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# $\alpha$ -Synuclein and biological membranes: the danger of loving too much

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The aberrant aggregation of  $\alpha$ -Synuclein ( $\alpha$ S), a disordered protein primarily localised at the neuronal synapses, is associated with a number of neurodegenerative disorders including Parkinson's disease (PD). The biological properties of  $\alpha$ S are strictly connected with its ability to bind synaptic membranes under both physiological and pathological conditions. Here we overview the recent studies on the structural and biological properties of the membrane interaction by  $\alpha$ S. The characterisation of this state is particularly challenging as the membrane binding of  $\alpha$ S is weak, transient and features a considerable degree of conformational disorder. Advancements in this area have been achieved through combinations of nuclear magnetic resonance (NMR), super-resolution microscopy, cryo-EM and cellular biophysics. Current data clarified the central role of the equilibrium between ordered and disordered states of  $\alpha$ S at the membrane surface, which regulates the membrane affinity, the aggregation into amyloid fibrils and the promotion of vesicle clustering. Recent results on toxic oligomeric species of  $\alpha$ S also revealed common features in the membrane interaction of functional and aberrant forms of this protein. These findings therefore evidence the challenging nature of identifying suitable therapeutics to target the aberrant aggregation of  $\alpha$ S in PD while leaving its normal physiological form unperturbed.

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## 1. Introduction

$\alpha$ S is a 14 kDa protein that is located predominantly at the presynaptic terminals and whose aggregation is associated with Parkinson's disease (PD).<sup>1–8</sup> Fibrillar aggregates of  $\alpha$ S have been identified as the major constituents of intraneuronal inclusions, known as Lewy bodies, that form in dopaminergic neurons of PD patients. Genetic traits also exist linking  $\alpha$ S and familial forms of early onset PD, including missense mutations as well as duplications or triplications of the  $\alpha$ S encoding gene (SNCA).<sup>9,10</sup> The aberrant aggregation of  $\alpha$ S is also associated with other neurodegenerative disorders such as dementia with Lewy bodies and multiple system atrophy,<sup>2,6</sup> whereas aggregates composed of the non-amyloid- $\beta$  component (NAC) region of the protein (residues 61–95) have also been found in association with Alzheimer's disease (AD).<sup>11</sup>

Despite the general consensus on the pathological relevance of  $\alpha$ S aggregation, the functional role of this protein remains highly debated.<sup>12</sup> Its prevalence at the presynaptic terminals suggests an involvement in the process of neurotransmission, with evidences pointing at a possible involvement in synaptic plasticity<sup>13</sup> and learning.<sup>14</sup> A consensus is gradually emerging

on a putative role of  $\alpha$ S in the regulation of the homeostasis of synaptic vesicles (SVs),<sup>15–20</sup> particularly in contexts where intense neuronal activity is required.<sup>21</sup> Additional studies have indicated the possibility of other functions of  $\alpha$ S, including a regulator of pools of SVs,<sup>22,23</sup> an inhibitor of the ER-to-Golgi vesicle trafficking,<sup>19,24</sup> and a binder of mitochondria that mitigates oxidative stress.<sup>25–28</sup> While these biochemical processes are fundamentally different, they share a common characteristic, namely the binding to cellular membranes as a critical step for  $\alpha$ S to attain biological activity. It is indeed becoming increasingly evident that the interaction with membranes is critical for the physiological behaviour of  $\alpha$ S.<sup>29</sup> Membrane binding of  $\alpha$ S appears to be tightly regulated *in vivo*<sup>30</sup> and has a relevant role also in the context of  $\alpha$ S aggregation and for the toxicity of its aggregates.<sup>1,31,32</sup>

In this feature article, we first summarize the current structural knowledge about the mechanism of membrane binding by  $\alpha$ S. We then discuss the current understanding of the biological relevance of the membrane interaction in  $\alpha$ S monomers and the aberrant aggregates (Fig. 1).

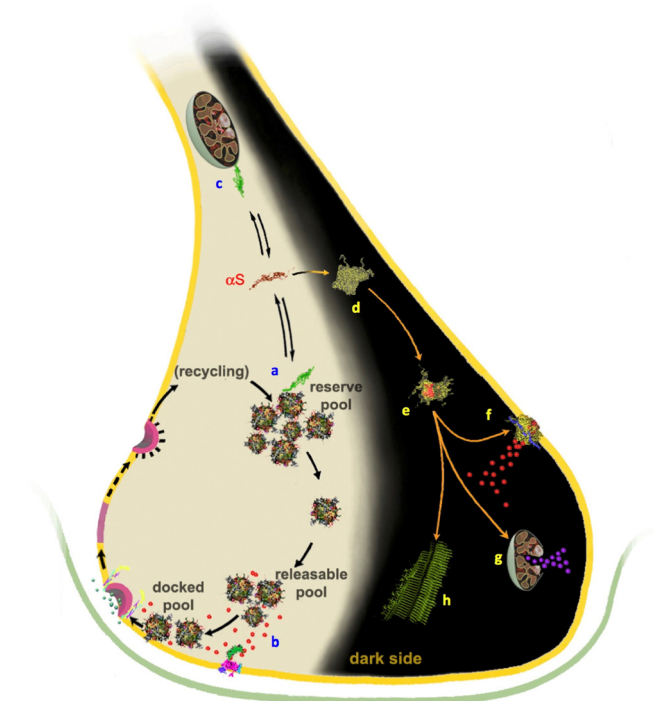
## 2. Conformational pathways of $\alpha$ S-membrane interaction

In its cytosolic form,  $\alpha$ S is monomeric and disordered.<sup>33,34</sup> Nuclear magnetic resonance (NMR) studies in aqueous

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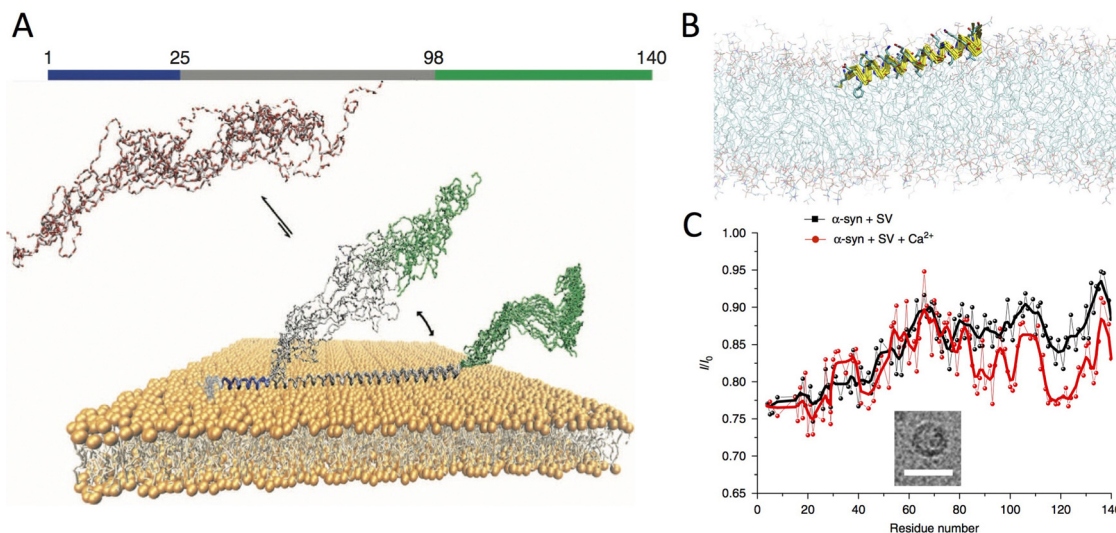
**Fig. 1** Membrane interactions of  $\alpha$ S in functional and pathological contexts. Under physiological conditions (left side of the synapse),  $\alpha$ S has been shown to interact with SVs and promote their clustering<sup>18–20</sup> into SV pools forming at the synaptic termini<sup>17,22–24</sup> (a). In addition, in the active zone  $\alpha$ S has been associated to a chaperone role of the SNARE formation<sup>15,16</sup> (b).  $\alpha$ S also binds to mitochondria where it has been associated with the mitigation of oxidative stress (c). Under aggregation-prone conditions (right “dark side” of the synapse),  $\alpha$ S is believed to aggregate first in amorphous early aggregates (d). Subsequently, these evolve into pre-fibrillar oligomeric species (e) that diffuse in the cellular milieu and establish aberrant interactions affecting the cellular viability.<sup>1</sup> The toxic oligomers are able to bind and disrupt the plasma membrane (f), causing calcium influx and metal dishomeostasis, and to disrupt the mitochondrial integrity (g) by provoking the release of cytochrome C. These oligomers ultimately evolve into mature fibrils, which have been shown to be the major constituents of Lewy bodies. Several literature evidences indicate that  $\alpha$ S fibrils are considerably less neurotoxic than oligomeric species,<sup>1</sup> however, mature amyloids can still act as pathogenic agents by acting as reservoirs of toxic oligomers that are released as a result of fragmentation.<sup>139</sup>

solution<sup>35,36</sup> and in the cellular milieu<sup>34</sup> indicated that this protein lacks any significant secondary and tertiary structure in its cytosolic state. Despite featuring a general structural disorder,  $\alpha$ S is unusually compact with respect to other intrinsically disordered proteins (IDP) as shown in NMR,<sup>37</sup> EPR<sup>38</sup> and MD<sup>39</sup> studies. This observation reflects the peculiar sequence of  $\alpha$ S, where only the C-terminal region (residues 98–140) presents the typical characteristics of IDPs, whereas the N-terminal 97 residues show some levels of propensity toward structural order.<sup>29</sup> This asymmetry in the  $\alpha$ S sequence is maintained in the membrane binding mechanism, which is driven by a disorder-to-order transition in the N-terminal region acquiring a character of an amphipathic  $\alpha$ -helix at the surface of lipid bilayers. The conformational switch from a purely disordered cytosolic monomer to a partially helical state is promoted by

7 imperfect sequence repeats of 11 residues located in the N-terminal region of  $\alpha$ S and encoding for amphipathic class A2 lipid-binding segments.<sup>35,40–42</sup> It was shown that these acquired helical segments of  $\alpha$ S lay on the membrane surface upon binding (Fig. 2).<sup>43</sup> The presence of multiple repeat modules in the membrane binding domain confers  $\alpha$ S the plasticity to interact with a variety of assemblies, including detergent micelles, small vesicles and planar lipid bilayers.<sup>40,41,44,45</sup> Upon interaction with detergent micelles,  $\alpha$ S was found to fold into a broken  $\alpha$ -helix composed of two helical segments (residues 3–37 and 45–92), with the C-terminal region remaining essentially disordered and not associated with the detergents. By contrast, EPR studies identified a single extended helix spanning the first 97 residues upon membrane binding.<sup>46</sup> Both topologies have in fact been found in association with detergent micelles or lipid membranes.<sup>44,47–50</sup> Using solid-state NMR (ssNMR) we clarified that, beyond the topological and structural properties, it is the dynamical nature of membrane-bound  $\alpha$ S to play a critical biological role.<sup>51</sup> Even upon membrane binding,  $\alpha$ S indeed retains a significant level of structural disorder with some regions existing in equilibrium between ordered-bound and disordered-unbound conformations. More specifically, the  $\alpha$ S sequence can be divided into three regions having distinct structural and dynamical properties when bound to the surface of lipid bilayers<sup>51</sup> (Fig. 2A). These regions have different roles for the membrane interaction, and influence in individual ways the thermodynamics and kinetics of the binding.<sup>52</sup>

The primary region of  $\alpha$ S to foster membrane binding is the N-terminus (residues 1 to 25), which acts as an anchor promoting the interaction with lipid bilayers (Fig. 2B).<sup>51</sup> In association with membranes, this region folds into an ideal amphipathic  $\alpha$ -helix characterised by a hydrophobic surface opposed to a lysine-rich hydrophilic surface. The ability to strongly anchor  $\alpha$ S to the membrane was associated to a partial insertion of the N-terminal 12 residues into the hydrophobic region of the lipid bilayer (Fig. 2B).<sup>43</sup> Moreover, the N-terminal acetylation of  $\alpha$ S has been shown to considerably increase the local membrane affinity of the protein,<sup>35</sup> and our studies clarified that this effect is not associated with topological-structural alterations of the membrane-bound state,<sup>53</sup> but likely arises from an enhanced helical character in the cytosolic state.<sup>35</sup> Following the N-terminal anchor, the central region (residues 26–97) was found to modulate the overall membrane affinity of  $\alpha$ S by acting as a “sensor” of the properties of the lipid bilayer.<sup>51</sup> This region, which includes the aggregation-prone non amyloid- $\beta$  component (NAC, residues 60–95), was found to exist in equilibrium between detached and membrane bound states, with the latter adopting an helical structure as indicated by EPR<sup>44,46</sup> and NMR<sup>40</sup> data. Enhanced coarse-grained simulations revealed that N-terminal and central regions of  $\alpha$ S have different modes of membrane binding. In the N-terminal region, a binding step through tethered-extended conformations is energetically possible, however, it is the folding into an amphipathic  $\alpha$ -helical structure to critically strengthen and “lock” the membrane interaction.<sup>54</sup> By contrast, in the central





**Fig. 2** Structural bases of  $\alpha$ S-membrane interactions. (A) The membrane bound state of  $\alpha$ S features three regions having distinctive structural and dynamical properties. These include a rigid N-terminal anchor (residues 1–25, blue), adopting the structure of an amphipathic helix, a central region (residues 26–98, grey) being in conformational equilibrium between membrane-tethered and membrane-detached states, and a C-terminal region (residues 99–140, green) remaining substantially disordered and poorly associated with the membrane. Adapted from ref. 51, with permission from Nature Publishing Group, copyright 2014. (B) The membrane-anchor was shown to adopt a topology where the amphipathic  $\alpha$ -helix lays parallel on the membrane surface by partially inserting the first 12 residues in the hydrophobic interior of the lipid bilayer. Adapted from ref. 43, with permission from Nature Publishing Group, copyright 2016. (C) Binding of calcium ions was shown to alter the modes of interaction with *ex vivo* SVs, as probed by NMR. A cryo-EM image of the SV is inserted in the CEST NMR plot (scale bar 50 nm). Adapted from ref. 60, with permission from Nature Publishing Group, copyright 2018.

region the energetics of disordered-tethered and helical-locked conformations are more similar, particularly in the segment 87–97 where these two binding modes have the same energy.<sup>55</sup> These fundamental characteristics have implications for the ability of a single  $\alpha$ S molecule to simultaneously bind multiple membranes (described below). Finally, the last portion of the  $\alpha$ S sequence, the highly negatively charged and proline-rich C-terminal region (residues 98–140), remains primarily disordered and poorly associated with lipid bilayers<sup>51</sup> or detergent micelles.<sup>41,56</sup> Using ssNMR in conjunction with paramagnetic relaxation enhancement (PRE), we observed that the C-terminal region establishes weak and transient interactions with the membrane surface,<sup>57</sup> a property that might have functional relevance in influencing protein–protein interactions, such as for example those established with synaptobrevin-2 upon SV binding.<sup>16,20</sup>

### 3. $\alpha$ S binding to synaptic vesicles

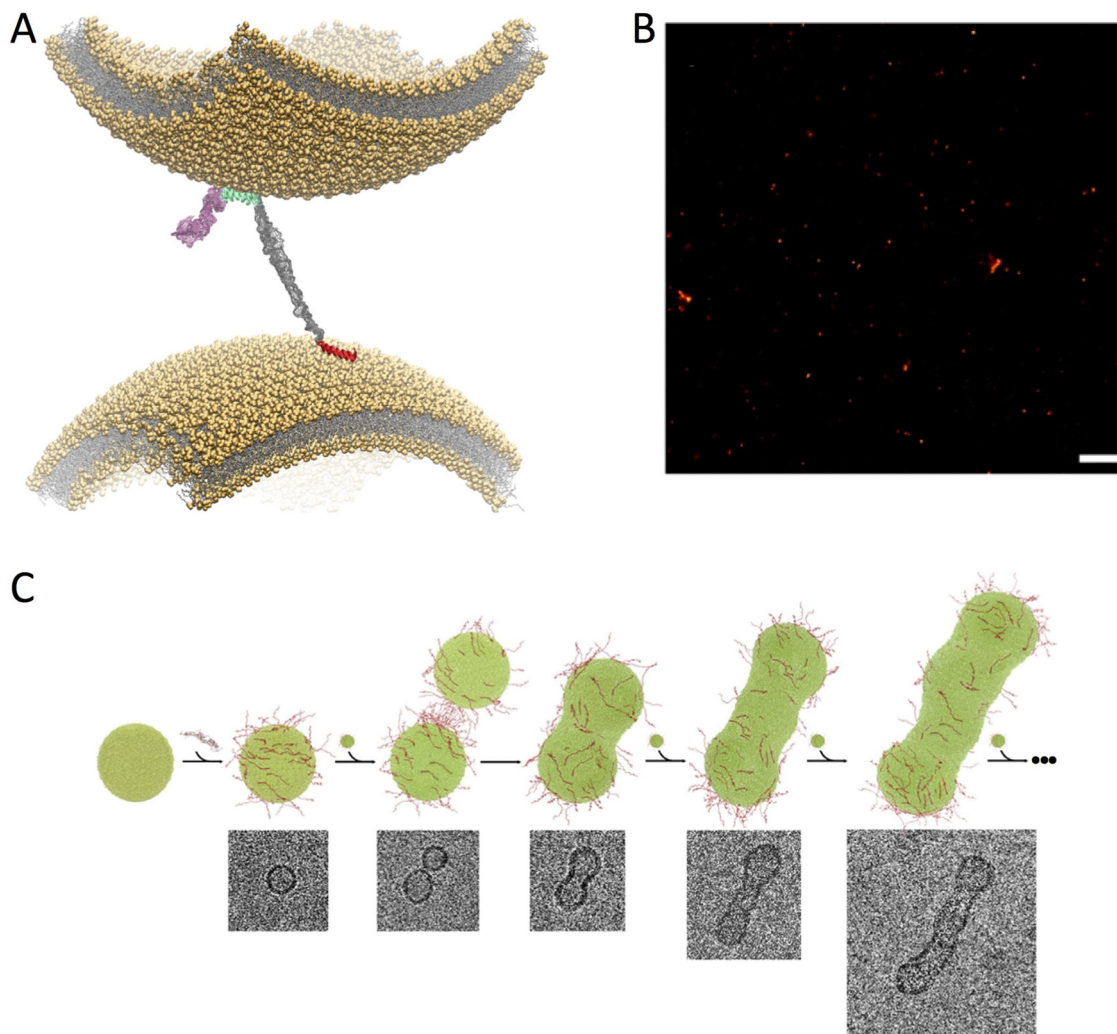
The localisation of  $\alpha$ S at presynaptic terminals, and a number of *in vivo* evidences, strongly indicate an involvement in SV trafficking.<sup>12</sup> In this context, it is generally considered that  $\alpha$ S binds SVs, despite initial proteomic studies did not found this protein in association to SVs.<sup>58</sup> Recently, ultra-definition experiments have, however, identified  $\alpha$ S as a “SV visitor” protein,<sup>59</sup> in line with the overall body of evidences supporting the weak and transient association of  $\alpha$ S with SVs. Super-resolution imaging of synaptosomes showed that  $\alpha$ S strongly colocalizes with SVs at the pre-synaptic terminals, and that this process is

regulated by calcium ions that weakly bind to the C-terminus of the protein (Fig. 2C).<sup>60</sup> In addition to this evidence for SV binding, an emerging idea is forming about a role for  $\alpha$ S as a chaperone of the assembly of the SNARE complex through direct interaction with synaptobrevin-2 on SV surface.<sup>15,16</sup> Indeed triple knock out mice lacking  $\alpha$ -,  $\beta$ -, and  $\gamma$ -synucleins were found to develop neuropathological phenotypes associated with impaired SNARE activity with ageing.<sup>61</sup> It was also shown that, as a result of the binding to SV,  $\alpha$ S is able to rescue the SNARE formation in mice lacking CSP $\alpha$ .<sup>62</sup>

Upon SV binding,  $\alpha$ S has the ability to promote their clustering,<sup>18–20</sup> a mechanism involved with the maintenance of SV pools at the synaptic termini.<sup>17,22–24</sup> We obtained experimental evidence for an underlying structural mechanism for the promotion of SV clustering, which is based on the subtle equilibrium between structured and disordered conformations adopted by  $\alpha$ S when bound to the surface of SVs. In particular, the study of the pathological  $\alpha$ S variants A30P and E46K revealed that the N-terminal (residues 1 to 25) and central (residues 65 to 97) regions of the protein are somewhat independent in their binding to the membrane surface.<sup>57</sup> This observation suggested that, in addition to interact with the same membrane, the two regions could bind simultaneously across two different membranes. The resulting “double-anchor” (Fig. 3A) provides a mechanism for the  $\alpha$ S promotion of indirect interactions between two separate vesicles. In aqueous solutions, this process can be quantified with assays monitoring the fusion of liposomes (Fig. 3C) and the clustering of *ex vivo* SVs (Fig. 3B).<sup>57</sup> By characterising these processes, it







**Fig. 3** Double-anchor mechanism. (A) Structural model of the double-anchor mechanism by which a single  $\alpha$ S molecule binds simultaneously two lipid vesicles that are up to 150 Å apart, thereby promoting their indirect interaction. A first anchor, which spans residues 1 to 25 in N-terminal region (red), binds a vesicle in an amphipathic helical conformation while a second anchor, spanning the region 65 to 97 (cyan) of  $\alpha$ S, binds a second vesicle. The C-terminal region (residues 99 to 140) and the linker (residues 26 to 59) are shown in pink and grey, respectively. (B) dSTORM imaging of the clustering of *ex vivo* SVs upon incubation *in vitro* with  $\alpha$ S. Scale bar 1  $\mu$ m. (C) Stepwise representation of vesicle fusion promoted by  $\alpha$ S *in vitro* as probed by cryo-EM.  $\alpha$ S molecules (red) bind dynamically the surface of lipid vesicles (green) by promoting their fusion in a “worm-like” assembly. All images adapted from ref. 57, with permission from Nature Publishing Group, copyright 2016.

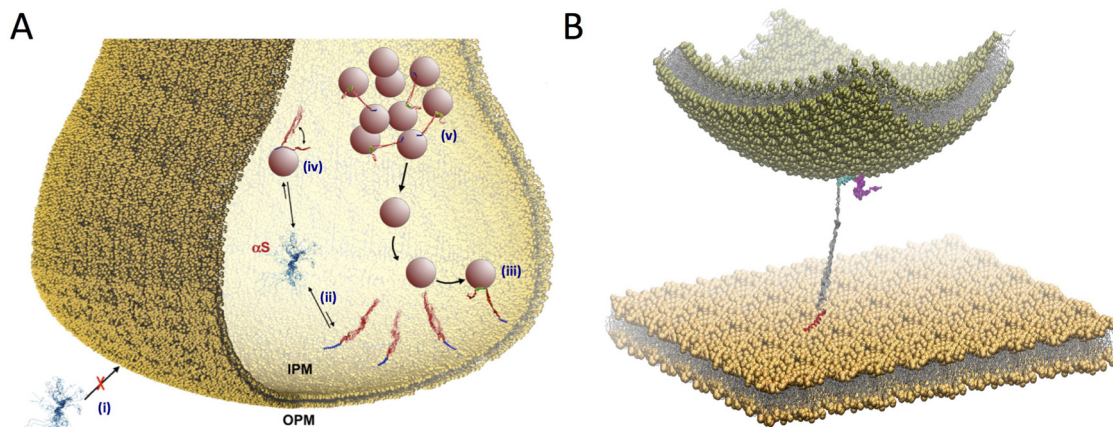
was found that the efficiency by which the double-anchor mechanism promotes vesicle-vesicle interactions depends on the amount of detachment of the central region of  $\alpha$ S from the membrane surface. Indeed the bound-unbound equilibrium can be altered by several factors such as the characteristics of the lipid bilayers, including curvature, charge,<sup>51</sup> levels of cholesterol,<sup>63</sup> packing defects and surface hydrophobicity,<sup>41,44,64–66</sup> as well as the properties of  $\alpha$ S, including mutations<sup>57</sup> and post-translational modifications.<sup>67</sup> The double-anchor mechanism clarifies a number of experimental observations where mutational variants affecting the membrane affinity of the two individual anchors impairs vesicle clustering, as observed in *S. cerevisiae*<sup>18</sup> or in aqueous solutions.<sup>20</sup> These investigations also identify a new link between functional and aberrant behaviour of membrane-bound  $\alpha$ S.<sup>57</sup> In particular,

the evolutionary pressure toward sequences of  $\alpha$ S that promote the detachment of the central region from the membrane surface may have a dual effect of (i) improving the efficiency of the double-anchor mechanism in vesicle clustering and (ii) enhancing the exposure of the amyloidogenic NAC region. The latter effect, however, may favour unwanted  $\alpha$ S self-assembly thereby enhancing its propensity to aggregate at the membrane surface.<sup>17,68–71</sup>

#### 4. Binding to other membranes and organelles

In addition to SV binding,  $\alpha$ S has been associated with a variety of other synaptic membranes. In particular, experimental





**Fig. 4** Multiple membrane interactions by  $\alpha$ S at the synaptic termini. (A) Scheme of competing interactions between  $\alpha$ S and biological membranes at the presynaptic terminal.  $\alpha$ S has negligible affinity to bind the outer PM (OPM) (i) but significantly higher affinity for the internal PM (IPM) (ii). The IPM bound state includes primarily the binding through the N-terminal anchor of  $\alpha$ S (blue), with the region 65–140 (red) being mostly dissociated from the IPM surface. This conformation is optimal to promote a double-anchor mechanism (first anchor in blue and second anchor spanning residues 65–97 in green) to stabilise the SV docking on the IPM surface (iii). Competing with this process, the binding to SVs (iv) promotes the clustering of SV in pools (v) slowing down the SV diffusion toward the active zone. (B) Double anchor mechanism by which  $\alpha$ S stabilizes SVs (green vesicle) on the IPM (flat yellow membrane) in a concentration dependent manner. The N-terminal region (red) binds the IPM while the central region (residues 65–97, cyan) interacts with the SV. The C-terminal region (residues 98–140) and the linker between the anchors (residues 26–64) are drawn in magenta and gray, respectively. Adapted from ref. 81, with permission from Nature Publishing Group, copyright 2021.

evidences have been obtained for  $\alpha$ S binding to mitochondrial membranes,<sup>72</sup> mitochondrial-associated membranes,<sup>73</sup> and presynaptic membranes (PM).<sup>74</sup> In the specific case of binding to the PM, monomeric forms of  $\alpha$ S have been shown to localise intracellularly near the PM,<sup>75,76</sup> while interaction with the outer PM leaflet has been associated with the cellular uptake of  $\alpha$ S monomers<sup>77</sup> or pathological aggregates.<sup>1,78</sup> We studied the modes of binding of  $\alpha$ S with the PM and found that the interactions with inner and outer leaflets (IPM and OPM) are significantly different (Fig. 4A). In particular,  $\alpha$ S was found to have poor affinity for OPM, although alterations of the content of gangliosides (GM) in the lipid bilayer, particularly GM1 and GM3, enhanced the affinity for this membrane. This finding has relevance in the context some neurodegenerative conditions where increased GM content occurs in the PM<sup>79</sup> as well as for lipid rafts,<sup>80</sup> where GM are highly abundant.<sup>81</sup> By contrast we found that  $\alpha$ S has considerable affinity for IPM, and that the structural properties of this binding make it ideal to promote a double-anchor mechanism between the IPM and SVs (Fig. 4B), which is in line with some literature hypotheses.<sup>21,82,83</sup> Using model vesicles, we also found that  $\alpha$ S stabilizes thermodynamically and kinetically the docking of SVs on the IPM in a concentration-dependent manner.<sup>81</sup>

The ability of  $\alpha$ S to induce both SV–IPM and SV–SV interactions explains, at least in part, some literature controversies about the promotion<sup>15,16</sup> or inhibition<sup>84–86</sup> of the SNARE activity. In particular, the promotion of SV clusters<sup>20</sup> downregulates the flow of vesicles toward the active zone<sup>17</sup> thereby negatively contributing to SV exocytosis. By contrast, the stabilisation of the SV docking on the IPM surface<sup>81</sup> as well as the role of chaperone for the SNARE formation<sup>16</sup> are expected to favour the exocytosis of SVs. These literature evidences, therefore,

delineate a subtle equilibrium between different roles of  $\alpha$ S in competing processes within the overall SV exocytosis (Fig. 4A). This balance can be perturbed by external factors such as for example the lipid dyshomeostasis leading to an increase of GM content in the PM<sup>79</sup> as well as calcium bursts,<sup>60</sup> post-translational modifications<sup>67</sup> (particularly Ser 87,<sup>87</sup> Ser 129<sup>88</sup> and Tyr 39<sup>82</sup>) or  $\alpha$ S aggregation. These recent data thereby provide a link between functional and pathological properties of  $\alpha$ S in the context of SV trafficking.

In addition to the PM,  $\alpha$ S binding to mitochondrial membranes is attracting considerable interest. Mitochondrial dysfunction is a hallmark of the pathogenesis of PD and other neurodegenerative disorders,<sup>89–91</sup> and alterations of the ATP and ROS production have been reported in the context of PD in addition to changes in the mitochondrial morphology and integrity. Studies have demonstrated that  $\alpha$ S localisation in mitochondria has an effect on their functions and integrity<sup>92–97</sup> and experimental evidences exist about the binding of  $\alpha$ S to both synthetic models of mitochondrial membranes and *ex vivo* mitochondria. It has been shown that  $\alpha$ S co-localizes with both outer and inner mitochondrial membranes, with some studies pointing to an exclusive interaction with OMM<sup>92</sup> or IMM,<sup>96,97</sup> and others indicating binding to both types of membranes.<sup>93,94</sup> In addition,  $\alpha$ S appears to interact also with mitochondrial associated membranes (MAMs).<sup>73</sup> Pathological point mutations of  $\alpha$ S, including A53T and A30P, have been shown to alter its mitochondrial interactions and abundance in isolated mitochondria.<sup>73,93</sup> It remains to be clarified whether the mitochondrial co-localization of  $\alpha$ S is promoted by protein–protein interactions, including chaperones<sup>98</sup> or membrane translocases such as TOM40,<sup>93</sup> TOM20<sup>99</sup> and VDAC<sup>100</sup> or only by its intrinsic affinity for membrane binding. Studies of  $\alpha$ S binding



to model synthetic mitochondrial membranes showed the key role of the N-terminal region of the protein as the deletion of the first 11 residues impaired this interaction.<sup>96</sup>

There is a debate about the role of  $\alpha$ S in mitochondrial morphology and dynamics,<sup>101</sup> with studies indicating that  $\alpha$ S induces mitochondrial fragmentation<sup>92,102,103</sup> particularly when in the form of the PD variant A53T.<sup>104</sup> The underlying mechanisms of  $\alpha$ S induced mitochondrial fragmentation are still unknown, with studies pointing to a direct involvement of the  $\alpha$ S-membrane interaction.<sup>102</sup> Other investigations, however, have revealed that  $\alpha$ S inhibits mitochondrial fusion<sup>92</sup> thereby posing new questions on its mitochondrial role in functional and pathological contexts. It is also now clear that in addition to the physiological monomeric form, aggregates of  $\alpha$ S are also involved in mitochondrial interactions under pathological conditions. A key question arises whether  $\alpha$ S aggregates induce mitochondrial dysfunction or it is the loss of mitochondrial function and integrity to trigger the formation of oligomeric  $\alpha$ S species. In this context, the increase of cardiolipin on the OMM has been shown to modulate the accumulation of  $\alpha$ S aggregates,<sup>105</sup> whereas  $\alpha$ S oligomers, but not monomeric or fibrillar species, were shown to damage isolated mitochondria<sup>106</sup> and mitochondrial-mimicking membranes<sup>107</sup> where  $\alpha$ S oligomers generated pores in the membranes. These and other studies also flagged the importance of cardiolipin in promoting the interaction of  $\alpha$ S with mitochondrial membranes<sup>97</sup> and in enhancing the pore-forming activity of its oligomeric species.

## 5. Pathological membrane binding by $\alpha$ S aggregates

The pathological relevance of  $\alpha$ S aggregation into Lewy bodies is now established and recent structural works have revealed that lipids represent also a significant component of these intraneuronal depositions forming in conjunction with PD.<sup>108</sup> A key focus therefore exists on the role of the membrane interaction in the process of  $\alpha$ S aggregation. Many studies have been performed on the role of membranes in altering the kinetics and pathways of  $\alpha$ S aggregation, with different results showing either inhibition or acceleration of this process depending on the experimental conditions and the membrane properties.<sup>17,71,109–111</sup> Literature data indicate that the membrane interaction has dramatic effects on the conformational ensemble of  $\alpha$ S, including the expansion from a compact state as observed in the cytosol<sup>34,112</sup> and the consequent exposure of the amyloidogenic NAC region at the membrane surface.<sup>113</sup> The mechanism by which membrane binding enhances  $\alpha$ S aggregation *in vitro* is indeed of particular interest in the context of synucleinopathies. Solid-state NMR studies have revealed that membrane-induced  $\alpha$ S aggregation passes through an  $\alpha$ -helical intermediate, which is not observed when the protein aggregates in aqueous solution.<sup>69</sup> By combining kinetics and structural studies, prefibrillar and early fibrillar intermediates of  $\alpha$ S forming on the surface of anionic vesicles

were isolated and characterised at high resolution using ssNMR.<sup>114</sup> This study showed a segmental folding process that enables the gradual build-up of the structural elements composing membrane-induced  $\alpha$ S fibrils. Moreover, structural studies of membrane-induced  $\alpha$ S aggregates have shown the role of lipids in directing the protofilament fold and in mediating the arrangement of protofilaments.<sup>115</sup> The involvement of membranes in the fibrillization of  $\alpha$ S may also explain the different polymorphic amyloids structures in post mortem analyses of patients affected by synucleinopathies.<sup>108,116</sup>

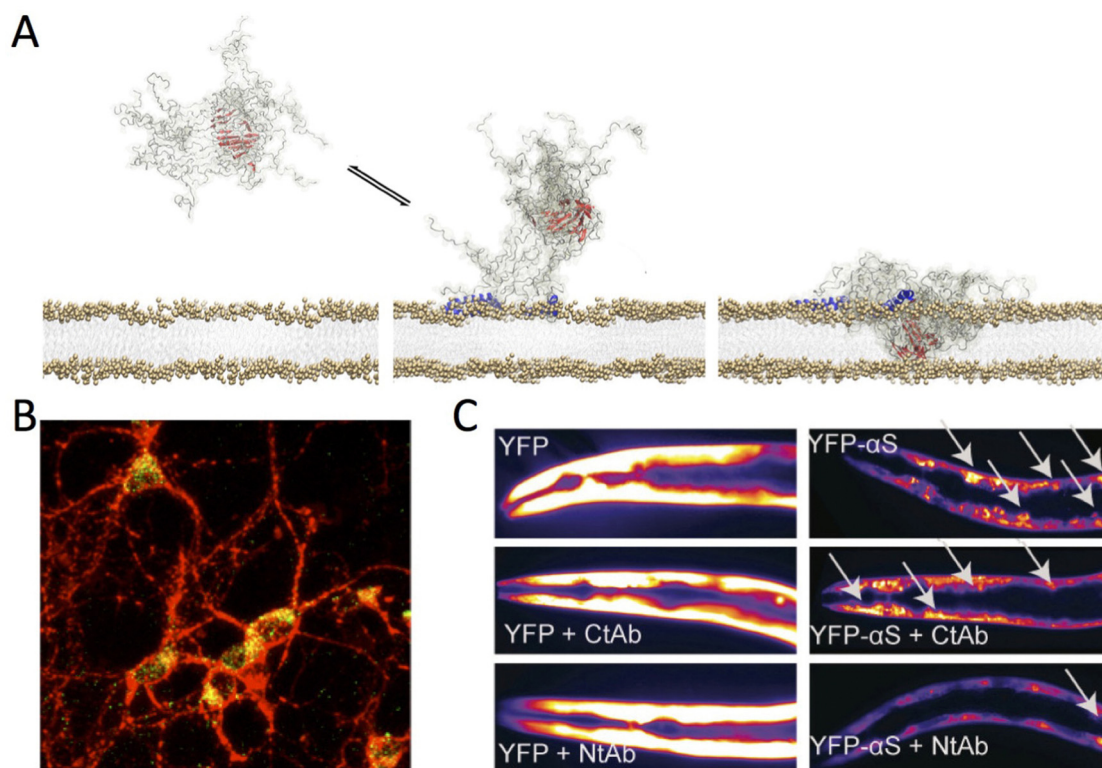
Despite the progress in the characterisation of fibrillar structures of  $\alpha$ S,<sup>117–122</sup> including WT and pathological mutants<sup>123,124</sup> as well as *ex vivo* aggregates,<sup>108,116</sup> the properties of oligomeric species of this protein remain still elusive. Understanding the nature of  $\alpha$ S oligomers is of critical importance as it has become evident both *in vitro*<sup>125–128</sup> and *in vivo*<sup>32,129</sup> that these small and diffusible aggregates are likely to be the key aggregates inducing neurotoxicity in PD.

To clarify the mechanism by which toxic  $\alpha$ S oligomers induce membrane disruption, we characterised the structural properties and membrane interactions of two types oligomers of exhibiting significantly different toxicity levels.<sup>1</sup> In particular, type-B\*  $\alpha$ S oligomers, in contrast to the type-A\* species, were found to induce toxicity when incubated with neuroblastoma cells and primary cortical neurons, including the generation of intracellular ROS, the increase in the basal calcium levels and the impairment of the mitochondrial activity.<sup>1</sup> When unilaterally infused into the *substantia nigra* of rats, type-B\*  $\alpha$ S oligomers triggered the deposition in neurons and microglia of fibrils of  $\alpha$ S with significant levels of S129 phosphorylation, as well as persistent neuroinflammatory response, early mitochondrial loss and a gradual nigrostriatal dopaminergic loss, associated with motor and cognitive impairment.<sup>130</sup> Further studies found that bilateral intracerebral infusion of the type-B\*  $\alpha$ S oligomers induced memory deficits in rats whereas spreading of toxic  $\alpha$ S was detected within anatomically interconnected areas of the brain.<sup>131</sup> The analyses showed neuroinflammation in distant cognition-relevant regions of the brain as well as a proinflammatory phenotype in microglia, as shown by increased levels of microglial tumor necrosis factor alpha (TNF- $\alpha$ ).<sup>131</sup>

ssNMR investigations of the type-B\*  $\alpha$ S oligomers clarified the critical role two elements of the aggregates in the process of binding and disruption of neuronal membranes (Fig. 5A and B).<sup>1</sup> The first element is the highly lipophilic N-terminal region of  $\alpha$ S, which is exposed at the surface of the type-B\* oligomers. This region promotes strong binding with neuronal membranes whereas a second region, the fibrillar core of the oligomers that is formed by a portion of the NAC region, inserts into the lipid bilayer thereby disrupting the membrane integrity. Mutations reducing the membrane affinity of the N-terminal region of  $\alpha$ S were found to suppress the toxicity of type-B\* oligomers when incubated with neuroblastoma cells and primary cortical neurons. Collectively these data suggest that targeting the membrane interaction of  $\alpha$ S aggregates provides a critical therapeutic opportunity to suppress their







**Fig. 5** Aberrant membrane disruption by  $\alpha$ S oligomers. (A) Toxic type-B\*  $\alpha$ S oligomers were found to possess both structured (red) and disordered (grey) regions. The oligomers bind the membrane surface using the N-terminal regions of  $\alpha$ S protomers in the assemblies (blue). The structured rigid core of the oligomers was found to insert into the lipid bilayer thereby disrupting its integrity. Adapted from ref. 1, with permission from the American Association for the Advancement of Science (licence number 5567500159295), copyright 2017. (B) Membrane interaction of type-B\*  $\alpha$ S oligomers with primary cortical neurons imaged using confocal scanning microscopy. Red and green fluorescence indicates the cell membranes and the  $\alpha$ S oligomers, respectively. Adapted from ref. 132, with permission from American Chemical Society, copyright 2019. (C) Effect antibodies targeting the N- and C-terminal regions of  $\alpha$ S (Nt-Ab and Ct-Ab) in *C. elegans* models of  $\alpha$ S toxicity. These worms overexpress YFP-tagged  $\alpha$ S in the muscles and the aggregation of the protein generates toxicity. Representative fluorescence microscopy images of worms at day 11 of adulthood expressing YFP (left) or YFP- $\alpha$ S (right) is shown in the absence (top) or presence of 0.4  $\mu$ M of Ct-Ab (middle) or Nt-Ab (bottom). The inclusions are indicated by white arrows. Adapted from ref. 132, with permission from American Chemical Society, copyright 2019.

toxicity. This hypothesis was tested using an antibody that specifically binds the N-terminal region of  $\alpha$ S protomers within the type-B\* oligomers,<sup>132</sup> showing a potent inhibition of the toxicity of the aggregates when incubated with neuronal cells. The antibody was also shown to rescue the pathological phenotype of *C. elegans* strains overexpressing  $\alpha$ S in muscles (Fig. 5C).<sup>132</sup> In addition to suppressing the toxicity derived from  $\alpha$ S aggregation in *C. elegans*, the antibody was shown to reduce the overall aggregation of the protein *in vivo*. In similar contexts, the use of small molecules such as Trodusquemine to displace toxic protein oligomers from cellular membranes has shown promising results.<sup>133</sup>

## 6. Conclusions

We are now beginning to uncover the connection between the normal and pathological forms of  $\alpha$ S and how specific structural elements of this protein, normally employed for its functional membrane interaction, can be recruited in processes associated with aberrant  $\alpha$ S aggregation. It has been demonstrated that toxic oligomeric species of  $\alpha$ S exploit the lipophilic

nature of the N-terminal membrane anchor to interact with biological membranes and promote unphysiologically strong binding that irreversibly disrupts the membrane integrity. These recent studies therefore revealed that the membrane interactions of physiological monomers and pathological aggregates of  $\alpha$ S, despite being involved in dramatically different cellular processes, share common features, including the mechanism of membrane anchoring promoted by the N-terminal region. The similarities in the interaction propensity of monomeric and aggregated species of  $\alpha$ S may be at the origin of the synaptic dysfunction observed in some pathological contexts.  $\alpha$ S oligomers were indeed shown to impair the SNARE formation by establishing aberrant interactions with the N-terminal region of synaptobrevin-2.<sup>134</sup> The concomitance of all PD familial variants in the membrane-binding region of  $\alpha$ S also indicate alterations in the membrane binding, as demonstrated *in vitro*,<sup>57,67,135</sup> which may in turn affect the biological behaviour of the protein in the context of SV trafficking.<sup>136,137</sup> Finally, a link between dysfunction and aggregation has been proposed whereby the fibrils growth induces toxic effects by depleting  $\alpha$ S monomers, leading to a loss of function of the protein.<sup>138</sup>



Taken together these recent literature studies uncover the nature of the tremendous challenges ahead in identifying suitable molecular strategies to target pathological aggregates of  $\alpha$ S in PD without interfering with its physiological monomeric form, as the biological properties of both  $\alpha$ S species are inextricably linked with similar strategies of membrane interaction. To achieve this critical milestone in PD research, it will be fundamentally important to advance our understanding of the physiological function of  $\alpha$ S at the neuronal synapses.

## Conflicts of interest

There are no conflicts to declare.

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