

## PERSPECTIVE OPEN

## Endogenous alpha-synuclein monomers, oligomers and resulting pathology: let's talk about the lipids in the room

Bryan A. Killinger<sup>1</sup>, Ronald Melki<sup>2</sup>, Patrik Brundin<sup>3</sup> and Jeffrey H. Kordower<sup>1\*</sup>

Alpha-synuclein is an intrinsically disordered, highly dynamic protein that pathogenically aggregates into inclusion structures called Lewy bodies, in several neurodegenerative diseases termed synucleinopathies. Despite its importance for understanding disease, the oligomerization status of alpha-synuclein in healthy cells remains unclear. Alpha-synuclein may exist predominantly as either a monomer or a variety of oligomers of different molecular weights. There is solid evidence to support both theories. Detection of apparent endogenous oligomers are intimately dependent on vesicle and lipid interactions. Here we consider the possibility that apparent endogenous alpha-synuclein oligomers are in fact conformations of membrane-bound alpha-synuclein and not a bona fide stable soluble species. This perspective posits that the formation of any alpha-synuclein oligomers within the cell is likely toxic and interconversion between monomer and oligomer is tightly controlled. This differs from the hypothesis that there is a continuum of endogenous non-toxic oligomers and they convert, through unclear mechanisms, to toxic oligomers. The distinction is important, because it clarifies the biological origin of synucleinopathy. We suggest that a monomer-only, lipid-centric view of endogenous alpha-synuclein aggregation can explain how alpha-synuclein pathology is triggered, and that the interactions between alpha-synuclein and lipids can represent a target for therapeutic intervention. This discussion is well-timed due to recent studies that show lipids are a significant component of Lewy pathology.

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## INTRODUCTION

Alpha-synuclein ( $\alpha$ Syn) is an intrinsically disordered, highly flexible protein, which plays an important role in the pathogenesis of several neurodegenerative diseases cumulatively referred to as synucleinopathies. In different synucleinopathies, neurons and/or glia bear the hallmark intracellular deposits of filamentous  $\alpha$ Syn<sup>1</sup> but the origin of this pathology remains unclear.  $\alpha$ Syn oligomers with  $\beta$ -sheet structure ( $\alpha$ SynO- $\beta$ ) are toxic to cells, possibly through physical disruption of cellular membranes.<sup>2,3</sup>  $\alpha$ SynO- $\beta$ /preformed fibrils (PFFs) generated in vitro or isolated from the brains of patients developing synucleinopathies can "seed" aggregates, especially in transgenic rodent models overexpressing  $\alpha$ Syn,<sup>4</sup> and initiate a toxic cascade reminiscent of that seen in disease.<sup>5–7</sup> However, not all oligomers are believed to be bad actors, as evidence suggests that various oligomers may not only exist in the cell but also have normal cellular functions.<sup>8–10</sup> Indeed, some conformers of  $\alpha$ SynO- $\beta$  do not seed pathology and are non-toxic.<sup>11–13</sup> Functional endogenous oligomers have been controversial, as their existence has been both confirmed and refuted by carefully executed studies.<sup>8,14,15</sup>

Are there benign  $\alpha$ Syn oligomers that have normal cellular functions, and if so, how do they transition to toxic  $\alpha$ SynO- $\beta$ ? Here, in this short review, we discuss how lipid- $\alpha$ Syn interactions might help explain the observation of apparent endogenous non-toxic oligomers and highlight alternative models that are monomer-centric. Furthermore, we will extend this viewpoint to consider its implications for synucleinopathy pathogenesis. There has been substantial work done in this area and several exhaustive reviews on lipid- $\alpha$ Syn interactions<sup>16–21</sup> and oligomerization<sup>22,23</sup> are available; hence, for the sake of clarity, we will not comprehensively discuss the literature.

## BIOLOGY OF LIPIDS IN NEURODEGENERATIVE DISEASE

The brain is ~60% lipids by weight.<sup>24</sup> Lipids have diverse cellular functions in biology including cell signaling, energy storage, and structural partitioning.<sup>25</sup> Phospholipids contain amphipathic characteristics with a charged hydrophilic phosphate group and a carbon chain of varying lengths.<sup>25</sup> Phospholipids spontaneously form bilayer structures in aqueous solutions that are the basis of cellular membranes. Lipids have not been as extensively studied as proteins in vivo, possibly because of their hydrophobicity, chemical complexity, and the fact that they are not gene products.<sup>25</sup> However, lipids are crucial for cellular function and are implicated in several neurodegenerative diseases including synucleinopathies.<sup>26</sup> Recent technological advances with lipidomic analysis have furthered the study of lipids.<sup>27–30</sup> Current lipidomic analyses, however, are focused on whole-cell lysates and therefore insensitive to cellular spatial and temporal dimensions, which are crucial for understanding lipid function.<sup>25</sup>

 $\alpha$ SYN-VESICLE MEMBRANE INTERACTIONS

Shortly following the discovery of  $\alpha$ Syn in Lewy pathology,<sup>31</sup> the lipid-binding properties of  $\alpha$ Syn were documented and the significance of disease-causing point mutations in lipid-binding domains was recognized.<sup>32</sup> Indeed, one of the earliest histochemical descriptions of Lewy bodies noted that they stain positively for phospholipids, particularly sphingomyelin.<sup>33</sup> Since then, interactions between  $\alpha$ Syn and vesicle lipids have been implicated in the initial generation of synucleinopathy.<sup>16–19,34–36</sup> The hypothesis that lipids control pathological  $\alpha$ Syn aggregation primarily stems from observations that lipids/vesicles influence aggregation kinetics in vitro,<sup>34,37–40</sup> and that disease-related missense

<sup>1</sup>Department of Neurological Sciences, Rush University Medical Center, Chicago, IL 60612, USA. <sup>2</sup>CEA and Laboratory of Neurodegenerative Diseases, Institut Francois Jacob (MIRGen), CNRS, 92265 Fontenay-Aux-Roses cedex, France. <sup>3</sup>Center for Neurodegenerative Science, Van Andel Research Institute, Grand Rapids, MI 49503, USA. \*email: [Jeffrey\\_Kordower@rush.edu](mailto:Jeffrey_Kordower@rush.edu)

mutations of *SNCA* alter residues within the N-terminal lipid-binding domain of *asyn*.<sup>41–46</sup> *asyn* may redistribute to lipid compartments early in disease pathogenesis.<sup>36</sup> Several disease-causing *asyn* mutants abnormally associate with intracellular vesicles and lipid droplets,<sup>47,48</sup> and dyshomeostasis of intracellular lipids are likely an early molecular event preceding pathology formation.<sup>49</sup> The key to pathogenesis lies within the lipid-binding domain of *asyn*.

*asyn* binding to vesicular membranes is important, because it influences oligomerization and pathological aggregation. Evidence suggests that non-pathological *asyn* is involved with vesicular dynamics in cells<sup>9,18,50–55</sup> and regulation of the presynaptic vesicle pool.<sup>56,57</sup> *asyn*–lipid interactions may have a vesicle tethering function. It has been proposed that the broken  $\alpha$ -helical N-terminus can function to tether intracellular vesicles via a “double-anchor” mechanism.<sup>58–61</sup> The biological significance of *asyn*-mediated clustering is unknown; however, it could serve to promote the exchange of lipids between adjacent vesicles<sup>62</sup> and possibly promote vesicle fusion.<sup>56–58,62–64</sup> *asyn* preferentially binds to membranes with lipid-packing defects<sup>65–68</sup> and high curvature.<sup>69–72</sup> In the neurons, *asyn* is densely clustered around intracellular vesicles and vesicular tubule structures, most prominently at the nerve terminal.<sup>73</sup> When incubated with small (~10–30 nm) unilamellar vesicles, the N-terminal of *asyn* adopts an extended  $\alpha$ -helical conformation as it coats the vesicular surface and a broken  $\alpha$ -helical conformation when interacting with micelles.<sup>59,74–80</sup> The interaction between the N-terminus of *asyn* with lipid membranes is driven by electrostatic interactions between positively charged residues and lipid phosphate head group.<sup>81</sup> When membrane bound, the N-terminus residues (1–26) of *asyn* rigidly bind to the membrane and the internal segment (residues 26–97) acts to sense lipid properties and regulates binding affinity.<sup>82</sup> Interestingly, the hydrophobic stretch of residues 71–82 are required for pathological aggregation of *asyn*<sup>83</sup> and, therefore, lipid-sensing properties of *asyn* and pathological aggregation occur through the same functional domain. It is not clear whether mutation of the N-terminus results in a toxic gain of function or loss of function.

Many cellular functions have been attributed to *asyn* and membrane interactions, including soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) complex assembly and exocytosis; however, the exact cellular function of *asyn* remains unclear.<sup>9,50,84</sup> *asyn* interacts with SNARE proteins at the vesicle surface.<sup>85–88</sup> *asyn* binding to membranes promotes SNARE complex formation and may function as a SNARE chaperone protein.<sup>9,88,89</sup> Vesicular membrane-binding promotes the oligomerization of *asyn*.<sup>9</sup> *In vitro* phospholipids can also increase the rate of pathological aggregation (i.e.,  $\beta$ -sheet confirmations) by decreasing lag time of primary nucleation.<sup>37,90</sup> The effect of lipids on *asyn* aggregation is dependent on lipid to protein ratio, with a low ratio promoting aggregation and higher ratio being inhibitory.<sup>74,91</sup> This bimodal phenomenon probably results from a lack of monomer available for oligomer elongation when the lipid ratio is too high. Interestingly, increasing *asyn* expression, presumably shifting the intracellular lipid to protein ratio, promotes aggregation of *asyn* in cells. Notably, it has been hypothesized that reducing monomeric *asyn* is an important therapeutic target.<sup>92–94</sup>

A confusing aspect to the literature is that binding of *asyn* to membranes has been reported to both inhibit<sup>95–97</sup> and to promote *asyn* aggregation.<sup>39,47</sup> This may be due to differences in assay conditions between studies, such as membrane lipid composition and *asyn* concentration. Indeed, recent studies using lipidomics implicated specific fatty acid oleic acid in the pathogenesis of Parkinson's disease.<sup>49</sup> In the model proposed by Fanning and colleagues<sup>49</sup>, soluble *asyn* binds to oleic acid, effectively sequestering the monomer to lipid membranes and ultimately culminating in pathological aggregate formation. As

they also observed an increase in oleic acid in response to *asyn* overexpression, there may be a toxic lipid dyshomeostasis that precedes aggregate formation. Their results suggest a complex origin of synucleinopathy where both lipid metabolism and *asyn* are central players.

*asyn* may have a more generalized cellular function as an effector of lipid dynamics, and not as a factor of a specific subprocess or pathway. To highlight this concept, consider the curious relationship between *asyn* and erythropoiesis (i.e., red blood cell differentiation) for which others have hypothesized *asyn* that may have an underlying redundant mechanism in the two cells of different lineage.<sup>98</sup> *asyn* is highly expressed in erythrocytes under the control of transcription factor GATA1.<sup>99,100</sup> During the terminal step of erythropoiesis, *asyn* expression dramatically increases and remains elevated in the mature erythrocyte.<sup>98</sup> *asyn* is then found associated with phospholipids and vesicle membranes in the mature erythrocyte.<sup>101</sup> Thus, which of the proposed cellular functions does *asyn* perform during erythropoiesis? One likely explanation is that *asyn* plays a role in the dramatic intracellular lipid organization, analogous to asymmetric cytokinesis, which occurs prior to the phenomenon of enucleation. Enucleation is the process by which organelles are condensed and extruded from the cell to form a mature erythrocyte. Indeed, just prior to enucleation *asyn* can be found associated with lipids of the cell, particularly the nucleus and ER, which are key players in enucleation. *asyn* accumulates at the site of nuclear extrusion,<sup>102</sup> suggesting it is directly involved with enucleation lipid dynamics. Concurrently, SNARE machinery is decreased in the erythrocyte, suggesting that the potential involvement of *asyn* in lipid dynamics during enucleation is independent of hypothesized SNARE functions.<sup>103</sup> However, if *asyn* is involved in this cellular phenomenon, it is non-essential or interchangeable with beta or gamma synucleins, as only minor phenotypic abnormalities of erythrocytes are observed in *asyn*-knockout models.<sup>99</sup>

## SOLUBLE OLIGOMERS DEVOID OF LIPID

There is good evidence of a naturally occurring metastable soluble *asyn* oligomer (i.e., tetramer) that is devoid of vesicle/lipid binding.<sup>8</sup> However, the existence of a soluble *asyn* tetramer is based mostly on results from crosslinking experiments.<sup>8,48,104–106</sup> The interpretation of crosslinking experiments is non-trivial. *asyn* tetramers are captured when using a permissive chemical crosslinker with spacer arm length (DSG spacer arm length 7.7 Å) and perhaps not with a shorter spacer arm (formalin spacer arm length ~2 Å).<sup>107</sup> A milieu of progressively larger oligomers are formed and captured even when purified recombinant *asyn* is incubated with glutaraldehyde.<sup>108</sup> The successful detection of an *asyn* tetramer in tissues and cells is dependent on sample preparation conditions and can be detected when cells remain intact prior to crosslinking or when tissue lysates are kept highly concentrated.<sup>107</sup> Indeed, purification of *asyn* prevents the detection of a soluble tetramer further suggesting a cofactor is required and this factor is likely of lipid origin.<sup>109</sup> Although the question remains which lipid cofactor might be responsible for the tetramer formation, the tetramer and *asyn*–lipid interactions are inextricably linked. This is highlighted when recently a transgenic mouse model (called “3K”) of tetramer deficiency was generated by introducing 3E->K mutations in *asyn*'s lipid-binding N-terminus.<sup>48</sup> These 3K mice exhibit aggressive *asyn* aggregation, loss of an apparent tetramer, and a motor phenotype that has some semblance to Parkinson's disease. The lipid-binding domain was mutated in the 3K mice and correspondingly lipid interactions were enhanced<sup>48</sup> and similar to what was observed with similar mutations in cells.<sup>110</sup> In both mice and cells, mutated 3K *asyn* clustered around vesicles and intact tissue crosslinking captured less soluble tetramer.<sup>48,106,110</sup> Was this due to less tetramer or alternatively less soluble tetramer? The results

could be explained either way, but if the captured  $\alpha$ syn species is truly an insoluble tetramer, one would expect less detection in the soluble fraction, as the authors observed. Indeed, the results appear to fit a scenario where folding on the vesicular membrane is driving  $\alpha$ syn pathology without the need for a soluble tetramer. (Fig. 1)

Detection of endogenous oligomers, including the tetramer, could be explained by compartmentalized  $\alpha$ syn residing on the vesical membrane (Fig. 1). Membrane interactions occur through electrostatic forces between positively charged lysine residues of  $\alpha$ syn and negatively charged phosphate group of lipids.<sup>41,75,81,111</sup> Interestingly, the same crosslinking chemicals used to capture tetramers chemically modify lysine side chains of  $\alpha$ syn and neutralize their charge.<sup>112</sup> Chemically modifying the lysine side chains during tissue crosslinking would presumably disrupt membrane binding, because it neutralizes the required electrostatic charge of lysine residues. Therefore, captured oligomers would dissociate from the membrane and would be detected in the soluble fraction, producing the characteristic gel-shift of the  $\alpha$ syn tetramer.<sup>106</sup> To highlight this concept, the neutralization of  $\alpha$ syn charge by chemical cross-linkers is routinely used to enhance the retention of  $\alpha$ syn on polyvinylidene difluoride membranes during western blotting protocols.<sup>112</sup> Together, adjacent  $\alpha$ syn molecules bound to intracellular vesicle surfaces (i.e., compartmentalized) might disassociate into the soluble fraction once chemically modified by the crosslinking reagent. This phenomenon would also help explain why the soluble tetramer has similar intermolecular n-terminal structure as the membrane-bound form.

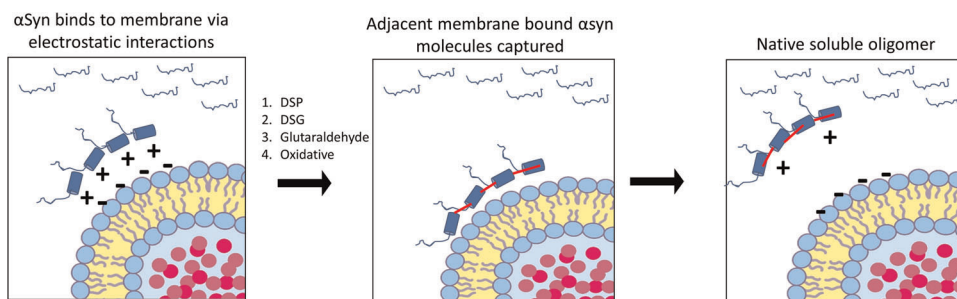
The apparent soluble tetramer may be stabilized by covalent bonds formed in the oxidative environment of the erythrocyte (i.e., the source from which it was originally isolated). Biochemical characterization of a putative  $\alpha$ syn tetramer was mostly done using erythrocyte derived  $\alpha$ syn.<sup>8</sup> Erythrocytes have millimolar concentrations of hemoglobin. Hemoglobin oxidatively catalyzes the formation of intramolecular dityrosine bonds resulting in a mixture of  $\alpha$ syn dimers and tetramers.<sup>113</sup> Dityrosine crosslink formation occurs rapidly<sup>113,114</sup> and would likely occur to some extent during  $\alpha$ syn purification from erythrocytes.  $\alpha$ Syn in erythrocytes associates with vesicles<sup>101,102</sup> and stable dityrosine  $\alpha$ syn occurs in clinical blood samples.<sup>115</sup> Together, it is probable that stable  $\alpha$ syn oligomers isolated from erythrocytes are due to oxidative crosslinking of adjacent  $\alpha$ syn molecules bound to vesicle membranes. Heat denaturation irreversibly abolished the tetramers  $\alpha$ -helix structure, indicating that the captured configuration was not in equilibrium but instead was a stabilized structure

originating from the tissue (i.e., vesicle bound). Lipid binding of the tetramer was enhanced when compared with the monomer, further suggesting it retained a lipid-binding confirmation.<sup>8</sup> Removal of lipids with Lipodex 1000 did not affect the tetramer detection, suggesting the tetramer was not associated with any stabilizing lipid structure. Stabilization of small oligomers via oxidative crosslinking can prevent progressive aggregation and might explain why the stable tetramer appears to resist aggregation.<sup>8,116</sup> Together, it is likely to be that the stable soluble  $\alpha$ syn tetramer purified from erythrocyte is a covalently stabilized membrane-bound confirmation similar to that captured using exogenous crosslinking agents.

Soluble  $\alpha$ syn oligomers have been detected using several imaging techniques. Förster resonance energy transfer (FRET) is a powerful technique used to determine intermolecular distances between molecules with 1–2 nm spatial resolution.<sup>117</sup> FRET has been employed to study various aspects of  $\alpha$ syn oligomerization mostly in vitro<sup>9,77,118–120</sup> but also ex vivo<sup>121</sup> and in vivo.<sup>122</sup> In vitro, purified  $\alpha$ syn forms distinct oligomer conformers, which then can spontaneously convert to protease resistant and toxic  $\alpha$ synO- $\beta$ .<sup>12</sup> Biomolecular fluorescence complementation (BiFC) technique uses fluorescent constructs to determine protein–protein interaction. BiFC constructs have been used to study  $\alpha$ syn aggregation in vivo,<sup>123,124</sup> however, the resolution of this technique cannot differentiate between small oligomers and complex formation (i.e., membrane bound). The method detects diffuse staining in neurons lacking pathology, suggesting either small aggregates or close association of  $\alpha$ syn molecules normally within the cytosol.<sup>124</sup>  $\alpha$ Syn comes into close proximity around synaptic vesicles and possibly forms multimers on the membrane.<sup>15</sup> Other BiFC techniques employing photoactivatable fluorescent molecules can increase the spatial resolution to several nanometers,<sup>125</sup> but this type of imaging has yet to be done with  $\alpha$ syn.

### SOLUBLE DISORDERED MONOMER DEVOID OF LIPID

There is also good evidence that  $\alpha$ syn exists predominantly as an intrinsically disordered monomer in the cytosol.<sup>10,14,126–130</sup>  $\alpha$ Syn purified from *Escherichia coli* behaves as an intrinsically disordered protein with a large stokes radius,<sup>129</sup> which may be why monomeric  $\alpha$ syn appears to have greater mass in some assays.<sup>126</sup> Non-denaturing purification procedures from several tissue sources also produce a disordered monomeric  $\alpha$ syn.<sup>126</sup> A disordered soluble monomer has been observed directly using in-cell nuclear magnetic resonance (NMR) imaging techniques.<sup>14</sup>



**Fig. 1** Endogenous soluble oligomers are inextricably associated with lipid/vesicle binding. Depicted is a possible explanation for the detection of a soluble  $\alpha$ syn oligomer. In the cytoplasm,  $\alpha$ syn exists in an equilibrium between a disordered slightly compact monomer and membrane-bound  $\alpha$ -helix confirmation. The N-terminus of  $\alpha$ syn binds to vesicle membranes via electrostatic interactions and adopts an  $\alpha$ -helix structure.  $\alpha$ Syn most likely binds to localized areas of vesicle surfaces with lipid-packing defects. Normally, in the cell ~5–10% of  $\alpha$ syn is interacting with vesicle surfaces. The same percentage is also proposed for soluble oligomers. Covalent bonds between adjacent  $\alpha$ syn molecules capture the confirmations bound to the vesicle surface. Covalent modification of amino-acid residue side chains, especially lysine, following chemical crosslinking neutralizes a portion of  $\alpha$ syn charge required for membrane binding. The captured species could then retain the membrane-bound confirmation and enter the aqueous phase for subsequent detection. Thus, endogenous soluble functional oligomers are unlikely, in agreement with several studies. Instead, endogenous oligomers may represent confirmations of membrane-bound  $\alpha$ syn. This hypothesis makes lipid-syn interactions at the membrane a crucial mediator of pathology initiation. DSP dithiobis(succinimidyl propionate), DSG disuccinimidyl glutarate



Specifically, investigators transfected cells with recombinant asyn labeled with  $^{15}\text{N}$  isotope to monitor individual asyn molecules within the living cells. Results showed that the majority of monomeric asyn maintained a disordered conformation in the cell, while becoming slightly more compact than in free solution, probably due to molecular crowding.<sup>14</sup> The compact structure observed *in vivo* likely prevents spontaneous aggregation in the cytosol.<sup>131</sup> Although this is compelling evidence that the majority of asyn in the cell occurs as a disordered monomer, the result does not rule out the existence of a tetramer. A tetramer that existed at low concentration would not be detected and it is possible that the recombinant asyn behaved dissimilarly to endogenous asyn. Importantly, this study demonstrated that the majority of asyn in the cell is cytosolic and monomeric, and suggests that membrane interactions are likely transient and highly dependent on local environment (e.g., nerve terminal). Correspondingly, it would be interesting if asyn persists as a monomer at axon terminals where its vesicle interactions are more prominent than in the cell body.<sup>73,132</sup> The  $\alpha$ -helix conformation asyn was recently described in HELA cells using FRET, where it was demonstrated that asyn assumes several conformations when interacting with vesicle surfaces.<sup>133</sup> Considered together, the majority of asyn in the cell exists as a relatively compact disordered monomer and adopts an  $\alpha$ -helix structure when interacting with vesicle membranes. The native state of asyn may not include an oligomer, whether free and soluble, or vesicle bound.

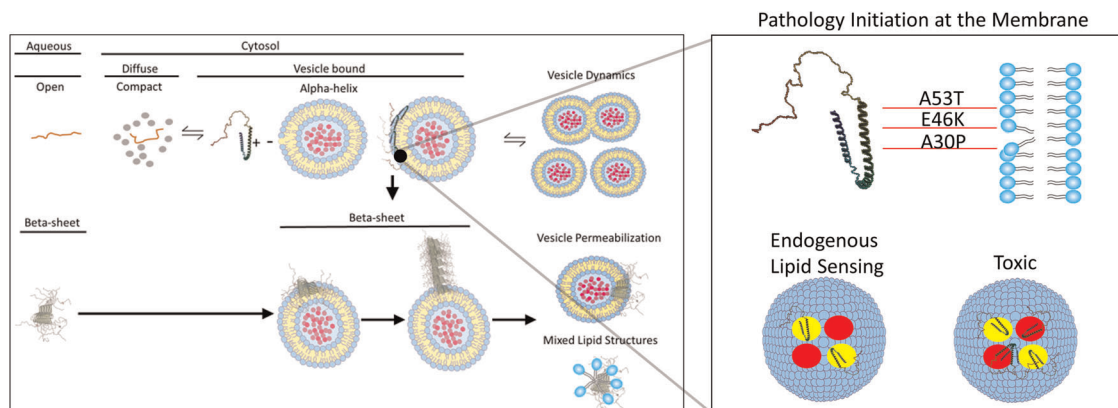
### ABERRANT VESICLE BINDING PROGRESSES TO PATHOLOGY

Assuming monomeric asyn is interacting with vesicle membranes, and remains monomeric at the vesicle surface under normal circumstances, how might pathology begin? (Fig. 2). One possible scenario involves vesicle surfaces acting as two-dimensional (2D) reactors that promote pathogenic intermolecular interactions of asyn.<sup>91,134–136</sup> In the cytosol, asyn remains monomeric and in a slightly compact configuration. Transient interactions with vesicle surfaces induce a conformational shift, but not necessarily oligomerization, and concentrate asyn molecules on the vesicle surface. This focal point on the vesicle surface is where opposing asyn molecules bind and might serve as the molecular origins for Lewy pathology. Numerous cellular and genetic factors converge

at this focal point in such a way that creates an environment conducive for the initiation of pathogenic asyn aggregation. Studies using sonicated asyn PFFs suggest that once the asynO- $\beta$  is present, progressive aggregation and toxicity follow.<sup>7,137</sup> Yet, studies that utilize PFF's to assess pathology are bypassing pathology generation and may be recapitulating downstream pathological events. Therefore, the use of PFF's to study synucleinopathy is likely to give valuable insight into the progression of these diseases and perhaps are not suitable to study the initiation of the disease.

At the membrane, asynO- $\beta$  might act similar to a “molecular shovel” inserting itself into the membrane with destructive, toxic, consequences.<sup>2,138</sup> Together with lipid/vesicle interactions at the center of asyn function, a route to pathophysiology might be the collapse of clustered lipids/vesicles into a pathological inclusion. Similar to a massive star transitioning to a black hole, at some point pathological asyn and lipids form a critically dense structure, and compact to form a Lewy body. Interestingly, the architecture of the Lewy pathology supports this interpretation.<sup>139–144</sup> Neuropathological examination of patient brains shows a mixture of asyn staining in neurons that consist of a pale diffuse, punctate irregular shape (i.e., uneven distribution), discrete body (i.e., pale body), and a massive dense structure with a pale core (i.e., Lewy body).<sup>139</sup> These structures have been hypothesized to be snapshots of a pathological process with Lewy bodies being the result. Indeed, pale bodies contain a mixture of granular and vesicular structures and are often found near mature LB.<sup>141,145</sup> Using a lipid centric view, punctate irregular “early” pathology might represent the initial vesicle clustering<sup>48</sup> or lipid droplet formation<sup>47</sup> before the characteristic pathology develops. Overexpression of asyn in yeast models produces lipid only inclusions, lacking the filamentous asyn that is the hallmark of synucleinopathies.<sup>146</sup> Lipids have been identified as a core component of Lewy pathology<sup>33,147,148</sup> but this has largely been ignored and the pathology is often considered “proteinaceous.”

Recent work by Shahmoradian and colleagues<sup>149</sup> provided substantial evidence that Lewy pathology consists of compacted lipid components from a variety of organelles with asyn oligomers interspersed. Their work strongly suggests that Lewy pathology is actually an inclusion of fragmented lipids, for which asyn–lipid interactions play a causative role. Ultrastructural characterization of Lewy pathology showed tubule vesicular, fragmented



**Fig. 2** Potential role of lipids in  $\alpha$ syn aggregate pathoetiology. In the cell,  $\alpha$ syn is partitioned between aqueous phase and the lipid phase via transient interactions at the vesicle surface. Endogenous  $\alpha$ syn probably exists in several states, including a compact monomer and a vesicle-bound monomer with an N-terminal  $\alpha$ -helix structure. Folding  $\alpha$ syn monomers on the vesicle surfaces likely plays a non-essential or redundant role in vesicle dynamics.  $\beta$ -Sheet confirmation of  $\alpha$ syn may begin at vesicle surfaces. Toxic effects of  $\beta$ -sheet oligomers included vesicle permeabilization or the formation of toxic mixed lipid–protein structures. Pathology initiation might involve specific configurations of  $\alpha$ syn folding onto a variety of membranes. Altered lipid-sensing properties by known disease-causing mutations (e.g., A30P, E46K, and A53T) might alter the affinity of  $\alpha$ syn for certain vesicle lipid components (depicted as yellow and red circles), or change the spatial arrangement of  $\alpha$ syn molecules on the vesicle surface. Resulting  $\beta$ -sheet oligomers may have different toxic or prion-like properties based the physicochemical details of the initial pathology development

membranous, and mixed lipid–protein structures, all of which can be formed from asyn interactions with vesicle membranes. Electron dense structures, consistent with lysosomes, were also observed throughout Lewy pathology.<sup>149</sup> Lysosomes are central mediators of lipid metabolism<sup>150</sup> and the conspicuous presence of lysosomes surrounded by fragmented membrane structures strongly suggests a deficit in lysosomal/autophagic pathways, specifically the removal of lipid membranes. Large-scale genome-wide association studies have implicated lysosomal/autophagy pathway in several neurodegenerative diseases, including synucleinopathies.<sup>151,152</sup>

A lipid-centric view of Lewy pathology is transformative in that it helps unify and identify disease-causing pathology of several molecular origins. Several neurodegenerative diseases are currently characterized by protein aggregation, when instead we may be missing the lipid components that are the core of the pathology. For example, clinical cases resembling synucleinopathies are documented without the presence of Lewy pathology (e.g., Parkin mutations with early-onset Parkinson's disease), as measured by asyn staining.<sup>153</sup> The presence of lipid inclusions in the absence of asyn are not generally considered when examining patient tissues.

#### DETERMINING $\alpha$ SYN–LIPIDS INTERACTIONS IN LIVING CELLS

$\alpha$ Syn interactions with lipids and vesicles has been investigated mostly in vitro and needs to be characterized in living cells. There are several promising strategies to start understanding lipid–asyn interactions. The first strategy uses synthetic bifunctional lipids to directly determine lipid–protein interactions.<sup>154,155</sup> This strategy offers flexibility with analysis and offers unambiguous evidence of direct asyn–lipid interactions in vivo. Captured lipid–asyn molecules can be subsequently labeled or purified for downstream analysis. Labeling the structures will help determine where asyn–lipid interactions are most relevant in the cell. Purification of the structures with subsequent analysis by liquid chromatography–mass spectrometry could determine specific asyn proteoforms involved with pathological lipid interactions, as well as global analysis of other proteins that are involved. However, the drawback to this strategy is the investigator can only assess one specific lipid species at a time and a synthetic bifunctional lipid must be available or developed for application. Recently, a bifunctional analog of glucosylceramide, a lipid implicated in synucleinopathy,<sup>156</sup> has become commercially available and could aid in these studies.

A shotgun lipidomic analysis may also be useful, but because of the complexity of whole-cell lipid determination, the data may not give insight into the localized asyn–lipid interactions that precede pathology formation. Recently, a shotgun lipidomic analysis was conducted on various asyn mutant models and asyn was found to have an effect on lipid metabolism.<sup>49</sup> However, it is difficult to draw distinct conclusions or find drug targets based on the description of a total lipid species. The resulting information is most useful in implicating lipid metabolism or catabolism pathways, and not the characterization of the specific localized lipids that may be involved with initiating pathology. To find a disease-relevant target, a focused lipidomic approach looking at specific organelles, or better yet, early Lewy pathology, will be the most illuminating. Lipidomic arrays can also be used to screen many lipid–protein interactions; however, they have the disadvantage of not representing in vivo binding conditions.

A key question remains: at what point during its interaction with cytoplasmic membranes and extracellular vesicles does asyn adopt a pathological confirmation? To answer this question, one needs to consider the membrane as a chemical reactor favoring molecular encounters.<sup>134</sup> This is the consequence that restrains monomeric or low-molecular-weight oligomeric asyn diffusion

from a three-dimensional to a 2D space upon interaction with the plasma membrane or extracellular vesicles plane.

#### CONCLUSIONS

Evidence for a soluble asyn oligomer might be best explained by folding intermediates on the plasma or vesicle membranes that remain soluble for subsequent extraction and detection. This interpretation does not require a soluble functional oligomer and seems to fit much of the experimental data. The distinction between a soluble native oligomer and vesicle-bound oligomers/folding intermediates is important, because it clarifies the origins of pathological aggregation of asyn. With this perspective, determining the molecular details of asyn–vesicle/lipid interactions is important for understanding the endogenous origins of synucleinopathy. Although there is consensus that aggregation of asyn is associated with neurological disease, the precise molecular origin of the aggregate pathology remains a mystery.

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## AUTHOR CONTRIBUTIONS

B.A.K. and J.H.K. conceived and prepared the manuscript. R.M. and P.B. provided valuable discussion, as well as reviewed and edited the manuscript.

## COMPETING INTERESTS

P.B. has received commercial support as a consultant from Axial Biotherapeutics, CuraSen, Fujifilm-Cellular Dynamics International, IOS Press Partners, LifeSci Capital, and Living Cell Technologies Ltd. He has received commercial support for grants/research from Lundbeck A/S and Roche. He has ownership interests in Acousort AB and Axial Biotherapeutics and is on the steering committee of the NILO-PD trial. J.H.K. has received commercial support as a consultant from Cellular Dynamics International, Inc., Michael J. Fox Foundation, Abbvie, Exicure, NSGENE, Guidepoint, Inhibikase, Axovant, and Seelos. B.A.K. and R.M. have no competing interests to disclose.

## ADDITIONAL INFORMATION

**Correspondence** and requests for materials should be addressed to J.H.K.

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