Exophagy of α-synuclein

Tubulin Polymerization Promoting Protein (TPPP/p25α) promotes unconventional secretion of α-synuclein through exophagy by impairing autophagosome-lysosome fusion

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Background: The mechanism of unconventional secretion of α-synuclein is unknown.

Result: Autophagy of α-synuclein followed by exocytosis of autophagy intermediates (exophagy) is increased by expression of TPPP/p25α.

Conclusion: Exophagy of α-synuclein is increased by lysosomal dysfunction and/or altered trafficking of autophagosomes.

Significance: Exophagy of α-synuclein might represent the first step in inter-neuronal spread of Lewy body disease.

SUMMARY

Aggregation of α-synuclein can be promoted by the tubulin polymerization-promoting protein (TPPP)/p25α, which we have here used as a tool in order to study the role of autophagy in the clearance of α-synuclein. In NGF-differentiated PC12 catecholaminergic nerve cells, we show that de novo expressed p25α co-localizes with α-synuclein, and causes its aggregation and distribution into autophagosomes. However, p25α also lowered mobility of autophagosomes and hindered final maturation of autophagosomes by preventing their fusion with lysosomes for final degradation of α-synuclein. Instead, p25α caused a four-fold increase in the basal level of α-synuclein secreted into the medium. Secretion was strictly dependent on autophagy and could be up-(trehalose, Rab1A) or down-regulated (3-MA, ATG5 shRNA) by enhancers or inhibitors of autophagy, or by modulating minus end-(HDAC6 shRNA) or plus end-directed (Rab8) trafficking of autophagosomes along microtubules. Finally, we show in the absence of TPPP/p25α, that α-synuclein release was modulated by dominant mutants of Rab27A, known to regulate exocytosis of late endosomal (and amphisomal) elements, and that both lysosomal fusion block and secretion of α-synuclein could be replicated by knock-down of the p25α target, HDAC6, the predominant cytosolic deacetylase in neurons. Our data indicate that unconventional secretion of α-synuclein can be mediated through exophagy, and that factors, which increase the pool of autophagosomes/amphisomes (e.g. lysosomal disturbance), or alter the polarity of vesicular transport of autophagosomes on microtubules, can result in an increased release of α-synuclein monomer and aggregates to the surroundings.

Parkinson’s disease (PD) is characterized by the progressive loss of dopaminergic neurons in the substantia nigra, although other neural populations...
of the central nervous system (CNS) are also affected. PD develops and progress over many years and affects an increasingly larger volume of the nervous system. Braak and co-workers have hypothesized that progression of PD correlates with a topographical spreading pattern of \(\alpha\)-synuclein inclusion body disease throughout the nervous system (1), which is also the case of spreading \(\alpha\)-synucleinopathy induced by injection of preformed \(\alpha\)-synuclein fibrils into the cortex or striatum of transgenic \(\alpha\)-synuclein-expressing mice (2). However, despite demonstrations that neurons are capable of secreting and internalizing \(\alpha\)-synuclein (3,4), and that Lewy body pathology can be transferred from recipient to engrafted embryonic stem cells or fetal mesencephalic dopaminergic neurons in patients or experimental animals (5-7), little is known about the inter-neuronal transmission mechanisms of \(\alpha\)-synuclein species.

Aggregated or modified forms of \(\alpha\)-synuclein are degraded by proteasomal activity and different forms of autophagy (8,9). During quality control (QC) macroautophagy a double-layered isolation membrane, also termed the phagophore, encloses a volume of cytosol containing damaged organelles or poly-ubiquitinated protein aggregates and thereby forms a vacuolar autophagosome (10). Generation of autophagosomes requires membrane lipids derived from endoplasmatic reticulum (ER), Golgi, or mitochondria (11) and is regulated by a set of conserved autophagy-related genes (ATG), including initiators (PI3K and Beclin-1) and elongators (conjugations systems ATG5-ATG12 and cytosolic light chain 3B (LC3B)) (12,13). The autophagic vacuole then quickly matures by fusion with compartments of the endosomal pathway before final fusion with lysosomes to generate an autolysosome where the luminal content of the autophagosome is degraded (14). Specifically, the fusion organelle of an autophagosome and an endosome (often a multivesicular body) is called an amphisome. In contrast to starvation-induced autophagy, which indiscriminately encloses a volume of cytosol and organelles for degradation and recycling of protein building blocks, QC autophagy typically accepts poly-ubiquitinated proteins, long-lived proteins, aggregates, and organelles as cargo. Selectivity is provided by ubiquitin-binding adaptor proteins p62/SQSTM1, NBR1, and HDAC6 (15,16), which link ubiquitinated cargo to LC3B, and in the case of HDAC6, additionally to dynein-dynactin motor proteins (16-20). Neurons depend on autophagy for differentiation and survival (21), and p62/SQSTM1 and HDAC6 are required for development of inclusion bodies and aggresomes by directing minus-end transport of ubiquitinated cargo on microtubuli (15,16).

Lewy bodies invariably contain modified and aggregated \(\alpha\)-synuclein as the main component along with a number of other nerve cell proteins typically highly ubiquitinated. The tendency of \(\alpha\)-synuclein to form cytosolic aggregates is influenced by a number of other proteins including synphilin-1 (22), protein interacting with NIMA 1 (PIN-1) (23), and TPPP/p25\(\alpha\) (hereafter referred to as p25\(\alpha\)) (24). The p25\(\alpha\) protein binds to microtubules and by doing so lowers their plus-end growth rate and protects them from depolymerisation (25-27). In addition, p25\(\alpha\) potently stimulates aggregation of \(\alpha\)-synuclein \textit{in vitro} and localizes to Lewy bodies \textit{in vivo} (24,28). In the CNS, p25\(\alpha\) is mainly expressed in oligodendrocytes and is required for their differentiation (29), but during PD progression p25\(\alpha\) becomes ectopically expressed in dopaminergic neurons. Conversely, \(\alpha\)-synuclein is up-regulated in p25\(\alpha\)-expressing oligodendrocytes of patients with Multiple System Atrophy (24), and both proteins co-localize in characteristic inclusion bodies of these diseases.

In the present study we have asked the question whether \(\alpha\)-synuclein species can be secreted by dopaminergic neurons following autophagy and exophagy. For this purpose we have used the well-known PC12 pheochromocytoma cell line as a model system of dopaminergic neuron-like cells to conditionally express the aggregation-prone \(\alpha\)-synuclein\(_{A30P}\) protein with or without p25\(\alpha\) as a tool to increase \(\alpha\)-synuclein aggregation and autophagic uptake. We show here that \(\alpha\)-synuclein monomer and high-molecular weight (High-Mw) species can be secreted by exophagy, and that p25\(\alpha\) further augments this process by inhibiting autophagosomal fusion with lysosomes.

**Materials and methods**

**Cell culture and neuronal differentiation**

The rat pheochromocytoma cell line PC12 (ATCC) was seeded on collagen-coated culture dishes and cultured in DMEM containing 10% horse serum, 5% fetal calf serum, and 1% penicillin and streptomycin (P/S) at 37°C in 5% CO\(_2\). For experiments PC12 cells were seeded at a density of 45,000 cells/cm\(^2\) and differentiated in DMEM medium containing 2% horse serum, 1% P/S, and 100ng/ml nerve growth factor (NGF) (2.5S subunit, Serotec) for two days.
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before transgene expression was induced for an additional two days with doxycycline (200 ng/ml). SH-SY5Y cells were cultured in DMEM containing 10% fetal calf serum and 1% P/S at 37°C in 5% CO2. For experiments SH-SY5Y cells were differentiated with 1 µM ATRA for 6-8 days to obtain a neuronal-like phenotype before transgene expression was induced by treatment with doxycycline (200 ng/ml) for an additional 24 hours.

Antibodies and chemical reagents

Antibodies used include mouse anti-α-synuclein mAb from BD Transduction Laboratories, mouse anti-α-synuclein mAb (LB509) from Abcam, rabbit anti-α-synuclein mAb (EP1646Y) from Abcam, and rabbit anti-α-synuclein polyclonal antibody from Sigma. rabbit anti-LC3B and anti-p62/SQSTM1 pAb’s, and mouse anti-acetylated tubulin mAb and mouse anti-β-actin mAb were from Sigma; rabbit anti-p25 pAb from Novus; rat anti-TPPP/p25α mAb (Enzo Life Sciences); rabbit anti-cleaved caspase-3 pAb (D175) and mouse anti-ubiquitin mAb (Cell Signaling); rabbit anti-LAMP1 pAb (Novus); mouse anti-HA mAb (Santa Cruz Biotechnology); rabbit anti-GFP pAb (Molecular Probes); mouse anti-ubiquitin mAb (Novus Biologicals). Alexa488- or 568-conjugated annexin-V, Alexa-conjugated phalloidin, and Alexa 488, -568 or -633-conjugated goat anti mouse or rabbit antibodies (Molecular Probes); Horse radish peroxidase (HRP)-conjugated swine or goat anti-mouse or rabbit secondary antibodies (DAKO); ToPro-3 iodide (Molecular Probes) for nucleus stain. The following chemicals were used: Bafilomycin A1; Leupeptin; Pepstatin A; Trichostatin A; Trehalose; HEPES (4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid); Trichloroacetic acid (TCA); Triton X-100, Tween20; phosphatase and protease inhibitor cocktail; (all from Sigma); 3-MA (from Calbiochem).

Lentivector construction and transduction

For conditional expression of transgenes, cDNA’s were PCR-cloned into BamHI/SalI-restricted lentiviral vector pLOX-cPPT.TW.W expressing cDNA under the control of a tetracycline-responsive promoter (tet-On). Already established and tested PC12-rtTA or SH-SY5Y-rtTA cell lines expressing the reverse tetracycline-controlled transactivator were superinfected with pLOX-cPPT.TW.W lentivectors expressing the cDNA’s of β-synuclein, α-synucleinwt, α-synucleinA30P, or p25α, or with the pHR-cPPT.CMV.W lentivector for constitutive expression of mCherry-eGFP-LC3B (kindly provided by Professor Terje Johansen, Institute of Medical Biology, University of Tromsø, Norway; (20)), 3xHA-tagged Rab1A, Rab3A or Rab8 (all human cDNAs from Missouri S&T cDNA Resource Center), or Rab7-GFP fusion constructs alone or in combination as specified. Dominant negative and positive Rab27A mutants Rab27A-T23N and Rab27A-Q78L, respectively, were expressed under the control of a CMV promoter in the pLenti6-V5-TOPO vector (30). For expression of polyglutamine tracts from exon 1 of human huntingtin, templates contained in plasmids pEGFP/HD-120Q and pEGFP/HD-18Q (31) were subcloned into the plasmid PCR2.1 (Invitrogen) by PCR using primers 5’-GGA AAA GGT TCA TGA GCA AGC -3’ and 5’-GGA TCC CCG GCT GAG GAA GCT GAG GAG-3’, and then BamHI cloned into the pHR-cPPT.CMV.GFP.W vector to express Huntington-115Q-GFP or -18Q-GFP. All constructs were verified by sequencing. Knock-down of HDAC6 expression was carried out by transducing PC12 cells 72 hours prior to analysis with Sigma MISSION™ HDAC6 shRNA in a pLKO.1 lentivector backbone (sense sequence AGGAAAGGTTCTCGAAGCA). PC12 cell populations knocked-down for ATG5 were generated by the combined constitutive expression of two ATG5 shRNA’s (sense sequence; CTGGGAACATCACAGTACA and CTGTTCACAGTCAGTCTAT) contained in the pGPIZ lentivector (Open Biosystems). At least 85% of cells carried transgenes (conditionally or constitutively) following creation of stable PC12 populations by lentiviral transduction, while transduction of differentiating PC12 cells yielded transduction efficiencies ranging from 40-60%. Vector production was carried out as previously described (32).

Western blotting

Cells were lysed in lysis buffer (100mM NaCl, 50mM Tris-HCl, 1mM EGTA, 10mM MgCl2, pH 7.2) containing 1% Triton X-100, phosphatase and protease inhibitor cocktail for 5 minutes at room temperature and hereafter kept on ice. Cell lysates were centrifuged at 16,100g for 5 minutes, 4°C and protein concentrations of the supernatant were determined with Dc Protein assay (BioRad), before addition of Laemml buffer and loading of equivalent protein quantities on SDS-PAGE gels for western blotting using chemiluminescent HRP detection substrate (Millipore).
**TCA protein precipitation**
Conditioned medium was harvested and centrifuged at 800g for 5 minutes, 4°C, before ice-cold TCA was added to the supernatant and incubated on ice for 10 minutes. The protein precipitates were pelleted by centrifugation (16,100g, 5 minutes, 4°C) and washed 4-5 times in ice-cold acetone until the pellet appeared clear white. The pellets were dried at 95°C for 10 minutes, dissolved in 2.5×4-5 times in ice-cold acetone until the pellet centrifugation (16,100g, 5 minutes, 4ºC) and washed for 10 minutes. The protein precipitates were pelleted by adding to the supernatant and incubated on ice for 10 minutes. The pellets appeared clear white. The pellets were dried at 95ºC, boiled for 20 minutes at 95ºC, and subsequently processed for western blotting.

**Exosome preparation**
Conditioned medium from 10 cm petri dishes with either PC12-α-synucleinA30P or α-synucleinA30P/p25α cells was pre-centrifuged at 1,200g for 10 minutes followed by another round of centrifugation at 10,000g for 30 minutes. The supernatants were then passed through 0.22μm filters before centrifugation at 100,000g for one hour in a Beckmann centrifuge with SW40Ti rotor. The pellet containing exosomes was washed once in PBS, and then aliquots of pellet and supernatant were separated by gel electrophoresis and processed for western blotting.

**Homogenization and sucrose gradient fractionation**
PC12 cells were suspended in PBS and pelleted by centrifugation at 800g, for 8 minutes, 4°C. Cells were washed once in hypotonic buffer (170mM sucrose, 75mM NaCl, and 10mM HEPES, pH 7.0) containing protease and phosphatase inhibitor cocktail, snap-frozen in liquid nitrogen, re-thawed in 200μl hypotonic buffer, and then repeatedly aspirated with a 27G-needle. The volume was diluted to 500μl with hypotonic buffer before centrifugation at 800g for 8 minutes, 4°C. Subsequently, the supernatant was centrifuged at 10,000g for 10 minutes, 4°C. The post-nuclear/mitochondrial supernatant was then applied to the top of a 15-45% sucrose gradient in gradient buffer (140mM NaCl, 25mM HEPES, pH 7.2) and ultracentrifuged in a Beckman centrifuge with a SW40Ti rotor head at 30,000rpm for 18 hours, 4°C. Twelve one-milliliter fractions were collected from the bottom with a peristaltic pump and aliquots thereof processed for western blotting.

**Immunofluorescence and electron microscopy**
PC12 cells were washed once in Hanks balanced saline solution (HBSS) and fixed in a phosphate buffer containing 2% paraformaldehyde (PFA), pH 7.4, for 30 minutes. Immunofluorescence was essentially performed as described previously (33), and images acquired with a Zeiss LSM510 confocal laser scanning microscope with a C-Apochromat ×63, 1.4 NA oil immersion objective, using the argon 488nm, the helium-neon 543nm and 633nm laser lines for excitation of Alexa 488, 568 and 633, respectively. Confocal sections of 0.8-1.0μm were collected and saved as 512x512-pixel or 1024x1024-pixel images at 12-bit resolution before import to Adobe Photoshop CS2 version 9.0.2, Zeiss LSM image browser, or ImageJ for compilation and quantification. When quantifying apoptotic cells, eight to twelve images (each containing around 200 cells) were acquired for each condition with a Fluor ×20, 0.75 NA water immersion objective. Cells positive for cleaved caspase-3 and the total number of cells (identified by ToPro-3 iodide) were quantified by a custom-made macro in ImageJ. For ultrastructural analysis PC12 cells were fixed in a phosphate buffer containing 2% PFA and 0.2% glutaraldehyde, pH 7.4, and processed for either epon embedding or cryo preservation and sectioning as previously described (33). Cryo-EM sections were incubated with monoclonal mouse anti-α-synuclein mAb LB509 and polyclonal rabbit anti-GFP (Molecular probes; A64655) followed by 7nm and then 14nm gold-conjugated goat-anti mouse or rabbit antibodies, respectively. Sections were examined in a Phillips CM 100 electron microscope equipped with a digital camera.

**Fluorescence recovery after photobleaching (FRAP)**
PC12 cells expressing mCherry-eGFP-LC3B were imaged in HEPES buffer, pH 7.4 at 37°C. A circular region of interest (ROI) measuring 35 pixels in diameter was defined and after three initial images mCherry fluorescence in the ROI was bleached with the 543nm laser (30 iterations, at 100% laser strength). Subsequently, fluorescence recovery in the ROI was measured over a period of 3.5 minutes every tenth second. The fluorescence recovery was adjusted for general photobleaching. Eight to ten cells were measured for each experiment and mean values were acquired from a minimum of three independent experiments.

**FACS analysis**
For cell death analysis PC12 cells were differentiated in 12-well culture plates and following incubated with 2μg/ml propidium iodide (PI) for 15 minutes at RT and subsequently placed on ice. The cells were immediately hereafter analyzed in an Accuri C6 Flow Cytometer using the 488nm laser for excitation and the FL3 optical filter (>670nm) for emission.
Detection. The lower fluorescence limit defining PI positive cells was set based on control PC12 cells that did not receive PI. When analyzing the endosomal degradation pathway, PC12 cells were incubated with 40µg/ml bovine serum albumin (BSA) conjugated to the green fluorescent BODIPY FL dye (DQ-BSA) (Molecular Probes) for 6.5 hours prior to flow cytometric analysis using the FITC filter. Mean fluorescence intensity of cells that did not receive DQ-BSA was subtracted from all sample values.

**Statistical analysis**

Data were analyzed with one-way analysis of variance (ANOVA) or students t-test, and significance levels defined as p-values less than 0.05 (* or #), 0.01 (** or ##), or 0.001 (*** or ###). The largest and the smallest variance were tested for homogeneity with F-test before test. Bar graph values are expressed as mean and error bars as standard error of the mean (SEM).

**RESULTS**

**Expression of p25α inhibits neurite extension and degradative capacity of PC12 cells**

We used lentiviral transduction to obtain conditional (tet-on) expression of α-synuclein or mutant α-synucleinA30P with or without co-expression of p25α in PC12 pheochromocytoma cells differentiated to catecholaminergic nerve cells with NGF. Unless otherwise specified, we adopted a differentiation protocol consisting of two days pre-treatment with NGF followed by two days of NGF and doxycycline treatment for transgene expression. At this time cells expressing α-synuclein, or control cells expressing the non-pathogenic but closely related β-synuclein, formed a fully extended neurite network (Figure 1A) although α-synuclein expression, in particular α-synucleinA30P, caused α-synuclein-positive neurite blebs (arrowheads, Figure 1B). In contrast, p25α expression alone or in combination with α-synuclein caused cell flattening and impaired neurite growth (Figure 1A and B). The tet-on system provided an equal expression level of transgenes in PC12 cells with only minor leakage from the promoter (Figure 1C). The anti-α-synuclein antibodies used throughout this study, unless otherwise stated, react only weakly with endogenous rat α-synuclein in western blotting applications. However, an alternative α-synuclein mAb (Abcam EP16464Y) with higher affinity to rat (α-synuclein detects endogenous rat α-synuclein, and (with the limitations of cross-species antibody detection) we estimate that PC12 cells differentiated with NGF for four days express transgene α-synucleinA30P ca. fifty-fold higher than the endogenous level of α-synuclein after 48 hours of doxycycline induction. Of note, differentiated PC12 cells express considerably lower levels of α-synuclein than primary nerve cells from mouse (data not shown). TPPP/p25α expression is usually absent in cell lines, and we could not detect it in PC12 cells. All western blot data in the following therefore pertain to transgene α-synuclein, although endogenous α-synuclein is also recruited (see Figure 4C-E). Lowering the serum concentration to initiate NGF-induced differentiation causes roughly 15% of β-synuclein-expressing control cells to die, but within the time frame α-synuclein and p25α co-expression only further augmented cell death through apoptosis by roughly 3% (Figure 1D and E). Huntingtin containing pathological poly-glutamine stretches is a substrate of both the proteasomal and autophagosomal degradation pathways, which both are known to be inhibited by modified forms of α-synuclein (34-36). Failure to degrade huntingtin results in the formation of large inclusion bodies called aggresomes. We therefore tested the effect of α-synuclein and/or p25α expression on the ability of PC12 cells to deal with co-expression of a polyglutamine tract (115Q) from huntingtin fused to GFP, or as control the non-pathogenic 18Q-GFP. As illustrated in Figure 1F and G, expression of either α-synucleinA30P or p25α alone or in combination increased huntingtin inclusion body formation up to two-fold, indicating that p25α and α-synucleinA30P expression interferes with the degradative machinery of the cells.

**Expression of p25α redistributes α-synuclein to cytosolic vesicles, and causes increased autophagy**

The majority of PC12 cells expressing α-synucleinA30P or α-synucleinA30P had a compact cell body and α-synuclein was distributed mainly in the cell periphery and in neurites, whereas p25α had a more diffuse somatic distribution (Figure 1B, and 2A and B). In contrast, when co-expressed p25α co-localized closely with α-synucleinA30P and induced its redistribution into cytosolic aggregates, inclusions, and/or vesicles (Figure 2C). In a subset of PC12-α-synucleinA30P/p25α cells (around 5%) large inclusion bodies with diameters up to 5µm could be observed (Figure 2D). PC12 cells expressing α-synuclein alone did not contain any distinguishable inclusion bodies (Figure 2A). Alpha-synuclein and its mutant forms are known to be degraded by macroautophagy.
we made use of PC12 cells constitutively expressing immunogold labeling for EM, and for this purpose light microscopical observations by cryo-as an autophagosomal substrate. We verified these substantially increases the suitability of autophagosomal adaptor protein p62/SQSTM1. This co-localization was increased considerably by autophagosomal markers LC3B (arrow, Figure 3A). To further pursue the involvement of autophagosomes we chose to focus on the aggregation prone mutant α-synucleinA30P (Figure 2E and F). Also a ca. three-fold increase in the LC3B-II/LC3B-I ratio was observed in differentiated SH-SY5Y cells expressing α-synucleinA30P with p25α (Figure 2G and H). We next subjected the cells to ultrastructural analysis to confirm an increased autophagic flux (Figure 2I-N). Co-expression of p25α increased the number of nascent autophagosomes in α-synuclein-expressing PC12 cells (black, open arrows, Figure 2I and M; J-L; and O), which correlated with an increased number of amphisomes, clearly containing luminal vesicles derived from fusion with late endosomes (white, filled arrows in Figure 2M and N; shows the fusion of a late endosome and an autophagosome to form an amphisome), and multilamellar bodies containing whorls of partially degraded lipids (white, open arrows, Figure 2M). Autophagosomes were evident in all parts of soma as well as neurites, and autophagosomes were observed to sequester various organelles including endosomes, dense core vesicles, and mitochondria (Figure 2J-L).

**Alpha-synucleinA30P co-localize with markers of autophagosomes**

To further pursue the involvement of autophagosomal protein clearance, we chose to focus on the aggregation prone mutant α-synucleinA30P, which is a more likely substrate for QC autophagy than α-synuclein wt. By indirect immunofluorescence of PC12 cells expressing α-synucleinA30P, we only observed few vesicular structures with overlapping fluorescence between α-synucleinA30P and the autophagosomal markers LC3B (arrow, Figure 3A). This co-localization was increased considerably by co-expression of p25α, which also to a lesser degree caused co-localization of α-synucleinA30P and the autophagosomal adaptor protein p62/SQSTM1 (arrows, Figure 3B and C), indicating that p25α substantially increases the suitability of α-synuclein as an autophagosomal substrate. We verified these light microscopical observations by cryo-immunogold labeling for EM, and for this purpose we made use of PC12 cells constitutively expressing an mCherry-eGFP-LC3B fusion construct (20). Alpha-synuclein detected by the monoclonal antibody LB509 could be co-localized with LC3B (detected with anti-GFP antibody) in autophagosomes in both PC12-α-synucleinA30P (Figure 3D-F) and to a higher extent in α-synucleinA30P/p25α-expressing cells (Figure 3G-I). In PC12-α-synucleinA30P cells, α-synuclein reactivity could also be detected in unidentified compartments without LC3B labeling (black, filled arrow in Figure 3F). In addition, α-synuclein and LC3B co-localized, in particular in PC12-α-synucleinA30P/p25α cells, in matured autophagosomal elements, likely representing amphisomes (Figure 3E, G and H; white, open arrows). For reasons discussed below we also consider (LC3B-positive) multimellar bodies (Figure 3G; white, open arrow) as amphisomes, although in the absence of functional antibodies for cryo EM-labeling of rat late endosomal markers we cannot conclusively say so. Cells expressing β-synuclein yielded only very sparse non-specific labeling (data not shown). Collectively, the data demonstrate that in the presence of p25α expression, over-expressed α-synucleinA30P becomes a suitable substrate for autophagy.

**Expression of p25α induces selective autophagy of α-synucleinA30P and also affects endogenously expressed α-synuclein**

The p25α-mediated inclusion of α-synuclein into autophagosomes was selective, inasmuch as pathogenic Htt-115Q co-expressed in the same cells, segregated entirely to p62/SQSTM1-positive aggresomes (Figure 4A and B). To rule out that the observed aggregation and autophagy of α-synuclein in the presence of p25α was due to over-expression of α-synucleinA30P, we also studied the effects on endogenous rat α-synuclein in PC12 cells expressing only p25α by immunofluorescence. As shown in Figure 4C and D, p25α caused a marked redistribution of endogenous α-synuclein from the cell periphery into ubiquitin-positive structures, which in themselves increased in numbers compared to control cells expressing β-synuclein. These structures were also immunoreactive for p25α, as well as late endosome/amphisome markers KA11 and mannose phosphate receptor (Figure 4E).

**Alpha-synucleinA30P is excluded from lysosomes in p25α-expressing cells.**

By immunofluorescence we observed considerable co-localization between α-synuclein and lysosomal-associated membrane protein 1 (LAMP1) in α-
synuclein$_{A30P}$-expressing PC12 cells after treatment with protease inhibitors leupeptin and pepstatin A (arrows, Figure 5A), indicating that vesicular α-synuclein observed by immunofluorescence and cryo-immunogold labeling eventually is degraded in lysosomes. However, in PC12 cells co-expressing p25$_{α}$, α-synuclein$_{A30P}$-positive vesicles and inclusions were conspicuously devoid of LAMP1 immunoreactivity suggesting impaired fusion of amphisomes with lysosomes (Figure 5B and C). Although some LAMP1-positive lysosomes (red arrows, Figure 5B) were distributed in close proximity to α-synuclein-positive vesicles (green arrows, Figure 5B), there was no evident overlap and this was also the case for larger inclusion bodies (white arrows, Figure 5C). To substantiate this further we first fractionated homogenates from α-synuclein$_{A30P}$- and α-synuclein$_{A30P}$/p25$_{α}$-expressing PC12 cells on sucrose gradients by velocity centrifugation. Most α-synuclein$_{A30P}$ was contained within low-density fractions (10 to 12) of the gradient corresponding to cytosolic proteins, including LC3B-I, and low-density membrane (Figure 5D). In PC12 cells expressing α-synuclein$_{A30P}$ a small accumulation of α-synuclein could be detected in the high-density fractions (1 to 4) of the gradient corresponding to where lysosomes sediment (we were not able to identify a functional anti-rat LAMP antibody for western blotting). These fractions contained only the LC3B-II form, which is incorporated into the membrane of autophagosomes. However, the high-density fraction content of α-synuclein was diminished in PC12 cells co-expressing p25$_{α}$ (Figure 5D). In addition, we performed a functional assay to investigate endosomal trafficking to lysosomes by incubating PC12 cells with fluorophore-conjugated BSA (DQ-BSA), which is internalized by endocytosis and becomes fluorescent only upon lysosomal proteolysis. While PC12 cells expressing β-synuclein or α-synuclein$_{A30P}$ showed a similar proficiency in DQ-BSA degradation, expression of p25$_{α}$ alone or in combination with α-synuclein$_{A30P}$ decreased the level of DQ-BSA fluorescence (Figure 5E). More dramatic decreases, that were largely cell line-independent, were obtained by either blocking lysosomal proteolysis with leupeptin/pepstatin A, or by blocking fusion of late endosomes/amphisomes with lysosomes using the V-ATPase inhibitor bafilomycin A1, which inhibits endosomal and lysosomal acidification. Collectively, the results indicate that p25$_{α}$ impairs the delivery of α-synuclein to lysosomes by interfering with the fusion of autophagosome maturation intermediates including amphisomes with lysosomes.

**Co-expression of p25$_{α}$ impairs autophagosome maturation**

We next analyzed the autophagosomal flux in greater detail by superinfecting PC12 cell lines with lentivector expressing the mCherry-eGFP-LC3B tandem construct (20). The construct is incorporated into the outer and inner leaflet of the isolation membrane of forming autophagosomes via LC3B. While eGFP fluorescence is pH-sensitive and decreases upon fusion with low-pH lysosomes, the mCherry fluorophore is pH-insensitive, which enables distinction of recently formed autophagosomes (co-localizing green and red dots) and autophagosomes, which have matured by fusion with lysosomes to form autolysosomes (only red dots). In PC12 cells expressing β-synuclein ca. 80% of vesicular LC3B was associated with exclusively red dots indicating a continuous flux of autophagosomes that fuse with lysosomes (Figure 6A, F and G). However, the ratio between mCherry- and mCherry/eGFP-positive vesicular compartments was decreased by both α-synuclein$_{A30P}$ and in particular p25$_{α}$, indicating an impairment of the autophagosomal flux, which could be mimicked in PC12 β-synuclein cells by treatment with bafilomycin A1 (Figure 6B, C, E-G). PC12 cells expressing p25$_{α}$ alone or together with α-synuclein$_{A30P}$ furthermore displayed large autophagosomes/amphisomes (>1µm) and a subset of cells (2%) displayed structures with diameters ranging up to 5µm (Figure 6D and H). These structures correlate in size with the α-synuclein$_{A30P}$-positive aggregates shown in Figure 2D, and could as above be instigated by bafilomycin A1 treatment of control cells alone (Figure 6E and H).

**Co-expression of p25$_{α}$ causes unconventional secretion of α-synuclein by exophagy**

Despite the ability of p25$_{α}$ to impair autophagosome maturation and autolysosomal degradation, p25$_{α}$ expression surprisingly correlated with an augmented clearance of α-synuclein (Figure 7A). In addition, clearance of α-synuclein in PC12 cells without p25$_{α}$ was not completely suppressed by addition of leupeptin/pepstatin A, which inhibits endosomal and lysosomal degradation. We therefore tested if α-synuclein was secreted to the surroundings by the PC12 cells. The conditioned medium after two days of transgene expression was precipitated with TCA and then analyzed for...
presence of α-synuclein by western blotting. When expressed alone both α-synucleinA30P and to a higher extent α-synucleinA30P was secreted into the medium, however, secretion of α-synuclein was greatly up-regulated by p25α co-expression (Figure 7B). TPPP/p25α was also secreted (Figure 7B), but had a longer cellular half-life than α-synuclein (data not shown).

In yeast exocytosis of autophagosomes constitutes an unconventional secretion pathway (37). Consistent with this and our observations above, addition of the autophagy inhibitor 3-MA, which inhibits class III PI3K activity required for formation of the isolation membrane, significantly reduced secretion of α-synuclein in PC12 cells co-expressing α-synucleinA30P and p25α (Figure 7B and F). Autophagy was effectively inhibited at the concentrations of 3-MA used, as the drug caused p62/SQSTM1 accumulation (15,20) (Figure 7B). In cells only expressing α-synucleinA30P, 3-MA on the contrary induced an increase in secretion of α-synuclein (Figure 7B and F). This is likely due to the persistent inhibitory effect of 3-MA on class I PI3K, (which normally activate mTOR pathway, and thereby opposes autophagy) and a transient inhibition of class III PI3K (required in both mTOR-dependent and -independent autophagy) as previously reported (38).

For space considerations western blots of conditioned medium have generally been cropped to show only monomer α-synuclein, but Figure 7C shows that SDS-insoluble oligomeric and High-Mw α-synuclein immunoreactive species were secreted alongside with monomers, and correlated in intensity with each other. By densitometry of western blots we estimate that between 6 and 12% of the total cellular α-synucleinA30P was present in the medium after two days of culture. This makes it difficult to see changes in cellular levels of α-synuclein by western blotting, but p25α expression markedly lowered the levels of endogenous α-synuclein in cell lysates, and this correlated with secretion of α-synuclein into the medium (Figure 7D). The low amounts of secreted endogenous α-synuclein in the medium could only be detected by immunoprecipitation and not by TCA precipitation.

TPPP/p25α-induced secretion of α-synuclein was not a consequence of the inability of p25α-expressing cells to form an extensive neurite network (see Figure 1A and B). If PC12 cells were fully differentiated with NGF for four to six days before induction of p25α expression with doxycycline, there was no subsequent effect on the neuritic network, and the cells maintained an augmented secretion of α-synuclein 4.9+/−1.3 (n=3) fold higher than control cells without p25α (data not shown). Secretion was however a function of NGF differentiation, as the amount of α-synuclein secreted from undifferentiated PC12 cells (with or without p25α expression) was less than 25% that of differentiated cells (data not shown).

### Chemical or genetic modification of the autophagosomal-lysosomal pathway affects secretion of α-synuclein

We further tested the effects of chemical modulation of the autophagosomal-lysosomal pathway on the secretion of α-synuclein by using a panel of inhibitors or enhancers of different forms of autophagy. As seen in Figure 7E and F, inhibition of lysosomal function by either leupeptin/pepstatin A or even more prominently with bafilomycin A1, which mimics the p25α-induced block of autophagosomal fusion with lysosomes, caused a clear up-regulation of α-synuclein secretion. This was also the case when PC12 cells with or without p25α expression were treated with trehalose, an mTOR-independent enhancer of autophagy (39), which correlated with marked increases in LC3B-II levels (Figure 7E). The increase in LC3B-II levels caused by bafilomycin and leupeptin/pepstatin is directly due to suppression of degradation of conjugated LC3B-II in lysosomes, while the increase mediated by trehalose is due mainly to increased conversion of LC3B. In contrast, rapamycin an enhancer of mTOR-dependent autophagy (starvation-induced autophagy), did induce transient conversion of LC3B (see below), but only increased secretion moderately in PC12-α-synucleinA30P cells, and not at all in p25α-expressing cells. Trichostatin, which inhibits HDAC6 and thereby deacetylation of cortactin required for fusion of QC autophagosomes with lysosomes (40), caused a modest up-regulation of α-synuclein secretion in PC12 cells with or without p25α expression.

The involvement of autophagosomes in secretion was verified by shRNA knock-down of ATG5, which is required for elongation and formation of the autophagosomal isolation membrane (12). Using a combination of two different shRNA constructs, ATG5 protein expression in PC12-α-synucleinA30P/p25α cells was reduced to 30%, which correlated with a decreased conversion of LC3B-I to LC3B-II and a 60% reduction in the amount of α-synuclein secreted (Figure 7G-I). Collectively, the data demonstrate that p25α mediates unconventional secretion of α-
synuclein through a pathway, which is dependent on autophagosome formation. In contrast, the levels of Htt-115Q-GFP detected in the conditioned medium of PC12 cell lines co-expressing huntingtin constructs were low and did not change significantly upon p25α expression (Figure 7J and K), likely reflecting the aggresome inclusion of Htt-115Q-GFP noted previously (Figure 4A and B).

**Trehalose and rapamycin-induced autophagosomes are differentially affected by p25α expression**

The different effects of trehalose and rapamycin on LC3B conversion and α-synuclein secretion after 48 hours made us more carefully examine the effect of these autophagy enhancers. Figure 8A shows the typical distribution and fluorescence of the mCherry-eGFP-LC3B construct in untreated PC12-α-synuclein/p25α cells. As expected 3-MA caused the number of autophagosomes to decrease, and apart from a few large vacuoles LC3B was mainly cytosolic. Trehalose induced an increased number of LC3B-positive structures, but as in the control situation, the autophagosomes were inhibited in their maturation (both red and green fluorescence). In contrast, the effect of rapamycin on LC3B conversion was transient (<24 hours) and did not differ significantly from untreated cells after 48 hours (Figure 8E and F; Figure 7E and F). However, maturation of rapamycin-induced autophagosomes in terms of acidification progressed unimpeded by p25α expression as assessed by the mCherry-eGFP-LC3B construct, which displayed increased number of vesicular profiles with only red fluorescence (Figure 8D). Collectively, the data therefore indicate that p25α expression specifically affects selective mTOR-independent autophagy.

**Secretion of α-synuclein depends on exocytosis of a compartment with late endosomal/amphisomal characteristics**

Exocytosis of late endosomal elements, including amphisomes, which arise from fusion with late endosomes, causes insertion of phosphatidylserine into the exoplasmatic leaflet of the plasma membrane and this can be detected by annexin-V staining (41). As binding of annexin-V to the cell surface is also used for detection of apoptosis we co-stained the cells with anti-caspase-3 antibodies to separate the two phenomena. As shown in Figure 9A and B, binding of Alexa-conjugated annexin-V to the cell surface was approximately three-fold increased up to 45% by the expression of p25α, while at this time point (two days of transgene expression) apoptosis detected by immunoreactivity for cleaved caspase-3 did not exceed 4% (Figure 9A, see also Figure 1E) indicating exocytosis of late endosomes and amphisomes. Exocytosis of late endosomes causes the release of small intraluminal vesicles, termed exosomes, to the extracellular medium (42), and it has recently been forwarded that α-synuclein can be taken up directly into late endosomes and secreted with exosomes (43). As shown in Figure 9C, PC12-α-synuclein/p25α cells expectedly secreted a higher amount of exosomes (identified by flotillin-1 expression) into the medium than α-synucleinA30P-expressing PC12 cells. However, almost all of secreted monomeric or High-Mw α-synuclein was soluble and did not associate with the exosome fraction, which we estimate contained less than 3% of the total secreted α-synuclein. It has recently been shown that the small GTPase Rab27A is essential for exocytosis of late endosomes by promoting the fusion of late endosomes with the plasma membrane (44). When we either silenced Rab27A by shRNA or expressed Rab27A mutants in PC12-α-synucleinA30P/p25α cells, we encountered an increased mortality, which precluded analysis of active secretion of α-synuclein. However, in PC12 cells expressing α-synucleinA30P alone, expression of dominant active (Q78L) or negative (T23N) mutants of Rab27A increased or decreased secretion of α-synuclein, respectively, despite a quite low efficiency of transduction with this pLenti vector (ca. 40% transduced cells for Rab27A-Q78L and 25% for Rab27A-T23N) (Figure 9D-F).

**HDAC6 deacetylase activity is decreased by p25α expression and HDAC6 shRNA knock-down promotes α-synuclein secretion.**

HDAC6 is the major cytosolic deacetylase in neurons and is required for QC autophagosome maturation at the level of lysosome fusion (40). It has recently been demonstrated that p25α inhibits the deacetylase activity of HDAC6 (27). In accordance herewith, the level of acetylated tubulin, a substrate of HDAC6, was increased by p25α expression in PC12 cells (Figure 10A), which could critically influence transport of mature organelles or autophagosome formation. As shown above, chemical inhibition of HDAC6 with trichostatin A increased secretion of α-synuclein (Figure 7E and F), so we started out by substantiating this correlation further by silencing HDAC6 with shRNA in PC12 cells only expressing α-synucleinA30P to mimic the p25α-induced inhibition of HDAC6. Figure 10B and C show that expression of HDAC6-specific shRNA
increased the level of acetylated tubulin more than two-fold in PC12-α-synucleinA30P cells without altering the total pool of α-tubulin. Although this was a modest increase in acetylated tubulin levels compared to α-synucleinA30P/p25α-expressing PC12, it translated into a disproportionally large increase in secreted α-synuclein (Figure 10B and D), probably reflecting that HDAC6 alters transport as well as fusion of autophagosomes with lysosomes. We further tested the effect of HDAC6 inhibition on autophagosomal maturation by flow cytometric analysis of the pH-sensitive eGFP moiety of the mCherry-eGFP-LC3B construct, which was expressed at equal levels in the PC12 cell populations (Figure 10E). As expected, expression of p25α increased eGFP fluorescence in the cells due to inhibited maturation and acidification, and this effect was replicated in PC12 cells either expressing β-synuclein or α-synucleinA30P by treatment with trichostatin (Figure 10F).

During live cell imaging of the mCherry-eGFP-LC3B construct, we noticed that the mobility of autophagosomes appeared severely reduced in p25α-expressing cells, and in order to quantitate this we applied Fluorescence Recovery After Photobleaching (FRAP) on PC12 cells constitutively expressing the mCherry-eGFP-LC3B construct. The percentage of recovered mCherry-fluorescence after bleaching in small regions of interest is shown in Figure 10G-I, where it is evident that p25α expression suppressed recovery with 80%. Although co-expression of α-synuclein depressed recovery further, α-synuclein alone had no effect. Inhibition of cytosolic deacetylase activity with either trichostatin or HDAC6 shRNA in α-synucleinA30P-expressing control cells replicated the effect of p25α expression (Figure 10I). The less efficient reduction in autophagosome mobility after HDAC6 knock-down when compared to trichostatin treatment, could be due to inefficient transduction as the knocked-down cells could not be identified before selecting cells for FRAP analysis. See supplementary materials for movie of autophagosome mobility in the β-synuclein- and α-synucleinA30P/p25α-expressing PC12 cells from Figure 10G and H.

**Rab GTPases modulate α-synuclein secretion and toxicity**

The Rab family of small GTPases are important regulators of vesicular transport and fusion events along the biosynthetic and endosomal pathway. Recent genetic screens in yeast and further experimentation in animal models showed that Rab1A and Rab8 alleviate α-synuclein-induced cytotoxicity (45-47). We therefore over-expressed Rab1A and Rab8 in PC12 cells to observe the effect on α-synuclein secretion (Figure 11A). Additionally, we expressed a Rab7-GFP fusion protein, as this GTPase is associated with maturation of late endosomal compartments by regulating fusion with lysosomes (48,49). Rab3A is known to regulate exocytosis of chromaffin-containing vesicles in PC12 cells and was used as a control. While expression of Rab1A increased secretion of α-synucleinA30P two-fold in PC12 cells only expressing α-synucleinA30P, it had no significant effect in PC12 cells co-expressing p25α (Figure 11B and C). However, most markedly, Rab8 increased α-synucleinA30P secretion several-fold in both cell lines without altering LC3B conversion significantly. To our surprise, expression of Rab7-GFP, which we expected to promote autophagosome maturation and lessen secretion, also modestly increased secretion. Interestingly, while promoting secretion, expression of Rab7 or Rab8 both reduced cytotoxicity of α-synuclein and p25α expression (Figure 11H and I), underscoring that within the time frame of analysis used throughout the study, release of α-synuclein is an active process and not correlated with cell death.

When we analyzed the effect of Rab expression on the distribution of α-synuclein by confocal microscopy, we observed that neither Rab3 (data not shown) nor Rab1A affected the distribution of α-synucleinA30P (compare Figure 11D with Figure 2C). In contrast, Rab8, which was mainly localized to subcortical areas of the cytosol, caused a change in distribution of α-synucleinA30P from cytosol to plasma membrane-near areas (Figure 11E). Rab8 could be seen to coat peripheral membrane elements also positive for LC3B as shown in Figure 11F, identifying them as autophagosomes/amphisomes. Interestingly, Rab8 consistently and specifically (compared to Rab3 as control) caused accumulation of particular material on the cell surface or substratum, which was immunoreactive with anti-KAI1 antibodies. A fraction of this material was also positive for α-synuclein, and likely represents the secreted and aggregated luminal contents of amphisomes and late endosomes (Figure 11G).

**Discussion**

The aim of the present study was to evaluate the role of autophagy in degradation and unconventional secretion of α-synuclein species from dopaminergic-like neurons in culture.
**TPPP/p25α impairs the autophagosomal degradation pathway**

Aggregated or modified forms of α-synuclein are substrates of all major degradative systems in vivo and in vitro including the ubiquitin proteasome system, chaperone-mediated autophagy, and macroautophagy (8,9,50,51). In this study we have used p25α expression as a tool to increase aggregation and autophagosomal uptake of α-synuclein. However, p25α is also in its own right an interesting protein in the context of α-synucleinopathy, because p25α is ectopically expressed in dopaminergic neurons in PD, and it is a component of Lewy bodies purified from post mortem brain of PD patients (24). Upon prolonged culture p25α-expressing cells eventually succumb to apoptotic cell death. However, within the time frame of experimentation used here α-synuclein or p25α expression only raised mortality associated with neuronal differentiation by a few percent (from 15% in NGF-differentiated control cells to 18% in α-synuclein/p25α-expressing cells).

It has recently been shown that p25α inhibits the deacetylase activity of HDAC6 (27), which is the predominant deacetylase in the cytosol of neurons and in accordance with this, p25α expression in PC12 cells increased tubulin acetylation about six-fold. Expression of HDAC6 shRNA in the absence of p25α afforded a two-fold increase in levels of acetylated tubulin, but this still correlated with a slowed maturation of autophagosomes and a three-fold increase in levels of secreted α-synuclein, suggesting that these effects of p25α expression are a consequence of HDAC6 inhibition. HDAC6 binds both poly-ubiquitinated cargo and the dynein-dynactin motor complex, and is required for the retrograde transport of ubiquitinated cargo and formation of aggresomes (18,52). However, under conditions of proteosomal inhibition HDAC6 is also an important mediator of the autophagosomal-lysosomal pathway of protein degradation in neurons, and when over-expressed HDAC6 can rescue neurodegeneration in a fly model (16). Expression of p25α could potentially influence autophagosomal processing due to HDAC6 inhibition on several levels (see Figure 12 for a schematic representation). First, p25α could interfere with the interactions between HDAC6 and dynactin, thereby uncoupling retrograde transport of poly-ubiquitinated cargo (18) and potentially autophagosomes (16). As estimated by FRAP, p25α expression severely reduced the mobility of autophagosomes, and direct inhibition of HDAC6 with trichostatin or shRNA knock-down similarly decreased mobility. We note that p62/SQSTM1-mediated retrograde transport of Htt-115Q-GFP to form conspicuous aggresomes was not disturbed by p25α expression, suggesting that hyper-acetylation of tubulin does not by itself affect retrograde transport. Secondly, HDAC6 has recently been pinpointed to an important role further down-stream in the autophagic pathway as it deacetylates cortactin, which in turn is specifically required for fusion of QC autophagosomes intermediates with lysosomes (40). This fusion event was severely affected by p25α expression, as evidenced by i) lack of co-localization between α-synuclein and LAMP1, ii) decreased amount of α-synuclein in heavy fractions following sucrose gradient fractionation, iii) decreased mCherry to eGFP fluorescence ratio of the LC3B fusion protein, and iv) decreased DQ-BSA degradation, which all testify to an inhibition of autophagosomal/amphisomal fusion with lysosomes. Therefore, by several mechanisms p25α expression caused the build-up of late autophagosomal elements. These likely include the multilamellar bodies observed in abundance by EM in α-synuclein/p25α-expressing cells as late endosomes and thereby amphisomes receive lysosomal hydrolases from the biosynthetic pathway to commence degradation of luminal contents.

**Alpha-synuclein is secreted by exophagy**

Even though p25α imposed a partial inhibition of the degradative capacity of the cell, the turn-over of α-synuclein was increased by p25α expression. We found that this phenomenon could be explained by a markedly increased secretion of α-synuclein into the external environment, and our data indicate that exocytosis of autophagosomes and amphisomes containing α-synuclein is responsible for this. In particular amphisomes may be involved in release of α-synuclein, as they constitute the compartment immediately preceding the p25α-mediated maturation block. Such a role also correlates well with the known physiological exocytosis of late endosomes (42), as well as the recently described unconventional secretion of the cytosolic Acb1 protein through exophagy of autophagosome intermediates in yeast (37,53). In partial agreement with a recent report (43) we find that a small fraction (<3% of total secreted α-synuclein) of monomer α-synuclein is associated with exosomes. The significance of this is at present unclear, but secretion of exosomes represent a physiological
mechanism for transferring cytosolic proteins in between cells that should be explored further in the context of disease transmission (54-56). Exosomes, though, are clearly not required to start the pathological cascade of inter-neuronal transmission of α-synuclein-derived prionoids, as α-synuclein aggregates made in vitro from recombinant α-synuclein is taken up by neurons in culture, and suffices to induce propagating inclusion disease in α-synuclein transgenic mice (2,5).

Formation of autophagosomes was a prerequisite for secretion of α-synuclein. First of all, by cryo-immunogold-labeling almost all vesicular α-synuclein was present in autophagosomes, amphisomes or autolysosomes. Secondly, expression of p25α correlated with increased levels of phosphatidylserine on the plasma membrane in the absence of apoptosis, indicative of exocytosis of late endosome/amphisome elements. Finally, secretion of α-synuclein could be controlled by chemical or genetic modulation of autophagy initiation (trehalose, 3-MA, Rab1), elongation (ATG5 shRNA), or autophagosome maturation (bafilomycin). Knock-down or introduction of mutants of Rab27A, which regulates fusion of late endosomes with the plasma membrane (59-61). When over-expressed in PC12 cells, Rab8 caused a pronounced redistribution of α-synucleinA30P elements to the plasma membrane, which was accompanied by a significantly increased secretion of α-synuclein with or without p25α expression. Rab1A also increased α-synuclein secretion in PC12 cells expressing only α-synucleinA30P, likely reflecting the reported role of Rab1A in autophagy initiation (36). Rab7 regulates dynamics and fusion events of late endosomal and autophagosomal elements (48,49,62). However, Rab7 over-expression did not circumvent the p25α-imposed fusion block and instead increased the secretion of α-synuclein, potentially as a consequence of Rab7-promoted plus end-directed microtubular transport of amphisomes through interactions with the LC3B-binding protein FYCO (63). Secretion of cytotoxic α-synuclein might be beneficial for the individual neuron as both Rab7 and Rab8, which promoted secretion, also reduced mortality significantly. However, secretion of α-synuclein may be a pathological mechanism of PD pathology transmission to nearby neurons (2,3,7), and in addition it can activate resident microglia, which mediate neuroinflammation and thereby exacerbate disease progression (64-66).

In conclusion, we propose that exophagy, with or without ectopic expression of p25α, may be an important physiological mechanism for unconventional secretion of α-synuclein from dopaminergic neurons, and we predict that lysosomal dysfunction observed in neurodegenerative diseases including PD (50) will aggravate such a release.

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Figure legends

Figure 1

**Conditional expression of p25α and α-synuclein in NGF-differentiated PC12 nerve cells.** PC12 cells were pre-differentiated with 100ng/ml NGF for 2 days and then transgene expression of β-synuclein (β-SNC), p25α, α-synuclein<sub>wt</sub> (α-SNC<sub>wt</sub>), α-synuclein<sub>A30P</sub> (α-SNC<sub>A30P</sub>), or α-SNC<sub>A30P</sub> and p25α was induced by doxycycline treatment for additionally two days. A) Bright field images showing p25α-mediated impairment of neurite outgrowth. Bars, 20µm. B) Indirect immunofluorescence of PC12 cells expressing α-SNC<sub>wt</sub>, α-SNC<sub>A30P</sub>, α-SNC<sub>A30P/p25α</sub>, or p25α alone with antibodies against α-SNC (BD Transduction Lab.) (green) or p25α (red). Arrowheads indicate neurite blebbing whereas arrows indicate α-SNC positive inclusions. Bars, 20µm. C) Representative western blots of transgene expression in doxycycline treated or non-treated PC12 cell lines analyzed with antibodies against p25α and α-SNC (BD Transduction Lab.) as indicated. D) Flow cytometry analysis of propidium iodide (PI) uptake as a measurement of cell death. The graph shows mean and SEM of PI-positive cells as a percentage of the whole population (n=3). E) Quantitation of caspase-3-positive cells detected by indirect immunofluorescence and counting. The bar graph shows mean and SEM values as percentage caspase-3-positive cells of the whole population (n=3). F) Differentiated PC12 cells lines were transduced with a lentivector expressing a pathogenic polyglutamine tract from exon 1 of huntingtin fused to GFP (Htt-115Q-GFP) and then induced with doxycycline. The images show microscopical fields containing ca. 250 cells, whereof a proportion is highly fluorescent due to inclusion body formation. Bar, 100µm. G) The bar graph shows the number of PC12 cells per microscopical field containing Htt-115Q-GFP inclusion bodies after four days, normalized to PC12 cells co-expressing β-synuclein (β-SNC). The data represent mean and SEM from three independent experiments.

Figure 2

**Expression of p25α promotes formation of α-synuclein-positive inclusions and alters the levels of proteins involved in autophagy.** A-D) Indirect immunofluorescence of α-synuclein (α-SNC) (BD Transduction Lab.) and p25α in NGF-differentiated PC12 cells expressing A) α-SNC<sub>A30P</sub>, B) p25α or C) and D) co-expressing α-SNC<sub>A30P</sub> and p25α. Note the pronounced co-localization between α-SNC and p25α (arrows) in both C) small vesicular profiles and D) large inclusions. Bars, 10µm. E) PC12 cell populations, as indicated, were differentiated as above and cell lysates processed for western blotting with anti-LC3B antibodies. F) The bar graph shows integrated optical density (IOD) ratio between western blot bands of LC3B-II and LC3B-I obtained from seven independent experiments. Error bars, SEM. G) Representative western blot of ATRA-differentiated SH-SY5Y cells expressing α-SNC<sub>A30P</sub> or α-SNC<sub>A30P/p25α</sub> using anti-LC3B antibodies. H) The bar graph shows IOD ratio between western blot bands of LC3B-II and LC3B-I obtained from three independent experiments. Error bars, SEM. I-N) PC12 cells expressing α-SNC<sub>A30P/p25α</sub> were fixed and processed for EM. Shown are micrographs of Epon-embedded sections. I) An area of the cell with many nascent autophagosomes (black open arrows) characterized by two outer membranes and a luminal content with an appearance very similar to cytosol. M, mitochondria. Bar, 500nm. J-L) Examples of autophagosomes containing different kinds of cargo including J) cytosol with endosomes, K) several dense-core vesicles contained in an amphisome located in a dendra, and L) mitochondria, as revealed by this cryo-EM section. Bars J-L, 250nm. M) An area with many autophagosome intermediates. Amphisomes, which have recently fused with late endosomes (white, filled arrows), are characterized by an outer membrane enclosing an autophagosomal body with limiting membrane in addition to material derived from fusion with endosomes (typically exosomes). In addition, many electron-dense, multi-lamellar bodies containing whorls of lipid were observed, often in addition to identifiable autophagosomal material (white, open arrows). A single nascent autophagosome is present (black open arrow) close to the Golgi apparatus (G), nucleus (N), and the extracellular space (Ex). Bar, 500nm. The inset N) shows a fusion event between a late endosome and an autophagosome. Bar, 100nm. O) The bar graph shows mean and SEM of the number of autophagosomes, amphisomes, and lamellar bodies in PC12 cells expressing either the doxycycline-binding rtTA transactivator alone (control) or together with α-SNC<sub>A30P</sub> or α-SNC<sub>A30P/p25α</sub>. From three independent EM experiments at least ten cell profiles of each cell population were counted.
Figure 3

**Autophagy markers LC3B and p62/SQSTM1 co-localize with α-synuclein.** Indirect immunofluorescence of PC12 cells expressing A) α-synuclein<sub>A30P</sub> alone (α-SNC<sub>A30P</sub>) or B) and C) together with p25α to visualize α-SNC (mAb LB509) distribution in relation to A) and LC3B or C) p62/SQSTM1. Co-localization between α-SNC and the respective markers are indicated with arrows. Bars A-C, 10 μm in the left panels and 5 μm in close-ups. D-F) PC12-α-SNC<sub>A30P</sub> or G-I) PC12-α-SNC<sub>A30P</sub>/p25α cells expressing mCherry-eGFP-LC3B were fixed and processed for cryo-immunogold labeling with mouse monoclonal anti-α-SNC (mAb LB509) antibodies and rabbit polyclonal anti-GFP antibodies, followed by secondary 14 nm or 7 nm gold-conjugated anti-mouse or anti-rabbit antibodies, respectively. E) The micrograph shows several autophagosomes containing only labeling for eGFP-LC3B (black, open arrows), and a single autophagosome containing both α-SNC and eGFP-LC3B (black, filled arrow), which is also true of an electron-dense autolysosome (open white arrow). The closed white arrow points to a cytosolic inclusion staining for both α-SNC and eGFP-LC3B. D) An autolysosome with both labels is also shown at higher magnification. F) A nascent autophagosome with both labels (black, open arrow) and a vacuole/autophagosome containing only aggregated label for α-SNC (black, filled arrow). In PC12 cells co-expressing α-SNC<sub>A30P</sub> and p25α (G-I) the number of autophagosomes was increased and recently formed autophagosomes (open black arrows) and amphisomes (white, open arrows) including lamellar bodies (white, open arrow in G) and electron-dense late autophagosomal elements (white, open arrows in H) contained label for both α-SNC and eGFP-LC3B. White, closed arrow in G) points to a late endosome devoid of immunoreactivity for either α-SNC or eGFP-LC3B, while the small, white arrow in H) points to extracellular α-SNC immunoreactivity associated with microvilli (MV) on the cell surface (white arrowheads). I) An electron-dense late autophagosomal element from H) shown at higher magnification. M, mitochondria. Bars in D-H, 500 nm.

Figure 4

**TPPP/p25α induced autophagy is selective, and also affects endogenous rat α-synuclein.** A) and B) PC12 α-synuclein<sub>A30P/p25α</sub> (α-SNC<sub>A30P/p25α</sub>) cells were transduced with lentivector pHR-cPPT.CMV.W-Htt-115Q-GFP and after two days α-SNC and p25α expression was induced with doxycycline for a further two days. Cells were then fixed and processed for indirect immunofluorescence to visualize A) Htt-115Q-GFP (blue), LC3B (red), and α-SNC (green; BD Transduction Lab.) or B) Htt-115Q-GFP (blue), p62/SQSTM1 (red), and α-SNC (green; BD Transduction Lab.). The rectangular box in A) is shown at higher magnification to the right. Note that α-SNC preferentially co-localizes with LC3B, while Htt-115Q-GFP co-localizes with p62/SQSTM1. Bars, 10 μm, in right panels 2 μm. C) NGF-differentiated PC12 cells expressing either β-synuclein (β-SNC) as control or p25α were analyzed by indirect immunofluorescence to localize poly-ubiquitin, endogenous α-SNC (Abcam mAb EP1646Y), and p25α as indicated. D) PC12 cells expressing p25α and stained as above at higher magnification. Arrows indicate co-localization of α-SNC with ubiquitin and p25α. Bars, 10 μm. E) Control (β-SNC) or p25α-expressing PC12 cells were processed for indirect immunofluorescence with rat anti-p25α mAb, mouse anti-α-SNC mAb LB509, and rabbit polyclonal antibodies against KAI1 and MPR. Arrows indicate vesicular structures with co-localization of α-SNC and KAI1/MPR, which to a certain extent also co-localize with p25α. Bars A-C, 10 μm.

Figure 5

**Expression of p25α prevents α-synuclein in reaching lysosomes.** A-C) Indirect immunofluorescence of leupeptin/pepstatin A-treated PC12 cells expressing A) α-synuclein<sub>A30P</sub> (α-SNC<sub>A30P</sub>) alone or B) and C) together with p25α, showing the distribution of α-SNC<sub>A30P</sub> (Abcam LB509) and the lysosomal marker LAMP1. Arrows in A) indicates co-localization between the antigens, whereas arrows in B) (red and green) and C) (white) indicate that, although closely apposed, α-SNC immunoreactivity is distinct from that of LAMP1. Bars A-C, 10 μm in the left panels and 5 μm in close-ups. D) Homogenates from PC12 cells expressing α-SNC<sub>A30P</sub> or α-SNC<sub>A30P/p25α</sub> were fractionated on a 15%-45% sucrose gradient and aliquots of collected fractions analyzed by western blotting of α-SNC (BD Transduction Lab.) and LC3B. The blots are representative of two independent experiments. Note that p25α decreases the amount of α-SNC present in heavy fractions 1-3, which also contains exclusively autophagosome-associated LC3B-II. E) Flow cytometric analysis of PC12 cells incubated with DQ-BSA for 6.5 hours with or without either bafilomycin A1 (100 nM) or leupeptin (50 μg/ml)/pepstatin A (67 μg/ml) treatment. DQ-BSA fluorescence intensity was decreased. This suggests that p25α prevents α-SNC from reaching lysosomes.
normalized to β-synuclein (β-SNC)-expressing PC12 cells and bar graph shows mean and SEM of relative fluorescence units (RFLU) (n=3). * denotes a statistic significant decrease in RFLU for untreated cell lines when compared to untreated β-SNC-expressing cells; # denotes statistic significant decrease within the same cell line after chemical treatment; NS = non-significant.

**Figure 6**
*Expression of p25α impairs the autophagosomal flux.* A-E) Confocal microscopy images of live PC12 cells co-expressing mCherry-eGFP-LC3B with either A) β-synuclein (β-SNC), B) α-SNCA30P, C) and D) α-SNCA30P/p25α, or E) β-SNC-expressing PC12 cells treated with 20nM bafilomycin A1 (Baf A1) for 6 hours. Bars, 10µm. F) The bar graph shows absolute mean and SEM of mCherry (red) and eGFP (green) fluorescent structures per cell profile of three independent experiments. In each experiment 25-30 cells from each cell line were analyzed. G) The same data as F) presented as the ratio between the absolute numbers of mCherry and eGFP fluorescent dots. H) mCherry- and eGFP-positive structures larger than 1µm in diameter were quantified according to size (1-2µm: grey; >2µm: black). The bar graph shows the number of inclusions per cell and represents mean and SEM of three independent experiments.

**Figure 7**
*TPPP/p25α induces α-synuclein secretion, which can be modified by regulators of the autophagosomal degradation pathway.* A) PC12 cells expressing α-synucleinA30P alone (α-SNCA30P) or with p25α (α-SNCA30P/p25α) were predifferentiated with NGF for two days and transgene expression induced for additionally two days. Doxycycline was then withdrawn and the cells chased for 6 days with or without leupeptin (50µg/ml) and pepstatin A (67µg/ml). Representative western blot for α-SNC (BD Transduction Lab.) shows decrease in α-SNC from cell lysates acquired from day 0, 2, 4 and 6 after doxycycline withdrawal. B) Representative western blot with anti-α-SNC (BD Transduction Lab.), p62/SQSTM1 (SQSTM1), and β-Actin of cell lysates and TCA-precipitated conditioned media obtained from PC12 cell lines expressing β-SNC, α-SNC wild type (α-SNCwt), or α-SNCA30P with or without p25α in the presence or absence of 3-MA (10mM) (n=3) C) Representative western blot of TCA-precipitated medium obtained from PC12 cells expressing α-SNCA30P alone or together with p25α using mouse anti-α-SNC (BD Transduction Lab.). Notice the presence of both monomeric (17kdA), and high molecular weight (High Mw) forms of secreted α-SNC. D) PC12 cells expressing β-SNC (as control) or p25α were treated or not with leupeptin/pepstatin A (50µg/ml and 67µg/ml) for the last 24 hours of culture before analysis of endogenous α-SNC in cell lysates and immunoprecipitates from conditioned medium (using BD Transduction Lab. and LB509 mAbs) by western blotting (using anti-α-SNC rabbit mAb EP1646Y) as indicated. Data are representative of three independent experiments. E) PC12 cells expressing α-SNCA30P or α-SNCA30P/p25α were treated with bafilomycin A1 (15nM), leupeptin (50µg/ml) and pepstatin A (67µg/ml), trehalose A (20µM), rapamycin (0.5µM), trehalose (100mM), or left untreated (control) for 48 hours concurrently with doxycycline induction before western blot analysis of TCA-precipitated conditioned media and cell lysates using antibodies as indicated. F) The bar graph shows fold increase in integrated optical density (IOD) of TCA western blot bands of secreted α-SNC (BD Transduction Lab.) obtained from B) and E) relative to untreated PC12-α-SNCA30P cells. Mean and SEM of three independent experiments are shown. G) TCA-precipitated conditioned medium or cell lysates from PC12-α-SNCA30P/p25α cells with or without stable co-expression of either control (pGIPZ) or ATG5 shRNA were analyzed by western blotting using anti-α-SNC (BD Transduction Lab.), ATG5, or LC3B antibodies as indicated. H) The bar graph shows IOD of ATG5 western bands normalized to PC12 cells expressing control shRNA (pGIPZ) and represents mean and SEM of three independent experiments. I) The bar graph shows IOD of α-SNC western bands (TCA samples) normalized to PC12 cells expressing control shRNA (pGIPZ) and represents mean and SEM of three independent experiments. J) and K) PC12 cell populations as indicated were transduced with either Htt-18Q-GFP or Htt-115Q-GFP for two days before transgene induction for a further two days. Conditioned medium was then TCA-precipitated and analyzed by western blotting with polyclonal rabbit anti-GFP antibodies. J) An approximately 50kDa band (upper arrow) immuno-reactive with anti-GFP antibodies is seen exclusively in the conditioned medium from Htt-115Q-GFP expressing cells, but not from control Htt-18Q-GFP cells. K) A similar secretion of Htt-115Q-GFP was observed in PC12 cells expressing β-SNC, p25α, or α-SNCA30P with or without p25α expression. The increased reactivity to monomer GFP to the right
on the blot is caused by overflow from an adjacent (not shown) cell lysate lane. Upper and lower arrows indicate HTT-115Q-GFP fusion protein and monomeric GFP, respectively.

**Figure 8**
*mTOR*-dependent and -independent autophagy enhancers rapamycin and trehalose, respectively, differentially affect the distribution and fluorescence properties of mCherry-eGFP-LC3B

A-D) PC12-α-synucleinA30P/p25α (α-SNCA30P/p25α) cells expressing mCherry-eGFP-LC3B were treated with 3-MA (10mM), trehalose (100mM), or rapamycin (0.5µM) as indicated, for the last 48 hours of culture, before live imaging with a Zeiss LSM510 confocal microscope. Note that 3-MA causes the diffusive cytoplasmic distribution of the mCherry-eGFP-LC3B construct, while trehalose induces the massive accumulation of large autophagosomal vacuoles that emit both mCherry- and GFP-fluorescence. In contrast, autophagy induced by rapamycin increased the proportion of autophagosomal vacuoles with predominant emission of only mCherry-fluorescence indicating correct acidification. Bars, 10µm, enlarged images 2µm. The images shown are representative of three independent experiments.

E) PC12-α-SNC/p25α cells were treated with 0.5 or 1µM rapamycin for different time intervals as indicated, and cell lysates were then analyzed by western blotting for conversion of LC3B and levels of β-actin (loading control). Samples from 48 hours time point were run on separate gel due to lack of wells. The western blot is representative of two independent experiments.

F) Integrated optical density (IOD) of LC3B-II bands from the experiment shown in E) were normalized to levels of β-actin and plotted over time.

**Figure 9**
Secretion of α-synuclein is mediated by compartments with late endosomal/lysosomal characteristics.

A) p25α-expressing PC12 cells were incubated with Alexa 568-conjugated annexin-V on ice before fixation and indirect immunofluorescence with antibodies against cleaved caspase-3. Note widespread annexin-V surface staining in the absence of caspase-3 immunoreactivity. Bar, 10µm. B) PC12 cell lines labeled with Alexa 488-conjugated annexin-V on ice were analyzed by flow cytometry. The bar graph shows percentage annexin-V positive cells of the whole cell population and represents mean and SEM of three independent experiments.

C) The conditioned medium (CM) from PC12 cells expressing α-synucleinA30P (α-SNCA30P) with or without p25α was centrifuged at 100,000g to obtain a pellet (containing exosomes) and a supernatant (sup). Aliquots were then western blotted with anti-α-SNC (BD Transduction Lab.) or anti-flotillin-1 (exosome marker) antibodies. The exosome fraction was applied on the gel at six-fold the relative load of supernatant and medium. The blot is representative of two independent experiments.

D-F) PC12-α-SNCA30P cells were differentiated for two days and transduced at two different MOI’s with lentivectors constitutively expressing FLAG-tagged Rab27A-Q78L (dominant positive) or -T23N (dominant negative). Control wells received a GFP-expressing vector (pLenti). D) After two days of transgene induction cell lysates were prepared to show expression of Rab27A using anti-Rab27A mAb 4B12 (arrow points to transgene) or anti-FLAG antibodies. E) The conditioned medium was TCA-precipitated and western blotted with anti-α-SNC antibodies (BD Transduction Lab.). F) The bar graph shows mean and SEM of the integrated optical density (IOD) of α-SNC western blot bands normalized to control-transduced cells (n=3). * and # denotes a statistic significant increase or decrease, respectively, in α-SNC secretion when compared to control-transduced PC12 cells.

**Figure 10**
HDAC6 inhibition mimics p25α-induced secretion of α-synuclein and reduces the mobility of autophagosomes.

A) Representative western blots showing cellular protein levels of acetylated tubulin (AcTub) and α-tubulin (α-Tub) obtained from PC12 cell populations as indicated. B) Cell lysates and conditioned medium from PC12-α-synucleinA30P (α-SNCA30P) or PC12-α-SNCA30P/p25α cells, transduced three to four days prior to analysis with lentivectors expressing control (pLKO.1) or HDAC6-specific shRNA, were subjected to western blotting using anti-AcTub and α-Tub antibodies (cell lysates) or anti-α-SNC antibodies (TCA-precipitated medium; BD Transduction Lab.). Integrated optical density (IOD) of western bands for C) AcTub in cell lysates, and D) secreted α-SNC (BD Transduction Lab.) in the TCA samples is presented as fold increase relative to untreated control cells. Mean and SEM of three independent experiments are shown.
* denotes a statistic significant increase in IOD within the cell line when co-expressing shRNA, whereas # denotes a statistic significant increase in IOD between the control cell lines not expressing shRNA. E) Cell lysates of PC12 cell populations as indicated, expressing mCherry-eGFP-LC3B were western blotted with anti-GFP antibodies to demonstrate an equal expression of the LC3B fusion protein. The 72kDa protein bands correspond to the size of the mCherry-eGFP-LC3B tandem construct. F) Flow cytometric analysis of untreated or trichostatin A-treated (10µM) PC12 cell populations expressing mCherry-eGFP-LC3B. The bar graph shows mean and SEM of eGFP relative light fluorescent units (RLFU) normalized to control cells expressing β-SNC and represents data from three independent experiments. * denotes a statistic significant increase in mean eGFP fluorescence intensity between the cell lines when compared to β-SNC-expressing PC12 cells, and # denotes a statistic significant increase after trichostatin treatment. G) and H) Live cell confocal microscopy images of PC12 cells expressing β-SNC or α-SNCA30P/p25a showing Fluorescence Recovery After Photobleaching (FRAP) time series before and after photobleaching (T=0 sec and T=50 sec), and after a defined recovery period (T=260 sec). The cells were bleached in the encircled region of interest (ROI) shown in close-ups just before the T=50 sec time point. Bars, 10µm. I) The bar graph shows percentage of FRAP in PC12 cells as indicated including PC12-α-SNCA30P cells treated with 20µM trichostatin A or transduced with HDAC6 shRNA. Mean and SEM were obtained from three to five independent experiments. * denotes a statistic significant decrease in FRAP between the cell lines when compared to β-SNC-expressing PC12 cells, whereas # denotes a statistic significant decrease after trichostatin treatment or HDAC6 shRNA co-expression when compared to PC12 cells expressing α-SNCA30P.

**Figure 11**
Expression of Rab8 enhances α-synuclein secretion while lowering cell death. A) Representative western blots showing expression levels of HA-tagged Rab1A, Rab3A or Rab8, and GFP-tagged Rab7 in PC12 cell lines expressing α-synucleinA30P (α-SNCA30P) or α-SNCA30P and p25a using anti-HA or -GFP antibodies. B) Representative western blots of TCA precipitated media and cell lysates obtained from PC12 cells expressing α-SNCA30P or α-SNCA30P/p25a with or without co-expression of Rab proteins as indicated using antibodies against α-SNC (BD Transduction Lab.) and LC3B. C) Quantified integrated optical density (IOD) of α-SNC western blot bands obtained in B) are presented as fold increase relative to α-SNCA30P-expressing PC12 cells. Mean and SEM of five independent experiments are shown. * denotes a statistic significant increase in IOD when compared to control cells within the respective cell line. D-F) Indirect immunofluorescence of PC12-α-SNCA30P/p25a cells co-expressing either D) HA-Rab1A, or E) and F) HA-Rab8. E) Rab8 was predominantly distributed towards the cell surface and caused a more peripheral distribution of α-SNCA30P (BD Transduction Lab.), and F) co-localized partly with LC3B-positive autophagosomes/amphisomes. Bars, 10µm. G) PC12-α-SNCA30P/p25a cells co-expressing HA-tagged Rab8 were fixed after two days of transgene induction, and then processed for immunofluorescence to visualize KAI1 (green), α-SNC (red), or F-actin detected with Alexa633-conjugated phalloidin (blue). Note that Rab8 expression results in accumulation of material on the cell surface, which is immunoreactive with anti-α-SNC and KAI1 antibodies (arrows). The figure is representative of three independent experiments. Bars, 10µm. H) The bar graph shows the percentage of caspase-3-positive PC12 cells of the whole population in indicated cell populations as determined by indirect immunofluorescence and counting. Data represents mean and SEM of four independent experiments. I) Flow cytometric analysis of PC12 cell populations as indicated stained with propidium iodide (PI) to measure cell death. The bar graph shows the percentage of PI-positive cells of the total population and represents mean and SEM of four independent experiments. In H) and I) # denotes a statistic significant decrease when compared to control cells within the same cell line.

**Figure 12**
Proposed mechanism for p25a effects and exophagy of α-synuclein. The diagram illustrates how p25a may alter trafficking pathways (marked by numbers) and autophagosome dynamics. Expression of p25a causes aggregation and autophagosomal uptake of α-synuclein (α-SNC), involving QC autophagosome adaptors p62/SQSTM1 and possibly HDAC6 (1). The autophagosome (AU) then fuses (2) with a late endosome (LE) to generate an amphisome (AM), which can travel retrogradely (3) to fuse with a lysosome (Lyso) thereby forming an autolysosome, which degrades α-SNC. Retrograde transport involves HDAC6-mediated interactions between LC3B and the minus end-directed dynein-dynactin motor complex, which may be
inhibited by p25α. Fusion of amphisomes with lysosomes is promoted by HDAC6 deacetylase activity, which is inhibited by p25α. Under conditions where pathway (3) is blocked (compromised HDAC6 activity, lysosomal dysfunction, and/or altered ratio of minus- to plus end-directed trafficking of amphisomes) anterograde transport of amphisomes towards the cell surface takes place (4), where a fraction of competent amphisomes can undergo exocytosis regulated by Rab27A (5), to release α-SNC in monomer and aggregated/modified forms to the extracellular environment. The extent to which autophagosomes may directly contribute to exocytosis is unclear (6).
Figure 1
Figure 2

A-C: Immunofluorescence microscopy images showing the expression of α-SNCA30P and p25α in SHSY5Y and PC12 cells. Arrows indicate the co-localization of α-SNCA30P and p25α.

E-G: Western blot analysis of LC3B-I and LC3B-II levels in PC12 and SHSY5Y cells transfected with α-SNCA30P and p25α constructs.

H: Quantitative analysis of LC3B-II/LC3B-I (IOD) levels in SHSY5Y cells treated with different constructs.

O: Bar graph showing the number of cells per profile in different conditions.

I-M: Electron microscopy images of SHSY5Y cells expressing α-SNCA30P and p25α, highlighting the formation of autophagosomes and autolysosomes.
Figure 4

A. α-SNC/p25α

B. α-SNC/p25α

C. β-SNC

D. p25α

E. β-SNC
Figure 5

A. α-SNC30P

B. α-SNC30P/p25α

C. α-SNC30P/LAMP1

D. Western blot analysis of α-SNC30P and α-SNC30P/p25α in different fractions.

E. Graph showing quantification of α-SNC, α-SNC30P, p25α, and α-SNC30P/p25α in untreated, Leu/Pepp, and Baflomycin conditions.
Figure 6
Figure 7

A

B

C

D

E

F

G

H

I

J

K
Figure 8

A. Untreated

B. 3-MA

C. Trehalose

D. Rapamycin

E. Table showing LC3B-I, LC3B-II, and β-Actin bands with corresponding kDa.

F. Graph showing LC3B IOD (normalized to β-Actin) over time with different concentrations of Rapamycin.
Figure 9

A

p25α

Caspase-3

Annexin-V

Merge

B

% Annexin-V+ cells

p25α

α

A

B C

72

17

150

50

High-Mw

Monomer

α-SNC

TCA

Flotillin

C

CM SUP CM SUP Exosomes kDa

High-Mw

High-Mw

α-SNC

TCA

Monomer

D

Rab27A

FLAG

kDa

28

28

E

α-SNC

TCA

100

100

100

100

pLenti Rab27A(Q78L)

pLenti Rab27A(T23N)

kDa

17

17

F

Secreted α-SNC

140

120

100

80

60

40

20

0

IOD norm. to control

Control Q78L T23N

pLenti Rab27A(Q78L)

pLenti Rab27A(T23N)

* #

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Figure 10

(A) 

(B) 

(C) 

(D) 

(E) 

(F) 

(G) 

(H) 

(I) 

**Figure 10**

(A) 

(B) 

(C) 

(D) 

(E) 

(F) 

(G) 

(H) 

(I)
Figure 11

A

\[
\begin{array}{c}
\alpha\text{-SNCA30P} \\
p_{25\alpha} \\
HA \\
GFP \\
HA-Rab1A \\
HA-Rab3A \\
Rab7-GFP \\
HA-Rab8 \\
\end{array}
\]

B

\[
\begin{array}{c}
\text{Cell lysate: TCA} \\
\alpha\text{-SNC} \\
\alpha\text{-SNC}_{\text{A30P}} \\
p_{25\alpha} \\
\text{LC3B-I} \\
\text{LC3B-II} \\
\end{array}
\]

C

D

\[
\alpha\text{-SNCA30P}/p_{25\alpha} \\
HA-Rab1A \\
HGFP \\
\alpha\text{-SNC} \\
\text{Merge} \\
\]

E

\[
\alpha\text{-SNCA30P}/p_{25\alpha} \\
HA-Rab8 \\
HGFP \\
\alpha\text{-SNC} \\
\text{Merge} \\
\]

F

\[
\alpha\text{-SNCA30P}/p_{25\alpha} \\
HA-Rab8 \\
HGFP \\
\alpha\text{-SNC} \\
\text{Merge} \\
\]

G

\[
\alpha\text{-SNC} \\
\alpha\text{-SNC}_{\text{A30P}} \\
p_{25\alpha} \\
\text{KAI1} \\
F\text{-actin} \\
\text{Merge} \\
\]

H

\[
\text{% PI+ cells} \\
\alpha\text{-SNC} \\
\alpha\text{-SNCA30P}_{\text{A30P}} \\
p_{25\alpha} \\
\text{Control} \\
\text{Rab1A} \\
\text{Rab3A} \\
\text{Rab7} \\
\text{Rab8} \\
\]

I

\[
\text{% Cleaved caspase-3 cells} \\
\alpha\text{-SNC} \\
\alpha\text{-SNCA30P}_{\text{A30P}} \\
p_{25\alpha} \\
\text{Control} \\
\text{Rab1A} \\
\text{Rab3A} \\
\text{Rab7} \\
\text{Rab8} \\
\]
Figure 12

Trehalose
Rab1A

3-MA

AM

Rab8

Rab27A

1

2

3

4

5

6

AU

LE

Autolysosome

β-synuclein monomers
Aggregated β-synuclein
Exosome
p62/SQSTM1 or HDAC6
LC3B-II
Microtubuli (polarized)
Dynein-dynactin

β-synuclein monomers
Aggregated β-synuclein
Exosome
p62/SQSTM1 or HDAC6
LC3B-II
Microtubuli (polarized)
Dynein-dynactin
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*Tubulin Polymerization Promoting Protein (TPPP/p25α) promotes unconventional secretion of α-synuclein through exophagy by impairing autophagosome-lysosome fusion*

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