DECIPHERING THE ROLE OF ALPHA-SYNUCLEIN STRAINS IN PRION-LIKE SYNUCLEINOPATHY INDUCTION AND SPREADING

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1. Summary

Parkinson’s disease (PD) is the second most prevalent neurodegenerative disorder after Alzheimer’s disease, affecting more than 7 million people worldwide. Its onset is linked to ageing, impacting up to 2% of people over 65 years old, thus its incidence is expected to grow in our ageing society. Despite the persistent effort of both public research and private institutions, PD remains incurable. Current treatments are only able to ameliorate the early motor symptoms of the disease. Moreover, these drugs become ineffective as the disease progresses. For this reason, there is an urgent need for a disease-modifying therapy for PD.

A prominent hallmark of Parkinson's disease (PD) is the presence of intraneuronal inclusions, the so-called Lewy bodies (LB), whose major component is misfolded and aggregated α-synuclein (a-syn). Mutations in α-syn cause rare familial PD forms, leading to an early onset of the disease. Although it is known that misfolded α-syn can propagate between neurons and disseminate LB pathology in the brain in a prion-like fashion, this phenomenon is still poorly understood. One of the most intriguing issues is the nature of the transmitted form of α-syn. It has been demonstrated that α-syn can assemble into different types of aggregates generating distinct strains which are thought to cause disease with specific characteristics.

The project pursued the objective of providing new information on the prion-like hypothesis for PD, characterizing the functional and conformational properties of α-syn aggregates, both in vitro and in animal models of the disease. The results indicate that in vitro generated α-syn amyloid strains and the aggregates present in patients suffering from different synucleinopathies propagate differently in the brain of model animals, strongly suggesting that the structural properties of α-syn aggregates are a key determinant of their brain propagation and their neuronal toxicity. The differential properties of these aggregates should be taken into account while trying to develop treatments for these devastating disorders.

The new mechanistic, structural and technical knowledge generated during the execution of this initial objective has allowed us to undertake a challenging new objective: The development of novel molecules able to stop the aggregation and especially the propagation of α-syn aggregates. In this context, we have identified a

novel compound that is effective against the aggregation and propagation of the different α-syn strains assayed so far, being a promising molecule for a disease-modifying therapy for PD.

2. Results

1. Production and purification of recombinant α-synuclein (α-syn) variants

During this project the group have successfully set up a protocol for the semipreparative production and purification of wild type (wt) α-syn protein, its mutants and some redesigned variants with a degree of purity and homogeneity that exceed those of the commercially available protein. The quality of the protein has been an important asset for the subsequent steps of the project. In particular we have produced the following PD familiar variants: A30P, H50Q, A53E, A53T, G51D, and E46K.

2. Generation of wt α-syn strains in vitro

After screening a number of combinations, we have set up the conditions to generate amyloid fibrils of α-syn under three different conditions: mildly low pH, neutral pH and low salt and neutral pH and high salt.

3. Characterization of wt α-syn strains

The three different amyloid species generated correspond to real strains, since they differ in their morphology, size, kinetics of aggregation, binding to amyloid dyes, quantity of aggregates per unit of protein and secondary structure. These structurally different and well characterized amyloid fibrils have been generated at semipreparative levels in unique batches and used to inoculate animal models of PD.

4. Generation and characterization of mutant α-syn strains

The three conditions that successfully rendered strains for the wt protein were employed to promote the aggregation of the A30P, H50Q, A53E, A53T, G51D, and E46K early-onset mutational variants. These generated a large and unprecedented number of conformationally different amyloid strains. An important conclusion of these still unpublished results is that in a given condition each particular mutant behaves differently. This has important implications since it indicates that the mutations

themselves can be the origin of different strains with differential toxic and propagative properties in patients. In addition, for any given mutation the aggregation conditions determine the features of the final protein aggregates in an unpredictable manner. Overall, these results converge to indicate that the conformational landscape of α-syn might be much more complex than previously assumed. For the new H50Q mutant we have shown that this is the case, since the unique combination of the neurodegeneration-related metal copper and the pathological α-syn mutation induces an alteration in the aggregation properties of α-syn that determine its propagative properties as well as its toxicity (Villar-Pique et al. PNAS 2016).

5. Generation and characterization of α-syn strains in the presence of membranes

It has been suggested that the aggregation of α-syn may be influenced by its binding to neuronal membranes. Therefore, we monitored for the wt protein and the A30P, H50Q, A53E, A53T, G51D, and E46K mutants, to see whether the presence of membrane mimics with different lipidic composition resulted in different amyloid strains. Effectively, the lipids influenced the kinetics of aggregation and the morphology of the final aggregates in a manner that was dependent on the specific mutation. Taken together with the previous data this clearly suggests that the environment in which the aggregation initiates is a crucial determinant of the structure of α-syn amyloids.

6. Generation and characterization of a synthetic α-syn variant to test α-syn regions relevant for amyloid propagation

The disordered nature of α-syn has hampered the use of structure-based protein engineering approaches to elucidate the molecular determinants of fibril formation and propagation. While the project was running, the 3D structure of a pathogenic α-syn fibril strain was published and provided a template for these studies, which complemented the originally designed ones. The structure supported the central NAC domain being a critical element in fibril propagation, since it constitutes the core of the fibril, delineating a Greek-key motif. We generated a synthetic variant in which we stapled the ends of this motif with a designed disulfide bond and evaluated its impact on the conformation, aggregation and toxicity of α-syn in different environments. Our study did not support the Greek-key motif being already imprinted in early α-syn assemblies, discarding it as a druggable interface to prevent the initiation of fibrillation

and its propagation. In contrast, it suggests the stabilization of native, compact ensembles as a potential therapeutic strategy to avoid the formation of toxic species and to target the early stages of PD. (Carija, et al. Redox Biology 2019)

7. Setting a PMCA assay for α-syn

We developed a protocol to implement the classical protein misfolding amplification assay used for the mammalian PrP protein to analyse human α-syn propagation. We achieved conditions in which both wt and mutant α-syn amyloid fibrils could be propagated in vitro in a prion-like fashion. However, the initial specific structural properties of the α-syn strains were lost during the amplification and therefore this protocol was discarded to generate material for in vivo studies in animal models. Nevertheless, as we will discuss in further sections, the developed protocol turned to be crucial in order to identify small molecules that can interfere with the aggregation and propagation of α-syn aggregates.

8. In vivo propagation properties of in vitro generated α-syn polymorphs.

From the large number of in vitro generated α-syn strains, those formed by wt, A30P, H50Q, and A53E variants were selected for in vivo studies in a mouse model of the disease. They account a total of 12 different strains, a significantly larger number than has been assessed in any previous study.

For in vivo studies we first established a colony of the PAC-Tg (SNCAWT) mice. In this animal, mouse α-syn is knocked-out and it bears a transgene encoding the human a-syn. This animal is used as control in many studies and does not have PD histopathological or behavioural phenotype, allowing us to ascribe any observed change to the injection of our in vitro generated amyloid strains. Animals were inoculated in the right parietal lobe or in the left striatum. Mice were monitored daily and their neurological status assessed twice a week. Mice were euthanized following a planned kinetic timeline or when showing evidence of undercurrent illness. During necropsy, brain was collected from each animal. Brains were sliced sagittally and the left hemibrain used for histopathological analysis, while the remaining right hemibrain was used for biochemical analysis.

Results obtained up to now showed differences in the onset of appearance of aggregation of phosphorylated α-syn, depending on the mutation of the recombinant

protein inoculated, but also on the buffer where those proteins were aggregated. In most of the inocula, the aggregation of phosphorylated α-syn started at 2 months post inoculation (mpi), to later disappear at 6 mpi, and finally rise again at 13 mpi, leading to the presence of two different curves of aggregation of phosphorylated α-syn in time. These kinetics have not been reported previously and might be important to understand the development of the disease.

α-syn started to aggregate as neurites and also as an accumulation of small abnormal intraneuronal inclusions bodies, spreading from the point of inoculation, the left striatum, caudally to the hippocampus and the cerebral cortex at that level, presenting a great accumulation. Overall, the results confirm that, as intended, the inoculated fibrils corresponded to functionally different strains.

9. In vivo propagation of α-syn aggregates from patients suffering from different synucleinopathies

Apart from PD, there are other related diseases generated by the accumulation of aggregates of α-syn, globally known as synucleinopathies, among them Lewy bodies dementia (LBD) and multiple systems atrophy (MSA). LBD is a type of progressive dementia that leads to a decline in thinking, reasoning and independent function, whereas MSA is characterized by a varying combination of symptoms and signs including parkinsonism, cerebellar ataxia and impaired functioning of the autonomic nervous system. The fact that despite the dramatically different symptoms, PD, DLB and MSA all result from the aggregation of α-syn in the brain has suggested that these diseases might indeed originate from different α-syn strains. To assess this hypothesis, brain extracts of patients who died after suffering any of these diseases where inoculated in PAC-Tg (SNCAWT) mice as described for in vitro strains and the propagation of α-syn aggregates present in these extracts analysed in the same manner.

The distribution of the phosphorylated α-syn seems to follow a similar pathway in a prion-like manner, regardless of the nature of the inoculum or its concentration, starting from the point of inoculation and spreading backwards through the whole brain, leading in similar phenotypes of accumulation. Several differences between the inocula have been found in the magnitude, dispersion and mainly in the onset of appearance of the first aggregates of phosphorylated α-syn. We can clearly

differentiate between a group comprising all variants of PD and DLB inocula, and another group that includes the MSA inocula. In MSA cases, inoculated animals presented a delayed appearance of aggregates and a lower magnitude and dispersion of those aggregates. The data are again consistent with a role of α-syn specific conformation in the propagation of the pathological signatures in the brain.

10. A high-throughput assay to identify modulators of α-syn aggregation

The present project allowed us to: i) produce highly pure α-syn protein, both wt and mutant, ii) implement a PMCA for α-syn amplification and iii) obtain highly reproducible aggregation kinetics. With these elements in hand we thought of implementing a new and ambitious objective, not present in the initial proposal: The discovery of molecules able to prevent the aggregation and propagation of α-syn aggregates. To this aim we first implemented a very robust methodology that allowed us to screen a library of more than 14,000 small compounds in the search for these activities (Pujols J, et al. Int J Mol Sci. 2017).

11. Identification of a novel small molecule that inhibits α-syn aggregation and its propagation

As a result of the screening campaign we have identified SynuClean-D (SC-D). It is a small molecule that interrupts α-syn aggregation, disentangles mature fibrils and, in addition, hampers the propagation of aggregated a-syn. Remarkably, SC-D is the only molecule described so far that agglutinates all these synergic and beneficial properties in a single small chemical scaffold. We have already demonstrated SC-D effect in vivo, where it is able to reduce the presence of amyloid aggregates and protect dopaminergic neurons from degeneration in a *Caenorhabditis elegans* model of PD (Pujol J, et al. PNAS 2018). Importantly, SC-D inhibits the aggregation of all the sequentially and environmentally generated α-syn strains tested so far. Thus, SC-D deserves further investigation as possible new avenue to treat PD and the other synucleinopathies.

3. Relevance and implications

The implications of the results in this project are numerous and range from methodological issues to the development of a potential novel therapy for the synucleinopathies.
In particular:

1) We have developed a system for the preparative production of highly pure and well behaved α-syn variants. A significant number of labs and companies will benefit from it, since this study and related ones could not have been possible with the commercially available protein.

2) We have developed a very robust high-throughput screening methodology that would make it possible to identify novel candidates for therapeutic intervention through the analysis of novel chemical libraries of natural or repurposed molecules.

3) We have generated a methodology to produce strains at a semipreparative level that will very probably be exploited in the field for further structural and functional studies.

4) We have provided information that confirms the phenomenon of prion-like behaviour of α-syn, and how different strains exhibit different propagating properties in the brain. This observation has profound implications for the treatment of the associated diseases, since it might imply that different treatments might be necessary depending on the kind of α-syn aggregate that initiates the disease. These divergence in the conformational properties of the aggregates can be originated by point mutations, but also by external genetic factors in patients or by the microenvironment in which the aggregation occurs. In this context, in our hands MSA seems to be a synucleinopathy that is clearly differentiated in terms of propagation from PD and LBD. This implies that what can give good results in the treatment of one synucleinopathy, would not necessarily work for another.

5) Despite all the above-mentioned concerns we have found a small molecule that seems to generically inhibit α-syn aggregation and propagation, disentangling pre-existing aggregates. The neuroprotective properties of this hit suggest that its

investigation might crystallise in a first molecule able to tackle these group of devastating disorders. In future projects we will continue to bring it to clinical practice.

4. Generated Literature

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