fixed between DIV 7-14. Cells were subsequently washed again then blocked with 20% Sea Block Blocking Buffer (37527; Thermo Scientific) containing 0.1% Triton X-100 (T8787; Sigma) for 1 h at 21 °C. Cells were stained at 10 µg/mL overnight at 4 °C with the following primary antibodies: anti-PIP5K1γ (gift from Dr. Pietro DiCamilli; (Di Paolo et al., 2002), anti-β-actin (MA1-91399; Invitrogen), anti-GAPDH (10494-1-AP; Proteintech), anti-α-synuclein [LB509] (ab27766; Abcam), anti-PLK1 (37-7000; Invitrogen), anti-IP_3R1

(75-035; Antibodies Inc.), anti-MAP2 (AB5622; Millipore), anti-MAP2 (ab11267; Abcam), anti-Ser(P)-129-α-synuclein (ab51253; Abcam), anti-VDAC1 (ab14734; Abcam). Cells were washed with PBS and incubated at 21 °C for 1 h with the following secondary antibodies at 1:1,000 in blocking solution: Alexa Fluor 647 goat anti-mouse (A21236; Invitrogen), Alexa Fluor 555 goat anti-mouse (A21424; Invitrogen), Alexa Fluor 647 goat anti-rabbit (A21245; Invitrogen), Alexa Fluor 555 goat anti-rabbit (A21429; Invitrogen). Z-stack images were collected using a Plan-Apochromat 63×/1.40 oil-immersion lens and a Zeiss 880 Airyscan microscope at room temperature (21 °C). Images were processed with Airyscan post-image processing using Zen software. Z-stacks were converted to a singular maximum intensity projection image in ImageJ. Analysis parameters Subtract Background, Median Filter, Threshold, and Particle Analysis were held constant for all images in ImageJ.

Single molecular localization microcopy

Undifferentiated SH-SY5Y cells were fixed in 4% PFA for 10 min, blocked with 20% SeaBlock containing 0.1% Triton X-100 for 1 h at 21 °C, and stained with anti-α-synuclein (ab27766; Abcam) overnight at 4 °C. Cortical neurons were treated with α-Syn fibrils or vehicle control for 72 hr, fixed between DIV 7-14, and stained for anti-PIP5K1γ (gift from Dr. Pietro DiCamilli; (Di Paolo et al., 2002)). Cells were incubated for 1 h in Alexa Fluor 647 donkey anti-rabbit (A31573; 1:1,000; Invitrogen) or Alexa Fluor 647 goat anti-mouse (A21236; 1:1000; Invitrogen) secondary antibody in blocking solution. Images were captured using a Leica Infinity TIRF super-resolution microscope equipped with a 163x

1.49 NA TIRF oil immersion objective and a Hamamatsu orca flash 4.0 camera. Particle Analysis in ImageJ was conducted using 20 nm pixel size.

Protein extraction and Western Blot

Protein from cultured fibroblasts was harvested and lysates were blotted as previously described (Tiscione et al., 2019). anti-PIP5K1γ (ABS190; 1:300; Sigma) and anti-β-actin (MA1-91399; 1:1000; Thermo-Fisher) were applied to transferred membranes overnight at 4 °C. Blot bands were detected by Sapphire Biomolecular Imager (Azure Biosystems) after 1 h incubation in the following secondary antibodies: goat anti-rabbit 680RD (P/N 926-68071, 1:10,000; LI-COR), goat anti-Mouse 800CW (P/N 925-32210, 1:10,000; LI-COR). Images were processed on ImageJ using the BioImporter plugin tool in order to calculate the protein expression for each band. Protein abundance was first normalized to beta-actin intensity then normalized to control cell intensity.

Ca^{2+} imaging

Cells were incubated in 2 mM Ca^{2+} Ringer's solution containing 5 μM Fluo-4 AM (F14201; Invitrogen) and 0.1% pluronic acid (P3000MP; Invitrogen) to permeabilize cells at 21 °C for 30 min, followed by deesterification in Fluo-4-free Ringer's solution for 30 min. Cells were bathed in 2 mM Ca^{2+} Ringer's solution and excited by a 488 nm laser and the resulting fluorescence was monitored using an inverted microscope with a Plan-Apochromat 40×/1.40 oil objective, connected to an Andor W1 spinning-disk confocal with a Photometrics Prime 95B camera. Image acquisition occurred at 21 °C every 5 s using Micromanager software. At 100 s, cells were perfused with 100 μM UTP, ATP, or

bradykinin in 2mM Ca^{2+} Ringer's solution for 100 s. At 200 s, cells were perfused again with 2 mM Ca^{2+} Ringer's solution and imaged until 400 s. Images stacks were analyzed in ImageJ. An ROI was drawn in the cytosol of Fluo-4-loaded cells and measured for fluorescence. Intensity was normalized to minimum intensity value before G_q agonist application. Amplitude and area under the curve measurements were made by GraphPad Prism.

IP_3R-VDAC1 colocalization assay

Mouse cortical neuron cultures were treated with or without α-Syn fibrils 72 hr prior to fixation and/or treated with UNC-3220 24 hr prior to fixation. Cultured cells were fixed, blocked, and stained as described in *Immunocytochemistry*. Coverslips were excited by a 488 nm or 633 nm laser, and resulting light was collected using a Plan-Apochromat 63×/1.40 oil-immersion lens and a Zeiss 880 Airyscan microscope at room temperature (21 °C). Z-stack Images were processed with Airyscan post-image processing using Zen software. Z-stacks were converted to a singular maximum intensity projection image in ImageJ. Analysis parameters Subtract Background, Median Filter, and Threshold were held constant for all images in ImageJ. Threshold images of each channel were converted to binary images and the resulting IP_3R channel was multiplied by the VDAC1. IP_3R-positive and VDAC1-positive (overlapping) pixels were divided by total number of IP_3R-positive pixels to obtain percent colocalization.

Mitochondrial Ca^{2+} assay

tsA-201 cells with or without α-Syn_{A53T} overexpression were transfected with Mito-RCamPh1. UNC-3230 and vehicle treatment groups were treated 24 hr prior to imaging. Cells were incubated in 2 mM Ca^{2+} Ringer's solution and excited by a 546 nm laser. Resulting light was collected using a Plan-Apochromat 63×/1.40 oil-immersion lens and a Zeiss 880 Airyscan microscope at room temperature (21 °C). Images were processed with Airyscan post-image processing using Zen software. After initial image capture, cells were perfused with 20 mM Ca^{2+} Ringer's solution containing 2.5 μM ionomycin for 5 min at 21 °C. Cells were excited by a 546 nm laser for a second time. Pre-inomycin and post-ionomycin images were compared for each treatment group. A threshold was applied to images in a consistent manner to obtain mitochondrial ROIs for RCaMPh1 fluorescence. The intensity ratio of post-inomycin / pre-ionomycin of mito-RCaMPh1 was taken for each cell, with higher ratios indicating less mitochondrial Ca^{2+}.

ROS assay

Mouse cortical neuron cultures were treated with or without α-Syn fibrils 72 hr prior to imaging and/or UNC-3220 24 hr prior to imaging. Coverslips were incubated in 2 mM Ca^{2+} Ringer's solution containing 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; D399; Invitrogen) for 20 min at room temperature (21 °C). Upon cleavage of acetate groups by ROS, non-fluorescent H_2DCFDA is converted to fluorescent 2',7'-dichlorofluorescein (DCF). Cells were excited with a 488 nm laser. Resulting light was collected using a Plan-Apochromat 63×/1.40 oil-immersion lens and a Zeiss 880 Airyscan microscope at room temperature (21 °C). Images were processed with Airyscan post-image processing using

Zen software. ROIs were drawn around resulting mitochondrial fluorescence and intensity was recorded for each cell. Intensity values for each group were normalized to control values.

Cell viability assay

Cell viability assay was conducted as per manufacturer's recommendations (K502-100; BioVision). Live DIV 13 cortical neurons were washed once with 2 mM Ca2+ Ringer's solution and loaded with 1 mL assay buffer containing 2 uL Live cell staining dye and 1 uL Dead cell staining dye. Cells were immediately excited using a 488 nm and 564 nm LED. Single-plane images were collected using a Plan-Apochromat 63×/1.40 oil-immersion lens and a Zeiss 880 Airyscan microscope at room temperature (21 °C). Images were analyzed using ImageJ. Ratio of green (live) to red (dead) fluorescence was taken for each neuron.

Reagents

Doxycycline (J60579; Alfa Aesar) was dissolved in diH2O and SH-SY5Y cells were treated at 3 ug/mL for 72 h. UNC-3230 (5271; Tocris) was dissolved in DMSO and cells were treated at 100 nM for 24 h prior to transient transfection or 24 h prior to fixation. Ro-3280 (5968; Tocris) was dissolved in DMSO and cells were treated at 100 nM 24 h prior to transient transfection or 24 h prior to fixation. Bradykinin acetate salt (B3259; Sigma-Aldrich) was dissolved in diH2O and perfused at 100 µM in 2 mM Ca2+ Ringer's Solution. Oxotremorine M (O100; Sigma-Aldrich) was dissolved in diH2O and perfused at 100 µM in 2 mM Ca2+ Ringer's Solution. UTP trisodium salt (U6625; Sigma-Aldrich) was

dissolved in diH2O and perfused at 100 µM in 2 mM Ca2+ Ringer's Solution. Active type 1 recombinant human α-Syn pre-formed fibrils (SPR-322; StressMarq Biosciences) were dissolved in PBS and sonicated for 10 min prior to treatment at 4 µg/mL for 72 h.

Data Analysis

Microsoft Excel, and GraphPad Prism were used to analyze all data. ImageJ was used to process and analyze images. For datasets with two treatment groups, parametric Student's *t* tests were conducted to determine significance. For datasets with more than two treatment groups, parametric one-way ANOVA tests were conducted, and significance was determined by comparing mean values of each group. Normality tests were conducted on all treatment groups, with groups not passing subject to nonparametric tests. P values < 0.05 were considered to be statistically significant.

3. α-Syn-dependent increases in PIP5K1γ produce more $PI(4,5)P_2$ perpetuating α-Syn aggregation

3.1 Introduction

α-synuclein (α-Syn) is one of three members of the synuclein family (α-, β-, and γ-) that is encoded by the *SNCA* gene and is abundantly expressed in neuronal tissue. A small (140-residue), natively unfolded protein, α-Syn was initially observed to localize in the nucleus and presynaptic terminals of neurons (Maroteaux, Campanelli, & Scheller, 1988). Based on its presynaptic localization, most of the postulated physiological roles of α-Syn have centered around its ability to influence neurotransmission and synaptic vesicle dynamics (Burre, Sharma, & Sudhof, 2014; Burré et al., 2010; Diao et al., 2013; Nemani et al., 2010; Sun et al., 2019; L. Wang et al., 2014); however it is now clear that α-Syn can also influence signaling reactions and membrane function of many organelles including the ER (Cooper et al., 2006; Mercado, Valdés, & Hetz, 2013; Paillusson et al., 2017), Golgi (Cooper et al., 2006; Thayanidhi et al., 2010), mitochondria (Guardia-Laguarta et al., 2014; Kamp et al., 2010), and endo/lysosomes (Dinter et al., 2016; Domert et al., 2016; Mazzulli, Zunke, Isacson, Studer, & Krainc, 2016). Despite this information, the physiological roles of α-Syn still remain relatively unknown.

Pathologically, missense mutations in *SNCA* lead to enhanced α-syn aggregation and cause inherited forms of Parkinson's disease (PD) (Chartier-Harlin et al., 2004; W. S. Kim et al., 2014; Krüger et al., 1998; Polymeropoulos et al., 1997; Singleton et al., 2003; Zarranz et al., 2004). In idiopathic PD, α-Syn aggregation leads to the formation of toxic α-Syn fibrils that constitute the building blocks of Lewy bodies, the deviant protein deposits that accumulate and propagate between neurons during neurodegeneration

(Giasson et al., 2002; Peng, Trojanowski, & Lee, 2020; Spillantini et al., 1997; Volpicelli-Daley, Luk, & Lee, 2014). Thus, from a genetic and idiopathic perspective, increased expression, and aggregation of α-Syn fibrils is a key pathological hallmark of PD. Despite clear neuropathological consequences for α-Syn aggregation, there is a lack of mechanistic information regarding the intracellular pathways, perturbed by α-Syn, that promote neuronal demise (Schekman & Riley, 2019).

The local membrane lipid environment has been suggested to play an important role for both the physiological and pathological effects of α-Syn (Cole et al., 2002; Fortin et al., 2004; Kubo et al., 2005; H. J. Lee, Choi, & Lee, 2002; Narayanan & Scarlata, 2001). α-Syn localizes to membrane – cytosol interfaces through interactions between its amphipathic N-terminal domain (Ferreon, Gambin, Lemke, & Deniz, 2009) and acidic phospholipids (Davidson, Jonas, Clayton, & George, 1998). Among lipids reported to interact with α-Syn are phosphoinositides (PIs) (Fortin et al., 2004; Jacob et al., 2021; Narayanan, Guo, & Scarlata, 2005; Schechter, Atias, et al., 2020); a family of low abundance, negatively charged phospholipids whose inositol ring can be phosphorylated or dephosphorylated at hydroxyl positions 3, 4, or 5 to generate 7 polyphosphoinositide (PPI) species from the parent PI molecule (for review see (T. Balla, 2013)). The signature PPI species of the plasma membrane (PM) is $PI(4,5)P_2$; here it is crucial for the control of a wide range of cellular processes, including ion channel function (T. Balla, 2013; Di Paolo & De Camilli, 2006; Hille, Dickson, Kruse, Vivas, & Suh, 2015). The majority of cellular $PI(4,5)P_2$ is produced through the enzymatic actions of Type I PIP kinases (PIPK1α, -β, -γ) on precursor PI(4)P pools. PIP5K1γ is the major isoform expressed in neuronal tissue and highly concentrated at synapses (Di Paolo et al., 2002). Linking PM $PI(4,5)P_2$ to PD

are mutations in the $PI(4,5)P_2$ metabolizing enzyme synaptojanin1 (SYNJ1) which cause inherited forms of PD (PARK20) (Cao et al., 2017), in addition to SYJN1 haploinsufficiency which causes increased $PI(4,5)P_2$ and drives dopamine neuron vulnerability (Pan et al., 2020). Despite these correlative associations, there remains a paucity of information on the molecular underpinnings that link α-Syn accumulation, $PI(4,5)P_2$ dysregulation, and neuronal cell death**.**

In this present study, we determine the molecular mechanism that links increases in α-Syn expression to neuronal cell death through PIP5K1γ-dependent increases in PM $PI(4,5)P_2$. This positive feedback loop precipitates α-Syn aggregation, potentiates ER-meditated Ca^{2+} release and leads to increases in mitochondrial Ca^{2+} levels which drives neuronal cytotoxicity. Inhibition or knockdown of PIP5K1γ, or other key regulators of this signaling pathway, normalizes PM $PI(4,5)P_2$, decreases α-Syn aggregates, and rescues neuron viability. These data suggest that PM $PI(4,5)P_2$ plays a crucial role in the pathological progression of PD and that modulation of enzymes that regulate $PI(4,5)P_2$ levels may provide a novel therapeutic target to treat and ameliorate the devastating consequences of PD or other synucleinopathies.

3.2 Results

3.2.1 α-Syn disease mutations or human α-Syn fibrils increase plasma membrane $PI(4,5)P_2$ across different brain regions.

It is important to note that although the etiology of sporadic and familial PD may be different, there is significant evidence to suggest that increased aggregation and fibrillar formation represents a key pathological hallmark of the disease (Reed 2019). It is

for this reason that throughout the present study, we use α-Synuclein$_{A53T}$, a mutation which exhibits an increased propensity to aggregate (Giasson et al., 2002; W. Li et al., 2004), and human pre-formed α-Syn fibrils (referred to hereafter as α-Syn fibrils), a model of synucleinopathy that seeds recruitment and aggregation of endogenous α-Syn leading to neuronal dysfunction and degeneration (Giasson et al., 2002; K. C. Luk et al., 2012; Volpicelli-Daley et al., 2011; B. Zhang et al., 2019), as our pathological cell models of PD to investigate molecular mechanisms of neurodegeneration.

Increases in α-Syn expression have previously been correlated with elevated cellular PI(4,5)P_2 levels in cell models of PD (Schechter, Atias, et al., 2020), yet the molecular mechanisms underlying these changes are unknown. To begin, we performed liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) to quantify absolute changes in PIP_2 levels using two cellular models that have altered α-Syn. First, using a model of familial PD, we found that patient cells harboring the α-Syn$_{A53T}$ mutation had ~2-fold higher cellular PIP_2 levels than age and sex matched controls (Figure 1A). Similarly, treating neurons with human pre-formed α-Syn fibrils (referred hereafter as α-Syn fibrils), also resulted in increased cellular PIP_2 levels relative to vehicle-treated control neurons (Figure 1A). Investigation of individual PIP_2 species in α-Syn fibril-treated neurons revealed that although all PIP_2 species were increased, PIP_2 38:4, which represents the most abundant species in primary cells (Kruse, Vivas, Traynor-Kaplan, & Hille, 2016; Traynor-Kaplan et al., 2017), exhibited the largest increase following treatment with α-Syn fibrils (Figure 1B).

Mass-spec analysis of PIP_2 levels is extremely quantitative, but does not provide (i) spatial information regarding intracellular pools of PIP_2, or (ii) which PIP_2 species

($PI(4,5)P_2$, $PI(3,4)P_2$, or $PI(3,5)P_2$) is increased following increases in α-Syn expression. Given the strong link between altered $PI(4,5)P_2$ and PD (Cao et al., 2017; Pan et al., 2020) and that $PI(4,5)P_2$ represents >80 % of the total cellular PIP_2 pool (Dickson & Hille, 2019), we quantified the intracellular distribution of $PI(4,5)P_2$ using two biosensors that report: (i) intracellular, non-nuclear $PI(4,5)P_2$ ($PH_{PLC\delta1}$), and (ii) nuclear $PI(4,5)P_2$ (3xNLS-PH; (Y. H. Wang et al., 2017)). Expression of $PH_{PLC\delta1}$ in neurons from three different brain regions that have been reported to undergo α-Syn-dependent neurodegeneration revealed that the vast majority of $PI(4,5)P_2$ reported by $PH_{PLC\delta1}$ was at the PM, with PM/cytoplasm ratios increased in the hippocampus (Figure 1C, D), cortex (Figure 1E, F), and substantial Nigra (Figure 1G,H) following expression of $\alpha\text{-Syn}_{A53T}$. Similar results were observed in HEK293, CHO, and fibroblasts (Figure S1), illustrating the cellular and molecular machinery responsible for α- Syn-dependent augmentation of $PIP(4,5)P_2$ is conserved in non-neuronal cells. In addition to the PM, the nucleus is also reported to contain a significant portion of cellular $PI(4,5)P_2$ (Mortier et al., 2005; Osborne, Thomas, Gschmeissner, & Schiavo, 2001; Watt, Kular, Fleming, Downes, & Lucocq, 2002). Using a nuclear-targeted 3xNLS-PH biosensor we determined that $PI(4,5)P_2$ levels were also increased, with a significantly higher nucleus/nucleoplasm ratio compared to control cells (Figure 1I,J). Taken together, these data determine that both PM and nuclear $PI(4,5)P_2$ levels are increased in neuronal and non-neuronal cells following overexpression, disease-mutations, or treatment with α-Syn fibrils.

3.2.2 Increased expression and distribution of PIP5K1γ drives α-Syn-dependent increases in $PI(4,5)P_2$.

In neurons, PIP5K1γ represents the major enzyme responsible for the production of $PI(4,5)P_2$ (Di Paolo et al., 2002; Wenk et al., 2001). With this in mind, we wanted to understand if changes in α-Syn expression or treatment with α-Syn fibrils could alter the distribution and/or abundance of PIP5K1γ. To begin, we performed western blot experiments and determined that fibroblasts harboring the $\alpha\text{-Syn}_{A53T}$ mutation and tsA-201 cells overexpressing $\alpha\text{-Syn}_{A53T}$ exhibited significantly higher PIP5K1γ protein levels compared to controls (Figure 2 A, B), but no change in PIP5K1α (Figure S2A,B). PIP5K1γ is a cytosolic protein that is recruited to cellular membranes to catalyze the production of $PI(4,5)P_2$ from precursor PI(4)P pools, therefore we also wanted to test if its localization was altered under different α-Syn conditions. Using AiryScan super-resolution confocal microscopy we expressed PIP5K1γ-GFP with or without $\alpha\text{-Syn}_{A53T}$ in tsA-201 cells and discovered that in control cells PIP5K1γ-GFP is localized to both the PM and cytoplasm with co-transfection of α-Syn shifting its distribution towards the PM (Figure 2C, D). To test if α-Syn alters endogenous expression of PIP5K1γ, we used a validated PIP5K1γ-antibody ((Di Paolo et al., 2002), Figure S2C) and found both the PM and nuclear fractions were increased following $\alpha\text{-Syn}_{A53T}$ expression (Figure 2E). α-Syn-dependent increases in PM PIP5K1γ were also observed in neurons, with quantitative analysis of single molecule localization microscopy experiments (resolution ~20-30 nm (Dickson et al., 2016)) conducted in TIRF mode determining that both the number and area of PIP5K1γ puncta were significantly increased when neurons were cultured with α-Syn fibrils (Figure 2F-H). Unlike PIP5K1γ, the cellular distribution of the phosphoinositide transfer protein

Nir2, whose knockdown has been shown to abrogate α-Syn-dependent increases in PM $PI(4,5)P_2$ (Schechter, Grigoletto, et al., 2020), was similar between control and α-Syn-expressing cells (Figure S2D,E).

The downstream consequence of increased abundance and localization of PM PIP5K1γ is elevated PM $PI(4,5)P_2$ levels, therefore we tested if knockdown or reduced recruitment of PIP5K1γ rescued α-Syn-dependent increases in PM $PI(4,5)P_2$. Consistent with this model, siRNA directed against PIP5K1γ (Figure 2I,J) or expression of a dominant negative Arf-6 (Arf6-HA-T27N), which reduces the recruitment of PIP5K to the PM (Hernández-Deviez, Roth, Casanova, & Wilson, 2004), normalized $PH_{PLC\delta1}$ levels in α-Syn_{A53T}-expressing cells back to control levels (Figure S2F,G).

In addition to increases in PIP5K1γ at the PM, α-Syn_{A53T}-expression increased levels of nuclear PIP5K1γ (Figure 2K,L), with inhibition of PIP5K1γ using the selective small molecule inhibitor, UNC-3230 (UNC) (Wright et al., 2014), normalizing 3xNLS-PH levels back to control levels (Figure 2M,N). Collectively, these data are consistent with the hypothesis that increased α-Syn levels leads to increased expression and membrane localization of PIP5K1γ and consequently increased membrane $PI(4,5)P_2$ levels.

3.2.3 PIP5K1γ influences size and abundance of α-Syn aggregates.

α-Syn has the ability to bind reconstituted $PI(4,5)P_2$ vesicles and its levels positively correlate with $PI(4,5)P_2$ at cellular membranes (Jacob et al., 2021). Given that increases in cellular α-Syn_{A53T} facilitate enhanced expression and PM localization of PIP5K1γ, we wanted to determine if the immediate downstream consequence of this reaction, namely increased $PI(4,5)P_2$, influences α-Syn levels (Figure 3A). To begin we

overexpressed PIP5K1γ and fixed and stained for endogenous α-Syn in tsA-201 cells. Quantitative analysis from resulting super-resolution images revealed that overexpression of PIP5K1γ-GFP increased both the density and area of α-Syn particles. In a complementary approach, we used a doxycycline-inducible SH-SY5Y cell line that allows for the controlled expression of α-Syn (Vasquez et al., 2018)(hereafter called α-Syn^{Dox} cells). α-Syn^{Dox} cells were cultured with vehicle control, doxycycline (to switch on α-Syn expression), the PIP5K1γ inhibitor UNC, or doxycycline in the presence of UNC before being fixed and stained for α-Syn. Analysis of the resulting confocal images revealed that doxycycline increased the expression of cellular α-Syn (Figure 3D,E; red), with co-treatment with UNC, normalizing α-Syn levels back into a control range (Figure 3D,E; purple). Similar observations were made using TIRF and super-resolution SMLM microscopy to visualize α-Syn at the PM (Figure 3F,G; Figure S3A,B). To further test if PIP5K1γ levels act as a rheostat for α-Syn, we knocked-down PIP5K1γ levels using siRNA and found that decreasing PIP5K1γ significantly reduced both the density and area of overexpressed α-Syn$_{A53T}$ puncta (Figure 3I). Therefore, PIP5K1γ expression correlates with α-Syn at cellular membranes, with its catalytic activity being essential for α-Syn aggregation.

3.2.4 PLK1 activity drives recruitment of PIP5K1γ to increase PM PI(4,5)P$_2$ and α-Syn aggregation.

The link between PIP5K1γ and α-Syn accumulation prompted us to investigate upstream elements that control PIP5K1γ and α-Syn aggregation at cellular membranes. Polo-like kinases (PLK) are Ser/Thr protein kinases act as key regulators of centrosome

maturation and bipolar spindle assembly in dividing cells (Strebhardt, 2010) and are enriched at healthy neuronal synapses where they influence synaptic plasticity (Kauselmann et al., 1999). PLK1, the most highly characterized family member, has the ability to bind $PI(4,5)P_2$ (A. E. Lewis et al., 2011; T. C. Lin, Kuo, Wu, Pan, & Yih, 2019), phosphorylate α-Syn at Ser-129 (Mbefo et al., 2010) which enhances membrane binding and α-Syn toxicity, and is also implicated in neurodegeneration through its elevated expression in susceptible hippocampal and cortical neurons of Alzheimer's Disease (AD) patients (Harris et al., 2000; Song et al., 2011). Accordingly, we examined if PLK1 is upstream of PIP5K1γ and regulates its expression/localization to influence levels of cytotoxic α-Syn phosphorylation at Ser-129 (Ser(P)-129) (Figure 4A).

To begin, we treated neurons with α-Syn fibrils and fixed and stained for PLK1 to determine if α-Syn can alter PLK1 levels. Analyses of Airyscan super-resolution confocal images revealed that treatment with α-Syn fibrils significantly increased PLK1 levels relative to vehicle controls (Figure 4B,C). To test if PLK1 can influence PIP5K1γ recruitment to the PM, we overexpressed PIP5K1γ-GFP in $\alpha\text{-Syn}^{Dox}$ cells and measured its distribution under three conditions: (i) PLK1 overexpressed (- Dox), (ii) α-Syn overexpressed (+ Dox), and (iii) co-expression of PLK1 and α-Syn (+ Dox). Quantification of images revealed equivalent increases in PM PIP5K1γ recruitment in the PLK1 overexpressed and α-Syn overexpressed groups, inviting the hypothesis that α-Syn-dependent recruitment of PIP5K1γ occurs in a PLK1-dependent manner. This model was further supported by experiments measuring the downstream product of PIP5K1γ, $PI(4,5)P_2$. Measurements of PM $PI(4,5)P_2$ in neurons using the $PH_{PLC\delta1}$ biosensor revealed that while (i) PLK1 overexpression alone, (ii) $\alpha\text{-Syn}_{A53T}$ overexpression, or (iii)

PLK1 and α-Syn$_{A53T}$ overexpression, all increased PI(4,5)P_2 to similar levels, inhibition of PLK1 using the selective inhibitor Ro-3280 (S. Chen et al., 2012), normalized PI(4,5)P_2 back to control levels (Figure 4F,G). Similar observations were made for endogenous PIP5K1γ levels, with PLK1 overexpression increasing PIP5K1γ while inhibition of PLK1 activity resulted in a decrease in PIP5K1γ (Figure S4A-D). To further probe the relationship between PLK1 and PIP5K1γ we immunoblotted for PIP5K1γ under conditions where PLK1 catalytic activity was inhibited in tsA-201 cells and determined that treatment of control cells with Ro-3280 decreased PIP5K1γ protein levels by 50 % and completely abolished α-Syn-dependent increases in PIP5K1γ (Figure 4H). These data suggest that elevations in α-Syn augment recruitment of PIP5K1γ to drive elevations in membrane PI(4,5)P_2 in a PLK1-dependent manner.

PLK1-dependent phosphorylation at Ser-129 of α-Syn has been reported to modulate membrane binding, localization, protein-protein interactions, and aggregation leading to neurotoxicity. Consequently, we assessed if PLK1-dependent recruitment of PIP5K1γ influences α-Syn Ser(P)-129. To begin we overexpressed PLK1-mCherry and quantified cellular α-Syn levels using immunofluorescence. Like reports from other groups, overexpression of PLK1 increased the number and size of α-Syn puncta (Figure 4I,J), with PLK1-dependent increases in α-Syn identical to those observed with overexpression of PIP5K1γ (Figure 4J, compare green and cyan bars). Next, we performed experiments immunoblotting for α-Syn or Ser(P)-129 α-Syn under conditions of altered PLK1 or PIP5K1γ activity and determined that overexpression of α-Syn$_{A53T}$ significantly increased WT and Ser(P)-129α-Syn levels with inhibition of PLK1 or PIP5K1Γ kinase activity reducing the amount of WT and Ser(P)-129 α-Syn (Figure 4K; Figure

S4E,F). Similar trends in Ser(P)-129 α-Syn were observed when neurons were concurrently treated with α-Syn fibrils and treated with PLK1 or PIP5K1γ inhibitors (Figure 4L,M). These data provide evidence that α-Syn-dependent increases in PLK1 recruit PIP5K1γ to elevate $PI(4,5)P_2$ and precipitate Ser(P)-129 α-Syn accumulation.

3.3 Discussion

α-Syn has been reported to play a vital role in the progression of PD pathology through its ability to form the building block of Lewy bodies, the deviant protein deposits that accumulate, deposit, and propagate between brain regions during neurodegeneration. Despite the correlation between α-Syn aggregation in both idiopathic and genetic forms of PD, the molecular connection(s) that link α-Syn accumulation and neurodegeneration remain unknown. Here, we provide evidence that the membrane lipid $PI(4,5)P_2$, a critical organizer of membrane events, is increased across several models of PD, including different brain neurons incubated with human α-Syn pre-formed fibrils. The consequences of elevated cellular $PI(4,5)P_2$ levels are enhanced α-Syn expression, aggregation of toxic α-Syn Ser(P)-129, and augmented IP_3-mediated Ca^{2+} release leading to mitochondrial cytotoxicity. Given the importance of $PI(4,5)P_2$ for orchestrating membrane events in neurons (T. Balla, 2013; Dickson, 2019), we expect other signaling reactions/cascades to be perturbed as well. The molecular events connecting α-Syn and $PI(4,5)P_2$ appear to involve PLK1-dependent increases in the $PI(4,5)P_2$ metabolizing enzyme, PIP5K1γ, as selectively targeting protein abundance or activity of each enzyme rescues cellular phenotypes. These data position PLK1 and PIP5K1γ as not only important regulators of PM identity in health, but also key targets to potentially uncouple

the destructive downstream consequences of α-Syn accumulation in PD and other synucleinopathies.

α-Syn is an intrinsically disordered protein that exists in a variety of conformations, with the prevailing, but not universal (Bartels et al., 2011; Narayanan & Scarlata, 2001; Zhu & Fink, 2003), hypothesis positing that its cumulative oligomerization at membranes correlates with toxicity (Jo et al., 2000; H. J. Lee et al., 2002; Roostaee, Beaudoin, Staskevicius, & Roucou, 2013). An important mediator that facilitates the binding of α-Syn to membranes are lipids with positive charges in the N-terminus of α-Syn electrostatically interacting with negatively charged lipid phosphate head groups (Jo et al., 2000). Interestingly, the lipid sensing regions of α-Syn and genetic mutations that facilitate its pathological aggregation occur at the same region, presenting a model were the local lipid environment could seed and nucleate the aggregation of α-Syn. Physiologically, stabilization of α-Syn by lipids promotes interactions with SNARE complex proteins (Burre et al., 2014; Diao et al., 2013; Sun et al., 2019) to influence synaptic vesicle endocytosis (Lautenschlager, Kaminski, & Kaminski Schierle, 2017; Logan, Bendor, Toupin, Thorn, & Edwards, 2017; Schechter, Atias, et al., 2020; Vargas et al., 2014; L. Wang et al., 2014). While pathophysiologically, lipid-protein interactions increase the propensity of α-Syn membrane aggregation (Galvagnion, 2017; Suzuki, Sango, Wada, & Nagai, 2018). In the present study, we demonstrate an increase in the plasma membrane lipid $PI(4,5)P_2$, which is in agreement with reports from other groups regarding an important role for this lipid in PD (Cao et al., 2017; Pan et al., 2020; Schechter, Atias, et al., 2020; Schechter, Grigoletto, et al., 2020). Given the importance of α-Syn for synaptic vesicle endocytosis and exocytosis, both $PI(4,5)P_2$-dependent

events (Di Paolo & De Camilli, 2006) that facilitate prion-like spread of Lewy bodies (Jucker & Walker, 2013; K. C. Luk et al., 2012; Vargas et al., 2014), and the ability of $PI(4,5)P_2$ to bind and aggregate α-Syn (Davidson et al., 1998; Jacob et al., 2021; Narayanan et al., 2005), indicates $PI(4,5)P_2$ may play an important role in the progression of α-Syn pathology. Supporting this hypothesis, data herein demonstrates that inhibition of PIP5K1γ, which is critical for mediating α-Syn-dependent increases in $PI(4,5)P_2$, normalizes α-Syn aggregates and rescues neurotoxic events. Thus, a picture is developing where the appropriate $PI(4,5)P_2$ – α-Syn ration is required for membrane targeting and orchestration of neuronal function, however, as this delicate balance shifts, such as during aging (Chu & Kordower, 2007) or synucleinopathies, it may lead to a local membrane environment that favors aggregation and supports cytotoxicity. Such a model would signify the importance of regulated $PI(4,5)P_2$ homeostasis for α-Syn membrane-assisted functions.

In addition to α-Syn dependent increases in PM $PI(4,5)P_2$, we also observed that $\alpha\text{-Syn}_{A53T}$ expression results in elevations in nuclear $PI(4,5)P_2$. Elevations in nuclear $PI(4,5)P_2$ appear to occur due to increased recruitment/expression of PIP5K1γ, as its knockdown, inhibition, or upstream targeting of PLK1 normalizes increases in $PI(4,5)P_2$ back to control levels. At the nucleus, $PI(4,5)P_2$–protein complexes are important regulators of nuclear organization and genomic function (Castano et al., 2019) including RNA Polymerase I and II-dependent transcription (Sobol et al., 2018; Yildirim et al., 2013). Increases in $PI(4,5)P_2$ could have implications on whole-cell mRNA and rRNA expression, potentially altering transcriptional products in pathological states. If this hypothesis is correct, elevations in nuclear $PI(4,5)P_2$ could act to modulate expression of neurotoxic

proteins to precipitate neurodegeneration. Future experiments are required to develop and test such a model.

In the present study we propose that α-Syn-mediated increases in $PI(4,5)P_2$ occur through increased expression and localization of the phosphoinositide-metabolizing kinase PIP5K1γ, a key regulator of synaptic vesicle trafficking (Di Paolo et al., 2004; Di Paolo et al., 2002). This finding is in accord with recent data demonstrating that chemical recruitment of PIP5K1γ to the PM increases α-Syn PM localization (Jacob et al., 2021). Further underscoring a connection between α-Syn and PIP5K1γ, inhibiting PIP5K1γ catalytic activity, rescues PM $PI(4,5)P_2$ and cellular phenotypes. We establish that PIP5K1γ expression/recruitment appears to be under the control of PLK1 as its overexpression is sufficient to recruit PIP5K1γ and elevate PM PIP_2 levels to a similar degree as $α\text{-}Syn_{A53T}$ overexpression, while PLK1 inhibition under conditions of α-Syn overexpression, rescue PIP5K1γ and $PI(4,5)P_2$ levels. Although most of the research into PLKs has focused on their important role in mediating mitotic processes (Schmucker & Sumara, 2014), they have also been reported to play crucial roles in neurons (Seeburg, Feliu-Mojer, Gaiottino, Pak, & Sheng, 2008). PLK2, which also can phosphorylate α-Syn at Ser-129 (Inglis et al., 2009; Mbefo et al., 2010), plays a critical function along with CDK5 in regulating synaptic plasticity during chronic elevations in intrinsic neuronal electrical activity (Seeburg et al., 2008). Neuronal hyperexcitability, which is a cellular phenomenon preceding SNl cell death in PD (Bishop et al., 2010; Carola et al., 2021) and other common neurodegenerative disorders (Brunet, Stuart-Lopez, Burg, Scekic-Zahirovic, & Rouaux, 2020; Ping et al., 2015; Siskova et al., 2014), induces PLK2-driven synaptic dampening, indicating higher enzymatic activity during times of continuous firing.

Given that PLK1-dependent upregulation of PIP5K1γ leads to increases in $PI(4,5)P_2$, perhaps PLK2, which shares a conserved catalytic domain and two polobox domains with PLK1, also plays an important role in PIP5K1γ recruitment during periods of enhanced synaptic activity. These data, paired with an essential role for PLK1 in mediating autophagic clearance of α-Syn (L. L. Chen et al., 2017) and facilitating β-amyloid-induced cell death (Song et al., 2011) suggest that its expression/activity should be tightly regulated in neurons and recommends more targeted clinical research may be required to determine if it is a potential target to slow neurodegenerative disease progression.

3.4 Figures

Figure 1

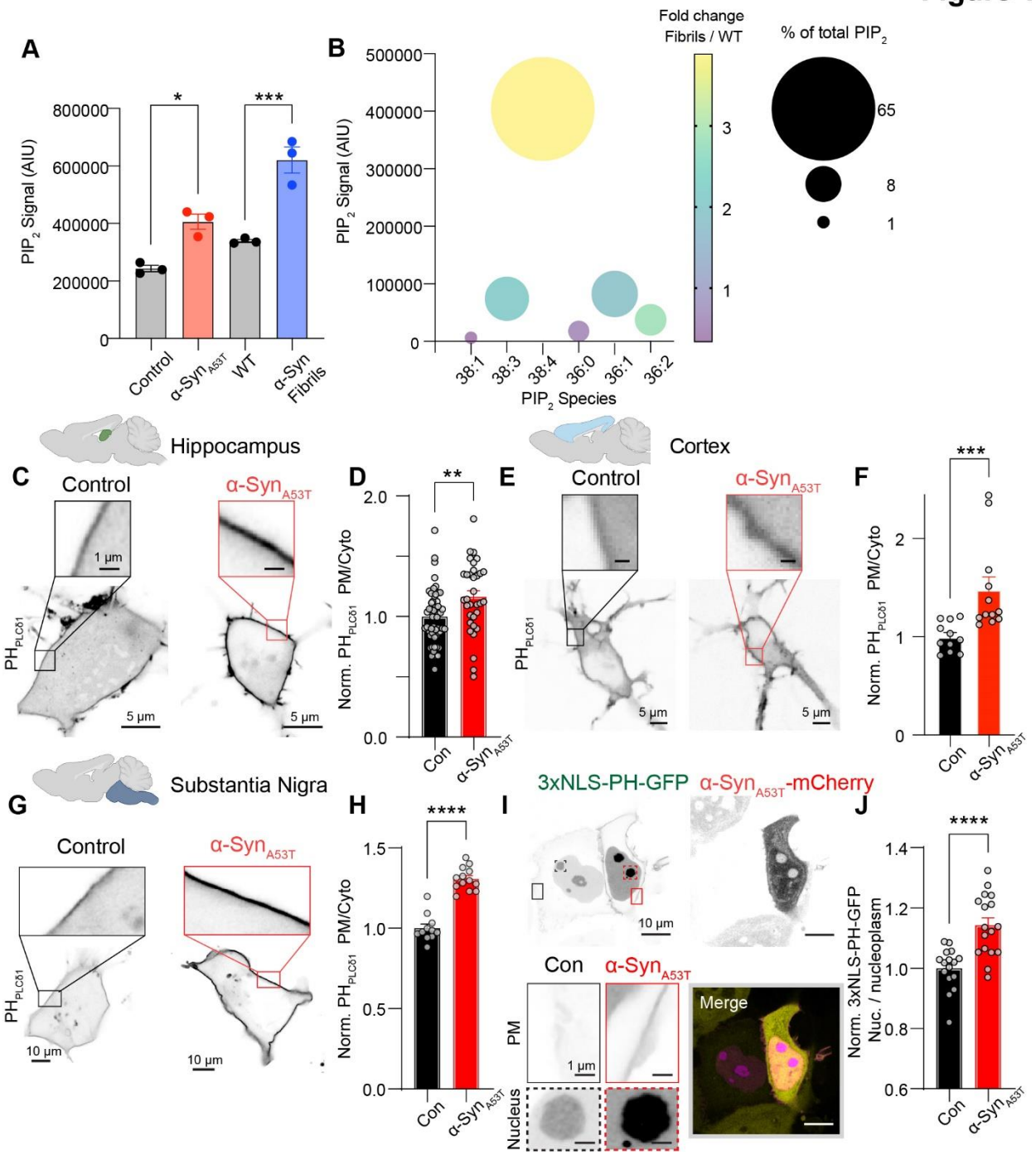


Figure 1. α-Syn expression or human α-Syn fibrils increase plasma membrane PI(4,5)P_2 across different brain regions. (A) Whole-cell PIP_2 levels measured by lipid mass spectrometry in fibroblasts from age- and sex-matched healthy control patients and PD patients harboring the A53T mutation in α-Syn, and DIV 12 mouse cortical neuron cultures with and without α-Syn fibril treatment. **(B)** Fold-change of different PIP_2 species from DIV 12 mouse cortical neuron culture compared to control culture. **(C)** Representative confocal images showing $PH_{PLC\delta1}$ localization in control and α-synuclein$_{A53T}$-transfected neurons and the quantification of PM/cytoplasm intensity of $PH_{PLC\delta1}$ respectively in mouse hippocampal (C and D), mouse cortical (E and F), and mouse substantia nigra cultures (G and H). **(I)** Representative confocal images of control and α-synuclein$_{A53T}$-transfected tsA201 cells transfected with 3xNLS- $PH_{PLC\delta1}$-GFP to show PM and nuclear PI(4,5)P_2 distribution. **(J)** Quantification of nucleoli/nucleoplasm intensity of $PH_{PLC\delta1}$ in tsA-201 cells. Error bars represent the standard error of the mean. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

Figure 2

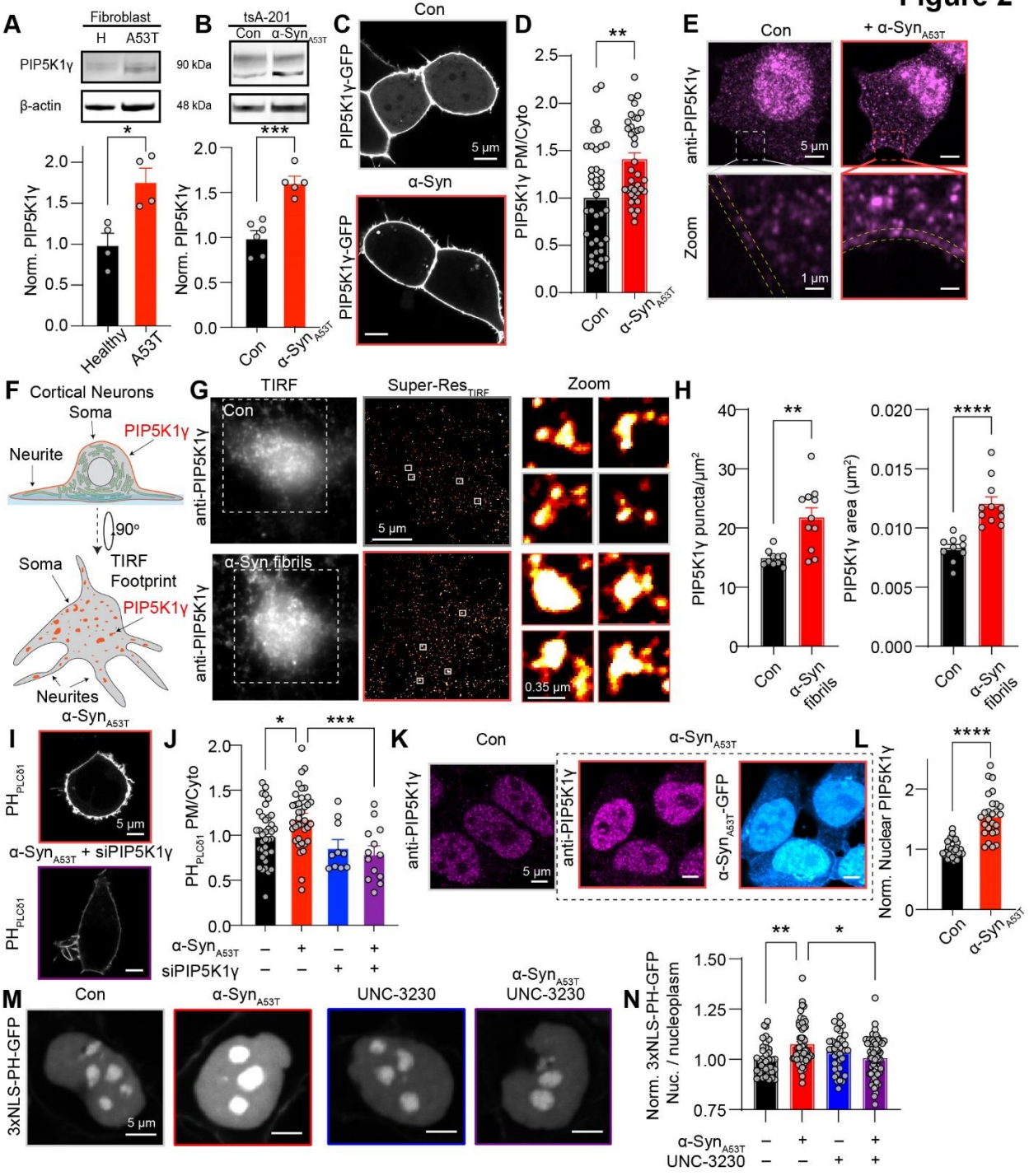

A
Fibroblast
H
A53T
PIP5K1γ
β-actin
Norm. PIP5K1γ
Healthy
A53T
B
tsA-201
Con
α-Syn A53T
90 kDa
48 kDa
Norm. PIP5K1γ
C
Con
PIP5K1γ-GFP
5 μm
α-Syn
D
PIP5K1γ PM/Cyto
E
Con
+ α-Syn A53T
anti-PIP5K1γ
Zoom
1 μm
F
Cortical Neurons
Soma
PIP5K1γ
Neurite
90°
TIRF Footprint
Neurites
α-Syn A53T
G
TIRF
Super-Res TIRF
Zoom
anti-PIP5K1γ
α-Syn fibrils
0.35 μm
H
PIP5K1γ puncta/μm²
PIP5K1γ area (μm²)
α-Syn fibrils
I
PH PLCδ1
α-Syn A53T + siPIP5K1γ
J
PH PLCδ1 PM/Cyto
α-Syn A53T
siPIP5K1γ
K
Con
α-Syn A53T
anti-PIP5K1γ
α-Syn A53T-GFP
L
Norm. Nuclear PIP5K1γ
M
Con
α-Syn A53T
UNC-3230
α-Syn A53T UNC-3230
3xNLS-PH-GFP
N
Norm. 3xNLS-PH-GFP Nuc. / nucleoplasm
α-Syn A53T
UNC-3230

Figure 2. Increased expression and distribution of PIP5K1γ drives α-Syn-dependent increases in $PI(4,5)P_2$. (A) Top: Representative western blot from control and α-Syn_{A53T} fibroblasts or (B) α-Syn_{A53T} tsa-201 cells stained for PIP5K1γ and β-actin. Bottom: Quantification of PIP5K1γ normalized to β-actin. **(B)** Same as **(A)** only in control and α-Syn_{A53T}-transfected tsA-201 cells. **(C)** Representative confocal images of control and α-Syn_{A53T}-transfected tsA201 cells transfected with PIP5K1γ-GFP. **(D)** Quantification of PM/cytoplasm intensity of PIP5K1γ. **(E)** Representative confocal images of control and α-$synuclein_{A53T}$-transfected tsA-201 cells stained for PIP5K1γ. **(F)** Schematic of TIRF imaging for PIP5K1γ in mouse neurons. **(G)** Representative TIRF images (diffraction-limited and super-resolution) of control and α-Syn fibril-treated mouse cortical neurons stained for PIP5K1γ. **(H)** Quantification of $Super\text{-}Res_{TIRF}$ images. **(I)** Representative images of α-Syn_{A53T} and $PH_{PLC\delta1}$-CFP-transfected tsA-201 cells transfected with or without siRNA targeting PIP5K1γ. **(J)** Quantification of PM/cytoplasm intensity of $PH_{PLC\delta1}$. **(K)** Representative confocal images of control and α-Syn_{A53T} -transfected tsA-201 cells stained for PIP5K1γ. **(L)** Quantification of PIP5K1γ nuclear intensity. **(M)** Representative confocal images of control and α-Syn_{A53T} -transfected tsA-201 cells, expressing 3xNLS-$PH_{PLC\delta1}$-GFP and treated with or without 100 nM UNC-3230 for 24 hrs. **(N)** Quantification of nucleoli/nucleoplasm intensity of $PH_{PLC\delta1}$ in tsA-201 cells. Error bars represent the standard error of the mean. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

Figure 3

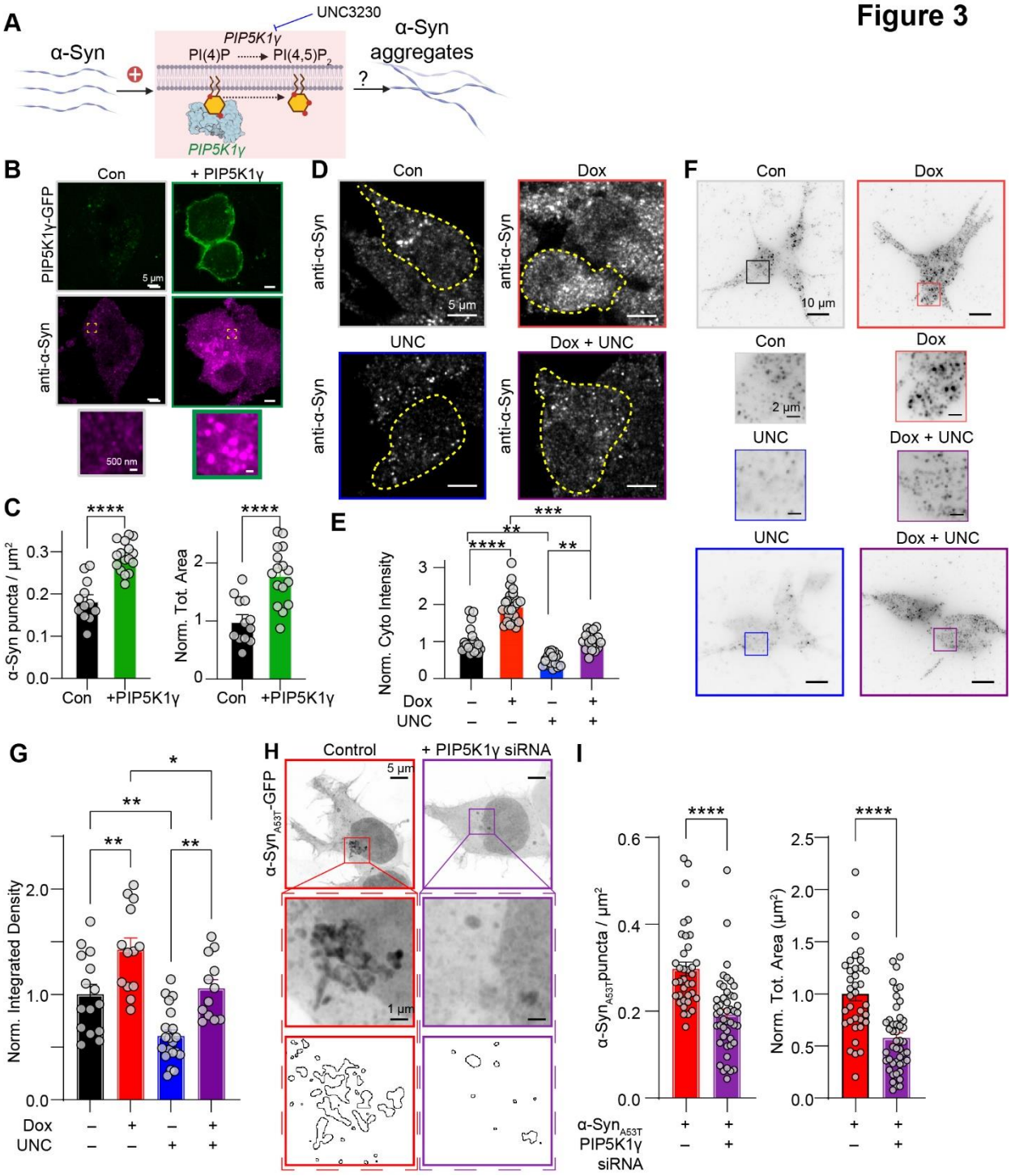
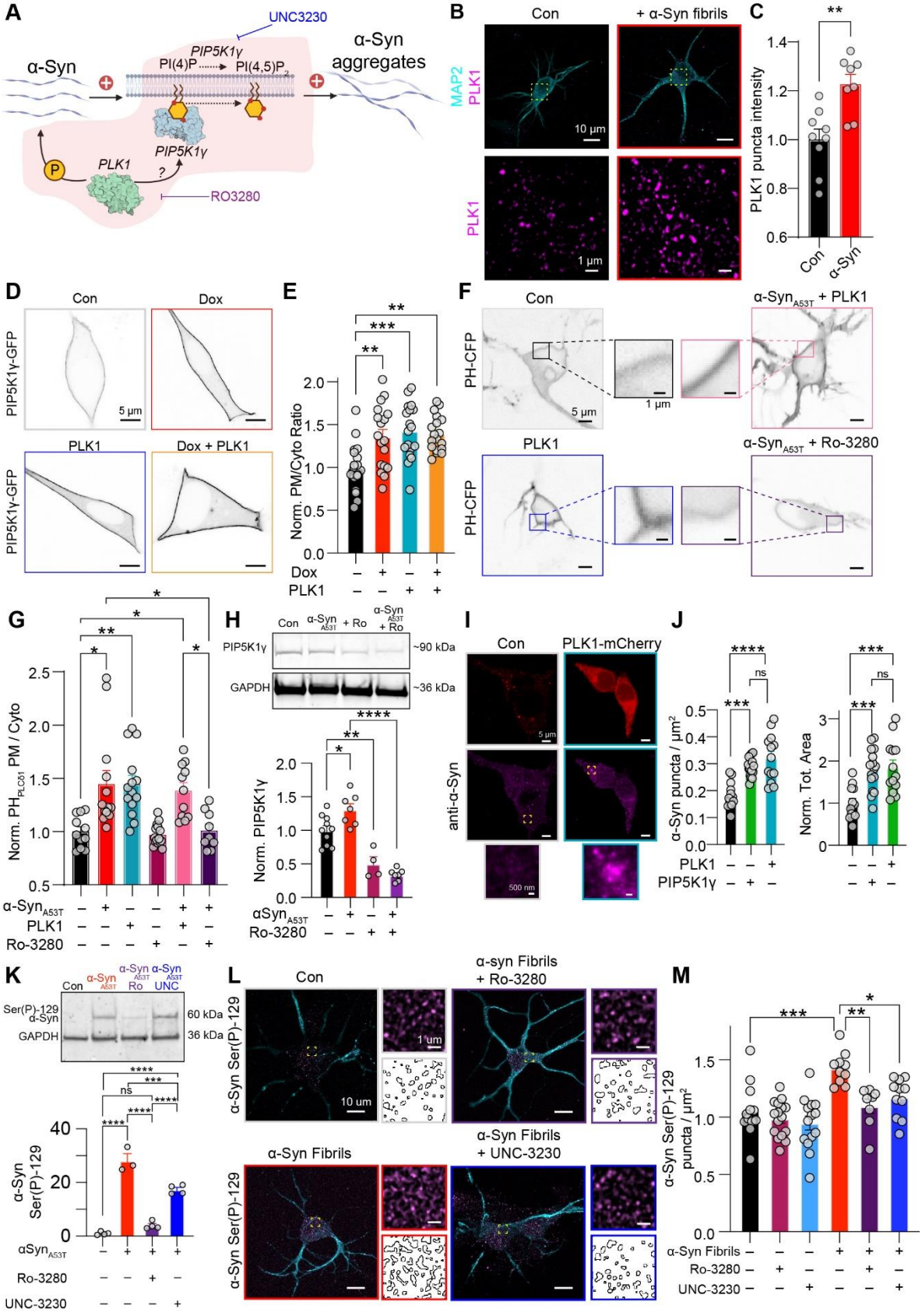

A
α-Syn
UNC3230
PIP5K1γ
PI(4)P
PI(4,5)P2
?
α-Syn aggregates
PIP5K1γ
B
Con
+ PIP5K1γ
PIP5K1γ-GFP
anti-α-Syn
5 μm
500 nm
C
α-Syn puncta / μm²
Norm. Tot. Area
Con
+PIP5K1γ
****
D
Con
Dox
UNC
Dox + UNC
anti-α-Syn
5 μm
E
Norm. Cyto Intensity
Dox
UNC
F
Con
Dox
UNC
Dox + UNC
10 μm
2 μm
G
Norm. Integrated Density
H
Control
+ PIP5K1γ siRNA
α-Syn A53T-GFP
5 μm
1 μm
I
α-Syn A53T puncta / μm²
Norm. Tot. Area (μm²)
α-Syn A53T
PIP5K1γ siRNA

Figure 3. PIP5K1γ influences size and abundance of α-Syn aggregates. (A) Schematic of hypothesis to be tested: α-synuclein-dependent increases in PIP5K1γ drive α-Syn aggregation. **(B)** Representative confocal images of control and PIP5K1γ-transfected tsA-201 cells stained for α-Syn. **(C)** Quantification of size and density of α-synuclein puncta. **(D)** Representative confocal images of undifferentiated control and doxycycline-induced α-Syn-overexpressing SH-SY5Y cells treated with or without 100 nM UNC-3230 and fixed and stained for α-synuclein. **(E)** Quantification of α-Syn puncta intensity. **(F and G)** Same as (D and E) only TIRF microscopy. **(H)** Representative confocal images of fixed, α-synuclein$_{A53T}$-transfected tsA-201 cells with or without transfection of siRNA targeting PIP5K1γ. **(I)** Quantification of size and density of α-synuclein puncta. Error bars represent the standard error of the mean. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

Figure 4


A
α-Syn
UNC3230
PIP5K1γ
PI(4)P
PI(4,5)P2
α-Syn aggregates
PIP5K1γ
PLK1
?
P
RO3280
B
Con
+ α-Syn fibrils
MAP2
PLK1
10 μm
PLK1
1 μm
C
PLK1 puncta intensity
Con
α-Syn
D
Con
Dox
PIP5K1γ-GFP
5 μm
PLK1
Dox + PLK1
PIP5K1γ-GFP
E
Norm. PM/Cyto Ratio
Dox
PLK1
F
Con
α-SynA53T + PLK1
PH-CFP
5 μm
1 μm
PLK1
α-SynA53T + Ro-3280
PH-CFP
G
Norm. PHPLC61 PM / Cyto
α-SynA53T
PLK1
Ro-3280
H
Con
α-SynA53T
+ Ro
α-SynA53T + Ro
PIP5K1γ
~90 kDa
GAPDH
~36 kDa
Norm. PIP5K1γ
αSynA53T
Ro-3280
I
Con
PLK1-mCherry
anti-α-Syn
5 μm
500 nm
J
α-Syn puncta / μm2
Norm. Tot. Area
ns
PLK1
PIP5K1γ
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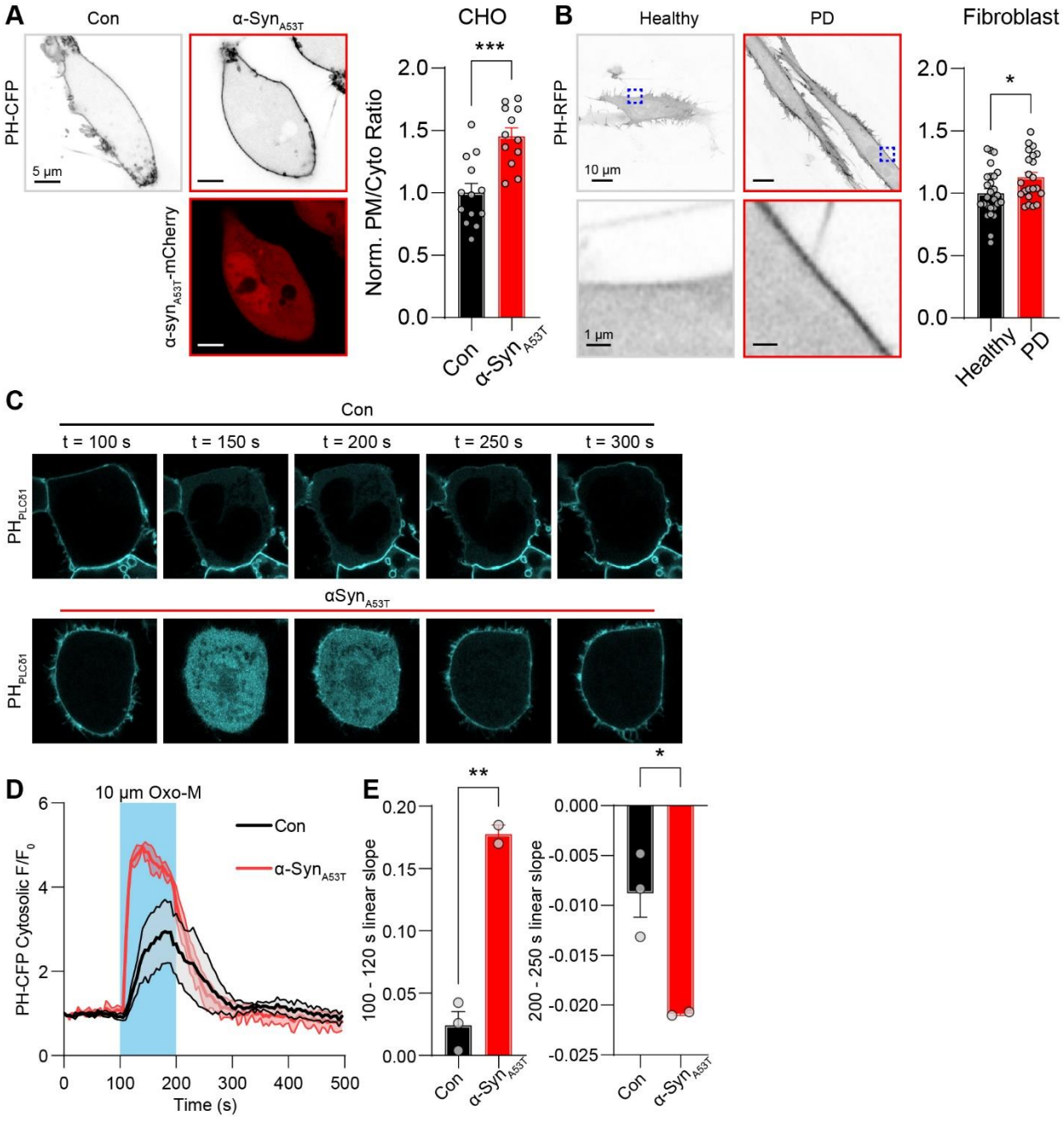
Con
α-SynA53T
α-SynA53T Ro
α-SynA53T UNC
Ser(P)-129 α-Syn
GAPDH
60 kDa
36 kDa
ns
α-Syn Ser(P)-129
αSynA53T
Ro-3280
UNC-3230
L
Con
α-syn Fibrils + Ro-3280
1 um
10 um
α-Syn Ser(P)-129
α-Syn Fibrils
α-Syn Fibrils + UNC-3230
α-Syn Ser(P)-129
M
α-Syn Ser(P)-129 puncta / μm2
α-Syn Fibrils
Ro-3280
UNC-3230

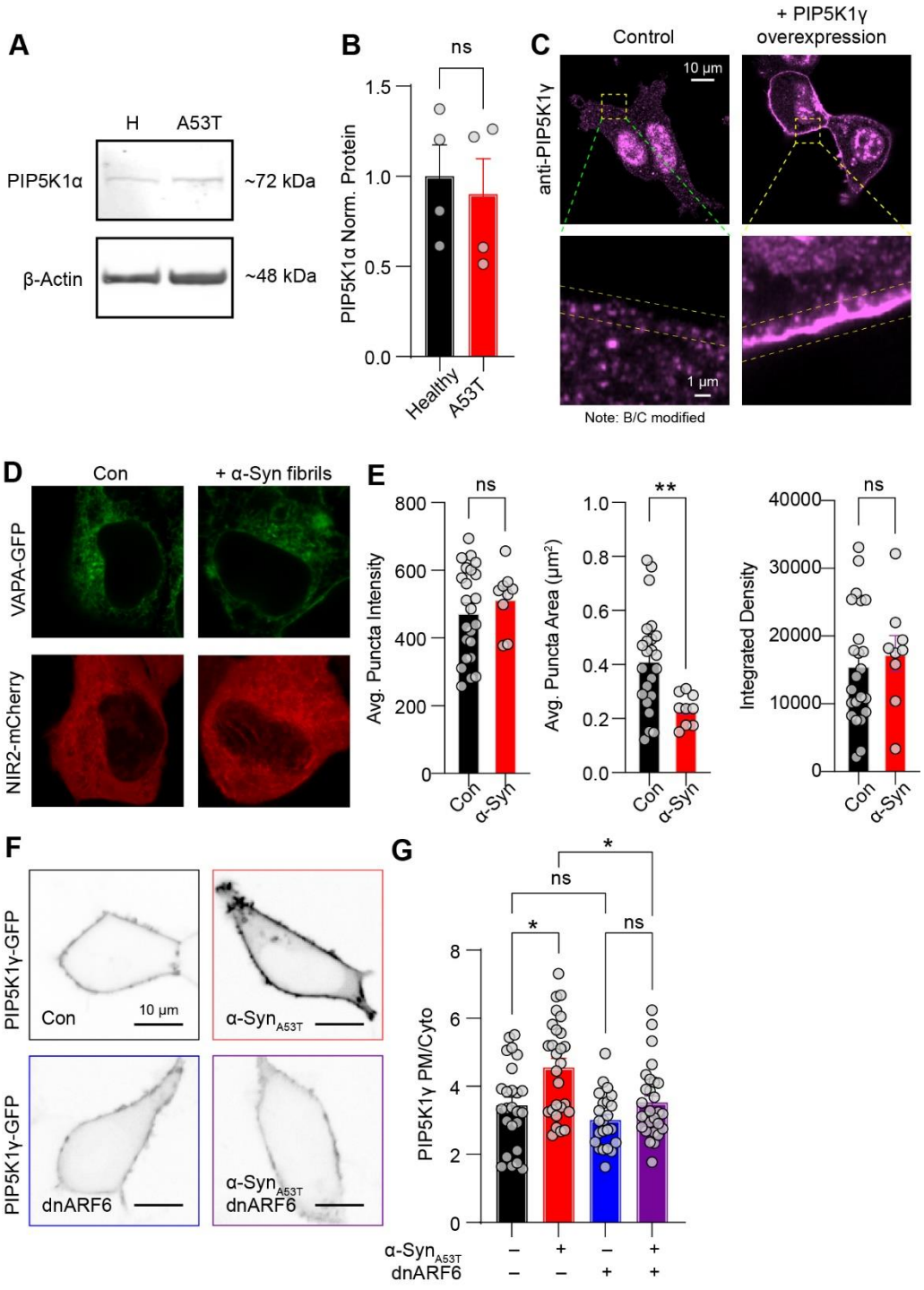
Figure 4. PLK1 activity drives recruitment of PIP5K1γ to increase PM $PI(4,5)P_2$ and α-Syn aggregation. (A) Schematic of hypothesis: PLK1-dependent increases in PIP5K1γ increase $PI(4,5)P_2$ and α-Syn aggregation. **(B)** Representative confocal images of control and α-Syn fibril-treated mouse cortical neurons stained for PLK1 (magenta) and MAP2 (cyan). Zoom shows only PLK1 staining. **(C)** Quantification of intensity of PLK1 puncta in cultured mouse cortical neurons. **(D)** Representative confocal images of undifferentiated control and doxycycline-induced α-Syn-overexpressing SH-SY5Y cells each transfected with PIP5K1γ or PIP5K1γ and PLK1. **(E)** Quantification of PM/cytoplasm intensity of PIP5K1γ. **(F)** Representative confocal images of control and $α\text{-Syn}_{A53T}$-transfected mouse cortical neurons each transfected with $PH_{PLCδ1}$-CFP, with or without PLK1 overexpression and with or without 24 hr treatment of 100 nM Ro-3280. **(G)** Quantification of PM/cytoplasm intensity of $PH_{PLCδ1}$. **(H)** Representative western blots and quantification of PIP5K1γ and GAPDH staining from protein lysates of control and $α\text{-synuclein}_{A53T}$-transfected tsA-201 cells with or without 24 hr treatment of 100 nM Ro-3280. **(I)** Representative confocal images of control and PLK1-transfected tsA-201 cells stained for α-Syn to show α-synuclein aggregation. **(J)** Quantification of size and density of α-synuclein puncta. PIP5K1γ-transfected cell images shown in Fig 3B; quantification shown for comparison. **(K)** Representative western blots and quantification of Ser(P)-129 α-Syn and GAPDH staining from protein lysates of control and $α\text{-Syn}_{A53T}$-transfected tsA-201 cells with or without 24 hr treatment of 100 nM PLK1 inhibitor Ro-3280, or 24 hr treatment of 100 nM PIP5K1γ inhibitor UNC-3230. **(L)** Representative confocal images of control and α-Syn fibril-treated mouse cortical neurons stained with Ser(P)-129 α-Syn (magenta) and MAP2 (cyan) with or without 24 hr treatment of 100 nM Ro-3280, or 24 hr treatment of 100 nM UNC-3230. Zoom and threshold images show only Ser(P)-129 α-Syn. **(M)** Quantification of density of Ser(P)-129 α-Syn puncta in mouse cortical neurons. Error bars represent the standard error of the mean. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

Figure S1


A
Con
α-Syn A53T
PH-CFP
5 μm
α-syn A53T-mCherry
CHO
***
Norm. PM/Cyto Ratio
2.0
1.5
1.0
0.5
0.0
Con
α-Syn A53T
B
Healthy
PD
Fibroblast
PH-RFP
10 μm
1 μm
*
Healthy
PD
C
Con
t = 100 s
t = 150 s
t = 200 s
t = 250 s
t = 300 s
PH PLCδ1
αSyn A53T
D
10 μm Oxo-M
Con
α-Syn A53T
PH-CFP Cytosolic F/F0
Time (s)
E
**
100 - 120 s linear slope
*
200 - 250 s linear slope

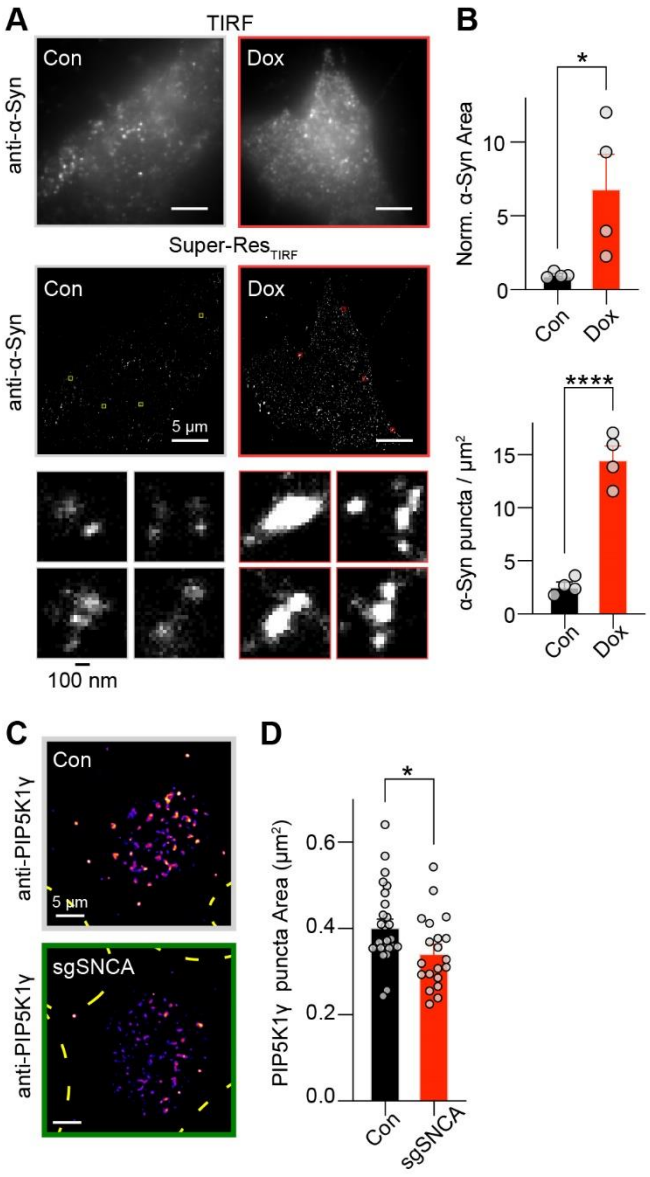
Supplementary Figure 1. α-Synuclein-dependent increases in PM $PI(4,5)P_2$. (A) Representative confocal images of control and $\alpha\text{-synuclein}_{A53T}$-transfected Chinese Hamster Ovary (CHO) cells transfected with $PH_{PLC\delta1}$-CFP. Quantification of PM/cytoplasm intensity ratio of $PH_{PLC\delta1}$ in CHO cells. α-Syn group values are normalized to control group values. **(B)** Representative confocal images of age-matched and sex-matched fibroblasts from a healthy control patient and fibroblasts from a PD patient transfected with $PH_{PLC\delta1}$-RFP to show PM $PI(4,5)P_2$ distribution. Quantification of PM/cytoplasm intensity ratio of $PH_{PLC\delta1}$ in fibroblasts. PD group values are normalized to control group values. **(C)** Representative confocal images of control and $\alpha\text{-Syn}_{A53T}$-transfected tsA-201 cells transfected with $PH_{PLC\delta1}$-CFP. 100 s to 200 s after initial recording, cells were perfused with 10 μM oxotremorine (Oxo-M). Images at 300 s after initial recoding show PM $PI(4,5)P_2$ recovery. **(D)** Normalized time series of cytosolic $PH_{PLC\delta1}$ intensity. **(E)** Quantification of the linear slope from normalized intensity between 100 s to 120 s and 200 s to 250 s showing rate of PM $PI(4,5)P_2$ hydrolysis and recovery, respectively. Error bars represent the standard error of the mean. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

Figure S2


A
H
A53T
PIP5K1α
~72 kDa
β-Actin
~48 kDa
B
ns
PIP5K1α Norm. Protein
1.5
1.0
0.5
0.0
Healthy
A53T
C
Control
+ PIP5K1γ overexpression
anti-PIP5K1γ
10 μm
1 μm
Note: B/C modified
D
Con
+ α-Syn fibrils
VAPA-GFP
NIR2-mCherry
E
ns
**
ns
Avg. Puncta Intensity
Avg. Puncta Area (μm²)
Integrated Density
Con
α-Syn
F
PIP5K1γ-GFP
Con
10 μm
α-Syn A53T
dnARF6
α-Syn A53T dnARF6
G
*
ns
*
ns
PIP5K1γ PM/Cyto
α-Syn A53T
dnARF6

Supplementary Figure 2. Increases in α-Syn-dependent PM PIP5K1γ does not appear to involve increased recruitment by NIR2. (A) Representative western blots from control and α-Syn$_{A53T}$ fibroblast lysates probed for PIP5K1α and β-actin. **(B)** Quantification of PIP5K1α. **(C)** Representative confocal images of control and PIP5K1γ-transfected tsA-201 cells stained for PIP5K1γ showing nuclear and PM staining. B/C of Con image is modified to view PM-specific PIP5K1γ staining. **(D)** Representative confocal images of control and α-Syn fibril-treated tsA-201 cells transfected with VAPA-GFP and NIR2-mCherry. **(E)** Quantification of VAPA puncta intensity, area, and integrated density. **(F)** Representative images of control and α-Syn$_{A53T}$-transfected tsA-201 cells transfected with or without dominant-negative ARF6 (dnARF6) and PIP5K1γ-GFP. **(E)** Quantification of PM/cytoplasm intensity of PIP5K1γ. Error bars represent the standard error of the mean. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

Figure S3


A
TIRF
Con
Dox
anti-α-Syn
Super-Res$_{TIRF}$
Con
Dox
anti-α-Syn
5 μm
100 nm
B
Norm. α-Syn Area
*
10
5
0
Con
Dox
α-Syn puncta / μm^2
****
15
10
5
0
Con
Dox
C
anti-PIP5K1γ
Con
5 μm
anti-PIP5K1γ
sgSNCA
D
PIP5K1γ puncta Area (μm^2)
*
0.6
0.4
0.2
0.0
Con
sgSNCA

Supplementary Figure 3. Knocking down α-Syn decreases PIP5K1γ. (A) Representative TIRF images and super-resolution localization maps (Super-Res$_{TIRF}$) of undifferentiated control and doxycycline-induced α-synuclein-overexpressing SH-SY5Y cells stained for α-synuclein. Zoom images show selected α-synuclein puncta. **(B)** Quantification of α-synuclein staining in Super-Res$_{TIRF}$ images. **(C)** Representative confocal images of control HEK293-Cas9 cells and HEK293-Cas9 cells transfected with scrambled sgRNA or sgRNA targeting at *SNCA*. Cells were fixed and stained for PIP5K1γ. **(D)** Quantification of PIP5K1γ puncta area. Error bars represent the standard error of the mean. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

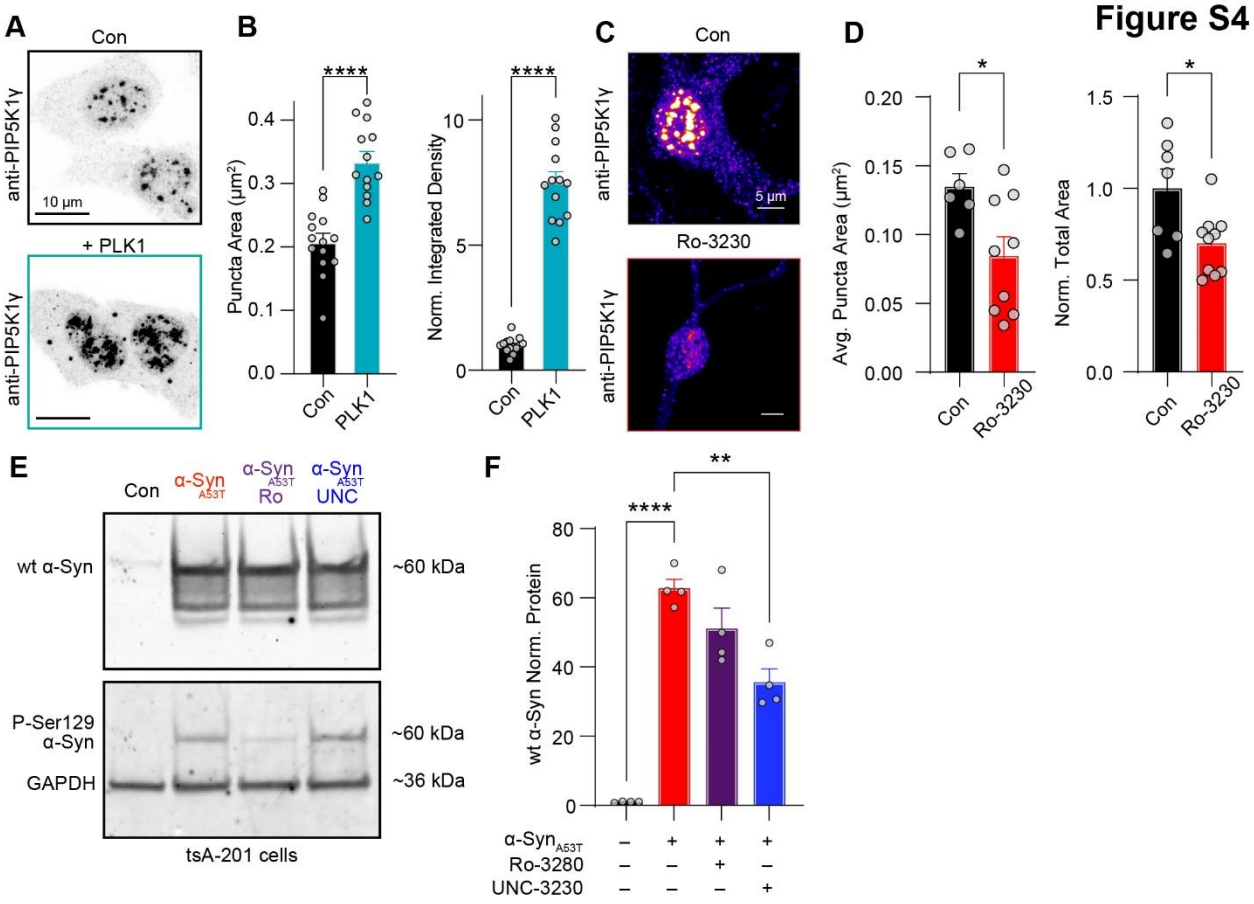


Figure S4
A
Con
anti-PIP5K1γ
10 μm
+ PLK1
anti-PIP5K1γ
B
****
Puncta Area (μm²)
0.4
0.3
0.2
0.1
0.0
Con
PLK1
****
Norm. Integrated Density
10
5
0
Con
PLK1
C
Con
anti-PIP5K1γ
5 μm
Ro-3230
anti-PIP5K1γ
D
*
Avg. Puncta Area (μm²)
0.20
0.15
0.10
0.05
0.00
Con
Ro-3230
*
Norm. Total Area
1.5
1.0
0.5
0.0
Con
Ro-3230
E
Con
α-Syn A53T
α-Syn A53T Ro
α-Syn A53T UNC
wt α-Syn
~60 kDa
P-Ser129 α-Syn
~60 kDa
GAPDH
~36 kDa
tsA-201 cells
F
**
****
wt α-Syn Norm. Protein
80
60
40
20
0
α-Syn A53T
Ro-3280
UNC-3230

Supplementary Figure 4. PLK1 activity drives recruitment of PIP5K1γ to increase α-Syn aggregation. (A) Representative confocal images of control and PLK1-transfected tsA-201 cells stained for PIP5K1γ. **(B)** Quantification of size and integrated density of PIP5K1γ puncta. **(C)** Representative confocal images of mouse hippocampal neurons stained for PIP5K1γ with or without 24 hr treatment of 100 nM Ro-3280. **(D)** Quantification of size and total area of PIP5K1γ puncta in mouse hippocampal neurons. **(E)** Representative western blots and **(F)** quantification of protein lysates from control and α-Syn$_{A53T}$-transfected tsA-201 cells with or without 24 hr treatment of 100 nM Ro-3280, or 24 hr treatment of 100 nM UNC-3230. Error bars represent the standard error of the mean. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

4. α-Syn-dependent production of $PI(4,5)_2$ enhances G_qPCR signaling cascades inducing neurotoxicity

4.1 Introduction

$PI(4,5)P_2$ plays a crucial role in many aspects of neuronal signaling (Dickson, 2019). One major role is acting as a precursor for the second messenger IP_3. Briefly, binding of neurotransmitters or hormones to G_qPCRs leads to activation of phospholipase C (PLC), hydrolysis of $PI(4,5)P_2$ and subsequent production of cytosolic IP_3 and membrane-bound DAG. Diffusible IP_3 is then free to bind IP_3 receptors (IP_3R) in endoplasmic reticulum (ER) membranes to initiate release of Ca^{2+} into the cytoplasm which serves as an instructional signal to alter the activity of Ca^{2+}-sensitive proteins (Figure 5A). Based on the finding that α-Syn increases cellular $PI(4,5)P_2$ levels (Figure 1 and (Schechter, Atias, et al., 2020)) and a the critical role for a splice variant of PIP5K1γ driving IP_3-mediated Ca^{2+} release (Y. J. Wang et al., 2004) we wanted to test if G_qPCR signaling is altered under conditions of enhanced pathological α-Syn (Figure 5A).

Furthermore, we have recently demonstrated that deviant increases in IP_3R type 1 (IP_3R1) clustering is a key player in neuronal cell death in the neurodegenerative NPC1 disease (Tiscione et al., 2021). IP_3R clustering is governed by several factors, including protein-protein interactions, phosphorylation, and IP_3 production. Clustering of IP_3R leads to enhanced cooperative gating, higher propensity to release Ca^{2+} leading to oscillations in intracellular Ca^{2+} levels. While these oscillations can govern the initiation of various Ca^{2+}-dependent pathways, release of Ca^{2+} can also shuttle the ion into other organelles that act as Ca^{2+} storage reservoirs. Interorganellar communication between endoplasmic reticulum and mitochondrial membranes facilitates the transfer of Ca^{2+} from the ER to

mitochondria at ER-Mito membrane contact sites (MCS) to influence bioenergetics and maintain cellular homeostasis (Cárdenas et al., 2010). Therefore, we wanted to elucidate if enhanced IP_3R1 clustering and subsequent Ca^{2+} release impacts neuronal fidelity.

4.2 Results

4.2.1 α-Syn-dependent increases in $PI(4,5)P_2$ enhance IP_3R clustering to augment G_q-mediated Ca^{2+} release.

To test if α-Syn alters G_q-mediated Ca^{2+} release we loaded several cellular models of altered α-Syn expression with the Ca^{2+}-sensitive indicator, Fluo-4 AM, and monitored changes in cytosolic Ca^{2+} levels after addition of the G_q-agonist, UTP (100 μM). Comparison of control and PD fibroblasts revealed that application of 100 μM UTP resulted in approximate doubling of the G_qPCR-mediated release of Ca^{2+} relative to controls (Figure 5B-D). Similar observations were observed for HeLa (Figure 5E,F) and tsA201 (Figure 5G) cells overexpressing α-Syn_{A53T}, and neurons treated with α-Syn fibrils (Figure S5). 24-hour treatment with the PIP5K1γ inhibitor, UNC-3230 normalized the Fluo-4 response in cells overexpressing α-Syn_{A53T} back into a control range (Figure 5G) indicating upstream increases in $PI(4,5)P_2$ was an important factor mediating increases in G_qPCR Ca^{2+} signaling. Following hydrolysis of $PI(4,5)P_2$, soluble IP_3 diffuses and binds to IP_3Rs to release Ca^{2+}. To test if IP_3R1, the most prevalent IP_3R isoform in neurons, mediates α-Syn-dependent increases in G_qPCR Ca^{2+} release, we transfected $IP_3R1^{-/-}$ HEK293 cells with α-Syn_{A53T}. We found that loss of IP_3R1 resulted in cells being refractory to α-Syn (Figure 5H,I) and exhibited smaller G_qPCR Ca^{2+} release. These data suggest augmented $PI(4,5)P_2$ levels increase G_qPCR-mediated Ca^{2+} release through IP_3R1.

Next, we loaded control and α-Syn$_{A53T}$ expressing HeLa cells with Fluo-4 AM and quantified oscillations in intracellular Ca^{2+} following GPCR activation. In control cells, simulation with the purinergic receptor agonist UTP (100 μM, 100 s) increased intracellular Fluo-4 intensity with an average of two oscillations in signal (Figure 6B,C). In comparison, cells transfected with α-Syn$_{A53T}$ had a significantly larger increase in Fluo-4 intensity that occurred with an average of five oscillations in signal (Figure 6B,C). To determine if enhanced oscillations in Ca^{2+} occurred due to increases IP$_3$R1 clustering, we took several complementary fluorescent approaches. First, using a cell line that has endogenous IP$_3$R1 tagged with GFP (eGFP-IP$_3$R1; (Thillaiappan et al., 2017)) we found that overexpression of α-Syn$_{A53T}$ significant increased IP$_3$R1 puncta intensity and area. (Figure 6D,E). Similar results were observed in MAP2-postive dendrites of hippocampal neurons transfected with α-Syn$_{A53T}$ compared to control neurons (Figure 6F,G). To test if α-Syn-dependent increases in PIP5K1γ and/or PI(4,5)P$_2$ were central drivers in enhanced IP$_3$R1 clustering, we treated cortical neurons with α-Syn fibrils under conditions of Ro-3280 (to inhibit PLK1 and consequently PIP5K1γ recruitment and PI(4,5)P$_2$ production (Figure 4 D-G)) or UNC-3230 (to inhibit PIP5K1γ production of PI(4,5)P$_2$ (Figure 2)) and fixed and stained neurons for the dendritic marker MAP2 and IP$_3$R1. Quantification of IP$_3$R1 clusters from MAP2-positive cells revealed that neurons treated with either UNC-3230 or Ro-3280 exhibited no significant difference in IP$_3$R1 immunostaining compared to control neurons, while neurons α-Syn fibril treatment demonstrated increased total puncta area and integrated density (Figure 6H-J). Co-treatment of α-Syn fibrils with Ro-3280 or UNC-3230 rescued IP$_3$R total puncta area and puncta integrated density back into a control range (Figure 6H-J). Collectively, these data present evidence that α-Syn-

dependent increases in $PI(4,5)P_2$ influences the distribution of IP_3R1 clusters to enhanced G_qPCR-mediated Ca^{2+} release.

4.2.2 α-Syn-dependent augmentation of IP_3R clustering increases mitochondrial Ca^{2+}, reactive oxygen species, and neuronal cytotoxicity.

The molecular elements that facilitate the transfer of Ca^{2+} at ER-Mito MCS are IP_3R and voltage-dependent anion channels (VDAC). Given the augmented IP_3R1 clustering observed in neurons with α-Syn fibril treatment (Figure 6), we tested the hypothesis that this would facilitate enhanced IP_3R1-VDAC1 interactions and amplify mitochondrial Ca^{2+} (Ca^{2+}_{Mito}) leading to cellular toxicity (Figure 7A). To begin we treated cortical neurons with α-Syn fibrils and fixed and immunostained against IP_3R1 and VDAC to map potential interactions between the proteins. Quantification of superresolution images revealed that α-Syn fibrils increased the fraction of IP_3R1 pixels colocalized with VDAC1 (Figure 7B,C). These data are aligned with previous reports of enhanced ER-Mito contacts with elevated α-Syn expression (Calì, Ottolini, Negro, & Brini, 2012). Next, we expressed a genetically-encoded Ca^{2+} indicator targeted to mitochondria (Mito-RCaMPh1) to ask if Ca^{2+}_{Mito} is altered following treatment with α-Syn fibrils. Quantification of Mito-RCaMPh1 intensities between tsA-201 cells with or without $α\text{-}Syn_{A53T}$ transfection revealed that α-Syn significantly increased Ca^{2+}_{Mito} (Fig. 7D), similar to what has been previously reported (Rosencrans, Rajendran, Bezrukov, & Rostovtseva, 2021). To understand if alterations in Ca^{2+}_{Mito} are accompanied by changes in cell health, we measured reactive oxygen species (ROS) and cell viability. Mitochondria are an important source of ROS with ROS production linked to mitochondrial damage in a range of pathologies, including

neurodegeneration. Measurement of ROS in control, PBS-treated neurons or neurons treated with α-Syn fibrils revealed a significant, 2-fold increase in mitochondrial ROS levels (Figure 7E) that correlated with a decreased in neuronal viability (Figure 7F). To test a role for the PIP5K1γ–$PI(4,5)P_2$–IP_3R1 signaling axis in mediating changes in mitochondrial health, we treated neurons with the PIP5K1γ inhibitor UNC-3230 which decreases α-Syn-dependent increases in $PI(4,5)P_2$ (Figure 2), PIP5K1γ (Figure 2,3), and IP_3R1 (Figure 5) and found that concurrent treatment of α-Syn fibrils with UNC-3230 normalized IP_3R1-VDAC colocalization (Figure 7B), Ca^{2+}_{Mito} (Figure 7D), mitochondrial ROS (Figure 7E), and cell viability (Figure 7F) back to control levels. Taken together, these data suggest that α-Syn-dependent increases in $PI(4,5)P_2$ initiate a damaging feedforward signaling cascade that alters intracellular Ca^{2+} signaling networks to perturb mitochondrial function and trigger neurotoxicity.

4.3 Discussion

One of the hallmarks of dopaminergic neurons of the SN is their vulnerability to sustained elevations in cytosolic calcium. Their poor endogenous calcium buffering capability (Foehring et al., 2009), autonomous oscillations in intracellular calcium concentrations (C. J. Wilson & Callaway, 2000), and elevated expression of voltage dependent $Ca_V1.3$ channels (Hurley et al., 2013) render the SN vulnerable to stressors that further elevated intracellular calcium levels. Furthermore, dysregulation of several proteins related to mitochondrial function are closely associated with α-Syn expression or familial variants of PD (Catoni, Calì, & Brini, 2019; Gomez-Suaga et al., 2017; Kumar et al., 2018; Lesage et al., 2016; Y. Liu et al., 2019; Ludtmann et al., 2018; Paillusson et al.,

2017). Our data contributes to this list of vulnerable features observed in neurons with altered α-Syn expression. We find that one of the downstream consequences of elevated $PI(4,5)P_2$ is enhanced G_qPCR Ca^{2+} release. Given that Ca^{2+} binding to the C-terminal tail of α-Syn (Lowe, Pountney, Jensen, Gai, & Voelcker, 2004; Nielsen et al., 2001) leads to α-Syn oligomerization, increases in G_qPCR Ca^{2+} release would not only be expected to augment α-Syn aggregation, but impact L-type voltage-gated calcium channel (LTCC) dependent oscillations in cytoplasmic Ca^{2+} that are essential for dopamine synthesis and ATP production (Hetzenauer, Sinnegger-Brauns, Striessnig, & Singewald, 2006; Surmeier, Guzman, Sanchez-Padilla, & Goldberg, 2010). Underlying enhanced G_qPCR Ca^{2+} release appears to be in part due to altered organization of IP_3R1 in ER membranes. We determine that that enhanced clustering of IP_3R1, a driver to neuronal death in neurodegenerative NPC1 disease (Tiscione et al., 2021), potentiates Ca^{2+}_{Mito}, ROS, and neuronal toxicity. Changes in IP_3R1 seem to be dependent on IP_3 generation as inhibiting $PI(4,5)P_2$ production abrogates neurotoxic phenotypes. That said, data from oocytes determined that PLK1 can phosphorylate IP_3R1 at residue Thr-2656 (Vanderheyden et al., 2009), resulting in increased IP_3 binding sensitivity resulting in more pronounced IP_3-induced ER calcium release. Although this relationship has yet to be investigated in neurons, our data indicates Ro-3280 treatment reduces neuronal IP_3R distribution in α-Syn-dependent and -independent states, suggesting that PLK1-mediated phosphorylation of IP_3R1 may be part of a mechanism for potentiating calcium cytotoxicity in PD. Nevertheless, we show for the first time that modulating $PI(4,5)P_2$ levels in α-Syn-expressing cells alters MCS integrity and mitochondrial Ca^{2+} handling, thus

demonstrating that PIP5K1γ may play a crucial role in arbitrating mitochondrial-dependent pathophysiology.

In summary, we present evidence that altered abundance of $PI(4,5)P_2$ leading to aberrant Ca^{2+} signaling has deleterious consequences for neuronal health in models of PD. Given the diverse range of cellular pathways that phosphoinositides integrate and control, and their reported role in other neurodegenerative diseases, our finding that they are critical rheostats for the development of PD pathophysiology invites further investigations to elucidate how careful modulation of their abundance and distribution could be exploited in the clinic as a means to slow PD progression.

4.4 Figures

Figure 5

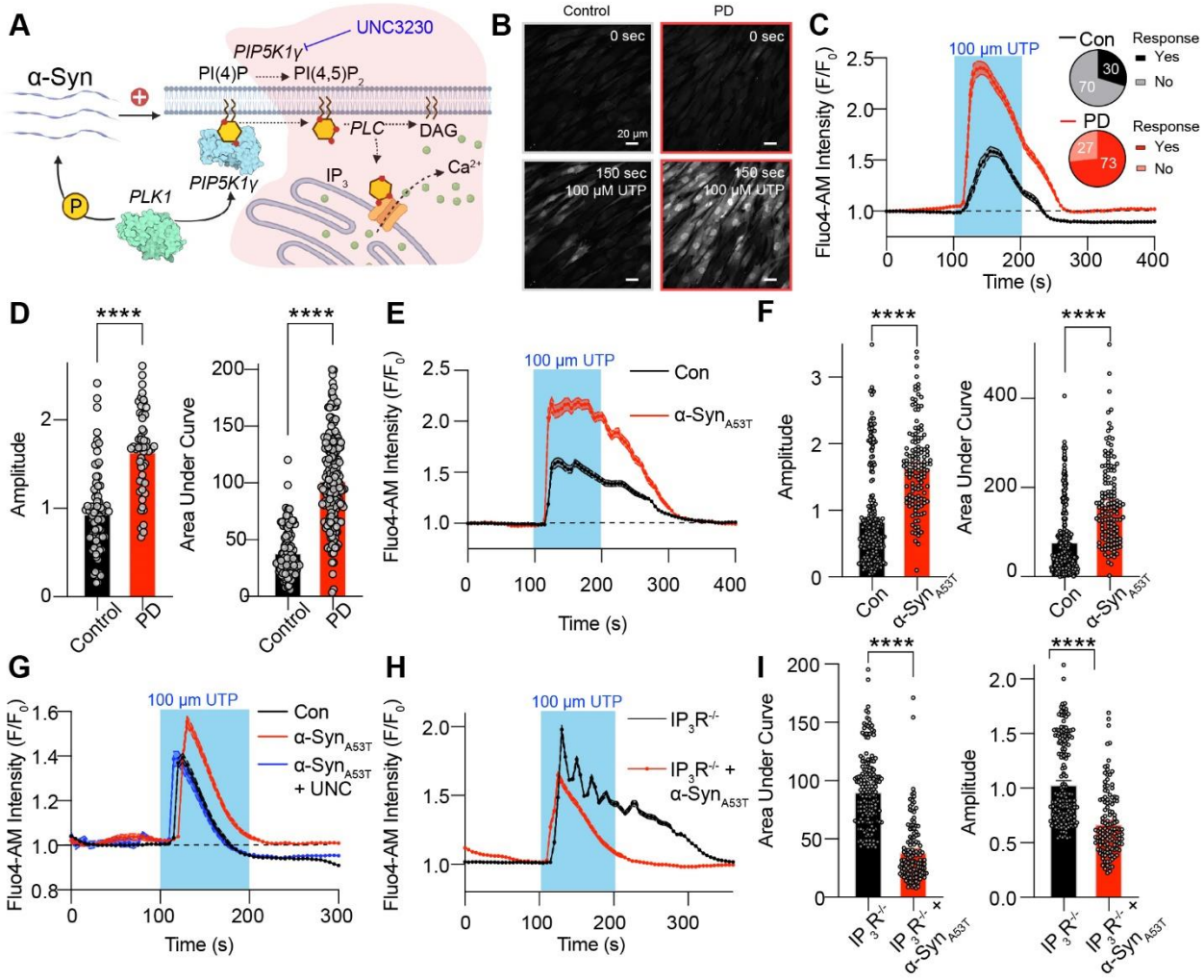


Figure 5. α-Syn augments G_q-mediated Ca^{2+} release. (A) Schematic to be tested: Does α-Syn-dependent increases in $PI(4,5)P_2$ increased ER Ca^{2+} release. **(B)** Representative confocal images of Fluo-4-loaded fibroblasts from age- and sex-matched healthy control patients and PD patients before and during 100 µM UTP application. **(C)** Quantification of normalized Fluo4-AM intensity in fibroblasts during UTP perfusion. Pie charts show percentage of fibroblasts responding to UTP application. **(D)** Quantification of amplitude and area under curve of Fluo4-AM response following UTP application. **(E)** Quantification of normalized Fluo4-AM intensity in control and $α\text{-Syn}_{A53T}$-transfected tsA-201 cells during UTP application. Confocal images not shown. **(F)** Quantification of amplitude and area under curve of Fluo4-AM response following UTP application. **(G)** Quantification of normalized Fluo4-AM intensity in control and $α\text{-Syn}_{A53T}$-transfected tsA-201 cells with or without 24 hr treatment of 100 nM UNC-3230 during UTP perfusion. **(H)** Quantification of normalized Fluo4-AM intensity in control and $α\text{-Syn}_{A53T}$ -transfected IP_3 type 1 knock-out HEK293 cells during UTP application. **(I)** Quantification of amplitude and area under curve of Fluo4-AM response following UTP application in IP_3R type 1 knock-out HEK293 cells. Error bars represent the standard error of the mean. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

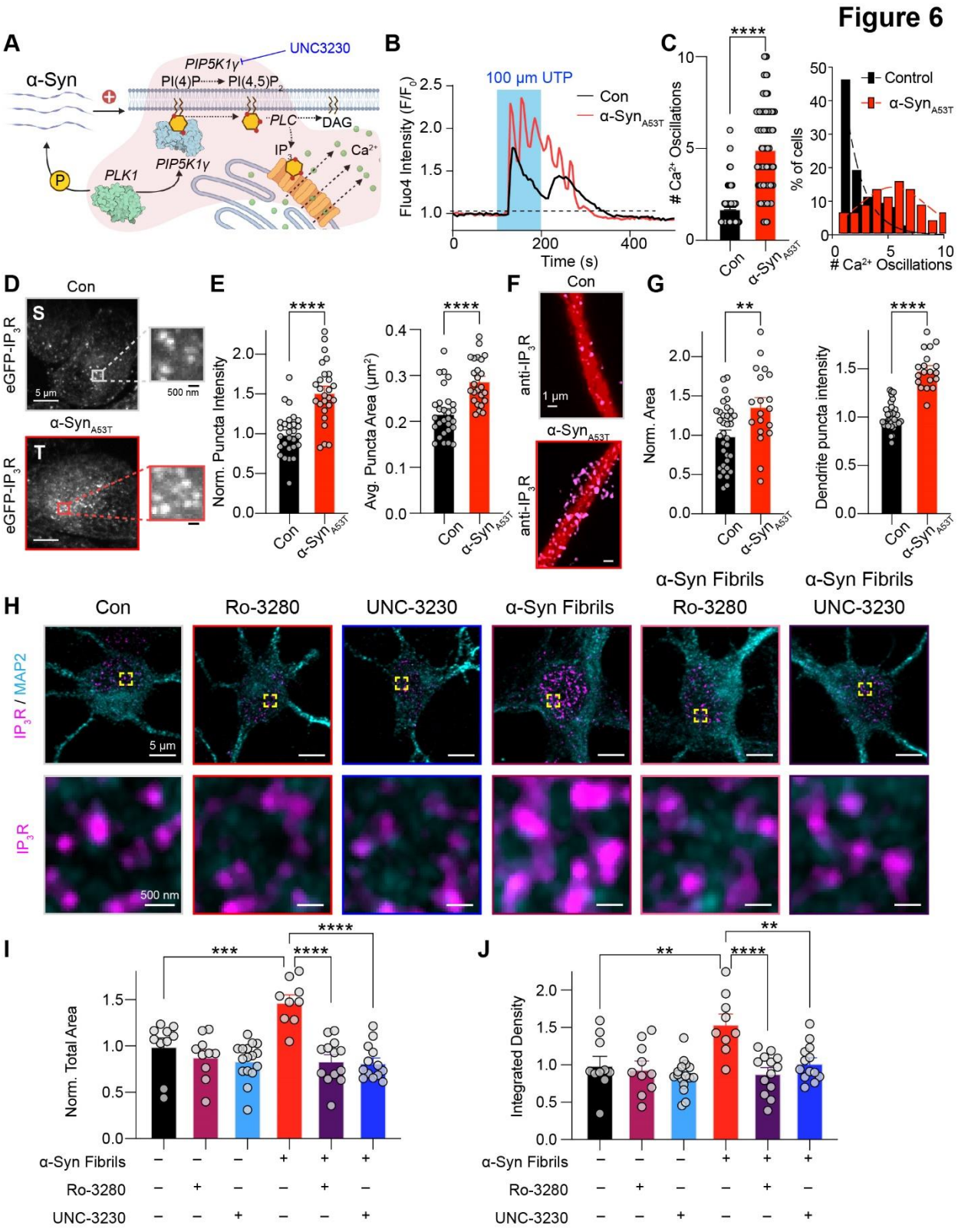


Figure 6
A
α-Syn
UNC3230
PIP5K1γ
PI(4)P
PI(4,5)P2
PLC
DAG
IP3
Ca2+
PIP5K1γ
PLK1
P
B
100 μm UTP
Con
α-SynA53T
Fluo4 Intensity (F/F0)
Time (s)
C
Ca2+ Oscillations
Control
α-SynA53T
% of cells
# Ca2+ Oscillations
D
Con
eGFP-IP3R
S
5 μm
500 nm
α-SynA53T
T
E
Norm. Puncta Intensity
Avg. Puncta Area (μm2)
F
Con
anti-IP3R
1 μm
α-SynA53T
G
Norm. Area
Dendrite puncta intensity
H
Con
Ro-3280
UNC-3230
α-Syn Fibrils
α-Syn Fibrils Ro-3280
α-Syn Fibrils UNC-3230
IP3R / MAP2
IP3R
5 μm
500 nm
I
Norm. Total Area
α-Syn Fibrils
Ro-3280
UNC-3230
J
Integrated Density

Figure 6. α-Syn-dependent increases in $PI(4,5)P_2$ enhance IP_3R1 clustering. (A) Schematic of hypothesis to be tested: α-Syn-dependent increases in $PI(4,5)P_2$ enhance IP_3R1 clustering. **(B)** Quantification of normalized Fluo4-AM intensity in HeLa cells during 100 µM UTP perfusion. **(C)** Quantification of number and distribution of cytosolic Ca^{2+} oscillations after UTP application. **(D)** Representative confocal images of control and α-Syn_{A53T}-transfected eGFP-IP_3R1 cells. **(E)** Quantification of size and intensity of IP_3R1 puncta. **(F)** Representative confocal images of control and α-Syn_{A53T} -transfected mouse hippocampal neurons with MAP2-labeled dendrites (red) stained for IP_3R type 1 (magenta). **(G)** Quantification of size and intensity of IP_3R puncta in hippocampal dendrites. **(H)** Representative confocal images of control and α-Syn fibril-treated mouse cortical neurons stained with IP_3R1 (magenta) and MAP2 (cyan), with or without 24 hr treatment of 100 nM Ro-3280, or 24 hr treatment of 100 nM UNC-3230. Zoom images show only IP_3R1. Quantification of **(I)** area and **(J)** integrated density of IP_3R1 puncta in mouse cortical neurons. Error bars represent the standard error of the mean. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

Figure 7

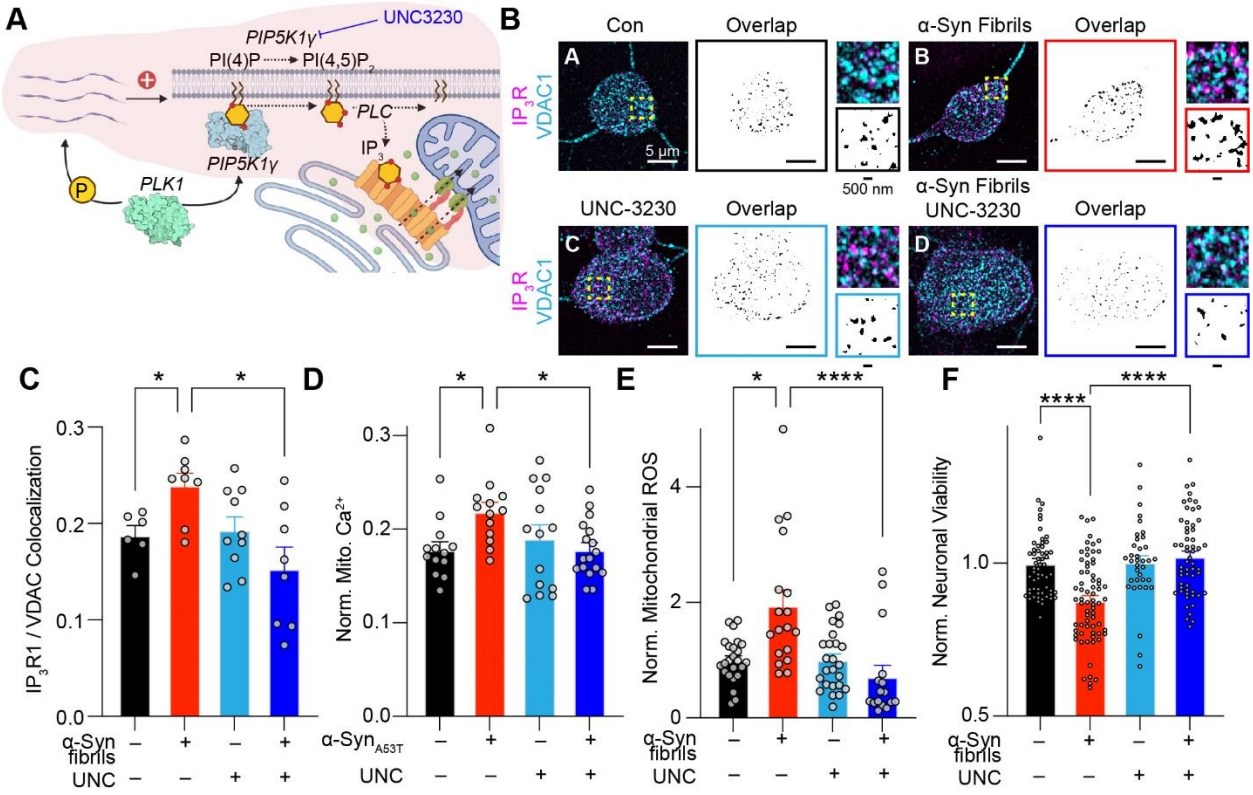
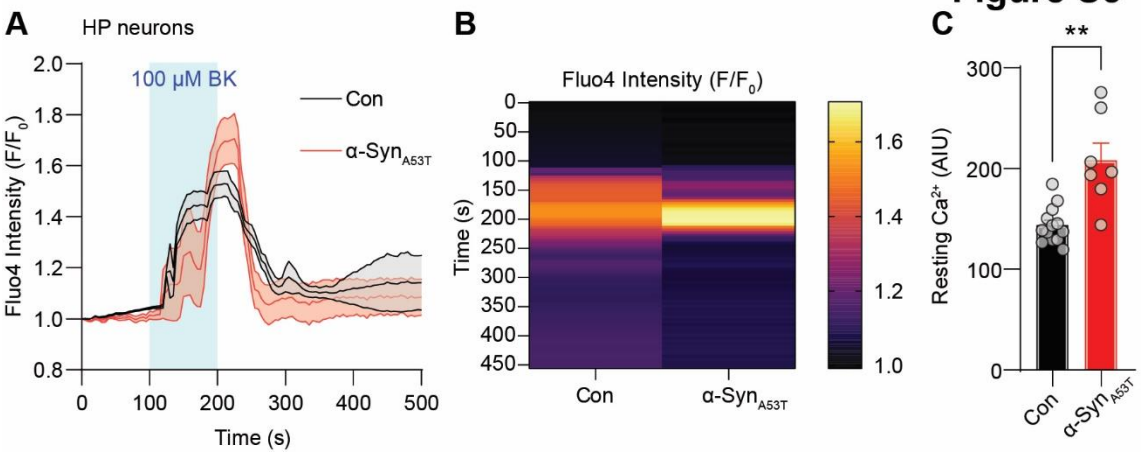

A
UNC3230
PIP5K1γ
PI(4)P
PI(4,5)P2
PLC
IP3
PIP5K1γ
P
PLK1
B
Con
Overlap
α-Syn Fibrils
Overlap
IP3R
VDAC1
5 μm
500 nm
UNC-3230
Overlap
α-Syn Fibrils
UNC-3230
Overlap
C
IP3R1 / VDAC Colocalization
D
Norm. Mito. Ca2+
E
Norm. Mitochondrial ROS
F
Norm. Neuronal Viability
α-Syn fibrils
α-SynA53T
UNC

Figure 7. α-Syn-dependent augmentation of IP_3R1 clustering increases mitochondrial Ca^{2+}, reactive oxygen species, and neuronal cytotoxicity. (A) Schematic of hypothesis to be tested: α-Syn dependent increases in ER Ca^{2+} potentiate mitochondrial Ca^{2+}. **(B)** Representative confocal images of control and α-Syn fibril-treated mouse cortical neurons with or without 24 hr treatment of 100 nM UNC-3230, stained for IP_3R1 (magenta) and VDAC1 (cyan). Overlap images show only pixels positive for IP_3R1 and VDAC1. **(C)** Quantification of the ratio of overlapping VDAC1/IP_3R pixels divided by all IP_3R1 pixels. **(D)** Quantification of normalized mitochondrial Ca^{2+} levels in control and $\alpha\text{-Syn}_{A53T}$-transfected tsA-201 cells, with or without 24 hr treatment of 100 nM UNC-3230. **(E)** Quantification of normalized mitochondrial reactive oxygen species (ROS) in control and α-Syn fibril-treated mouse cortical neurons with or without 24 hr treatment of 100 nM UNC-3230. **(F)** Quantification of cell viability assay in control and α-Syn fibril-treated mouse cortical neurons, with or without 24 hr treatment of 100 nM UNC-3230. Each data point represents an ROI in which the ratio of live neurons divided by all neurons was measured. Error bars represent the standard error of the mean. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

Figure S5


A
HP neurons
100 μM BK
Con
α-Syn A53T
Fluo4 Intensity (F/F0)
Time (s)
B
Fluo4 Intensity (F/F0)
Time (s)
Con
α-Syn A53T
C
**
Resting Ca2+ (AIU)
Con
α-Syn A53T

Supplementary Figure 5. Pathological α-Syn augments Gq-mediated Ca^{2+} release in cortical neurons *in vitro*. (A) Quantification of normalized Fluo4-AM intensity in control and α-Syn_{A53T}-transfected mouse hippocampal neurons perfused with 2 mM Ca^{2+} Ringer's solution before, during, and after application of 100 µM bradykinin (BK) from 100 s to 200 s. Fluo4-AM intensity values are normalized to t = 0 s value. **(B)** Heat map of Fluo4-AM assay in *(A)*. Heat map shows normalized Fluo4-AM intensity to initial recording in mouse hippocampal neurons. **(C)** Quantification of initial (t = 0 s) Fluo4-AM intensity recoding in mouse hippocampal neurons from Fluo4-AM assay in *(A)*. Measurements were taken by selecting a cytosolic ROI to show resting Ca^{2+} levels at t = 0 s before BK application. Error bars represent the standard error of the mean. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

5. Discussion and Future Perspectives

5.1 Therapeutic interventions

PD places a significant burden on individuals, caregivers, families, and friends of those with the disease. Given that there is currently no drug on the market to either cure or reverse the progression of PD, identifying therapeutic targets for the disease remains imperative for improving quality of life for patients. Due to its prevailing pathological role in both idiopathic and familial PD, α-Syn is a lucrative target for therapy intervention (Fields, Bengoa-Vergniory, & Wade-Martins, 2019).

5.1.1 Interference with prion-like spread

One of the most critical requirements for aberrant α-Syn aggregation is the presence of endogenous α-Syn itself. Indeed, exogenous α-Syn introduced into neuronal cultures seeds the exasperated aggregation through interactions with the residues of the non-amyloid component (NAC) domain of the protein (Volpicelli-Daley et al., 2011). Mechanisms by which α-Syn gains entrance into neurons to propagate seeding pathology include direct membrane penetration or endocytosis-mediated endo-lysosomal rupture, resulting in either endo-lysosomal-free aggregation or endo-lysosomal-mediated aggregation (Jiang, Gan, Yen, McLean, & Dickson, 2017). Due to differing physiological composition of all membranes, primarily by the varying presence of cholesterol (van Meer, Voelker, & Feigenson, 2008), some exogenous α-Syn aggregates may not be strong enough to penetrate the plasma membrane directly and thus must be endocytosed before penetrating the relatively less dense and less stable membrane of the endo-lysosomal system. Indeed, most Lewy-positive patients show high degree of colocalization between

α-Syn aggregates and galectin-3, a cellular marker for pathogenic lysis of organelles (Freeman et al., 2013; Jiang et al., 2017; Paz et al., 2010). It is apparent that strengthening the endo-lysosomal pathway may provide protection against α-Syn transmission from neighboring cells.

Modulating exogenous α-Syn uptake may act as a means to diminish the need for lysosomal degradation of the protein and decrease propensity for aggregation seeding. Lymphocyte-activation gene 3 (LAG3) is a extracellular plasma membrane receptor that demonstrates high specificity and affinity for α-Syn fibrils, and is required for exogenous α-Syn uptake and Rab5-dependent internalization. Ablation of LAG3 or immunotherapy targeted against LAG3 significantly decreases the endocytosis of α-Syn fibrils, as well as induced neurotoxicity (Mao et al., 2016). Another less specific mechanism for α-Syn uptake is mediated through interactions with heparan sulfate proteoglycans (HSPGs) on the cell surface, which also bind tau fibrils and other infectious prion-like pathogens (Horonchik et al., 2005). While fibrillar tau aggregates require HSPGs for internalization, α-Syn seems to have multiple mechanisms for endocytosis and propagation of seeding. In addition to targeting LAG3, treating cells with heparin and choral hydrate, which disrupt HSPG-mediated uptake, reduces the propagation of α-Syn seeding (Holmes et al., 2013). Clearly, there are multiple means by which α-Syn enters previously unaffected neurons, indicating the complex nature by which it is transmitted between cells and the difficulty in pinpointing treatment to a specific component of the endo-lysosomal pathway. Nevertheless, directing future research towards the mechanisms of α-Syn transmission and seeding may garner insights to slowing the pathoprogression of PD.

5.1.2 Reducing α-Syn production

As previously mentioned, transcriptional regulation of *SNCA* is under control of transcription factors GATA1, GATA2, and ZSCAN21, which may occupy intron 1 (GATA1, GATA2, ZSCAN21) or intron 2 (GATA2). Future studies should be directed at understanding regulatory mechanisms for these transcription factors, with particular emphasis on reduction of α-Syn transcription. Modulating translation of α-Syn has also garnered attention and produced successful results by several research groups. siRNA targeted against α-Syn *in vitro* in SH-SY5Y cells reduces intracellular α-Syn by 80% while supporting cell viability and protecting cells from MPP+-induced toxicity (Fountaine & Wade-Martins, 2007). Furthermore, RNA interference (RNAi) experiments targeting α-Syn *in vivo* using lentiviral-injected shRNA in rat striatum (Sapru et al., 2006), siRNA in mouse hippocampus (J. Lewis et al., 2008), and siRNA in primate SN (McCormack et al., 2010) support the notion that RNAi is an acceptable means for reducing the α-Syn expression. However, due to the important role α-Syn plays in healthy neurons, vast reductions in α-Syn expression could be deleterious to cellular physiology (Kanaan & Manfredsson, 2012). Indeed, AAV-delivered siRNA targeting α-Syn in rat SN induces dopaminergic cell loss and complementary behavioral deficits compared to delivery of control siRNA sequences (Gorbatyuk et al., 2010). Future research should consider the necessary levels of α-Syn required to carry out normal neuronal function before attempting to eliminate or reduce its production.

5.1.3 Inhibiting or reducing α-Syn aggregation

A more utile approach to lessening the pathological function of α-Syn without compromising physiological function may be targeting the reduction of fibrillar α-Syn aggregation. Multiple mechanisms for this therapeutic approach have already been investigated. One application involves endogenous molecular chaperones such as members of the heat shock protein (HSP) family that aid in adequate protein folding and prevent anomalous aggregation (Glover & Lindquist, 1998). In fact, in *Drosophila*, direct expression of HSP70 ameliorates dopaminergic cell loss, and interference with endogenous molecular chaperone activity potentiates toxicity, highlighting the effect these proteins have on the pathology of PD (Auluck, Chan, Trojanowski, Lee, & Bonini, 2002). Moreover, overexpressed HSP70 reduces aggregated α-Syn filaments and rescues cytotoxic adenylate release *in vivo* in mice and *in vitro* in human neuroglioma cells (Klucken, Shin, Masliah, Hyman, & McLean, 2004). However, a potential pitfall to this therapeutic approach is that during the accelerated time course of aggregation, HSPs are less effective at regulating protein misfolding (Cox, Selig, Griffin, Carver, & Ecroyd, 2016). Taken together with the positive effect that α-Syn concentration has on nucleation-dependent mechanisms of fibrillation (Wood et al., 1999), it appears that HSPs may only be effective at regulating the misfolding of the neurotoxic protein during early-onset cases of PD.

Small molecule inhibitors such as Anle138b, baicalein, and NPT100-18A among others have also demonstrated promise in treatment of PD and other synucleinopathies. The aim of these small molecules is similar to that of molecular chaperones such as HSPs, which is to prevent α-Syn aggregation, only their smaller structure enables them

to cross the blood-brain barrier and be used as an effective therapy for PD. Indeed, Anle138b ameliorates oligomer accumulation, neuronal degeneration, and disease progression, and also exhibits successful outcomes in mice after disease onset, which has led it to garner attention in clinical trials, advancing it to Phase I status in the United States (Levin et al., 2014; Wagner et al., 2013). Flavonoids, which are found in fruits and vegetables, are consumed daily in the United States and are an important component of herbal medicine. Many flavonoids, including baicalein, contain antioxidative properties and may combat the pathological phenotypes associated with oxidative stress produced in α-Syn pathology. Baicalein demonstrates these therapeutic properties by tightly binding α-Syn and promoting formation of physiological oligomers, as opposed to pathological fibrils, and even disperses existing α-Syn aggregates, demonstrating its potential for combating PD in both early and late onset patients (Zhu et al., 2004). Furthermore, structural modeling studies of the C-terminal domain of α-Syn, which is critical for its aggregative properties, have elucidated synthetic compounds that may bind the protein and mediate its biological interactions. Among these is NPT100-18A, which uniquely targets α-Syn dimers, preventing further cytosolic and lipid membrane-associated aggregation (Wrasidlo et al., 2016). Given that α-Syn has a high propensity to bind to biological membranes, future research should also be directed towards diminishing membrane interactions. Finally, molecular tweezers have been used to combat other neurodegenerative diseases in which protein misfolding persists (Sinha et al., 2011). These compounds bind at lysine-specific residues with high affinity and compete with other proteins to prevent aggregation due to the inhibition of the hydrophobic and electrostatic properties of the protein they bind to (Fokkens, Schrader, & Klärner, 2005;

Marshall et al., 2011). Lysine-specific molecular tweezer CLR01 is a very favorable candidate for PD prevention and treatment as it provides neuroprotective properties *in vitro* and *in vivo* by reducing aggregation, restoring motor behavior, and decreasing inflammatory glial activation (Bengoa-Vergniory et al., 2020). Certainly, many more small molecule compounds have yet to be identified and for PD therapy, offering a promising avenue for future investigation to improve neurodegenerative phenotypes.

Two independent classes of antibiotics have also been shown to remedy proteinaceous aggregation (Bi, Zhu, Jing, Liang, & Tao, 2013; Cankaya, Cankaya, Kilic, Kilic, & Yulug, 2019). Minocylcine, which is closely related to tetracycline, inhibits the Aβ fibril formation and microglial activation (Familian, Boshuizen, Eikelenboom, & Veerhuis, 2006). However, when investigated in the context of α-Syn and PD, the physiopathological ramifications of minocycline treatment remain unclear, as contradicting reports of *in vitro* have claimed to rescue and enhance dopaminergic neuronal death due to treatment of the antibiotic (Radad, Moldzio, & Rausch, 2010; L. Yang et al., 2003). Perhaps more convincing, and more well-documented, as a potential therapeutic approach, Rifampicin specifically disseminates existing α-Syn fibrils while preventing future aggregation and promotes formation of physiological oligomers. Rifampicin even exists in these oligomers suggesting that it inhibits α-Syn aggregation by likely occupying residues critical for the conformational change that occurs upon fibrillation (J. Li, Zhu, Rajamani, Uversky, & Fink, 2004). Whether Rifampicin treatment translates to a clinical setting remains uncertain as more pre-clinical data is required. However, the emergence of antibiotics as candidate for treating PD and other related

diseases illustrates the multi-faceted approach that researchers and clinicians take when considering novel therapies.

Arguably one of the most intriguing prospects for α-Syn aggregate mitigation is immunotherapy. One of the earliest accounts of immunotherapy successfully mitigating α-Syn accumulation and subsequent neuropathology utilized a human α-Syn vaccination. Transgenic human α-Syn mice were able internalize anti-human alpha synuclein antibodies and clear membrane-associated aggregates via the lysosomal activation, reducing total aggregation, preserving synaptic architecture, and ultimately slowing neurodegeneration (Masliah et al., 2005). More recently, two human monoclonal antibodies successfully passed Phase I clinical trials in the United States: PRX002 and BIIB054. These two antibodies are targeted against epitopes in the C-terminal and N-terminal regions, respectively, with a much higher propensity of binding aggregated forms of the protein. Doses of PRX002 were deemed to be safe and tolerable by both healthy patients and idiopathic PD patients, accompanied with up to a marked 97% reduction of free serum α-Syn levels, advancing it to Phase II clinical trial status (Jankovic et al., 2018; Schenk et al., 2017). BIIB054 selectively binds pathological forms of α-Syn in the SN in post-mortem PD patients and rescues pathological phenotypes in α-Syn fibril-inoculated mice, including reduction of motor impairment (Weihofen et al., 2019). The antibody performed well in human Phase I clinical trials in healthy volunteers and PD patients, demonstrating favorable tolerability and pharmacokinetic properties and generating recommendations for future clinical development (Brys et al., 2019). However, a potential pitfall to the use of antibodies in PD treatment is their high molecular weight, increasing the difficulty in penetrating the blood-brain barrier, due to its size-restrictive passage

properties to protect the brain against antigens and foreign molecules. Consequently, to circumvent this impedance, nanobodies may be used to gain entry into the brain for therapeutic means. Nanobodies express specific regions for antibody specificity, allowing them to utilize a much smaller and more efficient mechanism to elicit the same immune response (Siontorou, 2013). Similar to previous antibody studies, nanobodies NbSyn2 and NbSyn87 selectively bind to the C-terminal region of α-Syn to drastically inhibit and reduce formation of α-Syn fibrils *in vitro*, while promoting formation of less-stable multimers of which have higher physiological importance in healthy neurons (Iljina et al., 2017). Given the success clinicians have experienced using immunotherapy to treat other diseases such as cancer, this scope of therapeutics deserves further investigation for neurodegenerative disorders.

Finally, as demonstrated by this work and that of others, one of the emerging α-Syn interactions involves phospholipids. Given the propensity of α-Syn to associate with negatively charged acidic species, specifically PIP_2-containing vesicles, modulating PI expression and distribution presents a natural hypothesis for dispersing α-Syn aggregation. Indeed, overexpression of either INPP5E (non-specific inositol 5-phosphatase) or PTEN ($PI(3,4,5)P_3$ 3-phosphatase) to reduce PM levels of either $PI(4,5)P_2$ or $PI(3,4,5)P_3$, respectively, decreases immunostaining of α-Syn aggregation *in vitro* (Jacob et al., 2021). In this present dissertation, evidence is presented that pharmacological inhibition of PIP5K1γ, the predominant enzyme that catalyzes PM PIP_2 production in neuronal tissue, decreases WT and Ser(P)-129 α-Syn aggregation. Furthermore, pharmacological inhibition of PLK1, an upstream mediator of PIP_2 production capable of increasing recruitment and expression of PIP5K1γ, also reduces

WT and Ser(P)-129 α-Syn aggregation. These results warrant future investigation for the precise mechanisms leading to PIP_2-dependent neuronal dysfunction.

5.1.4 Reversing Ca^{2+} dyshomeostasis and mitochondrial dysfunction

The Ca^{2+} hypothesis of neurodegeneration states that neurodegenerative processes are accompanied by aberrant Ca^{2+} signaling and homeostasis (Berridge, 2010). Given that α-Syn is a Ca^{2+}-binding protein and Ca^{2+} positively mediates the propensity by which α-Syn binds lipid membranes (Lautenschläger et al., 2018; Nielsen et al., 2001), targeting Ca^{2+}-dependent cellular processes may alleviate pathological phenotypes observed in PD and other synucleinopathies. An early indication of this interaction was the discovery that the expression of calpain, a Ca^{2+}-dependent cysteine protease, is unproportionally increased in dopaminergic neurons in post-mortem PD patients (A. Mouatt-Prigent, Karlsson, Agid, & Hirsch, 1996) and is correlated with neurodegeneration via cleavage of its substrates (M.-s. Lee et al., 2000). Indeed, calpain inhibition rescues dopaminergic neurons from MPTP-dependent neurodegeneration. MPTP, the prodrug of neurotoxin MPP^+ that induces mitochondrial stress and dysfunction as well as PD-like clinical symptoms (Beal, 2001), alone is responsible for increased calpain levels in mice, suggesting that α-Syn-mitochondria interactions may also cause similar consequences (Crocker et al., 2003). Indeed, rescuing mitochondrial integrity has been identified as a potential therapeutic target for neurodegeneration, implying its importance in the pathogenesis and progression of PD and other neurodegenerative disorders (Rao, Carlson, & Yan, 2014). But are these pathological phenotypes solely due to elevated levels of intracellular Ca^{2+}? Due to the complex nature of neuronal Ca^{2+}

signaling, and its gravity in physiological processes such as synaptic vesicle release, long term potentiation, and cell survival, it is nearly impossible to completely eliminate all sources contributing to global Ca^{2+} elevation. However, targeting specific sources of cytosolic Ca^{2+} influx would aid in understanding some of the mechanisms underlying neurodegeneration. Work presented in this dissertation suggests that inhibiting PIP_2 production ameliorates faulty Ca^{2+} homeostasis in α-Syn-dependent pathology, likely via a means of G_qPCR signaling modulation. Another source of Ca^{2+} entry into neurons are VGCCs. A myriad of studies investigated the clinically positive effect of various VGCC inhibitors on SN neuronal viability (Ilijic, Guzman, & Surmeier, 2011), decreases in oxidative stress (Guzman et al., 2018), and reduction of α-Syn-positive vesicles (Lautenschläger et al., 2018), among other pathophysiological consequences (Leandrou, Emmanouilidou, & Vekrellis, 2019). Taken together, it is apparent that Ca^{2+} is a key regulator of α-Syn-mediated pathology and that perturbations in Ca^{2+} homeostasis may elicit negative cellular effects. This highlights the molecular and cellular components that influence aberrant Ca^{2+} signaling and dyshomeostasis as prime therapeutic targets for PD.

5.2 Increased knowledge of the physiological role of α-Syn

One of the major limits in the advancement of α-Syn-targeted therapeutics for disease is the relatively unknown physiological role of the cytotoxic protein. As previously stated, α-Syn has been posited to play a critical role in vesicle trafficking, synaptic plasticity, and even neurodevelopment, indicating it is required for neuronal function and viability. Yet, there lacks a consensus in the field whether α-Syn ablation results in age-

related pathology and cognitive impairments (W. Dauer et al., 2002; Greten-Harrison et al., 2010; Kokhan, Afanasyeva, & Van'kin, 2012; Specht & Schoepfer, 2001). Nevertheless, regulation of α-Syn remains paramount for investigating and treating pathological progression of PD and other related diseases. This is apparent upon investigation of the molecular structure of α-Syn. Intrinsic folding of the protein is a fundamental phenotypic feature, ultimately determining its physiological versus pathological function. Classically, α-Syn was described to be a natively unfolded protein, only demonstrating alpha-helical conformation upon membrane association (Davidson et al., 1998). Yet multiple lines of evidence now suggest that monomeric α-Syn drives pathological phenotypes, while endogenously derived tetramers of the protein resist aggregation (Bartels et al., 2011; Dettmer et al., 2015; S. Kim et al., 2018). Indeed, rescue of *GBA1* depletion leads to an increased α-Syn tetramer to monomer ratio, which is indicated with reduced cellular toxicity *in vitro* and improved motor and cognitive deficits *in vivo* (Glajch et al., 2021; S. Kim et al., 2018). Further investigation of the tetrameric to monomeric α-Syn shift is still required for other disease-modifying components of PD, such as those involved in PPI metabolism and other lipid dynamics. However, preliminary data suggest that reduction of only deleterious monomeric species, and perhaps not complete ablation of the protein, could have significant therapeutic benefit.

6. Concluding remarks

In summary, α-Syn, which has essential neuronal function, is pathologically dysregulated in PD and other Lewy-related pathologies. I present multiple lines of evidence that upregulation and perturbed distribution of $PI(4,5)P_2$ is implicated in α-Syn-dependent neurotoxicity including PIP5K1γ-induced cytotoxic aggregation, amplified G_qPCR-mediated ER Ca^{2+} release, augmented IP_3R clustering, elevated PLK1 activity, pathological Ser-129 αSyn phosphorylation, VDAC1 localization, increased mitochondrial Ca^{2+} load, and subsequent oxidative stress. Finally, reduction of PIP_2 via catalytic inhibition of PIP5K1γ promotes cell viability of cultured primary cortical neurons treated with pre-formed αSyn fibrils. Taken together, these data suggest modulation of PPI metabolism should be considered seriously as a future therapeutic target in α-Syn-dependent disease treatment. While future investigation of PIP_2 dynamics *in vivo* is still required before initiation of clinical studies, preliminary observations from this work demonstrate this avenue as an intriguing prospect for combatting PD.

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