Abstract:

Alpha-synuclein is known as a presynaptic protein that binds to small synaptic vesicles. Recent studies suggest that alpha-synuclein is not only attracted to these tiny and therewith highly curved membranes, but that in fact the sensing and regulation of membrane curvature is part of its physiological function. Moreover, recent studies have suggested that alpha-synuclein plays a role in the endocytosis of synaptic vesicles, and provided support for a function of alpha-synuclein during exo- and endocytosis, where curvature sensing and membrane stabilization are critical steps. This review aims to highlight recent research in the field and adds a new picture on the function of alpha-synuclein in maintaining synaptic homeostasis upon intense and repetitive neuronal activity.
Dear Jillian Shaw,

Many thanks for considering our manuscript “Alpha-synuclein – regulator of exocytosis, endocytosis or both?” for Trends in Cell Biology. The comments by the reviewers made clear that the review is highly topical and stimulates discussion, in line with the scope of TBC. We are very thankful to the reviewers for their interesting viewpoints, we think that our review article has improved significantly as a result and are delighted to resubmit our revised manuscript, which we hope meets with your approval.

Conflicts of interest:
JL was supported by a research fellowship of the “Deutsche Forschungsgemeinschaft” - DFG (LA 3609/2-1). CFK acknowledges funding from the Engineering and Physical Sciences Research Council (EPSRC) UK. GSK and CFK acknowledge funding from the Wellcome Trust, the Medical Research Council (MRC) UK, and Alzheimer Research UK (ARUK).

Yours faithfully
Janin Lautenschläger
Dear Jillian Shaw,

Many thanks for considering our manuscript “Alpha-synuclein – regulator of exocytosis, endocytosis or both?” for Trends in Cell Biology. The comments by the reviewers made clear that the review is highly topical and stimulates discussion, in line with the scope of TBC. We are very thankful to the reviewers for their interesting viewpoints, which we have addressed and incorporated as listed point-by-point below. We think that our review article has improved significantly as a result and are delighted to resubmit our revised manuscript, which we hope meets with your approval.

For Reviewer #1

Point 1. Yes, we agree with the reviewer, the studies on endocytosis, did not see any changes in the exocytosis, so we have adjusted this paragraph (p. 8). We clarified the fact that the cited studies, which report changes in endocytosis, have not reported any change in exocytosis.

Point 2. The reviewer argues that the function of alpha-synuclein is in clathrin-mediated endocytosis. Our review makes clear that the exact role of alpha-synuclein in endocytosis is not yet known. Our aim is to stimulate discussion and show possibilities, based on available evidence, on how alpha-synuclein function might be elicited via its membrane and curvature sensing properties. We have however more evenly balanced our discussion in line with the suggestions by the reviewer. The passage on CME and kiss-and-run has been rewritten (p. 10/11), and addresses the role of alpha-synuclein in both, slow or fast endocytosis pathways. The subtitle of this paragraph has been changed accordingly.

Point 3. We have included the comments raised by the reviewer on page 10/11 to present a more balanced discussion on the role of alpha-synuclein (see point 2).

Point 4. Figure 3 has not been changed, as we feel it is a balanced account of the current literature on the involvement of alpha-synuclein in CME as well as the kiss-and-run mechanism.

Point 5. We have included a short paragraph on alpha-synuclein and its relationship to Parkinson’s disease (PD) in the first part of the introduction (p. 2) and also when necessary for the understanding of the experimental settings described (p. 6, 10).

Points 6, 7 and 8. The tables 2 and 3 were merged into one, so that table 1 now summarises all alpha-synuclein overexpression studies, while table 2 includes all alpha-synuclein knock-out studies. We have not added a separate table for endocytosis data as these studies are discussed in much more detail in the text than the exocytosis data. “No change” has now been replaced by a simple dash in tables 1 and 2, as suggested by the reviewer.

Point 9. The data on alpha-synuclein and VAMP2 interaction are quite controversial, particularly so for alpha-synuclein mutants. We have made a suitable reference to these experiments but decided not to elaborate in detail, as it is not directly in focus with the topic of our review (p. 4).
Point 10. We have changed the expression used to describe FM4-64 to “applying lipophilic dyes, used for the visualization of exocytosis in live synapses.” (p. 8).

Point 11. Regarding the studies by Xu et al. and Busch et al., we have made clear which experiments have been done on synapses from transgenic mice (with overexpression of alpha-synuclein), and which experiments have been done with acute delivery of alpha-synuclein (p. 8/9).

Point 12. This was corrected to “in invertebrates” (p. 13).

For Reviewer #2

1. Point. We changed the sentence on p. 7, which was criticised by the reviewer. We summarize the data on the synaptic vesicle pool, but no direct link to the question of endocytosis is claimed.

2. Point. The study of Scott et al 2012 has already been cited in the text and we have now included the study by Wang et al. as well (p. 7).

3. Point. The study by Yavich et al. 2004 has been included in table 2 and cited in the text, the study by Abeliovich has been changed.

Yours sincerely,

Janin Lautenschläger
Alpha-synuclein - regulator of exocytosis, endocytosis, or both?

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Keywords: alpha-synuclein, exocytosis, endocytosis, LRRK2, kiss-and-run, ultrafast endocytosis

Abstract

Alpha-synuclein is known as a presynaptic protein that binds to small synaptic vesicles. Recent studies suggest that alpha-synuclein is not only attracted to these tiny and therewith highly curved membranes, but that in fact the sensing and regulation of membrane curvature is part of its physiological function. Moreover, recent studies have suggested that alpha-synuclein plays a role in the endocytosis of synaptic vesicles, and provided support for a function of alpha-synuclein during exo- and endocytosis, where curvature sensing and membrane stabilization are critical steps. This review aims to highlight recent research in the field and adds a new picture on the function of alpha-synuclein in maintaining synaptic homeostasis upon intense and repetitive neuronal activity.
Structure to function

From the time of its discovery, alpha-synuclein (see Glossary) has been known as a presynaptic protein [1]. The protein has attracted much interest as it is one of the major constituent of Lewy bodies [2] and mutations as well as gene duplications and triplications are linked to familial Parkinson’s disease [3–6]. But how much more do we know about this elusive protein today, nearly 30 years later? Structurally, alpha-synuclein is a 140 amino acid protein with 3 main regions, the N-terminal region, which forms an α-helix and is essential for binding to phospholipid vesicles, the NAC (non-Abeta component) region, relevant for aggregation, and the acidic C-terminal region, with chaperone like activity [7]. NMR-studies have recently determined the exact amino acid residues involved in membrane binding. The first 25 N-terminal residues of alpha-synuclein are designated as the membrane anchor region, binding very tightly to membranes. Amino acids 26-97, on the other hand, define a region that binds less tightly and is supposed to regulate the affinity of alpha-synuclein binding to membranes. The C-terminal region is shown to associate only weakly with membranes [8].

Recently, it has been proposed that alpha-synuclein is a curvature sensing protein [9,10] and it has been added to the class of amphipathic helix-containing proteins that sense and generate membrane curvature [11,12]. While these proteins are unstructured in solution, they start to fold their amphipathic helix in certain physicochemical conditions, a process that is strongly favoured by lipid packaging defects in curved membranes. In this way, such proteins are capable of sensing the curvature of membranes. And, upon insertion of the amphipathic helix into the lipid surface, the membrane curvature itself becomes stabilized (Figure 1).

Although, the amphipathic helix of alpha-synuclein is quite different structurally to the helices of other amphipathic lipid-packaging sensor (ALPS) proteins, alpha-synuclein seems to act in a similar way. Indeed, with its small hydrophobic residues and a more pronounced polar face,
the structure of alpha-synuclein appears to be inherently designed to bind to negatively
charged lipids and to interfere with small lipid packaging defects, occurring notably in synaptic
vesicles with a high content of polyunsaturated lipids (for an excellent review on the topic see
[13]).

In the past, alpha-synuclein has been viewed simply as a protein bound to vesicles. This
structure-function relationship is a potential paradigm shift from the traditional view of alpha-
synuclein, in that it suggests the notion that the protein is not only attracted to highly curved
membranes, but rather that the sensing and stabilization of curved membranes is inherent
part of its physiological function. The presynaptic terminal is a place of sustained membrane
remodelling as synaptic vesicles constantly approach the active zone of the presynaptic
plasma membrane and release their neurotransmitter content. Subsequently, both
membrane material as well as proteins have to be recycled, on the one hand, to clear the
active zone from excess material and, on the other hand, to form new synaptic vesicles, to be
loaded again with neurotransmitter molecules. Current efforts analysing the role of alpha-
synuclein in the regulation of exocytosis point to a regulatory function rather than a direct
involvement in the actual release machinery, so that the lipid binding properties come into
play. Recently, NMR studies found that alpha-synuclein is able to bind with the N-terminal
region (aa 1-25) as well as with the aa 65-97 region to lipid membranes, and a double-anchor
mechanism was proposed, where alpha-synuclein tethers either two vesicles to one another,
or vesicles to the plasma membrane, possibly to facilitate processes required for exo- and
endocytosis [14]. Moreover, general stabilization of membrane curvature could have a
modulating effect on exocytosis.
Alpha-synuclein in synaptic vesicle exocytosis

Alpha-synuclein and SNARE-complex assembly

A function of alpha-synuclein in synaptic vesicle docking and fusion has been proposed by studies showing that alpha-synuclein has a role in SNARE-complex assembly, a process essential for many membrane fusion events. For exocytosis of synaptic vesicles, the SNARE proteins from the plasma membrane, syntaxin and SNAP-25, and the SNARE protein anchored on the synaptic vesicle, VAMP2, assemble together to form a complex with four helices, which then zips the vesicles onto the plasma membrane, ready to undergo fusion following synaptic stimulation. There is still some controversy on how alpha-synuclein might act on this SNARE-complex. While one major study indicates increased SNARE-complex assembly [15], others argue for a negative regulatory function of alpha-synuclein [16,17]. A direct interaction of alpha-synuclein with VAMP2, the vesicular SNARE protein, has been described, but also the incorporation of alpha-synuclein within the four-helical SNARE protein-complex has been discussed (Figure 2a).

Alpha-synuclein and fusion of vesicles complemented by SNARE protein complexes

What can we learn from in vitro experiments studying the effect of alpha-synuclein on vesicle fusion? In assays reconstituting fluorescently labelled lipid vesicles with syntaxin and SNAP-25 in one case and with VAMP2 in the other, alpha-synuclein was clearly shown to inhibit vesicle fusion in a concentration dependent manner [18–20]. However, two of these studies argue that no direct binding of alpha-synuclein to the SNARE-complex is needed for this inhibition and that inhibition is merely due to the lipid interaction of alpha-synuclein [19,21]. On the other side, for alpha-synuclein oligomers, the binding to VAMP2 was found to be a critical step for inhibiting vesicle fusion [20]. Together, these data draw again quite a controversial picture.
of alpha-synuclein function on SNARE-complex formation, but, at least for alpha-synuclein monomer, a mechanism that relies on membrane curvature stabilisation, thus preventing premature fusion, seems likely. Studies analysing pure lipid vesicles lend further support to this hypothesis and clearly show a capability of alpha-synuclein to integrate into the lipid surface, which then enables the stabilization of lipid packaging defects of vesicles and thereby their membranes’ curvature [21,22] (Figure 2b).

In addition to a role in curvature sensing and stabilization, alpha-synuclein could potentially promote exocytosis through its double-anchor mechanism, binding to lipid membranes not only with the N-terminal region (aa 1-25), but also with the region aa 65-97 [14]. Therewith alpha-synuclein would be able to tether synaptic vesicles to the plasma membrane or even the active zone, which would influence exocytosis (Figure 2c). Even more, alpha-synuclein could this way influence the proximity of synaptic vesicles to voltage-gated calcium channels in the plasma membrane, which has been shown to regulate the mode of exo- and endocytosis by influencing local calcium environment [23].

Alpha-synuclein - inhibitory or facilitating function in vesicle fusion?

Does alpha-synuclein inhibit or facilitate vesicle fusion? The afore-mentioned in vitro studies largely point to an inhibitory role of alpha-synuclein. Furthermore, this is strengthened by experiments performed in cells in which alpha-synuclein overexpression has been shown to inhibit the docking and fusion of ER vesicles with Golgi membranes [17,24,25]. However, on the other side, there is strong evidence that alpha-synuclein is a positive modulator of SNARE-complex assembly, as shown in mice with a knockout of the cysteine-string protein-alpha (CSPα). In these mice a severe neurodegenerative phenotype is seen that has been linked to a decrease in the chaperoning of the SNAP-25 protein, which in turn decreases the efficacy of
the SNARE-complex assembly and exocytosis [26–28]. Intriguingly, there was an almost
complete rescue of this toxic phenotype when alpha-synuclein was overexpressed so that a
positive regulatory effect of alpha-synuclein on exocytosis was proposed. A simple
substitution of CSPα chaperone activity by alpha-synuclein was not seen, so it was suggested
that alpha-synuclein might instead be able to stabilize SNAP-25 within the SNARE complex and
thus make the protein less prone for degradation [27]. However, other, more subtle,
downstream effects could also come into play. The role of alpha-synuclein in stabilizing the
curvature of synaptic vesicles could reduce the capacity of vesicles to fuse fully with the
plasma membrane. Similarly, a faster membrane and protein retrieval after exocytosis could
improve overall protein homeostasis. These hypotheses could bring at a first glance
contradicting results into one line of thought. On one side alpha-synuclein is inhibiting vesicle
fusion via stabilisation of the curvature of synaptic vesicles. On the other side, alpha-synuclein
improves the recycling of synaptic vesicles, thus maintaining the exocytotic machinery in good
condition, overcoming the loss of CSPα chaperoning.

Function of alpha-synuclein on synaptic activity and transmitter release

What is the bottom line of studies looking at synaptic activity and transmitter release in alpha-
synuclein animal or cell models? Recent work in Parkinson’s disease models with
overexpression of wild type alpha-synuclein show mainly an inhibitory effect of alpha-
synuclein on neurotransmitter release [29–34], whilst two other studies come to the opposite
conclusions [35,36]. In one of the latter an increased synaptic activity was found in cultured
hippocampal neurons acutely injected with alpha-synuclein. In the other, a paired pulse
facilitation was seen in alpha-synuclein overexpressing mice (for detail on all studies see Table
1). What is happening upon alpha-synuclein knock-out? Here studies report decreased
exocytosis [33,35,37,38], no change [36,39], or even increased exocytosis [40–42] (Table 2). However, these studies need to be interpreted with care since alpha-synuclein is not the only synuclein isoform and a loss of physiological function could possibly be compensated by beta- or gamma-synuclein. Studies in mice with knock-out of alpha-, beta- and gamma-synuclein (triple knock-out mice) so far show an increase in synaptic transmission only in young, 3 months old, but not in adult mice [43]. However, a more recent study demonstrated an increase in dopamine release in one year old triple knock-out mice [44], which again argues for a negative regulatory function of alpha-synuclein in exocytosis (Table 2).

Several of the studies above also looked at the effect of alpha-synuclein on the maintenance of the synaptic vesicle pool. Here, alpha-synuclein knock-out was shown to decrease the number of synaptic vesicles, especially of the distal pool, which reduces their availability upon intense stimulation [38,45]. Other effects have also been reported upon alpha-synuclein overexpression. One study found an increase in synaptic vesicle size, another a decrease of vesicle re-clustering after endocytosis, and two other studies report decreased motility of synaptic vesicles [29,32,46,47]. Interestingly, alpha-synuclein overexpression has also been shown to increase the amount of vesicles docked to the membranes [31], which would be in line with the recently published double-anchor function of alpha-synuclein [14]. In summary, these studies show that alpha-synuclein seems to affect in the vesicle pool, but a uniform picture of the underlying mechanisms is still lacking.
**Alpha-synuclein in synaptic vesicle endocytosis**

**Alpha-synuclein and its emerging role in synaptic vesicle endocytosis**

The first indication that alpha-synuclein can lead to disturbances in the endocytosis of synaptic vesicles comes from studies applying lipophilic dyes, used for the visualisation of exocytosis in live synapses. Here a reduction in presynaptic loading efficacy was reported upon alpha-synuclein overexpression, which suggested a reduction of endocytosis [32,46].

Intriguingly, recent efforts now reveal that alpha-synuclein plays a role in the endocytosis of synaptic vesicle [48–50]. In mice with a simultaneous knock-out of alpha-, beta- and gamma-synuclein, synaptic vesicle exo- and endocytosis was monitored using a fluorescent sensor reporting on the recycling of synaptic vesicles. These data show a clear slowdown of vesicle endocytosis, while there was no observed change in the rate of synaptic vesicle exocytosis.

Expression of each individual synuclein isoform on the triple knock-out background was found to compensate this endocytotic failure, demonstrating the complementary function of the synuclein isoforms. A decrease in the rate of endocytosis was evident after acute stimulation, but also at the basal level, as reported by retrograde labelling, which revealed a diminished number of lately endocytosed synaptic vesicles [48].

A second study gives further confirmation of the role of alpha-synuclein in endocytotic membrane retrieval using patch-clamp capacitance measurements. The membrane capacity is a read out for the expansion of the presynaptic membrane upon fusion events, which was found to be similar for synaptic terminals from control mice and mice expressing the A53T mutant alpha-synuclein at the calyx of Held, indicating that there is no difference in the level of exocytosis. However, membrane retrieval was delayed in A53T alpha-synuclein expressing calyces. Furthermore, acute short term whole-cell dialysis of A53T as well as wildtype alpha-
synuclein delayed membrane retrieval, indicating a direct interaction with, and influence on membrane properties by alpha-synuclein [49].

A third study again analysed the vesicle pool in Lamprey synapses upon electrical stimulation [50]. When synapses acutely injected with alpha-synuclein protein were stimulated at 20 Hz, severe changes in the synaptic vesicle pool were seen, small synaptic vesicles were rare, and bulk membranous structures appeared within the presynaptic terminal. Controls exhibited a normal vesicle pool upon this stimulation protocol, and synapses acutely injected with alpha-synuclein also showed a normal vesicle pool when stimulated at 5 Hz. This indicates that alpha-synuclein is inducing a failure of synaptic vesicle endocytosis, however this seems to be dependent on neuronal activity, i.e. endocytotic failure only occurs upon increased synaptic vesicle turnover [50]. This would be supportive for a regulatory function of synuclein, maybe being not essential for basal neurotransmission levels but upon intense repetitive stimulation.

Mechanisms of synaptic vesicle endocytosis

From these studies the concept solidifies that alpha-synuclein has a function in endocytosis. There are four major modes of endocytosis to differentiate, referred to as, kiss-and-run of synaptic vesicles, estimated to occur in < 1 second time frame [51], clathrin-mediated endocytosis (CME), occurring in a 10-20 second time frame [52–55], ultrafast-endocytosis followed by endosomal budding (initial membrane retrieval 100 ms, vesicle recovery 5-6 second) [56], and bulk endocytosis [57]. Mechanisms of clathrin-mediated endocytosis are well established and many key players are known today, however mechanisms for the kiss-and-run mode are under sustained debate and a molecular identity involved in the process is still missing. Kiss-and-run endocytosis is also known as “flicker-fusion” since the synaptic vesicle does not fully collapse upon membrane fusion, instead the neurotransmitter is
released through a small fusion pore. In dopaminergic neurons, the neurons vulnerable in Parkinson’s disease, kiss and-run mediated exocytosis has been clearly shown [58,59]. However, in hippocampal neurons some studies argue that clathrin-mediated endocytosis is the main or even the only mechanism [54], while others report up to 60% of kiss-and-run endocytosis [60]. Clathrin-mediated endocytosis is one major mechanism of endocytosis, but there is still controversy on the involvement and relative importance of other mechanisms during “normal” synaptic vesicle endocytosis and it is not yet known if there are variations for different neuronal subtypes. Mechanisms of membrane retrieval might not be different between neuronal types per se, but maybe neurons rely on different mechanisms of endocytosis, depending on their electrophysiological activity and the accompanied need in vesicle recycling.

**Alpha-synuclein - a possible role in slow and fast endocytosis**

As discussed, it is now generally accepted that alpha-synuclein binds to synaptic vesicles. The evidence on its role in sensing and stabilizing the curvature of these tiny organelles is on the other hand much more recent and still emerging [9–11]. Regarding the function of alpha-synuclein in vesicle endocytosis, different views are emerging. On the one hand, alpha-synuclein is thought to be associated with clathrin-mediated endocytosis of synaptic vesicles (Figure 3a), as triple synuclein knock-out led to changes in CME protein expression in mice and as increased numbers of clathrin-coated pits have been seen after stimulation of triple synuclein knock-out synapses [48]. Also, early studies on receptor-mediated endocytosis of transferrin suggested a role of alpha-synuclein in CME [61–63]. Furthermore, the study in Lamprey synapses displayed disturbances of CME and increased bulk endocytosis [50]. However, as these changes have only been seen upon intense stimulation, another
interpretation would suggest that this is no direct effect in CME, rather it suggests that there is a compensatory upregulation of CME when alpha-synuclein related fast endocytosis mechanisms are impaired. Interestingly, endophilins, proteins well known for membrane curvature sensing and their function in CME, have been found to be upregulated in triple synuclein knock-out mice [11], supporting the idea that alpha-synuclein and endophilins have partially complementary function during endocytosis. Endophilin A, for example, is able to generate membrane tubulation [64], a phenomenon that has also been reported for alpha-synuclein [9,65], thus again pointing to a possible function of alpha-synuclein in inducing membrane curvature for vesicle endocytosis. However, whether this is part of CME or a clathrin-independent membrane retrieval mechanism remains open, as also endophilins have been proposed to have clathrin-independent functions [66,67].

So on the other hand, also a role of alpha-synuclein in fast endocytosis mechanisms like kiss-and-run has to be considered. Alpha-synuclein could play a role of stabilizing the curvature of small vesicles [11,21], thus making a full vesicle collapse during exocytosis less likely, such as required for kiss-and-run. Also, the proposed double-anchor mechanism could influence the tethering of vesicles to the plasma membrane, thereby regulating the likelihood of exocytosis and thus preventing the full collapse of synaptic vesicles [14] (Figure 3b). For the mechanism of ultrafast endocytosis, it has yet to be understood how membrane fission can occur in a time scale of tens of milliseconds, while normal fission is reported to be in the range of seconds or even tens of seconds [68]. Here, facilitation by additional factors, like actin, endophilins and membrane lipids has been discussed, raising the question if alpha-synuclein might also be involved in this process (Figure 3c).

It has been proposed that different endocytotic pathways can co-exist in one single synapse [69]. So it seems that different pathways are maybe not different for neuronal subtypes in
general, but that the usage of CME or fast membrane retrieval may be dependent on synaptic activity. Neurons with a high rate of exocytosis, like the dopaminergic neurons of the substantia nigra with their continuous pacemaker activity [70], may rely on fast endocytosis while other neurons are mainly relying on CME and are less affected when fast mechanisms of endocytosis are disturbed. The results on capacitance measurements show that alpha-synuclein is possibly directly involved in the step of membrane retrieval [49]. However, rather than speaking of fast and slow endocytosis, which make sense when looking at the time scale, one might more appropriately classify the processes into endocytosis upon intense stimulation and endocytosis upon moderate stimulation. While endocytosis can be maintained by CME at low frequency firing, membrane saving or fast retrieval mechanisms like the kiss-and-run or the ultrafast endocytosis are probably necessary to maintain the structure of the presynaptic membrane upon intense and repetitive stimulation, as only then further exocytotic events are possible. When these fast mechanisms fail, CME will still be possible to maintain presynaptic homeostasis to a certain level and this is, what leads to a slowing down of endocytosis [48], or, as seen in Lamprey synapses upon intense stimulation, bulk endocytosis will occur [50].

Intriguingly, overexpression of Rab proteins has been shown to rescue alpha-synuclein toxicity [24,25,71]. Rab proteins are known key players in the endosomal sorting of vesicles, so they are crucial for retrieving new functional synaptic vesicles. Whether it is a direct interaction of Rab proteins with alpha-synuclein or a general compensation of vesicle transport deficits remains elusive, however this outlines, once more, the important role of alpha-synuclein in maintaining the synaptic vesicle pool.

LRRK2 - role in synaptic vesicle endocytosis
Mutation in the LRRK2 gene are established as the most common genetic cause of Parkinson’s disease [72,73]. Intriguingly, cumulating evidence also indicates a role of LRRK2 in synaptic vesicle endocytosis, showing that LRRK2 knock-out as well as the common G2019S LRRK2 mutant slow down synaptic vesicle endocytosis [74]. Similar observations were made in Drosophila with LRRK2 loss-of-function mutants [64] and recently also in neurons from LRRK2 knock-out mice [75]. Furthermore, inhibitors of LRRK2 kinase activity seem to reduce synaptic vesicle endocytosis [76]. Up to now different mechanisms have been discussed, i.e. LRRK2 has been found to phosphorylate mammalian endophilin A1 [75] as well as Rab5b [74,77]. So far, there is still controversy on the mechanism, however a link to alpha-synuclein interaction with endophilin A or Rab proteins may reveal interesting new evidence related to Parkinson’s pathology.

Concluding remarks

The current picture on the function of alpha-synuclein points to a regulatory role in maintaining synaptic homeostasis upon intense neuronal activity. Since inhibitory neurons lack alpha-synuclein expression [78,79] and no synuclein homologs are expressed in invertebrates [80], alpha-synuclein does not appear to be essential for synaptic transmission per se. In fact, studies in songbirds indicate a possible function of alpha-synuclein during song development, pointing to a critical role upon intense synaptic activity [81].

The neurons most severely affected in Parkinson’s disease, the dopaminergic neurons of the substantia nigra, exhibit an autonomous pacemaker activity, meaning that they are facing continuous cycles of exo- and endocytosis all throughout their life [70]. So rather than a direct effect of alpha-synuclein on the basic machinery of exocytosis itself one might think of alpha-synuclein keeping the synapse in a good “shape” upon prolonged and intense synaptic activity.
Neurons with a high rate of exocytosis, like the dopaminergic neurons, may rely on fast endocytosis while other neurons can rely on CME, or at least, are less affected when the fast mechanisms of endocytosis are impaired.

When fast mechanisms fail, CME will probably still be capable of maintaining presynaptic homeostasis to a certain level and this is, what one might see as a slowing down of endocytosis/membrane retrieval [48,49], or bulk endocytosis may take over, as seen in Lamprey synapses upon intense stimulation [50]. Although, these studies clearly show a disruption of endocytosis, they do not point to any “pathway” alpha-synuclein is possibly involved in. One might presume a function of alpha-synuclein in the fast mechanisms of endocytosis, like kiss-and-run or the ultrafast membrane retrieval. Here, alpha-synuclein could act upon curvature stabilization or the double-anchor mechanism, making a full collapse of synaptic vesicles less likely, and thus favouring kiss-and-run. On the other hand, alpha-synuclein may assist ultrafast endocytosis influencing membrane curvature, as membrane tubulation properties similar to endophilin proteins have been shown for alpha-synuclein [9].

However, despite this exciting recent progress in the field, the exact role of alpha-synuclein is still far from understood and many details are yet to be clarified (see “Outstanding Questions Box”). Nevertheless, all intracellular mechanisms relying on reshaping and stabilization of membrane curvature seem to be interesting targets in the context of improving our understanding of the physiological implications of alpha-synuclein.
Acknowledgement

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Figure legends

Figure 1. Lipid packaging defects and membrane curvature sensing
In plane phospholipid bilayer membranes lipid packaging defects can occur when there is an imbalance between cylindrical and conical lipids (a). On the other side, a bending of membranes can induce less occupied regions as well, as cylindrical lipid are not able to fill the outer, larger radius (b). Proteins with an amphipathic lipid-packaging sensor (ALPS) fold their amphipathic helix if favoured by the presence of such lipid packaging defects and are thereby able to sense and stabilize membrane curvature (c).

Figure 2. Alpha-synuclein in exocytosis.
The current picture of the function of alpha-synuclein points to a regulatory role in maintaining synaptic homeostasis upon intense neuronal activity. A function in exocytosis has been suggested via mediating SNARE-complex assembly (a), curvature stabilization thus preventing premature fusion of vesicles (b) and a double-anchor mechanism tethering synaptic vesicles to the plasma membrane through two lipid binding regions (c).

Figure 3. Alpha-synuclein in endocytosis.
Latest studies indicate a role of alpha-synuclein in synaptic vesicle endocytosis. As a protein with curvature sensing properties the function of alpha-synuclein can be associated with several membrane forming processes. Clathrin-mediated endocytosis was the first to be proposed (a). However, a role in fast endocytotic pathways like kiss-and-run (b) or the just recently discovered ultrafast endocytosis (c) also seem favourable, especially in accordance with a role of alpha-synuclein upon intense and repetitive neuronal firing.
Membrane remodelling processes are critical steps for the exo- and endocytosis of synaptic vesicles.

From structure-function relationships alpha-synuclein has been proposed to be a curvature sensing and regulating protein, thus proposing a role in mechanisms of exo- and endocytosis.

A function in exocytosis has been suggested via mediating SNARE-complex assembly (a), curvature stabilization thus preventing premature fusion of vesicles (b) and tethering of synaptic vesicles via lipid binding of alpha-synuclein with both, its N- and a more C-terminal region (c).

Latest studies indicate a function of alpha-synuclein in synaptic vesicle endocytosis. A possible function in clathrin-mediated endocytosis has been proposed (a), but a function in fast endocytotic pathways like kiss-and-run (b) or the just recently discovered ultrafast endocytosis (c) also seems favourable.
• Alpha synuclein seems to have a major function in membrane curvature sensing and regulation. Is this function related to exocytosis, i.e. a stabilization of synaptic vesicles, preventing premature fusion of the vesicle? Would this simultaneously speak for a function in kiss-and-run? Or, on the other side, is the function maybe related endocytotic membrane retrieval at the presynaptic terminal?

• Would alpha-synuclein be able to initiate new budding events of vesicles or is it just facilitating the bending of membranes or even just stabilizing existing curved membranes?

• Molecular key players of kiss-and-run and ultrafast endocytosis are still missing, so how could one prove and visualize a possible overlap of alpha-synuclein with these elusive mechanisms. Would it be possible to demonstrate a decrease of kiss-and-run events in presynaptic dopaminergic terminals, while clathrin-mediated endocytosis is upregulated?

• How much do pathways of endocytosis coincide within one synaptic terminal? How do different mechanisms work together? Are there different needs in different neuronal subtypes according to their firing pattern?

• Are dopaminergic neurons especially vulnerable because of their autonomous pacemaking activity? Continuous cycles of exo and endocytosis, where the active zone has to be cleared from excessive membrane immediately and in time before the next round of exocytosis starts, would put a particular burden and special needs for fast endocytosis mechanisms...
Figure 3

(a) clathrin-mediated endocytosis

(b) vesicle pool
  - priming
  - docking
  - exocytosis
  - kiss-and-run

(c) endosomal sorting

ultrafast endocytosis

clathrin
AP2
dynamin

asyn
Table 1. Exocytosis - studies on alpha-synuclein overexpression.

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<td>Liu 2004 EMBO J</td>
<td>Neurons with α-syn injection</td>
<td>Synaptic activity ↑</td>
<td>Glutamate evoked mEPSCs, cultured hippocampal neurons</td>
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<td>Larsen 2006 J Neurosci</td>
<td>α-syn overexpression in PC12 and chromaffin cells</td>
<td>Evoked dopamine release ↓</td>
<td>HPLC, PC12 cells, KCl stimulation</td>
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<td>Exocytotic events per stimulus ↓</td>
<td>Amperometric recordings, chromaffin cells</td>
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<td>“docked” vesicles ↑</td>
<td>EM, PC12 cells</td>
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<td>Gureviciene 2007 Neurobiol Dis</td>
<td>α-syn overexpressing mice</td>
<td>Basal synaptic transmission -</td>
<td>fEPSP slope, PP, hippocampal slices, mice 4-5 months</td>
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<td>Watson 2009 Neuroscience</td>
<td>α-syn overexpressing mice</td>
<td>Basal synaptic transmission -</td>
<td>fEPSP, PP, corticostriatal slices, mice 2-6 months</td>
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<tr>
<td>Scott 2010 J Neurosci</td>
<td>Neurons from GFP α-syn overexpressing mice</td>
<td>Spontaneous synaptic activity ↓</td>
<td>mEPSC, cultured hippocampal neurons</td>
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<td>Endocytotic uptake ↓</td>
<td>FM4-64 loading</td>
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<td>Vesicle density, vesicle diameter ↓ / ↑</td>
<td>EM, cultured hippocampal neurons</td>
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<td>Presynaptic proteins ↓</td>
<td>Immunocytochemistry staining</td>
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<td>Nemani 2010 Neuron</td>
<td>α-syn KO neurons with α-syn overexpression / α-syn overexpressing mice</td>
<td>Exocytosis ↓</td>
<td>vGLUT1-pHluorin, cultured hippocampal and VM neurons</td>
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<td>Synaptic transmission ↓</td>
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<td>Recycling pool of synaptic vesicles ↓</td>
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<td>Wu J Neurosci 2010</td>
<td>α-syn overexpressing mice</td>
<td>Spontaneous synaptic activity ↓</td>
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<td>Janzic 2013 PNAS</td>
<td>α-syn overexpressing mice on α-syn KO background</td>
<td>Dopamine release in dorsal striatum ↓</td>
<td>FCV, striatal slices, mice 3-4 months, 12 months, 18 months</td>
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<td>Dopamine content dorsal striatum -</td>
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<td>Vesicle clustering ↑</td>
<td>EM, dorsal striatum, mice 3 months</td>
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1 mEPSCs - miniature excitatory postsynaptic currents, fEPSP – field excitatory postsynaptic potential, EM – electron microscopy, PP - Paired-pulse measurements, FCV - fast-scan cyclic voltammetry, HPLC – high performance liquid chromatography
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<td>Abeliovich 2000 Neuron</td>
<td>α-syn KO mice</td>
<td>Dopamine release: -</td>
<td>• FCV, PP, striatal slices, mice 6-8 weeks</td>
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<td>Dopamine / DOPAC level in the striatum: ↓ / -</td>
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<td>• fEPSP slope, PP, hippocampal slices, mice 4-5 weeks</td>
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<td>Paired-pulse facilitation: -</td>
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<td>Synaptic response prolonged repetitive stim.: ↓</td>
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<td>Martin 2004 Eur J Neurosci</td>
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<td>Senior 2008 Eur J Neurosci</td>
<td>αβ-syn DKO mice</td>
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<td>Striatal dopamine content: -</td>
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<td>• synaptosomes (3H-dopamine/14C-GABA), KC1, sucrose stimulation</td>
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Glossary

**Alpha-synuclein**: presynaptic protein and main constituent of Lewy bodies in Parkinson’s disease. The isoforms beta- and gamma-synuclein show high grade of homology to alpha-synuclein but are not associated with the disease.

**ALPS motif proteins**: proteins with amphipathic lipid-packaging sensor motif. These proteins fold into an amphipathic helix if favoured by the presence of lipid packaging defects, the amphipathic helix is inserted into the lipid membrane thereby stabilizing membrane curvature.

**Amphipathic helix**: \(\alpha\)-helical protein structure segregating hydrophobic and polar residues.

**Bulk endocytosis**: membrane uptake by large membrane invagination.

**CME**: clathrin-mediated endocytosis; endocytosis which is characterized by a coating of vesicles with the clathrin-triskelion scaffold.

**Curvature-sensing and stabilization**: curvature of biological membranes is a premise for many cellular processes. Major mechanisms of curvature-sensing and stabilization include: classic coats like the clathrin, arc-shaped BAR domain proteins and proteins with ALPS motif/amphipathic helix.

**Endocytosis**: uptake of molecules from the extracellular space into the cell by forming cell invaginations. In the case of endocytosis at the synaptic terminal the endocytosis is deemed to retrieve excess membrane rather than molecules.

**Exocytosis**: fusion of a vesicle with the plasma membrane leading to the release of the respective vesicle content into the extracellular space. In the case of synaptic vesicles exocytosis leads to the release of neurotransmitter into the synaptic cleft.

**Kiss-and-run**: mechanism of exocytosis, where the synaptic vesicle opens only a small fusion pore. This pore reseals easily afterwards and so the vesicle is kept intact. This in contrast to the full fusion event, where the synaptic vesicle collapses and both, vesicle and presynaptic membrane fuse together.

**LRRK2**: leucine-rich repeat kinase 2; enzymatic protein encoded by the PARK8 gene.

**Rab proteins**: proteins which present manifold involvement in membrane trafficking, including vesicle formation, transport and fusion. Up to date there are over 70 different Rab proteins identified.

**SNAP-25**: synaptosomal-associated protein 25; SNARE-protein of the presynaptic membrane contributing two alpha-helices to the SNARE-complex.

**SNARE-complex**: Soluble N-ethylmaleimide-sensitive-factor Attachment protein REceptor-complex; multiprotein complex bringing two membranes surfaces in close proximity, zipping them together and preparing the fusion of the two compartments. This mechanisms is involved in the fusion of synaptic vesicles with the presynaptic membrane but also for example for the fusion of vesicles from the ER with Golgi membranes.

**Syntaxin**: SNARE-protein anchored with the presynaptic membrane contributing one alpha-helix to the SNARE-complex.
**Ultrafast-endocytosis:** endocytosis at the presynaptic membrane with a very fast initial membrane retrieval in the 100 ms time frame. Vesicle recovery by endosomal budding occurs in about 5-6 seconds.

**VAMP2:** vesicles-associated membrane protein 2, also named synaptobrevin 2; SNARE protein anchored at the synaptic vesicle and contributing one alpha-helix to the SNARE-complex.