Functional Insights into Membrane Bound α -Synuclein:

Surface Density, Conformation, and Localization inside Cells

Mohammad Amin Abolghassemi Fakhree

2018

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FUNCTIONAL INSIGHTS INTO MEMBRANE BOUND

α -SYNUCLEIN:

SURFACE DENSITY, CONFORMATION, AND LOCALIZATION INSIDE CELLS

DISSERTATION

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to my family

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Chapter 1:

Introduction

1.1. Unstructured, yet important

According to the central dogma in biochemistry, genes are transcribed and translated into linear chains of amino acids. After or during translation process, the amino acids chain folds into a well-defined stable/functional form of the protein (Dyson, 2016). This stable functional form is the minimal energy conformation of the protein. The minimal energy structure is determined by the sequence of the amino acid chain. Depending on the sequence and types of amino acids in the chain, the conformational changes of amino acids in this linear chain result in secondary structures such as alpha-helix and beta-sheet. Physico-chemical properties of the secondary structures in the chain, cellular environment, and molecular chaperones result in further organization and the folding into the tertiary protein structure. Some proteins are functional as monomers and for these proteins the tertiary structure represents the functional state. Other proteins consist of two or more proteins and form complexes – known as quaternary structure of the proteins – in order to become functional.

During the second half of the 20th century, it was generally believed that proteins have to go through these folding processes in order to become functional (Liu et al., 2009, van der Lee et al., 2014). In order to understand structure-function relationships, x-ray crystallography was used to get information about the structure of the proteins. In the beginning, this was a challenging task. However, by improvements in sample preparation, light source, detectors, and computational tools, high quantity/quality structure determinations of the folded proteins was achieved (Campbell, 2002). In parallel to that, with the help of emerging computational molecular modeling techniques, the idea of sequence-structure/function relationship of proteins has been studied and confirmed. However, sequence-structure/function relationship found to be partial and does not include all of the proteins or whole sequence of a protein. For example, 44% of human protein-coding genes were reported to contain disordered regions with more than 30 amino acids in length, which are not stably folded (van der Lee et al., 2014). In the meantime, based on experimental findings it has become more and more clear that not all of the proteins need to adopt a single well defined structure to be functional. These unstructured regions are often have been reported important for the function of the protein (Linding et al., 2003, Uversky, 2015, Dyson, 2016, Babu, 2016).

Besides having important functions – such as signal transduction – in cells, unstructured proteins play role in diseases such as amyloidosis which involves the aggregation of certain proteins (Uversky and Fink, 2004, Mompean and Laurents, 2017). Currently, the treatment strategy of the most of these diseases is based on alleviating symptoms. This means that the reason for the disease is not addressed. In many of these cases, the unstructured nature of the proteins and limited knowledge of the function of the protein, hinders the development of a cure; because the molecular target(s) for treatments is not known (Uversky, 2016).

The absence of the tertiary structure, yet being functional, makes unstructured proteins an interesting subject of study. Further, involvement of unstructured proteins in diseases made it clear that disordered regions of proteins needs more attention than they have been given before. At the same period in which the importance of understanding the (dis)function of unstructured proteins became clear, the advancements in biomolecular techniques enabled researchers to study the proteins in their native and functional state inside living cells, or even whole organism. Consequently, in the recent millennium, the number of studies with focus on unstructured proteins has increased (Uversky, 2014, Uversky, 2016).

1.2. IDPs and IDRs

As mentioned earlier, it appears that many proteins remain partially or completely unfolded in the functional state. The Scheme 1.1 shows a spectrum of proteins ranging from being nearly completely structured to completely unstructured. Proteins which do not have any secondary/tertiary structure are called intrinsically disordered proteins (IDPs). The unstructured regions of the structured proteins are commonly referred to as intrinsically disordered regions (IDRs).

By folding, a structured protein reaches a “global” minimum energy state which makes the protein structure stable. Unlike structured proteins, the IDPs/IDRs do not have a “global” minimum energy state, rather, depending on the interaction with other molecules they can interchangeably adopt multiple structures to reach “local” minima energies. This implies that the IDPs/IDRs are flexible and can adopt more than one functional structure and may even have multiple functions (Burger et al., 2014).

Scheme 1.1. A spectrum of structured to unstructured proteins. Protein 1 shows an example of a structured protein. Protein 2 and 3 are examples of structured proteins with disordered regions. Protein 4 and 5 are examples of IDPs that does and does not adopt secondary structure based on the interacting partner, respectively.

On the one hand having structural flexibility can be advantageous, as single amino acid sequence can take part in various interactions and thus functions. On the other hand, the fact that IDRs/IDPs do not have an energy minimized stable structure in the absence of the interacting partner, makes unstructured proteins susceptible to undergo unwanted interactions. This, can disrupt the balance between their solution form and functional form. Which consequently gives rise to unwanted events such as self interaction and subsequently the formation of oligomeric/amyloid fibril species in amyloidosis (Uversky and Fink, 2004, van der Lee et al., 2014).

1.3. Functions of IDPs/IDRs

Similar to structured proteins, the presence of specific motifs in the amino acid sequence make IDPs/IDRs functional. Thus, it is necessary to understand the major functional features of IDPs/IDRs. The function of an unstructured protein is mainly resulted from the presence of one or more of the three following features (van der Lee et al., 2014):

- a. **Linear motifs** are short sequences of 3-10 AAs. These sequences provide a single or multiple low affinity recognition site(s) on the amino acid chain for their interacting partner. Further, the small size of the linear motifs makes them more accessible substrates. A good example of linear motifs in IDPs/IDRs, are the recognition sites for post translational modification (PTM) on the proteins (Babu, 2016, Lin et al., 2017).
- b. **Molecular recognition features (MoRFs)** are longer (10-70 AAs) sequences than linear motifs. Although unstructured in solution, the MoRFs can adopt alpha-helix (α -MoRF), beta-sheet (β -MoRF), and irregular but rigid (ι -MoRF) structures, or a mixture of mentioned structures upon binding the target (van der Lee et al., 2014). Similar to structured proteins, the newly formed structure depends on the AA sequence. As an example, N-terminal region of the protein p53 is an α -MoRF when it interacts with Mdm2 protein. The C-terminal region of the p53 can turn to an α -MoRF or ι -MoRF, upon interaction with S100B or Cdk2-cyclin A, respectively (Xue et al., 2013, Laptenko et al., 2016, Hsu et al., 2012).

c. **Intrinsically disordered domains (IDDs)** are the regions of the IDPs/IDRs that always remain unstructured. The IDD part provides a bulky, yet flexible part in the IDP/IDR. For IDDs being disordered is (part of) the functionality. Proteins with IDDs have diverse functions, mostly are associated with ribosomes, and binding of DNA, RNA, and proteins (van der Lee et al., 2014, Chen et al., 2006).

It is noteworthy to point out that even though the sequence of a single IDR is fixed, nonetheless it might show a spectrum of the abovementioned functional features. In other words, distinct classification of functional features based on sequence is not proper, because it depends both on the sequence of the unstructured protein and binding partner (e.g. sequence of the target).

Based on functional features, IDPs/IDRs can be categorized as following (van der Lee et al., 2014, Schulenburg and Hilvert, 2013).

a. **Entropic chains.** The disordered region acts as a flexible linker between domains of a protein, provides steric hindrance between macromolecules, or displays mechanical properties (such as elasticity) because of being disordered. Flexible linkers between structured domains of the proteins are an example of this type of IDR.

b. **Display sites.** Mainly consist of linear motifs that present the IDP/IDR to the target molecules. The sequence is short, not bulky, and unstructured, which makes it more and easier accessible to the interacting partner. Recognition sites for PTMs are included in this category.

c. **Chaperones.** The disordered regions of chaperones help proper folding of the RNA and protein molecules. Although the mechanism behind this interaction is not clear, nevertheless being disordered in nature and through weak and highly dynamic interaction with target molecules, make chaperones a multipurpose tool for the cell. It is noteworthy to mention that more than one third of protein chaperones and more than half of RNA chaperones contain disordered regions.

d. **Catalyzers/Enzymes.** Unlike structured enzymes which needs tertiary structure to form the active site for the catalytic activity, the unstructured enzymes can perform catalytic activity in their disordered form (Schulenburg and Hilvert, 2013). In comparison to the structured enzymes, the disordered enzymes provide an easier step of substrate binding and a faster product release.

Anhydrin of *Aphelenchus avenae*, a nematode, is an example of unstructured enzymes (Chakrabortee et al., 2010).

e. **Effectors.** IDPs/IDRs that bind to target molecule and change the activity of the target molecule. Typically this binding results in disordered-to-ordered transition of the disordered region. This transition is called coupled folding and binding. Calpastatin – endogenous inhibitor of calpain protease system – has two α -MoRF regions that undergo coupled folding upon binding and inhibiting calpain, is an example of effector IDPs (Dosztanyi et al., 2010).

f. **Assemblers.** These IDPs/IDRs provide an assembly platform for other target molecules. The disordered region does not have secondary/tertiary structure, hence inflict less steric shielding than folded proteins, which makes it easier to bring other macromolecules together. Ribosome, as a macromolecular assembly, is made up of ribosomal proteins, which are mainly disordered proteins (van der Lee et al., 2014).

g. **Scavengers.** These IDPs/IDRs bind to ligands. The purpose of this binding is to store and/or neutralize excess ligands. For example, calcium phosphate is stabilized/solubilized by casein, a highly disordered protein, in milk (van der Lee et al., 2014).

Depending on the conditions and/or interacting partners, a single IDP/IDR might have one or more of the mentioned functions. As mentioned earlier, these multiple interactions and functions of IDP/IDR depend on the local minima energies of the protein. Since these energy minima are dependent on the interaction partner, sequence-equal-to-function dogma becomes less practical for unstructured proteins. In this respect, the lack of a single well defined structure makes it harder to study, understand, and predict the function of the protein. The first step to overcome this difficulty is to look for the functional structures of the disordered protein depending on the interacting partner. This requires sub-cellular, even molecular level studying of the IDPs/IDRs inside cells. These structures might co-exist inside the cell, which makes it harder to isolate and study them.

1.4. α -Synuclein (α S) is an IDP

The protein α -synuclein was first identified and isolated from *Torpedo californica* (Maroteaux et al., 1988). Because of the protein's localization in

synapses and nuclei, it was named synuclein. Later it was shown that human precursor protein of non- $\alpha\beta$ component of Alzheimer's disease amyloid (NACP) is a homologue of rat alpha-synuclein (Campion et al., 1995). Human alpha-synuclein (α S) is made up of 140 amino acids and is present in high concentrations – 1-2 % w/w of total proteins – in neuronal cells.

α S goes in vitro

With finding more evidences that α S is involved in a series of neurodegenerative diseases (Wakabayashi et al., 1998), more systematic studies started on α S. As a first step, according to biochemistry dogma, it is crucial to determine the structure of the protein. However, NMR and CD experiments showed that, in buffer, α S lacks secondary structure and is mostly an IDP (Weinreb et al., 1996, Davidson et al., 1998). Nevertheless, this does not mean that α S is always disordered and behaves like a random coil polymer (Scheme 1.2).

In vitro experiments have shown that α S becomes more compact due to dynamic long range intra molecular interactions between the C-terminus with the N-terminus of the protein (Hoyer et al., 2004, Bertonecini et al., 2005, Dedmon et al., 2005). Furthermore, several long lived structures of α S have been reported and investigated in the presence of interacting partners (Wang et al., 2016, Mor et al., 2016, Dettmer et al., 2016, Deleersnijder et al., 2013). This possibility to adopt distinctly different structures from each other, originates from three major regions in the α S sequence: membrane binding region, NAC region, and C-terminal region.

The amino acids 1-95 host two of the three mentioned regions. This 95 amino acids long sequence is disordered in buffer, but in *in vitro* experiments it has been shown that it is susceptible to the formation of secondary structures (Davidson et al., 1998, Eliezer et al., 2001, Ulmer et al., 2005). The N-terminal region of α S allows it to bind to membranes. In the presences of micelles and lipid bilayers, α S adopts an amphipathic helical structure that inserts into the bilayer, with the hydrophobic amino acids in the helix facing the lipids and polar amino acids facing the aqueous solution (Galvagnion, 2017). The sequence of the membrane binding region of α S makes the protein organize into an imperfect helical structure. The membrane binding helix consist of 7 repeats composed of 3 turns per 11 amino acids (Shvadchak et al., 2011, Jao et al., 2008). *In vitro* NMR

Scheme 1.2. Various reported secondary structures for the IDP α S. Lack of predicted/measured secondary structures in buffer, disorder:order -promoting amino acids ratio of 2.5, and net charge of -9 in physiological pH, all together point toward disordered nature of α S. Yet, there are a series of secondary structures reported for α S in the presence of interacting partners. It is not clear how and what determines that α S switch between these structures.

studies have shown that the micelle bound forms a horseshoe like structure consisting of two anti-parallel helices of 9-52 and 57-89 connected by a short stretch of unstructured amino acids (Eliezer et al., 2001, Ulmer et al., 2005, Mazumder et al., 2013, Rao et al., 2010). However, organization into a broken, anti-parallel helix may be a result of the small size of the micelles. Indeed, α S has been reported to organize into a single helix upon binding to lipid bilayers (Cheng et al., 2013). Further studies have shown that whether α S organizes into broken helices or single helix, depends on the curvature, membrane packing defect, and charge of the lipid membranes, and the α S:lipid ratio (Shvadchak et al., 2011). It has been shown that membrane binding of the 6-25 amino acids is independent of the lipid composition and works as an anchor, where membrane binding of

26-90 amino acids is affected by the lipid composition of the membrane and work as a “membrane sensor” (Fusco et al., 2014, Bodner et al., 2009).

The second region of the α S is the hydrophobic non-amyloid component (NAC) region composed of amino acids 61-95 (Ueda et al., 1993). Though 61-95 amino acids are part of the membrane binding region, the NAC region is not essential for membrane binding, and considered to be involved in the modulating of the membrane activity of α S (Fusco et al., 2014). Being intrinsically disordered, this opens possibility for interaction with molecules other than lipids. For example under certain physicochemical conditions, this region is responsible for the formation of cross-beta-sheet fibrils upon self interaction. The amino acids 71-82 of the NAC region are necessary and sufficient for the aggregation of the protein into amyloid fibrils (Giasson et al., 2001, Rodriguez et al., 2015). In respect to its possible involvement in amyloidogenic diseases, the aggregation process of α S has been studied extensively, *in vitro* and *in vivo*. Yet, it is not clear whether the beta sheet formation by the NAC region is a functional feature of α S which is susceptible to pathogenesis, or, it is a pathological aspect of the protein. On the one hand there is no evidence to suggest a function for beta sheet formation of the NAC region inside cells. On the other hand, it does not make sense from the evolutionary point of view: why should a troublesome sequence be conserved? The membrane binding and the NAC regions of α S are highly conserved (Siddiqui et al., 2016).

The third region consists of the C-terminal part of α S (amino acids 96-140). This is a region rich in prolines and negatively charged amino acids. The high net charge and presence of prolines make this region intrinsically disordered under most conditions. However, this does not mean that it does not interact with other molecules. Samples from PD patients and *in vitro* aggregation experiments suggest that this charged entropic region acts as a “molecular bumper”, whereas repulsion between the C-terminal regions keeps α S from aggregating (Hoyer et al., 2004, Li et al., 2005, Anderson et al., 2006). Further, it has been shown that the C-terminal region of α S interacts with a series of biomolecules such as dopamine, VAMP2, and tau (Diao et al., 2013b, Burre et al., 2012, Burre et al., 2010, Conway et al., 2001, Mazzulli et al., 2006, Souza et al., 2000, Woods et al., 2007). Additionally, the C-terminal region can bind metal ions such as iron, copper, and zinc cations (Uversky et al., 2001, Binolfi et al., 2006, McDowall and

Brown, 2016, Peres et al., 2016, Lingor et al., 2017). Furthermore, NMR studies showed weak interactions between C-terminal region and lipid membranes (Fusco et al., 2014). These studies suggest that even though C-terminal region of α S is intrinsically disordered, yet it shows interactions with many different molecules.

In overall, the presence of many different functional interactions and (partial) folds of α S may seem puzzling, but demonstrates the functional versatility of the IDP α S.

α S goes in vivo

As a major player in synucleinopathies (e.g. Parkinson's disease, Lewy body dementia, and other diseases characterized by the presence of α S amyloid deposits) α S is one of the well studied, yet not well understood IDPs. Since discovery of α S in 1990s and proposing a role in synucleinopathies around 2000, about ten thousands PubMed indexed papers have been reported on synuclein/NACP in relation to its physiological or pathological aspects. Following the observed alpha helical structure of the membrane bound α S in model *in vitro* systems, (dis)functional interactions of α S with various membrane bound compartments of the cell have been studied. These compartments include but are not limited to the plasma membrane, synaptic vesicles (Lautenschlager et al., 2017), Golgi apparatus, endoplasmic reticulum, lysosomes (Moors et al., 2016), and mitochondria (Parihar et al., 2008, Guardia-Laguarta et al., 2014, Zaltieri et al., 2015, Nakamura, 2013). Yet, after nearly two decades of research, the exact physiological function(s) of α S is (are) not known. On the one hand this may be caused by α S being an IDP and the absence of well defined functional form; which means that the sequence/structure-function dogma is not applicable. On the other hand, in-vitro experiments hint towards the existence of an ensemble of secondary structures depending on interaction partners; which makes it hard to pinpoint the functional form.

The most well studied interaction partner of α S is lipid membranes. As outlined earlier in this section, *in vitro* studies show that the N-terminal region of α S adopts an amphipathic helix structure upon binding to anionic lipid bilayers and micelles. The insertion of the helix into the lipid membrane can modulate its physical properties, most importantly it induces membrane curvature (Braun et

al., 2012, Varkey et al., 2010, Shi et al., 2015). Increase in curvature can result in membrane remodeling processes such as tubulation or vesiculation. For this reason, it has been suggested that α S plays a role in vesicles fusion/fission processes inside cells. Based on this hypothesis, biochemical techniques were used to investigate the involvement of α S in vesicles trafficking inside cells. These experiments showed that the expression level of α S can affect the physiology of membrane-bound compartments in cells. In this respect, the interaction of α S with SNARE complexes and involvement in synaptic vesicles physiology have been studied (Burre, 2015, Burre et al., 2010, Bendor et al., 2013, Diao et al., 2013a, Choi et al., 2013). Furthermore, it has been shown that α S plays a role in endocytic/exocytic pathways (Lautenschlager et al., 2017), autophagy and apoptosis events (Xilouri et al., 2016, Jin et al., 2011).

Conversely, there are limited numbers of reports that directly show association of α S with membranes inside cells (Boassa et al., 2013, Spinelli et al., 2014, Fortin et al., 2004). Even though these studies showed that α S colocalizes/co-occurs with membranous structures in the cell, however, none provided direct evidence for the interaction of α S with the membrane. Being more specific the question which remains open is: is the *in vitro* binding to membrane has the same structure/mechanism as observed *in vitro*? An α -helical membrane bound α S inside the cell would mean a structured functional state for this “enigmatic” protein. However, the mechanism responsible for the observed colocalization/co-occurrence of α S with cellular membrane, and consequently function of α S inside the cell remains unknown. In order to look at the interactions of α S with membranes inside the cell, in-cell NMR and EPR approaches were used. Surprisingly, these works suggest that, in spite of the colocalization with intracellular membranes, α S mainly remains disordered in the cell (Cattani et al., 2017, Theillet et al., 2016).

As mentioned earlier, in-vitro data showed that α S induces membrane remodeling by helix insertion into the outer layer of the lipid bilayer. Further, in-vivo experiments suggest that α S is involved in membrane remodeling processes of the cell. However, direct interaction with membranes and the mechanism of this interaction inside cells remained unknown for α S. In this respect, a number of open questions can be presented:

Question 1: Does α S induce membrane curvature inside cell?

Question 2: If yes, what is the structure of membrane bound α S inside cell?

Question 3: What is the mechanism of this curvature induction? Is it direct or indirect?

Question 4: Is α S a general membrane modulator inside cell? Or is it specific for certain part(s) of cellular processes?

Question 5: Does α S *only* induce membrane curvature inside cell? Or does it also interact with other molecule(s)? Is it a single-purpose or a multi-purpose IDP?

1.5. Thesis overview

As discussed throughout this introduction, understanding the mechanism and role of the interaction between α S and membranes inside cells is of importance. For this reason, in the present work we focused on the interaction of α S with membranes inside and/or outside the cell, trying to answer the mentioned open questions.

In order to address **Question 1**, we looked into the local density of α S inside the cell. To do so, in Chapter 2 we counted the number of membrane bound α S on single vesicles inside cells using a photo-bleaching approach. The number of α S molecules per vesicle allowed us to deduce if local density of α S is high enough to remodel membranes inside the cell, as suggested by in-vitro experiments.

After establishing that α S is present on membranous structures inside cells, in order to address **Question 2**, in Chapter 3 we microinjected α S molecules labelled with donor and acceptor dyes and probed α S structure on cellular vesicles and in the cytoplasm based on their Förster resonance energy transfer.

Based on high density of membrane bound α S on cellular vesicles, and the possibility of inducing indirect membrane curvature, in Chapter 4 we investigated the contributions of the disordered region of α S to curvature generation and membrane remodeling. Results of this chapter addresses the **Question 3**.

To shed light on the specific pathway(s) that require(s) the membrane remodeling capacity of α S and answer **Question 4**, in Chapter 5 we looked at the involvement of α S in the endocytic pathway inside cells.

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Chapter 2:

Counting α S-GFP on cellular vesicles

The number of α -synuclein proteins per vesicle gives insights into its physiological function*

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Abstract

Although it is well established that the protein α -synuclein (α S) plays an important role in Parkinson's disease, its physiological function remains largely unknown. It has been reported to bind membranes and to play a role in membrane remodeling processes. The mechanism by which α S remodels membranes is still debated; it may either affect its physical properties or act as a chaperone for other membrane associated proteins.

To obtain insight into the role of α S in membrane remodeling we investigated the number of α S proteins associated with single small vesicles in a neuronal cell model. Using single-molecule microscopy and photo-bleaching approaches, we most frequently found 70 α S-GFPs per vesicle. Although this number is high enough to modulate physical membrane properties, it is also strikingly similar to the number of synaptobrevin, a putative interaction partner of α S, per vesicle. We therefore hypothesize a dual, synergistic role for α S in membrane remodeling.

Keywords: α -Synuclein; physiological function; membrane remodeling; synaptic vesicle; copy number; SNARE

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2.1. Introduction

α Synuclein (α S) is an intrinsically disordered protein of 14.3 kD that is abundant in the brain where it represents a considerable part of the cytosolic protein content (Stefanis, 2012). The protein α S has been suggested to play a key role in the development of Parkinson's disease (PD), a neurodegenerative disorder associated with loss of dopaminergic neurons in the substantia nigra (Polymeropoulos et al., 1997). Although the involvement of α S in PD pathology is well established, the exact physiological role of this protein remains an enigma.

α S is enriched in the presynaptic termini of dopaminergic neurons. At these termini the soluble protein is in equilibrium with vesicle bound α S (Spinelli et al., 2014). Membrane bound α S has been suggested to interact with other synaptic vesicle proteins. It has been reported to chaperone the formation of the SNARE complexes (Diao et al., 2013, Burre et al., 2014, Burre et al., 2010) and thereby mediate membrane fusion processes. *In vitro* experiments hint that, at high protein to lipid ratios, α S can also directly change the physico-chemical membrane properties. From *in vitro* experiments, α S has been reported to increase lipid packing (Ouberaï et al., 2013) and induce positive mean and negative Gaussian curvatures in phospholipid bilayers (Braun et al., 2012). Additionally, α S binding has been observed to cause tubulation and fragmentation of phospholipid vesicles at high α S concentrations (Braun et al., 2014, Varkey et al., 2010, Shi et al., 2015), and reduce membrane tension and increase undulations in small unilamellar vesicles (Braun and Sachs, 2015). Although these changes in membrane properties agree well with a role for the protein in membrane remodeling processes, it is still unclear if the changes in membrane properties observed *in vitro* are relevant *in vivo*.

The two suggested functions of α S – being an interaction partner for other proteins or changing the physico-chemical properties of the membrane – require distinctly different numbers of α S per vesicle. Whereas for specific interactions with synaptic vesicle proteins only one or a few membrane bound α S molecules may be needed, larger surface concentrations are required if the physiological function of the α S involves changing physico-chemical properties of the membrane. The copy number of most synaptic vesicle proteins is low. Interestingly, α S is absent in reports on the copy number of major protein constituents of synaptic vesicles (Takamori et al., 2006). This absence might

result from the equilibrium association of α S with membranes and the loss of bound protein during synaptic vesicle purification.

Here, we investigated the number of α S per vesicle in cells, thereby avoiding complications arising from vesicle purification. Using photo-bleaching experiments on α S-GFP expressing cells, we find a most frequent number of 70 α S-GFPs per vesicle. We discuss the observed surface concentration in relation to the possible physiological function of the protein.

2.2. Results

In images of primary neurons immunostained against α S, two distinct types of fluorescence signals are visible: a diffuse signal spread throughout the cell, and localized, small, high intensity α S puncta (Figure 2.1A, B). In these neuronal cells, the α S puncta possibly represent synaptic or endocytic vesicles(Boassa et al., 2013, Spinelli et al., 2014). The radius of synaptic vesicles has been reported to range from 15 to 37 nm with an average of 21 nm(Takamori et al., 2006), whereas endocytic vesicles in the synapse of neuronal cells have a typical radius of 40 nm(Watanabe et al., 2014). Clearly, these sizes are below the resolution limit of conventional optical microscopy, which is roughly 250 nm in radius. Hence, synaptic vesicles appear as diffraction limited sized features in the microscopy images.

Although immunostaining gives excellent results in visualizing and localizing α S in primary neurons, we not only aim to visualize α S, but also to determine the number of α S per vesicle. Unknown and varying labeling efficiencies plus possible steric hindrance due to the size of the antibodies preventing occupation of all possible binding sites, could easily lead to incomplete labeling of membrane bound α S which makes immunostaining unsuitable for quantitative studies.

Single molecule detection based techniques are by now well established in molecular life science (Hinterdorfer et al., 2012, Manzo and Garcia-Parajo, 2015, Klein et al., 2014, Grunwald et al., 2011, Joo et al., 2008). To determine the number of molecules in complexes or molecular aggregates, single molecule photo-bleaching of fluorescent labels has been shown to be a viable

Figure 2.1. α S distribution in primary neurons and differentiated SH-SY5Y cells. **A)** Confocal microscopy image of rat primary neurons immunostained for α S (green), actin filaments (red), and nuclei (blue). (scale bar is 20 μ m) **B)** Red channel of **A**, showing immunostaining for α S. Diffuse signal throughout the neurons and distinct bright α S puncta are visible throughout the neurites and soma of the neurons (scale bar is 20 μ m). **C)** Confocal microscopy image of differentiated SH-SY5Y cells immunostained for α S (green), actin filaments (red), and nuclei (blue). As expected for a neuronal cell model system, the similarities to primary neurons (see panel **A**) are evident. (scale bar is 20 μ m) **D)** Zoom of the area indicated with the red square in **C**. Isolated puncta of α S can be observed in the cell cytoplasm and extensions. (scale bar is 5 μ m) **E)** Single-molecule confocal microscopy image of α S-GFP signal in differentiated SH-SY5Y cells. The fluorescence is not

homogenously distributed throughout the cells. In both the cytoplasm and the extensions, high intensity α S-GFP puncta can be observed. (scale bar is 10 μ m). **F)** Zoom of the area indicated in **E**. Isolated puncta of α S-GFP can be observed in the cell extension. (scale bar is 1 μ m).

technique(Eggeling et al., 1998, Zijlstra et al., 2012, Zijlstra et al., 2014, Leake et al., 2006). In a cellular context, these photo bleaching based techniques require recombinant labeling of the protein of interest. These strategies, primarily based on GFP labeling of α S, have been proven successful before even in a mouse model of Parkinson's disease(Spinelli et al., 2014). A control experiment verifies that the GFP tag does not change the interaction between α S and membranes.

Circular dichroism experiments show that α S and α S-GFP bind phospholipid vesicles with comparable affinity (See Figure 2.S1), which is in agreement with literature (Nakamura et al., 2008). Therefore, we used α S tagged C terminally with GFP (α S-GFP) and expressed it in a SH-SY5Y cell model. The SH-SY5Y cell model has been shown to be, after differentiation, a suitable cell model system for neurons in neurodegenerative diseases like PD (Xie et al., 2010, Agholme et al., 2010). Indeed we find close resemblance between immunostained primary neurons (Figure 2.1A, B) and immunostained differentiated SH-SY5Y cells (Figure 2.1C, D). In all cell types we find both a diffuse distribution of α S and a localization of α S in well-defined puncta. For the photo-bleaching experiments, we subsequently expressed α S-GFP in the SH-SY5Y cell model system (See Figure 2.S2).

Since our study is based on the observation of bleaching steps from single GFP molecules, the use of ultrasensitive single-molecule fluorescence spectroscopy is required. In Figure 1 E and F we present typical images of our α S-GFP expressing cell line using ultrasensitive microscopy. We observed both a diffuse α S-GFP signal and a spotted pattern of α S puncta throughout the differentiated cells. The observation of both a diffuse signal and localized puncta agrees well with the observations from immunostained primary neurons and immunostained differentiated SH-SY5Y cells (Figure 2.1A-D). We attribute the diffuse background signal to cytosolic α S-GFP or α S-GFP associated with vesicles that cannot be resolved individually. The small, high intensity puncta are within the size range expected for diffraction limited structures and might hence represent α S-GFP associated with synaptic and/or endocytic vesicles. Since the size of these puncta cannot be further resolved with conventional confocal fluorescence microscopy, we used STED super-resolution microscopy. From STED microscopy images (see Figure 2.S3), we conclude that the structures giving rise to the puncta in our differentiated SH-SY5Y expressing α S-GFP model cell system are smaller than 80 nm, the resolution limit of the used STED setup, and are hence small enough to represent synaptic or endocytic vesicles.

To confirm that the puncta are indeed vesicles, we subsequently investigated the colocalization of the fluorescent α S-GFP puncta with membranes. To label membranes, we used the membrane marker wheat germ agglutinin, tagged with the fluorophore Alexa Fluor®647 (WGA-AF647). The GFP and AF647 fluorophores can be excited and detected independently and by

overlaying the images, colocalization can be visualized. To show colocalization of α S-GFP puncta with the membrane, WGA was incubated with the cells for six hours. Independent excitation and detection of GFP (excitation 485 nm, detection 550/88 nm) and WGA (excitation 640 nm, detection > 665 nm) resulted in colocalization of 40% of α S-GFP puncta with WGA-AF647 (see Methods).

In our colocalization experiments, upon excitation of GFP, we detected more intense emission above 665 nm than expected from direct excitation of AF647 by 485 nm laser light, and leakage of GFP emission into the detection window beyond 665 nm. Förster Resonance Energy Transfer (FRET) between the initially excited α S-GFP and WGA-AF647 could be the reason for this observation. When the two fluorescent markers, GFP and AF647, are in nanometer proximity, they form a FRET system in which the GFP (donor) fluorescence is efficiently quenched by the presence of AF647 (acceptor). The characteristic Förster distance for the GFP and AF647 pair is expected to be slightly smaller than the 5.6 nm reported for the spectroscopically similar AF488 and AF647 pair. This smaller distance results from the smaller quantum efficiency of GFP compared to AF488.

Moreover, in our experiments, a large fraction of puncta appeared only in the red channel (emission > 665nm, emission of AF647) after GFP excitation at 485 nm, indicating very efficient FRET between α S-GFP and WGA-AF647 (Figure 2.2, top panel). Furthermore, after photo-bleaching the FRET acceptor AF647 with high intensity laser light of 640 nm, the emission of the FRET donor, α S-GFP, appeared in the green channel (emission detection 550/88 nm) (Figure 2.2, low panels). After this acceptor bleaching of AF647, and resulting dequenching of α S-GFP, colocalization between α S-GFP and WGA-AF647 increased from 40% to 60%. The formation of such an efficient FRET system implies nanometer proximity between α S-GFP and WGA-AF647. This nanometer distance could result either from a direct interaction between WGA and α S or GFP, or juxta positioning due to high local concentrations. FRET experiments on solutions of mixtures of WGA and α S or GFP excluded relevant direct interaction between WGA and α S or GFP (see Figure 2.S4), suggesting that a high local density of α S-GFP near the membrane marker is most likely responsible for the observed FRET. Furthermore, nanometer proximity between α S-GFP and the membrane bound WGA-AF647 indicates that α S-GFP is in all likelihood membrane bound. This

Figure 2.2. FRET quenching of GFP emission by AF647. As long as the acceptor of the FRET pair (AF647 on WGA) is in close proximity to the donor (GFP on α S), there is no emission from the donor GFP, upper panels, before photo-bleaching. Photo-bleaching of AF647 removes the FRET acceptor, and energy can no longer be transferred away from GFP which results in dequenching of GFP, lower panels, after photo-bleaching. The scale bar is $2\mu\text{m}$ and refers to all images. The photo-bleaching experiments were done on puncta which appeared only in red channel upon 485 nm excitation.

conclusion is further supported by the observation that in some larger vesicular structures, α S-GFP outlines the vesicle contour (see Figure 2.S5). We hence conclude that the observed fluorescent puncta indeed represent vesicle bound α S-GFPs.

After establishing that the observed fluorescent puncta in the studied model cells originate from vesicle bound α S-GFP, the number of α S-GFPs per vesicle was determined. Recently, a number of methods have been developed that use single-molecule photo-bleaching to determine the number of fluorescently labeled subunits in a molecular complex or aggregate (Zijlstra et al., 2012, Eggeling et al., 1998, Zijlstra et al., 2014). For larger complexes, Leake et al. pioneered an elegant method to determine the number of labeled subunits per complex that is suitable for *in vivo* quantification (Leake et al., 2006). The number of fluorophores in a complex is determined by dividing the background subtracted initial fluorescence intensity by the average intensity per fluorophore, which is determined using a statistical analysis of each bleaching trace (see Methods). To confirm the applicability of this approach to our

experimental system with small cellular vesicles, we tested the approach on unilamellar vesicles (UVs) with an average radius of 50 nm and a known average number of membrane embedded fluorophores. We then experimentally determined the number of fluorophores per UV using single-molecule photo-bleaching and the analysis method described above. We find that the determined and expected values of fluorophores per UV agree very well (see Figure 2.S6). Hence, our control experiment verifies the suitability of the method to determine the number of fluorophores per vesicle.

To determine the number of α S-GFP per vesicle in our cell model, we recorded and analyzed bleaching traces obtained from more than 200 distinct puncta, a statistically relevant number. For our study we considered only α S-GFP puncta in the thin parts of the cell (Figure 2.1F) with a radius smaller than 350 nm in the fluorescence images (see Figure 2.S7 for size distribution). The chosen maximum punctum size relates to the punctum size expected from the convolution of the physical size of a vesicle and the convolution with the microscopes point spread function. Using the 350 nm maximum size criterion we exclude biasing our data by obtaining bleaching traces from large vesicular structures or the presence of multiple vesicles in the sampled volume simultaneously. After selecting an α S-GFP punctum that fitted these criteria, high intensity laser excitation (485 nm, 800 W/cm²) was used to completely photo-bleach the α S-GFP emission, while recording the fluorescence intensity over time (see example in Figure 2.S8A). The number of α S-GFPs per vesicle was determined from the bleaching traces of 243 distinct vesicles, the copy number was determined and assembled in the histogram presented in Figure 2.3.

Figure 2.3 Number of α S-GFPs per vesicle. Distribution of the number of α S-GFPs per vesicle in differentiated SH-SY5Y cells determined from photo-bleaching experiments (N=243). To minimize the influence of sampling more than one vesicle, puncta with a radius of less than 350 nm in the fluorescence image were selected from the thin parts of the cells. We find a distribution of the number of α S-GFPs per vesicle with a most frequent occurrence of ~ 70 α S-GFPs per vesicle.

The distribution of the number of α S-GFPs on the studied vesicles is broad, ranging from around 20 α S-GFPs per vesicle up to more than 300 α S-GFPs, with a most frequent value of approximately 70 α S-GFPs per vesicle. Finding a distribution of the number of α S-GFP per vesicles rather than a defined number matches the expectation, since it is well known that the size of vesicles is distributed (Takamori et al., 2006, Zhang et al., 1998, Mutch et al., 2011a). In reference (Takamori et al., 2006), the size of synaptic vesicles have been reported to range from 15 nm to 37 nm in radius, and with increasing vesicle surface area the number of α S-GFPs per vesicle – considering the stochastic nature of α S-GFP binding to the membrane – is also likely to increase. Interestingly, the shape of the vesicle surface area distribution obtained from the size distribution of synaptic vesicles (Takamori et al., 2006) and the distribution of the number of α S-GFPs per vesicle we determined, agrees very well. Copy numbers on the high end of the distribution might originate from more than one vesicle in the observation volume, in spite of using the selection criterion of puncta of less than 350 nm.

Using the reported sizes of synaptic vesicles and the number of α S-GFPs per vesicle obtained from the photo-bleaching experiments, it is possible to determine the average distance between α S-GFPs on a vesicle. For the most frequent value of 70 α S-GFPs on an average synaptic vesicle of 21 nm in radius, and neglecting multimerization or colocalization with other proteins, we find an average center-to-center distance of 10 nm between α S-GFPs on the vesicles. This average distance agrees well with our observation of efficient FRET between GFP and AF647 for some of the vesicles (see Figure 2). Considering the stochastic nature of both the binding of α S-GFP and the membrane marker WGA-AF647 to the vesicle, variations in energy transfer between α S-GFP and WGA-AF647 per vesicle are to be expected at this average α S density. For relatively few α S-GFPs per vesicle, the FRET partners are spaced further apart than the characteristic Förster distance. Hence for these low copy numbers colocalization of α S-GFP and WGA-AF647 is not accompanied by efficient energy transfer (i.e. 40% colocalization before photo-bleaching of the acceptor). For higher copy numbers, the distances between α S-GFP and WGA-AF647 are below the Förster distance, resulting in quenching of the FRET donor. The higher copy numbers therefore result in an underestimation of the colocalization of α S-GFP and WGA-

AF647 (i.e. an increase from 40 to 60% colocalization after photo-bleaching of the acceptor).

2.3. Discussion

We observed diffraction limited fluorescent puncta in a neuronal cell model system of differentiated SH-SY5Y cells expressing α S-GFP. We used STED microscopy and colocalization of the α S-GFP puncta with the membrane marker WGA-AF647 to show that these puncta are vesicles. Efficient FRET between GFP and the fluorescent label AF647 attached to WGA strongly indicates that α S-GFP is membrane bound, which is further supported by our observation that α S-GFP outlines larger membrane structures. We hence conclude that the α S-GFP puncta we observe in our neuronal cell model, are small vesicles with membrane bound α S. We used single-molecule photo-bleaching to determine the number of α S-GFPs per vesicle in the cells. We find a distribution of copy numbers per vesicle with a most frequent number of 70 α S-GFP per vesicle.

We exclude the possibility that the puncta represent the well-defined oligomeric species and amyloid fibrils that have been discussed in the context of Parkinson's disease (Stefanovic et al., 2014, Chaudhary et al., 2014, Lorenzen et al., 2014, Semerdzhiev et al., 2014). For example, the aggregation number of α S oligomers formed, *in vitro*, under different conditions was determined using single-molecule photo-bleaching experiments (Zijlstra et al., 2012, Zijlstra et al., 2014). These *in vitro* formed oligomers typically consist of 30 ± 5 monomers (Zijlstra et al., 2012, Stefanovic et al., 2015). Any fibrillar form of α S is expected to contain thousands of α S monomers. It can, however, not be excluded that vesicle bound α S is present as multimeric species.

The distribution in copy numbers reflects the distribution of vesicle sizes and the stochastic nature of binding of α S-GFP to vesicles. Considering that the SH-SY5Y cells were differentiated to neuron like cells, we assume that the studied vesicles are synaptic vesicles. The most frequent copy number of 70 α S-GFPs per synaptic vesicle, is rather high compared to the copy numbers found for most other synaptic vesicle proteins which are in the range of 1 to 10 per vesicle (Mutch et al., 2011b, Takamori et al., 2006). In this regard, since the number of α S per vesicle is high, the absence of α S in listings of the copy number of the major protein constituent of synaptic vesicles (Takamori et al., 2006) is most likely caused by the equilibrium nature of the association of α S with

membranes and detaching of α S from the vesicles during vesicle purification. Interestingly, 70 α S per vesicle is a high enough number to alter the physical membrane properties (Braun et al., 2012).

In the literature, α S has been reported to change the physical properties of phospholipid bilayers (Braun et al., 2014, Varkey et al., 2010, Braun et al., 2012). Based on experimental data and simulations, α S has been suggested to be involved in membrane curvature induction and binding of α S has been shown to cause tubulation and vesiculation of lipid bilayers (Braun et al., 2014, Varkey et al., 2010, Braun et al., 2012). At high molar ratios of protein to lipid; 1:10 to 1:40, α S induces vesiculation and tubulation in POPG lipid bilayers (Varkey et al., 2010). At lower molar ratios of 1:200, α S induces positive mean curvature and local negative Gaussian curvature in flat lipid bilayers (Braun et al., 2012). At this lower protein to lipid ratio, α S was reported to lower the membrane surface tension and increase membrane undulations in small unilamellar vesicles by decreasing the negative pressure in the headgroup region of the outer membrane leaflet and increasing the positive pressure throughout the hydrocarbon core (Braun and Sachs, 2015). If synaptic or endocytic vesicles in neurons are stressed by high curvature because of their size, the effect of α S binding on surface tension could stabilize the vesicles and prevent fusion.

Assuming that the observed puncta are synaptic vesicles with an average radius of 21 nm, the average copy number of 70 would result in a molar ratio of 1:250 between α S and lipids. Our data thus supports the idea that membrane binding of α S and the concomitant partial insertion of amphipathic α -helices can stabilize the membrane curvature and control fusion processes of vesicles with a radius smaller than 40 nm. Besides α -helix insertion, the large hydrodynamic radius of the charged unstructured C-terminal domain of the protein may contribute to the promotion of membrane curvature by generating steric pressure (Busch et al., 2015). Structural studies of α S bound to sodium dodecyl sulfate micelles show that the protein forms two antiparallel α -helices comprising residues 3-37 and 45-92, while residues 93-140 remain unstructured and are exposed to the solution (Bodner et al., 2009, Shvadchak et al., 2011). Assuming that the 48 residues at the C-terminus can adopt a free random walk with a persistence length l_p corresponding to roughly 3 amino acids, they would

form a globule with a radius of gyration of approximately $\sqrt{\langle R^2 \rangle} = \sqrt{\frac{Ll_p}{3}} =$

2.5 nm. Using the known average size of synaptic vesicles, we determined the average distance between α S-GFPs on the vesicle surface to be 10 nm. We therefore conclude that α S binding to the already crowded surface of synaptic vesicles may contribute to steric pressure. However, since we used a photo-bleaching approach to determine the copy number of α S-GFP per vesicle, we do not sample the number of endogenous α S. As an estimation for the number of endogenous α S, we used western blotting data as an indication about the ratio between α S and α S-GFP inside cells. Analysis of Western blots of cell lysate indicated that the ratio between the endogenous α S and α S-GFP in the differentiated SH-SY5Y cells was approximately 1:2 (see Figure 2.S2). We hence conclude that the total number of α S proteins, including α S and α S-GFP, associated with a single vesicle can be as much as 100. Insertion of the helical domain of α S and possibly, in conjunction with other membrane bound proteins, steric pressure exerted by the unstructured α S domain may thus considerably contribute to establishing and maintaining membrane curvature.

As mentioned earlier, α S has been reported to interact with other proteins. These reported interaction partners of α S include a variety of proteins, including, but not limited to, cytoskeletal proteins like tau(Jensen et al., 1999), chaperon proteins like HSP70(Auluck et al., 2002), and synaptobrevin^{6, 42, 43}, a SNARE protein involved in synaptic vesicle fusion. Most of the synaptic vesicle proteins are present in low copy numbers, the copy number of α S is high. The formation of complexes of α S and synaptic vesicle proteins in stoichiometric ratios of 1:10 or even lower seems unlikely. There is however an important exception to the low copy number found for synaptic vesicle proteins. Synaptobrevin, a v-SNARE protein involved in vesicle fusion(Sudhof, 1995), was reported to have an average number of 70 on a single synaptic vesicle (Takamori et al., 2006). The agreement between the number of synaptobrevin and α S molecules on the vesicle surface determined by us, is striking and supports a possible role for α S in SNARE complex formation. It has been suggested that α S promotes SNARE-complex formation by a non-classical chaperone activity involving binding to synaptobrevin(Burre et al., 2010). In this interaction the unstructured C-terminal domain of α S binds to the first 28 N-terminal residues of synaptobrevin, which are not involved in SNARE complex formation. This interaction is thought to catalyze the formation of SNARE complexes by presenting the vesicle bound synaptobrevin in the optimal conformation to interact with the plasma membrane bound SNAREs. We were not only able to confirm the interaction

between α S and the soluble part of synaptobrevin, but using microscale thermophoresis we were able to determine the K_d of the α S – synaptobrevin interaction to equal to 4.14 μ M (see Figure 2.9). The K_d for α S binding to synaptobrevin (4.14 μ M) is small enough to be considered as biomolecular interaction (Qi et al., 2014, Walter et al., 2010) and consistent with cellular α S concentrations (Shtilerman et al., 2002, Seo et al., 2002).

Based on our finding of a most frequent value of approximately 70 α S-GFPs per vesicle and the demonstrated direct interaction of α S with the soluble part of synaptobrevin, we hypothesize a possible dual purpose of membrane bound α S. α S possibly acts as both a modulator of the membrane's physical properties and interaction partner for synaptobrevin. Since interactions limit conformational freedom of the unstructured C-terminal domain of α S, the formation of a α S-synaptobrevin complex may not only result in a presentation of this v-SNARE to its t-SNARE partner but also reduce the steric pressure exerted by α S. We hypothesize that the interaction between α S and synaptobrevin is regulated by a third, yet unknown, factor. Switching on the interaction between α S and synaptobrevin with this factor, may not only result in the presentation of synaptobrevin, but also cause stabilization of the disordered part of α S resulting in a decrease of steric pressure and hence possible destabilization of the vesicle. The latter two events facilitate interaction between vesicle-SNAREs and target-SNAREs which finally results in the release of synaptic vesicle content.

2.4. Supporting Information

Figure 2.S1. Binding of recombinantly expressed GFP tagged (red triangles) and untagged α S (green circles) to POPC:POPS (1:1) SUVs. Membrane binding was quantified in CD spectroscopy experiments by following α S's structural transition from a random coil in solution to a membrane bound α -helix. The bound fractions were obtained from the mean residue ellipticity at 222 nm.

Figure 2.S2. Western blot for α S in wild type (ATCC®) and α S-GFP expressing SH-SY5Y cells in undifferentiated (no RA) and differentiated (10 μ M RA) cultures.

Figure 2.S3. Stimulated emission depletion (STED) super-resolution microscopy image of differentiated α S-GFP expressing SH-SY5Y cells, fixed and immunostained for α S (secondary antibody labeled with AlexaFluor®594). STED microscopy on the periphery of the cells resolves the puncta up to 80 nm, which is the resolution limit of the microscope (scale bar 1 μ m).

Figure 2.S4. Spectrofluorimetry of labeled WGA (5 $\mu\text{g}/\text{ml}$) in the presence of labeled αS ($\sim 3 \mu\text{M}$) or GFP ($\sim 3 \mu\text{M}$). The concentrations used were similar to the cell experimental conditions. After addition of the FRET acceptor to a solution of FRET donor molecules, there was no relevant reduction in donor emission intensity, or increase in acceptor emission intensity. The small reduction in donor emission intensity might be a dilution effect resulting from addition of the acceptor solution. These graphs indicate that, in solution, there is no relevant FRET between WGA labeled with AlexaFluor[®]647 and αS labeled with AlexaFluor[®]488 or GFP. Since there is no FRET, we conclude there is no direct interaction between WGA and αS , or GFP.

Figure 2.S5. Fluorescent signal originating from αS -GFP in differentiated SH-SY5Y cells (scale bars are 4 μm). Besides small αS -GFP vesicles which appear as diffraction limited puncta, larger vesicular structures are visible in the soma of the cells. The αS -GFP is located at the periphery of these large vesicular structures. This further hints toward a localization of αS on the surface of vesicles.

Figure 2.S6. Distribution of the number of fluorophores per UV (N=71). POPG UVs with an average radius of 50 nm doped with 0.05 % rhodamine labeled DOPE, were used as a control model system for the photo-bleaching experiments and data analysis. The calculated average number of fluorophores per UV is 50, and the experimental results obtained from analyzing 71 photo-bleaching traces shows an average number of 54 fluorophores per UV.

Figure 2.S7. Determined size distribution of the studied puncta (N=243). To have a defined selection of vesicles that are diffraction limited in size, we only studied puncta with radius of less than 350 nm, based on a Gaussian fit to the measured intensity profile of the punctum.

Figure 2.S8A. Photo-bleaching trace for α S-GFPs on a vesicle. α S-GFPs on single vesicles observed in SH-SY5Y cells, was photo-bleached using high intensity excitation light (485nm, 800 W/cm²). Photo-bleaching was followed in time until there was no significant reduction in fluorescence intensity anymore. Because of the high intensity bleaching light, generally after 60 seconds there was no further reduction in fluorescence intensity.

Figure 2.S8B. Power spectrum obtained from a single photo-bleaching trace. From the photo-bleaching traces (Fig S7A), power spectra were obtained of the pairwise difference distribution of fluorescence intensity. In the power spectrum, the step size associated with the photo-bleaching of a single α S-GFP was assigned to the first significant peak against the background noise (indicated by red arrow). In the assignment of the step size, the presence of the second order peak (indicated by blue arrow) was included in the selection criteria.

Figure S9. Binding of recombinantly expressed α S to the soluble part of the SNARE protein synaptobrevin. Complex formation was quantified in a microscale thermophoresis experiment at a synaptobrevin concentration of 30 nM. The data points represent the average of 3 measurements. The red line represents a fit through the data assuming a bimolecular binding with an equilibrium dissociation constant $K_d = 4 \mu\text{M}$.

Table 2.S1. List of the antibodies used in this study

Primary antibody			
protein	epitope	host species	source
α S	15-123	rat	BD biosciences
α S	121-125	mouse	Santa Cruz
Secondary antibody			
host	anti	conjugated dye	source
goat	rat	AlexaFluor®594	Invitrogen
goat	mouse	AlexaFluor®555	Invitrogen

2.5. Materials and Methods

Primary neuronal cells. The extraction and culturing of primary neurons was performed as described elsewhere (Stegenga et al., 2009). In short, cells were obtained from newborn (P1) Wistar rat pups. Both (cortical) cerebral hemispheres were isolated in a sterile environment, minced and trypsinized. The minced hemispheres were dissociated by trituration after which the cells were plated on polyethylenimine coated coverslips (Sigma-Aldrich, USA) to 60 % density. After 2 hours, adhered cells were washed with DMEM (Invitrogen, USA) and cultured in 900 μ l serum and antibiotics-free R 12 medium at 37 °C with 5 % CO₂ (Romijn et al., 1984). All research involving animals has been conducted according to Dutch law (as stated in “Wet op de dierproeven”), and approved by DEC, the Dutch Animal Use Committee.

Construct design for α S-GFP DNA plasmid. For construction of human α S-GFP plasmid, the complete human α S cDNA was obtained from pT7-7 human α S via a PCR reaction (van Raaij et al., 2006). For this, a forward primer 5'-GAAGATCTAAGATGGATGTATTCATGAAAGGAC-3' and reverse primer 5'-GAAGATCTCCGGCTTCAGGTTCTAGTCTTG-3' were used on a pT7-7 human- α S template. The reverse primer was designed in such a way that the natural human α S stop codon was removed. Following digestion with *Bgl*II, the human α S cDNA was isolated and ligated into the *Bgl*II and *Bam*HI sites of pEGFP-N1 plasmid. The sequence of the construct was confirmed by DNA sequencing analysis.

Cell culture, transfection and selection of SH-SY5Y cells. The culturing, differentiation, and transfection of SH-SY5Y cells was performed as described elsewhere (Raiss et al., 2016). In short, SH-SY5Y cells were grown in proliferation medium GlutaMAX™ supplemented with 10% heat inactivated FBS, 1% non-essential amino acids, 10mM HEPES buffer, and 1% Penicillin/Streptomycin, all obtained from Gibco®. For differentiation, we seeded SH-SY5Y cells to 60% confluency and induced differentiation by adding starvation medium containing 1% FBS and 10 μ M retinoic acid for 3 days. All chemicals were obtained from Invitrogen, USA if not indicated otherwise.

For transfection, SH-SY5Y cells were seeded, grown until 30%-50% confluency, and transfected with α S-GFP. DNA (250 ng/cm²) was diluted in Opti-

MEM in reduced Serum medium (GIBCO®) including Lipofectamine® LTX Reagent with PLUS™ Reagent. The mix was incubated for 5 minutes at room temperature before adding Lipofectamine LTX. For every 250 ng of DNA, 0.5 μ l of Lipofectamine LTX and 0.19 μ l of Lipofectamine PLUS reagent were mixed, according to manufacturer's protocol. After Lipofectamine LTX addition, a 30 minute incubation at room temperature was performed. The medium of the cells was changed and the DNA, Lipofectamine LTX, and Lipofectamine® LTX Reagent with PLUS™ Reagent mix were added. After one day, the medium was changed to proliferation medium. Two days after transfection, cells were trypsinized and re-seeded in conditioned medium. The next day, G418 (500 μ g/ml) was added and cells were grown in G418 supplemented conditioned medium until selection by fluorescence-activated cell sorting (FACS). The α S-GFP positive cells were expanded in culture dishes and stocks were stored in liquid nitrogen.

Immunocytochemistry and cell staining. Cell samples were washed with PBS and fixed in 3.7% paraformaldehyde/PBS solution for 30 min at room temperature (RT). For immunolabeling, primary neurons were permeabilized with 0.3% Triton X-100 and 0.1% BSA in PBS for 2 hours at RT. The permeabilization step for differentiated SH-SY5Y cells was done for 5min at RT. Autofluorescence was quenched with 50 mM NH_4Cl in PBS for 15 min at RT. Aspecific binding sites were blocked by incubation of the sample with goat serum dilution buffer (16% goat serum, 0.3% Triton X-100, 0.3 M NaCl in PBS) for 30 minutes at RT. Thereafter, primary antibodies (Table 2.S1) were diluted 1:200 in goat serum dilution buffer and incubated with the sample at RT for 1 hour. Then cells were washed with 0.3% Triton X-100 and 0.1% BSA in PBS 3 times, each time for 5 min at RT, and the appropriate secondary antibodies (Table 2.S2) were diluted 1:200 in 0.3% Triton X-100 and 0.1% BSA in PBS and incubated with the sample for 1 hour at RT. For actin staining, cells were incubated with 2 U/ml phalloidin-Alexa Fluor®488 (Invitrogen, USA) in PBS for 15 minutes. Nuclear counterstaining was performed by incubation in 300 nM 4',6-diamidino-2-phenylindole (DAPI) in PBS for 10 minutes. After washing with PBS, samples were mounted with mounting medium (ibidi, Germany).

To label membranes, we used the membrane marker wheat germ agglutinin (WGA) tagged with the fluorophore AlexaFluor®647 (AF647)

(Invitrogen, USA). WGA is a lectin protein that binds to N-acetyl-D-glucosamine and sialic acid on cell membranes (Chang et al., 1975). For WGA-AF647 labeling, living differentiated GFP-SH-SY5Y cells were incubated with 5.0 $\mu\text{g/mL}$ WGA-AF647 in medium for 10 minutes to 6 hours. Subsequently, cells were fixed and imaged as described above. Although previous reports (Kim et al., 1993, Schwarz et al., 1999) suggest that incubation with WGA-AF647 might induce apoptosis, we did not observe any detectable increased apoptosis associated with either the amount of WGA-AF647 or incubation time in our experiments.

Confocal fluorescence microscopy. Confocal laser scanning microscopy images were obtained using a commercial Nikon A1 confocal microscope with a 100 x oil immersion objective (NA=1.4, Nikon, Japan) and laser sources of 405 nm, 488 nm, and 561 nm. Emission was detected using appropriate dichroic mirrors and filter sets.

Single-molecule imaging and photo-bleaching experiments. The photo-bleaching experiments were performed using an ultra sensitive custom-built inverted confocal microscope, described in detail elsewhere (Zijlstra et al., 2014). In short, as excitation source, we used a pulsed diode laser operating at 485 nm at a repetition rate of 20 MHz (LDH-D-C-485, Picoquant, Germany). An epi-illumination configuration was used, i.e., the illumination and emission are collected through the same microscope objective (UPLSAPO 60XW, 60X, 1.2NA, Olympus). The remaining excitation light in the detection path was suppressed with a long pass filter (RazorEdge[®], 488 nm, Semrock, USA) and a notch filter (StopLine[®], 488/14 nm, Semrock, USA). An additional band pass filter for the green channel (BrightLine[®], 550/88 nm, Semrock, USA) and a long pass filter for the red channel (RazorEdge[®], 664 nm, Semrock, USA) were used. The emission was spatially filtered using a 15 μm pinhole and was subsequently focused onto a single photon avalanche diode (SPCM-APQR-16, PerkinElmer), connected to a photon counting module (PicoHarp300, Picoquant, Germany). The sample was first scanned at a high scanning speed, 1 ms per pixel, and low excitation power, $\sim 80 \text{ W/cm}^2$, to prevent dye bleaching. In the initial scan, we located individual αS -GFP puncta in PFA-fixed cells, localized them in the focus of the objective, and sequentially collected fluorescence intensity time traces from distinct αS -GFP

puncta. To obtain the intensity time trace we used higher excitation powers, $\sim 800 \text{ W/cm}^2$, to make sure that all fluorophores of a punctum photo-bleached within 1 minute.

After acquiring the photo-bleaching intensity time trace for the punctum (see Figure 2.S8A), a previously reported data analysis has been used (Leake et al., 2006). In short, a forward-backward non-linear filtering was used to de-noise the trace. Then, a power spectrum of the pairwise difference distribution function for each of the traces was obtained (see Figure 2.S8B). The unitary step size – average effective emitted photon count from a single GFP until it photo-bleaches in each punctum – has been determined based on the power spectrum following the method by Leake and coworkers (**Nature** 2006 355-358), where the first significant peak against the background noise gives the step size. In the assignment of the step size, the presence of the second order peak was included in the selection criteria. Using the determined unitary step size, the total number of emitters per punctum was calculated.

Intensity profiles of the puncta were determined with Symphotime 64 (Picoquant, Germany) by applying a script that applies a Gaussian fit and calculates the Full Width Half Maximum (FWHM).

Calculating colocalization percentage between α S-GFP puncta and membrane marker. Two images were made sequentially using the 485 nm and 640 nm excitation of the single-molecule sensitive microscope. To quantify the colocalization between α S-GFP puncta and the membrane marker WGA, the ratio of puncta in the green channel (485 excitation) which co-localized with signal in the red channel (640 excitation), to all puncta in the green channel, was calculated. Then, each punctum that appeared in the red channel (485 excitation) image, but not in the green channel, was photo-bleached using the 640 nm laser light. After photo-bleaching, the number of puncta that additionally appeared in the green channel of 485 excitation were added to the numerator and denominator of the earlier ratio calculation, and the colocalization percentage was re-calculated.

Recombinant α S-GFP production. α S-GFP producing vector was prepared by insertion of the WT α S coding DNA in the pRSETa-EGFP vector (Volkmer et al., 2000). Stop codon was removed from the WT α S in the pT7 plasmid (Paleologou

et al., 2008). NheI overhang was created with DNA linkers. pRSETA-EGFP and the α -synuclein with NheI overhang were cut with NheI and BglII. α S was ligated into the pRSETA-GFP, creating a 6His-syn-GFP producing vector. The vector was transformed into BL21(DE3)pLysS. An overnight culture (37 °C, 200 rpm) was diluted 100x and 1mM IPTG (Thermo Fisher Scientific Inc., US) was added at an OD_{600nm} of 0.6 to induce the expression of the protein. Temperature was decreased to 20 °C during the expression. After 18 hours bacteria were pelleted by centrifuging 10 minutes at 6000g. The bacteria were lysed by 6 freeze-thaw cycles in liquid nitrogen and water and resuspended in a solution of 50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole. The cells were sonicated on ice at a power of 6 for 10 seconds (Branson 250 sonicator) before centrifugation for 20 minutes at 10000g. Supernatant was incubated with Ni-NTA agarose (Qiagen) for 1 hour at 4 °C while stirring. This mixture was poured in an empty PD-10 column (GE) and washed twice with a solution of 50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole. Protein was eluted with a solution of 50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole. The buffer was exchanged for 10 mM Tris pH 7.4, 50 mM NaCl on a PD-10 column (GE). The protein concentration was determined using Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., US) assuming a molar extinction coefficient of 56000 M⁻¹cm⁻¹ for the GFP at 507 nm.

STED microscopy. Stimulated emission depletion (STED) microscopy (Klar and Hell, 1999) was employed for sub-diffraction limit resolution fluorescence imaging on a custom-made setup. The system's implementation is based on a supercontinuum laser source, and similar to the setup described elsewhere (Wildanger et al., 2008). It is capable of acquiring one channel with confocal and two channels with STED resolution quasi-simultaneously. The supercontinuum laser source was a SC450-PP-HE system running at 1 MHz (Fianium Ltd, UK). For beam-scanning, we used a YANUS IV scan head from Till Photonics, Germany. The objective was a Leica 100x/1.4. For imaging green fluorescent protein (GFP), Alexa Fluor® 594 and Alexa Fluor® 647, we used excitation/emission wavelengths of 488±3 nm/520±14 nm, 586±7 nm/624±40 nm and 637±5 nm/685±20 nm, respectively, using optical filters (AHF, Germany). The STED wavelengths for Alexa Fluor® 594 and Alexa Fluor® 647 were set to 720±10 nm, and 750±10 nm, respectively. Beam powers for acquisition were 1 - 5 μ W for the excitation beams, as measured in front of the objective. STED beam powers

amounted to 1 - 2 mW. To reduce crosstalk, pulses for various channels were separated in time by varying optical path lengths. A home-built electronic gating device transmitted detector signals occurring at the correct time to the acquisition hardware, and rejected crosstalk signals occurring at other times. Dichroic mirrors and filters were purchased from AHF, Germany.

POPG UV model system. 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho (1'-rac-glycerol) (sodium salt) (POPG) and Rhodamine-6-G labeled 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (Rhod-DOPE) lipids were purchased from Avanti Polar Lipids (Alabaster, AL) and were used without any further purification. Lipid mixtures were prepared from a 10 mg/ml stock solutions in chloroform. The labeled lipid, Rhod-DOPE, was added to the POPG solution to end up with a mixture containing 0.05% of labeled lipids. To prepare Unilamellar Vesicles (UVs) the membrane extrusion method was used following the protocol of the provider (Avanti Polar Lipids). In short, a mixture of lipids dissolved in chloroform, was dried using a nitrogen stream. After drying of the lipids, an appropriate volume of phosphate buffer saline (PBS) was added to the dry lipid layer to reach final lipid concentration of 500 μ M. This suspension of lipids, was mixed and subjected to 12 cycles of freeze/thawing using liquid nitrogen and 37 $^{\circ}$ C water. The thus obtained lipid dispersions were filtered at least 11 times using 1000 μ l MICROLITER syringes through an Avanti Polar Lipids mini Extruder containing a Nuclepore track-Etch Membrane with a pore size of 0.1 μ m. This resulted in UVs with diameter of approximately 100 nm. The UV sample was diluted 1:200 in PBS and used in single-molecule microscopy setup for photo-bleaching experiments.

We calculated the average number of lipids and labels per UV as follows:

The area per monolayer of a vesicle with a radius of 50 nm equals $A = 4\pi r^2 = 31415 \text{ nm}^2$. This corresponds to an area of $\sim 62830 \text{ nm}^2$ for the lipid bilayer of one UV. The head group size of POPG is estimated to be 0.628 nm^2 (Murzyn et al., 2005), and therefore a single UV consists of approximately $62830/0.628 = 100000$ lipids. With 0.05 % of labeled lipids, this corresponds to 50 labeled lipid per UV.

POPC:POPS SUV model system. Stock solutions of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) were purchased from (Alabaster, AL) and were used without any further purification. Stock solutions of POPC and POPS were mixed in a 1:1 molar ratio, dried under a stream of nitrogen and placed under vacuum for 1 hour. The dried lipid films were subsequently rehydrated in a 10 mM Tris, 100 mM KCl solution and vortexed for 4 minutes. Small unilamellar vesicles (SUVs) were prepared by sonicating the rehydrated liposome solution for 40 minutes using a Branson tip sonicator. Thereafter SUVs were centrifuged at 13200 rpm to remove possible residue from the sonicator probe. The SUVs were stored at 4 °C and used for experiments within 2 days.

Circular dichroism (CD) spectroscopy. CD spectra were obtained on a Jasco J-175 spectropolarimeter. POPC:POPS (1:1) SUVs were titrated to a protein concentration of 3 μ M. Upon binding to vesicles, the initially unstructured (random coil) α S undergoes a structural transition to an α -helix. Membrane binding can therefore be visualized by following this structural transition. For this purpose we followed the change in absorbance at 222 nm that is indicative of α -helix formation as a function of the lipid concentration. To obtain the bound fraction, the calculated mean residue ellipticity (MRE) values were normalized assuming that the plateau MRE values represent saturation of protein binding sites on the lipid membrane. CD spectra of samples in 1 mm thick cuvettes were recorded with a step size of 1 nm and a scan speed of 10 nm/minute at room temperature.

Microscale thermophoresis. Synaptobrevin (soluble part) was labelled using an amine-reactive labelling kit, Monolith NT™ Protein Labelling Kit BLUE-NHS (NanoTemper® Technologies GmbH, München, Germany). Labeled synaptobrevin was diluted to a concentration of 30 nM in the final solution measured and mixed with α S of different concentrations, see Figure S9. The buffer used for the microscale thermophoresis experiments consisted of 10 mM Tris pH 7.4 (tris(hydroxymethyl)aminomethane, Sigma-Aldrich Chemie, Munch, Germany), 0.5 mg/ml BSA (NanoTemper® Technologies GmbH, München, Germany), 0.05% Tween (NanoTemper® Technologies GmbH), 10 mM NaCl

dissolved in MilliQ-water. Microscale thermophoresis was performed using a Monolith NT.115 MST (NanoTemper® Technologies GmbH) equipped with the Nano –BLUE/GREEN filter combinations. The data was recorded using NT Control 2.0.2.29 (NanoTemper® Technologies GmbH) and processed using NT Analysis 1.5.41 (NanoTemper® Technologies GmbH).

2.6. References

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Chapter 3:

α S conformation on cellular vesicles

Different conformational subensembles of the intrinsically disordered protein α -Synuclein in cells*

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Abstract

The intrinsically disordered protein α -synuclein (α S) is thought to play an important role in cellular membrane processes. Although *in vitro* experiments indicate that the initially disordered protein obtains structure upon membrane binding, NMR and EPR studies in cells could not single out any conformational subensemble. Here we microinjected small amounts of α S, labelled with a FRET pair, into SH-SY5Y cells to investigate conformational changes upon membrane binding. Our FRET studies show a clear conformational difference between α S in the cytosol and when bound to small vesicles. The identification of these different conformational subensembles inside cells resolves the apparent contradiction between *in vitro* and *in vivo* experiments and shows that at least two different conformational subensembles of α S exist in cells. The existence of conformational subensembles supports the idea that α S can obtain different functions which can possibly be dynamically addressed with changing intracellular physicochemical conditions.

KEYWORDS FRET; Microinjection; Alpha-synuclein; Intrinsically disordered protein; SH-SY5Y cell line.

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3.1. Introduction

α Synuclein (α S) is an intrinsically disordered protein (IDP) of 140 amino acids abundantly present in neurons. Its physiological functions are not well understood but it has been suggested to act as an interaction hub for different binding partners. Its aggregation is involved in the death of neurons in degenerative disorders such as Parkinson's disease and Lewy body dementia.

The term "intrinsically disordered" implies a lack of both secondary and tertiary structure. However, in solution, long range contacts between residues cause α S to adopt an ensemble of significantly more compact structures.(Dedmon et al., 2005) *In vitro* experiments show that α S can organize into different folds that depend on binding partners.(Uversky, 2003) α S has been reported to preferentially bind metal cations in a C-terminal region with residual structure(Binolfi et al., 2006) and to associate with several cytoplasmic proteins.(Sousa et al., 2009, Esposito et al., 2007, Xu et al., 2002) The best characterized α S fold is the membrane bound alpha-helical conformation.(Ferreon et al., 2009, Veldhuis et al., 2009) α S binds anionic lipid bilayers and membranes of zwitterionic lipids in the liquid-ordered and gel phase.(Nuscher et al., 2004, Pfefferkorn et al., 2012, Shvadchak et al., 2011) Membrane binding is accompanied by folding of α S into helices that are oriented parallel to the membrane surface.(Chandra et al., 2003, Jao et al., 2008, Drescher et al., 2008) The membrane bound conformation is thought to represent a functional fold of the protein as reviewed in (Snead and Eliezer, 2014). By inserting amphipathic α -helices into the membranes α S is thought to support curvature and (re)cluster vesicles.(Nemani et al., 2010, Varkey et al., 2010) Membrane bound α S may additionally act as a non-classical chaperone in SNARE mediated fusion processes.(Burre et al., 2010)

However, in spite of the distinct conformations and conformational diversity of α S observed *in vitro*, NMR and EPR studies seem to indicate that in cells the disordered nature, observed for monomeric α S in solution, is preserved.(Cattani et al., 2017, Theillet et al., 2016) Considering the well-defined α -helical conformation of α S on membranes in *in vitro* experiments and the high number of α S molecules associated with cellular vesicles,(Fakhree et al., 2016) it seems unlikely that all of the α S retains a disordered conformation inside cells.

This controversy is a subject of intense discussion in the current literature as reviewed by (Pauwels et al., 2017) Here we set out to resolve this contradiction and turned to imaging Förster resonance energy transfer (FRET) to discriminate distinctly different conformational ensembles inside cells.

3.2. Results and discussion

In agreement with literature on the sub-cellular distribution of α S in primary neurons that overexpress the protein, immuno-stained images of primary rat neurons show endogenous α S in two distinct localization patterns (Figure 3.1 A,B).(Boassa et al., 2013, Spinelli et al., 2014) Cytosolic endogenous α S is visible as a diffuse background while the membrane bound form of the protein appears as distinct high intensity puncta. To investigate possible differences of the protein conformation between the protein in the cytosol and the puncta we chose to microinject small amounts of fluorescently labelled α S into SH-SY5Y cells, a well-established neuronal cell model.(Xicoy et al., 2017) We observe the same α S distribution of puncta and diffuse background in cells injected with α S (Figure 3.1C).

Figure 3.1. Localization of α S in cells. **A)** Confocal microscopy image of rat primary neurons immuno-stained for α S (red), actin filaments (green), and nuclei (blue) (scale bar is 10 μ m). **B)** The α S fluorescence from image A., shown in a separate image, clearly shows the presence of both a diffuse background fluorescence and distinct puncta. **C)** Confocal microscopy image of a single SH-SY5Y cell that was microinjected with α S-AF488. The α S localization pattern is the same as that of primary neurons, it is visible as a diffuse background and distinct high intensity puncta (scale bar is 5 μ m).

After confirming that the puncta in the images indeed represent α S on vesicles, using the method reported in (Fakhree et al., 2016) (Figure 3.2), an α S FRET probe designed to identify the membrane bound α -helical conformation was introduced. *In vitro* experiments have shown that α S binds lipid vesicle membranes and micelles by organizing into an amphipathic α -helix. (Pfefferkorn et al., 2012) This membrane bound structure consists of two α -helical segments comprising residues 3-37 and 45-92, joined by a flexible linker. (Bussell and Eliezer, 2003, Chandra et al., 2003, Jao et al., 2008, Lokappa and Ulmer, 2011) To discriminate between the membrane bound α -helical and unstructured form of α S using changes in FRET efficiency, the distance between the labelled residues has to be markedly different in both forms. A maximum distance difference is achieved by labelling at amino acid positions 9 and 69 (Fig 3A). In the anti-parallel α -helical form these residues are very close and thus show high FRET, while in the unstructured form the average distance between the residues is larger resulting in lower FRET. Previous *in vitro* experiments confirmed the ability of this probe to discriminate between the membrane bound and unstructured form of α S (Veldhuis et al., 2009).

Cells microinjected with α S, labelled with the AF488 FRET donor and AF568 FRET acceptor, show clear differences in FRET between different cellular structures or compartments (Figure 3.3B). In the composite image of the donor and acceptor emission intensity, low FRET is visible in green. With increasing FRET, the color in the composite image changes to yellow and red. The cytoplasm of the cells is visible in green, which represents low FRET and thus the cytoplasm contains α S in unstructured form. In the cytoplasm yellow and red puncta can be observed, originating from increased FRET. Clearly at least two distinctly different α S conformations are present in the cell. Since we (Fakhree et al., 2016) and others (McLean et al., 2000) confirmed that the puncta represent α S on small vesicles (Figure 3.2) we can even go one step further and conclude that the increased FRET in the puncta results from the membrane bound α -helical conformation of α S.

For a more in depth analysis, beyond single images, the FRET data has to be quantified. To quantify, the intensities in both the FRET donor (green) and acceptor (red) channel need to be related. However the signal in both of these channels is a combination of the FRET signal and the cell's autofluorescence.

Figure 3.2. Membrane bound α S. To confirm that the distinct puncta in the cells represent the vesicle bound α S population, colocalization with the membrane marker WGA-AF647 was studied. **A)** Confocal fluorescence image of SH-SY5Y cells microinjected with α S-AF488 (green) and stained with WGA-AF647 (red). Independent excitation and detection of the injected α S-AF488 (excitation 485 nm, detection 550/88 nm) and WGA-AF647 (excitation 640 nm, detection >665 nm) resulted in moderate colocalization. However, the two dyes form an efficient FRET pair and this may render a fraction of the α S-AF488 invisible. **B)** Scatter plots of the WGA-AF647 fluorescence intensity versus α S-AF488 fluorescence intensity. Photobleaching of WGA-AF647 dequenched the emission of α S-AF488 confirming the formation of a FRET pair and thus the nanometer proximity of the two dyes. The dequenching of the α S-AF488 fluorescence is visible as a shift of the intensity distribution. The original distribution presented in green changes upon bleaching to the distribution presented in orange. Data points were obtained per pixel. **C)** Colocalization image obtained by combining the image of the initial WGA-AF647 fluorescence with the image of the α S-AF488 fluorescence after dequenching. Colocalization of α S-AF488 with the membrane is visible in yellow. In both images the position of the nucleus is indicated with a blue oval, the scale bar is 5 μ m.

The autofluorescence of the SH-SY5Y cells, with excitation at 485 nm, is not constant but varies both in and between cells. The ratio between the autofluorescence in the red and green channel, or autofluorescence index, is distributed. The ratio between the autofluorescence in the red and green channel, or autofluorescence index, is distributed as we show in the cumulative histogram (Figure 3.3D). The distribution of the autofluorescence index prevents the quantification of the data from the FRET images in terms of a FRET efficiency. Therefore the data was collected in FRET index histograms for α S in the cytoplasm and on vesicles. The FRET index is given by the ratio of the acceptor emission intensity over the donor emission intensity. The FRET index will be low for low FRET, and high for high FRET.

Figure 3.3. **A)** Schematic of the antiparallel helices (left) and a representation of a disordered conformation of the FRET labelled α S. Differences in distance between the red and green emitting fluorophores give different FRET, here as a cartoon depicted by different relative sizes of the donor and acceptor emission halos. **B)** Composite donor and acceptor fluorescence image of a single cell microinjected with the FRET labelled α S. Regions of low FRET (green) and high FRET (yellow-red) can be discriminated. The position of the nucleus is indicated with a transparent blue oval (scale bar is 5 μ m). **C)** Histogram of the FRET index for α S in the cytoplasm (green) and on vesicles (red). **D)** Cumulative probability histogram of the FRET index for α S in the cytoplasm (dotted green), on fibrils (dot-dashed blue), and on vesicles (dashed red). The autofluorescence index of unlabeled cells is indicated in solid black.

For both the membrane bound α S and α S in the cytoplasm, the FRET index is distributed. For α S in the cytoplasm the peak FRET index is found at 0.22 while for α S on vesicles a distinctly different peak FRET index of 0.45 is observed (Figure 3.3C). The shift to higher FRET indices for the vesicle bound α S is even more clearly visible in the cumulative histogram (Figure 3.3D). The FRET index histogram of cytoplasmic α S is narrower than that of membrane bound α S. This narrow distribution may be a result of compaction of the protein in the crowded environment of the cytoplasm as was observed in in-cell NMR

experiments.(Theillet et al., 2016) The broader FRET index distribution of vesicle bound α S might be a result of the flexibility of the linker connecting the two α -helical regions of membrane bound α S, resulting in a distribution of distances between the FRET pairs.(Bussell and Eliezer, 2003, Lokappa and Ulmer, 2011, Robotta et al., 2011) Additionally in imaging small vesicles, below the optical resolution, the sampled volume will always also contain cytoplasm. This last factor together with the cellular autofluorescence index, partly overlapping with the FRET signal, prevents direct translation into a FRET efficiency. Hence these *in vivo* measurements cannot be directly compared with FRET studies on *in vitro* model systems. Control experiments in which a mixture of α S-AF488 and α S-568 was injected into the cells show that the observed high FRET on vesicles does not result from inter-molecular FRET due to crowding of the labelled protein on the membrane surface or inter-molecular interactions (Figure 3.S1).

To highlight the ability of our FRET probe to discriminate different α S conformations we included data on α S fibrils in the cumulative probability histogram (Figure 3.3D). The FRET index of the fibrils is rather narrow and peaks at 0.27. The different FRET index peak value and shape of the histogram indicate that a 3rd conformational subensemble of the protein can be discriminated using these labelling positions and that the microinjected α S did not aggregate into amyloid fibrils in the cells.

In contrast to what has been previously reported our data shows that the disordered nature of monomeric α S is not preserved in cells. *In vivo* NMR and EPR studies may have overlooked the membrane bound conformation. The membrane bound form of α S has been reported to be only a small fraction of the total α S in brain.(Lee et al., 2002) The NMR study already indicated that it may not be possible to detect and discriminate lowly populated α S states with this bulk method.(Theillet et al., 2016) In the NMR experiment the α S concentration increases to 10s of micro-molars which may saturate membrane binding sites resulting in an additional accumulation of unstructured α S in the cytoplasm. This excess of unstructured cytoplasmic α S may mask the presence of the membrane bound population. The EPR studies were conducted on stage V/VI *Xenopus laevis* oocytes at even higher α S concentrations.(Cattani et al., 2017) These cells are in an inactive state which does not require much membrane trafficking, trafficking vesicles will therefore be largely absent. The

cytoplasm mainly contains yolk granules and the absence of membrane bound α S in these oocytes is therefore not surprising.

The sensitivity and ability to image and laterally resolve conformational differences makes our method very well suited to single out conformational subensembles. Given the widely observed membrane bound α -helical conformation in *in vitro* experiments, the existence of this conformational subensemble inside cells confirms our expectations. The used FRET probe was designed to identify the membrane bound α -helical conformation of α S. Other probes can be designed to identify subpopulations representing α S to metal cations,(Binolfi et al., 2006) synaptobrevin,(Burre et al., 2010) 14-3-3,(Perez et al., 2002) actin,(Esposito et al., 2007) and other proteins as reported in *in vitro* experiments. These multiple interactions may represent different conformational subensembles that coexist in a network of coupled binding equilibria. This distribution of α S over these different subensembles is probably tightly balanced. The sensitivity of these interactions to the changes in the intracellular conditions may make α S a hub in interaction networks.

3.3. Supporting information

Inter-molecular FRET of α S on vesicles. The FRET observed on single vesicles in the SH-SY5Y cells does not result from a single labelled α S molecule. Multiple FRET labelled α S proteins are bound to the vesicles. Close proximity of the labelled α S proteins therefore possibly also results in inter-molecular FRET. To test if the observed high FRET on vesicles was a result of inter- rather than intra-molecular FRET the cells were microinjected with a 1:1 mixture of α S-AF488 and α S-568. Note that in this experiment the fluorophore concentration was kept constant, the concentration of protein is twice as high as in the experiments with double labeled protein. As observed for the FRET labelled protein (Figure 3.3) the FRET index distribution of the mixture of proteins differed between the cytoplasm and on vesicles (Figure 3.S1). Following expectations inter-molecular FRET was higher on vesicles. However, the FRET index distributions for inter-molecular FRET are shifted to much lower values compared to intra-molecular FRET. The observed inter-molecular FRET index values deviate from for a number of reasons. 1) When α S-AF488 and α S-568 share the excitation volume, direct excitation of the AF-568 amounts to approximately 5% of the AF-488 excitation. This results in a non-zero FRET index. 2) The cellular autofluorescence mainly contributes to the red (acceptor) channel. This also increases the FRET index. 3) When labelled proteins are bound to a vesicle a small amount of FRET is expected due to intermolecular proximity. We therefore conclude that the FRET data presented in Figure 3.3 results from intra-molecular FRET. The observed FRET is a result of a specific protein conformation rather than a signature of inter- α S-interactions.

Figure 3.S1. Comparison of intra- and inter-molecular FRET in SH-SY5Y cells microinjected with appropriately labeled α S in a cumulative histogram. As observed for intra-molecular FRET, the inter-molecular FRET index distributions observed in cells in which both α S-AF488 and α S-AF568 were injected also differs between the cytoplasm (blue) and on vesicles (black). The FRET index distribution for intra-molecular FRET (red: on vesicles, green: in cytoplasm) is also shown. The inter-molecular FRET index distributions are shifted to lower FRET index.

3.4. Materials and Methods

Cell culture and membrane labeling. The SH-SY5Y cells (ATCC, USA) were grown in proliferation medium DMEM-F12 GlutaMAX™ supplemented with 10% heat inactivated FBS, 1% non-essential amino acids, 10 mM HEPES buffer, and 1% Penicillin/Streptomycin, all obtained from Gibco® (Invitrogen, USA). For labeling the membranes of the SH-SY5Y cells, 5.0 µg/ml wheat germ agglutinin (WGA) tagged with the fluorophore AlexaFluor®647 (AF647) (Invitrogen, USA), was incubated with living cells for 2 hours. The labelled cells were used for membrane colocalization imaging after microinjection of AlexaFluor®488 (AF488) labelled α S. After microinjection experiments, cells were washed with PBS (3x) and fixed in 3.7% paraformaldehyde/PBS solution for 10 minutes at room temperature (RT). After washing with PBS, samples were mounted using mounting medium (ibidi, Germany).

Preparation of FRET labeled α S. An α S modification in which the amino acids at position 9 and 69 were replaced with cysteines was prepared and labelled as described before (Veldhuis et al., 2009). In short, the cysteines were reduced with 1mM DTT, then an equimolar concentration of the maleimide derivative of AF488 was added to 0.5 ml of 200 µM α S(9C/69C). After 1 hour incubation at RT, the AF488 labelled α S(9C/69C) was desalted using a Zeba Spin desalting column (Pierce Biotechnology) to remove unreacted AF488 and DTT, followed by immediate incubation with 330 mg prewashed Thiopropyl Sepharose 6B (GE Healthcare Life Sciences). This step separates α S(9C/69C) labelled with two AF488 dyes from protein containing one or zero labels. The single-labeled and/or unlabeled α S that was bound to the column was subsequently eluted using 10-15 ml of 10 mM Tris-HCl, pH 7.4 buffer, containing β -mercaptoethanol. After pooling the eluted fractions were concentrated to a volume of about 0.5 ml and desalted. Then a 2-3x molar excess of AF568 was added. After incubation for 1 hour at RT, free dye was removed using two desalting steps and the solution was filtered through a Microcon YM100 filter (Millipore, Bedford, MA). The stoichiometry of the dyes on the protein (AF488:AF568) was obtained from UV/Vis absorption spectra (Nanodrop, Thermofisher, USA) using the known extinction coefficients of the dyes. The ratio between AF488 and AF568 on α S(9C/69C) was determined to be 0.83:1. The excess of AF568 agrees with the removal of α S containing two AF488 dyes

during the labeling procedure. This procedure ensured that the final sample only contained the AF488 and AF568 FRET labeled α S and some α S with two AF568.

Preparation of labeled α S. A cysteine mutant of α S (α S18C) was used to prepare single labeled α S. In short, the cysteines were reduced with 1mM DTT, then excess concentration of the maleimide derivative of either AF488 or AF568 was added to 0.5 ml of 200 μ M α S. After 1 hour incubation at RT, the labelled α S was desalted using a Zeba Spin desalting column (Pierce Biotechnology) to remove unreacted dyes and DTT. The concentrations of the labeled proteins were determined using UV/Vis absorption spectra (Nanodrop, Thermofisher, USA) using the known extinction coefficients of the dyes.

Preparation of FRET labeled α S(9C/69C) fibrils. FRET labeled fibrils were prepared as described elsewhere (Sidhu et al., 2014), with minor modifications. In short, a final concentration of 100 μ M α S (99 μ M wild type α S + 1 μ M AF488 and AF568 FRET labeled α S) was incubated in 400 μ l 10 mM Tris-HCl buffer (pH=7.4) in 2 ml Lo-Bind round bottom centrifuge tubes (Eppendorf, Germany) in an incubator (Termaks, Norway) at 37 °C with 500 rpm shaking (Shaker, Titramax100, Heidolph, Germany) for 7 days. Then the content of the tube was slightly concentrated by centrifugation at 3000 $\times$ g for 10 minutes, and 20 μ l from the bottom of the tube was deposited on a clean coverslip for imaging.

Microinjection setup. Microinjections were performed using a FemtoJet® (Eppendorf, Germany), equipped with a manual hydraulic 3D micromanipulator (Narishige, Japan). For injection, pre-pulled borosilicate glass micropipettes with an inner filament and inner diameter of 400 nm (WPI, USA) were used. In order to decrease the adherence of the cells to the surface of the capillaries, capillaries were UV-O₃ cleaned prior to use. The microinjections were performed using micropipettes back filled with 300 nM FRET labeled α S (in 10mM Tris, pH 7.4) and monitored on a Nikon TE2000 microscope (Nikon, Japan). For performing microinjections, an injection pressure, a constant pressure, and a duration of the injection of 150 hPa, 15hPa, and 0.1 second were used respectively.

In order to find back the position of the microinjected cells, the cells were seeded on 35 mm imaging dishes with a glass bottom imprinted with 50 μ m grids (ibidi, Germany). The glass bottom dishes were coated manually with collagen IV prior to seeding. The injections were done after the cells reached a confluency of 80-90%.

Chow et al. used comparable microinjection settings (capillaries with an inner diameter of 500 nm, an injection pressure and a duration of the injection equal to 150 hPa and 0.1second) and reported that these settings resulted in an injection volume of 500 fl per delivery(Chow et al., 2016). Assuming a similar injection volume in our setup, we diluted 2% w/v FluoSpheres™ (100 nm diameter yellow-green fluorescent nano-beads; Invitrogen, USA), equivalent to a concentration of 3.6×10^{13} particles/ml according to the manufacturer's description, 15,000 times in 10 mM Tris, pH 7.4 to obtain ~1 particle per 500 fl injection volume with the settings used. The resulting dilution was microinjected in cells and resulted in the expected Poisson distribution of the number of beads per injection, confirming that the volume of injection in our experiments is ~500 fl. Although the microinjection resulted in inflation of the cell, the cells rapidly recovered to their original shape and volume.

Imaging setup and configurations. Fluorescence images were taken using an ultra-sensitive custom-built inverted confocal microscope, described in detail elsewhere(Zijlstra et al., 2012) with minor modifications. In short, as excitation source, a pulsed diode laser operating at 485 nm at a repetition rate of 20 MHz (PDL800-D, PicoQuant, Germany) was used. The sample was excited and the emission was collected through the same microscope objective (Plan Apo VC, 60X, 1.2NA, Nikon). A dichroic mirror (405/488/594nm BrightLine® triple-edge laser-flat Dichroic Beamsplitter, Semrock, USA) was used to reflect the excitation light towards the objective, and to transmit the emitted light towards the detectors. The remaining excitation light in the detection path was suppressed with a long pass filter (RazorEdge®, 488 nm, Semrock, USA) and a StopLine®, 488/14 nm filter (Semrock, USA). The emission was separated into two spectral channels with a 585 nm dichroic beam splitter (T585lpxr , Chroma, USA): a green channel for detection at wavelengths shorter than 561 nm (RazorEdge®, 561 nm short pass, Semrock, USA) and a red channel for detection from 590 nm to 770 nm (590 nm long pass, Olympus, Japan in combination with a BrightLine®, 770nm short pass, Semrock, USA). The emission was spatially filtered using a 15 μ m pinhole and was focused onto a single photon avalanche diode (MPD-5CTC, PicoQuant, Germany), connected to a photon counting module (PicoHarp300, PicoQuant, Germany).

Microinjected cell samples were imaged at a rate of 1 ms per pixel at a low excitation power of $\sim 80 \text{ W/cm}^2$, to prevent bleaching. To collect enough photons, the intensities in each channel were summed over 5 consecutive images for each area of interest. Fibrils were imaged with the same settings, but with only one scan per area of interest. Autofluorescence of the SH-SY5Y cells, the non-treated cells, was imaged with a higher excitation power of $\sim 800 \text{ W/cm}^2$.

FRET analysis. To determine the FRET indices for membrane bound αS , regions of interest (ROIs) with an average of ~ 25 pixels/punctum, were defined around the high intensity puncta. These ROIs enclosed the fluorescent puncta identified in both channels, and the corresponding red to green ratio was calculated. For cytoplasmic αS , ROIs with an average of 267 pixels/ROI were drawn. The ROIs in the cytoplasm were selected to include as much diffuse signal in the cytoplasm of the cells as possible, while excluding any signal from the high intensity puncta. The histograms of the FRET index are based on average values per ROI. In the cumulative histograms the FRET index of individual pixels in the ROIs was considered and cumulated.

Membrane colocalization. As an excitation source for AlexaFluor® 647 (AF647) dye, a pulsed diode laser operating at 640 nm at a repetition rate of 20 MHz (LDH-D-C-640, PicoQuant, Germany) was used. The emission filters for the green and red channels were changed to a 550 nm band pass (BrightLine®, 550/88 nm, Semrock, USA), and a 664 nm long pass (RazorEdge®, 664 nm long pass, Semrock, USA) filter, respectively. The imaging was done sequentially using 485 nm ($\sim 80 \text{ W/cm}^2$) and 640 nm ($\sim 1 \text{ W/cm}^2$) excitation light sources. Then the puncta which appeared in the red channel image with 485 nm excitation, were photobleached by maximum power of the 640 nm laser ($\sim 1000 \text{ W/cm}^2$). After photobleaching of the acceptor, another set of images were sequentially obtained with the 485 nm and 640 nm excitation light sources.

Primary neuronal cells and staining. Cells were obtained from the cortical cerebral hemispheres of newborn (P1) Wistar rat pups as described elsewhere (Stegenga et al., 2009). The isolated cells were allowed to adhere to polyethylenimine coated coverslips (Sigma-Aldrich, USA) and cultured in 900 μl serum and antibiotics-free R12 medium (Romijn et al., 1984). All research

involving animals has been conducted according to Dutch law (as stated in “Wet op de dierproeven”), and approved by DEC, the Dutch Animal Use Committee.

The cell samples were stained using standard immunostaining protocols. In brief, after fixation and permeabilization, the autofluorescence was quenched and aspecific binding sites were blocked. For staining of α S a primary mouse anti- α S (121-125) (Santa Cruz) antibody was used in combination with an AF555 labeled secondary goat anti-mouse antibody (Invitrogen, USA). For actin staining, cells were incubated with phalloidin-Alexa Fluor®488 (Invitrogen, USA) and nuclear counterstaining was performed with DAPI. Samples were mounted in mounting medium (ibidi, Germany).

3.5. References

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Chapter 4:

α S membrane remodeling mechanisms

Cooperation of helix insertion and lateral pressure to remodel membranes*

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Abstract

Nature has developed different protein mediated mechanisms to remodel cellular membranes. One of the proteins that is implicated in these processes is α -synuclein (α S). Here we investigate if besides α S's membrane bound amphipathic helix the disordered, solvent exposed tail of the protein contributes to membrane reshaping. We produced α S variants with elongated or truncated disordered solvent exposed domains. We observe a transformation of opaque multi lamellar vesicle solutions into non-scattering solutions containing smaller structures upon addition of all α S variants. Experimental data combined with model calculations show that the cooperation of helix insertion and lateral pressure exerted by the disordered domain makes the full length protein decidedly more efficient in membrane remodeling than the truncated version. Using disordered domains may not only be cost efficient, it may also add a new level of control over vesicle fusion/fission by expansion or compaction of the domain.

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4.1. Introduction

α Synuclein (α S) is a 140 amino acid long intrinsically disordered protein (IDP) that has been associated to membrane remodeling processes, vesicle trafficking and synaptic transmission.(Nemani et al., 2010, Snead and Eliezer, 2014, Murphy et al., 2000) α S has been observed to localize at the synaptic terminal where it binds to the surface of synaptic vesicles.(Maroteaux et al., 1988, Jensen et al., 1998) At the synaptic terminal, vesicle bound α S is thought to mediate membrane fusion processes by acting as a non-conventional chaperone for the V-SNARE protein, synaptobrevin.(Burre et al., 2010) The contribution of α S to membrane remodeling may however be much more direct. The IDP α S has been reported to bind net negatively charged model membranes.(Rhoades et al., 2006, Stoeckl et al., 2008) Upon binding membranes, the ~90 amino acids at the N-terminal side of the protein undergo a disorder-to-order transition; in both *in vitro* experiments and in cells they fold into an amphipathic α -helix.(Eliezer et al., 2001, Fakhree et al., 2018) The insertion of amphipathic α -helices into one of the membrane leaflets is a well-known mechanism of generating curvature.(Campelo et al., 2008) The area difference between the inner and outer membrane leaflet that results from partial insertion of helices contributes to the curvature generating properties of proteins such as epsin(Ford et al., 2002) and endophilin.(Isas et al., 2015) Accordingly, the insertion of α S into lipid bilayers has been reported to stabilize a positive mean curvature(Braun et al., 2012) and to convert flat membranes into highly curved vesicles and tubules.(Varkey et al., 2010)

Besides the asymmetric insertion of membrane helices, the asymmetric grafting of polymers, including DNA, has been shown to generate spontaneous membrane curvature.(Nikolov et al., 2007, Lipowsky, 2002) Several membrane remodeling proteins, including epsin (Stachowiak et al., 2012), that bind membranes via amphipathic α -helices contain additional, polymer like, disordered domains. It has been argued that these long and relatively bulky, disordered domains can contribute to the curvature generating mechanism of these proteins.(Busch et al., 2015) At high surface densities, the collisions between bound proteins generate a lateral steric pressure that causes membranes to bend.(Stachowiak et al., 2012) Compared to well folded proteins of an equally long amino-acid chain, intrinsically disordered proteins (IDPs) are relatively bulky and therefore particularly effective in creating

Scheme 4.1. Cartoon of the mechanisms that may contribute to generating curvature by binding of α S to membranes. Curvature can be induced by helix insertion (α S₁₋₁₀₈) (left), and be further enhanced by lateral pressure exerted by the IDR of membrane bound α S (wt- α S) (right). The green circles indicate the volume occupied by the IDR.

lateral pressure. In this respect, the 568 and 431 amino acid long disordered adaptor domains of AP180 and epsin1 respectively efficiently drive membrane bending.(Busch et al., 2015) The C-terminal disordered domain of membrane bound α S is more than 10 times shorter, but highly negatively charged. If the lateral pressure generated by the hydrophilic domains of monomeric membrane bound proteins contributes or interferes with other curvature generating mechanisms is still under debate (Kozlov et al., 2014). If the relatively short disordered domains of membrane bound α S at physiological surface charge densities is high enough to contribute to curvature generating mechanism at all is still an open question.

4.2. Results and Discussion

To obtain insight into the contribution of the disordered domain of α S to the membrane remodeling capacity of α S, we compare the full length wild type α S (wt- α S) with a variant in which the disordered C-terminal tail is truncated (α S₁₋₁₀₈) (Scheme 4.1). To differentiate the membrane reshaping capability of the proteins we used a phospholipid vesicle clearing assay. Non extruded multi-lamellar phospholipid vesicle (MLV) solutions strongly scatter light because of the large size of the vesicles and hence appear opaque. The conversion of the large vesicles into highly curved smaller structures by interacting proteins, results in a decrease of scattering and clearing of the solution. The decrease in scattering by the formation of small, non-scattering structures, can be quantita-

Figure 4.1. a) POPG MLV clearing assay. Upon the addition of wt- α S (blue squares) and α S₁₋₁₀₈ (red circles) a reduction in OD measured at a wavelength of 500 nm is visible as a result of forming small, non-scattering structures. The OD of the control, non-treated sample (black triangles) does not change significantly in 24 hours. After addition of protein the OD was followed for 24 hours. After 24 hours the OD remained constant. b) The ellipticity at 222

nm as a function of the phospholipid concentration due to α S binding to POPG LUVs observed by CD spectroscopy. The CD spectra were obtained at a protein concentration of 25 μ M, the POPG concentration in form of vesicles was varied. Symbols show experimentally determined ellipticity at 222 nm (CD_{222nm}) as readout for membrane bound wt- α S (blue squares) and α S₁₋₁₀₈ (red circles). Black line shows the global fit to the data to determine the dissociation constant, K_d .

tively followed using UV/VIS spectroscopy and is visible as a decrease in optical density (OD).

α S preferentially binds net negatively charged phospholipid bilayers in the liquid disordered state. We therefore selected membranes composed of POPG as a model system. To test if the wt- α S and α S₁₋₁₀₈ differ in their ability to clear the MLV solution, the POPG MLVs were aliquoted into three samples. To the first two samples equimolar amounts of wt- α S and α S₁₋₁₀₈ were added respectively, while the third sample served as a control (Figure 4.1a). The change of scattering of these samples was recorded over time. While the control shows only a minor decrease in OD, the MLV solution shows an initial sharp decrease in OD in the presence of both wt- α S and α S₁₋₁₀₈. This initial decrease is followed by a slower decrease in optical density. After 24 hours we do not observe changes in OD anymore. Although both wt- α S and α S₁₋₁₀₈ are able to clear the solution, identical concentrations of wt- α S result in lower OD values. Wt- α S seems to be more efficient in MLV clearing than α S₁₋₁₀₈.

The higher clearing efficiency of wt- α S compared to α S₁₋₁₀₈ could either result from the lateral pressure exerted by the disordered tails or may be the result of a higher membrane binding affinity, and thus a higher number density of wt- α S

on the membrane surface. To test if there is a difference in membrane binding affinity for wt- α S and α S₁₋₁₀₈ we obtained binding curves for both proteins from CD spectra. In these experiments, we kept the α S concentration constant and followed the formation of α -helical structure, representing the membrane bound state of the protein, by measuring CD spectra at increasing POPG vesicle concentration (Figure 4.S1). From these CD spectra we obtained the ellipticity at 222 nm as function of the POPG concentration. With increasing concentration of POPG vesicles we clearly observe the signature of α -helix at 222 nm evidencing α S binding to the membrane (Figure 4.1b). The data points for both wt- α S and α S₁₋₁₀₈, presented in Figure 4.1b, show strong overlap and can be globally fitted with a single apparent equilibrium dissociation constant, K_d of 0.4 μ M, $n=25$ with n being the number of lipids associated to one protein. Apparent K_d values in the micromolar range are in good agreement with literature.(Shvadchak et al., 2011, Braun et al., 2014) We conclude that the observed difference in clearing efficiency does not primarily result from a difference in membrane binding affinity. Since the membrane binding affinity is identical, the protein number density on the membrane is identical for all protein concentrations and the clearing assays in the presence of either wt- α S or α S₁₋₁₀₈ can be directly compared. Considering that the membrane binding α -helical domains are identical and intact in both wt- α S and α S₁₋₁₀₈, this is not unexpected.

To further investigate the role of steric lateral pressure in membrane remodeling, we measured the OD of the MLV solution as a function of the α S concentration (Figure 4.2). For this purpose the equilibrium OD values were recorded 24 hours after addition of the protein. At low protein concentrations no clearing is observed. With increasing protein concentrations clearing sets in and levels to almost complete clearing. The OD versus protein concentration curves for wt- α S and α S₁₋₁₀₈ are similar in shape but the wt- α S curve is shifted to lower concentrations. 50% clearing is reached at 1.8 times lower concentrations for wt- α S compared to α S₁₋₁₀₈. It is observed at 3.8 μ M for wt- α S and at 6.8 μ M for α S₁₋₁₀₈. This shift to lower α S concentrations shows that the clearing capacity of wt- α S is decidedly higher.

Towards a more mechanistic understanding, we quantified the initial protein exposed membrane area in our clearing experiments. The clearing assays were performed on MLV. The inner layers of MLV are protected from binding protein by the outer layer, hence only a fraction of the total membrane surface

Figure 4.2. αS MLV clearing assay. Change in opacity of 500 μM POPG MLV solutions upon addition of wt-αS (blue squares) and αS₁₋₁₀₈ (red circles). The optical density (OD) was measured 24 hours after protein addition at a wavelength of 500 nm. At low protein concentrations, no decrease in opacity is observed, with increasing protein concentration clearing sets in. Wt-αS is more efficient in clearing than αS₁₋₁₀₈. Black lines serve as guide to the eye.

area is available for protein binding. To determine the fraction of solvent accessible membrane area at the start of the experiment, we prepared POPG vesicles labelled with NBD-PE. After measuring the initial fluorescence intensity of a solution of these vesicles, the fluorescence quencher dithionite was added. Dithionite cannot penetrate intact membranes, hence only NBD at the outer solvent accessible surface is quenched. Upon adding dithionite, we observe a 13% decrease in fluorescence intensity corresponding to 13% protein accessible membrane area (Figure 4.S2). The total concentration of lipid used and POPG head group area (Dickey and Faller, 2008) gives the total accessible surface area. The addition of the surfactant Triton-X100 results in micellization of the bilayer and a total loss of fluorescence.

At low protein concentrations the coverage of the accessible membrane surface area is low, hence no clearing of the MLV solution is observed. With increasing protein concentration the MLV surface facing the solution becomes covered with protein. Above a certain protein coverage, membrane remodeling sets in, the outer MLV bilayer breaks up into less or non-scattering small vesicles. This consumption of the outer layers results in a reduction in the observed optical density of the MLV solution and is visible as the onset of clearing. At the same time new MLV surface becomes available for binding protein. Clearing will continue until the protein is depleted from the solution. At 13% protein exposed membrane area we estimate from our clearing assay for wt-αS the onset of clearing to be ~0.22 μM while for αS₁₋₁₀₈ the onset is shifted to ~0.43 μM. The (accessible) lipid-to-protein ratio at these onset concentrations is >1000 and the inter-protein distance at the remodeling onset concentration is therefore

>10nm. For other membrane binding proteins membrane solubilization and transitions to non-lamellar phases are typically observed at an order of magnitude lower lipid-to-protein ratios (Killian et al., 1996). We therefore conclude that at the clearing onset concentrations the coverage of the accessible surface area is high enough to bend the membrane into membrane into non-scattering vesicles but too low solubilize the membrane. At lipid to protein ratios of ~ 0.1 α S has been reported to form ellipsoidal discoid-like lipoprotein particles with a diameter of 19-28 nm (Eichmann et al., 2016). With increasing concentration of α S the solubilization of the membrane into wormlike micelles or protein lipid particles therefore probably contributes to the decrease in OD. Here we will focus on the mechanism responsible for membrane remodeling near the clearing onset concentrations.

Using the measured protein exposed membrane areas and assuming that clearing follows the mechanism outlined above, we estimated the magnitude of the contributions of helix insertion and lateral pressure. We based our calculations on existing models (SI) and used the size of the amphipathic α -helix and the solvent exposed disordered domain of α S as input (SI). In our model we do not correct for the binding affinity, based on the K_d determined and the concentrations used we assume that all proteins bind to the membrane. From the calculations we obtain the radius of curvature as a function of the protein concentration. In Figure 4.3 we show the radius of curvature of the small vesicles induced by the α -helix alone, the disordered domain alone and the combined effect of helix insertion and lateral pressure. As expected the modelling shows that at low concentrations insertion of amphipathic α -helices is much more efficient in generating curvature than lateral pressure. Only at high concentrations lateral pressure is more efficient. The combined effect of helix insertion and lateral pressure further decreases the radius of curvature.

At the concentrations at which we observe the onset of clearing, at approximately 0.22 and 0.43 μ M for wt- α S and α S₁₋₁₀₈ respectively, the curvature radius amounts to about 80 and 50 nm, according to the model we use. This agrees well with the mean vesicle radii measured using DLS (Figure 4.S3) and thus confirms the validity of the assumptions made in the calculations. Small vesicles of that size would not scatter and remodeling membranes into such vesicles would thus be visible as a decrease in OD. The correspondence in the radii found at the clearing onset concentrations of both wt- α S and α S₁₋₁₀₈

Figure 4.3. Calculated spontaneous membrane curvature radii. The red line denotes the curvature generated by helix insertion. It represents α S₁₋₁₀₈ in our experiments. The dashed blue line gives the spontaneous curvature as a result of lateral pressure only, while the solid blue line represents the combined effect of helix insertion and lateral pressure. It represents wt- α S. The dashed vertical lines indicate the experimentally observed clearing onset

concentrations.

confirms that modelling and experiment agree well. Helix insertion and lateral pressure seem to synergistically contribute to curvature induction.

The model calculations predict that the clearing onset concentration depends on the available protein-exposed membrane surface area, as well as on the size of the disordered domain. To test these dependencies, we constructed an α S variant with a much larger disordered domain by quadruplicating the C-terminal 111-140 amino acids of the full length protein (α S_{4xC}). After confirming that the presence of the larger disordered domain did not affect the membrane binding affinity of the protein (Figure 4.S4), we performed clearing assays on MLV preparations with different protein exposed surface areas. We performed clearing assays with all three protein constructs for 32% and 50% protein exposed membrane area. As expected, the larger exposed area results in a shift of the clearing onset concentration to larger values, while the larger disordered coil results in a lower clearing onset concentration (Figure 4.S5). All experimental data and model predictions are combined in Figure 4.4.

In Figure 4.4 we plot the calculated protein concentration required to obtain vesicles with a radius between 50 and 100 nm for the three protein constructs as a function of the available membrane surface area. To this graph, we added the experimentally observed clearing onset concentrations. For α S₁₋

¹⁰⁸ and wt- α S the clearing onset concentrations mostly fall into the protein concentration range for which vesicles with a radius between 50 and 100 nm are

predicted. Interestingly, the α S_{4xC} clearing onset concentrations clearly fall outside this size range. The clearing onset is found at α S_{4xC} concentrations for which our model predicts much larger vesicle radii than for the clearing onset concentrations of α S₁₋₁₀₈ and wt- α S. This either indicates that larger vesicles are formed, or, it could mean that the disordered part of α S_{4xC} does not behave as a self-avoiding random walk. Intra-chain interactions could result in a non-spherical shape of the disordered domain and most likely exist within the C-terminal region of the protein (Dedmon et al., 2005). One can imagine that when the longest dimension of this disordered domain is preferentially oriented parallel to the membrane surface clearing will set in at lower concentrations than predicted by our much simplified model.

Translating our *in vitro* findings to the function of α S *in vivo* we consider known interactions of α S with vesicles in cells. The synaptic and endocytic vesicles found in neurons have been shown to bind α S and have a radius between 20 and 40 nm. Our model calculations estimate the number of wt- α S protein molecules required to obtain vesicles of 30 nm to be 75. This number fits surprisingly well in the number distribution of α S-GFP on single vesicles in cells. (Fakhree et al., 2016) At the same time the coverage is low enough (~30%) to guarantee accessibility of other membrane proteins and functional interactions.

Summarizing, we conclude that the combined effect of helix insertion and lateral pressure synergistically contribute to curvature induction. Considering that disordered protein domains occupy considerably larger volumes than folded proteins of the same length, the combined action of amphipathic α -helix insertion and lateral pressure may represent efficient use of material. Longer helices or a higher number of helices may generate the same effect as the disordered domains but because of their compactness require longer amino acid chains. The failure of our simplified model to include the behavior of α S_{4xC} indicates that additional factors such as intramolecular interactions encoded in protein sequence may have to be accounted for. These encoded interactions may also add to the biological function of the protein. An external trigger may induce compaction or expansion of the disordered domain, thereby releasing lateral pressure thus assisting vesicle fusion/fission events.

4.3. Supporting Information

Figure 4.S1. Binding of wt- α S to POPG vesicles monitored with CD spectroscopy. The CD spectra show the conformational transition of wt- α S from a random coil to a α -helix upon increase of the POPG concentration. This change in conformation reflects the transition from the random coil conformation of the protein in solution to the (partially) α -helical form in the membrane bound state.

Figure 4.S2. NBD-PE fluorescence quenching experiment to determine the exposed, outer MLV area. The fluorescence NBD-PE in POPG MLVs was quenched by the addition of dithionite. The resulting relative drop in fluorescence intensity, here 13%, reflects the percentage of NBD-PE, and thus lipids, present in the most outer leaflet of the MLV. The addition of Triton X-100 results in dissolution of the

bilayer and complete quenching of the fluorescence.

Figure 4.S3. Size distribution obtained from dynamic light scattering experiments on POPG MLVs exposed to α S₁₋₁₀₈ (red), wt- α S (blue), α S_{4xC} (green). The DLS intensity distributions of vesicle diameters were obtained at a final POPG concentration of 15 μ M and a α S concentration of 0.3 μ M.

Figure 4.S4. The CD signal at 222 nm as a function of the phospholipid concentration to follow α S binding to POPG vesicles. The CD spectra were obtained at a protein concentration of 25 μ M, the POPG concentration in form of vesicles was varied. Symbols show experimentally determined ellipticity at 222 nm as readout for membrane bound α S₁₋₁₀₈ (blue), wt- α S (red), α S_{4xC} (green). Black line shows the global fit to the data to determine the dissociation constant, K_d .

Figure 4.S5. Change in opacity of a 500 μM POPG MLV solution upon addition of α S. The optical density (OD) was measured 24 hours after protein addition at a wavelength of 500 nm. At low protein concentrations, no decrease in opacity is observed, with increasing protein concentration clearing sets in. a) MLV clearing assays for α S₁₋₁₀₈ at 13% (●), 32% (○), and 50% (○) protein exposed MLV area. With increasing exposed surface area the clearing curves shift to higher α S concentrations. b) MLV clearing assays for α S1-108 (red), wt- α S (blue), α S4xC (green) at an exposed MLV area of 32%. With increasing size of the disordered domains the clearing curves shift to lower α S concentrations. In both figures the black lines serve as a guide to the eye.

4.4 Materials and Methods

Preparation of alpha synuclein. α S₁₋₁₀₈, wt- α S, and α S_{4xC} were expressed in Escherichia coli strain BL21(DE3) using the pT7-7 expression plasmid and wt- α S and α S_{4xC} were purified as previously reported (van Raaij et al., 2006). The α S₁₋₁₀₈ (NH₄)₂SO₄ pellet was dissolved in 50 mM glycine buffer pH 3.3 and purified on a Resource S cation exchange column (GE healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK). To quadruplicate amino acids 111-140 of the full length protein and create the α S_{4xC} construct we made use of the Apol restriction site that is present in the unstructured C-terminal tail of the protein. The unstructured tail C-terminal tail of α S was extended in two steps. First amino acids 111-140 of wt- α S C-terminal tail were multiplied with PCR and Apol restriction sites were created. The PCR fragment was cut with Apol and ligated into the pT7-wt- α S plasmid using the corresponding restriction site. This resulted in the α S_{2xC} construct. The last of the now two Apol restriction sites was subsequently removed by mutagenesis and the 2x 111-140 amino acid construct of α S_{2xC} was multiplied with PCR and an Apol restriction site was created. This PCR fragment was again cut with Apol and ligated into the pT7- α S_{2xC} plasmid using the corresponding restriction site.

Before doing the experiments, the freshly thawed α S solutions were spin-filtered using prewashed Pierce® Spin-Cups filters (Thermoscientific, Rocford, IL, USA) at 3000g for 5 minutes at 4 °C (IEC MicroMax RF, Needham Heights, MA, USA). Next, the concentration of the filtered alpha synuclein was determined using UV/Vis absorption (Nanodrop ND-1000, Thermofisher Scientific Inc., USA) with known extinction coefficients of 5600 M⁻¹ cm⁻¹ and 1400 M⁻¹ cm⁻¹ for wt- α S and α S₁₋₁₀₈ at 276 nm, respectively. For clearing assays dilutions were done with HEPES buffer (20 mM HEPES, 10mM NaCl, pH 7.4). For CD measurements, dilutions were done with modified PBS buffer (Na₂HPO₄ 10mM, KH₂PO₄ 1.8 mM, NaCl 13.7 mM, KCl 2.7 mM, pH 7.4). In order to prevent non-specific binding of α S to microtubes, which can alter the effective concentration, Protein LoBind tubes (Eppendorf, Germany) were used.

MLV and LUV preparation. POPG (1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA), aliquots were prepared and dried under nitrogen flow. For preparing POPG MLVs, the protocol from the producer is used. In short, 76 μ l of

10 mg/ml chloroform solution of the lipid were added into 1ml chloroform under nitrogen flow. By rotating the glass tube under nitrogen flow, the lipid solution was dried as even layer on the inner wall of the glass tube. This resulted in 0.76 mg lipid films. In the next step for rehydration of the lipid films, 1 ml HEPES buffer for clearing assays, or 1 ml modified PBS buffer for CD measurements, were added to the dried lipid layers under nitrogen flow. Following the addition of the aqueous buffer, the cap of the glass tube was sealed and vortexed 5 times at maximum shaking power for 1 minute using Vortex Genie 2 (Scientific Industries Inc., Bohemia, NY, USA). This resulted in a final concentration of 1 mM POPG MLVs for the clearing assays. For the CD experiments 4mM POPG MLV solutions were prepared.

For preparation of 100 nm LUVs, the content of the glass tube went through a 10 times freeze-thawing cycles. This made the suspension less turbid and more homogenous. Next, extrusion is used to make the 100nm LUVs. This was done by extruding the suspension of POPG vesicles through a polycarbonate Nuclepore™ membrane filter (Whatman®, GE Healthcare, USA) with pore size of 100 nm, with two drain discs as filter supports (Whatman®, GE Healthcare, USA), located inside a manual extruder (Avanti Polar Lipids Inc., Alabaster, AL, USA). In order to make sure that the final size of the LUVs was homogenous, the extrusion done 21 times. Size distribution of the LUVs was checked by dynamic light scattering (Zetasizer Nano ZS, Malvern Panalytical Ltd., UK) and found to be a narrow size distribution centered at 100nm.

Clearing assay. To a final concentration of 500 μ M POPG MLVs, dilutions of α S were added, resulting in the final concentrations in the clearing S-curves. For the time dependent experiments, immediately after addition of the protein, the mixture was transferred to a 1cm path length, 60 μ l quartz cuvette, and optical density (OD) was measured at 500 nm using a UV-Vis spectrophotometer (UV-2401PC, Shimadzu, Japan). The slit size of 1nm was used to collect the data. After measuring the OD values for the first 5 minutes, the sample was transferred back to the low-bind microtube and stored at 4 °C. Finally, after 24 hours the OD values were measured as the end points of the clearing assays.

Dynamic light scattering. Dynamic light scattering experiments were performed using Zetasizer Nano ZS (Malvern Panalytical Ltd., UK) with a laser

beam of $\lambda = 632.8$ nm at back scattering mode. Refractive index of the sample was set at 1.4, and absorbance was set to zero. Experiments were performed at fixed temperature of 25 °C. Disposable polystyrene cuvettes were used for each sample. Size distributions were calculated using Zetasizer software from sample measurements with at least 10 sub runs per each measurement.

CD measurements. A Jasco J-1500 Circular Dichroism Spectrometer (Jasco Inc., Easton, MD, USA) was used to perform the measurements. Spectra were measured in the wavelength range of 190 to 260 nm, step size of 1nm, bandwidth of 1nm, dwell time of 1 s/step, and averaging of 8 scans per sample with 1mm path length at 21 °C. Samples of αS (final concentrations of 25 μM) with 100nm LUVs (final concentrations of 0, 6.5, 11.4, 20, 37, 65, 114, 200, 370, 650, 1140, and 2000 μM) in the modified PBS buffer were mixed and after 2 hours the corresponding CD spectra were measured.

To obtain the apparent equilibrium dissociation constant K_D , the fraction of vesicle bound protein X_B as a function of the POPG concentration was determined from the measured ellipticities at 222 nm.

$$X_B([L_{total}]) = \frac{[\alpha S L_{bound}]}{[\alpha S_{total}]} = \frac{ellipticity_{observed} - ellipticity_{initial}}{ellipticity_{final} - ellipticity_{initial}}$$

In this expression the initial and final values refer to the plateau ellipticities at low and high lipid concentrations respectively. Assuming that the equilibrium binding can be described with the following reaction:

where αS represents the free αS concentration, L the free lipid concentration, n the number of lipids associated with a single protein, and $\alpha S L_{bound}$ the concentration of lipid bound protein, the fraction bound can be described in terms of the known total concentrations of lipid (L_{total}) and αS (αS_{total}) following the law of mass action. In this equation we assume that all lipid-binding sites are equivalent, we do not take into account any cooperativity in binding (Boersma et al., 2015, Iyer et al., 2014).

$$X_B = \frac{(K_D + [\alpha S_{total}] + \frac{[L_{total}]}{n}) - \sqrt{(K_D + [\alpha S_{total}] + \frac{[L_{total}]}{n})^2 - \frac{4[\alpha S_{total}][L_{total}]}{n}}}{2[\alpha S_{total}]}$$

Fitting the measured $X_B([L_{total}])$ to this equation gives K_D and n .

Dithionite experiment. Dithionite experiments were done as described elsewhere (McIntyre and Sleight, 1991). In short, POPG MLVs were prepared with 1% (mol/mol) embedded NBD labeled PE (Avanti Polar Lipids Inc., Alabaster, AL, USA) and diluted to final POPG concentration of 10 μ M. Next, using a fluorescence spectrophotometer (FluoroMax-4, HORIBA Jobin Yvon, Edison, NJ, USA) the sample was excited at 470 nm and fluorescence collected every second at 540 nm. After measuring the emission for a short time to see that fluorescence is not changing, dithionite was added to the sample to a final concentration of 10mM. The drop in fluorescence was followed in time. Following reaching a steady fluorescence signal, Triton X-100 was added to the sample to a final concentration of 1.25% w/w which resulted in complete dissolution of the lipid layers, and consequently 100% quenching of the NBD dyes.

Calculations. To obtain an estimate for the radius of gyration of the highly negatively charged solvent exposed disordered domain of membrane bound α S we calculated the Flory radius of a sphere from a self-avoiding polymer chain in good solvent. With a diameter of 0.5 nm and a Kuhn length of 1 nm, the properties of this polymer chain aim to reflect the properties of the amino acid chain. We assumed that the disordered domain of membrane bound wt- α S comprises 48 amino acid residues (Ulmer et al., 2005) while the construct with the elongated the disordered domain was 139 amino acids in length. For the disordered domains of wt- α S and α S4xC this resulted in radii of 3.9 and 7.5 nm respectively.

The contribution of steric lateral pressure to the spontaneous curvature was calculated following the method reported by Stachowiak et al. with a small modification (Stachowiak et al., 2012). Instead of assuming that lateral pressure resulted in the formation of membrane tubules, we argue for the formation of vesicles. The pressure exerted by the spherical disordered domain of α S is isotropic hence we adjusted the expression for the free energy to account for the appearance of vesicles. This results in the following expression for radius of the vesicles:

$$R = \frac{4K_{bend}}{p\sigma} - \frac{\sigma}{2}$$

In this expression the membrane's bending rigidity, K_{bend} , was assumed to be $10 k_B T$, σ represents 2x the radius of gyration of the disordered domain, and p is the pressure obtained from the Carnahan-Starling equation (Stachowiak et al., 2012).

$$p = \frac{\eta}{\sigma^2 \pi} \left[1 + 2\eta \frac{1-0.44\eta}{(1-\eta)^2} \right] k_B T$$

Since p is a function of the fraction of the membrane area covered by proteins (η) this term contains the dependence of R on protein concentration.

To obtain an estimate for the (contribution of) helix insertion to the spontaneous curvature we took into account the area difference between the inner and outer membrane leaflet of a spherical vesicle as a result of helix insertion. We determined the spontaneous curvature from the ratio between the inner and outer membrane as a function of the protein concentration. In our calculations we did not account for the equilibrium dissociation constant (which was measured to be the same for all constructs used) but assumed that all proteins were membrane bound. To obtain the spontaneous curvature we estimated the area occupied by the helix to be 15 nm^2 (Braun et al., 2012) and assumed the POPG head group area and bilayer thickness to be 0.66 nm^2 and 3.7 nm respectively (Kucerka et al., 2012).

4.5. References

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Chapter 5:

α S and the endocytic pathway

The localization of alpha-Synuclein in the endocytic/exocytic pathways

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Abstract

Alpha-synuclein (α S) is an intrinsically disordered protein, involved in synucleinopathies, but its exact cellular function is not known. The observed *in vitro* membrane remodeling by α S along with studies using various biochemical methods suggest that α S might be involved in cellular events such as endocytosis, exocytosis, and autophagy. Here we studied the involvement of α S in the endo/exocytic pathways. As a first step, the colocalization between α S and a selection of endo/exocytic markers, namely clathrin, caveolin, EEA1, Rab5, Rab7, and Rab11 is investigated. We find colocalization of α S with caveolin and EEA1, and to less extent with Rab7 and Rab11. We do not observe significant colocalization with clathrin and Rab5. Based on the observed pattern of colocalization between α S and the studied endocytic markers, we suggest that α S is involved in endocytic uptake of a specific receptor, such as transferrin receptor.

5.1. Introduction

Even though alpha-synuclein (α S) is considered a major player in diseases collectively known as synucleinopathies, the exact cellular function of this elusive intrinsically disordered protein (IDP) remains unknown. *In vitro* experiments have shown that the N-terminal part of α S undergoes a disordered-to-helix transition upon membrane binding. Following the structure-function dogma of biochemistry, this may imply that the functional state of α S is the membrane bound state. This is supported by the observation that α S possesses membrane remodeling capacities, it can induce positive mean and/or negative Gaussian curvatures in phospholipid bilayers (Braun et al., 2012) (see Appendix Curvature for more details). Adopting a combination of a positive mean and negative Gaussian curvature is necessary in specific stages of cellular vesicle formation and fusion events. Accordingly, there are a number of studies suggesting that α S is involved in cellular processes that require membrane remodeling, such as formation, processing, and coalescence of trafficking vesicles (Xilouri et al., 2016, Lautenschlager et al., 2017, Burre, 2015).

Membrane tubulation, budding, invagination, and fusion are the most common membrane remodeling events required for the formation of trafficking vesicles and their later coalescence with other membrane bound compartments. These membrane remodeling events require the generation of curvature. The formation, maintenance, and release of membrane curvature is attained using various cellular mechanisms including the asymmetric insertion of amphipathic protein domains (e.g. epsin N-terminal homology domain (Ford et al., 2002)), scaffolding (e.g. clathrin (Rejman et al., 2004)), and the generation of lateral steric pressure (e.g. epsin C-terminal random coil domain (Busch et al., 2015)). An overview of cellular mechanisms that are involved in membrane curvature generation are shown in Appendix Curvature. In the case of the IDP α S, a combination of two mechanisms, amphipathic insertion and the generation of lateral pressure are responsible for its role in membrane remodeling processes (Chapter 2, 4).

Trafficking vesicles, which include vesicles found in endocytic/exocytic pathways, enable the communication between membrane enclosed compartments inside the cell. At the plasma membrane these vesicles are responsible for receptor/ligand uptake. After uptake, vesicles evolve into early/sorting/recycling endosomes, late endosomes/lysosomes/phagosomes,

and are used to traffic material to/from the Golgi apparatus, endoplasmic reticulum, and mitochondria. Interestingly, there are numerous reports pointing towards a function of α S in endocytic/exocytic pathways(Lautenschlager et al., 2017). In this respect, it has been suggested that α S regulates endocytic/exocytic pathways(Lautenschlager et al., 2017). Most research effort into unravelling the role of α S in endo- and exocytosis has focused on the regulation of the synaptic vesicle pool. However, α S is probably involved in more membrane trafficking pathways than the regulation of the synaptic vesicle pool(Barbour et al., 2008). Nevertheless, with which specific pathways α S is associated and how α S regulates membrane remodeling processes is not well understood. Important open questions include: Is α S involved in the vesicular uptake/secretion(Lautenschlager et al., 2017, Baksi et al., 2016)? Does α S interact directly with endocytosis/exocytosis specific proteins such as Rab proteins(Lautenschlager et al., 2017, Hasegawa et al., 2011)? Or does α S indirectly regulate specific pathways via the sensitivity of the α S-membrane interaction on membrane composition(Snead and Eliezer, 2014, Jo et al., 2000, Narayanan et al., 2005)? Or is the regulation an outcome of an α S-dependent downstream signaling pathway(Narayanan et al., 2005, Schnack et al., 2008)? In order to take a first step towards understanding the role of α S in the endocytic pathway, we investigated the colocalization between α S and vesicle bound proteins that mark specific stages of the endocytic pathway in differentiated SH-SY5Y cells by immunostaining against α S and endocytic markers. Fluorescence confocal laser scanning microscopy was used to collect images for colocalization analysis.

Vesicular endocytosis starts at the plasma membrane where membrane is internalized by splitting off small vesicles. This vesiculation process is enabled by membrane modulating proteins. Clathrin and caveolin mediated endocytosis are two of the major receptor mediated vesicular membrane uptake routes that are enabled by membrane modulating proteins(Rejman et al., 2004). In clathrin mediated endocytosis, a cage of clathrin triskelia assembles on the inner plasma membrane. The cage is linked to the membrane via adaptor proteins. This indirect membrane bending mechanism is known as scaffolding, and causes a vesicle to bud into the cytosol(Rejman et al., 2004). Unlike clathrin, the protein caveolin bends membranes directly, by partial insertion of amphipathic residues into the inner leaflet of the plasma membrane. Furthermore, it has been suggested that membrane inserted caveolin proteins oligomerize and work as an

adaptor for scaffolding proteins such as cavins(Cheng and Nichols, 2016, Chaudhary et al., 2014, Ludwig et al., 2016). In our α S colocalization study, we chose clathrin and caveolin as markers for different vesicular endocytosis routes (see Scheme 1).

Once the clathrin/cavin coated vesicles are budded from the plasma membrane as endocytic vesicles, known as endosomes, their coat disassembles. The disassembly of the coat is known as uncoating. After uncoating, the lipid and protein composition of the outer membrane of the endosomes undergoes a series of changes, for example the Rab5 and phosphatidylinositol (PI) content increases significantly(He et al., 2017). Subsequently increased Rab5 on these early endosomes increases phosphorylation of PI, which results in an increased phosphatidylinositol 3-phosphate (PI3P) content of the early endosome. The increased PIP3 content of the early endosomes results in binding of proteins such as early endosome antigen 1 (EEA1), which is an effector protein of Rab5(Christoforidis et al., 1999). With this in mind, we selected Rab5 and EEA1 as markers for early endosomes (see Scheme 1). The presence of both Rab5 and EEA1 on early endosomes, enables them to bring two early endosomes close together which facilitates SNARE mediated vesicle fusion, and results in an increase in the radius of the early endosomes. Changes in size along with adaptations in the lipid and protein content of the early endosomes outer membrane, determines further interactions between early endosomes and motor-proteins, which results in sorting of the cargo of the early endosome to the correct destination inside the cell. Depending on the Rab protein content, the early endosomes will subsequently be sorted towards recycling endosomes or late endosomes by motor proteins(Zerial and McBride, 2001).

The recycling endosomes are used to recycle membrane and receptor proteins. An example of a specific Rab protein found on recycling endosomes is Rab11. Depending on the Rab11 interacting adaptor proteins on the endosome, three different motor proteins can bind to the vesicle(Campa and Hirsch, 2017, Welz et al., 2014). Starting from sorting endosomes, in the presence of adaptor protein protrudin, GDP bound Rab11 interacts with a kinesin-like motor protein, KIF5, that moves towards the cell periphery, resulting in the directional transport of early endosomes towards the plasma membrane(Matsuzaki et al., 2011).If the adaptor protein is FIB2 instead of protrudin and Rab11 is GTP bound, myosin-V

Scheme 5.1. Schematic representation of possible endocytic/exocytic routes for a cargo. As explained in the main text, clathrin, caveolin, EEA1, Rab5, Rab7, and Rab11 can be used to mark major parts of endocytic/exocytic pathways. See Scheme 5.S1 for a chronological changes in these markers on endosomes.

which moves on actin filaments binds to Rab11 along with adaptor proteins, and moves towards the plasma membrane (Pylypenko et al., 2016). Another slower recycling route happens when the adaptor protein is FIB3, then GTP bound Rab11 interacts with a dynein motor protein that moves on microtubules towards the peri-centrosomal recycling endosomes. After cargos are pooled in the peri-centrosomal recycling endosomes, they are sent back towards plasma membrane via myosins or motor proteins of the kinesin super family (Horgan et al., 2010). As outlined above, Rab11 can be used as a marker in colocalization studies to visualize most of the recycling endosome species (see Scheme 1).

Late endosomes are the result of maturation of early endosomes. During this process, the membrane protein composition of the endosomes changes and the cargo is exposed to degrading pH and enzymes. The surface concentration of the protein Rab5 decreases while an increase in the protein Rab7 is observed.

Rab7 is a GTPase protein that facilitates binding of the endosome to the motor proteins dynein or kinesin which depends on the adaptor proteins(Wijdeven et al., 2015, Hyttinen et al., 2013). Dynein transports late endosomes toward lysosomes where the cargo will be degraded, while kinesin transports multi-vesicular bodies (MVBs) towards the plasma membrane where the cargo is secreted. Here we used Rab7 antibodies to mark the presence of late endosomes (see Scheme 1).

As outlined above, endosomal vesicles go through a series of membrane remodeling events. Since α S is a membrane remodeling protein, and biochemical experiments indicate it may be involved in several parts of the endocytic pathway, we studied the colocalization between α S and caveolin, clathrin, EEA1, Rab5, Rab7, and Rab11 to pinpoint in which endocytic pathway α S plays a role. When there is direct interaction and/or structural association between α S and endocytic markers, this should be visible as colocalization of the respective proteins in immunostained cells. The other way around, the absence of colocalization rules out direct interaction or structural association between α S and the endocytic marker. This is a simple, yet crucial step towards understanding cellular localization and function of α S.

5.2. Results and discussion

Differentiated SH-SY5Y cells were used as neuronal cell model. Immunostainings were performed for both α S and each of the studied endocytic markers, separately. Images were taken as three-color z-stacks from approximately 10 distinct fields of view. Each field of view contained a few cells. In total 40-60 cells per marker were imaged and analyzed. As it can be seen from Figure 5.1, the fluorescence signal of the immunostained samples appears as low intensity diffuse cytosolic background and high intensity punctuated patterns inside cells. The size of the puncta varies from diffraction limited to 1 to 2 μ m. For Rab11 and EEA1, and to some extent Rab7 markers, the bigger structures appear in close proximity to nuclei. The primary antibody against α S recognizes an epitope in the C-terminus of α S, hence it can bind to free and membrane bound α S, and based on our previous work(Fakhree et al., 2018, Fakhree et al., 2016) we assume that the diffraction limited puncta of α S represent vesicles (α S⁺ vesicles) while diffuse background signal represent free α S (Figure 5.1).

Since we are looking at the colocalization of two proteins on a single vesicle, the close proximity of these proteins may result in Förster resonance energy transfer (FRET). We used secondary antibodies labeled with AlexaFluor 488 and AlexaFluor 568, which are known as a FRET pair (see **Chapter 3**). In this respect, it is important to determine if there is FRET between the two fluorophores, before looking into colocalization. For example, in the previous chapters we observed efficient FRET between fluorescently labelled α S and the membrane marker WGA (Fakhree et al., 2018, Fakhree et al., 2016). In this chapter, however, the proteins of interest are visualized using primary and fluorescently labelled secondary antibodies. Each of these antibodies have a size of ~ 10 - 15 nm, which adds up to a structure with height of ~ 20 - 30 nanometers above the surface of the antigens. With an $R_0 = 6.2$ nm for the AF488 and AF568 dye pair, the FRET efficiency between the labeled antibodies would thus be close to zero (see Appendix FRET for more details). The absence of FRET between the immunolabeled proteins was investigated by excitation of the donor dye (AF488) and looking to emission channel of the acceptor dye (AF568) (data not shown). In this experiment no FRET was observed and we therefore conclude that FRET does not affect the imaging of the different structures after immunolabeling, and consequently this labeling strategy can be used to quantify the colocalization between α S and endocytic markers.

In a first visual inspection of the images, we can see that the degree of colocalization varies from partial colocalizing channels to the almost complete absence of colocalizing signals. Whereas signal of the individual proteins is visible in green and red, colocalization is depicted in yellow. For all proteins, studied cells contain vesicles on which only one of the proteins of interest can be observed. For α S and caveolin, cells additionally contain vesicles on which these proteins colocalize, whereas vesicles that contain α S, almost never contain clathrin. For α S and EEA1, a few vesicles on which the proteins colocalize are visible. In the case of α S and Rab proteins, there is some but not much colocalizing signal between the channels. From the images it is clear that the overlap between the individual endocytic markers and α S is not 0% or 100%. Although visual inspection allows for a quick and qualitative assessment of colocalization, clearly insight into the role of α S in endocytosis requires a more reliable way to assess the amount of colocalization.

Figure 5.1. Overview of images of immunostained SH-SY5Y cells against α S and endocytic markers. In these images the following color code was used: α S (Channel 1, green) and endocytic markers (Channel 2, red), and DAPI counterstaining for nuclei (blue). Images are maximum z intensity projections which are brightness/contrast adjusted to visualize both the diffuse signal and fluorescent puncta. The fluorescence signal for α S (A, D, G, J, M, and P), caveolin (B), clathrin (E), EEA1 (H), Rab5 (K), Rab11 (N), and Rab7 (Q) appear as a combination of a diffuse and punctuated pattern throughout the cells. The colocalization between α S and endocytic markers appears in yellow in the merged images. Even though z intensity projection is a very simple way to depict the data, which gives the first insight on the signal distribution, diffused and punctated, laterally and axially throughout the cell, however this presentation has limited use: only usable for low density of structures. For high density of structures, z intensity projections might be misleading for colocalization. For this reason, orthogonal cross sections are presented in the remaining figures. Scale bars are 10 μ m.

To better assess the possible colocalization or exclusion between α S (channel1) and endocytic markers (channel2), we constructed scatterplots. In colocalization studies a scatterplot is a 2D histogram of the fluorescence intensity values of channel one (axis x), versus the corresponding fluorescence intensity values of the other channel (axis y), per pixel. If two molecules fully colocalize with each other, the scatterplot of the two colocalizing channels will appear as points on a $y = ax$ line. However, due to noise and the stochastic nature of the distribution of fluorophores on vesicles in cells, the scatterplot of two colocalizing molecules appears as dots scattered around this line. In **Chapter 3**, we presented an example of this colocalization trend in Figure 3.2. In the case that vesicles either contain α S or the endocytic marker, and the presence of one completely excludes presence of the other, the scatterplot will appear as two sets of points on the x and y axis. Once more, as mentioned above, due to noise of imaging and the stochastic nature of the distribution of the membrane bound protein in cell samples, an exclusion will appear as “clouds” stretched in the direction of the x ($y = b$) and y ($x = c$) axis (Figure 5.2 Clathrin). For two molecules that interact or structurally associate with each other on a fraction of the vesicles, the scatterplot will be something between these two extreme cases (Figure 5.2 α S-Caveolin).

The cells shown in Figure 5.1 contain vesicles that contain either one of the protein species and vesicles on which both proteins are presents. The scatter plots are therefore expected to contain both the signatures of exclusion and colocalization (Figure 5.2, α S-EEA1). Colocalization of proteins, visible as a correlation between the fluorescence intensities of α S (Figure 5.1, Channel1) and endocytic markers (Figure 5.1, Channel2) is most convincingly visible for caveolin and EEA1 compared to the other studied endocytic markers. For clathrin and Rab5, most fluorescence intensity is observed parallel to the x and y axis, this suggests that there is no colocalization of α S with clathrin and Rab5. For Rab7 and Rab11, the fluorescence intensities of both channels are scattered somewhat in the middle part of the quadrant. It is however not clear if this is a result of functional colocalization of the two proteins or coincidentally overlapping of the signals. By looking at the scatterplots, it is hard to draw conclusion on the degree of colocalization. There is no clear correlation between α S and any of the studied markers. Even though scatterplots are a useful tool to visualize highly colocalizing/correlating signals, like Figure 3.2 between α S and membrane marker, it is qualitative and becomes complicated in the case of

Figure 5.2. Scatterplots. Typical scatterplots from a middle slice of z-stack images for each studied marker. In the case of α S-Clathrin and α S-Rab5, it appears that scatterplots showing exclusion. For α S-Caveolin and α S-EEA1, it appears that a fraction of signals are correlating with each other. Finally, no clear correlation/exclusion between signals of α S-Rab7 and α S-Rab11 is visible.

mixed populations/events. This makes the quantitative assessment of the colocalization problematic. In order to avoid the correlation problem of scatterplots, the amount of the colocalization is quantified using the overlap between two channels.

Manders' colocalization coefficients ($m_{Chan.1-Chan.2}$ and $m_{Chan.2-Chan.1}$) are used as a tool to quantify the amount of intensity-weighted overlap between two channels. The intensity-weighted fraction of *non-zero* pixels of the channel 1 which overlaps with *non-zero* pixels of the channel 2, is $m_{Chan.1-Chan.2}$. The intensity-weighted fraction of *non-zero* pixels of channel 2 which overlaps with *non-zero* pixels of the channel 1, is $m_{Chan.2-Chan.1}$. Since most of the times the signal distribution of the channel 1 is different from the signal distribution of the channel 2, $m_{Chan.1-Chan.2}$ and $m_{Chan.2-Chan.1}$ values are not necessarily same numbers. A mathematical description is presented in Materials and Methods section, and a schematic example is presented in the Appendix Manders.

Looking at the definition, it becomes clear that $m_{Chan.1-Chan.2}$ and $m_{Chan.2-Chan.1}$ are sensitive to a *non-zero* background signal in the images, such as the diffuse cytoplasmic α S in the SH-SY5Y cells. In this respect, it is critical to remove the background signal prior to the determination of $m_{Chan.1-Chan.2}$ and $m_{Chan.2-Chan.1}$. For

that purpose, the images were thresholded using their corresponding puncta masks (see Materials and Methods for details). Even though the thresholding levels were chosen to include as much signal from puncta as possible, this might cut off the low intensity signal of the structures at the flanks of the intensity distributions, and thus affect colocalization. However, in order to use Manders' colocalization coefficients, the background has to be filtered out, thus the low intensity signal cut-off is unavoidable. In other words, the calculated $m_{Chan.1-Chan.2}$ and $m_{Chan.2-Chan.1}$ values might underestimate the actual colocalization.

Since we want to look at the amount of overlap between signals of two channels representing vesicle bound proteins, and the images are crowded with vesicles, random overlap is likely to occur (Figure 5.1). This random "filling" effect should be taken into account. To ensure that the measured amount of

Figure 5.3. Effect of signal density on the amount of random colocalization. Lower signal density per cell area (top panel) results in less random colocalization than higher signal density per cell area ($m_{Chan.1-Chan.2 Rand}=0.05$ vs $m_{Chan.1-Chan.2 Rand}=0.49$). Random re-distribution of fluorescence signal inside the cell area can be seen in the right panel images, while the original images are shown in the left panel. Assuming that the punctuated pattern is a special case of spatial intensity distribution for both images, signal randomization was performed by re-distributing the pixels, and the puncta were not treated as separate objects. Note that for clarity, the images are shown as masks (1bit images), but m values are intensity weighted.

colocalization was not caused by random placement of puncta in the cell, pixel intensity values of masked channels were randomly distributed over the area of the cell. Next, the $m_{Chan.1-Chan.2}$ and $m_{Chan.2-Chan.1}$ for the randomized image were determined as $m_{Chan.1-Chan.2 Rand.}$ and $m_{Chan.2-Chan.1 Rand.}$, and compared to $m_{Chan.1-Chan.2}$ and $m_{Chan.2-Chan.1}$ of the original images. The result of randomization of images is shown for two sample images, with very dilute and crowded vesicle densities (Figure 5.3).

In agreement with expectations, the higher puncta density result in a larger random overlap. This shows that the calculated overlap between two channels should always be compared with the amount of overlap in the random signal distributions in the cell which is factor of signal density per cell area.

Is α S involved in the vesicular uptake?

In the case of clathrin images, thresholding was not enough to effectively remove the non-homogeneous diffuse cytoplasmic signal. To remove the non-homogenous background we therefore, first subtracted the median filtered image from original image(Dunn et al., 2011), and then applied a threshold to make a mask for clathrin puncta. Next, using this mask we calculated the level of colocalization between α S and clathrin to be $m_{\alpha S-Clathrin} = 0.06$ and $m_{Clathrin-\alpha S} = 0.12$, and random colocalization of $m_{\alpha S-Clathrin Rand.} = 0.03$ and $m_{Clathrin-\alpha S Rand.} = 0.06$ (see Table 1 for statistics). Comparing the distributions of $m_{\alpha S-Clathrin}$ and $m_{Clathrin-\alpha S Rand.}$ (Figure 5.4 B, C), we conclude that most of the observed colocalization can be attributed to random signal overlap. In the same way, most of $m_{Clathrin-\alpha S}$ is due to random colocalization (Figure 5.4C).

According to the literature, α S is involved in receptor mediated endocytosis(Lautenschlager et al., 2017, Cheng et al., 2011) and more specifically in endocytosis of the transferrin receptor(Baksi et al., 2016). In the papers cited above it is argued that this receptor mediated endocytosis is clathrin dependent, we therefore expected to observe colocalization between α S and clathrin. This inconsistency between our observations and literature reports might have been caused by the fact that not all of the receptor mediated endocytosis happens via a clathrin dependent route. For example, a clathrin-independent pathway has been reported for the endocytic uptake of the transferrin receptor(Aoki et al., 2007). α S may be involved in this clathrin independent receptor mediated

uptake pathway. Furthermore, if the interaction between α S and clathrin is very short lived and happens in scarce parts of the cell, the fraction of colocalizing structures might be so low that it will appear as non-colocalizing signals. For example, it is known that after internalization of the endosomes, uncoating happens and endosomes loose clathrin. So, if the association between α S and clathrin is limited to clathrin coated vesicles, as suggested in (Lautenschlager et al., 2017), then it is not surprising to see only a small amount of colocalization between α S and clathrin. Finally, even though differentiated SH-SY5Y cells are suitable neuron-like cell model(Xie et al., 2010, Agholme et al., 2010, Fakhree et al., 2016), it is possible that they lack a series of cellular processes – such as

	z-stacks	Slices	Cells	α S cell density*	Endocytic marker cell density*	$m_{\alpha S\text{-marker}}$	$m_{\alpha S\text{-marker Rand.}}$	$m_{\text{marker-}\alpha S}$	$m_{\text{marker-}\alpha S Rand.}$
Clathrin	8	201	40	6.5 $\pm 2.0\%$	3.1 $\pm 1.1\%$	0.06 ± 0.03	0.03 ± 0.01	0.12 ± 0.05	0.06 ± 0.02
Caveolin	9	205	48	7.1 $\pm 1.8\%$	22.8 $\pm 5.8\%$	0.53 ± 0.10	0.22 ± 0.06	0.18 ± 0.04	0.07 ± 0.02
EEA1	11	242	96	4.7 $\pm 1.8\%$	5.7 $\pm 2.4\%$	0.31 ± 0.11	0.05 ± 0.02	0.20 ± 0.05	0.04 ± 0.02
Rab5	10	225	57	5.5 $\pm 2.2\%$	4.9 $\pm 1.4\%$	0.11 ± 0.04	0.04 ± 0.02	0.10 ± 0.03	0.05 ± 0.02
Rab7	10	193	50	11.0** $\pm 3.2\%$	4.3 $\pm 2.1\%$	0.14 ± 0.05	0.04 ± 0.02	0.33 ± 0.09	0.10 ± 0.03
Rab7exc.	6	134	26	8.1 $\pm 2.5\%$	4.5 $\pm 2.3\%$	0.15 ± 0.05	0.04 ± 0.02	0.27 ± 0.08	0.08 ± 0.02
Rab11	11	209	66	7.5 $\pm 2.6\%$	9.0 $\pm 2.8\%$	0.15 ± 0.06	0.07 ± 0.02	0.12 ± 0.05	0.05 ± 0.02

Table 1. Statistics of the colocalization studies. Signal density and colocalization values are calculated after median filtering for Clathrin, Rab5, and Rab7, and after image thresholding for all of the studied images.

* α S/Marker cell density is the percent of the number of pixels in puncta masked images of each channel, divided by the number of pixels of the cell mask.

** For α S-Rab7 images, the number of z-stack images that contain cells with high α S signal density per cell area are more than the other α S-endocytic markers. Rab7exc. row shows summary of the excluded high α S signal images. As it can be seen, after excluding z-stack images with higher α S signal density per cell area, the $m_{\alpha S\text{-Rab7}}$ does not change, so we used Rab7 images without excluding images with higher α S signal density per cell area.

synaptic vesicle release and recycling – that are dependent on co-operation between α S and clathrin (Lautenschlager et al., 2017). Hence, we observe no colocalization between α S and clathrin in our cell model.

For α S and caveolin, based on the analyzed images (see Table 1 for statistics) the average values of the Manders' colocalization coefficient were determined to be $m_{\alpha S-Caveolin} = 0.53$ and $m_{Caveolin-\alpha S} = 0.18$, after randomization these numbers changed to $m_{\alpha S-Caveolin\ Rand.} = 0.22$ and $m_{Caveolin-\alpha S\ Rand.} = 0.07$. For the colocalization of α S and caveolin the distribution of $m_{\alpha S-Caveolin}$ values significantly differs from the $m_{\alpha S-Caveolin\ Rand.}$ distribution (Figure 5.4E). We therefore conclude that most of the observed colocalization is not random. This means that approximately 50% of α S vesicles, have overlapping caveolin signal. For caveolin puncta, the amount of αS^+ vesicle signal is around 20% (Figure 5.4F). This shows that most of the caveolin dependent endocytosis events take place in the absence of α S.

Considering our hypothesis that α S is involved in the membrane remodeling of endosomes, this observation can be interpreted as follows: In differentiated SHSY-5Y cells half of the αS^+ vesicle pool also contains caveolin, it is therefore likely that α S plays a role in specific (receptor mediated) caveolin dependent endocytosis events. On the other half of the α S positive vesicles caveolin is absent. For this absence two possible explanations can be given. Either these $\alpha S^+/Caveolin$ -negative (Cav^-) vesicles do not take part in caveolin dependent endocytosis but are involved in other vesicle trafficking pathways or these $\alpha S^+/Cav^-$ vesicles were internalized by caveolin dependent endocytosis but have proceeded further along this vesicle trafficking pathway. As a consequence of vesicle sorting and maturation processes, $\alpha S^+/Cav^+$ vesicles have lost their caveolin while the α S remained associated with the vesicles during this process (see Scheme 5.S1).

For our observation of $m_{Caveolin-\alpha S}$ of around 20%, we can think of two possible explanations. First, it is plausible that α S is associated in only one or a few of caveolin dependent endocytic uptake or signaling pathways. For example, α S might be only involved in caveolin dependent uptake of the transferrin receptor or Cdc42 signaling, and not in many of the other caveolin dependent vesicle uptake/signaling pathways. Second, it is possible that only a specific step of caveolin dependent endocytosis is α S dependent. The presence of α S may only be required during the vesicle budding/fusion processes of caveolin

Figure 5.4. Colocalization between α S and clathrin or caveolin. **A,D)** Orthogonal cross section of a sample z-stack image of differentiated SH-SY5Y cells immunostained for α S/Clathrin or α S/Caveolin. Examples of colocalizing signals are indicated with white symbols: open arrows for overlapping diffraction limited signals, stealth arrows for diffraction limited α S⁺ inside or around larger endocytic marker, and triangles for bigger α S⁺ structure than the endocytic marker. Scale bars are 10 μ m. **B,E)** Distribution of the calculated Manders' colocalization coefficients for α S⁺ vesicles that have punctated clathrin or caveolin signal. The distributions represent the data obtained from each of the analyzed slices (see Table 1 for details). **C,F)** Distribution of the calculated Manders' colocalization coefficients for clathrin⁺ or caveolin⁺ puncta that have punctated α S signal, in each of the analyzed slices. The amount of colocalization between α S and caveolin is significantly more than random colocalization while the colocalization between α S and clathrin is comparable to the random distribution.

dependent endocytosis. Considering the tissue-specific expression level of α S(Lautenschlager et al., 2017), along with α S being a non-vital protein(Greten-Harrison et al., 2010), it is most probable that α S is associated in only one or a few of the caveolin dependent endocytic paths. This path may act as an extra/helper route of endocytosis of highly demanding cells such as neurons.

Observing colocalization of around 50% of α S⁺ vesicles with caveolin is in good agreement with our previous observation. WGA is known to be endocytosed via caveolae (Bonilla and Moggio, 1987, Mo and Lim, 2004) and in previous experiments we observed 59 ± 6 % of the α S⁺ vesicles to colocalize with WGA(Fakhree et al., 2016). There is more evidence that α S may play a role in caveolin dependent endocytosis. In a report by Madeira et al. (Madeira et al., 2011), it has been shown that α S and caveolin directly interact with each other. Furthermore, both α S and caveolin have been reported to colocalize with cholesterol rich domains on the plasma membrane(Hooper, 1999, Fortin et al., 2004). Overall, our observation of colocalization between α S and caveolin and previous reports on direct interactions hint toward a functional colocalization of α S and caveolin.

In order to confirm that there is a functional colocalization of α S with Cav⁺ vesicles, cells were treated with genistein. Treatment with genistein – a tyrosine-kinase inhibitor – results in disruption of the actin cytoskeleton and inhibition of dynamin II action in caveolae dependent endocytosis(Vercauteren et al., 2010, Tsutsui et al., 2003, Linford et al., 2001). Hence, if the observed colocalization between α S and caveolin is part of caveolae dependent endocytosis, the percentage of colocalization is expected to change after treatment with genistein. The amount of colocalizing α S vesicles with caveolin changes from $m_{\alpha S-Caveolin} = 0.53$ to $m_{\alpha S-CaveolinGen.} = 0.45$ after genistein treatment (203 slices, 7 z-stacks). However, the amount of caveolin colocalizing with α S changes from $m_{Caveolin-\alpha S} = 0.18$ to $m_{CaveolinGen.-\alpha S} = 0.23$. The differences between $m_{\alpha S-Caveolin}$ and $m_{\alpha S-CaveolinGen.}$, and $m_{Caveolin-\alpha S}$ and $m_{CaveolinGen.-\alpha S}$ are small, yet still significant. Overall, it seems that that α S⁺/Cav⁺ vesicles are not very sensitive to genistein treatment. This can have two possible reasons. The first possibility is that the association of α S and caveolin is not limited to the dynamin-dependent vesiculation process at the plasma membrane. The association may mainly be important for a dynamin independent caveolar uptake mechanism. The second possible explanation is that the final concentration and/or duration of genistein

treatment was not enough to severely affect the caveolin dependent cargo uptake and to reduce the observed colocalization to the random colocalization level. However, higher concentration of genistein and/or longer duration of the treatment have been reported to affect the viability of the cells (Vercauteren et al., 2010, Tsutsui et al., 2003, Linford et al., 2001).

Is α S associated with endocytic/exocytic proteins?

Not all of α S⁺ vesicles are associated with clathrin or caveolin. Hence, α S⁺ vesicles may be involved in events further along the endocytic pathway. Since the newly formed endosomes pass through early/sorting endosomes, we looked at colocalization between α S and early endosome markers, Rab5 and EEA1. For Rab5 images, we not only observe distinct puncta but also a non-homogeneous cytoplasmic diffuse signal in the images immunostained cells. To remove this background, a median filter was applied before thresholding the signal, as described for clathrin. The amount of observed colocalization between α S and Rab5 is $m_{\alpha S-Rab5} = 0.11$ which is more than random colocalization with $m_{\alpha S-Rab5}^{Rand.} = 0.04$. For Rab5⁺ structures which show signal overlap with α S, $m_{Rab5-\alpha S}$ is 0.10 in comparison to random colocalization with $m_{Rab5-\alpha S}^{Rand.} = 0.05$ (see Table 1 for statistics). The $m_{\alpha S-Rab5}$ and $m_{Rab5-\alpha S}$ values compared to the random values indicate that there is little, yet still a significant amount of overlap between two channels. This is better visible in distribution of $m_{\alpha S-Rab5}$ and $m_{Rab5-\alpha S}$ at higher values than random Manders' coefficients presented as histograms in Figure 5.5B, C.

In contrast to our observations for α S-Rab5, we observed a higher level of colocalization between α S vesicles and EEA1. Besides α S⁺/EEA1⁺ vesicles, α S⁺ vesicles that are colocalizing with EEA1⁺ structures bigger than diffraction limit are observed (Figure 5.5D). These EEA1⁺ structures can be enlarged early endosomes. Most of these bigger structures have at least one α S positive vesicles inside or next to them. For the Manders' colocalization coefficient we measured $m_{\alpha S-EEA1} = 0.31$ ($m_{\alpha S-EEA1}^{Rand.} = 0.05$) and $m_{EEA1-\alpha S} = 0.20$ ($m_{EEA1-\alpha S}^{Rand.} = 0.04$) (see Table 1 for statistics and Figure 5.5E, F for distributions). The broad distribution in $m_{\alpha S-EEA1}$ and $m_{EEA1-\alpha S}$ values, characterizes the mixture vesicles in which α S and EEA1 do not colocalize and in which they do colocalize. Additionally, the

Figure 5.5. Colocalization between α S and Rab5 or EEA1. **A, D)** Orthogonal cross section of a sample z-stack image of differentiated SH-SY5Y cells immunostained for α S/Rab5 or α S/EEA1. Examples of colocalizing signals are indicated with white symbols: open arrows for overlapping diffraction limited signals, stealth arrows for diffraction limited α S⁺ inside or around larger endocytic marker, and triangles for bigger α S⁺ structure than the endocytic marker. Scale bars are 10 μ m. **B, E)** Distribution of the calculated Manders' colocalization coefficients for α S⁺ vesicles that have punctated Rab5 or EEA1 signal, in each of the analyzed slices (see Table 1 for details). **C, F)** Distribution of the calculated Manders' colocalization coefficients for Rab5⁺ or EEA1⁺ puncta that have punctated α S signal, in each of the analyzed slices. The amount of colocalization between α S and EEA1 is significantly more than random colocalization and larger than the colocalization between α S and Rab5.

distribution of $m_{EEA1-\alpha S}$ is further broadened by the presence of αS vesicles inside the larger EEA1 positive structures.

Concluding Rab5 and EEA1, both are markers for early endosomes and we observe colocalization between αS and EEA1 positive structures, but to lesser extent between αS and Rab5. The difference in colocalization of αS with Rab5 and EEA1 is puzzling. In order to make sure that anti-Rab5 immunostaining is not the problem, colocalization with Rab5-GFP was investigated. After transfecting the SH-SY5Y cells with Rab5-GFP plasmid, they were observed to express Rab5-GFP. The transfected cells were immunostained for αS , and imaged as described above. As for the images of immunostained αS -Rab5, it was necessary to apply a median filter before thresholding αS -Rab5-GFP images to remove the inhomogeneous background. By looking at the images and analyzing Manders's colocalization coefficient between αS and Rab5-GFP ($m_{\alpha S-Rab5GFP} = 0.23 \pm 0.11$ versus $m_{\alpha S-Rab5GFP Rand.} = 0.13 \pm 0.04$), we observe similar amounts of non-random colocalization of αS and Rab5 (distributions are shown in Figure 5.S1) as in the non-transfected anti-Rab5 immunostained cells. We therefore conclude that there is less colocalization between αS and Rab5 than between αS and EEA1.

One possible explanation could be that certain types of early endosomes contains less Rab5 than others (Leonard et al., 2008). In endocytic uptake of transferrin or transferrin receptor (TfR) pathway, the level of Rab5 has been reported to be lower than in endosomes of epidermal growth factor receptor (EGFR) uptake. The difference in colocalization of αS with Rab5 and EEA1, and the observation that αS is probably involved in a specific caveolin dependent uptake mechanism, suggests that, following the work of Leonard et al. (Leonard et al., 2008), αS colocalizes with a specific type of early low Rab5 density endosomes. Since these vesicles have low Rab5 density, their corresponding fluorescence signal might not be distinguishable from the diffuse signal for Rab5 in the studied images. Hence, their corresponding signal would not be included in the calculations after background removal. This could result in an apparent lower colocalization.

In the next step of vesicle trafficking, early endosomes sort the vesicle cargo towards elimination or recycling routes. In the case of the elimination route, the Rab7 content of endosomes increases. For recycling endosomes, Rab11 can be used as a general marker. By looking at the images, it appears that αS^+ vesicles localize around bigger Rab7 and Rab11 positive structures (Figure 5.6, A, D). In

the case of Rab11, these bigger structures can be peri-centrosomal recycling endosomes. For Rab7, the bigger structures might be multivesicular bodies (MVBs), lysosomes, and autolysosomes. In order to apply thresholding to Rab7 images, first, median filtering was applied. The calculated $m_{\alpha S-Rab7} = 0.14$ is significantly larger than random colocalization $m_{\alpha S-Rab7 Rand.} = 0.04$. The $m_{Rab7-\alpha S}$ is 0.33 whereas we calculated the Manders' coefficient of random colocalization to be $m_{Rab7-\alpha S Rand.} = 0.10$ (see Table 1 for statistics). Looking at the distribution of $m_{\alpha S-Rab7}$ and $m_{Rab7-\alpha S}$ values (Figure 5.6 B, C), we can see that a significant number of slices shows moderate colocalization between two channels. In the case of colocalization between α S and Rab7, $m_{Rab7-\alpha S}$ is more than $m_{\alpha S-Rab7}$. By looking at the distribution of $m_{Rab7-\alpha S}$ it is possible to see that Rab7⁺ structures colocalize with α S⁺ vesicles, more than the other studied markers. This might mean that a fraction of Rab7⁺ structures require α S for their structure/function. There are a number of cellular structures that have Rab7 on their outer membrane including MVBs, lysosomes, and autolysosomes. Several reports suggest that there is an association between α S and the mentioned Rab7⁺ compartments (Moors et al., 2016, Kunadt et al., 2015). Yet it is not clear what type of association there is in action.

In the case of Rab11, the calculated $m_{\alpha S-Rab11} = 0.15$ in comparison to $m_{\alpha S-Rab11 Rand.} = 0.07$, and related distributions, suggests a low level of colocalization between α S and Rab11. By looking at $m_{Rab11-\alpha S} = 0.12$ versus $m_{Rab11-\alpha S Rand.} = 0.05$, related distributions and images (Figure 5.6 D-F), we can see less association of α S⁺ vesicles with Rab11⁺ signal. Comparison between $m_{\alpha S-Rab7}$ of 0.14 and $m_{\alpha S-Rab11} = 0.15$ suggests that almost equal amounts of α S⁺/Rab7⁺ and α S⁺/Rab11⁺ vesicles exist in our studied cells. The vesicles are apparently sorted from early endosomes, EEA1⁺ structures, 50:50% to recycling and late endosomes. Even though this is a rough estimate, it can be used as an indicator for the specific endocytic pathway that α S⁺ vesicles are involved. For example, this specific pathway can be the uptake of transferrin/TfR rather than EGF/EGFR (Jackle et al., 1991, Leonard et al., 2008). The work reported in ^{42,43} shows that EGF/EGFR mainly ends up in late endosomes and to lesser extent in recycling endosomes, but transferrin/TfR ends up in both recycling and late endosomes.

Figure 5.6. Colocalization between αS and Rab7 or Rab11. **A,D)** Orthogonal cross section of a sample z-stack image of a SH-SY5Y cell immunostained against αS /Rab7 or αS /Rab11. Examples of colocalizing diffraction limited αS^+ signal inside or around larger endocytic marker are indicated with white stealth arrows. Scale bars are 10 μm . **B,E)** Distribution of the calculated Manders' colocalization coefficients for αS^+ vesicles that have punctated Rab7 or Rab11 signal, in each of the analyzed slices (see Table 1 for details). **C,F)** Distribution of the calculated Manders' colocalization coefficients for Rab7 $^+$ or Rab11 $^+$ puncta that have punctated αS signal, in each of the analyzed slices. The amount of colocalization between αS and Rab7 or Rab11 is more than random colocalization.

General picture

Considering that we are looking at snapshots of membrane remodeling by α S in endocytic pathways, the complete α S⁺ vesicle pool should add up to 100% if the complete pathway is covered by our studies. The sum of colocalization between α S and caveolin, clathrin, EEA1, Rab7, and Rab11 adds up to about 120% (Scheme 2). This is not far off from 100%, but probably would add up to an even higher number had it not been necessary to threshold the images. There are two possible reasons that colocalization of α S⁺ vesicles in endocytic pathway is this high. Firstly, based on the presented distributions, we can see that a fraction of our measured colocalization values is due to random overlap of the signals between two channels. This is mostly the case for clathrin, to some extent Rab11, and the least random ones are caveolin, EEA1, and Rab7. Secondly, in reality, different parts of endocytic pathway overlap with each other. For example, a fraction of α S⁺/EEA1⁺ vesicles are probably also caveolin⁺ vesicles (see Scheme 2). Or, a fraction of α S⁺/EEA1⁺ vesicles might contain Rab7 (Girard et al., 2014, Vonderheit and Helenius, 2005). Although we do not know how much the overlap between different markers is in our cell line, nevertheless it seems that a major fraction of α S⁺ vesicles colocalize with general endocytic markers.

It is interesting that the association between endocytic markers and the secretion of α S has been studied in relation to the development of synucleinopathies such as Parkinson's disease. For example, it has been reported that cargo/aggregates of α S colocalize with Rab5, Rab7, and Rab11. This colocalization has been interpreted as being a result of the secretion of α S (Chutna et al., 2014, Hasegawa et al., 2011). The partial colocalization between α S and Rab5, Rab7, and Rab11 is in agreement with our data. However, there are reasons we think that in the cell model of our study, α S is not the cargo, but is localized on the outer membrane of the endosomes. Firstly, we have shown in Chapters 2 and 3 that α S is on the membrane of vesicles (Fakhree et al., 2018, Fakhree et al., 2016). Secondly, if α S is the cargo of Rab11⁺ vesicles, then how does it interact with Rab11 which is on the outer membrane of the vesicles (Chutna et al., 2014)? Thirdly, in the diseased case of cellular overload with α S, as in the reported works in the literature, it does make sense to secrete surplus α S and thus lower the cellular α S concentration. However, in the cell model of our study, we do not induce over expression of α S. We therefore

Scheme 5.2. Schematic overview of the observed colocalization between α S and the studied endocytic markers. The numbers are percentage of α S⁺ vesicles that colocalize with endocytic marker. Based on our colocalization observation, the probable endocytic pathway in which α S is involved is indicated with black arrows.

conclude that this study reports on the functional state of the α S rather than on α S elimination routes in disease related conditions.

In conclusion, the observed colocalization pattern between α S and the studied markers, suggests that α S is involved in a *specific* caveolin dependent endocytic uptake and/or signaling in differentiated SH-SY5Y cells. Most probably, the specificity of this pathway originates from the presence of a specific receptor/protein (The red star in Scheme 2). According to reported works on the involvement of α S in trafficking of vesicle and signaling pathways, clathrin-independent transferrin/TfR uptake and recycling, and Cdc42 signaling pathway, are the most likely candidates. An interesting aspect of association between α S and caveolin is that single/double/triple knock-out of either of α / β / γ -synucleins or caveolins-1/2/3 is not lethal (Lautenschlager et al., 2017, Le Lay and Kurzchalia, 2005, Razani et al., 2001, Razani et al., 2002, Park et al., 2002, Greten-Harrison et al., 2010). This might suggest that either α S/caveolin dependent endocytosis

is not essential for the whole organism and is an extra/helper endocytic route. Or, assuming that both proteins are helper proteins in modulating membrane curvature, α S or caveolin can do the job in the absence of the other protein. In that case, knockout of both caveolin and α S might appear as a synthetic-lethal condition for the organism.

5.3. Supporting information

Scheme 5.S1. Schematic representation of the chronological order and timescale on which the studied membrane remodeling proteins are physically associated with the endosomes. Color indicates that the proteins are associated with the endosomes, white indicates that the protein is absent. In the case of clathrin, the association with endosomes is short lived. Since caveolin is inserted into the membrane, it stays on endosomes until they mature into early endosomes. It is not clear if caveolin remains part of endosomes' membrane after pooling into the early endosomes. After endosomes are formed, the Rab5 and EEA1 content of the vesicles membrane increases, reaches a peak, then after sorting, the early endosome marker content decreases. At the same time that the early endosome marker content decreases, depending on the route, either the late endosome marker (Rab7) or the recycling endosome marker (Rab11) increases.

Figure 5.S1. Colocalization between αS and Rab5-GFP. Distribution of the calculated Manders' colocalization coefficients distributions for αS^+ vesicles that have punctuated Rab5-GFP (Left) and Rab5-GFP puncta that have αS signal, in 111 slices. Similar to αS and Rab5 most of the observed colocalization appears as random colocalization. Manders' colocalization coefficients for both original and randomized signal are higher than those between αS and Rab5 (Figure 5.5 B,C) which can be attributed to over-expression of Rab5-GFP.

5.4. Materials and Methods

SH-SY5Y cells culture, differentiation, and transfection. Please refer to Materials and Methods section of the Chapter 2.

Immunocytochemistry and cell staining. The cells were fixed in 3.7% paraformaldehyde in PBS for 30 min at room temperature (RT) and permeabilized with 0.3% Triton X-100 in PBS for 5 min at RT. Autofluorescence was quenched with 50 mM ammonium chloride in PBS for 15 min at RT. Aspecific binding sites were blocked with 16% goat serum, 0.3M NaCl and 0.1% TX100 in PBS for 30 min at RT. The sample was incubated for at least 1 hour at RT with the primary antibody (endosomal marker kit, Cell Signaling, number 12666) diluted in blocking solution. The primary antibodies were diluted according to the manufactures protocol: caveolin 1:400, clathrin 1:50, Rab5 1:200, EEA1 1:200, Rab11 1:100, and Rab7 1:100. After incubation with the primary antibody, the sample was incubated for at least 1 hour at RT with the secondary antibody (Goat Anti-Rabbit IgG, labeled with Alexa Fluor 488, obtained from Life Technologies™, catalog number: A-21071) diluted 1:200 in 0.1% TX100 in PBS. A serial dilution of the secondary antibody was made in order to find the optimum concentration of secondary antibody.

For anti- α S immunostaining a primary antibody against α S raised in mouse, obtained from Santa Cruz Biotechnology (Heidelberg, Germany), was diluted 1:100 and coupled with the secondary antibody (Goat anti-mouse conjugated with Alexa Fluor 568, Life Technologies) as described above.

Nuclear counterstaining was performed by incubation in 300 nM 4',6-diamidino-2-phenylindole (DAPI) in PBS for 10 minutes. After washing with PBS, samples were mounted with mounting medium (ibidi, Germany).

Imaging. Please refer to Materials and Methods section of the Chapter 2. Imaging conditions (scan speed, pixel size, detector sensitivity, laser power) were kept the same for all of the samples.

Image analyses. Nikon z-stack images were imported in (FIJ)ImageJ software. The bottom and top slices were selected to include at least one clear and sharp

α S or endocytic marker punctum. Next, images were separated to channel1 (α S), channel2 (endocytic marker), and channel3 (DAPI). Scatterplots were drawn using original images of channel1 and channel2. The intensity value of the pixel_{x,y} of the channel1 (α S) is considered as x value of the scatterplot, and corresponding pixel intensity value of the channel2 (endocytic marker) was taken as y of the scatterplot. By doing this for all of the pixels of the image, the scatterplots were drawn.

In order to quantify the colocalization between puncta of two channels Mander's colocalization coefficients were calculated. To correctly calculate the Manders' colocalization coefficient it is necessary to remove diffuse and background signal of the images. In order to limit images to puncta only signal, masks were applied. To make masks, thresholds were applied. For making puncta masks of the channel1 and channel2, first ImageJ's "IJ_IsoData" threshold algorithm was used. Second, by going through z-axis and manually adjusting the lower limit of the threshold, the final value was set and threshold applied to the whole images of one z-stack of one channel. In the case of clathrin, Rab5, and Rab7, it was necessary to apply median filtering prior to thresholding(Dunn et al., 2011). Median filtering was applied by a probing array with radius of 25 pixels which is bigger than diffraction limited punctum with radius of 5 pixels in our images. This ensures that no new features are introduced in the images because of filtering(Dunn et al., 2011). The median filtered image was subtracted from the original image, the resulting image was later used for thresholding. Channel2 (marker channel) was used to make cell area masks. In order to exclude nucleus area from analyses, DAPI nucleus counterstaining masks were subtracted from the cell area masks. This was done for all of the 61 analyzed z-stacks and channels, separately. The procedure is shown in the next page.

Flow chart of preparing images to calculate Manders' colocalization coefficients

After applying masks on original images, by pixels-wise multiplication of masks with the corresponding channel, the images were limited to puncta intensity only. Manders' colocalization coefficients were calculated using these masked images. The mathematical definition of $m_{Chan.1-Chan.2}$ and $m_{Chan.2-Chan.1}$ per two-channel image is as follows:

$$m_{Chan.1-Chan.2} = \frac{\sum_i I_{1,i,Coloc.}}{\sum_i I_{1,i}} \quad I_{1,i,Coloc.} = \begin{cases} I_{1,i} & \text{if } I_{2,i} > 0 \\ 0 & \text{if } I_{2,i} = 0 \end{cases}$$

$$m_{Chan.2-Chan.1} = \frac{\sum_i I_{2,i,Coloc.}}{\sum_i I_{2,i}} \quad I_{2,i,Coloc.} = \begin{cases} I_{2,i} & \text{if } I_{1,i} > 0 \\ 0 & \text{if } I_{1,i} = 0 \end{cases}$$

where $I_{1,i}$ and $I_{2,i}$ are intensity values of the i^{th} pixel of channel1 and channel2; and $I_{1,i,Coloc.}$ and $I_{2,i,Coloc.}$ are intensity values of the i^{th} pixel of channel1 and channel2 that has non-zero signal intensity in the other channel, respectively. The calculated $m_{Chan.1-Chan.2}$ and $m_{Chan.2-Chan.1}$ values of each slices of the studied z-stacks were used to construct histograms of Manders' colocalization coefficients between αS and each one of the studied endocytic markers.

Randomization of the signal intensity values of puncta limited images throughout the cell area (CellMask) was done as follows. First, two arrays of random numbers with the size of cell area (non-zero CellMask elements) were generated. Next, the CellMask-corresponding pixel intensity values of masked channels (non-zero intensity puncta signal along with zero intensity pixels that are within cell area), were sorted based on the order of the elements of the earlier generated two arrays of random numbers. This results in randomized intensity distribution of puncta limited signal throughout the cell area. The corresponding $m_{Chan.1-Chan.2\ Rand}$ and $m_{Chan.2-Chan.1\ Rand}$ were calculated after randomization. A Matlab code for Manders' colocalization coefficient calculation, along with randomization of signal, is available in Appendix Matlab.

5.5. References

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Concluding remarks and future outlook

Why did we do it?

During the past two decades, intrinsically disordered proteins (IDPs) and the intrinsically disordered regions of structured proteins (IDRs) have received an increasing amount of attention. There are two major reasons for the increased research efforts in this direction: 1) IDPs/IDRs are recognized to play a role in the development of diseases, 2) IDPs/IDRs do not have secondary structure which makes it harder to determine their function, and makes their study a challenging task. To complicate matters, not only do IDPs/IDRs not have a fixed structure, they also interact with a range of ligands possibly resulting in multiple functions. Hence IDPs are often “mysterious” proteins (van der Lee et al., 2014). It is interesting to mention that higher order organisms such as eukaryotes have higher amounts of IDPs/IDRs than prokaryotes (van der Lee et al., 2014, Liu et al., 2009). This might be interpreted that IDPs/IDRs are not as essential for building blocks of life as structured proteins. In other words, organisms appear to be able to survive down-regulation/knockout/mutation of IDPs/IDRs. Their being non-essential is perhaps also the reason that, clinical manifestations of IDP-related disorders start later in life with mild symptoms, continue as a chronic condition, and finally causes severe morbidity/mortality among old people. In this context, the age dependency of these diseases could be inferred as failure of essential cellular events, and lack of the helper/supporter IDP/IDR system.

In order to understand the connection between IDPs/IDRs and diseases, and try to cure IDP related disease it is necessary to “define” the IDP/IDR. The

first step to define IDPs/IDRs is to determine what their interaction partners are. As explained in the **Introduction** chapter, most of IDPs adopt (partial) secondary structure upon binding interaction partners. So, one should determine possible interacting partners of the IDP/IDR and look at the affinity and/or structural association between them, separately or in combination with other interacting partners. By thus characterizing and quantifying the different interactions of a single IDP species, it should be possible to derive the protein/ligand network in which the IDP/IDR serves as a hub. This interaction network represents the physiological role(s)/function(s) of the IDP/IDR of interest. In the present work, we have shown how a number of questions regarding defining the functional form of an IDP, namely alpha-synuclein, can be addressed.

Knowledge of the interaction network becomes important in the design of therapeutic interventions in IDP/IDR related diseases. Since the structure-function dogma is not applicable to IDPs/IDRs, the classic lock-and-key approach to design and target a specific protein with single function as the receptor, will not work and needs adjustment. This adjustment should include specific interaction with one of the many functional forms of the one IDP/IDR, without disrupting the required balance in the protein/ligand interaction network of the IDP/IDR.

What is achieved? How did we do it?

In the present work, we investigated the biophysical functional features of alpha-synuclein, as a model IDP, in a cellular context. In **Chapter 2** we measured the surface density of α S on cellular vesicles and discussed that based on the determined surface density, α S can act as a membrane remodeling agent. Moreover, based on recent reports on the role of intrinsically disordered domains of protein in the induction of membrane curvature, we suggested a new mechanism of action for membrane remodeling capacity of α S. Furthermore, we linked the determined surface density to the surface density of a suggested interaction partner of α S, synaptobrevin. We thereby made a new possible functional link in the interaction network of α S. As one can see, based on the knowledge from *in vitro* experiments, determining the cellular copy number of an IDP helped to explain a role, propose a new mechanism of action, and obtain support for one of the suggested interaction partner for the IDP, α S. This was

doable because of the techniques that were developed during late 20th century such as expressing fluorescent proteins and single molecule imaging.

In **Chapter 3**, we went one step further, and showed that similar to *in vitro* experiments, α S changes its structure upon binding to cellular vesicles. This is important in two aspects. First, it shows the existence of more than one specific conformationally different form of the IDP inside cells. Since also in the cellular context there is structure, this structure can help us understand the function of the IDP, for example membrane remodeling. Second, our report provided a cellular evidence for the *in vitro* observations of membrane bound α S with a structure that is distinctly different from the structure in solution. This distinctly different structural sub-population of α S was not resolved in previous reports by other groups using bulk methods such as NMR and EPR. Again, the higher sensitivity of the used single molecule technique made it possible to differentiate populations within cells.

After observing membrane bound structure of α S inside cells, and knowing that α S can modulate membranes, in **Chapter 4** we tested our hypothesis on the second membrane remodeling mechanism of α S, inducing curvature by generating lateral steric pressure. In order to avoid complications by the other possible interaction partners inside cell, such as synaptobrevin and Ca^{2+} , we used an *in vitro* membrane model. We were able to show that both truncation and elongation of the disordered region of α S, affects the ability of the IDP to remodel membranes into small vesicles. This shows that the disordered domain of the IDP α S plays a role in membrane remodeling. Moreover, we were able to provide a theoretical model for dual mechanism of α S in membrane remodeling. This suggests that the hypothesis posed in **Chapter 2** on the membrane remodeling mechanisms of α S is correct.

By careful examination of the literature on the numerous interaction partners of α S, which appears puzzling because it interacts with almost everything inside cells, and following the argumentation of the first four chapters of the presented work, in **Chapter 5** we proposed that α S is involved in cellular events that require membrane remodeling, such as endocytosis and exocytosis. So, we went another step further and investigated the colocalization between α S and general parts of endocytosis/exocytosis pathways. Our colocalization findings suggest that α S is mainly associated with the endocytic pathway, from vesicular uptake to recycling and elimination inside cell. And it appears that α S

is involved in a specific vesicular uptake/recycling path. Based on our observed colocalization pattern and reported works from literature, this specific path most probably is the uptake of transferrin. The endocytosis path in which α S plays a role generated in this chapter helps to put the puzzle pieces of the function of α S inside cell together. Most of the reported interacting proteins/lipids with α S, and organelles affected by α S, are linked to endocytosis and exocytosis.

What are the next steps? How does this help?

The major reason for studying α S is to try to understand its function/dysfunction and the resulting contribution of α S to pathogenesis of synucleinopathies such as Parkinson's disease. In this respect, isoforms of α S, beta- and gamma-synucleins, and mutants of alpha-synuclein, such as A30P, E46K, and A53T, would be the next subject of studies similar to the work reported here. Another possible follow up research direction would be to study α S in the presence of additional interaction partners such as synaptobrevin and Ca^{2+} . For example, in **Chapter 2** we discussed that synaptobrevin and Ca^{2+} are the most probable effectors of the steric pressure generated by the c-terminus random coil of α S. For the colocalization chapter, co-immunoprecipitation studies on α S and the studied marker proteins and immunostainings to pinpoint specific pathway more precisely by looking at the transferrin receptor or Cdc42, is recommended. It may also be a good idea to move to a more biologically relevant model; the use of induced pluripotent stem cells and/or primary neurons are possible options. In this respect, systems that over-express α S should be avoided. In this direction, it seems that evolving technology like CRISPR will be highly beneficial, which allows the recombinant attachment of a fluorescent protein to the endogenously expressed protein.

The methods presented in this thesis can be used to study other IDPs/IDRs and even structured proteins in more detail. For example it is most probable that similar to α S, disordered domains of reticulons (RTNs) and ADP-ribosylation factor GTPase-activating protein 1 (ARFGAP1) contribute to remodeling membranes, in addition to the hairpin and amphipathic alpha-helix insertion of RTNs and ARFGAP1, respectively. Another example could be the copy number of Rab proteins on endosomes. It appears that early endosomes have very different densities of one specific protein on their surface (Leonard et al., 2008). Different densities of Rab proteins possibly determine the cellular fate of these

endosomes. Photobleaching experiments on cells in which these proteins are fluorescently labelled can be used to quantify the surface density. Photobleaching analysis may thus be a useful technique to look in more detail at the protein characteristics of such endosomes. More generally, the number of a specific protein in cellular compartments, can possibly be used to identify sub-populations of the organelles inside cells. This can help to better understand cellular processes that are highly similar, specialized, but doing different tasks. Early endosomes and synaptic vesicles are example of such compartments(Leonard et al., 2008, Crawford and Kavalali, 2015).

In summary, protein quantification and labeling seem to be two complementary and viable approaches to study the function of structured or IDPs. This shows the importance of application, further improvement, and development of quantitative protein labeling and characterization techniques in research on disordered proteins.

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Appendices

Appendix FRET

Förster resonance energy transfer (FRET) is the non-radiative energy transfer between two fluorophores via dipole-dipole coupling. Energy is transferred from an initially excited fluorophore, the donor (D), to another fluorophore, the acceptor (A), resulting in an excited acceptor. De-excitation of the acceptor can result in the emission of a photon which will be detected in the acceptor channel. The efficiency of this energy transfer is highly dependent on the distance between the two fluorophores. The formula to calculate FRET efficiency based on the distance between the donor and the acceptor is as follows:

$$E_{FRET} = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6}$$

where E_{FRET} is the FRET efficiency, r is the distance between the donor and the acceptor, and R_0 is the fluorophore specific distance between the donor and the acceptor where $E_{FRET} = 0.5$. Typically R_0 values are in the range of $< 10\text{nm}$. As it can be seen from the formula, the FRET efficiency is dependent on the sixth power of the distance between the donor and the acceptor. Because of the sixth power and R_0 values, FRET is highly sensitive to changes in distance between the donor and the acceptor and happens in distances shorter than 10 nm.

In practice, when there is FRET between two fluorophores, upon excitation of the donor, partial or all of the emission of the donor is quenched by the acceptor. This will appear as lower or no signal in the emission channel of the donor in comparison to samples that FRET does not happen. The energy transfer via FRET works to excite the acceptor (instead of direct excitation with light), and consequently, results in the emission of the acceptor. A simple way to remove the quenching effect of the acceptor, is to photobleach it. This has been explained in Chapter 2 for the FRET between $\alpha\text{S-GFP}$ and WGA-AF647 species.

Appendix Membrane curvature

Curvature is defined as the inverse of the radius of curvature:

$$C = \frac{1}{R} \quad \text{Eq.1}$$

where C and R are curvature and radius of curvature, respectively. Any surface can be locally characterized by two orthogonal radii of curvature, R_1 and R_2 (See Figure Appendix Membrane curvature1). These two radii of curvature determine the mean curvature and Gaussian curvature of a given surface:

$$J = C_1 + C_2 = \frac{1}{R_1} + \frac{1}{R_2} \quad \text{Eq.2}$$

$$K = C_1 \times C_2 = \frac{1}{R_1} \times \frac{1}{R_2} \quad \text{Eq.3}$$

where J and K are mean and Gaussian curvature, respectively. Figure Appendix Membrane curvature1 shows two examples of surfaces with different mean and Gaussian curvatures. Membrane remodeling events such as vesicle budding, tubulation, and fusion typically involve changes in mean and Gaussian curvature.

Figure Appendix Membrane curvature1. A surface curvature can be defined by two orthogonal radii of curvatures R_1 ($1/C_1$) and R_2 ($1/C_2$). The surface of a spherical object such as vesicles is an example of positive curvature ($J > 0$, $K > 0$). The surface of a saddle, such as the neck of a fusing/budding vesicle, is an example of negative curvature ($J \approx 0$, $K < 0$).

One can categorize membrane remodeling events to direct and indirect mechanisms. The direct mechanisms are the ones which changes curvature by directly and asymmetrically changing the outer and/or inner area of the lipid bilayer. The indirect mechanisms are the ones which affect membrane curvature without directly affecting the area of the leaflets. A series of these mechanisms are presented in Figure Appendix Membrane curvature2 along with their examples. Depending on the pathway, cell uses one or a combination of these mechanisms to induce changes in the mean and Gaussian curvatures of lipid bilayers.

Figure Appendix Membrane curvature2. Direct and indirect mechanisms of membrane curvature induction. To change membrane properties, high densities of modulating agents are required. Various mechanisms can be involved in remodeling into specific membrane structures including changes in lipid composition, amphipathic insertion, and scaffolding in the formation of a typical clathrin coated vesicle.

Appendix Manders' colocalization coefficients

Chan.1	Chan.2	Merge		Chan.1	Chan.2	Merge
$m_{Chan.1-Chan.2} = 1$ $m_{Chan.2-Chan.1} = 1$				$m_{Chan.1-Chan.2} = 0.6$ $m_{Chan.2-Chan.1} = 0.6$
$m_{Chan.1-Chan.2} = 0.6$ $m_{Chan.2-Chan.1} = 1$				$m_{Chan.1-Chan.2} > 0.6$ $m_{Chan.2-Chan.1} > 0.6$
$m_{Chan.1-Chan.2} = 0.33$ $m_{Chan.2-Chan.1} = 0.33$				$m_{Chan.1-Chan.2} < 0.6$ $m_{Chan.2-Chan.1} < 0.6$
$m_{Chan.1-Chan.2} = 0$ $m_{Chan.2-Chan.1} = 0$				$m_{Chan.1-Chan.2} < 0.6$ $m_{Chan.2-Chan.1} > 0.6$

Schematic Manders. Left part of the schematic, shows the effect of the amount of overlapping signal among two channels, on the value of $m_{Chan.1-Chan.2}$ and $m_{Chan.2-Chan.1}$. In the case of fully colocalizing signal, $m_{Chan.1-Chan.2} = m_{Chan.2-Chan.1} = 1$. For full exclusion, $m_{Chan.1-Chan.2} = m_{Chan.2-Chan.1} = 0$. For partial overlap, $0 < m < 1$. In the right part of the schematic, the effect of the intensity distribution on $m_{Chan.1-Chan.2}$ and $m_{Chan.2-Chan.1}$ is shown. Since $m_{Chan.1-Chan.2}$ and $m_{Chan.2-Chan.1}$ are intensity weighted, m values change depending on the intensity distribution among overlapping signals.

Appendix Matlab code for image analyses

```

%% colocalization between two channels as zStack
%Uses codes for "tif" file processing. So, Matlab 2009 or higher should be used
clear
clc
%%
%.\Name of the folder which z-stack images are saved\':
MainDir = '\Cav\Cav1\';
% Name of the first channel (AntiaS)
Channel1name = 'AntiaS';
% Name of the second channel (e.g. one of the endocytic markers):
Channel2name = 'Cav';
% Name of the folder where scatter plots will be saved:
Scatters = 'Scatters';

% MainDir\Filenames of the channels 1 and 2
Channel1tif = strcat(MainDir,Channel1name,'.tif');
Channel2tif = strcat(MainDir,Channel2name,'.tif');
% MainDir\Filenames of the channels 1 and 2 puncta masks, and cell mask
PunctaMask1tif = strcat(MainDir,'PunctaMask1.tif');
PunctaMask2tif = strcat(MainDir,'PunctaMask2.tif');
CellMaskTif = strcat(MainDir,'CellMask.tif');
DAPImaskTif = strcat(MainDir,'DAPImask.tif');

% Reads number of images per tif stack file (assuming equal slice for both channels)
StackInfo = imfinfo(Channel1tif);
Slice = numel(StackInfo);

% Preparing file format for final output
ColocStat = zeros((Slice+1),20);
ColocStat = num2cell(ColocStat);
ColocStat(1,:) = {'Slice','non-zero1','non-zero2','Min1','Min2','Max1','Max2','Mean1','Mean2','Mode1','Mode2','M1','M2','PCC','M1rand','M2rand','PCCrand','CellArea','M1randCal','M2randCal'};

% For the number of slices in the stack, performs the image processing
for i = 1:Slice
    SliceText = num2str(i);
    % Read i'th Slice of the stacks
    Channel1 = imread(Channel1tif, i);
    Channel2 = imread(Channel2tif, i);
    PunctaMask1 = imread(PunctaMask1tif, i);
    PunctaMask2 = imread(PunctaMask2tif, i);
    CellMask = imread(CellMaskTif, i);
    DAPImask = imread(DAPImaskTif, i);

    % % Unmarking this section, exclude thresholding by the masks
    % PunctaMask1(:) = 1;
    % PunctaMask2(:) = 1;
    % CellMask(:) = 1;
    % DAPImask(:) = 1;

    % Masks are 8 bit, images are 16 bit. Turn masks to 16 bit, 1/0
    PunctaMask1 = uint16(PunctaMask1);
    PunctaMask1 = PunctaMask1/max(PunctaMask1(:));
    PunctaMask2 = uint16(PunctaMask2);

```

```

PunctaMask2 = PunctaMask2/max(PunctaMask2(:));
CellMask = uint16(CellMask);
CellMask = CellMask/max(CellMask(:));
DAPImask = uint16(DAPImask);
DAPImask = DAPImask/max(DAPImask(:));
% Applies nuclei mask to puncta and cell masks to exclude nuclei area
PunctaMask1 = PunctaMask1 .* DAPImask;
PunctaMask2 = PunctaMask2 .* DAPImask;
CellMask = CellMask .* DAPImask;

% Limit signals of each channels to their puncta
MaskedChannel1 = Channel1 .* PunctaMask1;
MaskedChannel2 = Channel2 .* PunctaMask2;

% Find the size of the image, and calculate the number of pixels
[X1,Y1] = size(Channel1);
ImagePixels = X1*Y1;

% Turns images to 1D array
MaskedChannel11D = reshape(MaskedChannel1,ImagePixels,1);
MaskedChannel21D = reshape(MaskedChannel2,ImagePixels,1);

% Calculate Mander's colocalization coefficient (MCC) between channel1 and channel2 (M1) and channel2
and channel1 (M2)
SumChannel1 = sum(MaskedChannel11D(:));
SumChannel2 = sum(MaskedChannel21D(:));
SumColocChannel1 = 0;
SumColocChannel2 = 0;
M1 = 0;
M2 = 0;
for j = 1:ImagePixels
    % Sums up all signals of channel1 which has >0 signal in channel2
    if MaskedChannel21D(j,1)>0
        dM1 = double(MaskedChannel11D(j,1))/double(SumChannel1);
        M1 = M1 + dM1;
    end
    % Sums up all signals of channel2 which has >0 signal in channel1
    if MaskedChannel11D(j,1)>0
        %SumColocChannel2 = SumColocChannel2 + MaskedChannel21D(j,1);
        dM2 = double(MaskedChannel21D(j,1))/double(SumChannel2);
        M2 = M2 + dM2;
    end
end
% Turns numerical M1 M2 values to text, for output file
M1text = num2str(M1); M2text = num2str(M2);

% Calculate Pearson's correlation coefficient
PCCMaskedChannel1 = double(MaskedChannel1);
PCCMaskedChannel2 = double(MaskedChannel2);
PCC = round(corrcoef(PCCMaskedChannel1,PCCMaskedChannel2)*1000)/1000;
PCCtext = PCC(1,2);
PCCtext = num2str(PCCtext);

% Plot scatterplots between channel1 and channel2 pixels
PlotName = strcat('Slice#',SliceText,' (PCC=',PCCtext,')');
figure1 = figure('name',PlotName);
plot(MaskedChannel11D,MaskedChannel21D,'MarkerSize',5,'Marker','.', 'LineStyle','none');

```

```

title(PlotName);
xlabel(strcat(Channel1name,' (M1=', M1text, ')'));
ylabel(strcat(Channel2name,' (M2=', M2text, ')'));
CellColocScatter = figure(1);
ScatterDir = strcat(MainDir,Scatters,'\');
    if exist(ScatterDir) ~= 7
        mkdir(ScatterDir);
    end
ScatterSliceFIG = strcat(MainDir,Scatters,'\SliceText','fig');
saveas(CellColocScatter, ScatterSliceFIG);
ScatterSliceEMF = strcat(MainDir,Scatters,'\SliceText','emf');
saveas(CellColocScatter, ScatterSliceEMF);
close(CellColocScatter);

% PCC Randomization Randomization Randomization Randomization Randomization
% this returns matrix index of non-zero elements of the Mask array
MaskMatrixIndices1 = find(PunctaMask1);
MaskMatrixIndices2 = find(PunctaMask2);
MaskMatrixIndicesChn1 = MaskMatrixIndices1;
MaskMatrixIndicesChn2 = MaskMatrixIndices2;
SizeMaskMatrixIndices1 = size(MaskMatrixIndicesChn1);
SizeMaskMatrixIndices2 = size(MaskMatrixIndicesChn2);
RandG = rand(SizeMaskMatrixIndices1);
RandR = rand(SizeMaskMatrixIndices2);
% Sort MaskMatrixIndicesChn1&2 like RandG&R. RandG&R are randomly ordered. So this will randomize the
order of Matrix indices.
[~, RandGorder] = sort(RandG);
MaskMatrixIndicesChn1order=sort(MaskMatrixIndicesChn1);
MaskMatrixIndicesChn1(RandGorder)=MaskMatrixIndicesChn1order;
[~, RandRorder] = sort(RandR);
MaskMatrixIndicesChn2ord=sort(MaskMatrixIndicesChn2);
MaskMatrixIndicesChn2(RandRorder)=MaskMatrixIndicesChn2ord;
% sets values of old Channel1&2 to new random channel1&2 (randomization within puncta area)
RandomizedChannel1 = MaskedChannel11D;
RandomizedChannel1(MaskMatrixIndices1) = MaskedChannel11D(MaskMatrixIndicesChn1);
RandomizedChannel2 = MaskedChannel21D;
RandomizedChannel2(MaskMatrixIndices2) = MaskedChannel21D(MaskMatrixIndicesChn2);
% Reshape 1D to 2D of Randomized Masked Channels
RandomizedChannel12D = double(reshape(RandomizedChannel1, [512,512]));
RandomizedChannel22D = double(reshape(RandomizedChannel2, [512,512]));
% Calculate Pearson's correlation coefficient (PCC) between two randomized channels
% PCCrandMaskedChannel1 = double(RandomizedChannel12D(find(PunctaMask1)));
% PCCrandMaskedChannel2 = double(RandomizedChannel22D(find(PunctaMask2)));
PCCRandomized = corrcoef(RandomizedChannel12D,RandomizedChannel22D);
PCCrandtext = PCCRandomized(1,2);
PCCrandtext = num2str(PCCrandtext);

% MCC Randomization Randomization Randomization Randomization Randomization
% this returns matrix index of non-zero elements of the Mask array
MaskMatrixIndices = find(CellMask);
MaskMatrixIndicesChn1 = MaskMatrixIndices;
MaskMatrixIndicesChn2 = MaskMatrixIndices;
SizeMaskMatrixIndices = size(MaskMatrixIndicesChn1);
% two sets of random number
RandG = rand(SizeMaskMatrixIndices);
RandR = rand(SizeMaskMatrixIndices);

```

% Sort MaskMatrixIndicesChn1&2 like RandG&R. RandG&R are randomly ordered. So this will randomize the order of Matrix indices.

```
[~, RandGorder] = sort(RandG);
MaskMatrixIndicesChn1order=sort(MaskMatrixIndicesChn1);
MaskMatrixIndicesChn1(RandGorder)=MaskMatrixIndicesChn1order;
[~, RandRorder] = sort(RandR);
MaskMatrixIndicesChn2ord=sort(MaskMatrixIndicesChn2);
MaskMatrixIndicesChn2(RandRorder)=MaskMatrixIndicesChn2ord;
% sets values of old Channel1&2 to new random channel1&2 (randomization)
RandomizedChannel1 = MaskedChannel11D;
RandomizedChannel1(MaskMatrixIndices) = MaskedChannel11D(MaskMatrixIndicesChn1);
RandomizedChannel2 = MaskedChannel21D;
RandomizedChannel2(MaskMatrixIndices) = MaskedChannel21D(MaskMatrixIndicesChn2);
% Reshape 1D to 2D of Randomized Masked Channels
RandomizedChannel12D = reshape(RandomizedChannel1, [512,512]);
RandomizedChannel22D = reshape(RandomizedChannel2, [512,512]);
```

% M1rand & M2rand for randomized channels

```
SumColocChannel1 = 0;
SumColocChannel2 = 0;
M1rand = 0;
M2rand= 0;
for j = 1:ImagePixels
    % Sums up all signals of channel1 which has >0 signal in channel2
    if RandomizedChannel2(j,1)>0
        dM1 = double(RandomizedChannel1(j,1))/double(SumChannel1);
        M1rand = M1rand + dM1;
    end
    % Sums up all signals of channel2 which has >0 signal in channel1
    if RandomizedChannel1(j,1)>0
        %SumColocChannel2 = SumColocChannel2 + MaskedChannel21D(j,1);
        dM2 = double(RandomizedChannel2(j,1))/double(SumChannel2);
        M2rand = M2rand + dM2;
    end
end
M1randtext = num2str(M1rand); M2randtext = num2str(M2rand);
```

% Write statistics of the colocalization to ColocStat matrix

% 'Slice' 'non-zero1' 'non-zero2' 'Min1' 'Min2' 'Max1' 'Max2' 'Mean1' 'Mean2' 'Mode1' 'Mode2' 'M1' 'M2'
'PCC'

```
ColocStat(i+1,1) = num2cell(i);
ColocStat(i+1,2) = num2cell(length(MaskedChannel11D(find(MaskedChannel11D(:)))));
ColocStat(i+1,3) = num2cell(length(MaskedChannel21D(find(MaskedChannel21D(:)))));
ColocStat(i+1,4) = num2cell(min(MaskedChannel11D(find(MaskedChannel11D(:)))));
ColocStat(i+1,5) = num2cell(min(MaskedChannel21D(find(MaskedChannel21D(:)))));
ColocStat(i+1,6) = num2cell(max(MaskedChannel11D(find(MaskedChannel11D(:)))));
ColocStat(i+1,7) = num2cell(max(MaskedChannel21D(find(MaskedChannel21D(:)))));
ColocStat(i+1,8) = num2cell(mean(MaskedChannel11D(find(MaskedChannel11D(:)))));
ColocStat(i+1,9) = num2cell(mean(MaskedChannel21D(find(MaskedChannel21D(:)))));
ColocStat(i+1,10) = num2cell(mode(MaskedChannel11D(find(MaskedChannel11D(:)))));
ColocStat(i+1,11) = num2cell(mode(MaskedChannel21D(find(MaskedChannel21D(:)))));
ColocStat(i+1,12) = num2cell(M1);
ColocStat(i+1,13) = num2cell(M2);
ColocStat(i+1,14) = num2cell(PCC(1,2));
ColocStat(i+1,15) = num2cell(M1rand);
ColocStat(i+1,16) = num2cell(M2rand);
ColocStat(i+1,17) = num2cell(PCCrandomized(1,2));
```



```

ColocStat(i+1,18) = num2cell(length(find(CellMask(:))));
ColocStat(i+1,19) =
num2cell(length(MaskedChannel21D(find(MaskedChannel21D(:)))/length(find(CellMask(:))));
ColocStat(i+1,20) =
num2cell(length(MaskedChannel11D(find(MaskedChannel11D(:)))/length(find(CellMask(:))));
end

% Write statistics of the z-stack colocalization t a text file (space separated)
FileName = strcat(MainDir, 'ColocStat.txt');
fileID = fopen(FileName,'w');
formatSpec = '%s %s %s %s %s %s %s %s %s %s %s %s %s %s %s %s %s %s\n';
fprintf(fileID,formatSpec,ColocStat{1,:}
);
formatSpec = '%f %f %f %f %f %f %f %f %f %f %f %f %f %f %f %f %f %f\n';
[nrows,ncols] = size(ColocStat);
for row = 2:nrows
    fprintf(fileID,formatSpec,ColocStat{row,:});
end
fclose(fileID);

```

List of publications/presentations resulted from this work:

Papers:

M.A.A. Fakhree, N. Zijlstra, C.C. Raiss, C.J. Siero, H. Grabmayr, A.R. Bausch, C. Blum, M.M.A.E. Claessens. The number of α -synuclein proteins per vesicle gives insights into its physiological function. *Sci Rep.* 2016;6:30658.

M.A.A. Fakhree, I.S. Nolten, C. Blum, M.M.A.E. Claessens. Different conformational subensembles of the intrinsically disordered protein α -synuclein in cells. *J Phys Chem Lett.* 2018;9(6):1249-1253.

M.A.A. Fakhree, S.A.J. Engelbertink, K.A. van Leijenhorst-Groener, C. Blum, M.M.A.E. Claessens. Cooperation of helix insertion and lateral pressure to remodel membranes. 2018 *Biomacromolecules* (*Submitted*).

Oral presentations:

M.A.A. Fakhree, C. Blum, M.M.A.E. Claessens. Cytosolic puncta of alpha-synuclein: membrane co-localization and copy number. Dutch biophysics 2015, Veldhoven.

M.A.A. Fakhree, C. Blum, M.M.A.E. Claessens. Cytosolic puncta of alpha-synuclein: membrane co-localization and copy number. CHAINS 2015, Veldhoven.

M.A.A. Fakhree, C. Blum, M.M.A.E. Claessens. Vesiculation by α -Synuclein on lipid membranes. MESA+ meeting, Enschede.

M.A.A. Fakhree, C. Blum, M.M.A.E. Claessens. Disordered and ordered subensembles of the unstructured protein α -synuclein in cells. MESA+ meeting 2018, Enschede; Dutch biophysics 2018, Veldhoven.

Poster presentations:

M.A.A. Fakhree, C.C. Raiss, N. Zijlstra, J. Kole, C. Blum, M.M.A.E. Claessens. In-vivo α -synuclein diffraction limited clusters and its colocalization with cellular membrane: a FRET study. Dutch biophysics 2014, Veldhoven.

M.A.A. Fakhree, C.C. Raiss, N. Zijlstra, J. Kole, C. Blum, M.M.A.E. Claessens. Diffraction limited intracellular α -synuclein clusters colocalize with cellular membranes. MESA+ meeting 2014, Enschede.

M.A.A. Fakhree, C. Blum, M.M.A.E. Claessens. The number of α -synuclein proteins per vesicle gives insights into its physiological function. MIRA day 2016, Enschede; CHAINS 2016, Veldhoven; Dutch biophysics 2016, Veldhoven; Dutch biophysics 2017, Veldhoven.

M.A.A. Fakhree, C. Blum, M.M.A.E. Claessens. The number of α -synuclein proteins per vesicle gives insights into its physiological function. IUPAB 2017, Edinburgh, UK.

M.A.A. Fakhree, C. Blum, M.M.A.E. Claessens. Indications for physiological function by single molecule photo-bleaching to determine protein copy numbers on cellular vesicles. MAF 2017, Brugge, Belgium.

M.A.A. Fakhree, C.C. Raiss, N. Zijlstra, J. Kole, C. Blum, M.M.A.E. Claessens. Functional insights into membrane bound α -Synuclein: Surface density, conformation, and mechanism of action. MESA+ meeting 2018, Enschede.

Selected publications prior to PhD:

M.A.A. Fakhree, A. Shayanfar, W.E. Acree Jr, A. Jouyban. Solubility of Phenanthrene in Binary Mixtures of C1– C4 Alcohols+ 2-Propanol and Ethanol+ Methanol at 298.2 K. J Chem Eng Data 2009;54(4):1405-1408.

M.A.A. Fakhree, W.E. Acree Jr, A. Jouyban. Solubility of Phenanthrene in Binary Mixtures of C1– C4 Alcohols at 298.2 K. J Chem Eng Data. 2009;55(1):531-534.

M.A.A. Fakhree, W.E. Acree Jr, A. Jouyban. Solubility of Phenanthrene in Ternary Mixtures of C1–C4 Alcohols at 298.2 K. Ind Eng Chem Res. 2010;49(13):6238-6242.

M.A.A. Fakhree, D.R. Delgado, F. Martínez, A. Jouyban. The importance of dielectric constant for drug solubility prediction in binary solvent mixtures: electrolytes and zwitterions in water+ ethanol. AAPS PharmSciTech. 2010;11(4):1726-1729.

M.A.A. Fakhree, S. Ahmadian, V. Panahi-Azar, W.E. Acree Jr, A. Jouyban. Solubility of 2-hydroxybenzoic acid in water, 1-propanol, 2-propanol, and 2-propanone at (298.2 to 338.2) K and their aqueous binary mixtures at 298.2 K. J Chem Eng Data. 2012;57(11):3303-3307.

Samenvatting

Intrinsiek ongeordende eiwitten (IOEs) en de intrinsiek ongeordende regio's van eiwitten met een structuur (IORs) hebben steeds meer wetenschappelijke aandacht gekregen in de afgelopen twee decennia. Er zijn twee belangrijke redenen voor deze toename: 1) IOEs/IORs worden nu erkend als factoren in de ontwikkeling van ziektebeelden en 2) IOEs/IORs hebben geen secundaire structuur wat het moeilijk maakt om hun exacte functie te bepalen. Naast het gebrek aan een secundaire structuur hebben IOEs/IORs ook een groot aantal mogelijke liganden en mogelijk meerdere functies. Om deze redenen worden IOEs ook wel 'mysterieuze' eiwitten genoemd.

Om de relatie tussen IOEs/IORs en ziektes te begrijpen en deze ziektes te kunnen behandelen is het noodzakelijk om de IOE te karakteriseren. De eerste stap om IOEs/IORs te karakteriseren is om te bepalen wat de interactiepartners zijn. De meeste IOEs nemen een (gedeeltelijke) secundaire structuur aan bij het binden met hun interactiepartners. Men moet dus mogelijke interactiepartners van IOEs/IORs identificeren en naar de affiniteit en structurele associatie tussen deze kijken. Zowel apart als in combinatie met andere interactiepartners. Door de interacties van een specifieke IOE te karakteriseren en kwantificeren zou het mogelijk moeten zijn om het eiwit ligand netwerk af te leiden waarin de IOE/IOR als hub dient. Dit interactienetwerk representeert dan de fysiologische rol(len)/functie(s) van het specifieke IOE. In dit proefschrift hebben we laten zien hoe een aantal vragen over de functionele vorm van een IOE, namelijk alpha-synucleïne (α S), opgelost kunnen worden.

Het interactienetwerk is belangrijk voor de ontwikkeling van therapeutische interventies in ziektes gerelateerd aan IOEs/IORs. Het klassieke slot-en-sleutel model dat volgt uit de structuur-functie dogma van een eiwit met een enkele functie als receptor is niet van toepassing op IOEs/IORs en zal daarom aangepast moeten worden. Deze aanpassing moet rekening houden met de specifieke interactie met een van de vele vormen van de IOE/IOR zonder de balans te verstoren in het eiwit/ligand netwerk van de IOR/IOR.

In dit proefschrift hebben we de biofysische functionele eigenschappen van alpha-synucleïne onderzocht in een cellulaire context. We hebben de

oppervlaktedichtheid van α S op cellulaire vesikellen gemeten en besproken dat gebaseerd op deze metingen α S als membraanhermodelleerder kan dienen. Ook suggereren wij een nieuw mechanisme voor de membraan hermodelleringcapaciteit van α S gebaseerd op recente verslagen over de rol van intrinsiek ongeordende domeinen van eiwitten in de vervorming van membraankromming. Bovendien hebben we de oppervlaktedichtheid gekoppeld aan de oppervlaktedichtheid van synaptobrevin, een gesuggereerde interactiepartner van α S en hiermee een nieuwe mogelijke functionele connectie toegevoegd aan het interactienetwerk van α S. Gebaseerd op kennis opgedaan in *in vitro* experimenten stellen we een nieuw mechanisme en rol voor voor een van de gesuggereerde interactiepartners van α S. Deze experimenten waren mogelijk dankzij in de late 20ste eeuw ontwikkelde technieken zoals het tot expressie brengen van fluorescente eiwitten en microscopie op het niveau van individuele moleculen.

Vervolgens zijn we een stap verder gegaan en hebben we aangetoond dat net als in *in vitro* experimenten, de structuur van α S veranderd bij het binden aan een cellulair vesikel. Dit is belangrijk om twee redenen. Ten eerste toont het aan dat er meer dan één verschillende conformatie van de IOE mogelijk is binnen cellen. Omdat er in deze context een structuur aanwezig is, helpt deze structuur ons de functie van de IOE (zoals membraan hermodellering) beter te begrijpen. Ten tweede geven wij bewijs dat in cellen, net als *in vitro* experimenten, de membraan gebonden structuur van α S anders is dan de structuur van het eiwit in het cytoplasma. Deze andere structuur is niet gevonden in voorgaand onderzoek door andere groepen met bulk methodes. Door gevoeliger single-molecule technieken te gebruiken is het mogelijk om α S populaties met verschillende structuren van elkaar te onderscheiden binnen cellen.

Gebruik makend van observatie dat α S de membraankromming kan veranderen en een specifieke membraan gebonden structuur heeft hebben we vervolgens de hypothese dat α S membraankromming kan induceren door laterale druk uit te oefenen getest. Om de invloed van andere interactiepartners van α S zoals synaptobrevin en Ca^{2+} in de cel uit te sluiten hebben we hiervoor een *in vitro* membraanmodel gebruikt. We hebben laten zien dat inkorting en verlenging van het ongeordende domein van α S effect heeft op de mogelijkheid van de IOE om het membraan te hermodelleren naar kleinere vesikellen. Dit

toont aan dat het IOR van α S een rol speelt in membraanhermodellering. We hebben dit idee vervolgens getoetst in een eenvoudige model berekening waarin we het effect van zowel helix insertie als laterale druk op de membraan kromming meenemen. De overeenkomst tussen experimenten en berekeningen suggereert dat onze hypothese over de membraanhermodelleringmechanismes van α S juist is.

Wat betekenen deze observaties voor α S in de cellulaire context? Door de interactiepartners van α S uit literatuur te onderzoeken en onze eigen biofysische vindingen, stellen wij voor dat α S betrokken is bij cellulaire activiteiten waar membraanhermodellering nodig is, zoals endo- en exocytose. Om dit idee te testen hebben we de colocalisatie van α S en onderdelen van de endo-/exocytose pathway onderzocht. De resultaten van deze studie suggereren dat α S vooral is betrokken bij endocytose van vesikelopname tot recycling en vernietiging binnen de cel. Gecombineerd met een literatuuronderzoek lijkt het dat α S specifiek betrokken is bij de opname van transferrine. Deze vindingen helpen om de rol van α S binnen de cel duidelijker te maken. De meeste van de gevonden eiwitten en lipiden die interactie aangaan met α S en organellen die beïnvloed worden door α S zijn betrokken bij endo- en exocytose.

Samengevat, we hebben aangetoond dat α S een rol speelt bij evenementen met processen zoals endo- en exocytose. Mogelijk als deel van een α S/ligand interactienetwerk. Verder onderzoek is nodig om het hele interactienetwerk en de functies van α S aan het licht te brengen.

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