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COMMENTARY

N-Terminal Fusion Potentiates α-Synuclein Secretion

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Parkinson's disease is a progressive neurodegenerative disease characterised histologically by aggregates of misfolded α-synuclein. Large accumulations of α-synuclein aggregates are termed Lewy bodies. Based on the distribution of Lewy bodies in the brain, Braak suggested about 15 years ago that α-synuclein aggregates spread through the brain along neuronal projections (Braak et al. 2003). In this theory, Lewy bodies are first found in the brainstem and olfactory bulb and subsequently spread to midbrain and other structures, reaching cortex in late stages of the disease. Support for this theory came from the demonstration that Lewy pathology is transferred from diseased brain into grafts of fetal mesencephalic neurons (Kordower et al. 2008; Li et al. 2008), and from the clinical description of "prodromal" or "premotor" stages of PD with olfactory deficits, gastrointestinal problems and specific sleep disturbances that plausibly correspond to Lewy pathology in the olfactory bulb and brain stem areas (Postuma et al. 2015; Klingelhoefer and Reichmann 2017).

Preclinical models provide additional evidence for spread of α-synuclein aggregates. For instance, α-synuclein aggregates generated in vitro (pre-formed fibrils, PFFs) are able to induce α-synuclein aggregates when added to the culture medium of neurons or injected into mouse brain (Masuda-Suzukake et al. 2014; Volpicelli-Daley et al. 2014; Peelaerts et al. 2015). Such propagation of pathology by seeding, axonal transport, and trans-synaptic spread is not limited to PD but similarly found for Alzheimer's disease and amyotrophic lateral sclerosis (Stopschinski and Diamond 2017).

Still, many aspects of α-synuclein transfer have remained unclear. For instance, spread of α-synuclein aggregates through rodent brain does not require endogenous α-synuclein (Helwig et al. 2016), indicating that transport, transfer, and seeding of new aggregates are independent processes. Furthermore, the molecular mechanism of uptake, transport, and secretion has not been fully elucidated.

These questions require measuring—and in many cases visualising—α-synuclein. Fusion with fluorescent proteins is one of the most commonly used strategies to detect α-synuclein (Falkenburger et al. 2016). Yet, fusion of fluorescent proteins to α-synuclein can alter its propensity to form aggregates (Opazo et al. 2008) and further biophysical and cell biological properties. An elegant approach to monitor α-synuclein oligomers as opposed to monomers is the fusion of α-synuclein to the N-terminal, respectively, C-terminal fragments of the fluorescent protein Venus or to fragments of luciferase (Danzer et al. 2012; Dimant et al. 2013; Lázaro et al. 2014). In this "bimolecular fluorescence complementation" (BiFC) approach, α-synuclein oligomers are detected by the formation of functional fluorescent proteins upon formation of α-synuclein oligomers. In addition, BiFC was used to detect transfer of α-synuclein molecules from one cell to another, by transfecting α-synuclein tagged by different fragments in co-cultured or synaptically connected cells (Bae et al. 2014).

α-Synuclein can be secreted in different forms (Fig. 1). It can be secreted as monomers or in the form of aggregates, and it can be secreted as "free" α-synuclein or inside extracellular vesicles (Emmanouilidou et al. 2010; Danzer et al. 2012; Kunadt et al. 2015). In recent work published in this issue of CEMN, Gustafsson et al. scrutinised the secretion of α-synuclein using transiently transfected SH-SY5Y cells, a catecholamine producing cell line frequently used as a PD model. Extracellular vesicles (EVs) were isolated by ultracentrifugation, and α-synuclein detected by ELISA in the cytosol, in the EV fraction, and in cell culture supernatant without vesicles (Gustafsson et al. 2018). The authors compared secretion of different α-synuclein constructs and found that the extracellular medium with

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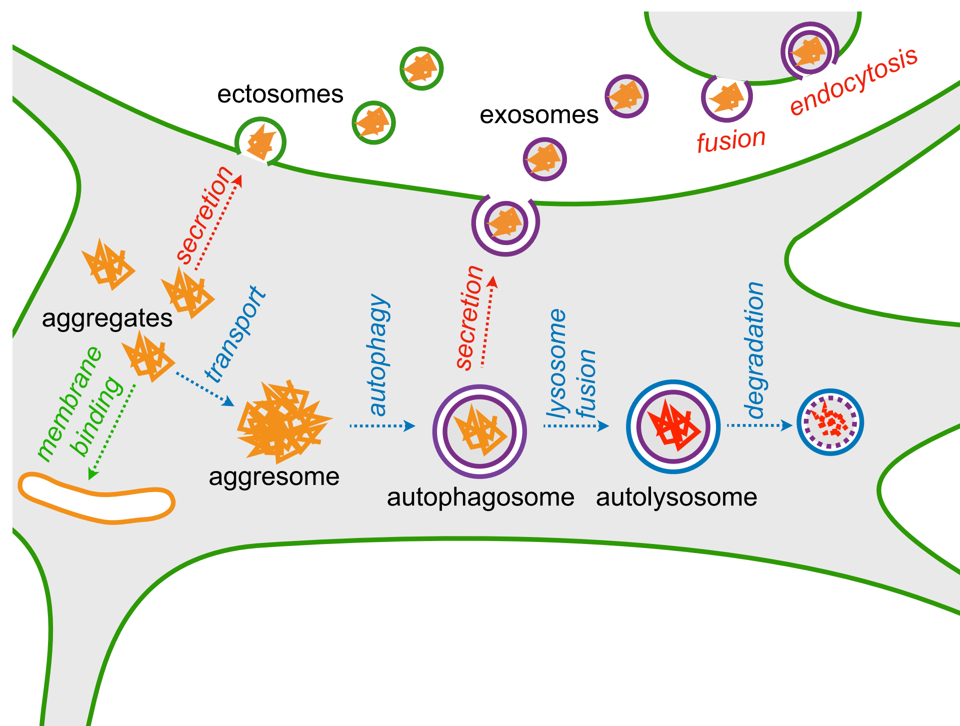

Fig. 1 Pathways of synuclein secretion. This is a cartoon view of current hypotheses about synuclein degradation and secretion. Degradation pathway (blue): Aggregates are transported towards the perinuclear region where they form aggresomes. Aggresomes are hubs for autophagy, i.e. the engulfment of aggregates by cellular membranes, leading to double-membrane vesicles. These autophagosomes can fuse with lysosomes to degrade their cargo. Formation of extracellular vesicles (red): Ectosomes are vesicles shedded from the plasma membrane. They contain cytosol and thus may include aggregates. Exosomes result from fusion of double-membrane compartments with the plasma membrane, such as multivesicular bodies and autophagosomes. Extracellular vesicles are taken up by neighbouring cells by either fusion with the plasma membrane or endocytosis. Membrane binding (green): Synuclein binding to cellular membranes is likely to impair processing through the transport—autophagy—secretion/degradation pathway

the highest proportion of EV-associated α-synuclein showed the greatest amount of α-synuclein uptake. This is consistent with the hypothesis that vesicular α-synuclein is taken up more readily by receiving cells than "free" α-synuclein, making vesicular α-synuclein the relevant species for cell-to-cell transfer. Accordingly, neurons take up extracellular vesicles from PD or DLB patients, inducing α-synuclein aggregation (Stuendl et al. 2015; Ngolab et al. 2017).

α-Synuclein secretion requires calcium (Emmanouilidou et al. 2010), indicating that it occurs through exocytosis and not through direct permeation of the plasma membrane. Interestingly, disruption of membranes in the extracellular vesicle (EV) fraction by RIPA buffer did not change the amount of α-synuclein much (Gustafsson et al. 2018), suggesting that α-synuclein is not localised mainly inside EVs but to a large extent on the outside. This finding is consistent with previous reports by others (Danzer et al. 2012), but it is difficult to reconcile with a specific secretion mechanism. Disruption of EVs during the preparation procedure could account for this finding but electron microscopy confirmed intact vesicles in the EV fraction (Gustafsson et al. 2018).

One plausible pathway to mediate EV-associated α-synuclein secretion is through exosomes, extracellular vesicles resulting from fusion of the outer membrane of multivesicular bodies or autophagosomes with the plasma membrane (Raposo and Stoorvogel 2013; Cocucci and Meldolesi 2015). Indeed, amyloid beta secretion was reduced in the brains of mice deficient for Atg7, a protein required for autophagy (Nilsson et al. 2013) indicating that autophagy is involved in secretion. This process was termed "secretory autophagy" and its molecular constituents recently identified (Kimura et al. 2017). α-Synuclein secretion is enhanced by impaired autophago-lysosome fusion (Ejlerskov et al. 2013; Lee et al. 2013; Poehler et al. 2014), consistent with a "secretory autophagy" mechanism in which exosome secretion is an alternative fate for autophagosomes if fusion to lysosomes fails. In this theory, however, α-synuclein is expected to reside inside EVs, consistent with the observation that EV-associated α-synuclein is not susceptible to trypsin (Kunadt et al. 2015). The finding that the amount of α-synuclein in EV fraction as measured by ELISA is unaltered by the addition of RIPA buffer (Gustafsson et al. 2018; Danzer et al. 2012) therefore remains unexplained.

Secretion of α-synuclein in the EV fraction was greatly increased when α-synuclein was fused on its N-terminus to the first 157 amino acids of Venus (“V1S”). This construct showed not only increased overall secretion but a selective increase in the ratio of vesicular as compared to non-vesicular extracellular α-synuclein, indicating that the N-terminal modification changes intracellular trafficking of α-synuclein. α-Synuclein secretion in EVs was previously shown to depend on SUMOylation (Kunadt et al. 2015), but the main SUMOylation sites are K96 and N102 (Krumova et al. 2011). N-terminal fusion of the Venus fragment thus unlikely alters α-synuclein secretion by this mechanism.

Recently α-synuclein was demonstrated to be acetylated on K6 and K10 (de Oliveira et al. 2017). Acetylation reduces α-synuclein aggregation and prevents its binding to membranes, consistent with previous findings that it is the α-synuclein N-terminus that primarily binds to artificial membranes (Fusco et al. 2014). Glycation was also found to affect primarily the N-terminus of α-synuclein and to reduce membrane binding (Vicente Miranda et al. 2017), but it is unclear to what extent glycation occurs in cell lines with transient α-synuclein expression. Collectively, these recent findings suggest that N-terminal modifications, including fusion of the Venus fragment, decrease α-synuclein binding to intracellular membranes and increase secretion by extracellular vesicles.

What does this finding tell us about the mechanism of α-synuclein secretion? Increased secretion with decreased membrane binding argues against a passive mechanism by which α-synuclein is trapped in membranes that eventually become secreted. Rather, this finding is consistent with a mechanism where protein aggregates are detected by specific adaptor molecules and directed towards a degradation/secretion pathway. The only secretion pathway for which we know specific adaptor molecules is through autophagy (Fig. 1). This pathway starts with the accumulation of misfolded proteins at the perinuclear microtubule organising centre (MTOC), leading to aggresomes if degradation cannot keep up with aggregate transport (Kopito 2000). Protein misfolding was recently identified as a sufficient trigger for aggresome formation (Bersuker et al. 2016), and aggresome formation was demonstrated to be the first step of Rab7-induced aggregate clearance (Saridaki et al. 2018). V1S and acetylation-deficient α-synuclein mutants are thus interesting models to provoke EV-associated α-synuclein secretion and study this process in more detail.

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