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**REVIEW ARTICLE**

# Rationale for Therapeutic Silencing of Alpha-Synuclein in Parkinson's Disease

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The purpose of this paper is to provide the rationale for therapeutic silencing of the alpha-synuclein gene (SNCA) in Parkinson's disease (PD). The paper reviews the public health significance of PD; the causal links between rare SNCA variants and familial PD; the association of common SNCA variants and PD susceptibility; the association of SNCA variants also with age at onset and motor and cognitive outcomes in PD; therapeutic strategies targeting SNCA in PD; and preliminary findings and considerations on small interfering RNA-based therapies and PD.

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**Key Words:** Parkinson's disease, Alpha-synuclein, RNA-based therapies.

## Public Health Significance

Parkinson's disease (PD) is a common aging brain disorder characterized by progressive motor and cognitive impairment.[1,2] The burden of PD to society is staggering. Two percent of people develop PD during their lifetime, and the prevalence of PD is expected to double by 2030.[3,4] PD patients have a seven-fold increased risk of nursing home placement, and a two-fold increased risk of death.[5,6] The total annual cost of PD in the United States was estimated as $23 billion and is expected to grow.[7] Yet we do not know what factors determine motor and cognitive outcomes in PD, and currently there are no established therapeutic targets or disease modifying therapies for PD. These are major data gaps and public health priorities.

## Parkin Genes and Parkinson's Disease

Major progress in understanding the causes of PD, including its motor and cognitive outcomes, can be attributed to the mapping of parkin (PARK) locus genes via linkage analyses. More than a dozen genomic loci have been causally linked to PD in multiplex families. For six of these loci, causal gene mutations (including sequence variations and copy number variations) have been identified. These gene loci include alpha-synuclein (SNCA), parkin (PARK2), ubiquitin carboxy-terminal hydrolase L1 (UCHL1), PTEN-induced putative kinase 1 (PINK1), oncogene DJ-1 (DJ1), and leucine-rich repeat kinase 2 (LRRK2).[8,14] However, SNCA is unique because it is the only PARK locus gene that encodes a protein that is a major component of Lewy bodies (the pathological hallmark of PD).[15] Further, SNCA is the only PARK locus gene for which the pathogenesis has been clearly linked to an over-expression mechanism (amenable to gene silencing therapeutic strategies, the subject of this paper).[16]

## SNCA and Parkinson's Disease

In June 1997, the PD field was transformed by the discovery that a point mutation in the SNCA gene is a cause of familial parkinsonism.[8] While the mutation discovered was very rare, within weeks it was discovered that alpha-synuclein is a principle protein component of Lewy bodies, the pathological hallmark of PD.[15] For several years to follow, it was unclear whether al-

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pha-synuclein mutations caused PD on the basis of a loss of function or a toxic gain of function or another mechanism. In 2003, an additional major breakthrough came with the discovery that triplication of the SNCA locus was a cause of autosomal dominant parkinsonism in an Iowan family.[16] This finding demonstrated that over-expression of wild type SNCA was sufficient to cause PD, elucidating a possible mechanism by which SNCA contributes to the pathogenesis of sporadic PD also (because patients with sporadic as well as familial PD demonstrate alpha-synuclein immunostaining pathology).

## SNCA and Susceptibility to Parkinson's Disease

It has since been demonstrated that while the SNCA point mutations or multiplication mutations that cause familial parkinsonism are very rare, that common variations in the SNCA gene are reproducibly associated with susceptibility to PD in populations worldwide.[17] Specifically, the Genetic Epidemiology of PD (GEO-PD) Consortium demonstrated that short, intermediate, and long allele length variations in the dinucleotide repeat sequence REP1, within the core 5' promoter of the SNCA gene, occurred with different frequencies in PD cases versus controls. Genotypes defined by short alleles were associated with a reduced risk for PD, while genotypes defined by long alleles were associated with an increased risk for PD. Indeed, there was a two-fold difference in PD susceptibility between persons homozygous for short alleles versus persons homozygous for long alleles. Transgenic experiments in humanized mice have demonstrated that short alleles are associated with reduced SNCA mRNA and protein expression levels in brain tissues (two-fold differences also!).[18] Similar patterns of genotype-specific mRNA and protein expression levels have also been observed in human brain tissues.[19] Hence, just as for rare multiplication mutations, common promoter variations in the SNCA gene are associated with PD susceptibility, via a mechanism of over-expression.

## Genome-Wide Association Studies, SNCA, and Parkinson's Disease

The genetic association of SNCA and susceptibility to PD was originally demonstrated using a hypothesis driven, or "candidate gene", approach. Specifically, it was hypothesized that if rare SNCA variants cause familial parkinsonism via a mechanism of over-expression, that common SNCA variations confer susceptibility to sporadic PD via a similar mechanism.[17] However, candidate gene studies have fallen out of favor because the reported associations rarely replicate and because the p-values reported are not adjusted for millions of possible variations across the entire human genome. Hence, genome-wide association studies have become the favored study design for evaluating the association of common genetic variations with complex traits such as PD.[20]

The initial three genome-wide association studies of PD were statistically underpowered and yielded no significant findings after adjusting for multiple comparisons.[20,22] However, there have been five genome-wide association studies since that have been adequately powered to detect single nucleotide polymorphism (SNP) associations with small effect sizes, even after adjusting for multiple comparisons.[23,27] Remarkably, only one genomic locus was independently and unequivocally mapped by all five studies: SNCA! Further, these studies demonstrated that beyond variable number tandem repeat allele variability in the 5' region (REP1) of SNCA, that SNP variability in the 3' region of SNCA is also associated with PD. While the mechanism by which 3' SNP variability is associated with PD is less clearly established than for REP1, a recent study suggested that the mechanism is again related to differences in SNCA expression levels.[28] We have studied the association of more than 20 common variations in the SNCA locus and PD, in a case-control series of 1,103 matched pairs. Both REP1 and the 3' SNP rs2736990 are independently associated with susceptibility to PD in multipoint analyses, with equivalent though small main effects.[29]

## SNCA and Age at Onset of Parkinson's Disease

It is reasonable to expect that if SNCA causes familial parkinsonism and confers susceptibility to PD via a mechanism of over-expression, that PD cases who express higher levels of SNCA might have younger ages at onset than PD cases who express lower levels of SNCA. The relationship of SNCA expression levels and age at onset of PD is best demonstrated in kindreds with multiplication mutations. In families with triplication of the SNCA locus, the age at onset of PD is typically before age 40; while in families with duplication of the SNCA locus, the age at onset of PD is typically in the 40's and later.[30] In the remarkable "Lister" family, triplication of the SNCA locus occurred in one branch while duplication of the SNCA locus occurred in another branch! In that family, cases with triplication mutations developed PD at an average age of 31 years, while cases with duplication mutations developed PD at an average age of 71 years.[31] Because common variations in the SNCA gene share a similar mechanism of pathogenesis as multiplication mutations (over-expression), it was hypothesized that genotypes defined by REP1 or 3' SNP alleles might also associate with age at onset of PD (in addition to susceptibility). Hadjigeorgiou and colleagues first reported in a small sample of 178 PD cases that persons with longer REP1 alleles had an earlier age at onset of PD.[32] However, in a much large series of 2,692 PD cases, the GEO-PD consortium reported no association of genotypes defined by REP1 allele length variants and age at onset of PD.[17] In 1,103 Mayo clinic cases, we recent-

ly reported no significant association of more than 20 different SNCA variants and age at onset of PD, including REP1 and 3' SNPs.[29] Importantly, all cases prospectively enrolled in that study had age at onset uniformly defined (by contrast to the studies conducted in consortia or in small convenience samples). Only one of eight genome-wide association studies of PD reported a genomically significant association of SNCA variability and age at onset of PD.[24] It is possible that because the onset of PD is insidious, that age at onset estimates are inaccurate (accounting for disparate findings from study to study).

## SNCA and Motor Impairment

It is reasonable to also hypothesize that if over-expression of SNCA is a cause of familial parkinsonism or associated with susceptibility to sporadic PD, that SNCA over-expression genotypes are also associated with motor severity of PD. With regard to families with multiplication mutations, historical data on motor severity are limited. There is no clear correlation of SNCA multiplication dose (triplication versus duplication) and motor severity in such families.[30,31] To date, there have been no longitudinal studies of REP1 or 3' SNP variations and motor severity in sporadic PD.

## SNCA and Cognitive Impairment

It is reasonable to also hypothesize that if over-expression of SNCA is a cause of familial parkinsonism or associated with susceptibility to sporadic PD, that SNCA over-expression genotypes are also associated with cognitive impairment in PD. Indeed, the severity and distribution of alpha-synuclein immunostaining pathology has been correlated with dementia in PD.[33] Within families with multiplication mutations, it has been noted that dementia occurs invariably and at an early age when the SNCA locus is triplicated, but only variably and at a later age when the SNCA locus is duplicated.[30,31]

By contrast, two studies of common SNCA variants failed to reveal an association with cognitive impairment in PD (measured crudely by the Mini Mental State Examination).[34,35] Those studies were also limited by very small sample sizes and by either cross sectional study designs or very short intervals of longitudinal follow up. Interestingly, two studies of asymptomatic carriers reported an association of SNCA duplication mutations and SNCA risk haplotypes with performance on quantitative tests of stimulus-reward learning (paradigms sensitive to basal ganglia function; PD is characterized by striatal dopamine deficiency).[36,37]

## Rationale for Therapeutic Targeting of SNCA in Parkinson's Disease

In summary, the rationale for therapeutic targeting of SNCA in PD includes: 1) SNCA point and copy number mutations cause familial parkinsonism; 2) the brains of patients with familial and sporadic PD are characterized by alpha-synuclein immunopathology; 3) common variations in the SNCA gene are unequivocally associated with PD susceptibility worldwide; 4) the mechanism by which SNCA gene variability causes or confers susceptibility to PD has been defined (over-expression); 5) SNCA variability may also be associated with disease severity, as measured by age at onset or motor and cognitive tests.

## Therapies Targeting SNCA in Parkinson's Disease

Nearly 14 years have passed since the landmark genetic discoveries linking the SNCA locus with familial parkinsonism and identifying alpha-synuclein immunopathology as the hallmark of PD. There is no genomic locus that is more clearly associated with PD susceptibility than SNCA. A mechanism by which SNCA contributes to the pathogenesis of PD (over-expression) has been defined. Yet remarkably, there has never been a single clinical trial of a therapy that specifically targets SNCA in PD! Given that there is no established method of meaningfully slowing or halting the progression of PD, and given the public health significance of PD, this is an unacceptable failure on the part of the academic scientific community and the pharmaceutical and biotechnology industries.

This said, a number of preclinical studies have explored different methods of therapeutically targeting SNCA in PD and related synucleinopathies. Some of these methods attempt to reduce alpha-synuclein protein aggregation; others attempt to promote alpha-synuclein sequestration; and others attempt to reduce SNCA expression.[38,40] This paper will focus on therapies to reduce SNCA expression, because the pathogenetic mechanism of over-expression is clearly defined.

## Therapeutic Silencing of SNCA in Parkinson's Disease

Following the observation that SNCA multiplication mutations cause familial PD via a mechanism of over-expression, it was envisioned that PD could be treated via targeted brain delivery of small interfering RNAs (siRNAs) that silence SNCA expression. SiRNAs silence the expression of genes by binding gene-specific mRNA transcripts to RISC complexes that destroy the transcript ("killing the messenger").[41,42]

It has been demonstrated in cultured catacholaminergic neurons that SNCA expression can be silenced. It has also been demonstrated in infused mice hippocampi that SNCA can be silenced and in the absence of adverse behavioral effects or inflammation. SiRNA silencing of SNCA in mice brains is highly selective and lasts for about two weeks in duration.[40]

More recently, several experiments in infused monkey substantia nigras have demonstrated siRNA silencing of SNCA expression by 40-50% and in the absence of inflammation or alteration of the dopaminergic neuronal phenotype.[43]

## Considerations on Small Interfering RNAs Delivery

There are limitations to the therapeutic strategy of siRNA silencing of SNCA in PD. SiRNAs must be delivered to the brain directly, as they are not delivered to the bloodstream when administered orally and as they are not delivered to the brain tissues when administered intravenously (blood-brain barrier). There is some evidence that siRNAs administered intrathecally silence gene expression in adjacent tissues.[44,46] However, it would appear unlikely that siRNAs injected intrathecally would be effectively delivered to the substantia nigra, other pigmented brainstem nuclei, or the limbic and neocortex (brain regions remote from the cerebrospinal fluid system and demonstrating alpha-synuclein immunopathology in PD).

While it is possible with available surgical technologies to infuse siRNAs directly to the parenchyma of targeted brain regions such as the substantia nigra, it must be acknowledged that the pathology of PD is distributed to multiple regions of the brain over time.[47] Therefore, while silencing SNCA expression in the substantia nigra may slow motor progression in PD, it may not prevent cognitive impairment in PD (which is correlated with neuropathology in cortical brain regions). While SNCA silencing in mice and primate experiments lasts several days, it is clear that intermittent or continuous delivery of siRNAs would be required to therapeutically silence SNCA in PD. Hence, retroviral delivery of siRNAs should not be an effective method of sustained reductions of SNCA expression.

However, there are many advantages to the therapeutic strategy of siRNA silencing of SNCA in PD. This approach is agnostic to the mechanism by which alpha-synuclein triggers cell death in PD. By simply reducing the expression of SNCA, we can reduce the quantity of alpha-synuclein protein, which should result in reduced neuronal death and disease progression (certainly in patients with multiplication mutations or with overexpression genotypes, if not in all patients with alpha-synuclein immunopathology). By directly infusing siRNAs to brain parenchyma, the blood brain barrier is no longer a limitation. There is a precedent for surgical therapies for PD, including experimental infusion therapies (e.g., glial cell line-derived neurotrophic factor).[48] Hence therapeutic strategies to silence SNCA need not be limited to oral or intravenous administration of small molecules (which lack tissue or brain region specific selectivity). By contrast to vaccine therapies, siRNA therapies do not produce brain inflammation and CNS toxicity.[49] Further, direct brain delivery of siRNAs should not produce systemic toxicities. For example, SNCA is expressed in red blood cells.[50] Might systemic therapies targeting SNCA expression cause hematological complications? Recent studies have associated anemia and PD, and it can been speculated that in some patients anemia and PD may be pleiotropic manifestations of altered SNCA expression.[51]

Presently, deep brain stimulation (DBS) is a highly effective treatment to reduce advanced motor symptoms of PD and complications of dopaminergic therapies. However, it does not slow PD progression. Further, there is a reluctance to perform early DBS surgeries because of the associated risks and costs. This is despite evidence that early DBS therapy may provide better outcomes than early medical therapy (and reduced long term costs).[52] SiRNA infusion therapy may provide a solution to this barrier to early DBS surgery. Namely, DBS probes are typically positioned with the caudal tip in or just above the substantia nigra, while the stimulating electrodes are typically positioned a few millimeters rostrally in the subthalamic nucleus. What if instead of implanting a solid probe, we implanted a cannula probe? The open end of the cannula would be positioned in the substantia nigra, so that with one surgery patients would be eligible to receive both intraparenchymal siRNA infusion therapy (neuroprotection) and DBS (palliation). Because neuroprotection is most appealing when administered early, the combination of intraparenchymal siRNA infusion therapy and DBS would make a compelling case for earlier surgery in PD.

Finally, there is evidence that alpha-synuclein protein is transmitted neuron to neuron and that this transmission is time dependent.[53] It has been suggested that neuron-to-neuron transmission of SNCA may account for the specific pattern of caudal to rostral spread of the brain pathology. Therefore, early infusion of siRNA to the substantia nigra may not only slow motor progression in PD, but may also prevent neuron-to-neuron transmission of alpha-synuclein and hence also cognitive impairment (by sparing rostral cortical brain regions of alpha-synuclein toxicity and pathology).

## Is It Safe to Reduce SNCA Expression?

The function of the alpha-synuclein protein in man is not well known. Alpha-synuclein was first functionally described as a protein that is regulated during the critical period for song learning in the zebra finch.[54] Because of its localization to the synaptic terminal of neurons (“synuclein”), and because it is upregulated in the adult mammalian brain following trauma or toxic insults, it is possible that alpha-synuclein plays a role in neuronal repair.[55,56] Similarly, because of its localization to the synaptic terminal, alpha-synuclein may play an important role in synaptic transmission.

Accordingly, what is the evidence that it is safe to reduce SNCA expression? There is no evidence in human subjects. However, there is evidence in other mammals. When SNCA is

knocked out in mice, the mice are viable, fertile, and motorically normal; however, one study suggested that SNCA knock out mice also exhibit increased brain reward due to disinhibition of the dopaminergic limbic system.[57] In human subjects, might reducing SNCA expression lead to addictive or compulsive behaviors or other psychiatric states? This is a relevant question because dopamine agonist therapies in PD are associated with compulsive behaviors.[58]

## Is It Effective to Reduce SNCA Expression?

Short of a clinical trial, it can't be determined if therapeutic silencing of SNCA is an effective treatment for PD. There are no satisfying experimental models for PD, within which siRNA therapies targeting SNCA can be evaluated. In monkey experiments, siRNAs targeting SNCA reduced MPTP induced neuronal toxicity by about 20% (D. Bumcrott 2009, personal communication). However, nonhuman primate models of PD are not without limitations.[59]

Therefore, we have invented a concept called "virtual clinical trials" to provide proof of principle for therapeutic silencing of SNCA. Specifically, within defined PD cohorts, one can employ available genotypes defined by SNCA REP1 alleles to stratify the cohorts into three groups: reduced expression genotypes (homozygous, short allele), intermediate expression genotypes (heterozygous, short allele), and increased expression genotypes (null, short allele). One can then measure survival free of time dependent outcomes in the cohorts, such as motor impairment (e.g., survival free of Hoehn and Yahr stages 4 or 5) and cognitive impairment (e.g., survival free of Mini Mental State Examination Score < 26 or TICS-M score ≤ 28 or AD-8 ≥ 2). One can then perform Cox proportionate hazard analyses to determine whether reduced expression genotypes are associated with statistically significant, lower hazard ratios. Presently we are conducting virtual clinical trials of SNCA reduced expression and survival free of PD motor and cognitive outcomes, in a cohort of 1,103 PD cases ascertained at the Mayo Clinic in Rochester, MN; and of survival free of death, in a cohort of > 6,000 PD cases ascertained at 20 GEO-PD consortium sites within 13 countries and 4 continents. Fig. 1 is a visual illustration of the principles underlying these virtual clinical trials.

Virtual clinical trials have several advantages over clinical trials. First, they do not require exposure of subjects to an experimental therapeutic agent (no risk). Second, they do not require a manufactured therapeutic agent (only one that is conceptualized). Third, they may provide data for large samples over extended periods of times (beyond the scope of clinical trials and at much lower costs). As the results of virtual clinical trials become available, we hope that a compelling case can be made to the pharmaceutical and biotechnology industry to bring therapies targeting SNCA expression forward as clinical trials.

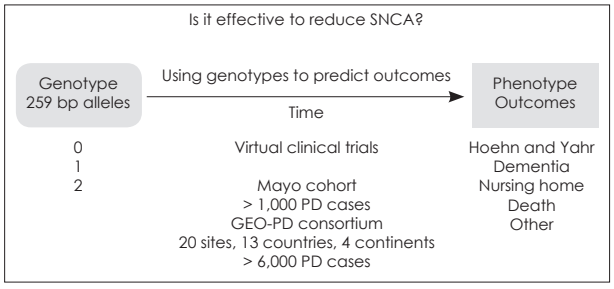


**Figure 1.** Illustration of the concept of virtual clinical trials as a method of predicting the efficacy of therapies to reduce SNCA expression of Parkinoson's Disease.

The first clinical trials specifically targeting SNCA will represent a historic event and will hold great promise as methods to slow and halt the progression of PD.

### Conflicts of Interest

The author received more than $10,000 in royalty payments from Alnylam Pharmaceuticals, Inc., regarding a method to treat PD through reduced SNCA expression. The author also received an investigator initiated, industry sponsored grant from Alnylam Pharmaceuticals, Inc. and Medtronic, Inc. to conduct virtual clinical trials in PD.

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