The Localization of Alpha-synuclein in the Endocytic Pathway

Mohammad A. A. Fakhree, Irene B. M. Konings, Jeroen Kole, Alessandra Cambi, Christian Blum and Mireille M. A. E. Claessens

α-synuclein (αS) is an intrinsically disordered protein (IDP) that is abundantly present in the brain and is associated with Parkinson’s disease (PD). In spite of its abundance and its contribution to PD pathogenesis, the exact cellular function of αS remains largely unknown. The ability of αS to remodel phospholipid model membranes combined with biochemical and cellular studies suggests that αS is involved in endocytosis. To unravel which route(s) and stage(s) of the endocytic pathway αS is associated, we quantified the colocalization between αS and endocytic marker proteins in differentiated SH-SY5Y neuronal cells, using an object based colocalization analysis. Comparison with randomized data allowed us to discriminate between structural and coincidental colocalizations. A large fraction of the αS positive vesicles colocalizes with caveolin positive vesicles, a smaller fraction colocalizes with EEA1 and Rab7. We find no structural colocalization between αS and clathrin and Rab11 positive vesicles. We conclude that in a physiological context, αS is structurally associated with caveolin dependent membrane vesiculation and is found further along the endocytic pathway, in decreasing amounts, on early and late endosomes. Our results not only shed new light on the function of αS, they also provide a possible link between αS function and vesicle trafficking malfunction in PD. © 2021 The Author(s). Published by Elsevier Ltd on behalf of IBRO. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

Key words: alpha synuclein, endocytosis, colocalization, membrane, caveolin, EEA1.

INTRODUCTION

Alpha-synuclein (αS) is a 140 amino acid, intracellular, intrinsically disordered protein (IDP) that is abundantly present in the brain. Having a disordered nature probably allows αS to adapt its structure to changing conditions and to interact with many different cellular constituents. The exact cellular function of αS remains largely unknown probably due to its multifunctionality. The main function of αS has been suggested to involve membrae remodeling in processes such as the formation, processing, and coalescence of trafficking vesicles found in endo and exocytosis, especially at the synaptic terminals of neurons (Burre, 2015; Xilouri et al., 2016; Lautenschlager et al., 2017; Logan et al., 2017; Ramezani et al., 2019). For this function, the ability of αS to bind vesicles and to induce membrane curvature is thought to be important. In vitro experiments have shown that the N-terminal part of αS undergoes a conformational change into an amphipathic helix upon membrane binding (Davidson et al., 1998). Additionally, it has been observed that αS binding remodels membranes by inducing positive mean and/or negative Gaussian curvature in phospholipid bilayers (Braun et al., 2012). Such combination of a positive mean and negative Gaussian curvature is necessary in specific stages of cellular vesicle fission and fusion (Siegel, 1999).

Most research on the involvement of αS in endo- and exocytotic pathways has focused on Parkinson’s disease where the aggregation of αS is implicated in disturbed neurotransmission and cell death (Sung et al., 2001; Cooper et al., 2006; Gitler et al., 2008; Liang et al., 2008; Liu et al., 2009; Higashi et al., 2011; Yin et al., 2014; Stefanovic et al., 2015; Dinter et al., 2016; Hassink et al., 2018; Masaracchia et al., 2018). These studies indicate that endo and exocytotic pathways get disturbed by an excess of αS. Moreover, endo and exocytosis play a role in the disease related transmission of αS species between cells (Park et al., 2009; Gonçalves et al., 2016; Delenclos et al., 2017).

In a functional context the role of αS in different parts of vesicle trafficking pathways remains unclear. Previously we observed colocalization between αS and wheat germ agglutinin (WGA) in differentiated SH-SY5Y neuroblastoma cells (Fakhree et al., 2018). WGA binds...
glycoproteins on the plasma membrane and is internalized via endocytosis in time. The colocalization of WGA with αS thus suggests that αS plays a role in endocytosis. With which endocytic process αS is associated is under debate. In the literature conflicting reports on the involvement of αS in either clathrin or caveolin dependent endocytosis can be found (Hashimoto et al., 2003; Jin et al., 2007; Liang, et al., 2008; Ben Gedalya et al., 2009; Park, et al., 2009; Cheng et al., 2011; Madeira et al., 2011; Kisos et al., 2014; Delenclos, et al., 2017; Medeiros et al., 2017; Soll et al., 2020). To unravel with which route (s) and stage (s) of the endocytic pathway αS is associated, we investigated the colocalization between αS and vesicle bound proteins that mark specific routes and stages of the endocytic process in differentiated SH-SY5Y cells. Using an object based colocalization analysis, we quantified αS colocalization with caveolin, clathrin, EEA1, Rab7, and Rab11 in confocal microscopy images. Comparison with randomized data allowed us to discriminate between structural and coincidental colocalizations. We show that αS is associated with caveolin mediated endocytosis and also colocalizes to a lesser degree further along the endocytic pathway with EEA1 positive early endosomes and Rab7 positive late endosomes. Quantifying the colocalization is a step towards understanding cellular localization and function of αS.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Differentiation:** Culturing and differentiation of SH-SY5Y cells was performed as described elsewhere (Raiss et al., 2016). In short, SH-SY5Y cells were obtained from ATCC (US) and grown in proliferation medium GlutaMAX™ supplemented with 10% heat inactivated FBS, 1% non-essential amino acids, 10 mM HEPES buffer, and 1% Penicillin/Streptomycin. For the experiments the SH-SY5Y cells were differentiated (Lopes et al., 2010; Raiss, et al., 2016). For differentiation the SH-SY5Y cells were seeded in collagen IV coated 6 channel μ-Slides (ibidi, Germany) to reach 60% confluency. Subsequently the differentiation was induced by changing the proliferation medium to starvation medium for 3 days. The starvation medium is prepared similar to the proliferation medium, except it contains only 1% FBS and additionally 10 μM retinoic acid (Sigma). All materials were obtained from Invitrogen, USA if not indicated otherwise.

**Immunostaining:** Cells were fixed in 3.7% paraformaldehyde in PBS for 15 min at room temperature (RT) and permeabilized with 0.3% Triton X-100 in PBS for 5 min at RT. Autofluorescence of the samples was quenched with 50 mM ammonium chloride in PBS for 15 min at RT. Aspecific binding sites were blocked by incubating the samples with 16% goat serum, 0.3 M NaCl and 0.1% TX100 in PBS for 30 min at RT. The samples were incubated over night at 4 °C with the primary antibodies diluted in blocking solution (See Table 1 for details). In Fig. 1 we show an overview of the investigated pathways and chosen endocytic markers. After the incubation with the primary antibodies, the samples were washed 3 times 5 min with wash buffer (0.3% TritonX100 and 0.1% BSA in PBS) at room temperature. Later, the samples were incubated for at least 60 min at RT with the secondary antibodies (See Table 1 for details). A serial dilution of the primary and secondary antibodies was made in order to find the optimum concentration for the immunostainings. Following 3 times 5 min washing with the wash buffer, nuclear counterstaining was performed by incubating cells with 300 nM 4',6-diamidino-2-phenylindole (DAPI) in the wash buffer for 10 min at RT, followed by 3 times 5 min washing with the wash buffer. Finally, samples were washed 2 times 5 min with PBS, and stored in mounting medium (ibidi, Germany). All materials were obtained from Sigma, Germany if not indicated otherwise.

**Imaging:** Confocal laser scanning microscopy images were obtained using a commercial Nikon A1 confocal microscope with a 60× water immersion objective (NA = 1.2, Nikon, Japan) and laser sources of 405 nm, 488 nm, and 561 nm. Emission was detected using appropriate dichroic mirrors and filter sets. Images were recorded using the Nyquist mode of the NIS software (Nikon, Japan). Using the Nyquist option for the XY plane, a pixel size of ~110 nm was used to record images. Sections along the z axis were recorded with ~1 μm distance between slices. For image analysis purposes, the bottom and top slices of the z-stacks were determined such that they included at least one clear and sharp αS or endocytic marker punctum. Images were recorded using an image depth of 12 bit,

| Table 1. List of antibodies used in this study |
|----------------------------------------------|
| **Primary antibodies**                        |
| **Host species**                              |
| **Dilution**                                  |
| **Source**                                    |
| Alpha synuclein (211)                         |
| Mouse                                        |
| 1‘100                                        |
| Santa Cruz Biotechnology                      |
| Caveolin-1 (D46G3) XP®                       |
| Rabbit                                       |
| 1‘100                                        |
| Cell Signaling                               |
| Clathrin HC (D3C6)                           |
| Rabbit                                       |
| 1‘50                                         |
| Cell Signaling                               |
| EEA1 (C4S810)                                |
| Rabbit                                       |
| 1‘100                                        |
| Cell Signaling                               |
| Rab11 (D4F5) XP®                             |
| Rabbit                                       |
| 1‘100                                        |
| Cell Signaling                               |
| Rab7 (D95F2) XP®                             |
| Rabbit                                       |
| 1‘100                                        |
| Cell Signaling                               |
| **Secondary antibodies**                     |
| **Host species**                              |
| **Dilution**                                  |
| **Source**                                    |
| Goat anti-mouse conjugated with Alexa Fluor 568 (A-11004) | Goat | 1‘200 | Life Technologies |
| Goat anti-rabbit conjugated with Alexa Fluor 488 (A-11008) | Goat | 1‘200 | Life Technologies |
and scanning settings were optimized to avoid intensity saturation in the images. Image analyses: The analysis of the images was performed per slice, unless mentioned otherwise. For each endocytic marker approximately 200 confocal slices originating from at least 40 cells were analyzed to obtain the nearest neighbor distance distributions. To only include fluorescent signal that originates from the cell interior, a cell mask was applied to all images. Fluorescence from the endocytic marker channel was used to determine the shape of the cell mask using the ImageJ software (Fiji version). Since we are only interested in signal coming from the cytoplasm, signal from the nucleus was removed by applying an additional nuclei mask. This mask was based on the images of the counterstained nuclei, a threshold for this mask was created using the Huang auto-threshold (ImageJ). Next, the TrackPy (Allan et al., 2019) object finder function was used to identify the fluorescence puncta within the cell shape in the images. In the identification, only fluorescent puncta of a size of $>5$ pixels and minimum mass equal to Average $+ 10 \times \text{SD}$ of the image intensity were taken into account. For both channels, the pixel coordinates of all identified fluorescent puncta (objects) were extracted. Next, the distance from each $\alpha S$ positive object to the nearest marker protein positive object was determined. To determine these distances, the “knnssearch (I)” function of the Matlab 2019b package was used. The angle between the two nearest neighbor objects in the two channels was calculated using the arctangent function on the XY coordinates of the two points. To discriminate between structural colocalization and coincidental overlap, we randomized the position of the identified objects within the cell mask. We accounted for the excluded volume that is not accessible to the vesicles by increasing the density of vesicles. This decrease in the available volume results in an increase in the apparent density of vesicles. The excluded volume is in first approximation randomly distributed in the cell’s cytoplasm. We therefore used an increased density of the objects in the nucleus excluded cell volume when randomizing the data to match the experimentally obtained distance distributions. Distances between objects in the randomized data sets were determined as described above.

RESULTS

As observed before (Fakhree et al., 2016, 2018), after staining $\alpha S$ in differentiated SH-SY5Y cells with fluorescently labeled antibodies, vesicle bound $\alpha S$ is visible as small puncta with sizes that approach the diffraction limit (Fig. 2 B, E) and cytoplasmic $\alpha S$ gives some diffuse signal. Here we investigate the involvement of $\alpha S$ in endocytosis, we neglect the diffuse signal and only take into account fluorescence signal originating from the puncta. Since both clathrin and caveolin mediated endocytosis proceed via the formation of small vesicles, we focused on these uptake pathways. Staining clathrin and caveolin in differentiated SH-SY5Y cells using fluorescently labeled antibodies results in a distribution of small fluorescent puncta in the cell, as observed after staining for $\alpha S$. Fig. 2 C, F show the overlay of signal from the $\alpha S$ channel and the caveolin and clathrin channels respectively. In Fig. 2 C, a considerable fraction of the $\alpha S$ positive puncta colocalizes with the caveolin positive structures. Many of the $\alpha S$ positive vesicles do however not colocalize with caveolin positive puncta. For clathrin and $\alpha S$ hardly any colocalizations are observed (Fig. 2 F). Considering the high density of puncta, the apparent colocalization in the z-projected data could be coincidental. To discriminate between structural colocalization and coincidence, we determined the distance and direction between each $\alpha S$ positive vesicle and its nearest caveolin or clathrin positive vesicle based on their position in individual confocal slices.

To judge if colocalization events are coincidental or not, only short distances between $\alpha S$ and caveolin/clathrin positive vesicles should be taken into account. We therefore plotted the angle dependent nearest neighbor distribution for distances from $\alpha S$ to caveolin and from $\alpha S$ to clathrin up to 1.5 $\mu m$ (Fig. 3 A, B). The distributions are markedly different. Whereas the nearest distances between $\alpha S$ and caveolin cluster below 0.3 $\mu m$, there is no such cluster visible for the
Population of microscope, evidences colocalization for a sub-caveolin distances within the diffraction limit of the remaining population of caveolin or clathrin seem to be randomly distributed. To verify if this distribution is random, we modeled the data nearest neighbor distribution. This signifies that there is no offset due to misalignment can be excluded. Due to the diffraction limit nearest neighbor distances \( r < 0.3 \mu m \) occur within one bin.

In the center of the polar plot \( (r < 0.3 \mu m) \), the clusters of short distances between \( \alpha S \) and caveolin, and between \( \alpha S \) and clathrin represent objects that are present in the same detection volume. At first glance, \( \alpha S \) and caveolin thus seem to colocalize. However, at the observed vesicle densities, the presence of \( \alpha S \) and clathrin or caveolin in the same detection volume may be coincidental and not represent a structural colocalization on the same vesicle. To quantitatively assess if the observed short distances between \( \alpha S \) and caveolin or clathrin represent structural colocalization or are coincidental, we plotted the nearest neighbor distance distribution up to 10 \( \mu m \) in histograms (Fig. 3C, D). In the histogram for caveolin (Fig. 3C), we observe that 48% of the \( \alpha S \) positive vesicles are located within 0.3 \( \mu m \) from caveolin. The nearest neighbor distance of the remaining 52% is broadly distributed and peaks at approximately 1.2 \( \mu m \). The large fraction of \( \alpha S \) to caveolin distances within the diffraction limit of the microscope, evidences colocalization for a sub-population of \( \alpha S \) with caveolin positive vesicles. For the remaining population of \( \alpha S \) vesicles, the distances to the nearest caveolin seem to be randomly distributed. To verify if this distribution is random, we modeled the data based on the density of non-colocalizing vesicles (excluding the first bin in the histogram Fig. 3C). We calculated the probability density function for distances between randomly distributed \( \alpha S \) and caveolin at the experimentally observed density. The calculated probability density function significantly deviates from the experimentally observed one (Fig. 3C, inset, dashed line). The calculated function peaks at 1.8 \( \mu m \) and neither reproduces the high occurrence of short distances nor the long tail of longer distances. However, two important aspects are ignored in this calculation. In the calculation we assume an isotropic environment whereas the cell has a shape. Additionally, not the whole cell volume is accessible to the vesicles: the cell is a very crowded environment. Effectively there is a volume from which the vesicles are excluded because it contains i.a. other vesicles, endoplasmic reticulum or the Golgi apparatus. In our simulation of randomized positions we take both the nucleus excluded cell shape and excluded volume into account (Materials and Methods). We find very good agreement between the experiments and the simulations when we assume that 56% of the nucleus excluded volume is not accessible to the vesicles (Fig. 3C, D). For \( \alpha S \) to caveolin distances, the peak position in the experimentally determined distance distribution is reproduced in the simulations with a small overshoot in probability. The probability of finding long distances is slightly underestimated in the simulations. Coincidental colocalizations (distances < 0.3 \( \mu m \)) are rare and comprise roughly 2% of the total probability distance distribution. We therefore conclude that, for our experiments, 46% of the total \( \alpha S \) positive vesicle population structurally or functionally colocalizes with caveolin positive vesicles.

Fig. 2. Colocalization of \( \alpha S \) with caveolin and clathrin. Representative images of differentiated SH-SY5Y cells immunostained against \( \alpha S \) and the endocytic markers caveolin (A–C) and clathrin (D–F). In the merged images (C, F), the following color code was used: \( \alpha S \) (green) and endocytic markers (red), and DAPI counter-staining for nuclei (blue). Images are maximum z-intensity projections which are brightness/contrast adjusted. Scale bars are 10 \( \mu m \). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
colocalizing αS population of nearest neighbor distances could again be simulated well by a random distribution in the nucleus excluded cell shape assuming that 56% of the cell volume was not accessible to the vesicles. Interestingly, the experimental data shows higher probabilities for distances < 1 μm than the simulated randomized data. Revisiting the images show that in several cases large EAA1 positive early endosomes colocalize with smaller αS positive vesicles (Fig. 4A). The fact that the EAA1 positive endosomes are much larger than the αS positive vesicles implies that the criterion for colocalization needs to be adjusted. The criterion that the distance between the center of mass of the two structures should be < 0.3 μm (or one diffraction limit) no longer holds. Merged early endosomes typically reach diameters up to 1 μm (Murk et al., 2003; Ramanathan and Ye, 2012; Kaur and Lakkaraju, 2018), for our analysis this means that distances up to ~1 μm (the first 3 bins of our analysis) may indicate structural colocalization. Colocalization of small αS positive vesicles with large early endosomes therefore explains the difference between the experimental and simulated data in the 2nd, and 3rd bin of the histogram. Summing the difference in the probability between the experimental and simulated data in the first 3 bins indicates that 22% of αS vesicles structurally colocalize with EAA1 positive early endosomes.

Early endosomes can develop into recycling endosomes or late endosomes. To visualize recycling endosomes, we stain for the presence of Rab11, an often used marker protein for this stage of endocytosis (van Ijzendoorn, 2006). In the images, colocalization events are rare (Fig. 5A). To discriminate structural colocalization from coincidental colocalization we again assemble the nearest neighbor distances in a histogram (Fig. 5C) and compare the experimental data to simulated randomized data. The simulation shows very good agreement with the experimental data. We see no signs for structural colocalization between αS and Rab11, all observed colocalizations can be explained by random overlap.

Alternatively, early endosomes further develop into late endosomes. To visualize late endosomes, we stain for the late endosome marker protein Rab7 (Vanlandingham and Ceresa, 2009). As observed for the early endosomes, some Rab7 positive structures are large compared to the αS positive vesicles (Fig. 5B).
In the images, apparent colocalization events between αS and Rab7 positive structures can be observed as an overlap between the αS and Rab7 signal. In some cases, more than one αS positive vesicle localizes with one large Rab7 positive structure. The overlay of the measured and simulated nearest neighbor distance histogram shows that the randomized data does not represent the experimental distribution well. Especially the probability to find short nearest neighbor distances, < 1 μm, is higher for the experimental data. Taking into account the relatively large size of some of the Rab7 positive structures, this is a signature of structural colocalization. The experimentally observed probability to find nearest neighbor distances up to 1 μm originates from a combination of structural colocalization and coincidental colocalization. To obtain a better agreement between the measured and simulated data at distances > 1 μm, we simulate randomized distributions in the cell mask without considering the colocalizing fraction at distances < 1 μm.

DISCUSSION

To deduce with which part of the endocytic pathway αS is associated, we studied the object based colocalization of αS positive vesicles with proteins that mark different routes and stages of the uptake process. In order to quantify the data and to discriminate between structural and coincidental colocalization, we additionally compared experimental findings to simulated, randomized data. The results of this colocalization study are summarized in Table 2. For objects that do not structurally or functionally colocalize but overlap incidentally, we find very good agreement between randomly distributed objects and the experimental data when we assume that 56% of the total cell volume is not accessible to the vesicles. This non-accessible volume is consistently required to match the different sets of experiments to the distance distributions based on simulated, randomized data. The existence of non-accessible volume can easily be rationalized: vesicles are only present in the cytoplasm which typically accounts for ~50% of the nucleus excluded cell volume (Luby-Phelps, 2000).

The colocalization experiments clearly show that in differentiated SH-SY5Y cells, αS is associated with caveolin. Immunoprecipitation experiments reported in the literature even indicate that αS and caveolin directly interact (Madeira, et al., 2011). The localization of αS on caveolin positive vesicles accounts for approximately half of the αS positive vesicle population. The large fraction of colocalizations and the previously observed high αS copy number on vesicles (Fakhree et al., 2016) strongly suggests that αS has a membrane remodeling function in the caveolin dependent pathway. In vitro studies show that αS induces curvature in model membranes (Varkey et al., 2010; Braun et al., 2014; Fakhree et al., 2019), it may play a similar role at the early stages of endocytosis and suggests that the colocalization may be functional. In Parkinson’s diseases, αS induced dysregulation of the extracellular signal-regulated kinases (ERK) that assemble onto caveolin scaffolds has been suggested to play a role (Hashimoto, et al., 2003). The structural colocalization between αS and caveolin observed here indicates...
that there is a direct link between αS function and vesicle trafficking malfunction.

In differentiated SHSY-5Y we do not find evidence that αS plays a role in clathrin dependent endocytosis. Although we do find apparent colocalization between αS positive and clathrin positive vesicles, careful analysis and comparison to randomized data shows that these colocalizations are coincidental. Additional αS colocalization experiments with fluorescently labelled transferrin support this finding. Transferrin is taken up by cells via clathrin dependent endocytosis and does not structurally colocalize with αS (see SI Fig. 1).

Currently, there is no consensus in the literature on the involvement of αS in clathrin dependent endocytosis under normal physiological conditions. While some exclude the involvement of αS (Kaur and Lee, 2020), others deduce that αS has a functional role in clathrin dependent endocytosis (Ben Gedalya, et al., 2009; Dijkstra et al., 2015; Chung et al., 2017). The complexity of the experiments and promiscuity of αS interactions may provide an explanation for this conflicting data. Most of the evidence for the involvement of αS in clathrin dependent endocytosis is indirect and relies on e.g. specific

### Table 2. Percentage of αS positive vesicles that colocalize with endocytic marker proteins. Coincidental colocalizations are excluded

| Endocytic marker | % positive colocalizations |
|------------------|---------------------------|
| Clathrin         | –                         |
| Caveolin         | 46%                       |
| EAA1             | 22%                       |
| Rab7             | 10–15%                    |
| Rab11            | –                         |

![Fig. 5. Colocalization between αS and Rab11 and Rab7. For both Rab 11 (A) and Rab7 (B) endocytic markers representative orthogonal cross-sections of immunostained differentiated SH-SY5Y cells are shown. In the images αS is visible in green and Rab11 and Rab7 in red. Cell nuclei are counterstained with DAPI (blue). Examples where αS and Rab7 signal overlaps are indicated with white arrows. Scale bars = 10 μm. (C, D) Histograms of the αS to nearest Rab11 and Rab7 neighbor distances, respectively. Experimental data is shown in green, simulated data in gray. For the simulated data the localizations were randomized in the cell shape taking into account the non-accessible volume. For the Rab7 data non coincidental localizations in the first bins, counting up to 10–15%, were excluded in the randomization. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image-url)
receptors and cargo uptake, interactions with proteins that are also involved in clathrin dependent endocytosis, and downstream effects (Liang, et al., 2008; Dijkstra, et al., 2015; Baksi et al., 2016; Chung, et al., 2017; Soll, et al., 2020).

In the context of αS overexpression and disease the involvement or interference of αS with clathrin dependent endocytosis appears to be more pronounced (Jin, et al., 2007; Ben Gedalya, et al., 2009; Cheng, et al., 2011). IDPs are known to be promiscuous in their interactions with other proteins, and in overexpression systems non-functional interactions of moderate to low affinity may result in colocalizations (Vavouri and Lehner, 2009; Monty, 2015; Macosay-Castillo et al., 2019; Uversky, 2019). Excess αS in disease may thus disturb endocytosis. Additionally, clathrin dependent endocytosis may play an important role in the transmission and exchange of αS species between cells in Parkinson’s disease etiology (Medeiros, et al., 2017; Soll, et al., 2020; Yang et al., 2020).

Moving further along the endocytic uptake process, αS colocalizes with EEA1 positive early endosomes. From the images and our analysis, it becomes apparent that the early endosomes are often much larger than the αS positive vesicles. Generally we do not observe colocalization of large αS and large EEA1 positive structures, colocalization is found between small αS structures and large EEA1 structures. This observation may indicate that we are mainly capturing αS positive vesicles at docking and fusion events with the early endosomes. We observe a similar pattern for αS positive vesicles and large Rab7 positive structures and therefore hypothesize that αS positive vesicles might also fuse with Rab7 positive structures. The presence of αS at these stages of the endocytic pathway may again be functional. αS has been suggested to play a role in vesicles fusion, either directly by bridging the membranes of different compartments (see works by Dikiy and Eliezer (2012) and Fusco et al. (2016)) or indirectly by chaperoning SNARE mediated fusion (Burre et al., 2010; Burre, 2015).

In our experiments, we do not see indications for structural colocalization of αS positive vesicles with Rab11 positive recycling endosomes. Apparently, in differentiated SH-SY5Y cells αS does not play a role in the membrane remodeling processes required for the transition of early endosomes to recycling endosomes under physiological conditions. However, colocalization between αS and Rab11 may play a role under non-physiological conditions as colocalization has been observed in the context of αS overexpression and Parkinson’s disease related uptake and/or clearance of αS (Liu, et al., 2009; Chutna et al., 2014; Poehler et al., 2014; Breda et al., 2015; Gonçalves et al., 2016; Maekawa et al., 2016).

We conclude that in a physiological context, αS is structurally associated with caveolin dependent membrane vesiculation and is found further along the endocytic pathway, in decreasing amounts, on early and late endosomes. The decrease is possibly linked to the change in the membrane composition along the endocytic pathway. Studies on phospholipid vesicles show that the αS binding affinity strongly depends on membrane composition (Middleton and Rhoades, 2010; Shvadchak et al., 2011; Pfefferkorn et al., 2012).

Clearly with the current selection of endocytic markers we do not account for the whole population of αS positive vesicles. To establish the lower boundary of the size of the unaccounted fraction, we assume that the vesicle populations that are screened with the different markers, do not overlap. Under this assumption, the structural colocalizations with caveolin, EEA1 and Rab7 account for 80–85% of the αS positive vesicle population. At least 15–20% of the αS positive objects are unaccounted for in our analysis. This is not surprising considering that αS has also been suggested to play a role in SNARE mediated fusion with the plasma membrane (Burre, et al., 2010; Kaur and Lee, 2020) and to localize in mitochondria (Faustini et al., 2017; Wu et al., 2017).

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**APPENDIX A. SUPPLEMENTARY DATA**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuroscience.2021.01.017.

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