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Perspective

Alpha-synuclein preformed fibrils: a tool to understand Parkinson's disease and develop disease modifying therapy

Piotr Chmielarz, Andrii Domanskyi*

Parkinson's disease (PD) is the second most common neurodegenerative disorder characterized by multiple motor and non-motor symptoms, which include, among others, constipation, sleep disturbance, bradykinesia, gait and balance abnormalities, muscle stiffness and resting tremor. The motor symptoms are caused by progressive age-related death of dopaminergic neurons and in the vast majority of patients suffering from age-related idiopathic PD the cause of dopaminergic neurodegeneration is unknown. Even in the familial early-onset PD where genetic mutations have been identified, the molecular mechanisms driving degeneration of dopaminergic neurons are far from clear. Consequently, there is no clinically approved disease-modifying therapy capable of stopping or at least slowing down the disease progression.

Intracellular accumulation of insoluble protein deposits emerged as a common element of major age-related neurodegenerative disorders. Such deposits are commonly observed in histopathological examination of brains from patients with Alzheimer's disease (AD) (α -synuclein (α Syn), β -amyloid peptide and Tau deposits); amyotrophic lateral sclerosis (SOD1, TDP43 and FUS deposits); multiple systems atrophy (MSA), dementia with Lewy bodies (DLB) and PD (α Syn deposits in all three). Moreover, protein aggregation pathology is not limited to neurodegenerative diseases, but can also occur in peripheral organs, such as the gastrointestinal tract and kidneys, for example, in various amyloidoses. Despite being common denominator of most prevalent neurodegenerative disorders, the role of pathological protein aggregation in the etiology of AD, amyotrophic lateral sclerosis, MSA, DLB, and PD remains unclear, with very few molecular mechanisms confirmed in patients. Moreover, several clinical trials have targeted protein aggregates in neurodegenerative disorders (e.g. treatments targeting β -amyloid peptide in AD) with limited success, leading to a view that protein aggregation might be just epiphenomenon accompanying neurodegeneration. However, in our opinion it is rather the lack of mechanistic understanding of pathological protein misfolding, spread and disturbances it causes to cells that hindered successful development of disease-modifying therapies (Chmielarz and Saarma, 2020). Recently developed models utilizing α Syn pre-formed

fibrils (α Syn PFFs) have rapidly progressed our understanding of protein aggregation in PD, aiming for the development of more evidence based and ultimately effective treatments.

α Syn is one of the main components of proteinaceous neuronal inclusions called

Lewy Bodies characteristic for DLB and PD (Fouka et al., 2020). Lewy Bodies contain α Syn crowded together with fragments of membranous organelles and vesicles (Shahmoradian et al., 2019) and their formation has been recently proposed to drive neuronal pathology (Mahul-Mellier et al., 2020; **Figure 1**). Mutations and polymorphisms in the gene encoding α Syn are known risk factors and, in some cases, even direct causes of familial forms of PD. During PD progression, insoluble aggregates are increasingly observed in multiple brain regions, suggesting that misfolded α Syn spreading through interconnected neuronal networks possesses prion-like properties. Supporting the hypothesis, host-to-graft spread of Lewy Body pathology was also observed in fetal midbrain-derived neurons transplanted to the brains of PD patients.

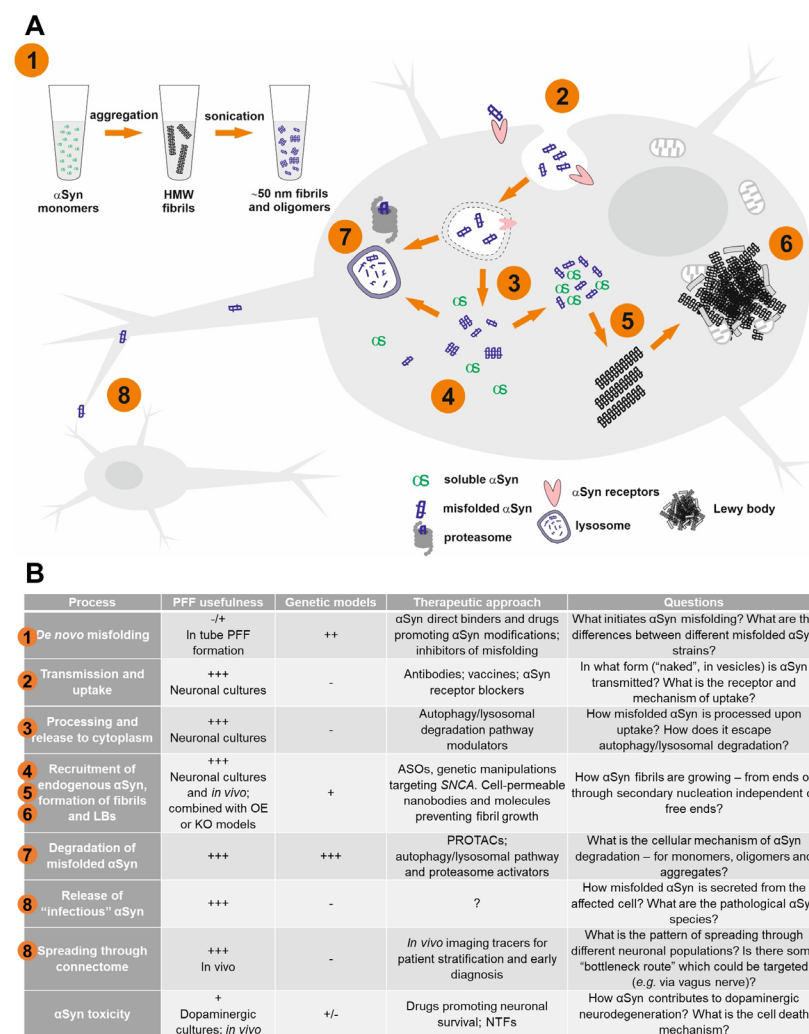


Figure 1 | Preparation and application of α -synuclein preformed fibrils to reconstruct multiple steps of Lewy body formation and pathological protein transmission.

(A) Major steps in PFF-induced α Syn aggregation model: (1) formation of α Syn fibrils *in vitro*; (2) cellular uptake of α Syn PFFs; (3) processing and release to cytoplasm; (4) recruitment of endogenous α Syn; (5) seeding of endogenous α Syn misfolding, formation of oligomers and insoluble α Syn fibrils; (6) maturation of fibrils and formation of Lewy Bodies containing fragment of lipid membranes and mitochondria; (7) lysosomal and proteasomal degradation of misfolded α Syn oligomers and fibrils; (8) release and propagation of misfolded α Syn. (B) Application of α Syn PFFs and genetic models to study molecular mechanisms and develop therapies targeting pathological α Syn aggregation, and the remaining questions. Numbers correspond to major PFF-induced α Syn aggregation steps depicted in Figure 1. ASOs: Anti-sense oligonucleotides; HMW: high molecular weight; KO: knockout; LBs: Lewy bodies; NTFs: neurotrophic factors; OE: overexpression; PROTACs: proteolysis targeting chimeras.

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Yet, despite that Lewy Bodies in PD have been described more than 100 years ago and for more than 20 years we know that α Syn is their main component, the causative role of α Syn in PD progression and degeneration of dopaminergic neurons has not been conclusively proven. For example, it is still not clear which conformation(s) and oligomeric state(s) (e.g. soluble oligomers or short fibrils) of misfolded α Syn are the most active in propagation and seeding aggregation in neurons and glial cells in the human brain. The molecular mechanisms for uptake of misfolded α Syn are also not fully elucidated. In addition to genetic and pathological evidence, application of sensitive protein misfolding cyclic amplification or real-time quaking-induced conversion assays demonstrated the presence of misfolded protein species capable of seeding α Syn aggregation in cerebrospinal fluid (Shahnawaz et al., 2020) and even in skin samples of PD patients (Manne et al., 2020). Inoculation with Lewy Body extracts collected post-mortem from PD patients' brains induced the development and spread of α Syn pathology not only in rodents, but also in non-human primates (Arotcarena et al., 2020). Supported by such data, α Syn has emerged as a promising target for disease-modifying therapy in PD, MSA and, possibly, DLB. Proposed therapeutic approaches targeting α Syn include interfering with initial aggregation by small molecules stabilizing correctly folded protein conformation, directly binding misfolded fibrils and promoting either their dissociation or inhibiting their growth; enhancing degradation of intracellular α Syn by stimulating lysosomal activity or by small molecules – proteolysis targeting chimeras (PROTACs) – promoting proteasomal degradation; lowering cellular α Syn levels by anti-sense oligonucleotides (ASO) and cell-penetrating nanobodies; inhibiting uptake and targeting transmission of extracellular α Syn with active and passive immunization. Many of such treatments are currently in clinical trials (see <https://clinicaltrials.gov/> for a regularly updated list) and some have shown good safety profiles (e.g. anle138b, NPT200-11, ENT-01, nilotinib, memantine and several other small molecules and active and passive immunotherapies targeting α Syn misfolding and spread), however, conclusive evidence of their efficacy is still to be demonstrated. Interestingly, encouraging results have recently been reported from PASADENA phase II clinical trial of monoclonal humanized antibody designed to stop misfolded α Syn transmission. While not meeting its primary endpoint, this trial demonstrated clear improvement in both motor and cognitive symptoms, convincing enough to prompt Roche and Prothena to support moving into the next phase of clinical development (see <https://ir.prothena.com/news-releases/news-release-details/roche-and-prothena-will-advance-prasinezumab-late-stage-clinical>). Further development of successful α Syn targeting therapies would

benefit immensely from a comprehensive understanding of the entire process of the pathology transmission, Lewy Body formation and resulting cellular damage. Significant progress in our understanding of these questions has been made in preclinical studies, thanks to multiple α Syn transgenic models (Airavaara et al., 2020) and the development of α Syn PFFs able to seed aggregation of endogenous α Syn in cultured cells and *in vivo* (Hijaz and Volpicelli-Daley, 2020).

α Syn PFFs model allows studying multiple steps of Lewy Body pathology development: uptake of pathogenic fibrils, their internalization, transport, processing and recruitment of endogenous α Syn, its post-translational modifications (e.g. phosphorylation of Ser129), formation and maturation of Lewy Body-like inclusions (**Figure 1A**) and, finally, effects of these processes on cell function and survival. Furthermore, with α Syn PFFs we can study the mechanism of pathology transmission between neurons and *in vivo* spreading through neuronal connectome as well as motor and behavioral outcomes of accumulation of α Syn deposits in different brain structures (Hijaz and Volpicelli-Daley, 2020). Application of α Syn PFFs as well as genetic models allowed pre-clinical testing of multiple approaches aiming to develop disease-modifying PD therapy (**Figure 1B**). Additionally, the formation of fibrils may be templated with pathological α Syn species from the brain and/or cerebrospinal fluid samples of PD and MSA patients. In such case the fibrils assume pathological conformation of templating patient material, allowing studying the differences and discriminating pathological α Syn aggregates in PD and MSA (Shahnawaz et al., 2020).

α Syn PFFs are purified prion-like, self-templating and transmittable aggregates of misfolded α Syn, which can induce formation of Lewy Body-like inclusions and cellular dysfunction both in *in vitro* and *in vivo* models (Er et al., 2020; Hijaz and Volpicelli-Daley, 2020). α Syn PFFs are formed by bacteria-expressed purified recombinant monomeric α Syn incubated at high concentration at 37°C with shaking for up to seven days, during which time it folds into elongated fibrils, exhibiting amyloid and self-templating properties. Obtained fibrils are subsequently sonicated to form short, about 50 nm in length, fragments, most efficient at seeding pathology (Hijaz and Volpicelli-Daley, 2020; **Figure 1A**).

Formation of fibrils *in vitro* can be easily monitored to investigate initial steps in this putatively nucleation-dependent process. For example, conversion of monomeric α Syn into pathogenic α Syn PFFs involves the preceding step of liquid-liquid phase separation, which can be fostered by PD linked mutations or heavy metal interaction (Ray et al., 2020). Understanding of the *de novo* α Syn PFFs formation and ability to monitor it is of obvious value for the development of drugs

targeting this process. *In vitro* PFF model is also useful for testing compounds aiming to dissociate fibrils or inhibit their growth to avoid formation of putatively more toxic α Syn oligomers. Several such compounds (e.g. anle138b, NPT200-11, ENT-01, memantine and others) are currently in different phases of clinical trials.

α Syn PFFs were also used to study neuronal uptake of misfolded α Syn, which was shown to be receptor-mediated, albeit alternative hypotheses have also been proposed, such as direct penetration of membrane, adsorptive-mediated endocytosis or transmission through tunneling nanotubes or exosomes (Fouka et al., 2020). After uptake, α Syn PFFs localize in lysosomal compartment and partial lysosomal processing might actually enhance or even be required for α Syn PFFs pathogenicity in cells (Hijaz and Volpicelli-Daley, 2020; **Figure 1**). However, it is still not clear how exactly α Syn PFFs are processed and how they are released to cytoplasm and, possibly, also to extracellular space. Several studies highlight the role of extracellular exosomes in promoting the spread of misfolded α Syn; moreover, elevated levels of α Syn in neuronal exosomes isolated from patients' serum emerge as a promising diagnostic biomarker of early PD (Hijaz and Volpicelli-Daley, 2020; Jiang et al., 2020). However, additional studies are needed to resolve what type of autophagy/lysosomal pathway modulation will be beneficial and which might be ineffective or even harmful. We have recently utilized α Syn PFFs *in vitro* in primary dopaminergic neurons and *in vivo* in mouse to demonstrate protection against formation of α Syn deposits by stimulation of GDNF/RET signaling at early time points after α Syn PFF uptake, presumably through modulation of endo-lysosomal pathway (Chmielarz et al., 2020). Indeed, several molecules, such as ambroxol and venglustat, modulating lysosomal function by affecting levels and activity of lysosomal glucocerebrosidase are currently being tested in clinical trials for PD treatment.

When applied to neuronal cultures, including primary and human induced pluripotent stem cell derived neurons, α Syn PFFs induce accumulation of insoluble intracellular deposits which mature over time, increasing their resemblance to Lewy Bodies (Mahul-Mellier et al., 2020). Application of α Syn PFFs to neurons cultured in microfluidic chambers allows to dissect cell-to-cell transmission of pathological aggregates and to demonstrate the effectiveness of antibodies targeting extracellular α Syn or putative α Syn receptors in blocking this process. When α Syn PFFs are injected into the brain parenchyma, similar deposits are observed in anatomically connected areas (Arotcarena et al., 2020; Hijaz and Volpicelli-Daley, 2020), resembling the spreading behavior of pathology in PD patients (Horsager et al., 2020). Importantly, formation and spreading of α Syn PFFs induced deposits through neuronal

connections is dependent on endogenous α Syn, and the extent of pathology varies between neuronal types (Hijaz and Volpicelli-Daley, 2020). Application of α Syn PFFs to primary mouse midbrain neuronal cultures or human stem cell-derived dopaminergic neurons allows monitoring accumulation of α Syn deposits and their effects on survival of PD-relevant dopaminergic neurons *in vitro* and in humanized rats (Er et al., 2020; Hoban et al., 2020). Sensitivity of α Syn PFF induced pathology to endogenous α Syn levels makes α Syn PFF based models ideally suited to test the effectiveness of ASOs, PROTACs or gene therapy aimed at lowering expression or promoting degradation of α Syn.

The biggest limitation of α Syn PFFs model is that seeded deposits only modestly impair neuronal function and survival, the latter only after prolonged period of time (Mahul-Mellier et al., 2020), complicating testing of survival-promoting agents. Different research groups have reported variable effects of PFFs on cell survival. This could be partially because of technical difficulties in standardizing PFF preparations and batch-to-batch variations. However, delayed and modest effects on cell survival seem to be more in line with actual slow PD progression in humans. Actually, the mechanism by which pathological α Syn cause cell death remains elusive, although recent data suggest that this process is driven by formation of pathological deposits resembling Lewy Bodies containing not only α Syn but also fragments of membranous organelles (Mahul-Mellier et al., 2020). Understanding how α Syn pathology contributes to neuronal death could allow to design therapies aimed at increasing survival of cells already afflicted with α Syn pathology, such as neurotrophic factors or small molecules mimetics (Chmielarz et al., 2020). From the perspective of drug discovery, α Syn PFF models have advantages of being adaptable to multi-well plate formats with automatized quantification in dopaminergic neurons (Er et al., 2020). While still not feasible for use in high throughput screening campaigns, they can allow for functional validation of promising therapeutics in a physiologically relevant model allowing for monitoring α Syn aggregation and dopaminergic cell survival. Subsequently, therapies can be further validated in α Syn PFF based models in stem cell-derived human dopaminergic neurons and *in vivo* for long term effectiveness at slowing the spread of pathology and for behavioral outcomes (Hoban et al., 2020). Thanks to progressive nature of α Syn PFF *in vivo* model, it could also be used to identify a time window for effective treatment. Moreover, by varying the site of α Syn PFF injection (e.g. brain versus duodenum) (Arotcarena et al., 2020), we can verify the effectiveness of treatments in putative brain-first or body-first subtypes of PD (Horsager et al., 2020).

Lastly, *in vivo* α Syn PFF models will be useful for development of PET tracers for

longitudinal assessment of α Syn pathology in PD patients. When developed, such imaging tools would be extremely useful both for monitoring of clinical trials and early diagnosis of the disease.

Overall, α Syn PFFs allow to study both pathophysiology of PD and the effectiveness of therapeutic approaches in physiologically relevant cellular and *in vivo* models, amenable to both meticulous investigations of mechanisms driving Lewy Body formation and spread, and screening and efficacy validation of compounds blocking these processes. While we still have many questions related to α Syn misfolding and pathology progression (Figure 1B), α Syn PFFs have been and will be very instrumental for helping researchers to find answers to these questions. Not surprisingly, α Syn PFFs elicit great hopes as a reliable and robust tool to model pathological protein aggregation in PD research and have seen extensive adoption in the field.

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