The small GTPase Rab11 co-localizes with α-synuclein in intracellular inclusions and modulates its aggregation, secretion and toxicity

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Alpha-synuclein (aSyn) misfolding and aggregation are pathological features common to several neurodegenerative diseases, including Parkinson’s disease (PD). Mounting evidence suggests that aSyn can be secreted and transferred from cell to cell, participating in the propagation and spreading of pathological events. Rab11, a small GTPase, is an important regulator in both endocytic and secretory pathways. Here, we show that Rab11 is involved in regulating aSyn secretion. Rab11 knockdown or overexpression of either Rab11a wild-type (Rab11a WT) or Rab11a GDP-bound mutant (Rab11a S25N) increased secretion of aSyn. Furthermore, we demonstrate that Rab11 interacts with aSyn and is present in intracellular inclusions together with aSyn. Moreover, Rab11 reduces aSyn aggregation and toxicity. Our results suggest that Rab11 is involved in modulating the processes of aSyn secretion and aggregation, both of which are important mechanisms in the progression of aSyn pathology in PD and other synucleinopathies.

INTRODUCTION

Alpha-synuclein (aSyn), a 140-amino-acid protein, is a key molecule involved in the pathophysiology of several neurodegenerative diseases, including Parkinson’s disease (PD) and Dementia with Lewy bodies (DLB), collectively known as synucleinopathies (1–3). Missense mutations and multiplications of the SNCA gene encoding for aSyn are linked to familial forms of PD (4). Furthermore, misfolded and aggregated aSyn is found in Lewy bodies (LB) and Lewy neurites (LN)—pathognomonic cytoplasmic inclusions characteristic of both PD and DLB (2). Although the mechanisms underpinning the pathophysiology of PD are not clearly understood, many studies indicate that aSyn aggregation is a critical event involved in this pathology (5,6). aSyn is natively unfolded, but it acquires the α-helical structure on its N-terminal region upon binding to membranes, both in vitro and in vivo (7–9). Under pathological conditions, aSyn molecules associate to form oligomers that grow into protofibrils and, finally, form mature amyloid fibrillar structures. The identification of the cytotoxic aSyn species remains a subject of intense investigation. Nonetheless, there are several studies suggesting that oligomeric intermediates might constitute the most toxic aSyn species (10–12).

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While aSyn lacks an endoplasmic reticulum signal peptide and has therefore been considered a purely intracellular protein, recent studies have found that it can be actively secreted (13–16). This is in agreement with the presence of aSyn in the cerebrospinal fluid and blood plasma of both PD patients and healthy subjects (17, 18). Notably, aSyn can be externalized via non-classical exocytosis and, in part, in association with exosomes (16). In enteric neurons, aSyn seems to follow a classical, ER-Golgi network-dependent pathway (19). There is evidence that aSyn secretion is calcium-regulated and can be increased under conditions of cell stress (20); however, the exact mechanisms regulating this process remain unclear.

aSyn pathology progresses from the lower brain stem through the midbrain to the cerebral cortex (21), leading to the suggestion that a neurotropic pathogen may cause the spreading of LB and LN pathology during PD progression. This hypothesis is in agreement with clinical observations of aSyn pathology found in neuronal grafts in PD patients several years after transplantation (22). There is mounting evidence suggesting that aggregated aSyn is the key agent for propagation of PD pathology by a prion-like mechanism, where misfolded aSyn is released from a donor cell and is taken up by a recipient cell where it seeds aggregation of endogenous aSyn (23–25). Additionally, extracellular aSyn is known to stimulate pro-inflammatory activity in microglia, which in turn can lead to a further increase in neurotoxicity and pathology progression (26–28). Therefore, understanding the regulatory mechanisms involved in aSyn secretion might be highly relevant for therapy aimed at attenuating or halting the progression of PD pathology.

Rab11 is a member of the Rab small GTPase protein family which plays critical roles in regulating transport, docking and fusion of vesicles with their target membranes (29, 30). Rab11 associates with recycling endosomes, trans-Golgi membranes and secretory vesicles (31–34). As is the case with Rab5, Rab11 is localized to synaptic vesicles in neuronal cells (35). Apart from a well-documented function in endosomal recycling, several studies indicate that Rab11 also plays a role in exocytic secretory pathways. It has been described to be involved in Ca\textsuperscript{2+}-regulated and constitutive exocytosis (35), in insulin granule secretion (36), in exosome release (37) and in stretch-regulated exocytosis (38). These studies suggest that Rab11 is an important regulator in the crosstalk between endocytic and secretory pathways.

aSyn has recently been detected in endosomal compartments, co-localizing with Rab5a, Rab7 and Rab11a—markers of early, late and recycling endosomes, respectively (39). Notably, Rab11 regulates the secretion of aSyn from neurons, after internalization from the extracellular milieu, back to the extracellular space (40) and a portion of endogenous aSyn is trafficked via the recycling pathway regulated by Rab11 (39). Interestingly, recent work has found that Rab11 is neuroprotective in an in vivo model of Huntington’s disease (HD), another neurodegenerative disease with pathological protein aggregation (41, 42). Rab11 was sequestered in LC3-positive amphisome-like structures in dendritic spines in the presence of mutant huntingtin (HTT) aggregates, followed by impairment of Rab11-dependent endosomal recycling (41). In addition, Rab11 overexpression rescued dendritic dysfunction, dystrophy and neurodegeneration caused by mutant HTT aggregation, providing a neuroprotective effect in a Drosophila model of HD (41, 42). Moreover, Rab11 dysfunction was shown to slow trafficking of the neuronal glutamate transporter EAAC1 to the cell surface, causing oxidative stress and cell death in HD (43).

In the present study, we investigated the role of Rab11 in modulating aSyn secretion and aggregation. We found that secretion of intracellular aSyn can be regulated by Rab11, and that Rab11 physically interacts with aSyn and co-localizes with aSyn in intracellular inclusions. Our results also suggest that Rab11 is involved in modulating aSyn aggregation. In total, our study provides molecular support for the protection afforded by Rab11 against aSyn-mediated behavioral and functional deficits in flies (Breda et al., submitted for publication), highlighting its potential as a therapeutic target in synucleinopathies.

RESULTS

Rab11 interacts with aSyn in vivo and modulates aSyn secretion

Co-localization of aSyn with Rab5a, Rab7 and Rab11a in endocytic compartments has recently been described in HEK293T and SH-SY5Y cells (39). In order to study if there is a direct interaction between Rab11 and aSyn in vivo, we performed a co-immunoprecipitation (co-IP) analysis of aSyn and Rab11 proteins from rat brain lysate. Following the immunoprecipitation of endogenous aSyn, endogenous Rab11 was detected using a Rab11-specific antibody (Fig. 1A). This result suggests that these two proteins interact in vivo in addition to being present in the same subcellular compartment. Rab11 is an important regulator of various trafficking steps at the interface between endocytic and secretory pathways. Recently, it has been suggested that the endocytic pathway is involved in aSyn secretion (16, 39). Thus, we next investigated whether Rab11 is involved in this process.

To determine whether Rab11 plays a role in aSyn secretion, we used SH-SY5Y cells expressing wild-type (WT) aSyn under control of the Tet-off regulatory expression system (16, 44). First, we knocked down Rab11 expression using three adeno viral vectors encoding for Rab11 miRNAs and measured the levels of intracellular as well as extracellular aSyn in the supernatant of the conditioned media (CM) by immunoblot analysis (Fig. 1B). Rab11 knockdown led to a parallel decrease in intracellular aSyn and an increase in levels of aSyn in the CM (Fig. 1B and C). To assess whether this increase in extracellular aSyn was due to increased release of aSyn from dying cells, we measured the release of lactate dehydrogenase (LDH) into the CM as an indicator of cell-membrane permeability/dysfunction, which is typical of dying cells (Fig. 1D). We found that Rab11 knockdown modestly increased LDH levels in the CM when compared with the control; however, this was not correlated to the increase in extracellular aSyn levels. Moreover, the construct for Rab11 knockdown leading to the highest aSyn extracellular levels displayed LDH levels comparable with control (kd 3, Fig. 1B and D). These data suggest that the increase in extracellular aSyn levels in Rab11 knockdown condition occurs due to an active secretory process.

Next, we investigated the effect of Rab11 on aSyn secretion by expressing EGFP-tagged wild-type Rab11a (Rab11a WT), or the GDP-bound, dominant negative Rab11a mutant (Rab11a S25N). While in the case of Rab11 knockdown, we decreased the total...
Figure 1. Rab11 interacts with endogenous aSyn in vivo and modulates aSyn secretion. Rat brain lysate was analyzed for aSyn and Rab11 protein interaction by co-immunoprecipitation. (A) Following the immunoprecipitation (IP) of endogenous aSyn, the co-IP with endogenous Rab11 is demonstrated with a Rab11-specific antibody. Highlighted are the unspecific signals from the heavy chain (HC) and light chain (LC) in the IGG control sample. Rab11 knockdown leads to increased secretion of aSyn into the CM. (B) Representative immunoblot of cell lysate (intracellular) and CM (extracellular) of Rab11 knockdown SH-SY5Y cells overexpressing aSyn WT is shown. (C) Graphical representation of aSyn secretion (extracellular levels/intracellular levels normalized to GAPDH). (D) LDH levels in the CM of Rab11 knockdown SH-SY5Y cells overexpressing aSyn WT. (E and F) Overexpression of Rab11a WT and dominant negative mutant (Rab11a S25N) leads to increased aSyn secretion. (G) LDH levels in the CM of SH-SY5Y cells overexpressing aSyn WT transfected with EGFP, EGFP-Rab11a WT or EGFP-Rab11a S25N. All the data shown are representative of at least three independent experiments (mean ± SD, *P < 0.05, **P < 0.01, ***P < 0.001).
levels of endogenous Rab11, Rab11a S25N altered the Rab11 function by introducing a GDP-bound Rab11a mutant that competes with the endogenous active Rab11 GTPase, therefore eliminating its activity. The levels of aSyn in the cell lysates, as well as in the 48 h—CM, were measured by immunoblot analysis (Fig. 1E). In agreement with the results from Rab11 knockdown, the amount of externalized aSyn was significantly increased in the presence of Rab11a S25N (Fig. 1F), compared with the EGFP control. Interestingly, we also observed significantly higher levels of aSyn in the CM of Rab11a WT expressing cells (Fig. 1F). These results are consistent with previous findings showing increased secretion of overexpressed human growth hormone (hGH) in PC12 cells upon co-expression of Rab11a WT or Rab11a S25N, with Rab11a WT having a moderate and Rab11a S25N a more pronounced effect on hGH secretion (35). To further confirm that the increase in extracellular aSyn was not due to increased cell death, we performed LDH assays to assess its levels in the CM (Fig. 1G). There was no significant difference in the LDH levels in the CM of the EGFP, EGFP-Rab11a WT or EGFP-Rab11a S25N transfected cells, indicating that the expression of Rab11a WT or its dominant negative mutant form leads to increase in aSyn secretion due to an active process and not due to cell death.

Rab11-mediated increases in aSyn secretion do not occur via the endocytic recycling pathway

To investigate whether the increased secretion of aSyn observed upon co-expression of Rab11a WT or Rab11a S25N occurred through changes in endocytic recycling, we measured endocytic recycling dynamics using fluorescently labeled human transferrin. Transferrin is internalized by endocytosis after binding to its receptor on the cell surface and is recovered to the extracellular milieu by endocytic recycling (45). Twenty-four hours post-transfection with EGFP-Rab11a WT, EGFP-Rab11a S25N or EGFP alone, aSyn expressing SH-SY5Y cells were loaded for 15 min with Alexa-546-labeled human transferrin and pulse-chased for 10 min with non-labeled human transferrin (Fig. 2A). We evaluated the percentage of Alexa-546-transferrin positive cells in each condition by fluorescence microscopy, as a measure of endosomal recycling dynamics. Compared with control transfected cells, there was no significant difference in the percentage of fluorescently labeled transferrin cells in the case of EGFP-Rab11a WT expression (Fig. 2B). In contrast, we observed a significantly higher proportion of Alexa-546-transferrin-labelled cells expressing EGFP-Rab11a S25N when compared with EGFP expressing cells (Fig. 2B). These results indicate that endocytic recycling is impaired in the presence of the dominant negative, GDP-bound Rab11a mutant, as less transferrin was secreted from the cells, while the expression of Rab11a WT did not affect endocytic recycling. Therefore, we conclude that increased secretion of aSyn from SH-SY5Y cells mediated by Rab11a is not due to increased trafficking of aSyn via the endosomal recycling pathway.

aSyn secretion by exosomes is not increased in the presence of Rab11a WT and Rab11a S25N

It has been previously shown that aSyn can be secreted from cells in association with exosomes (16,46,47). Exosomes are small
vesicles of various sizes (40–100 nm in diameter) that are formed as intra-luminal vesicles by budding into multivesicular bodies (MVBs) and are released by fusion of MVBs with the plasma membrane (PM) (48). Because of their endosomal origin, exosomes are characterized by the presence of endosome-associated proteins such as Rab GTPases, SNAREs, annexins and flotillin, some of which are involved in MVB biogenesis (Alix and Tsg101) (48). Rab11 modulates MVB fusion and exosome release in erythroleukemic cell lines, but the exact step in which it is involved is not known (37).

To test the hypothesis that Rab11a WT or Rab11a S25N expression leads to increased secretion of aSyn by exosomes, CM from EGFP, EGFP-Rab11a WT or EGFP-Rab11a S25N expressing cells was subjected to an established protocol of serial centrifugation steps for exosomal extraction (16). The pellet resulting from the last 100 000 g centrifugation step containing exosomes was subjected to immunoblot analysis using antibodies against the exosomal marker TSG101, Rab11 and aSyn (Fig. 3). Quantification of aSyn in the exosomal fraction revealed lower levels of aSyn in exosomes in cells expressing EGFP-Rab11a S25N (≏30% of the control), while in cells expressing EGFP-Rab11a WT aSyn exosomal levels were comparable with control (≏90%) (Fig. 3). Rab11a was also found present in the exosomal fraction (Fig. 3), as expected by the endosomal origin of exosomes.

These results show that the Rab11a dominant negative mutant reduces the levels of aSyn released in association with exosomes, while Rab11a WT does not have a major effect on exosomal release of aSyn. Therefore, we concluded that Rab11a regulates aSyn secretion by another, independent pathway.

Rab11a modulates aSyn aggregation and co-localizes with aSyn in intracellular inclusions

Although the process of aSyn aggregation has been extensively studied in vitro, it is still unclear which cellular pathways are involved. First, to investigate the effect of Rab11 on aSyn aggregation, recombinant aSyn was incubated in the presence of total protein lysates from cells overexpressing either EGFP-Rab11a

Brefeldin A treatment leads to increased release of aSyn and this effect is attenuated by Rab11a WT and Rab11a S25N expression

Several studies showed that treatment with Brefeldin A (BFA)—a fungal metabolite blocking classical, ER/Golgi-to-PM secretory pathway—does not block aSyn secretion (13,16,20). Based upon these findings, it was suggested that aSyn is secreted from neuronal cells via an unconventional, ER/Golgi-independent pathway. However, results from a recent study show that in entheal neurons aSyn is secreted via conventional, ER/Golgi-dependent exocytosis sensitive to BFA inhibition (19).

Thus, to investigate whether Golgi-dependent exocytosis contributes to aSyn secretion in the presence of Rab11a WT and Rab11a S25N expression, aSyn expressing SH-SY5Y cells were transfected with EGFP-Rab11a WT or EGFP-Rab11a S25N and 24 h post-transfection were treated with 1 μg/ml BFA for 6 h. The levels of extracellular aSyn in the CM were measured by immunoblotting (Fig. 4A). At the same time, the CM was used for LDH assay to assess cell death (Fig. 4C). Similar to previous reports, we verified that BFA treatment did not block aSyn secretion (Fig. 4A). In fact, we observed higher levels of extracellular aSyn following BFA treatment. This observation could be attributed to increased cell death after BFA treatment, as we also observed an increase in LDH activity in CM upon BFA treatment (Fig. 4C). However, despite a similar increase in cell death in all conditions, the levels of aSyn in the CM did not increase significantly in case of Rab11a WT and S25N expression in contrast to the control (Fig. 4A and B). These results might indicate that in the presence of Rab11a WT and S25N expression, there is an inhibition of aSyn secretion when the classical secretory pathway is blocked by BFA and this effect is masked by leakage of aSyn from dying cells. Altogether, our data suggest that Rab11a plays a role in regulating aSyn secretion.

Rab11a modulates aSyn aggregation and co-localizes with aSyn in intracellular inclusions

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**Figure 3.** Rab11a S25N inhibits aSyn secretion by exosomes. aSyn WT expressing SH-SY5Y cells were transfected with mock (EGFP), EGFP-Rab11a WT or EGFP-Rab11a S25N expressing plasmids. Twenty-four hours post-transfection, the culture medium was replaced with 2% FBS exosome-depleted medium and conditioned for 48 h. CM was subjected to sequential centrifugation with final step at 100 000 g to extract exosomal pellet. Exosomal pellet was resuspended in RIPA buffer and analysed by immunoblotting using antibodies for the indicated proteins. A representative immunoblot of cell lysates and exosomal pellet is shown. Graph represents immunoblot quantification of aSyn levels in exosomal fraction. All the data shown are representative of at least three independent experiments.
or EGFP, as a control. aSyn fibrillization was followed by monitoring ThT fluorescence at 482 nm. ThT is an amyloid-specific dye whose fluorescence dramatically increases upon binding to cross-\(\beta\) sheet structures such as those formed during aSyn self-assembly. We found that aSyn aggregation was clearly decreased in the presence of the EGFP-Rab11a cell lysate but not in the presence of the EGFP control cell lysate (Fig. 5A). Since the composition of the cell lysis buffer might alter protein assembly and ThT fluorescence, we included a control containing the corresponding amount the cell lysis buffer but no cell lysate, which resulted in a similar kinetics to that obtained in the presence of EGFP cell lysate (Fig. 5A). This indicated that, under the conditions tested, the presence of cellular proteins had negligible effects on aSyn fibrillization and, therefore, the effect of Rab11a was specific. Additional controls with cell lysates in the absence of aSyn were included to discard any significant aggregation process or binding of the cell extracts to ThT.

We further validated the ThT data by assessing the distribution of aSyn between the soluble and insoluble fraction in the aggregation reactions. Dot blot analyses showed that the presence of Rab11a decreased the amount of aSyn in the insoluble fraction (Fig. 5A, inset).

Next, we used an established cell model that enabled us to assess aSyn inclusion formation in an intracellular context. EGFP-Rab11a WT, EGFP-Rab11aS25N or EGFP alone was co-expressed in H4 human neuroglioma cells along with a C-terminal modified version of aSyn (synT) and synphilin-1. This is an established paradigm of aSyn aggregation that results in the formation of LB-like inclusions (49–51). In this model, we counted the percentage of cells presenting aSyn inclusions versus cells that presented homogeneous aSyn staining, with no inclusions (Fig. 5B). We found that both Rab11a WT and Rab11a S25N decreased the percentage of cells with aSyn inclusions, with a higher proportion of cells presenting homogenous aSyn staining without the presence of intracellular inclusions (Fig. 5C). Interestingly, we observed the opposite effect when Rab11 was knocked down, as this resulted in an increased percentage of cells displaying aSyn inclusions (Fig. 5D). Together, these results suggest that Rab11a can modulate aSyn aggregation.

To study the sub-cellular localization of aSyn and Rab11 in the absence or presence of aSyn aggregation, EGFP-Rab11a was co-expressed together with wild-type aSyn (aSyn WT) or with synT/synphilin-1 in H4 cells, as described above. In the presence of aSyn WT, Rab11a was normally distributed in the cell, as in the control situation (Fig. 6A). Strikingly, the subcellular localization of Rab11a was changed in the presence of aSyn inclusions (Fig. 6A). We found that Rab11a was co-localized inside these inclusions, together with aSyn (Fig. 6B).

**Rab11a reduces aSyn cytotoxicity**

Considering the neuroprotective effect of Rab11 against mutant HTT in HD (41,42), we investigated whether Rab11 protected against aSyn toxicity in a cell model (50,52). H4 cells were
transfected with a plasmid expressing aSyn WT or mock-transfected with empty vector (control), together with EGFP, EGFP-Rab11a WT or EGFP-Rab11a S25N. aSyn-induced toxicity was significantly reduced in the presence of Rab11a WT or Rab11a S25N (Fig. 7A). Conversely, we observed a significant increase in aSyn toxicity upon Rab11 knockdown (Fig. 7B).

Additionally, we also investigated cytotoxicity in the aSyn aggregation model described above. Similarly to the results in cells expressing aSyn WT, Rab11 knockdown increased aSyn cytotoxicity (Fig. 7D). However, expression of either Rab11a WT or Rab11a S25N did not significantly affect aSyn toxicity in this model (Fig. 7C).

Altogether, these results show that Rab11 modulates aSyn aggregation and toxicity and that Rab11 changes its sub-cellular localization in the presence of aSyn inclusions, co-localizing with aSyn in these inclusions.

**DISCUSSION**

Several recent studies indicate that a large number of proteins without an N-terminal signal sequence for ER entry are efficiently released from cells. These include proteins such as IL-1β, acyl-CoA binding protein (AcbA), ubiquitin carboxy-terminal hydrolase, visfatin and also aSyn (53). Several mechanisms have been proposed for the transfer of molecules from the cytoplasm to the extracellular space, such as direct translocation through pores in the PM, uptake into the internal vesicles of
MVBs (subsequently released as exosomes), passage via recycling endosomes or autophagosomes, incorporation into microvesicles budding outward from the PM and export via secretory lysosomes (53). aSyn has been observed inside cells in vesicles of unknown identity (13) and is known to be actively secreted into the extracellular space either in free or vesicle-bound form.
However, little is known about the route(s) aSyn follows to leave the cell or the mechanisms regulating aSyn secretion. It has been suggested that an endocytic pathway is involved in aSyn secretion (13,15,16). Indeed, blocking the endosome-lysosomal pathway by methyalmine or chloroquine leads to increased aSyn secretion (16). Exosomes, small secreted vesicles originating from the endocytic pathway, have also been shown to carry aSyn (16), although it seems that only a small portion of aSyn is secreted by this route (16,20,39). In addition, impairment in MVB formation has been found to increase aSyn secretion (39). Notably, aSyn localization has been observed in endocytic compartments, including the recycling endosomes (39).

Here, we first investigated whether aSyn and Rab11 interact in vivo. Co-immunoprecipitation analysis of rat brain lysate demonstrates that endogenous aSyn protein does indeed interact with endogenous Rab11 (Fig. 1). We next wished to explore whether Rab11 modulates aSyn secretion. It has been shown that Rab11 regulates the re-secretion of extracellularly added aSyn back into the extracellular space after its uptake by the cell (40). Furthermore, increased aSyn secretion caused by block of MVB formation using a dominant-negative mutant of vacuolar protein 4 could be restored to normal levels by simultaneous expression of Rab11a S25N (39). These results point at the involvement of Rab11-regulated recycling in aSyn secretion. Therefore, we investigated the role of Rab11 in aSyn secretion by manipulating its function in the cell, either by knocking it down or expressing the Rab11a WT or the GDP-bound inactive form of the protein. We observed that both Rab11 knockdown and expression of Rab11S25N—which both impairs Rab11 function—lead to increased aSyn secretion. Surprisingly, the same effect, although to a lesser extent, was observed by expressing Rab11a WT. One possible explanation is that overexpression of Rab11a WT does not lead to an overall increased Rab11 function, as it may be competing with the endogenous Rab11 for the interacting molecules, which can be limiting.

Figure 7. Rab11 modulates aSyn toxicity. (A and B) H4 cells were transfected with aSyn WT or empty vector (control) and co-transfected with EGFP-Rab11a WT, EGFP-Rab11a S25N or EGFP. For Rab11 knockdown, cells were transduced with an adenovirus containing miRNA construct against Rab11a or scrambled construct (control). LDH extracellular levels were measured to assess cytotoxicity. (A) Rab11a WT and Rab11a S25N decrease aSyn toxicity. (B) Rab11 knockdown increases aSyn toxicity. (C and D) Cytotoxicity was assessed in the aSyn aggregation model (synT + synphilin-1) described above. (C) Rab11a WT and Rab11a S25N do not affect aSyn cytotoxicity in the aSyn aggregation model. (D) Rab11 knockdown increases aSyn toxicity in the aSyn aggregation model. All the data shown are representative of at least three independent experiments (mean ± SD, *P < 0.05, **P < 0.01, ***P < 0.001).
factors for normal Rab11 function. This is supported by the results of the transferrin-recycling dynamics in our model. While Rab1a S25N impairs transferrin recycling to the extracellular space, expression of Rab1a WT did not have any effect on this process. These results together suggest that increased aSyn secretion observed after expression of Rab1a WT or Rab1a S25N does not occur via endosomal recycling in SH-SY5Y cells. A similar effect was observed using Rab1b WT or Rab1b S25N in PC12 cells expressing hGH (35). Both Rab1b forms increased the secretion of hGH in these cells, with Rab1a S25N having a more pronounced effect. It has been suggested that despite leading to similar effects of increasing the constitutive exocytosis of hGH, WT and S25N Rab1b have distinct mechanisms of action. Expression of Rab1a S25N decreased the excessive release of aSyn following a block in MVB-formation back to normal levels (39). This suggests that aSyn can be secreted by the way of recycling endosomes in a Rab1a-function dependent manner. Our results show that impairing Rab1a function by knockdown or expression of Rab1a S25N leads to increased secretion of aSyn, suggesting that aSyn secretion follows other pathway(s), independent of recycling endosome when Rab1a function is impaired.

It was previously demonstrated that Rab11 has a distinct function in exocytosis depending on the cell type (35). While in neuronal (PC12) cells, GTP- and GDP-bound Rab11b stimulated constitutive exocytosis of hGH, in non-neuronal (HEK) cells GTP- and GDP-bound Rab11b inhibited constitutive exocytosis and caused an accumulation of cellular hGH (35). In this study, we have used human neuroblastoma SH-SY5Y cells, in contrast to HEK cells used by Hasegawa et al. (35). Therefore, this might be one reason for the different effects on aSyn secretion observed in these two studies. Another possible explanation is that aSyn can employ different pathways for its exocytosis, depending on the state of the cell. When a block in one of the pathways occurs, aSyn could be directed to other pathway(s). This would allow aSyn release to be carried out by distinct mechanisms, in response to the state of the functioning of the cell. This is supported by an observation of changes in aSyn release in response to cellular stress conditions (20).

Rab11 has been implicated in regulating exosomal release in K562 erythroleukemia cells; however, the exact step remains unknown (37). Since aSyn was shown to be secreted in association with exosomes, we have investigated the impact of Rab1a function on exosomal aSyn secretion. We have observed lower levels of aSyn in the exosomal fraction in cells expressing the dominant negative Rab1a S25N mutant, while in the case of Rab1a WT the exosomal levels of aSyn were similar to control levels (Fig. 4). At the same time, Rab1a S25N did not lead to an overall decrease in exosome release, judged by the levels of the exosomal marker TSG101 (Fig. 4). These results together might indicate that impaired Rab1a function prevents aSyn entering the MVBs and exosomes, while promoting exit of aSyn from the cell through an independent pathway.

It was suggested that aSyn leaves the cell by a Golgi-independent transport route. This notion is based upon results showing that aSyn secretion is not blocked by BFA, a drug that disassembles the Golgi stacks (13,16,20). However, insensitivity to BFA treatment by itself does not unequivocally mean that a protein normally reaches the cell surface via a non-conventional route. It is possible that certain molecules take a Golgi bypass route when the pathway they normally employ is no longer operational. Moreover, results from a recent study show that in enteric neurons aSyn is secreted via conventional, ER/Golgi-dependent exocytosis sensitive to BFA inhibition (19). Furthermore, although BFA treatment reduced aSyn secretion in enteric neurons, it did not block it completely. Therefore, one might hypothesize that aSyn can use different pathways for exocytosis, depending on the cell type and cell condition.

We studied the involvement of Golgi-dependent pathway in aSyn secretion in the presence of Rab1a WT or Rab1a S25N by analysing extracellular aSyn levels following BFA treatment. Although we observed a similar increase in cell death following the BFA treatment in all conditions, aSyn extracellular levels were not significantly increased in the case of Rab1a WT or Rab1a S25N expression. Therefore, we concluded that part of aSyn can be secreted by classical ER-Golgi secretory pathway when Rab11 function is altered. Overall, our results indicate that aSyn secretion can be modulated by Rab1a and that aSyn can be secreted by different secretory pathways, depending on the condition of the cell.

Interestingly, intravesicular aSyn is more prone to aggregation than aSyn found in the cytosol (13). Moreover, exposing cells to stress conditions promoting accumulation of misfolded protein leads to increased translocation of aSyn into vesicles and the consequent increase in aSyn secretion (20). Furthermore, a recent study found that inhibition of the autophagy/lysosome pathway leads to increased aSyn aggregation and exocytosis (34). These studies indicate that there is a connection between aSyn aggregation and aSyn secretion. Increased secretion could be a protective mechanism by the cell to dispose of misfolded and aggregated aSyn. We addressed the role of Rab1a on aSyn aggregation in vitro by monitoring aSyn fibrilization using ThT fluorescence. We observed a significant decrease in aSyn fibrillation in the presence of cell lysates from cells expressing Rab1a WT when compared with control. This result was further validated by dot-blot analysis of soluble and insoluble fraction, showing decreased amount of aSyn in the insoluble fraction in the presence of Rab1a. Additionally, we studied the role of Rab1a on aSyn aggregation using a cell model characterized by formation of aSyn-positive intracellular inclusions, and observed a reduction in aSyn aggregation in the presence of Rab1a WT or Rab1a S25N. Since knocking down Rab11 resulted in an increased proportion of cells presenting aSyn aggregates, our results suggest a GTPase independent effect of Rab11 on aSyn aggregation. Moreover, Rab1a was found to co-localize with aSyn-positive inclusions, in contrast to its normal intracellular localization in the endocytic recycling compartment, as observed in the presence of non-aggregating aSyn.

Furthermore, we addressed the effect of Rab11 on aSyn toxicity. While the presence of Rab1a WT or Rab1a S25N significantly decreased aSyn-induced toxicity, Rab11 knockdown resulted in a marked increase in cytotoxicity in aSyn WT expressing cells. A similar effect was observed in the aSyn aggregation model, where Rab11 knockdown lead to increase in aSyn toxicity. Interestingly, Rab1a WT and Rab1a S25N had no effect on aSyn toxicity in this model. Since in this model Rab11 was observed to be localized in intracellular inclusions together with aSyn, it is therefore possible that Rab11 was unable to exert a protective effect because it was being recruited from its original subcellular localization and was sequestered inside the inclusions.
Altogether, our results show, for the first time, that Rab11 interacts with aSyn inside the cell, co-localizes with aSyn in intracellular inclusions and, furthermore, modulates aSyn aggregation and toxicity, while regulating the exit of aSyn from the cell. Since we also found that Rab11 modulates aSyn-mediated behavioral deficits in vivo (Breda et al., submitted for publication), our studies strongly suggest Rab11 holds great potential as a therapeutic target in PD and other neurodegenerative disorders.

MATERIALS AND METHODS

Cell culture

For aSyn secretion studies, we used SH-SY5Y cell inducibly expressing aSyn wild-type (SH-SY5Y aSyn WT) previously described (44). SH-SY5Y cells overexpressing aSyn WT were cultured in the RPMI 1640 medium (Life Technologies) containing 10% fetal bovine serum (FBS), penicillin/streptomycin (100 U/ml), streptomycin (100 μg/ml) and 2 mM L-glutamine in the presence of 250 μg/ml G418 and 50 μg/ml hygromycin B and doxycycline (1 μg/ml; Clontech Laboratories). Expression of aSyn WT was switched on by the removal of doxycycline from the media as described previously (44). For aSyn aggregation and aSyn cytotoxicity studies, we used human H4 neuroglioma cells. H4 were maintained in OPTI-MEM I (Life Technologies) supplemented with 10% FBS in the presence of penicillin (100 U/ml; Life Technologies) and streptomycin (100 μg/ml; Life Technologies).

SH-SY5Y aSyn WT cell line transfection and Rab11 knockdown

SH-SY5Y aSyn WT cells were grown in the absence of doxycycline for 6 days to induce aSyn WT expression. Cells were then seeded onto 100 mm diameter dishes (1.5 × 10⁶ cells/dish) in RPMI 1640 medium containing 10% FBS 24 h prior to transfection or transduction. For Rab11 knockdown, cells were transduced with adenovirus with three distinct Rab11 miRNA constructs and incubated for 48 h before changing the medium for conditioning. For Rab11 overexpression, cells were transduced with pEGFP Rab11a WT, pEGFP Rab11a S25N (kind gift from Dr Chiara Zurzolo, Institut Pasteur, Paris) or empty pEGFP vector using Lipofectamine 2000 (Life Technologies). Four hours after transfection, medium was replaced with fresh growth medium.

Preparation of CM, LDH cytotoxicity assay and preparation of cell extracts

Twenty-four hours after transfection or 48 h after transduction, the medium was changed to RPMI 1640 medium containing 2% FBS and conditioned for 48 h. The CM from transfected or transduced cells was collected and centrifuged at 4000 g for 10 min at 4°C to remove cell debris. For western blotting, the CM was concentrated using 3 kDa cutoff Amicon Ultra filters (Merck Millipore). CM without concentration was used to determine the membrane integrity of cells used in the experiments by measuring released LDH as described in the manufacturer’s instructions (Clontech Laboratories). For extraction of cellular proteins, cells were washed 2 × with cold PBS and lysed in NP-40 buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40) supplemented with protease inhibitor cocktail tablet (Roche Diagnostics).

Preparation of exosome-depleted medium and purification of exosomal fraction

The depletion of the medium from bovine serum-derived exosomes was performed as described previously (16). Briefly, RPMI 1640 medium containing 20% FBS, penicillin/streptomycin and L-glutamine was centrifuged at 100 000 g for 16 h at 4°C. The supernatant was carefully removed and sterilized by filtering through a 0.2 μm filter (Whatman) and stored at 4°C until additional use in exosome preparation. Exosomal fraction from the CM was prepared as described previously (16). Briefly, SH-SY5Y aSyn WT cells were seeded in three 100 mm dishes in 10% FBS and 24 h later transfected as described above. Twenty-four hours post-transfection, the culture medium was replaced with exosome-depleted medium diluted 10-fold with RPMI 1640 medium and conditioned for 48 h. Culture supernatants of cells were collected and spun at 300 g for 10 min to remove cells. The supernatants were then sequentially centrifuged at 2000 g for 10 min, 10 000 g for 30 min and 100 000 g for 90 min. The pellet containing exosomes was washed once with cold PBS and centrifuged again at 100 000 g for 90 min. The resulting pellet was resuspended in 30 μl of radioimmuno-precipitation assay (RIPA) buffer (50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 0.5% Na deoxycholate and 0.1% SDS). All centrifugations were performed at 4°C.

Western blotting

Protein concentration in cell extracts and concentrated CM was quantified using BCA protein assay kit (Thermo Scientific). Equal amount of total protein (250 μg for CM and 15 μg of cell lysate) was loaded on a 15% polyacrylamide separation gel and separated by SDS–PAGE using a Tetra cell (Bio-Rad). For immunoblot analysis of exosomes, whole fraction from single exosomal extraction (30 μl) was used each time. After separation by SDS–PAGE, proteins were transferred to nitrocellulose membranes using standard procedures with a Mini Trans-Blot system (Bio-Rad). Mouse anti-a-synuclein-1 antibody (BD Biosciences, 1:1000), mouse anti-Rab11 (BD Biosciences, 1:1000), mouse anti-GAPDH (Life Technologies, 1:4000) and mouse anti-TSG101 (Abcam, 1:1000) were used. Secondary anti-mouse antibody coupled to horseradish peroxidase (GE Healthcare, 1:10 000) was used. Membranes were incubated with ECL Chemiluminescent HRP Substrate (Millipore). Densitometry analysis of the corresponding bands was performed using the ImageJ software.

BFA treatment

SH-SY5Y cells expressing aSyn WT transfected with pEGFP, pEGFP-Rab11a WT or pEGFP-Rab11a S25N were pre-treated with BFA (1 μg/ml; SIGMA-ALDRICH) for 1 h before the medium was changed to RPMI 1640 medium containing 2% FBS and conditioned in the presence of BFA for further 5 h. CM was collected and processed for western blot and LDH analysis as described above.
Transferrin pulse-chase

SH-SY5Y cells expressing aSyn WT were seeded on glass cover slips 24 h prior to transfection with pEGFP, pEGFP Rab11a WT or pEGFP Rab11a S25N. Twenty-four hours post-transfection, cells were washed with PBS and incubated with human Alexa-546-Transferrin (50 μg/ml; Life Technologies) at 37°C for 15 min. Cells were then washed 2 × with cold PBS and incubated with unlabeled human holo-transferrin (5 mg/ml; SIGMA-ALDRICH) at 37°C for 10 min. Cells were washed 2 × with cold PBS, fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature (RT) and then mounted on glass microscopy slides in GVA mounting media (Genemed Biotechnologies). Cells were analyzed using Zeiss Axiovert 200M widefield fluorescence microscope. The percentage of transfected cells (EGFP-positive) positive for Alexa-546-Transferrin was counted using the ImageJ software. Minimum of 100 cells were counted per each condition.

H4 cell line transfection, Rab11 knockdown, immunocytochemistry, microscopy analysis and cytotoxicity assays

For intracellular aSyn aggregation experiments, H4 cells were seeded on 35 mm glass bottom imaging dishes (ibidi GmbH) 24 h prior to transfection. For Rab11 knockdown, cells were transfected with adenovirus with miRNA against Rab11 or with scrambled control (Scr). Cells were then co-transfected with synT (aSynEGFP deletion mutant WT/SynEGFPΔ155) and synphilin-1 in 1:1 ratio as described previously (49,50) using FuGENE6 (Roche Diagnostics). For Rab11 overexpression, 24 h post first transfection with synT and synphilin-1, cells were further transfected with pEGFP, pEGFP-Rab11a WT or pEGFP-Rab11a S25N. Twenty-four hours later, cells were fixed with 4% PFA for 10 min at RT, washed 2 × with PBS and subjected to immunocytochemistry analysis. Briefly, cells were permeabilized with 0.5% Triton X-100 in PBS for 20 min at RT, blocked for 1 h at RT with 1% normal goat serum in 0.1% Triton X-100 in PBS, incubated with primary antibody against aSyn (mouse anti-aSyn 1:1000; BD Biosciences) at 4°C overnight followed by secondary antibody incubation (1:1000, goat anti-mouse IgG-Alexa568, Life Technologies) for 2 h at RT and incubated for 2 min with DAPI 1:1000 in PBS (SIGMA-ALDRICH). Cells were then subjected to microscopy analysis using Zeiss Axiovert 200M widefield fluorescence microscope. The proportion of cells displaying aSyn-positive intracellular inclusions in the aSyn-positive cell population was determined by counting at least 100 cells in each condition using the ImageJ software.

For Rab11a and aSyn co-localization studies, H4 cells were transfected either with pSI-α-syn, a plasmid encoding for aSyn WT (gift from Dr Bradley T. Hyman), with empty pSI plasmid or co-transfected with plasmids encoding for synT and synphilin-1 as described above. Twenty-four hours post first transfection, cells were further transfected with pEGFP-Rab11a WT and 24 h later cells were fixed and subjected to immunocytochemistry for aSyn as described above. Cells were analyzed for Rab11a and aSyn colocalization using Zeiss LSM 510 META confocal microscope followed by analysis using the ImageJ software. Sequential multi-track frames were acquired to avoid any potential crosstalk from the two fluorophores.

For aSyn cytotoxicity assay, H4 cells were transduced with adenovirus for Rab11 knockdown or transfected with pEGFP Rab11a WT, pEGFP Rab11a S25N or pEGFP as described above and co-transfected with pSI-α-syn, a plasmid encoding for aSyn WT (gift from Dr Bradley T. Hyman), or with empty pSI plasmid. Twenty-four hours post-transfection, culture media were used to determine the levels of released LDH as described in the manufacturer’s instructions (Clontech Laboratories). LDH levels in the culture media were measured in the presence of Rab11a overexpression or Rab11 knockdown in the aSyn aggregation model described above (H4 cells transfected with synT and synphilin-1) in the same manner.

Rab11 and aSyn co-immunoprecipitation analysis

For co-IP experiments, brain tissue from WT Sprague–Dowley adult female rats was used. Whole-brain tissue lysates were prepared with immunoprecipitation buffer (50 mM Tris–HCl pH 7.5; 0.5 mM EDTA; 150 mM NaCl; 0.05% NP40), supplemented with protease inhibitor cocktail (Roche Diagnostics) using a HT 24 bead beating homogenizer (OPS Diagnostics). Approximately 6 mg of total protein lysates were pre-cleared by incubation with 20 μl of protein G beads (Invitrogen) for 30 min at 4°C in rotation. Supernatants were recovered and incubated overnight at 4°C in rotation, with 2 μg of the immunoprecipitation antibody, anti-aSyn (C-20, Santa Cruz Biotechnologies). The next day, 40 μl of protein G beads were added for 3 h in a rotator at 4°C. Beads were washed 5 × with immunoprecipitation buffer, then re-suspended in 20 μl of protein sample buffer (50 mM Tris–HCl pH 6.8; 2% SDS; 10% glycerol; 1% β-mercaptoethanol; 0.02% bromophenol blue) and boiled at 95°C for 5 min. Supernatants were resolved on a 15% SDS–PAGE gels. Proteins were transferred overnight to nitrocellulose membranes and blocked in 5% non-fat dry milk in TBS-Tween for 1 h. In order to test the co-IP with Rab11, the membranes were incubated overnight at 4°C with the primary antibody for Rab11 (BD Biosciences, 1:1000). Immunoblots were washed with TBS-Tween and incubated for 1 h at RT with the corresponding mouse-HP secondary antibody (GE Healthcare, 1:10 000). Immunoreactivity was visualized by chemiluminescence using an ECL detection system (Millipore) and subsequent exposure to auto-radiographic film. To prove the efficiency of α-synuclein immunoprecipitation, the same membrane was incubated with anti-α-synuclein (syn-1, BD Biosciences 1:1000) for 3 h at RT and developed as described above.

Recombinant aSyn expression and purification

WT aSyn was cloned in a pET21 vector (Novagen) for bacterial expression in Escherichia coli BL21 (DE3). Protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside for 3 h at 37°C. Cells were harvested and protein purification was performed as previously described (55) with minor modifications. Briefly, cells were boiled and the resulting cell debris was discarded by centrifugation. 10% solution of streptomycin sulphate (136 μl/ml of solution) and glacial acetic acid (228 μl/ml solution) were added to the supernatant. The sample was centrifuged and the supernatant, enriched in aSyn,
was recovered. The protein was precipitated with saturated solution of ammonium sulphate at 4°C (1 ml/ml solution). aSyn was collected by centrifugation and precipitated once more with ammonium sulphate followed by an additional precipitation step with 100% ethanol. Pelleted aSyn was resuspended in 25 mM Tris–HCl buffer pH 8.0 for further purification using an anion exchange chromatography. Sample was applied to a HiTrap Q HP column (GE Healthcare) and eluted with 10 column volumes of a 0–1 M NaCl gradient. WT aSyn integrity and purity was checked by mass spectrometry.

**aSyn in vitro aggregation**

Recombinant aSyn was assembled in vitro in the presence of cell lysates obtained from transfecting H4 cells with pEGFP plasmid or pEGFP-Rab11a WT plasmid, as described above. Cells were lysed 48 h after transfection in PBS supplemented with 0.5% NP-40 and protease inhibitors cocktail for 20 min on ice. Subsequently, samples were submitted to two cycles of 10 s sonication at 4°C, cell debris was discarded by centrifugation and protein quantification was performed as described above.

aSyn was resuspended in PBS and filtered through a 100 kDa Amicon Ultra centrifugal filter (Millipore) to remove preformed high molecular weight species. Assembly reactions were prepared in PBS containing 0.5 μg/ml of cell lysate, 70 μM of recombinant aSyn, 20 μM Thioflavin T (ThT) and 0.02% sodium azide. Respective controls were prepared accordingly. Samples were incubated at 37°C and 600 rpm and at the indicated time points ThT fluorescence was recorded in Infinite 200 PRO plate reader (Tecan) using excitation and emission wavelengths of 450 and 482 nm, respectively.

Complementarily, aggregating samples were also analysed by Dot blotting. Twenty-five microliters of aliquots were collected and centrifuged for 2 h at 15 000 g. The supernatant (soluble fraction) was diluted in 200 μl of PBS and the pellet (insoluble fraction) was resuspended in 225 μl of PBS. Both fractions were boiled for 5 min and applied to a nitrocellulose membrane (GE Healthcare). The membrane was subsequently blocked with 5% of skimmed milk powder and incubated with mouse anti-aSyn antibody (BD Biosciences, 1:1000) for 2 h at RT. The membrane was further processed as described above.

**Data analysis and statistics**

Statistical analyses were performed using Prism 6 (GraphPad Software). All values in the figures are represented as the mean ± SD. All the data shown are representative of at least three independent experiments. For transferrin pulse-chase and aSyn aggregation assay, minimum of 100 cells were analysed per condition. Statistical analysis was performed using one-way ANOVA with Bonferroni’s post hoc comparison and two-tailed Student’s t-test for unpaired data (*P < 0.05, **P < 0.01, ***P < 0.001).

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