Review Article

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Alpha-synuclein as therapeutic target in Parkinson's disease

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Abstract: It took 180 years from James Parkinson's descriptions in "An essay on the shaking palsy" to the discovery of alpha-synuclein as key player in Parkinson's disease (PD). The identification of a PD causing mutation in the gene of alpha-synuclein was followed immediately by detection of its presence in Lewy bodies, inclusions found in the brains of patients. While many open questions remain, findings on how alpha-synuclein pathology emerges, propagates and causes neuronal death provide hope for development of disease-modifying therapeutics beyond the current dopamine replacement therapy. The recent hypothesis of a prion-like transmission of alpha-synuclein pathology raises controversy but also inspired numerous exciting research avenues, partially already translating into novel drug targets. This review summarizes evidence for a critical role of alpha-synuclein in PD pathogenesis followed by a discussion of current promising treatment avenues.

Keywords: synucleinopathy, prion, neurodegeneration, neuroprotection, dopamine

Zusammenfassung: 180 Jahre vergingen zwischen James Parkinson's "An essay on the shaking palsy" und der Entdeckung der zentralen Rolle von alpha-synuclein in der Pathogenese von Parkinson's disease (PD). Der Identifikation einer PD verursachenden Mutation im alpha-synuclein Gen folgte rasch der Nachweis des Proteins in Lewy Körperchen, den charakteristischen Proteineinschlüssen im Gehirn der Patienten. Trotz vieler ungeklärter Fragen, Forschungsergebnisse zur Entstehung, Ausbreitung und Neurotoxizität der alpha-synuclein Pathologie geben Hoffnung auf die Entwicklung einer Krankheits-modifizierenden Therapie, über die Dopaminersatztherapie hinaus. Die Hypothese, dass alpha-synuclein Pathologie sich ähnlich wie ein Prion ausbreitet, wird kontrovers diskutiert, und initiierte viele interessante neue Forschungsansätze und

therapeutische Zielstrukturen. Dieser Übersichtsartikel fasst die Evidenz für eine zentrale Rolle von alpha-synuclein in der Pathogenese der PD zusammen, gefolgt von einer Diskussion neuer Therapiestrategien.

Lack of disease-modifying therapeutics for Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder, affecting more than 1% of the population above 60 years of age. While a growing number of gene mutations define familial PD, the vast majority of cases is sporadic, meaning that the cause of the disease remains elusive for most patients. Neurodegeneration in PD affects diverse neuronal subtypes, including dopamine neurons of the substantia nigra pars compacta (Tretiakoff, 1919). These neurons project to the caudate nucleus and putamen, where loss of dopamine results in cardinal signs of this movement disorder, just as described 200 years ago by James Parkinson, i. e. bradykinesia, rigidity, tremor and gait disturbance (Parkinson, 1817). Of note for therapeutic interventions: dopaminergic neurons have extensively degenerated and striatal dopamine is largely depleted during the first years after diagnosis (Kordower et al., 2013).

There is currently no treatment to stop or halt progressive neurodegeneration in PD. Arvid Carlsson, using the vesicular monoamine transporter inhibitor reserpine in rodents and rabbits, identified a major role of dopamine in brain and the potential of its precursor 3,4-dihydroxyphenylalanine (DOPA) to counteract the "tranquillizing" effects of reserpine in vivo (Carlsson et al., 1957; Carlsson et al., 1958). In 1960 Oleh Hornykiewicz made the landmark discovery of dopamine loss in PD brains (Ehringer and Hornykiewicz, 1960). Only one year later he demonstrated the astounding effect of L-DOPA administration to PD patients. L-DOPA, which crosses the blood brain barrier, offered an immediate and substantial relief from akinesia, which tremendously improved the quality of

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life for patients and remains the current gold standard of drug treatment. Unfortunately, long-term treatment elicits uncontrolled involuntary movements, termed dyskinesia. Furthermore the drug does not improve many motor and non-motor symptoms which severely reduce the quality of life, including for example some speech deficits, tremor, cognitive decline, anxiety and sleep dysfunction. Notably, dopamine replacement does not halt progression of the disorder and its symptoms (Schapira et al., 2014). Consequently, the main focus of research in PD is to develop novel treatments that address these shortcomings.

Development of such disease modifying therapeutics requires knowledge on cause and mechanisms of neurodegeneration. Despite intensive research it is still not known why dopaminergic neurons die predominantly in PD. It has been proposed that dopamine and its metabolites represent a toxic burden for neurons making them more vulnerable to additional impacts of environmental toxins such as pesticides (Chesselet, 2003). In fact some toxins are taken up specifically by the dopamine transporter and cause mitochondrial dysfunction and oxidative stress, which may explain mitochondrial deficiency in PD (Schon and Przedborski, 2011). In addition, there is considerable involvement of glial activation around the site of neurodegeneration which could contribute to neuronal susceptibility (Ouchi et al., 2009). Unfortunately, similar to many other neurological diseases, prospective novel therapeutics targeting these mechanisms have so far failed in clinical trials (Schapira et al., 2014). Many reasons have been proposed for such failure, including the inadequacy of animal models, methods and evaluation criteria used in pre-clinical studies, which are often followed by clinical trials with low power to detect neuroprotective effects in patients. Apart from redesigning of preclinical and clinical trials, there is a need for basic research on the therapeutic targets by studying how gene mutations or risk factors initiate or contribute to the cascade of events that causes neurodegeneration.

The rise of alpha-synuclein as key therapeutic target

At present, one protein is heavily targeted for development of novel therapeutics: alpha-synuclein. Its role first emerged with the discovery of a mutation in the gene encoding alpha-synuclein (*SNCA*) in familial PD (Polymeropoulos et al., 1997). Shortly after, alpha-synuclein was found to be a main component of Lewy bodies,

a pathological hallmark of sporadic PD first described by Friedrich Lewy (Lewy, 1912; Spillantini et al., 1997). Additionally, elevated expression of alpha-synuclein due to gene multiplications or nucleotide polymorphisms can cause PD or significantly increase the risk to develop the disease (Devine et al., 2011). While the crucial role of alpha-synuclein is thus established, it is still not fully understood how alpha-synuclein pathology connects to neurodegeneration, and why specific neuronal subtypes, such as the dopaminergic neurons of the substantia nigra, are preferentially affected in PD.

Curiously, alpha-synuclein pathology is involved in other neurodegenerative diseases, together frequently referred to as synucleinopathies. In multiple system atrophy, alpha-synuclein aggregation affects mainly oligodendroglia (Papp-Lantos bodies). Lewy bodies are also found in neurocognitive disorders such as Dementia with Lewy bodies (DLB). DLB is commonly distinguished from Parkinson's disease dementia (PDD) based on arbitrary defined earlier onset of cognitive impairment compared to motor symptoms. Both PD and DLB overlap in many clinical features, genetics, neuropathology, and management and are therefore currently regarded as subtypes of an alpha-synuclein-associated disease spectrum (Jellinger and Korfczyn, 2018). Simple malfunctions of the protein are unlikely to cause such complex and diverse disease development. But what may trigger heterogeneous yet specific pathology? Is there a causative link between alpha-synuclein aggregation and disease progression, or are Lewy bodies merely by-products of neurodegeneration? Sufficiently answering these most critical questions will facilitate the development of urgently-required disease modifying therapy for synucleinopathies.

The answers may be found in the propensity of alpha-synuclein to associate into more or less toxic protein assemblies. Physiologically, the 140 amino acid protein alpha-synuclein, as indicated by its name, concentrates in nerve terminals and is also found in the nucleus. The protein was suggested to form alpha-helically folded tetramers, probably membrane-bound, but there is also compelling evidence for a monomeric state in mammalian cells (Theillet et al., 2016). The physiological function of alpha-synuclein is poorly understood. It is involved in transmitter release at nerve terminals and appears to be able to remodel membranes (Bendor et al., 2013). Mice lacking the alpha-synuclein gene show only modest differences in transmitter release, which could be somewhat aggravated by additional knockout of the beta- and gamma-synucleins, supporting some functional redundancy (Anwar et al., 2011). However, the detrimental role of the protein in neurodegeneration undoubtedly involves "gain

of toxic function". Probably triggered by factors such as higher expression, disturbance in metabolism, or interaction with other agents (environmental toxins, infections), alpha-synuclein becomes prone to oligomerization and ultimately forms the amyloid fibril with a cross beta-sheet quaternary protein structure which constitutes Lewy bodies. During this process, alpha-synuclein forms multiple kinds of species, or strains, which seem to differ in their capacity to spread out and cause acute cell death (Winner et al., 2011; Luk et al., 2012; Peelaerts et al., 2015). The proportion of nigral neurons bearing Lewy bodies seems stable (about 3,6 %), supporting that the harboring neurons die while new bodies are forming in the remaining neurons (Greffard et al., 2010). Mutations in the gene for alpha-synuclein, described as cause for familial PD, increase alpha-synuclein aggregation or elevate the level of assembly-prone free alpha-synuclein by reducing its ability to associate with membranes (Burre et al., 2015). Thus, it is conceivable that in synucleinopathies different triggers, or pathological alterations in specific cells, shift alpha-synuclein assembly towards the more toxic species which then drive the disease progression.

The prion-like concept applied to alpha-synuclein pathology

The idea of propagation of alpha-synuclein pathology with disease progression is substantiated by the highly influential work of Heiko Braak and colleagues (Braak et al., 2003). By analyzing brains of subjects with clinical diagnosis of PD and nigral Lewy body pathology versus subjects without reference to PD symptoms but Lewy body pathology versus subjects with neither PD symptoms nor Lewy body pathology they were able to conceive a staging procedure based upon the readily recognizable topographical extent of the lesions. Lewy bodies first emerge in the olfactory bulb and brain stem (stage 1–2), followed by the substantia nigra (stage 3, symptomatic PD), the temporal cortex and the neocortex (stage 4–6, cognitive decline). Subsequently alpha-synuclein pathology was even demonstrated in the peripheral and enteric nervous system of PD patients, which could explain why non-motor symptoms such as constipation characterize the prodromal, early phase of PD. Pathology may actually be initiated in the periphery and spread to the central nervous system, because there is evidence for transport across nerves to central neurons. Truncal vagotomy was reported to reduce the risk to develop PD (Svensson et al., 2015; Liu et al., 2017), but others did not arrive at the same

conclusion (Tysnes et al., 2015). As such, there is controversy about the "PD starts in the gut" hypothesis, which requires further research. A recent study demonstrated that colonizing the gut of alpha-synuclein overexpressing mice with microbiota of PD-affected patients enhances the behavioral impairments, while antibiotic treatment was protective, supporting a pivotal role of gut bacteria in PD pathogenesis (Sampson et al., 2016).

Support for a prion-like spread came from findings of Lewy bodies in fetal mesencephalic dopaminergic neurons that had been transplanted into the putamen and caudate nucleus of patients with advanced PD (host-to-graft propagation) (Li et al., 2008). Alpha-synuclein strains injected into rodent brain can induce aggregation and pathology that propagates, with specific strains being more toxic and invasive, perhaps explaining diversity in disease progression in patients (Luk et al., 2012; Peelaerts et al., 2015). Together these studies show that alpha-synuclein can adopt alternative conformations which self-assemble into toxic species. Those transfer across cells and recruit further protein, with the result of self-propagation of pathology characteristic for prion diseases (Fig. 1). Regardless of the evidence, this theory is not undisputed, but the toxicity of certain alpha-synuclein assemblies to neurons has been sufficiently demonstrated. If this process indeed starts in the periphery and/or the olfactory bulb, one would expect those neurons to be preferentially exposed to some (unknown) triggering insult, or harbor a specific intrinsic vulnerability. Interestingly, while inoculation of nigral Lewy body-enriched fractions from postmortem PD brains in mice promoted alpha-synuclein pathology and dopaminergic neurodegeneration (Recasens et al., 2014), the same approach using alpha-synuclein-containing Lewy body extracts purified from peripheral postmortem stellate ganglia did not trigger respective pathology (Recasens et al., 2018). For a definite answer on how alpha-synuclein pathology propagates, further research is required to dissect mechanisms underlying the different pathogenic capacity observed in the aforementioned studies, also including alpha-synuclein aggregates from other peripheral regions, and at different disease stages. Several studies aiming to define a biomarker for PD have reported decreased extracellular alpha-synuclein levels; however, others found increased levels (Malek et al., 2014). Recently alpha-synuclein was reported in extracellular vesicles which are released from neurons and other CNS cells and may present a reservoir for biomarkers (Gamez-Valero et al., 2019). Furthermore, development of specific PET-imaging tracers to track different forms of pathological alpha-synuclein in the periphery and CNS in patients could greatly advance the field (Lionnet et al.,

2018). Of note, novel sensitive protein assays for detection of misfolded alpha-synuclein in cerebrospinal fluid of patients have the potential to be effective tools for the early diagnosis of synucleinopathies (Paciotti et al., 2018). Clearly, PD is a complex disease and there may be multiple alternative pathogenic avenues, differing between patients, which ultimately cumulate in significant neuronal death causing symptomatic PD. Alpha-synuclein may be a common denominator along these avenues which makes it very attractive as therapeutic target.

Therapeutic targeting of alpha-synuclein

For preclinical testing there are several cellular (Lazaro et al., 2017) and animal models (Chesselet and Richter, 2011) of alpha-synuclein pathology available. These models are genetically altered to overexpress the human wildtype or a mutated form of alpha-synuclein, or more recently, were injected with exogenous alpha-synuclein (e.g. synthetic fibrils). There is no perfect model that represents all features of PD, but the model selected for a specific drug trial should obviously harbor the pathomechanism which is targeted (e.g. aggregation of alpha-synuclein), and for the *in vivo* trials provide a set of related pathological and ideally behavioral readouts to measure drug efficacy (Chesselet and Richter, 2011).

Therapeutic interventions directly targeting alpha-synuclein pathology aim to (i) reduce expression, (ii) inhibit aggregation, (iii) prevent spreading and (iv) increase metabolism (Fig. 1). The following chapters describe examples of compounds and strategies in development for each aim.

Reduce expression

Overexpression of wild-type alpha-synuclein by gene multiplication or polymorphisms in the promotor region is capable of causing disease in a dosage dependent manner (triplication causes a more severe and early onset disease than duplication) (Devine et al., 2011). Furthermore, nigral dopaminergic neurons of sporadic PD patients express about 7-fold higher levels of alpha-synuclein mRNA (Grundemann et al., 2008). The cause of alpha-synuclein mRNA and protein accumulation is still elusive for the majority of patients, but the resulting downstream pathology clearly correlates with disease progression. Specific reduction of gene expression can be achieved by using synthetic

non-coding small interfering RNAs (siRNA) against the target mRNA, thereby taking advantage of endogenous RNA interference (RNAi). However, efficient delivery of siRNA into neurons *in vivo* remains challenging due to biological barriers, degradation, low transfection and insufficient distribution. Infusion of naked siRNA against alpha-synuclein into the substantia nigra of nonhuman primates reduced the protein level close to the injection site significantly (McCormack et al., 2010). Packaging of siRNA into nanoparticles increases stability, distribution and transfection rate which allows injection into cerebral spinal fluid with widespread protein knock-down in brain evident 5 days after a single application (Helm-schrodt et al., 2017). Similarly, antisense oligonucleotides (ASO) against alpha-synuclein mRNA and conjugated with a monoamine uptake inhibitor achieved protein knock-down in brainstem of mice up to three days after 4 consecutive days of intranasal application (Alarcon-Aris et al., 2018). An alternative avenue could be reduction of mRNA expression by discovery of regulating pathways. Recently, agonists of the beta 2 receptor were associated with a reduction of alpha-synuclein gene expression and a lower risk to develop PD (Mittal et al., 2017). The extensive experience with these drugs can facilitate clinical development. Interestingly, iron was shown to upregulate alpha-synuclein expression at the translational level. Iron accumulates in the substantia nigra of PD patients and thereby increases oxidative stress burden to the neurons (Berg et al., 2002). The combination of oxidative stress and alpha-synuclein accumulation is likely to accelerate protein misfolding and its propagation. Therefore, iron chelators, already approved for other diseases, were tested preclinically with positive results and are now moving forward in clinical trials (Martin-Bastida et al., 2017).

Inhibit aggregation

Given the above described alpha-synuclein self-assembly into toxic species, stabilizing the protein in its physiologic non-toxic (monomeric?) form represents a rational target. Small-molecules that cross the blood brain barrier and interact with alpha-synuclein were shown to improve behavioral, neuropathological and biochemical endpoints in preclinical trials (Levin et al., 2014; Wrasidlo et al., 2016; Richter et al., 2017). Such compounds are for example selected out of large drug libraries using high-throughput screening for ability to reduce oligomer formation (Levin et al., 2014). In that case the precise mechanism(s) of action is(are) determined at later stages. Alternatively

Fig. 1: Therapeutic targeting alpha-synuclein pathology by (i) reducing expression of alpha-synuclein monomers (blue) through RNA silencing (RNAi), (ii) inhibiting the formation of toxic oligomers/strain assembly with small molecules (purple/pink), (iii) prevent spreading of a toxic assemblies through binding to specific antibodies and degradation in microglia, (iv) increase of lysosomal degradation (autophagy pathway) by stabilizing the conformation of specific enzymes from the endoplasmic reticulum to the lysosome (pharmacological chaperoning).

small molecules are chosen based on specific activities, such as shielding of Lys residues with low affinity, thereby interrupting formation of oligomers without interfering with the physiological protein function (Richter et al., 2017). The effects of these drugs *in vitro* and *in vivo* are also highly informative on the mechanism of alpha-synuclein toxicity. For example, one compound was developed de novo by molecular modelling methods targeting a C-terminus domain of alpha-synuclein, which is important for dimerization and membrane penetration. As expected, the compound reduces binding of alpha-synuclein to membranes, which, however, was later shown to represent a physiological and likely protective mechanism, at least in synaptic vesicles. Regardless, the compound is highly effective in reducing protein aggregation and toxicity *in vivo* without overt side effects. The current hypothesis is that it might work by preventing toxic interaction of alpha-synuclein with the plasma membrane and other intracellular organelles, without altering the physiological alpha-synuclein associated with synaptic vesicles (Wrasidlo et al., 2016). These examples show that effects of interference with alpha-synuclein oligomerization are complex and difficult to predict. Therefore these compounds are tested in several different *in vitro* and *in vivo* models to provide a comprehensive picture before moving into clinical trials. It will remain difficult to predict whether targeting the initial process of alpha-synuclein pathology can improve endpoints at the symptomatic stage of PD, where Lewy bodies are widespread and neurons extensively degenerated. Interference with higher order aggregates is usually avoided as it could be deleterious, because increase in the concentration of lower molecular weight assemblies may produce toxic strains.

Prevent spreading

Transfer of misfolded alpha-synuclein and thus propagation of pathology across the brain is targeted via active (alpha-synuclein mimicking peptides) and passive (antibodies against human alpha-synuclein) immunotherapies. Despite numerous challenges, such as achieving blood brain barrier penetration without targeting intracellular alpha-synuclein, avoidance of unspecific inflammatory responses, and the need for repeated applications, there are several candidates currently in clinical trials. Results so far support brain penetration and acceptable safety profiles, however, the recent failure of a similar strategy in a phase 3 trial with Alzheimer's disease patients cautions to wait for data on clinical endpoints. Knowledge on the exact (disease specific) spectrum of toxic alpha-syn-

uclein species or strains could allow an even more specific antibody to be developed, which is already in progress. Further targets for specific antibodies could be proteins or receptors that specifically facilitate the entry of fibrillary alpha-synuclein into neurons, as recently demonstrated for lymphocyte-activation gene 3 (Mao et al., 2016).

Increase metabolism

Apart from reducing its expression, increasing the metabolism of alpha-synuclein, ideally of its more toxic assemblies, represents another heavily targeted mechanism. Alpha-synuclein degradation pathway involves the lysosome (autophagy). Disturbance of lysosomal function is thought to increase the concentration of aberrant protein assemblies thus contributing to neurodegeneration. Notably, mutations in *GBA1*, the gene for the lysosomal hydrolase acid β -glucosidase, are the most common known genetic risk factor for PD, and the protein is reduced in the substantia nigra of PD patients. Compounds that increase stability and thus activity of this lysosomal protein (Richter et al., 2014; Migdalska-Richards et al., 2017) or generally increase autophagy were successfully tested in preclinical models (extensively reviewed in (Moors et al., 2017)). Among these, Ambroxol, used as expectorant for over 30 years, was surprisingly found to increase the activity of the lysosomal enzyme in a drug screening assay and is now rapidly moving forward in clinical trials.

Summary and outlook

While the current picture of its role in pathogenesis of PD is still incomplete, the high validity of alpha-synuclein as target in PD is beyond doubt. While targeting specific toxic strains is at risk to miss the most relevant species in a patient (or stage of progression), overall downregulation of alpha-synuclein expression will certainly reduce the pathological burden. However, dosage has to be titrated with a potential underappreciated physiological role of the protein in mind. The diversity of the disease likely requires a patient specific combination of strategies in the future. Still, cumulative effects and side effects are difficult to predict. On top of this remains the challenge to measure alpha-synuclein specific pathological endpoints in clinical trials, the hope that the current strategies will not require decades before beneficial effects emerge, and the efforts to diagnose patients early in disease progression prior to overt neuronal loss. Regardless of these caveats,

substantial progress has been made in understanding the disease pathogenesis that hopefully can be translated into disease-modifying therapy for patients in the near future.

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Bionotes

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