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Implication of Alpha-synuclein Transcriptional Regulation and Mutagenesis in the Pathogenesis of Sporadic Parkinson's Disease

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IMPLICATION OF ALPHA-SYNUCLEIN TRANSCRIPTIONAL REGULATION AND MUTAGENESIS IN THE PATHOGENESIS OF SPORADIC PARKINSON’S DISEASE

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Burnett School of Biomedical Sciences in the College of Medicine at the University of Central Florida Orlando, Florida

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Major Professor: Yoon-Seong Kim
ABSTRACT

Parkinson's disease (PD) is an age-related neurodegenerative disorder characterized by selective loss of dopaminergic neurons (DA neurons) from the substantia nigra (SN) of the mid-brain. PD is classically associated with cytoplasmic inclusion of aggregated proteins called Lewy bodies. alpha-synuclein (α-SYN) coded by the gene SNCA, is one of the major components of Lewy body and neurite along with several other proteins like ubiquitin, neurofilament to name a few. PD is broadly categorized into two groups based on their incidence of occurrence. First is the familial form that occurs due to known genetic aberrations like mutation, gene duplication/triplication in important PD associated gene like SNCA which in turn leads to early-onset PD (EOPD). Second is the late-onset idiopathic or sporadic form, whose origin of occurrence is often unknown. Interestingly, more than 90%-95% of reported PD cases belong to the latter category. Although, the familial and the idiopathic form of PD are different in their respective cause of occurrence, aggregation of α-SYN into Lewy body is a common pathologic hallmark seen in both. Aggregation of α-SYN in turn is strongly implicated by the transcriptional upregulation of the gene as seen in both familial forms as well as idiopathic forms.

In this thesis, we first describe the designing and functioning of a novel tool to monitor real-time SNCA transcription in Human Embryonic Kidney (HEK) 293T cells. In the next part, we shed light into a novel transcriptional deregulation phenomenon called transcriptional mutagenesis, which leads to accelerated aggregation of α-SYN as seen in sporadic PD. In brief, the focus of this work is to highlight the importance of
transcriptional regulation of SNCA gene, through development of a tool and a mechanism affecting the fidelity of transcription under pathologic condition.

In the first study, we developed a stable cell line in HEK293T cells in which \( \alpha \)-SYN was tagged with Nanoluc luciferase reporter using CRISPR/Cas9-mediated genome editing. Nanoluc is a small stable reporter of 19KDa size, which is 150 fold brighter compared to firefly and Renilla luciferase, thus making it a very good candidate for endogenous monitoring of gene regulations. We successfully integrated the Nanoluc at the 3’end of the SNCA before the stop codon. Successful integration of the Nanoluc was demonstrated by the fusion \( \alpha \)-SYN protein containing the Nanoluc. This allowed efficient monitoring of \( \alpha \)-SYN transcription keeping its native epigenetic landscape unperturbed which was otherwise difficult using exogenous luciferase reporter assays. The Nanoluc activity monitored by a simple two-step assay faithfully reflected the endogenous deregulation of SNCA following treatment with different drugs including epigenetic modulators and dopamine which were already known to up-regulate SNCA transcription. Interestingly, use of exogenous promoter-reporter assays (firefly luciferase assays) failed to reproduce the similar outcomes. In fact, exogenous system showed contradictory results in terms of the \( \alpha \)-SYN regulation which aroused from spurious effects of the drug on the reporter system. To our knowledge, this is the first report showing endogenous monitoring of \( \alpha \)-SYN transcription, thus making it an efficient drug screening tool that can be very effectively used for therapeutic intervention in PD.

In the next study, we investigated the effect of oxidative DNA damage in the form of 8-hydroxy-2-deoxyguanosine (8-oxodG, oxidized guanine) on aggregation of \( \alpha \)-SYN
through a novel phenomenon called transcriptional mutagenesis. It is already known that 8-oxodG is repaired by a specific component of the base excision repair machinery of the cell called 8-oxodG-DNA glycosylase 1 (OGG1). If left unrepaired, 8-oxodG can lead to misincorporation of adenine instead of cytosine (C→A transversion) in the synthesized mRNA during transcription for post-mitotic cells like neurons. This phenomenon is called transcriptional mutagenesis (TM) and can generate novel mutant variants of any functional protein. α-SYN, which is implicated very strongly in the pathogenesis of PD, has been shown to become aggregation prone by specific point mutation. Previous studies have shown that certain point mutations can make α-SYN more prone to aggregation and can affect the aggregation of the parental protein as a template directed misfolding mechanism. We used SNCA as a model gene and predicted the generation of forty-three different positions that can be mutated by the TM event. We investigated the generation of three out of the forty-three possible TM mutants from the SN of post-mortem PD and age-matched control brain cohorts based on their potential to aggregate as predicted by aggregation prediction software TANGO. The three mutants were Serine42Tyrosine (S42Y), Alanine53Glutamate (A53E) and Serine129Tyrosine (S129Y). We confirmed the presence of all the three mutant α-SYN (S42Y, A53E and S129Y) in SNCA mRNA from the SN of human post-mortem PD brain using a PCR-based detection technique. As expected, analysis of the overall distribution of the three mutants showed a higher rate of occurrence in the PD cohort compared to the age-matched controls. Sequencing genomic DNA of the same PD sample from the same region of α-SYN revealed no mutations at the genomic DNA level, thus implying
its generation during transcription. Although we could detect the presence of S42Y, 
A53E and S129Y α-SYN in the cohort of PD patients, we focused to analyse the 
contribution of S42Y towards the aggregation of wild-type (WT) α-SYN parental protein 
based on its higher potential to aggregate. By using cell-based biochemical and 
recombinant protein assays, we saw that S42Y-α-SYN can accelerate the aggregation 
process involving the WT protein even when present in significantly lower proportion 
(100 times less compared to the WT). Importantly, we developed antibody to specifically 
detect the S42Y α-SYN in human PD cohort. Immunohistochemical analysis of serial 
post-mortem PD brain sections with Hematoxylin and Eosin staining (H&E), anti- 
ubiquitin staining and anti-S42Y α-SYN staining, showed Lewy bodies that stained 
positively with S42Y α-SYN. To our knowledge, this is the first report about TM related 
mutations of α-SYN in Parkinson's disease and their role in the pathogenesis.
Dedicated to

To my Parents (Mr. Sudipta Basu & Mrs. Sipra Basu)

&

To my nieces Oishi & Shreyangshi
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LIST OF ABBREVIATIONS

5-azadC – 5-aza cytidine
8-oxodG- 8-hydroxy-2-deoxyguanosine
BER – Base excision repair
CRISPR – Clustered regularly interspaced short palindromic repeats
DA- Dopaminergic neurons
DNMT1 – DNA methyl transferase 1
EOPD – Early onset Parkinson’s disease
GWAS – Genome wide association studies
HDAC – Histone deacetylase
HDR – Homology dependent repair
LB – Lewy bodies
LOPD – late onset Parkinson’s disease
NAC – Non-amyloid component of alpha-synuclein
OGG1- 8-oxodG-DNA glycosylase 1
PD – Parkinson’s disease
RNA Pol II – RNA polymerase subunit II
ROS – Reactive oxygen species
SNCA- Gene encoding alpha-synuclein
SNpc – Substantia nigra pars compacta
TM- Transcriptional Mutagenesis
CHAPTER 1: GENERAL INTRODUCTION TO PARKINSON’S DISEASE

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1.1 Parkinson’s disease

Parkinson’s disease (PD) is an age-related progressive neurodegenerative disorder affecting approximately 60,000 people every year with a total of one-million people affected alone in the United States (Statistics from Parkinson’s Disease Foundation). The first clear description of the medical relevance of the disease as a neurological syndrome was established by James Parkinson in the year 1817. He described the very early beliefs about the disease in his essay called “An essay on the Cerebral Palsy” where he described Parkinson’s disease as “Involuntary tremulous motion, with lessened muscular power, in parts, not in action and even when supported; with a propensity to bend the trunk forward, and to pass from a walking to a running pace; the senses and intellects being uninjured.” ¹.

1.1.1 History of Parkinson’s disease

Jean-Martin Charcot was particularly thorough in his description of Parkinson’s disease and also distinguished bradykinesia or tremor at rest as an integral feature of the disease. In his teaching, he wrote “Long before rigidity actually develops, patients have significant difficulty performing ordinary activities: this problem relates to another cause.
In some of the various patients I showed you, you can easily recognize how difficult it is for them even though rigidity or tremor is not the limiting feature. Instead, even a cursory exam demonstrates that their problem relates more to slowness in execution of movement rather than to real weakness. Inspite of tremor, a patient is still able to do most things, but he performs them with remarkable slowness. Between the thoughts and the action there is a considerable time lapse. One would think neural activity can only be affected after remarkable effort. “ 2. Charcot and his co-workers noted the two most important spectrum of the disease, namely, the tremor and the rigidity. Charcot is also accredited as the first person to suggest the use of the term “Parkinson’s disease”, rejecting the earlier designation of shaking palsy as coined by James Parkinson’s 2. In the year 1888, William Gowers, contributed an important study to the demographics of Parkinson’s disease where he described his personal experience with 80 patients suffering from the disease. He also identified the slight bias of male patients suffering from the disease and also studied the joint deformities seen typically in patients. The details of his work were described in his “Manual of Diseases of the nervous system” 3. More detailed clinical descriptions of the disease and the pathological changes related to it were reported by Richer and Meige in the year 1895. In their description, Richer and Meige et al. provided morphological details about the progressive stages of the disease. They also gave a valued pictorial representation and statues that remain as most important documents related to the discovery of PD till date. The strange motor fluctuations as seen in the disease were first reported by Babinski in 1921.
However, it was Brissaud in the year 1925, who first showed that damage to the SN as the anatomical change that occurs during PD. This discovery led to further pursuing of pathological studies of the midbrain during PD in the late 1920’s. In the year 1953, Greenfield and Bosanquet came up with the most complete analysis in relation to the pathology seen during PD conditions. They are accredited with delineating, the progression of the lesion seen in the brain stem. Based on this discovery, Hoehn and Yahr in the year 1965 came up with their internationally recognized staging system to study progression of PD. In this time dependent staging system, they delineated the stages as: Unilateral (Stage I), Bilateral (Stage II - IV) and development of postural reflex impairment (Stage III) as significantly key stages in the disease.

1.1.2 Neuropathology of Parkinson’s disease

PD is clinically characterized by bradykinesia, tremor, rigidity and postural instability. However, there are an array of disorders of the nervous system that can share some or all of these clinical features. Together, the clinical syndrome is referred to as “parkinsonism”. PD can also have non-motor symptoms associated with it, such as cardiovascular or gastrointestinal symptoms, hyposmia and olfactory dysfunctions, anxiety, depression and dementia. Taken together, the disorders that comprise Parkinsonism syndrome are collectively called parkinsonian disorders, which may be degenerative or non-degenerative in nature, depending on the fact whether it leads to degeneration of the affecting neurons. Pathologists classify most degenerative parkinsonian disorders into two major classes based on their underlying mechanism of
accumulating the pathologic microtubule associated protein (MAPT/Tau) or presynaptic protein alpha-synuclein (α-SYN) - (1) tauopathies or (2) alpha-synucleinopathies \(^5\). The other degenerative parkinsonian disorder characterized by α-SYN inclusion is multiple system atrophy (MSA). However, in MSA, not only the nigrostriatal pathway, but also the cortical afferent pathways are affected. The α-SYN inclusions of neurons seen in MSA are only a minor component of the pathological feature unlike PD. This disorder is rather characterized by glial cytoplasmic inclusions and also α-SYN inclusions within the nucleus, a feature not seen in PD\(^7,8\). Neuronal inclusions composed of tau protein are most commonly seen in progressive supranuclear palsy (PSP). In PSP, inclusions of tau are also seen in the glial cells comprising both the microglia and astrocytes\(^9\). PSP and MSA are often referred to as “Parkinsonian plus” disorder since both these disorders consistently include other neurologic features which are not clearly outlined under Parkinsonism such as eye movement problems, dementia, autonomic dysfunction and also sleep disorders\(^10\).

In terms of pathology, PD is characterized by loss of dopamine (DA) producing neurons from the substantia nigra pars compacta (SNpc) region of the mid brain. Positron emission tomography or PET imaging have clearly shown that the very initial symptoms of PD starts appearing after the loss of 60-80% DA neurons\(^11\). The pathological hallmark of PD is cytoplasmic proteinaceous neuronal inclusion composed primarily of the pre-synaptic protein, α-SYN. These inclusions are often present in the perikarya, and are collectively termed as Lewy bodies (LB’s). Similar proteinaceous processes are also seen in the DA producing neurons, which are called Lewy neurites. The
combination of Lewy bodies and Lewy neurites are collectively referred to as Lewy body pathology, which is an integral part of the PD pathologic feature 12 (Figure 1).

A detailed analysis of the neuropathology of PD has been done both at the macroscopic and microscopic level. Tissue sections of brain stem from PD patient samples have revealed loss of dark black pigment in the SN region and the locus ceruleus (LC). The loss of this dark colored pigment indicates the loss of pigment (neuromelanin) containing DA neurons in the SN and noradrenergic neurons in the LC. In case of PD, atrophy is mostly seen in the midbrain region encompassing the nigrostriatal pathway. Frontal cortical atrophy is usually not found in case of PD patients5.
Figure 1 Pathological hallmark of Parkinson’s disease showing the aggregated Lewy body of Substantia nigra in PD.

(Left) Hematoxylin & Eosin staining of SN section from PD brain shows the presence of Lewy Body (shown with the arrow) like structure under 40X resolution. (Right) Microscopic image for ubiquitin (Ub) immunohistochemistry (1:250) showing Lewy body like structure.
As described earlier, microscopically LB’s are pathologic hallmark of PD. Hematoxylin and Eosin (H&E) staining of LB’s have shown these inclusions to be hyaline like in appearance, whereas in less vulnerable neurons like cortical neurons, the α-SYN inclusions appear as a pale staining and poorly circumscribed \(^{13}\). It is believed based on supporting literature that pale bodies and cortical LB’s may precede the formation of classical LB’s. However, in certain severe cases of PD, matured LB’s can also be detected in the adjoining areas such as amygdala and cortex, mostly in the limbic cortex \(^5\). It has been shown by ultrastructural analysis that LB’s are mainly composed of dense and granular material which form straight filament like structure approximately 10-15nm in diameter \(^{14,15}\). The exact compositions of LB’s are widely being researched even today. α-SYN is a robust component of the LB’s, particularly in the halo of the LB’s \(^{12}\). However, some of the components that have been shown to be present consistently in LB’s are neurofilament protein \(^{16}\), Ubiquitin \(^{17}\) and the ubiquitin binding protein p62 \(^{18}\). A small population of LB’s show positive immunoreactivity for tau protein also, and these neurons are often vulnerable to tau pathology \(^{19}\).

### 1.1.3 Distribution of pathology in PD

The hallmark of any neurodegeneration associated disease like PD, MSA, PSP or Alzheimer’s disease (AD) involves selective loss of neurons from a particular region of the brain. Moreover, along with this degenerative process there are a lot of accompanied changes brought about in the affected region. These changes primarily include reactive microglia and astrocytes which starts expressing markers of activation
like class II major histocompatibility antigen HLA-DR (for microglia) and glial fibrillary acidic protein (for astrocytes). Besides, the glial activation process, it has also been seen that α-SYN containing LB’s can spread far beyond the SN region of the midbrain thus affecting other regions also.

Based on the distribution of pathology related to α-SYN, Braak and coworkers proposed a staging scheme for its progression. According to this scheme of staging, the neuronal pathology starts early in the dorsal motor nucleus in medulla and olfactory nucleus in the olfactory bulb. With progression of the disease, it spreads to LC in the pons and then onto the dopaminergic neurons in the substantia nigra. In later stage of the disease, it spreads to the basal forebrain, amygdala and temporal lobe. Finally the cortical areas get affected at the very last stage. It has been reported that the motor symptoms associated with PD generally starts from Stage 3 onwards when the amygdala and SN gets affected. Most of the cognition related problems starts appearing once the cortex and neocortex gets affected.

A limiting factor for the Braak staging scheme is that, it is based on the distribution of the aberrant α-SYN deposition and not on the neuronal loss. How this staging procedure relates to the neuronal loss as seen in PD has not been studied in details. This scheme was based on individuals who showed early medullary pathology thus biasing the staging progression. However, in more recent studies on PD patients, the Braak staging does not always hold true. It has been observed that for some individuals the LB’s are confined to the olfactory bulb or the amygdala only, especially if it is associated with Alzheimer’s type pathology. Conversely, some individuals who are
normal show sparse but widespread LB pathology involving even the cortex\textsuperscript{24,25}. This observation fits better to a theory of multicentric disease process from the onset. Based on the cases studied, it was concluded that progression of PD stages depends solely on the individuals where the progression is studied\textsuperscript{26}.

The progression of PD as predicted and hypothesized by Braak and coworkers fed to the long standing debate about cell-to-cell transmission of unknown prion like disease factors such as α-SYN aggregates as shown in the study where fetal mesencephalic intrastriatal transplants to treat PD have been shown to develop LB pathology\textsuperscript{27,28}. Recent studies have shown that intrastriatal injection of recombinant mouse α-SYN assemblies into WT mice led to the formation of aggregated pathology and brain dysfunction as seen in PD\textsuperscript{29}. This observation was supported by studies which have shown that intrastriatal injection of α-SYN assemblies into WT mice and WT primates resulted in the formation of inclusions comprising endogenous α-SYN\textsuperscript{30,31}.

To support the early non-motor symptoms of PD, studies have reported the existence of Lewy pathology in the in the enteric nervous system, with a possibility that the pathology spreads from the enteric nervous system (ENS) to the central nervous system (CNS) through peripheral nervous system (PNS)\textsuperscript{32,33}.

1.1.4 Animal models in Parkinson’s disease

To understand the pathogenic mechanism that underlies PD and to evaluate the therapeutic interventions of PD, numerous animals models have been developed over the years. These animal models have proven to be an extremely important tool in PD
research, and understanding the strengths and limitations of these models can impact the choice of model for a study and related experimental designs.

An ideal model for PD should reflect both the clinical as well as the pathological hallmarks of the disease which involves both dopaminergic and non-dopaminergic system, central nervous system, peripheral nervous system and also the motor symptoms. Moreover, the model should also be reflective of an age dependent and progressive characteristic of PD. Unfortunately, all the PD animal models till date have failed to simulate the actual clinical and pathological features of PD. Yet, over years the contribution of PD animal models in deciphering the mechanism of pathogenesis and therapeutic interventions has been tremendous.

Current animal models of PD can be classified broadly into two categories- namely the genetic model and the neurotoxic model. Genetic models are created primarily on the basis of identified targets that can serve as a potential mechanism of PD pathogenesis in humans (for example A53T alpha-synuclein overexpressing animals or the G2019 Leucine rich repeat kinase -2 (LRRK2) animals)\textsuperscript{34,35}. Most of the genetic models of PD have been characterized to show motor symptoms due to the behavioral deficits observed in them. However, these models fail to display an appreciable neurodegeneration as seen in PD\textsuperscript{36}. Some of the important genetic models of PD are listed:

**Alpha-synuclein transgenic PD model:** Familial mutations (as discussed in the genetics of SNCA section) and increased levels of α-SYN due to gene duplication or triplication, lead to increased aggregation of the protein as seen in Lewy body like structures\textsuperscript{37,38}. 
**LRRK2 transgenic PD model:** LRRK2 is a large protein kinase, which has GTPase and kinase domains in addition to leucine rich-repeat domains. Dominant mutations in the LRRK2 protein such as G2019S, R1441C and R1441G are considered to be one of the most common causes of familial PD, which leads to reduced or aberrant kinase activity of the protein\(^49\).

**PARKIN genetic model:** Mutations in the PARKIN (*PRKN*) gene have been strongly associated with autosomal recessive juvenile PD\(^54\). This led to the development of several knock out animals of *PRKN* gene trying to model PD\(^54\)-\(^56\).

**PINK1 genetic model:** PINK1 is a serine/threonine protein kinase which is important for the recruitment of the parkin E3-ligase. Important disease-associated mutations in *PINK1* block the ability of PINK1 to recruit parkin and perform the physiological functions. Moreover, PINK1 has an amino terminal mitochondrial targeting sequence, which definitely indicates an important dysfunctional nexus of PINK1 and mitochondria in PD\(^61\).

**DJ-1 genetic model:** Investigators in the past have researched the role of DJ1 in cell based models. The data collected from these experiments indicated DJ-1’s role as a redox sensitive molecular chaperone that provides protection against cellular stresses\(^64\)-\(^66\).
### Table 1: Autosomal dominant gene based animal models of PD

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Transgene</th>
<th>Cell death</th>
<th>Motor function</th>
<th>Pathological features</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF-β</td>
<td>WT α-SYN</td>
<td>No Neurodegeneration</td>
<td>Rotarod performance decrease, TH and fiber density decline</td>
<td>Inclusion body (positive for α-SYN and Ub)</td>
</tr>
<tr>
<td>mThy-1</td>
<td>A30P α-SYN</td>
<td>Neuronal loss from brainstem and SC</td>
<td>Severe leading to paralysis @8-12months</td>
<td>LB-like inclusion</td>
</tr>
<tr>
<td>mPrP</td>
<td>A53T α-SYN</td>
<td>Neuronal loss from red nuclei, brainstem and SC</td>
<td>Severe motor deficit @12 months</td>
<td>LB-like inclusion with mitochondrial dysfunction</td>
</tr>
<tr>
<td>Rat TH</td>
<td>A30P/A53T α-SYN</td>
<td>Progressive DA neuronal loss</td>
<td>Decline in locomotor activity and reduced DA metabolites</td>
<td>No inclusion/diffuse α-SYN in DA neurons</td>
</tr>
<tr>
<td>CamKII-rTA (tet off)</td>
<td>WT/A53T α-SYN</td>
<td>Trend seen for TH neurons loss in SN and hippocampus (WT)/ non-DA neuronal loss (A53T)</td>
<td>Decline in Rotarod performance and motor learning</td>
<td>No inclusion</td>
</tr>
<tr>
<td>BAC</td>
<td>LRRK2 (R1441G)</td>
<td>No</td>
<td>Rearing @12months, decreased DA transmission in striatum, Decreased fiber density (SNr)</td>
<td>Increase in Tau and Phospho-tau</td>
</tr>
</tbody>
</table>

TH: tyrosine hydroxylase; Ub: Ubiquitin; SC: Spinal Cord; DA: Dopamine; SNr: Substantia nigra pars reticulata (Adapted from *Cold Spring Harb Perspect Med* 2012)
Table 2: Autosomal recessive gene based animal models of PD

<table>
<thead>
<tr>
<th>PD gene knock-out</th>
<th>Motor deficit</th>
<th>Electrophysiological/neurochemical dysfunction</th>
<th>Biochemical changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parkin</td>
<td>Decline in voluntary activity, beam traversing, startle response</td>
<td>Increased DA in striatum and limbic region, decrease in NE (OB and SC), decreased striatal neuronal excitation</td>
<td>Increased levels of reduced glutathione and Parkin substrates, decreased mitochondrial and antioxidant proteins</td>
</tr>
<tr>
<td>PINK1</td>
<td>Age-dependent decline in spontaneous activity</td>
<td>Decreased DA release and content in striatum</td>
<td>Increased large mitochondria, mitochondrial respiration, ATP generation, decreased acotinase activity in the striatum</td>
</tr>
<tr>
<td>DJ-1</td>
<td>Age-dependent decline in locomotor activity, rearing</td>
<td>Increased DA re-uptake and DA content in striatum</td>
<td>Decreased mitochondrial peroxidase activity, Normal amounts of TH, DAT and oxidized protein</td>
</tr>
</tbody>
</table>

OB: Olfactory bulb; Ub: Ubiquitin; SC: Spinal Cord; DA: Dopamine; DAT: Dopamine transporter (Adapted from *Cold Spring Harb Perspect Med 2012*)
From the description of the genetic models of PD, it can be concluded that a tremendous demand of genetic PD mouse models still exist that can reflect the degeneration of DA neurons in a manageable time frame to develop therapeutic interventions to prevent the neuronal loss. Once the PD related genetic defects can be modeled in mice achieving the significant loss of DA neurons, the overall picture of the signaling events directly relevant to the neuronal loss can be deciphered. Moreover, these limitations of the genetic models can be complemented by the advent of the neurotoxic models in which DA neuronal death is achieved by use of damaging molecules found in the environmental insecticides, pesticides and other form of toxins. The major neurotoxic models of PD are described below in the format of a chart, which includes 6-hydroxydopamine, MPTP, Paraquat, Maneb and Rotenone.
Table 3: Table describing the various neurotoxic animal models of PD with their distinctive pathology and behavioral deficits.

<table>
<thead>
<tr>
<th>Models</th>
<th>Pathology</th>
<th>Behavioral deficits</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nigrostriatal damage</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SN cell body</td>
<td>Striatal damage</td>
</tr>
<tr>
<td></td>
<td>Striatal damage</td>
<td>Striatal dopamine</td>
</tr>
<tr>
<td></td>
<td>Extranigral pathology</td>
<td>Lewy bodies</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Motor</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nonmotor</td>
</tr>
<tr>
<td>6-OHDA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat (stereotaxic injection to SN, Str)</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>MPTP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-human primates (i.p., i.m., intracarotid infusion)</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>Mouse (acute, subacute i.p.)</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>Chronic (osmotic minipump)</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>Paraoquat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse (i.p.)</td>
<td>YES</td>
<td>Sometimes</td>
</tr>
<tr>
<td>Paraoquat/Maneb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mouse (i.p.)</td>
<td>YES</td>
<td>Notdetermined</td>
</tr>
<tr>
<td>Rotenone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat (infusion via minipump)</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>i.p. injection</td>
<td>YES</td>
<td>YES</td>
</tr>
</tbody>
</table>

ND: Not determined; LC: Locus Coeruleus; GI: gastro-intestinal (Adapted from *Cold Spring Harb Perspect Med* 2012)
1.2 Role of alpha-synuclein in PD

α-SYN is a presynaptic protein that contributes to the pathogenesis of PD both genetically as well as neuropathologically. The very initial demonstration of the molecule being an important causative factor of PD was established by the fact that genetic defects in the α-SYN gene (SNCA) could lead to early onset PD (EOPD). In 1990, an Italian-American family was reported to have EOPD with confirmed Lewy pathology. In the year 1996 Nussbaum and colleagues identified genetic markers in chromosome 4 (q21-q23) that caused the EOPD. In 1997, Polymeropoulos mapped the discrepancy in chromosome 4 to SNCA that was mutated in the Italian-American family, leading to the first ever report that linked SNCA to PD. This discovery lead to a series of ground breaking studies to investigate the genetic basis of the disease, culminating in Genome wide Association studies (GWAS) and also the pathologic role of the molecule in PD.

The importance of targeting α-SYN as a potential lead in PD developed from parallel studies which showed abnormal α-SYN depositions in cytoplasm of DA neurons from PD specimens as well as other synucleinopathies (a collective term for all neurodegenerative condition where α-SYN deposits are seen). Gradually, these aggregated deposits of α-SYN became a hallmark of PD.

1.2.1 Structure of alpha-synuclein

α-SYN is a member of synuclein family of proteins, which includes β and γ synucleins also. Structurally, α-SYN is a 140 amino acid protein and natively unfolded. It can exist
as random-coil state as well as β-sheet conformation upon aggregation or α-helical conformation upon binding to membranes. Sequence of α-SYN can be divided into three regions with distinct characteristics (Figure 2): 1) the amphipathic lysine-rich N-terminus (residues 1–60), which is mainly involved in membrane interactions; 2) the middle hydrophobic region (non-β component of amyloid plaques (NAC), residue 61-95), which is prone to β-sheet formation and fibrillation; and 3) the C-terminus (residues 96–140), which is a highly acidic and proline-rich region and primarily controls the nuclear localization and interaction with other proteins. Synuclein family of proteins is primarily neuronal proteins that localize in the presynaptic terminals. However, it is the NAC domain of the α-SYN that sets it apart from the other two members of its family. α-SYN contains seven imperfect repeats of 11-amino acids with a 6-amino acid core of KTKEGV. The core amino acid repeats are separated by intermediate five amino acids except for repeat number four and five which has nine intermediate amino acids.
Figure 2 Structure of full length α-SYN and its functional components.

N-terminal (1-65), NAC domain (66-95) and C-terminal (96-140); Seven KTKEGV motif is present throughout the molecule (white color labelled 1-7); Point mutations reported in familial PD (autosomal dominant form) are present in the N-terminal. NAC domain contains most hydrophobic residues and 3 of the 7 KTKEGV motifs. NAC domain promotes aggregation of the molecule. C-terminal has three nitration sites and one phosphorylation site (T125, 133, 136) & S129.

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1.2.2 Function of alpha-synuclein

α-SYN is a widely expressed protein in the nervous system comprising of almost 1% of total cytoplasmic proteins. Immunohistochemical analysis in normal brain tissue samples has shown a presynaptic localization of the protein with a consistent puncta like staining pattern. The staining pattern is comparatively less in the neuronal soma than the synapse. Moreover, studies investigating the localization of α-SYN have clearly demonstrated that the protein is closely located to synaptic vesicles but not with the vesicle. α-SYN is also abundant in erythrocytes and platelets, the reason for which is still being researched. Glial cells also contain a basal level of α-SYN, but whether it is present in all physiological condition or induced particularly under certain condition is debated.

It is believed that α-SYN expression is induced during neuronal development, following differentiation and during development of synapse. Expression of α-SYN is also altered during neuronal injury or following plasticity of the cell. Based on these evidences, α-SYN is considered a modulator of synaptic transmission. It has also been shown that knock out or overexpression of α-SYN can greatly affect the release of neurotransmitter. Moreover, data also supports that α-SYN may act a break for neurotransmitter release under conditions of continuous firing by the neuron.

A study by Fortin et al. showed that α-SYN is transiently attached to synaptic vesicles and during neuronal firing, it moves away from the vesicle and again returns gradually. Further investigation into α-SYN’s function has demonstrated that the protein may also be involved in vesicle biogenesis through phosphatidic acid metabolism. In line with
these reported findings, α-SYN extensively controls the phenotype of mice null for a presynaptic protein CSP-a (Cysteine string protein a) which underwent synaptic degeneration. In the same study it was also shown that α-SYN may have a chaperone like function by acting together with CSP-a in the assembly of SNARE complex\(^9^1\). Overall, although a topic of extensive research, α-SYN is believed to be playing an important role in the release of neurotransmitter by modulating assembly of SNARE complex. Moreover, the physiological function of α-SYN may provide some insight into the abnormal function seen during diseased condition like PD.

1.2.3 α-SYN post-translational modification

α-SYN can undergo a variety of post-translational modifications like phosphorylation, nitrosylation, glycosylation and oxidation. The most researched post-translational modification amongst all the other modification is phosphorylation. Multiple sites of phosphorylation have been identified which includes Serine at 87 and 129\(^{th}\) position, tyrosine at 39\(^{th}\), 125\(^{th}\), 133\(^{rd}\) and 136\(^{th}\) position. Antibodies specific to detect Serine129 and tyrosine39 have identified the existence of these post-translationally modified forms of the protein in Lewy bodies of PD patients\(^9^2\)\(^-\)\(^9^4\). In recent years, several studies have contradicted the very original hypothesis that Serine129 phosphorylation leads to generation of more neurotoxic oligomeric species of α-SYN\(^9^5\). In fact, one of the studies has shown that the event of phosphorylation occurs after the formation of the Lewy bodies\(^9^6\)\(^,\)\(^9^7\). Similar findings were also seen recently in human postmortem samples, where there was an accumulation of phosphotyrosine-39 α-SYN in brain tissues and
Lewy bodies of PD patients compared with age-matched controls. Although, in vitro studies have shown that phosphorylation of α-SYN at tyrosine-39 enhances α-SYN aggregation, no clear evidence could be provided to support the prevalence of the two events i.e. whether the aggregation takes place first followed by the phosphorylation, or the other way round. However, increased phosphatase activity has shown a protective role against α-SYN mediated neurotoxicity, thus suggesting a detrimental role of phospho α-SYN in the PD process. The phosphorylation event described for the Serine 129th and tyrosine 39th position does not always hold true for the other positions, which have been observed to be phosphorylated in PD condition but with no clear pathological significance.

A number of past studies using cell-free and cell based systems have shown that the protein can be oxidized by iron and dopamine like agents to drive its oligomerization. One of the potent oxidant HNE (4-hydroxy-2- nonenal) promoted formation of stable soluble oligomers, which are believed to inhibit the formation of fibrillar forms. Such stable oligomers have been shown to cause neurotoxicity in cell models of PD. Nitrosylated α-SYN could be detected in Lewy bodies of PD patients using nitration-specific α-SYN antibodies. The effect of nitration on α-SYN has been shown to bear a very complex effect, leading to fibril formation under certain conditions (monomeric and dimeric nitrated α-SYN) and oligomer formation under others.

Another important modification of α-SYN implicated very strongly in PD is the C-terminal truncation of α-SYN by calpain like enzymes. This event has been shown to generate α-SYN lacking the C-terminal acidic tail. Several recombinant protein assays and cell-
based experiments have shown the increased propensity of the truncated form to fibrillization\textsuperscript{105}.

### 1.2.4 SNCA genetics linked to PD

The first reported genetic aberration in relation to the occurrence of PD was reported in 1997 in an Italian cohort and a small Greek cohort in SNCA. The single point mutation corresponded to G209A of the SNCA locus, which resulted in the generation of A53T $\alpha$-SYN (\textit{Alanine 53 Threonine}). Studies showed that the A53T $\alpha$-SYN was autosomal dominant in its inheritance pattern and resulted in early onset of PD (EOPD). The advent of this mutation faced considerable debate since in rodent’s SNCA homolog (\textit{snca}) the threonine is present in the 53\textsuperscript{rd} position instead of the alanine. However, this initial skepticism was further supported by the discovery of two more autosomal dominant inherited mutations in the SNCA gene leading to A30P (\textit{Alanine 30 Proline}) and E46K (\textit{Glutamate 46 Lysine}). Mutant $\alpha$-SYN reported in familial form of PD, A30P, H50Q, A53T, A53E and E46K, have been involved in template directed misfolding in the preinitiation stage of aggregation. The mutant proteins are transformed into amyloid fibrillar species (consisting of $\beta$-sheets), which have properties to serve as template to drive normal physiological proteins to adopt similar structural changes\textsuperscript{106-108}.

The next important landmark discovery that linked SNCA to PD was the triplication of SNCA gene locus in families with autosomal dominant inheritance that led to EOPD. Triplication of the gene locus led to the generation of double load $\alpha$-SYN protein in such patients which was confirmed later by a separate study. Following the path, other families were subsequently described to have SNCA gene duplication which too was
autosomal dominant and resulted in EOPD. Interestingly, a dosage effect of α-SYN was studied in these separate cohorts of PD patients, where triplication cases were reported to have earlier onset and developed a much severe form of PD. Studies and discovery on the genetic defect in the SNCA locus leading to EOPD gave way to extensive research being conducted trying to link α-SYN defects to sporadic PD also with the hope of identifying some polymorphisms that may be linked using association studies. Data from the association studies revealed Rep1 polymorphic region located around 10Kb upstream of the SNCA transcriptional start site. It was seen through initial association studies and later large meta-analysis of cohorts that polymorphism in this site risked individuals to developing sporadic PD; GWAS studies showed that the 3’ region of the gene has been a consistent hit to link SNCA to onset of PD. The genetic studies focusing on α-SYN concluded that SNCA can be linked to both rare familial form and sporadic form of PD from a genetic standpoint. Based on these findings, genetic variations in SNCA have now been extended to extensively study to MSA and PSP.
1.2.5 Aggregation potential of α-SYN

Since the discovery of α-SYN as an integral component of LB’s, several studies have focused to decipher the aggregation potential of the protein. The ability of α-SYN to form β-sheet structures under pathological condition like PD generated a lot of curiosity to study the protein in details in order to come up with a unified pathogenic basis of the disease. Moreover, the discovery of plaques of β-amyloid as seen in Alzheimer’s disease gave way to parallel studies on this path to unravel the condition which triggered the aggregation of α-SYN.

In the year 2000, a landmark study by Conway et. al., showed that α-SYN can form amyloid like fibrils on incubation in solution. This was applicable for both the wild type and the disease related mutants A53T, A30P, E46K, A53E, H50Q and G51D. Since then a lot of other studies have tried to characterize the aggregation potential of newly discovered α-SYN protein using recombinant protein technology. This was a breakthrough discovery in terms of unraveling the conditions of α-SYN aggregation process, since mature fibrillar α-SYN were also a component of LB’s and LN’s. This same study along with others showed the kinetics of α-SYN aggregation both as wild-type and disease associated species. It was observed that single point mutations as seen in the disease associated variants can greatly affect the aggregation of α-SYN and also the kind of intermediates formed during the aggregation.

Over the years, it has been shown that aggregation of α-SYN is a complex process involving a multitude of intermediate forms. Initially, monomeric α-SYN form soluble oligomers, which assume spherical, ring like and string like characteristics as seen
under electron microscope. These structures are collective referred to as “protofibrillar species”. Gradually with increasing time, the protobrils acquire a more matured fibrillar form which is insoluble in nature. Khurana et al. using AFM studies for the characterization of α-SYN fibrils morphology, hypothesized a hierarchical model for fibrils: α-SYN monomers, in aggregating conditions, first assembly in protofilaments (3.8±0.6 nm); after protofilaments elongation, two of them intertwine and form protofibrils (mean height 6.5±0.6 nm), in order to reduce the exposure of hydrophobic interfaces. Finally, two protofibrils intertwine into mature fibrils as seen in LB’s (9.8±1.2 nm)\textsuperscript{117,118}.

The intermediate aggregates of α-SYN were found to be very transient and thus may not be always resolved using cell based experiments. However, their biochemical recombinant counterparts which are also termed as soluble oligomeric species are mostly stable and can retain their conformation on SDS-PAGE gels. In 2004 Pountney et. al., showed the presence of α-SYN annular oligomers from inclusions in patients of MSA. These equivalent species are on-pathway α-SYN oligomeric species which were then identified \textit{in vitro} using aggregation assays, however only recently they gained importance in PD because they were proposed as the most toxic aggregation intermediate in the α-SYN fibrillization pathway\textsuperscript{74}.

The soluble oligomers and protofibrils showed annular or elliptical shapes, probed by atomic force microscopy and electron microscopy, with diameters going from 4.5 to 55 nm. The estimation of the average number of α-SYN monomers constituting these oligomers goes from 16 to more than 42 as well \textsuperscript{76,109,119,120}. 

25
The major ongoing debate about α-SYN aggregation is its contribution to toxicity as seen during degeneration of the DA neurons in PD.

A53T or A30P α-SYN has been shown to form more protofibrils compared to the WT α-SYN, whereas A53T alone can form more matured fibrils. However, E46K, forms less protofibrils compared to the other disease related species. In-vitro studies have demonstrated that A53E can reduce α-SYN aggregation and amyloid formation without affecting the major secondary structure and initial oligomerization tendency. Similarly, the newly discovered H50Q α-SYN has been shown to accelerate the fibrillation process when compared to the WT α-SYN alone. On the contrary, G51D α-SYN has been shown to attenuate in-vitro aggregation and membrane binding of the protein. Taking together all these data from the in-vitro studies, it is difficult to conclude a simple correlation between the α-SYN mutants and the aggregation potential which can eventually lead to neurotoxicity. Moreover, recent studies have also shown that α-SYN aggregation as LB’s and LN’s can be a cell’s protective mechanism to dump toxic oligomeric species into a larger non-toxic form \(^{74, 103-107}\).

In the initial years of PD research, α-SYN had been primarily characterized as an intracellular protein, since it did not have any signaling sequence for secretion. However, over the years this notion changed because α-SYN could be detected in the conditioned medium of cells and also in extracellular fluids such as plasma and CSF\(^{121,122}\). Although, the mechanism of α-SYN secretion is not clearly elucidated, it appears to mediate through a secretory pathway by making use of exosomes, endocytic vesicles which are released during calcium influx \(^{122,123}\). Interestingly, this secreted α-
SYN can have detrimental effect on neuronal homeostasis, sometimes leading to death, even at concentrations as low as physiological levels but appearing to be soluble oligomeric in form. Moreover, extracellular α-SYN can also trigger inflammatory response in glial cells, thus adding to the detrimental effect. Most of the studies conducted to research the extracellular α-SYN have often used moderately high to high concentration of recombinant α-SYN that may not be physiologic in nature.

A related issue of α-SYN pathology that has garnered huge attention in recent times, is its apparent ability to be uptaken by cells, thus stimulating the hypothesis of propagation of pathology as observed in PD. Till very recently, the mechanism governing this cell-to-cell transmission of pathologic α-SYN was not known. However, a recent study has demonstrated the important role of a novel receptor called LAG3 (Lymphocyte activation gene 3) to bind prefibrillar (PFF) α-SYN and initiate the process of endocytosis, transmission and toxicity. Another very interesting observation related to this mechanism, showed oligomeric-aggregated α-SYN species to have increased potential to be uptaken by cells and initiate a “seed” based fibrillation event of the endogenous α-SYN. Two landmarks studies performed in vivo support this “prion-like” propagation theory. In the first study it had been shown, progenitor cells transplanted in the hippocampus of α-SYN transgenic mice incorporated host α-SYN in their cell cytoplasm. Another study showed LB pathology in fetal dopaminergic grafts in human with PD, several years after implantation, although there was no confirmation whether the α-SYN has been derived from the host. A major breakthrough study showed, intragastric injection of mitochondrial complex 1 inhibitor rotenone led to
enteric α-SYN pathology, which eventually spread to central nervous system including DA neurons. This study concluded that misfolded α-SYN has the potential to assume independent propagating seeding and neurotoxic potential 32.

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1.3 Oxidative stress-mediated damage in neurodegeneration

Oxygen is an essential component to the survival of all living beings. But the greatest paradox remains in the fact that production of reactive oxygen species (ROS) as a by-product of oxygen metabolism, are highly toxic to cells. Reactive oxygen species (ROS) are molecules that can react with cellular macromolecules and impair their functions. It can include both free radicals like superoxide, hydroxyl radical and nitric oxide (containing highly reactive unpaired electrons) and molecules like hydrogen peroxide and peroxynitrite. Post-mortem brain tissues from patients of Parkinson’s disease, Alzheimer’s disease, and Amyotrophic Lateral Sclerosis (ALS) have clearly demonstrated higher amount of ROS in the selective areas that undergoes neurodegeneration75.

Oxidative stress originates when the rate of ROS production is significantly higher compared to its elimination from the system. Several markers of the oxidized cellular macromolecules have been identified under conditions of neurodegeneration. For example, elevated levels of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), which are markers of oxidized lipids, have been identified in the cortex and hippocampus of patients with AD, substantia nigra of patients with PD and spinal fluid of
patients with ALS\textsuperscript{132-135}. Oxidative modification of unsaturated fatty acids can result in the generation of lipid peroxides which can further cause oxidation of the unsaturated fatty acids in a chain-like event, finally leading to the disruption of plasma membranes and membranes of other cellular organelles like mitochondria\textsuperscript{136}. The level of protein carbonyls, a marker of protein oxidation, have been reported to be consistently elevated in the hippocampus and neocortex of individuals with AD, SNpc in case of PD and motor neuron in case of ALS\textsuperscript{137-140}. Oxidation of proteins can disrupt the active site of enzymes, lead to conformational change, disrupt protein-protein interactions, and alter their binding capacity to DNA which in all can be detrimental to the survival of cells. Increased levels of oxidative damage to DNA and RNA bases have been consistent with the neurodegenerative conditions like PD, AD and ALS. Guanine, which is the most susceptible base to oxidative damage, and gets readily oxidized to form 8-hydroxy-2-deoxyguanosine (8-oxodG) and serves as a marker for oxidative damage\textsuperscript{141}. However, beside 8-oxodG, several other forms of oxidative modifications of bases have also been seen in PD and AD, like hydroxyadenine (8-OH-adenine), Fapyadenine, 5-OH-cytosine\textsuperscript{142}. In AD, the level of nuclear DNA damage is significantly higher compared to age matched controls considering three regions of the frontal, parietal and temporal lobe\textsuperscript{142}. Overall, it can be concluded that oxidative stress is consistently associated with neurodegeneration and plays a critical role in mediating the process.
The origin of oxidative stress and subsequent accumulation of damage can not only be attributed to the generation of ROS, but also to the inefficient defense and repair machinery in the cell which are specifically associated with oxidative stress and associated damage\textsuperscript{136,143}. The defense machinery refers to the antioxidant enzymes like Superoxide Dismutase (SOD), Glutathione Peroxidase (GSHpx), Glutathione reductase (GSHrd), and catalase among many others whose primary function is to scavenge the very reactive ROS generated in the cell. For example, glutathione peroxidase (GSHpx) detoxifies hydrogen peroxide using reduced glutathione. Oxidized glutathione (GSSG) can be subsequently reduced and reused. SOD converts superoxide to hydrogen peroxide which is subsequently converted to molecular oxygen and water by catalase \textsuperscript{136}. A number of reports have shown reduced activity of the antioxidant machinery in AD\textsuperscript{144,145}. In familial ALS, mutation in the copper and zinc containing SOD, leads to a toxic gain of function that leads to the conversion of SOD itself to a pro-oxidant protein involved in ROS generation\textsuperscript{146,147}. PD is also characterized by significant loss of thiol reducing agent glutathione (GSH) in the SN, which is one of the earliest known indicators of nigral neuron degeneration \textsuperscript{148}. Together, these evidences comprehensively indicate that reduced antioxidant potential might be a critical factor towards increased oxidative stress that is associated with these disorders.

As discussed before, accumulation of ROS-induced DNA damage, like oxidation of bases and single strand breaks (SSBs) have been implicated in the etiology of AD, PD, ALS and other neurological disorders. This accumulation of the DNA damage directly implies the defect in the DNA repair machinery of the cell which is designated to perform specific functions. It has been shown that ROS induced DNA damages are
primarily repaired via highly conserved base excision repair pathway $^{149,150}$. Neuronal dysfunctions have been linked to mutations or differential expression of Base excision repair (BER) enzymes like OGG1, XRCC1 $^{151-153}$, Single strand break repair enzymes (SSBR) like TDP1, aprataxin $^{154,155}$ and double strand break repair enzymes like ATM, NBS1$^{156}$. Furthermore, it is reported that OGG1 knockout mice exhibits age-associated loss of nigrostriatal pathways and make the animal more sensitive to MPTP treatment (a Parkinsonian animal model) $^{157}$. Together, all these evidences suggest very strongly that oxidative stress mediated damage to cellular macromolecules like DNA coupled with inefficient repair leads to progressive neurodegeneration as seen in AD, PD, ALS and others.

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1.3.1 Mitochondria and generation of oxidative stress

Mitochondria are highly dynamic intracellular membrane enclosed organelles present abundantly in eukaryotic cells. Mitochondria are responsible for carrying out a number of crucial functions such as pyruvate oxidation, Kreb’s cycle, metabolism of amino acids, fatty acids and importantly generation of energy in the form of adenosine triphosphate (ATP). Generation of ATP is done through the mitochondrial electron transport chain and the oxidative phosphorylation system. Apart from synthesis of energy in form of ATP, mitochondria perform two other very important duty of regulating the calcium homeostasis and control of programmed cell death (PCD) $^{158}$. 
Mitochondria are very unique organelles in the sense that these have their own DNA encoded by their genome; thus having their own machinery for RNA and protein synthesis. Each of the mitochondrion contains several circular mitochondrial genomes in their matrix that encode for thirteen mitochondrial proteins that primarily function in the oxidative phosphorylation. Although mitochondrion has this unique genome, most of the other functions performed by the mitochondrion require other proteins that are encoded by the nucleus. These nuclear proteins have mitochondrial targeting sequence by which they are targeted and imported into the mitochondria to perform specific functions designated to each.

In neurons, mitochondria are dispersed throughout the length of the neuron including axons and dendrites to facilitate their functioning in subcellular compartment distant from the cell body. Neurons are particularly vulnerable to dysfunctional mitochondria because they depend for energy on the mitochondrial metabolism of pyruvate produced from glucose using the glycolytic cycle. Moreover, dopaminergic neurons of the SNpc region have been shown in previous reports to have compromised respiratory chain activity (activity of complex 1) under PD like condition. This impairment is expected to make the DA neurons more vulnerable to Bax-induced apoptosis (programmed cell death), thus contributing to the death and dysfunction of cells during the PD disease process. Another important finding that adds to the damaging effects of mitochondrial impairment is the build-up of oxidative stress in the neurons causing damage to both the mitochondrial machinery and also the cellular machinery.

Mitochondrial dysfunctions have been shown to cause increased oxidative stress. This leads to damaged lipids, proteins and DNA, including decreased antioxidant glutathione.
levels in PD. This piece of evidence gives a plausible link between oxidative damage and formation of the Lewy body protein aggregates which may impair proteasomal ubiquitination pathway and degradation of proteins. A recent study showed increased plasma 8-hydroxydeoxyguanosine (8-OHdG) levels in patients with PD than in age matched controls.\textsuperscript{159}

Moreover, rodent and primate models of PD have shown that dopamine neuronal degeneration, a-synuclein pathology and motor deficits can be induced by the systemic and local administration of inhibitors of mitochondrial electron transport chain like rotenone, MPTP, and annonacin, paraquat, manebr, dieldrin, heptachlor. From these reports it can be concluded that modulation of mitochondrial pathways lead to parkinsonian-like syndromes. In addition to this, mitochondrial dysfunctions have also been reported in muscle tissues, platelets, lymphocytes, and fibroblasts of PD patients, thus suggesting mitochondrial dysfunction as a more global and important feature of PD. As discussed earlier, familial mutations in PINK-1, which is a mitochondrial kinase involved in fission, mitophagy, and quality control of the cell can lead to PD like symptoms. The need of increased oxidative metabolism in PD brains as shown by magnetic resonance studies, in conjunction with energy imbalance, can be considered to be indicative of mitochondrial dysfunction mechanisms that may be present in the brain of patients with PD even in the absence of overt clinical manifestations\textsuperscript{74,158,159}.

1.3.2 Oxidative DNA damage in the form of 8-oxodG in Parkinson’s disease

Oxidative stress has been classically linked to the etiology of Parkinson’s disease. The high metabolic activity of neurons along with their long span of life makes them highly
susceptible to damage by oxidative overload\textsuperscript{160,161}. Moreover, the dopaminergic neurons of SN, which undergoes neurodegeneration in the disease, are particularly vulnerable to oxidative stress. It is because, Dopamine (metabolite produced by these neurons) can generate very toxic molecules, if not properly handled\textsuperscript{127}. Dopamine has the capacity to auto-oxidize at normal pH into toxic Quinone species, producing superoxide and hydrogen peroxide\textsuperscript{162}. Monoamine oxidase (MAO) can also enzymatically deaminate dopamine into non-toxic metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) and hydrogen peroxide\textsuperscript{163}. Hydrogen peroxide can in turn be broken down into cytotoxic hydroxyl radical in a reaction catalyzed by iron. The level of iron is reportedly higher in the nigral dopaminergic neurons as compared to the other regions of the brain, owning to its binding affinity to neuromelanin\textsuperscript{133,164,165}. Thus, dopamine when synthesized or transported into cell from extracellular space is rapidly stored into synaptic vesicles which provide a stable environment for dopamine without MAO and low pH. Under conditions of PD, nigral neurons appear to be in an exaggerated state of oxidative stress, causing severe damage to cellular macromolecules. Damage to nucleic acids is particularly very hazardous amongst all the cellular macromolecules, because it can change genetic information present in both nuclear and mitochondrial genome\textsuperscript{166}. DNA damage by oxidative stress can result in the production of either non-bulky DNA lesions like 8-oxodG which can be repaired by components of the base excision repair (BER) machinery or bulky lesion which are primarily targets of the Nucleotide excision repair (NER) pathway\textsuperscript{167}. In PD, oxidation of DNA base is one of the most common damage incurred by the cell. The most frequent DNA lesion caused by oxidative stress is 8-oxo-7, 8-dihydroguanine (8-oxodG), the oxidized form of
guanine, often associated with neurodegenerative diseases including PD. Immunocytochemical analysis of 8-oxodG revealed a significant increase of this DNA oxidation marker in the SN of patients with PD although, the extent of nuclear 8-oxodG accumulation is not as high as mitochondrial 8-oxodG. Despite the presence of 8-oxodG specific DNA repair enzyme OGG1 (8-oxodG DNA glycosylase), a significant percentage of this DNA lesion remains unrepaired and accumulates under disease conditions. This observation can also be supported by the fact that OGG1 protect neurons from oxidative damage and cell death caused during ischemic conditions in a mouse model. Moreover, it is reported that the overall activity of OGG1 in brain decreases over ageing in a mouse model. The 8-oxodG lesions generated by direct oxidation of DNA, can base pair with both adenine and cytosine during replication and thus, cause G:C to T:A transversion mutation. Thus, 8-oxodG remains the extensively studied route for mutagenesis in proliferating cells. However, majority of the cells in our body, including neurons, exist in non-proliferating quiescent state. Neurons being post-mitotic cells, face a major challenge of DNA repair during transcription. Failure to maintain both transcriptional and translational fidelity is expected to result in functional degeneration of the cells. Thus, quiescent cells like neurons may not be protected from the potential deleterious effect of DNA damage, as many of these non-bulky lesions present on the transcribing strand of a gene is reported to be bypassed by RNA polymerase (RNAP) during transcription, and misinsert an incorrect base in a the growing mRNA, producing mutant RNA molecules. This phenomenon referred as transcriptional mutagenesis, has been implicated in different stages of tumor development. In the next few sections, the perspective of this phenomenon in
contributing to the pathogenesis of Parkinson’s disease with SNCA (α-SYN) as a model gene will be discussed.

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1.3.3 8-oxodG-mediated transcriptional mutagenesis

DNA damage-mediated mutagenesis in a replication based model has provided a wide range of information for better understanding of origin of mutation and subsequently its contribution to many biological events that account for variation in genetic structure of an organism. However, as discussed briefly in the last section, majority of cells in the natural environment are not frequently engaged in division and do not undergo continuous cycles of replication. Most of the multicellular organs of eukaryotes, like brain or heart are mainly comprised of non-dividing cells. Under condition of no cell division, these organs primarily depend on the fidelity of both transcription of DNA and translation of mRNA to maintain their complex physiological function. DNA damage in the form of 8-oxodG or other non-bulky lesion can result in production of erroneous transcripts of a gene, through efficient bypass of the lesion coupled with misinsertion of a ribonucleotide in the nascent mRNA chain. This event, referred as transcriptional mutagenesis has the potential to produce significantly high amount of mutant transcripts and subsequently erroneous proteins when compared to other DNA replication independent pathways of producing mutant proteins. Using in vitro system, it has been shown that under TM condition, each round of transcription produce a mutant transcript (with a targeted change in the sequence at the site of lesion) as long
as the damage is not repaired. This event is expected to generate a fairly large population of pure mutant transcript which will be translated multiple times leading to a relatively large amount of the mutant protein. As a result of this, TM might have a significant effect on physiological function and phenotype, especially in a non-dividing cell like neuron.

A number of studies have shown, a plethora of DNA damage that can lead to transcriptional mutagenesis event and consequently the outcome on the mRNA. Broadly, the two types of DNA damage considered are either non-bulky lesion (8-oxodG, dihydrouracil), which can effectively get bypassed by RNA PolII during transcription or the bulky lesions (cyclobutane pyrimidine dimers, cyclo-deoxyadenosine) which pose strong but incomplete block to transcription. The structural analysis of yeast RNA Pol II at an 8-oxodG lesion revealed the possible mechanism of transcriptional mutagenesis. In this study, it has been shown that 8-oxodG can mispair with adenine instead of cytosine through a Hoogsteen bond with the lesion at the polymerase active center, thereby escaping the proofreading of the polymerase and is maintained in the RNA as a result of this event.

For the first time, the potential of 8-oxodG to cause TM event had been demonstrated in a bacterial system using Escherichia coli as a model organism and its role in the etiology of developing antibiotic resistance. Further, in another study it has been shown using a luciferase based reporter system in mammalian cells, that TM event is strongly affected by factors such as promoter strength of the gene, flanking sequence around the lesion and position of the lesion with reference to the promoter. In the same study, it has been shown that TM event occurs more frequently in OGG1 null
condition as opposed to the cells which lack transcription coupled repair machinery, thus confirming the fact that 8-oxodG once incorporated in the DNA does not pose a blockade for transcription and its associated repair\textsuperscript{184}. A major step forward in this field of research involved replacement of the luciferase gene with an actual oncogene \textit{HRAS} in which 8-oxodG replaced guanine at codon 61. If the 8-oxodG inserted in this position, underwent TM event, it resulted in the production of constitutively active mutant form of \textit{HRAS} (Q61K). Moreover, it also demonstrated that under condition of OGG1 null background in mouse embryonic fibroblast, sufficient quantity of the mutant protein got generated to activate components of the MAPK pathway including ERK phosphorylation\textsuperscript{177}.

An important aspect of the TM event reflects the transient phenotypic change that is brought about in a cell because of the production of a large pool of mutant proteins. As compared to the large infrequent DNA damages, 8-oxodG-mediated TM event should occur at a much higher rate, thereby successfully showing some phenotypic change. However, it is yet to be established how the TM-mediated erroneous protein generation can contribute to conditions like Cancer, neurodegeneration or cardiovascular disease \textit{in vivo}.

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Figure 3 8-oxodG-mediated Transcriptional mutagenesis event.

The figure depicts TM event which occurs when 8-oxodG present on the transcribing strand (3’ → 5’) of a gene can misinsert an adenine instead of a cytosine in the growing mRNA chain, thus introducing a mutation at the mRNA level.

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1.3.4 Potential of TM event to cause α-SYN aggregation as seen in sporadic Parkinson’s disease

This section of the introduction links the experimental mechanism of α-SYN aggregation and why TM event may possibly play a role in its aggregation process as seen under conditions of idiopathic PD. A growing body of experimental data implicates multiplication of SNCA gene and the mutant variants of α-SYN protein in accelerating the process of aggregation, linked mostly to dominant inherited familial PD. As discussed in the last few sections, increased oxidative stress is a key contributor to the pathogenesis of PD. Several in vitro and in vivo experiments have linked oxidative stress to α-SYN aggregation. This observation has been supported by several recombinant and cell based studies, where SH-SY5Y cells exposed to ferrous ions, hydrogen peroxide, MPP⁺, Nitric oxide, and superoxide all promoted the aggregation process. In vivo studies also corroborated the same idea using rat models which have been systemically exposed to pesticides like rotenone or 6-OHDA (6-hydroxydopamine).

The over-expression or misfolding of α-SYN and particularly its mutant variants has been shown to increase the aggregation. In misfolded state, α-SYN is characterized by twisted, non-branched filaments of β-sheets. Mutant α-SYN reported in familial form of PD, A30P, H50Q, A53T, A53E and E46K, have been involved in template directed misfolding in the preinitiation stage of aggregation. The mutant proteins are transformed into amyloid fibrillar species (consisting of β-sheets), which have properties to serve as template to drive normal physiological proteins to adopt similar structural changes. Thus, how 8-oxodG-mediated TM event might play a critical role in the process of
aggregation depends entirely on the species of mutant molecules generated by this mechanism and whether these species can acquire a misfolded state themselves, which will eventually lead to template directed misfolding process of the wild type protein also. If the mutant species generated through 8-oxodG-mediated TM event is more stable in the β-sheet form, then the event can promote prion like nucleation event\(^{191,192}\). This idea of template directed nucleation process have also been supported by \textit{in vitro} recombinant studies showing that a small amount of the mutant protein can act as a seed towards the nucleation process of the wild type species which is present in excess\(^{193}\). Overall, to test this model of aggregation, the key would be to analyze the mutant mRNA species of α-SYN that itself have more potential to form misfolded β-sheet structure.

In paraquat based animal model, it has been shown that nuclear 8-oxodG accumulation in the \textit{substantia nigra} clearly correlated with increase in proteinase-K resistant species of α-SYN indicating more aggregation\(^{194}\). A recent study indicates that genomic distribution of 8-oxodG is not a random event; instead it is localized preferentially to specific areas of the chromosome\(^{195}\). This fact implies that TM could affect any gene at random, and mutant species originating from TM event should be outnumbered by the normal physiological form. For most proteins, generation of small portion of non-functional TM species would not be likely to cause entire functional impairment. However, α-SYN could become cytotoxic in the nucleation-dependent oligomerization process in which small addition of mutant α-SYN species to WT population may initiate the seeding process and fibrillogensis\(^{129,193}\). This biochemical feature of α-SYN would strongly support the feasibility of the proposed model.
In Figure 3, all the possible mutant mRNA species of SNCA are shown that can be generated through TM event (when an adenine is replaced in place of a cytosine during transcription). In this hypothetical form, all the cytosine residues are changed to adenine and then their respective change in amino acid sequence is listed. Structural analysis of α-SYN protein revealed that disruption of the alpha-helical structure of the protein is key towards the aggregation process\(^{196}\). The three familial mutations, A30P, E46K and A53T also cause the hydrophobic regions in α-SYN to acquire β-sheet configuration, to fibrillize, form amyloid that cause cytotoxicity and subsequent neurodegeneration\(^{197}\). The mutation A53E is located on the second alpha helix of the polypeptide\(^{196}\) and based on the amino acid change of hydrophobic alanine to hydrophilic aspartate; it might also result in the disruption of the alpha-helical structure. From the list of TM generated mutants, a number of mutants are seen to exist in the same position as the familial forms like A30E (familial form A30P), H50N (familial form H50Q), and A53E (familial forms both A53E & A53T). Based on the literature of the familial mutant forms and their potential to aggregate, it would be an important perspective to study these TM generated mutants. Apart from these forms, there are several others residues which on getting mutated might disrupt the native structure of the protein also and make it more prone towards aggregation. For example, using a statistical mechanics algorithm, TANGO, to predict protein aggregation, the mutants on the N-terminal L38I & S42Y, Q62K in the NAC domain all seemed to affect the aggregation potential score positively (Figure 4)\(^{198}\). Recent studies have identified residues within protein sequence that promote ordered aggregation and amyloid formation as seen for α-SYN\(^{199}\). The algorithm TANGO identifies such aggregating regions within a protein sequence based
on the hydrogen bonding potential, physico-chemical parameters and how well each such segment can populate each of the conformational states according to Boltzmann distribution (frequency of existence of a structural state relative to its energy distribution). Post translational modification like phosphorylation on Serine 87 & 129 may also affect the aggregation process. TM event can change the serine at 129th position to a tyrosine. Based on the above literature and generation of predicted TM species, it would be interesting to study the profile of the TM species in PD patients and whether these species contribute to the pathogenesis of the disease (Figure 5).

The majority of idiopathic PD cases are a late-onset sporadic form with cytoplasmic α-SYN aggregates, which indicate that increasing degree of aggregation do not depend only on genetic mutations in SNCA. However, till date, the approaches to understand the molecular mechanism of α-SYN aggregation have focused primarily on the biochemical properties of mutant protein species that were identified in rare familial form of PD and their behavior within the cells. The proposed model will give insight into a novel mechanism called transcriptional mutagenesis that might lead to generation of mutant mRNA species (mutations introduced in mRNA because of oxidative stress related DNA lesion and not polymorphism) from the accumulated 8-oxodG lesion in the protein coding area of the SNCA gene. If the proposed hypothesis is proven true then it will show the direct effect of 8-oxodG lesions on SNCA to the generation of TM-derived mutant species of α-SYN and its contribution to aggregation. It will definitely add a new dimension to the understanding of α-SYN pathology in conjunction with oxidative stress.
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Figure 4 Predicted mutant amino acid sequences of α-SYN generated by 8-oxodG-driven TM.

Upper line (Black), reference amino acid sequence; Lower line, predicted amino acid changes arising from C to A transversion through transcriptional mutagenesis (Red); Green, reported familial α-SYN mutants (A30P, E46K, H50Q, A53E & A53T); reported site for phosphorylation at Serine 87 and Serine 129 (blue)

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Figure 5 Proposed model for aggregation of Wild type α-SYN protein by TM generated mutant α-SYN species.

The figure depicts a possible mechanism by which mutant α-SYN generated through TM event may contribute to nucleation-dependent aggregation of the predominantly higher amount of WT species as seen in Lewy bodies of sporadic PD patients, where no genomic mutation is observed in the DNA.  

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CHAPTER 2: DESIGNING A NOVEL TOOL FOR MONITORING ENDOGENOUS ALPHA-SYNUCLEIN TRANSCRIPTION BY NANOLUCIFERASE TAG INSERTION AT THE 3’END USING CRISPR/CAS9 GENOME EDITING TECHNIQUE

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**Abstract**

α-synuclein (α-SYN) is a major pathologic contributor to Parkinson’s disease (PD). Multiplication of α-SYN encoding gene (SNCA) is correlated with early onset of the disease underlining the significance of its transcriptional regulation. Thus, monitoring endogenous transcription of SNCA is of utmost importance to understand PD pathology. We developed a stable cell line expressing α-SYN endogenously tagged with NanoLuc luciferase reporter using CRISPR/Cas9-mediated genome editing. This allows efficient measurement of transcriptional activity of α-SYN in its native epigenetic landscape which is not achievable using exogenous transfection-based luciferase reporter assays. The NanoLuc activity faithfully monitored the transcriptional regulation of SNCA following treatment with different drugs known to regulate α-SYN expression; while exogenous promoter-reporter assays failed to reproduce the similar outcomes. To our knowledge, this is the first report showing endogenous monitoring of α-SYN transcription, thus making it an efficient drug screening tool that can be used for therapeutic intervention in PD.

**Keywords:** α-synuclein, endogenous NanoLuc luciferase, transcription
2.1 Introduction

α-SYN is a key protein involved in the progression and pathogenesis of Parkinson's disease (PD)\textsuperscript{74}. Familial PD studies have revealed that multiple copies of the gene encoding α-SYN (SNCA) cause severe early onset PD, highlighting the importance of its tight transcriptional control \textsuperscript{37,75,186,201}. However, little is known about the transcriptional dysregulation of SNCA. Recently, Gründemann et al. confirmed significant increase in SNCA mRNA levels in individual dopamine neuron from idiopathic PD brains using laser capture microdissection, implying a significant transcriptional de-regulation in pathologic conditions\textsuperscript{202}. Moreover, recent progress in research on epigenetic influences on SNCA transcription revealed that hypomethylation of SNCA regulatory region play a significant role towards its higher expression in idiopathic PD\textsuperscript{203-205}.

Advances in genome mapping and the completion of ENCODE project (Encyclopedia of DNA Elements) highlighted the importance of epigenetic architecture governing transcriptional regulation of a gene\textsuperscript{206,207}. In light of these discoveries, complete understanding of SNCA expression in pathologic conditions may require a molecular tool/system that can detect changes in transcription and also account for changes brought about by endogenous epigenetic modulation of the gene. Currently, the most widely used tool for understanding transcriptional activity of a gene is by using luciferase reporter fused to the promotor of a gene of interest\textsuperscript{208}. However, the plasmid-based exogenous reporter systems largely ignore the comprehensive aspect of gene expression regulation by complex interaction between different epigenetic factors, transcription factors and various cis elements by artificially limiting investigation on a putative promoter region. To overcome this limitation of exogenous reporter system, we
developed a novel tool where a reporter construct is tagged at the 3’ end of SNCA endogenously, allowing us to monitor transcriptional activity of the gene keeping its epigenetic architecture unperturbed. The NanoLuc luciferase reporter used in this study is 150-fold brighter and significantly smaller in size than firefly or Renilla luciferase, thus making it an ideal tag for even low expressing genes. Recent breakthrough in genome editing techniques like CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats) has made specific genome editing simple and scalable. Tagging SNCA endogenously with the NanoLuc using CRISPR/Cas9 method allows sensitive and real-time measurement of changes in transcriptional activity under various conditions of stimuli. This strategy can help to shed light on the transcriptional regulation of SNCA, and may serve as a very strong tool for screening of drugs to limit the progression of PD.

2.2 Results

2.2.1 Generation of a stable cell line endogenously tagged with functional NanoLuc luciferase reporter at the 3’end of SNCA.

To introduce the NanoLuc reporter at the 3’end of the SNCA, a double-strand DNA break (DSB) was introduced on the reverse strand with the –NGG protospacer adjacent motif sequence (PAM) directly abutting the stop codon located in the exon 6. This was achieved in human embryonic kidney cell line (HEK293T) by transient transfection with the pSpCas9 (BB)-2A-Puro vector. Along with the CRISPR/Cas9 construct, a donor vector containing the NanoLuc sequence was co-transfected to take advantage of the cell’s homology-directed repair (HDR). This donor construct
contained two flanking homology domains each of about 800 base pairs, corresponding to the upstream and downstream of the DSB target site (Fig. 6a). The NanoLuc sequence was cloned between these two domains to precisely introduce this reporter construct right before the stop codon of SNCA. Potential positive clones were identified by PCR amplification of the genomic DNA using the NanoLuc forward and reverse primers (Table 2) (Fig. 6b; Fig. 10). To confirm NanoLuc insertion at the target location in the SNCA gene, a second PCR using “Insertion Confirmation Primers” (Table 2) was performed and later sequenced (Fig. 10). The wild-type (WT) allele generated a 280 base pair (bp) PCR amplicon while the NanoLuc-tagged allele generated a 805 bp band, indicating a heterozygous insertion of the reporter construct (Fig. 6c). To overcome the PCR amplification bias towards the shorter allele, a separate amplification for NanoLuc-tagged allele was performed using a forward primer (NanoLuc Internal Forward Primer) on the NanoLuc insert in combination with the same reverse primer (cDNA sequencing Reverse Primer) on the 3′ UTR, generated a comparable amplification of 356 bp product for NanoLuc insert (Fig. 6c, lane 2). The PCR using NanoLuc internal forward primer failed to amplify any band in wild type HEK293T cells (Fig. 6c, lane 4). A second potential positive clone was found to have an incomplete insertion of the NanoLuc reporter tag (colony 14, Fig. 6b), and thus not used any further. To confirm the expression of NanoLuc-tagged SNCA allele tagged with the NanoLuc in the cell line, hereafter referred to as 293T-SNCA-3’NL, RT-PCR was conducted using primers encompassing the entire coding region of the gene and the 3′ UTR. The amplicon was then sequence verified to confirm the presence of NanoLuc insertion (Fig. 6d, Fig. 11).
Following confirmation of the NanoLuc insertion in the SNCA genomic region and the presence of mature mRNA, we sought to confirm protein expression and functional activity of the NanoLuc. Western blot analysis of cell lysates with a polyclonal anti α-SYN antibody confirmed the presence of both wild type α-SYN (~15 KDa) and a NanoLuc-tagged protein (~34 KDa) matching α-SYN fused with the NanoLuc (19.1 KDa) (Fig. 7a, Fig. 13). We performed luciferase activity assay on 293T-SNCA-3’NL cells by measuring luminescence after addition of substrate furimazine. 293T-SNCA-3’NL cells produced a considerably high signal distinguishable from cells without the NanoLuc incorporation or 293T-SNCA-3’NL cells without furimazine (Fig. 7b). Titration of cell counts from 2,500 to 50,000 produced a linear increase in luminescence activity ($R^2 = 0.95$), indicating that the luminescence of the SNCA-tagged NanoLuc reporter is internally consistent (Fig. 7c).

Taken together, these results show that SNCA was successfully tagged with the NanoLuc construct at the 3’end, and that expression of the NanoLuc-tagged allele leads to generation of a fusion protein.

2.2.2 α-SYN-NanoLuc luciferase activity reflects SNCA transcriptional regulation

To validate whether this system would be able to monitor changes in endogenous SNCA transcription, 293T-SNCA-3’NL cells were treated with known epigenetic modulators like DNA methyltransferase 1(DNMT1) inhibitor (5-AzadC), histone deacetylase (HDAC) inhibitors (sodium butyrate) and also dopamine which may
have a toxic effect beyond a certain threshold concentration \(^{214}\). SNCA harbors CpG islands at the regulatory regions encompassing the promoter and intron1\(^{204,205}\). The CpG island in the intron1 of SNCA in HEK293T remains completely methylated which upon demethylation can increase gene expression\(^{205}\). We treated the 293T-SNCA-3'NL cells with 5-AzadC for 72 hours to allow more than one round of cell division. A significant increase in the NanoLuc activity was observed in cells treated with 5-AzadC as compared to vehicle treated ones (3.68 times increase; \(p \leq 0.0001\)) (Fig. 8a). SNCA transcript level from sister cultures correlated well with the observed increase in the NanoLuc activity (Fig. 8a). Changes in methylation of the SNCA-intron1 CpG island were detected using bisulfite sequencing as done by Jowaed et al. \(^{204}\). Ten clones from each sample were analyzed and a significant reduction in mean intron 1 methylation by 31.7\% \((p<0.05)\) was observed (Fig. 8b). The reduction of cytosine methylation in the intron1 positively correlated with increase in SNCA transcript, as we saw with the increased NanoLuc activity.

It has already been reported that dopamine at 100µM concentration can enhance SNCA transcription in HEK293T cells without inducing subsequent toxicity\(^{205,214}\). Likewise, we treated 293T-SNCA-3'NL cells with 100 µM dopamine for 48 hours and a significant 1.31 times increase in the NanoLuc activity was observed as compared to the controls \((p<0.0001)\) (Fig. 3c). Again this increase in the NanoLuc luciferase activity complied with an increasing trend in α-SYN/Nanoluc mRNA and protein expression after dopamine treatment as seen by RT-PCR and western blot analyses (Fig. 8c, Fig. 14). Hyper-acetylation of histone is expected to unwind underlying DNA, which in turn favors transcription\(^{215,216}\). To test whether HDAC inhibition in 293T-SNCA-3'NL cells would
faithfully monitor transcription in response to histone hyper-acetylation, cells were treated with sodium butyrate (class I and IIa inhibitor of HDAC) at concentrations 2.5 mM and 5.0 mM for 24 hours\textsuperscript{215}. This treatment paradigm significantly increased the NanoLuc activity by 1.5 and 2.35 times respectively as compared to the controls (p<0.001) (Fig. 8d). α-SYN/NanoLuc transcript levels also corroborated well with the activity measurement and showed a dose-dependent increase (Fig. 8d).

**2.2.3 Exogenous promoter reporter assays failed to reproduce transcriptional activation of SNCA as seen in endogenous conditions.**

*Firefly* luciferase-based promoter assay is considered a gold standard for assessing promoter activity of a target gene, which in turn reflects the transcriptional activity of the gene\textsuperscript{217}. We compared the reporter activity of SNCA transcription between transient transfection-based luciferase system and the endogenous NanoLuc system that we designed. HEK293T cells were co-transfected with pGL3 basic plasmid containing SNCA promoter-intron1 region cloned upstream of luciferase coding sequence and CMV-\textit{Renilla} (transfection control). Twenty-four hours later, transfected cells were treated exactly with the same modulators of SNCA expression as described in the previous result. We observed a significant decrease in normalized reporter activity upon 5-AzadC treatment post 72hrs (p<0.0001) (Fig. 9a) contrary to the increased NanoLuc activity and transcript expression (Fig. 8a). The CMV promoter-driven \textit{Renilla} luciferase activity which was used as an internal control significantly varied upon 5-AzadC treatment, while the SNCA promoter-intron1 driven *firefly* luciferase activity remained largely unaffected, thereby leading to reduction in the normalized reporter
activity (*firefly/Renilla*) (Fig. 9a). Next, to compare the effect of dopamine on the exogenous luciferase activity driven by *SNCA* promoter-intron1 and the endogenous *SNCA* behavior, the transfected cells were treated with 100 µM dopamine for 48hrs, exactly following paradigm followed for the 293T-*SNCA*-3’NL cells. Interestingly, we did not observe any significant change in the normalized luciferase activity (p=0.22) (Fig. 9b). In this treatment, no significant change was observed for either *firefly* luciferase (p=0.15) or *Renilla* luciferase activity (p=0.16) (Fig 9b), although an increasing trend could be seen for both. This data again failed to demonstrate the endogenous state of regulation upon dopamine treatment (Fig 8c).

We also investigated the effect of HDAC inhibition (for 24hrs) on the *SNCA* promoter-intron1 driven luciferase activity. Similar to 5-AzadC treatment, normalized *firefly* activity showed a significant decrease from control (p< 0.0001) (Fig. 9c). This time we saw a significant increase in the *firefly* luciferase activity (p<0.05), along with a significant increase in the *Renilla* activity (p<0.001) thereby causing an artefactual reduction in *SNCA* promoter activity after normalization (p<0.0001). This observation is again opposite to what we found with the NanoLuc-based endogenous system after addition of HDAC inhibitor (Fig. 8d). Together, these observations showed that the exogenous luciferase-based reporter system largely failed to demonstrate original state of endogenous transcriptional activity of *SNCA*. 
2.3 Discussion

In the present study, we have successfully incorporated the NanoLuc reporter construct at the 3’ end of SNCA in HEK293T cells using CRISPR/Cas9 technology and monitored changes in expression induced by two epigenetic modulators and dopamine which are known to deregulate the gene’s expression. The newly emerging endogenous reporter system represents a significant paradigm shift in the study of gene regulation and may provide new and exciting opportunities for both basic and translational research. Such strategies allow to insert a reporter directly into the targeted genome, enabling us to investigate endogenous gene regulation while keeping the epigenetic structure intact.

This particular feature is extremely relevant in studying SNCA expression, as this gene has been shown to get extensively regulated by its epigenetic structure\textsuperscript{203-205}. Moreover, α-SYN is a molecule whose level of expression is directly correlated with the severity of the PD pathogenesis, thus making this tool very helpful for developing potential therapeutic options\textsuperscript{37,186,201,202}. Recent studies have demonstrated that epigenetic regulation by 5-AzagC increases SNCA expression in SK-SN-SH and SH-SY5Y cells\textsuperscript{204,218}. This cytidine analogue passively demethylates by irreversibly trapping DNA methyltransferase I (DNMTI), which positively correlates with higher expression of various genes\textsuperscript{219}. SNCA harbors CpG islands at the regulatory regions encompassing the promoter and intron1\textsuperscript{204,205}. It has been shown that hypomethylation of the intron1 CpG island but not the promoter CpG island is strongly associated with PD\textsuperscript{204}. So, we sought to check the methylation status of the 23 CpG sites\textsuperscript{204} in the SNCA-intron1 by bisulfite sequencing. As expected, a decrease in methylation of intron1 CpG correlated
with an increase in transcript levels as measured by the NanoLuc activity. Conventionally, to study the effect of methylation on the promoter's transcriptional efficiency, that promoter-reporter construct is fully methylated \textit{in vitro} and the reporter activity is then compared with the unmethylated one\textsuperscript{204,220}. Consistent with our observation for endogenous $\alpha$-SYN with NanoLuc activity, Jowaed et.al. showed that in-vitro methylation of regulatory promoter-reporter construct of $\alpha$-SYN reduces transcription of $\alpha$-SYN. Studying gene expression this way could lead to different outcome than actual transcriptional state of the target gene as the regulatory region of that gene might exist as partially methylated condition endogenously, which cannot be replicated using the exogenous system. Moreover, comprehensive regulation of the target gene promoter with inputs coming from other local epigenetic modifications such as histone post translational modifications (PTMs)\textsuperscript{221} and \textit{trans} factors which are usually present endogenously, might not be able to regulate the exogenously introduced promoter of the same gene.

During genetic typing, we saw that the intensity of the NanoLuc-tagged allele after PCR is relatively weaker when compared to the wild-type PCR product for both the genomic DNA and cDNA (Fig. 6c and Fig. 11a). This may be a result of PCR bias towards the amplification of the shorter allele over the longer ones involving reaction in a single tube\textsuperscript{222}. This problem of preferential amplification was overcome by using separate primer set to amplify only the NanoLuc-tagged allele (not encompassing the wild-type allele, Fig. 6c and Fig15a, b) which could amplify the NanoLuc-tagged allele very efficiently and comparably to the wild-type allele (Fig.6c and Fig. 14b). In addition to using separate set of primers, we used equal number of cycles (30 cycles) to ensure
that the PCR products are not saturated, which is indicated by the increase in expression after dopamine treatment. However, the western blot shown in the Fig. 2a indicated that the NanoLuc-tagged α-SYN protein (band shown at ~34 KDa) is higher in intensity than the wild type protein (band at ~15 KDa) when probed with α-SYN specific antibody. This unequal distribution of protein bands between wild type (low) and NanoLuc-tagged allele (high) may indicate that either that the Nanoluc luciferase has been targeted to α-SYN in more than one allele of the locus, or it is also possible that fusion of a highly stable NanoLuc luciferase protein with α-SYN may affect the stability of the target protein positively. HEK293T cells are not typically diploid, and are instead complex hypo-triploid in nature, containing less than three times the number of chromosomes of a normal diploid human cell\(^\text{223}\). Our results show that the nanoluc reporter construct is precisely targeted to the end of SNCA and faithfully reports the transcriptional changes of α-SYN.

The superiority of using an endogenous reporter system was highlighted by the data obtained from conventional reporter assays after treatment with different drugs such as 5-AzadC, dopamine and sodium butyrate.

As 5-AzadC inhibits DNA methylation in dividing cells therefore we previously observed that the drug reduced endogenous SNCA-intron1 methylation and increased transcription or NanoLuc activity in our endogenous reporter system. However, we did not observe such increase in exogenous SNCA-promoter/intron1 firefly reporter activity upon drug treatment, may be due to lack of DNA methylation in the exogenous plasmid (Fig. 9a first panel). Surprisingly, we observed a significant increase in Renilla luciferase activity upon 5-AzadC treatment (Fig. 9a second panel) and a significant decrease in
normalized reporter activity (Fig. 4a third panel). However, the observed decrease in reporter activity can be attributed to CMV-Renilla luciferase activity which, although equally transfected, significantly varied upon 5-AzadC treatment. The mechanism of this apparent variation in Renilla luciferase activity is yet unknown. However, it is known that Renilla luciferase activity can vary significantly upon treatment paradigm depending on the promoter driving its activity. This could lead to erroneous interpretation of the observed data after normalization to Renilla as is a standard protocol for these type of assays.\textsuperscript{224}

Moreover, it was also shown that dopamine can enhance α-SYN transcription in HEK293 cells.\textsuperscript{205} Similarly, we also observed a significant increase in the NanoLuc activity upon dopamine treatment to 293T-SNCA-3’NL cells (Fig. 8b) unlike exogenous promoter-reporter assay (Fig. 9b), which further fortifies the reliability of our endogenous tagging system. This apparent discrepancy between the outcome coming from endogenous and exogenous systems may be attributed to either lack of appropriate crosstalk between cis/trans elements in exogenous reporter system or due to lack of methylation structure in the SNCA promoter-intron1 construct in the plasmid-based luciferase system. Since, the aim of our study was to design a tool that can faithfully monitor changes in endogenous α-SYN transcription at physiological level, the question that we seek to address was whether this NanoLuc-tagged α-SYN reflected accurately the change in transcription of wild-type α-SYN (although present in higher copy) after treatment with dopamine. The comparable increasing trend of mRNA and protein of both the wild-type and the tagged α-SYN after treatment with dopamine (Supplementary
Fig. 9b, c) indicated that the engineered cell line could efficiently replicate transcriptional changes of endogenous wild-type α-SYN.

Next we investigated how HDAC inhibitor, sodium butyrate can regulate transcription of SNCA. As the dynamics between histone acetylation or chromatin relaxation and histone deacetylation or chromatin condensation play an important role in regulation of gene transcription\textsuperscript{225,226}, it can be envisaged that the application of HDAC inhibitor would result in hyper-acetylated condition in the gene promoter, which in turn might favor induction in transcription. As expected, we observed a significant increase in the NanoLuc activity in response to sodium butyrate (p<0.001) (Fig. 8d). However, a significant opposite observation was noticed using exogenous reporter system (Fig. 9c). This prominent decrease in the normalized reporter activity can again be attributed to the significant increase of Renilla luciferase activity following sodium butyrate treatment. Interestingly, it has been shown that this HDAC inhibitor can change the overall chromatin structure of the cell and functions through the butyrate response elements on the gene regulatory regions that encompass Sp1/Sp3 binding sites\textsuperscript{227}. Since, SNCA regulatory region contains multiple Sp1/Sp3 binding sites, it is reasonable to assume that recruitment of acetylated Sp1/Sp3 can enhance SNCA expression. Analysis of CMV promoter sequence revealed that it harbors around 5 cognate sequences for Sp1/Sp3 binding, suggesting a possible mechanism for sodium butyrate mediated significant increase in the Renilla luciferase activity. Since, Renilla luciferase plasmid is usually used as endogenous control for this kind of conventional luciferase assay, it is very important to interpret the reporter assay results with caution using this type of transfection controls\textsuperscript{224}. 
Transcriptional upregulation of SNCA has long been a concern in PD pathogenesis. Most intellectual effort has been focused on aggregation behavior, resulting in a lacuna of information on transcriptional regulation of this gene despite its immense importance. In this study we developed a novel screening tool that can efficiently monitor SNCA transcript levels under different treatment conditions known to up-regulate transcription. This tool can provide a new diagnostic platform for drug development and testing of compounds believed to regulate SNCA in the cell in an inexpensive and precise way. As the epigenetic environment for this gene regulation is kept unchanged, the effects of treatments would more closely mimic the state seen in the cells than has previously been available. It is also worth mentioning that the stability and sensitivity of this NanoLuc luciferase reporter makes it suitable to monitor very low expressing genes which are unlikely to be achievable by any other conventional reporter systems.

2.4 Materials and methods

2.4.1 Cell Culture

HEK293T LVX cells were maintained in DMEM (Dulbecco’s Modified Eagle Medium) supplemented with heat-inactivated 10% fetal bovine serum and 1% Penicillin/Streptomycin (Gibco, 10000U/mL). Cells were maintained at 37°C in humidified incubators with 5% CO₂ and passaged following trypsinaization with 0.25% Trypsin/0.53 mM EDTA (ThermoFisher Scientific).
2.4.2 Designing SNCA specific short guide RNA (sgRNA)

The vector for cloning of the sgRNA pSpCas9 (BB)-2A-Puro (PX459) was a gift from Feng Zhang Lab (Addgene plasmid # 48139\textsuperscript{212}). The sgRNA targeting the stop codon area of the SNCA gene was ligated into the pSpCas9 (BB)-2A-Puro vector as previously described with minor modifications\textsuperscript{212} (Table 1). Briefly, sgRNA oligos corresponding to 20 base pairs immediately upstream of –AGG PAM sequence located on the reverse strand at the SNCA stop codon were ligated into pSpCas9(BB)-2A-Puro vector using Fast-Digest BbsI (ThermoFisher Scientific) and T7 ligase (New England Biolabs) by cycling between 37°C and 21°C 10 times for 6 minutes. Ligation reaction was treated with Plasmid-Safe ATP-Dependent DNase (Epicentre) and transformed into CaCl\textsubscript{2} competent cells DH5a E. coli by heat shock (42°, 50 sec). Plasmid was purified using Gene-Jet plasmid Miniprep Kit (ThermoFisher Scientific) according to manufacturer’s protocol and successful insertion of the sgRNA was confirmed by sequencing.

2.4.3 Cloning of NanoLuc-homology donor vector

Primers used for amplification, addition of restriction sites and insert confirmation are listed in Table 1. Approximately 800 bp-length homology domains were amplified from HEK293T genomic DNA using Q5-Polymerase (New England Biolabs). Upstream homology arm (Chromosome 4, 89,727,231-89,728,021, reverse strand GRCh38:CM000666) was modified to include a 5' NotI restriction site and a 3' SacI restriction site (Fig. 1). Downstream homology arm (Chromosome 4, 89,726,457-89,727,235, reverse strand GRCh38:CM000666) was modified to include a 5' HindIII cut site and 3' NotI/AatII cut site. The NanoLuc luciferase sequence was amplified from
pNL1.1 vector (a gift from Promega Corporation). To allow for sequential plasmid ligation, NanoLuc coding sequence was modified to include terminal 5’ SacI and 3’ HindIII/AatII restriction enzyme sites. Upstream homology arm and the NanoLuc were ligated together and cloned into NotI and AatII digested pGEM -T Easy vector (Promega Corporation; cat no. A137A) and transformed into competent DH5a cells. Plasmid was purified using Gene-Jet Plasmid Miniprep Kit according to manufacturer’s protocol (ThermoFisher Scientific). Downstream homology arm was then cloned into AatII-digested vector. Completed homology sequence was digested with NotI to release sequence from pGEM -T Easy vector and delete AatII sequence, then ligated into NotI digested pAAV-IRES-hrGFP backbone (2,846 bp) and transformed into chemically competent DH5a cells. Presence of insert was confirmed by sequencing.

2.4.4 Generation of HEK-293T cell line stably expressing SNCA-NanoLuc (293T-SNCA-3′NL)

Vector constructs were transfected into HEK293T LVX (CloneTech) cells using X-Fect Polymer (CloneTech) according to manufacturer’s protocol. Briefly, HEK293T LVX cells were seeded in a 6-well plate with 1x10^6 and allowed to grow to 80% confluency. X-Fect Polymer was mixed with 1.25 µg pSpCas9 (BB)-2A-Puro with SNCA sgRNA and 1.25 µg NanoLuc-homology arm donor vector and incubated with cells for 4 hours, followed by a change with fresh media. After 48 hours, cells were subjected to 5 µg/mL puromycin (ThermoFisher Scientific, cat no. AC227420100) selection for 48 hours. Surviving puromycin resistant cells were diluted to a single cell level and plated in 96
well plate then allowed to propagate for approximately two weeks. 15 pure colonies were recovered and grown to 50% confluency before passaging them to 1:2 in a 24 well plate.

**2.4.5 Confirmation of stable integration of the NanoLuc reporter at the 3' end of SNCA**

Genomic DNA of the puromycin resistant colonies was extracted by 16 hours incubation in lysis buffer (10 mM Tris-HCl pH 7.6, 0.5 mM EDTA, 0.67% SDS, 132 µg/ml Proteinase-K) at 55°C, followed by precipitation in 100% ethanol with 150 mM NaCl. Presences of the NanoLuc insert was confirmed by PCR using NanoLuc forward and reverse primers and identity of insert was confirmed by gene specific confirmation primers from cDNA using RT-PCR as described below. The details of the primers are listed in Table 1.

**2.4.6 Western blotting**

For Western blotting, protein was extracted using RIPA buffer (PBS, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% PMSF, 100 ng/ml protease inhibitor, dH2O) and 40 µg protein were ran on 10% denaturing SDS gel and transferred to PVDF membrane (Millipore cat no. IPFL00010). Following the transfer process, the membrane was blocked with Odyssey Blocking Buffer (LI-COR cat no. 927-50000) mixed 1:1 with TBS. Specific protein bands were detected using rabbit anti-a-synuclein antibodies (Santa Cruz Biotechnology, Lot SC-7011R, 1:1000 dilution) overnight and followed by goat anti-rabbit secondary antibodies for fluorescent detection at 680 nm (LI-COR, cat no. 926-68020).
2.4.7 Assay for the NanoLuc luciferase activity

To measure the NanoLuc luciferase activity, 200,000 cells were seeded per well of a 24 well plate. After 24 hours, appropriate treatments were done and incubated for designated periods of time in duplicate. Cells were detached by trypsinization and two wells were combined in a 1.5 mL microcentrifuge tube and pelleted at 2,000 X g for 3 minutes. Cell pellets were resuspended in 500 µL colorless DMEM and counted using an automated cell counter (BIORAD, USA) three times and then luciferase activity was monitored in technical triplicate using the Nano-Glo Luciferase Assay System according to manufacturer’s protocol with minor modifications (Promega corporation, cat no. N1110). Briefly, 5,000 cells in 30 µL colorless DMEM were assayed using 30 µL of assay buffer mixed 1:100 with NanoGlo substrate. Plates were incubated for 5 minutes and then luminescence was recorded in triplicate on a multi-plate reader (EnVision, PerkinElmer). This experiment was performed three times independently.

2.4.8 SNCA promoter-reporter assay

To generate the SNCA promoter luciferase construct, SNCA promoter-intron1 region (-2,200 to +118 bp; with respect to ATG) was amplified from HEK293T genomic DNA and cloned into XhoI/HindIII sites of promoter less pGl3 basic vector (Promega, USA, cat no. E1751). The presence of insert was validated by sequencing. For the promoter-reporter assays, 200,000 HEK293T LVX cells were co-transfected with SNCA-pGL3 and CMV-pRL (as a transfection control) vectors in a 24 well plate format. Each well were co-transfected with 500 ng of SNCA-pGL3 and 10 ng of CMV-pRL plasmids using X-Fect polymer as described above and incubated 24 hours before
proceeding to any chemical/drug treatments. Following the drug treatments for appropriate time HEK293T LVX cells were collected for lysis. Briefly, cell pellet was lysed in 100 µL of Lysis Buffer (25mM Tris-Phosphate Buffer pH 8.0, 4 mM EGTA, 2 mM DTT, 20% Glycerol & 1% Triton X-100) for 10 minutes at room temperature with occasional shaking, then centrifuged at 16,000 X g for 5 minutes and the lysate was collected in a separate tube. 10 µL of this lysate was assayed with 140 µL of luciferase assay buffer (25 mM Tris-Phosphate Buffer pH 8.0, 4 mM EGTA, 20 mM MgSO₄, 1 mM DTT, and 2 mM ATP). Firefly luciferase activity (0.75 mM luciferin, 10 mM DTT) and Renilla luciferase activity (1.5 µM Coelenterazine, 100 mM NaCl, 25 mM Tris pH 7.5) were measured in triplicate using a multi-plate reader (EnVision, PerkinElmer). Relative luciferase activity was measured by normalizing the firefly luciferase activity to Renilla luciferase activity (F/R).

2.4.9 Cell treatment paradigm

All the toxin treatments were performed in duplicate wells. Compounds used in the study are as follows: Sodium butyrate (Alfa Aeser; cat no. A11079), (5-AzadC) (Sigma, cat no. A3656) and Dopamine hydrochloride (Sigma, cat no. H8502). Sodium butyrate stock was prepared in distilled water at a concentration of 500 mM and cells were treated with sodium butyrate for 24 hours at 2.5 mM and 5.0 mM. 5-AzadC stock of 5 mM was prepared in 50% acetic acid and immediately aliquoted and frozen. Cells were treated with 10 µM 5-AzadC for 72 hours, refreshed every 8-12 hours²⁰⁴. Dopamine stock was prepared in distilled water at a concentration of 10 mM. Cells were treated
with 100 µM dopamine for 48 hours, refreshed every 24 hours. Control wells were treated with respective vehicles for indicated times.

2.4.10 Bisulfite sequencing

Genomic DNA from 293T-SNCA-3’NL cells following 5-AzadC treatment was extracted as mentioned above. Approximately, 500 ng high quality DNA (260/280>1.8) was used for bisulfite conversion using EZ DNA Methylation-Direct Kit (Zymo Research cat no. D5020) then used as template for PCR amplification of SNCA intron1 region using specific primers designed for bisulfite modified DNA as described in article by Jowaed et al. EpiMark Hot Start Taq DNA Polymerase (NEB Inc; cat No. M0490S) was used for PCR and the amplicon was cloned into pGEM -T Easy vector (Promega Corporation). 10 positive colonies were selected for sequencing analysis using T7 universal primer. The sequenced products were analyzed for degree of methylation using BISMA and QUMA software.

2.4.11 Semi quantitative reverse transcriptase PCR (RT-PCR)

RNA was extracted using TRIzol Reagent according to manufacturer protocols (Life Technologies Inc.; cat no. 15596-026). Complementary DNA (cDNA) was generated by conversion of 1 µg total RNA using amfiRivert cDNA synthesis Platinum Master Mix according to manufacturer’s protocol (GenDEPOT; cat no. R5600-50). cDNA was diluted 1:1 with nuclease free water before PCR. The amplification of SNCA expression was done using primers against the NanoLuc sequence and normalized by amplification of an endogenous control gene, β-actin. The details of all the primers are listed in the Table 1.
2.4.12 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc.). Data are presented as mean ± SEM. To get statistically meaningful data, all experiments were performed in technical triplicates. The times change in the NanoLuc luciferase activities in treated groups were calculated by normalizing the value with respective control for each experiment. All the experiments were independently repeated three separate times using separate batches of cells and the means of each experimental set were analyzed. In case of exogenous luciferase reporter assays, minimum of four independent repeats were performed. Statistical significance was determined by comparing means of different groups and conditions using unpaired 2-tailed Student’s t test, and one-way ANOVA. Multiple corrections were made using post-hoc Tukey test, Bonferroni test and Scheffe’s test whenever it was required. Significance was assessed at 95% level.
2.5 Figures
a

SNCA Gene
5’

1a 1b 2 3 4 5 6

3’

Intron 5 6 3’ UTR

Homology Donor Vector

Sacl HindIII

NanoLuc

HDR

NanoLuc Tagged SNCA Gene
5’

1a 1b 2 3 4 5 6

3’

b

b

293T WT

5’

10 11 12 13 14 15

pNL1.1

SNCA Ref
CTACGAACCTGAAGCCCTAA

NanoLuc Ref
ATGGTCTTCACACTGGAAGATT

293SNCA3’NL
CTACGAACCTGAACGCCAGCTCGTCTTGACTCTGAGGATT

5’ junction

Cell Type Primer NLuc A NLuc B WT A WT B

1.0 kb -

0.3 kb -

3’ junction

SNCA Ref
GAACGCTTTCTGGCGTAA

NanoLuc Ref
GAACGCTTTCTGGCGTAA

293SNCA3’NL
GAACGCTTTCTGGCGTAA

Sacl

HindIII
Figure 6 Development of 293T-SNCA-3’NL cells.

(a) Schematic representation of cloning strategy. SNCA gene map showing exons (1a and 1b non-coding, 2-6 coding) and the 3’UTR. Transfection of sgRNA targeting the 3’ end of exon 6 induces a DSB near the stop codon (TAA). Donor vector design contains 5’ homology arm of 790 bp encompassing part of intron 5 and exon 6 upstream from the stop codon and the NanoLuc-3’ homology arm of 800 bp downstream of the stop codon containing part of the 3’UTR. Co-transfection of donor vector with the CRISPR/Cas9 construct precisely incorporated the NanoLuc right before the stop codon by HDR of the SNCA gene. (b) Following puromycin selection and single cell dilution, genomic DNA from all surviving isogenic colonies were screened for the NanoLuc insert with pNL1.1 NanoLuc vector and HEK293T LVX cells as controls. From 15 colonies recovered, two were positive for the NanoLuc insertion. (c) Gene specific PCR with primers in the intron 5 (A) and the 3’UTR of SNCA showed colony 9 had a heterozygous insertion in 293T-SNCA-3’NL cells (Lane 1); PCR with forward primer on the NanoLuc (B or NanoLuc Internal Forward Primer) and the same 3’UTR reverse primer (cDNA sequencing Reverse Primer) showed comparable amplification of the NanoLuc tagged allele (Lane 2); PCR of the wild-type α-SYN and NanoLuc from the HEK293T LVX as controls (Insertion Confirmation Forward Primer and cDNA sequencing Reverse Primer) (Lanes 3 and 4) (d) Excerpt of Sanger sequencing results showing insertion of the NanoLuc sequence with restriction sites precisely before the stop codon of SNCA and with correct continuation of the 3'UTR after the NanoLuc sequence.
Figure 7 Functional expression of the NanoLuc luciferase.

(a) Lysates from HEK293T LVX, HEK293T SNCA K/O, and 293T-SNCA-3’NL cells were Western blotted with anti-α-SYN antibody. As expected, a wild-type band appeared in HEK293T LVX and 293T-SNCA-3’NL cells, but in the 293T-SNCA-3’NL cells an additional band was identified at approximately 34 kDa corresponding to α-SYN fused with the NanoLuc that was absent in non-NanoLuc tagged cells. (b) Luminescence activity of HEK293T LVX cells compared with 293-SNCA-3’NL cells. Only 293T-SNCA-3’NL cells generate significant NanoLuc activity in the presence of furimazine as the substrate. (c) Luminescence of 293T-SNCA-3’NL cells follows a linear trend with increasing cell numbers ($R^2$=0.9957). All the experiments have been performed in triplicates.
Figure 8 293T-SNCA-3’NL cells having the NanoLuc integration can be used to model deregulated SNCA as seen in sporadic PD.

(a) 293T-SNCA-3’NL cells treated with 10 µM 5-AzadC for 72 hours which induced a significant increase in the NanoLuc activity as compared to the control. The NanoLuc activity was corroborated by increase in α-SYN transcript as shown in the RT-PCR. (b) Methylation status of 23 CpG sites on the SNCA intron1 was determined by bisulfite sequencing. Amplified PCR products were cloned into pGEM-T Easy vector and 10 clones were sequenced. (Left) Comparison of vehicle and 5-AzadC treatment (10 µM, 72 hours) showing unmethylated (open circles) and methylated (closed circles)
cytosines for all 10 clones (y-axis) at each of the 23 CpGs in intron1 (x-axis). (Right) Scatter plot showing overall decrease in methylation by 31.7% compared to the control. (c) Similarly, 293T-SNCA-3′NL cells treated with dopamine at 100 µM concentration for 48 hours, increased NanoLuc activity significantly. Increase in the NanoLuc activity was confirmed by RT-PCR after dopamine treatment. (d) Following HDAC inhibitor (sodium butyrate) treatment at concentrations of 2.5 mM and 5.0 mM for 24 hours, the 293T-SNCA-3′NL cells showed a significant dose dependent increase in the NanoLuc activity. This dose-dependent increase in the NanoLuc activity was also confirmed by RT-PCR following same treatment paradigm. β-actin amplification was used as an internal control for all the PCRs. Error bars show the mean from three technical repeats. p values are given for t-test (5-AzadC, dopamine), one-way ANOVA (Sodium butyrate) where * represents p<0.05, ** represents p<0.01, *** represents p<0.0001.
Figure 9 Exogenous overexpression of SNCA driven firefly and Renilla luciferase failed to replicate endogenous SNCA behavior after similar paradigm of drug treatment.

(a) HEK293T LVX cells were co-transfected with SNCA-pGL3 (firefly luciferase) and CMV-pRL (Renilla luciferase) constructs. Twenty-four hours after transfection, cells were subjected to 10 µM 5-AzadC treatment for 72 hours. No significant change in firefly luciferase activity was seen, however the Renilla luciferase activity showed a significant increase. Thus overall normalized luciferase assay showed a significant decrease in the activity (F/R) after treatment with 5-AzadC. (b) Similarly, HEK293T LVX cells were also co-transfected with SNCA-pGL3 and CMV-pRL plasmids, treated dopamine at concentration of 100 µM for 48 hours. No significant change was observed either in firefly luciferase activity or Renilla luciferase activity. Thereby, the normalized luciferase activity (F/R) also did not show any significant change after treatment with dopamine. (c) Treatment with sodium butyrate at concentrations of 2.5 mM and 5.0 mM respectively for 24 hours, gave comparable results (like 5-AzadC) in normalized luciferase activity (F/R) which showed a significant decrease. This decrease in the normalized luciferase was attributed to the significant increase in firefly luciferase activity and Renilla luciferase activity. Error bars show the mean from three technical repeats. p values given for t-test (5-AzadC, dopamine), one-way ANOVA (Sodium butyrate) for three independent experiments where * represents p<0.05, ** represents p<0.01, *** represents p<0.0001.
Table 4- Sequences of the primers used for the generation and confirmation of 293T-SNCA-3’NL cells.

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<thead>
<tr>
<th>Name of the oligos</th>
<th>Sequence (5’ to 3’)</th>
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<td>SNCA sgRNA Top Strand</td>
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</tr>
<tr>
<td>SNCA sgRNA Bottom Strand</td>
<td>AAACAAAGAAATATCTTTGCTCCCCAC</td>
</tr>
<tr>
<td>Upstream Homology Forward</td>
<td>TATGGCGGCCGCTTAGGAACAAGGAAAAT</td>
</tr>
<tr>
<td>Upstream Homology Reverse</td>
<td>AGTGAGCTCGGCTCCAGGTTTCGTAGTC</td>
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<tr>
<td>NanoLuc Forward</td>
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</tr>
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<tr>
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</tr>
<tr>
<td>NanoLuc Internal Forward</td>
<td>AAGGTGATCCTGCACCATATGGCA</td>
</tr>
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Figure 10 Sequence alignment of wild type SNCA against 293T-SNCA-3’NL genomic DNA sequence.

Part of the sequence alignment is shown depicting the 3’ end of the SNCA of 293T-SNCA-3’NL. The wild type (WT) SNCA genomic DNA sequence (reference sequence: GRCh38.p7) was aligned with the sequencing data from 293T-SNCA-3’NL to check for the proper incorporation of the NanoLuc construct at the end of the coding sequence of SNCA. Sequence 1 is expected SNCA genomic DNA, sequence 2 is 293T-SNCA-3’NL sequence. The last exon of SNCA is highlighted in blue. The start and stop codons of NanoLuc are highlighted by yellow and red colors respectively. The aligned bases between both the sequences are marked by star (*). The in-frame incorporation of the NanoLuc can be seen in between the two restriction enzyme sites, SacI and HindIII (underlined) in 293T-SNCA-3’NL cells (2). Sequence alignment was done using Clustal Omega program.
a

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************************************************************
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CATGGTGTG
************************************************************
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************************************************************
GGCTTTGTCAAAAAGGACCAGTTGGGCAAGAATGAAGAAGGAGCCCCACAGGAAGGAATT
************************************************************
CTGGAAGATATGCCTGTGGATCCTGACAATGAGGCTTATGAAATGCCTTCTGAGGAAGGG
************************************************************
GAGGGAGTTGTGGCTGCTGCTGAGAAAACCAAACAGGGTGTGGCAGAAGCAGCAGGAAAG
GAGGGAGTTGTGGCTGCTGCTGAGAAAACCAAACAGGGTGTGGCAGAAGCAGCAGGAAAG
************************************************************
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b

```
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2  TGGTGTAAAGGAATTACATTAGCCATGGATGTATTCATGAAAGGACTTTCAAAGGCCAAG
   ************************************************************
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2  TGGTGTAAAGGAATTACATTAGCCATGGATGTATTCATGAAAGGACTTTCAAAGGCCAAG
   ************************************************************
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2  TGGTGTAAAGGAATTACATTAGCCATGGATGTATTCATGAAAGGACTTTCAAAGGCCAAG
   ************************************************************
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Figure 11 293T-SNCA-3'NL cDNA indicates correct insertion of NanoLuc sequence.

(a). PCR of SNCA from 293T-SNCA-3'NL cDNA using “cDNA sequencing primers" (Table 1) produces two bands, one matching expected wild-type size and one matching expected size for wild-type with NanoLuc insertion (NanoLuc Tagged α-syn). b. Part of the sequence alignment is shown depicting the coding region of the gene and incorporation of the Nanoluc sequence in 293T-SNCA-3'NL cell line. The WT SNCA mRNA sequence (reference sequence: NM_007308.2, transcript variant 4) was aligned with the sequencing data from 293T-SNCA-3'NL to check for the proper incorporation of the NanoLuc construct at the end of the coding sequence of SNCA. Sequence (1) is WT SNCA mRNA transcript sequence (2) is 293T-SNCA-3'NL sequence. The start and stop codons in the WT SNCA mRNA sequence are highlighted by green and red colors respectively. The aligned bases between both the sequences are marked by star (*). The in-frame incorporation of the NanoLuc can be seen in between the two restriction enzyme sites, SacI and HindIII (underlined) in 293T-SNCA-3'NL cells (2). The NanoLuc insert is unique to 293T-SNCA-3'NL cell line and not found in WT SNCA mRNA sequence. The new stop codon after the NanoLuc sequence in 293T-SNCA-3'NL cell is marked by yellow. “N” denotes unread base in the sequence. Sequence alignment was done using Clustal Omega program.
Figure 12 Sequence alignment of SNCA – NanoLuc fusion with colony 14.

Part of the sequence alignment file is shown depicting the coding region of the gene and incorporation of Nanoluc sequence to check for the proper incorporation of NanoLuc construct at the end of the coding sequence of SNCA for 293SNCA 3’ NanoLuc colony 14. Sequence (1) is expected sequence of SNCA gene around exon 6 after NanoLuc incorporation, sequence (2) is 293T-SNCA-3’NL colony 14 sequence. The aligned bases between both the sequences are marked by star (*). The partial deletion of NanoLuc sequence and 6 bases of SNCA exon 6 can be seen. The sequence alignment was done using Clustal Omega program. Due to this, colony 14 was not selected for downstream studies.
Figure 13 Full western blot of 293T-SNCA-3’NL cells showing NanoLuc tagged and WT α-synuclein.

Full western blot image from Fig. 2a. α-SYN was detected using polyclonal rabbit antibody (Santa Cruz, SC-7011-R). Wild-type α-SYN is shown at approximately 16 KDa and is present in both wild-type 293T cells and 293T-SNCA-3’NL cells, but absent from 293T-SNCA-knockout cells. NanoLuc-fused α-SYN is shown at approximately 34 KDa, and only appears in 293T-SNCA-3’NL cells. The unmarked bands present in the blot are non-specific bands that commonly appear with this particular polyclonal antibody.
a

Wild Type PCR

5'-------------------\(\text{SNCA}\)-------------------3'

NanoLuc PCR

5'-------------------\(\text{SNCA}\)---------------\(\text{NanoLuc}\)-------------------3'

b

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<tr>
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PBS Treated     DA Treated

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Figure 14 Comparable increase of endogenous and NanoLuc-tagged SNCA levels following dopamine treatment.

(a). Schematic representation of PCR strategy to avoid preferential amplification issues from heterozygous allele sizes. For WT amplification, primers used were “SNCA Exon 4 Forward” and “Insert Confirmation Reverse” (Table 1) to give an amplification product size of 338 base pairs. For NanoLuc specific amplification, primer pair used were “NanoLuc Internal Forward” and “Insert confirmation reverse” to get a product size of 356 base pairs. Elongation time was restricted for NanoLuc specific amplification to prevent competitive wild-type amplification. b, c. Qualitative image of RT-PCR and western blot analyses of 293T-SNCA-3′NL cDNA and protein under vehicle and dopamine (100uM) treated condition show comparable increasing trend for both wild-type and NanoLuc tagged α-SYN.
 CHAPTER 3: IMPLICATION OF 8-OXODG MEDIATED TRANSCRIPTIONAL MUTAGENESIS IN THE PATHOGENESIS OF SPORADIC PARKINSON’S DISEASE

(This chapter contains unpublished data by Sambuddha Basu)

Abstract

Parkinson’s disease is an age-related progressive neurodegenerative disorder pathologically characterized by intra-cytoplasmic proteinaceous aggregates called Lewy bodies primarily consisting of a pre-synaptic protein called alpha-synuclein (α-SYN). Structural studies of α-SYN have shown the sensitivity of the molecule towards amino-acid changes which makes it more aggregation prone, thereby leading to PD like condition. Oxidative DNA damage in the form of 8-oxo-7,8-dihydroguanine (8-oxodG, oxidized guanine) is increased in the genomic DNA of PD patients compared to age-matched subjects. 8-oxodG being a non-bulky DNA lesion does not stall the RNA Pol II and can lead to misincorporation of adenine instead of cytosine in mRNA during transcription of a gene, a phenomenon known as transcriptional mutagenesis (TM). We hypothesized using SNCA (alpha-synuclein) gene as a model, that 8-oxodG-mediated TM event can generate novel variants which contribute to the aggregation of the wild-type protein as seen in Lewy bodies. We predicted the generation of 43 possible positions that can be mutated, but focused on a few which had the highest potential towards aggregation (structural analysis by algorithm TANGO). We confirmed the presence of three of the predicted mutations (Serine42Tyrosine (S42Y), Alanine53Glutamate (A53E) and Serine129tyrosine (S129Y)) in α-SYN mRNA from the SN of human post-mortem PD brain using RNaseH2 PCR. Sequencing genomic DNA of
the same PD sample and same region of α-SYN revealed no mutations at the DNA level. By using cell-based biochemical assays and recombinant protein assays we have seen that S42Y α-SYN can accelerate the aggregation process involving the wild-type protein even when present in significantly lower amount relative to the WT parental protein. Importantly, we developed antibody to specifically detect the S42Y α-SYN. Immunohistochemical analysis of serial post-mortem PD brain sections with H&E staining, anti-ubiquitin staining and anti-S42Y α-SYN staining, showed Lewy bodies that stained positively with S42Y α-SYN. To our knowledge, this is the first report about TM related mutations of α-SYN in Parkinson's disease and their role in the pathogenesis.

*Keywords*: 8-oxodG, transcriptional mutagenesis, alpha-synuclein, aggregation
3.1 Introduction

Parkinson’s disease (PD) is the second most common neurodegenerative disorder after Alzheimer’s disease (AD)\textsuperscript{230}. PD is an age-dependent progressive disease characterized by loss of dopamine producing neurons (dopaminergic neurons, DA) from the \textit{substantia nigra pars compacta region} (SNpc) of the ventral mid-brain \textsuperscript{127}. In PD, the dopamine metabolism associated with the dopaminergic neurons generate reactive oxygen species (ROS) that render these neurons very susceptible to degeneration\textsuperscript{168,169}. Pathologically, PD is associated with cytoplasmic proteinaceous aggregates which are the hallmark of the disease. These aggregated proteins are called Lewy Bodies and Lewy neurites \textsuperscript{231}. Like all other neurodegenerative disease, PD can be broadly classified into familial form (genetic aberrations like mutations or multiplication of gene, early onset form) and the idiopathic form with unknown etiology (late-onset form). More than 95% of the PD cases are reported as sporadic or idiopathic form \textsuperscript{232}. Among all the different important genes (and proteins) reported to play a role in the pathogenesis of PD, alpha-synuclein (α-SYN) encoded by the gene \textit{SNCA} has been the most researched protein. It is because, α-SYN is an integral component of the Lewy bodies and neurites owning to its inherent potential to aggregate into β-sheet rich structures as seen in PD\textsuperscript{75}. α-SYN is known to play a critical role in the pathogenesis of both the familial and the idiopathic form of the disease and has thus attained a hallmark status to study PD and its progression\textsuperscript{169}.

Earlier reports have strongly implicated oxidative stress as a key mediator of PD pathogenesis. Increased oxidative stress is often accompanied with the damages incurred by the macromolecules of cell like DNA, RNA, proteins and lipids whose
functions are indispensable for survival. Among the known oxidative damages, 8-oxo-7,8-dihydroguanine (8-oxodG), the oxidized form of guanine, is the most commonly occurring and is often linked with several disease conditions like neurodegenerative disorders and cancer to name a few.\textsuperscript{127,168} 8-oxodG is known for its strong mutagenic potential because it's a non-bulky DNA lesion which often goes unrecognized by the repair machinery of the cell.\textsuperscript{168,233} 8-oxodG can misincorporate adenine instead of cytosine through Hoogsteen bonding during replication (in the daughter DNA strand) and also in the nascent mRNA (during transcription), thereby causing G: C\textrightarrow:T:A transversion mutation.\textsuperscript{178,233} In mRNA, when 8-oxodG misincorporate adenine instead of cytosine, it leads to an event called transcriptional mutagenesis (TM), which in turn lead to the generation of mutated species of protein.\textsuperscript{178,184} In post-mitotic cells like neurons, the pathogenic effect caused by mutated species generated through 8-oxodG-mediated TM event may get accumulated over life and thus make them more vulnerable.\textsuperscript{184}

In addition, several reports have documented the accumulation of 8-oxodG in the ageing brain which gets further increased in brains of patients with PD.\textsuperscript{168,234} In this study we hypothesize that 8-oxodG-mediated TM may generate novel pathological mutants of α-SYN that drive misfolding of the native monomeric α-SYN, thus contributing to the fibrillation process and pathogenesis. Like other reported pathological mutants of α-SYN, the contribution of TM to α-SYN fibrillation will depend on whether the mutant species can acquire a misfolded state by themselves that may eventually drive the aggregation of the native protein.\textsuperscript{193} We analysed in-silico that TM event can generate 43 different α-SYN species.\textsuperscript{75} On predicting the aggregation potential of all
the mutants individually using the aggregation prediction software TANGO, we focused on the mutant *Serine 42 Tyrosine* (S42Y) α-SYN which had the highest score amongst all the other 43 mutants. *Alanine 53 glutamate* (A53E) α-SYN which is a reported familial mutation could also be generated through TM. Lastly, *Serine 129 tyrosine* (S129Y) α-SYN which can be generated through TM event overlaps with a very important site for phosphorylation and aggregation of α-SYN\textsuperscript{169}. In this study we investigated the generation of the three TM-related α-SYN mutants in human post-mortem PD and control cohort and further analysed the contribution of the S42Y α-SYN in aggregation of the WT α-SYN.

### 3.2 Results

#### 3.2.1 Increased 8-oxodG levels in the substantia nigra of PD patients

The first aim of this study was to evaluate the difference in 8-oxodG level from the genomic DNA of PD and control post-mortem samples in the SNpc region. This section of the result was performed in collaboration with Dr. Fernando Cardozo-Pelaez. We observed a significant increase in the 8-oxodG level in the PD cohort \( n=8 \) when compared to the age-matched controls \( n=9 \) \( p<0.0004 \) (fig 15a). This was in line with the increased oxidative stress reported in PD patients. Initially, it was expected that 8-oxodG accumulation in the PD condition may be a cumulative effect of the increased oxidative stress and the failure of the base excision repair machinery designated for 8-oxodG repair. However, mRNA analysis of the 8-oxodG repair enzyme, 8-oxodG DNA glycosylase (OGG1) did not show any significant difference between the two cohorts.
Interestingly, when we investigated the activity of OGG1 protein from the SN region of the cohort in repairing 8-oxodG lesion, no significant difference in OGG1 activity was observed between the SN region of control and PD group (fig 15c,f). Even though, no significant difference between the two cohorts was seen when comparing the OGG1 activity, significant correlation was observed between 8-oxodG levels and OGG1 activity in the control group only (p=0.0101) ($R^2 = 0.6347$) (fig 15d) which was not observed for the PD cohort (p=0.06) ($R^2 = 0.4747$) (fig 15e). This indicated that under moderate oxidative stress condition as seen during normal ageing, OGG1 can actively repair the 8-oxodG lesion. However, when the oxidative stress gets severely increased during PD like condition, dopaminergic neurons try to compensate the damage by up regulating the activity of OGG1, which eventually fails to keep up with the accumulation of 8-oxodG. This observation was consistent with previous reports which showed that OGG1 protein expression was up regulated in dopaminergic neurons of SN region\textsuperscript{235} and it was a response to the elevated oxidative stress and not a cause for the 8-oxodG accumulation. Taken together, this figure shows that the significant increase in accumulation of 8-oxodG in the genomic DNA in the SN during PD condition can be attributed to the severe increase in oxidative stress and not due to defect in the repair machinery associated with 8-oxodG. Overall, this figure strongly fortifies the foundation of our hypothesis to investigate 8-oxodG mediated TM event in PD.
3.2.2 Detection of TM generated mutant α-SYN mRNA from human post-mortem PD and control brain samples

To explore the generation of TM-mediated α-SYN mutants in sporadic PD patients, we designed RH-primers (for RNaseH2 PCR) specific to detect three mutants S42Y, A53E and S129Y α-SYN (fig 17a). Specificity of the RNaseH2 PCR was stringently optimized using plasmid and cDNA based system harboring the mutants (fig 17b-d). With the optimized PCR conditions, we screened human post-mortem control (n=11) and PD samples (n=15) for the presence of S42Y, A53E and S129Y α-SYN (fig 16a, 17e). We observed that the S42Y α-SYN was detected in 3 out of 11(approximately 27%) control samples compared to 8 out of 15 (approximately 53%) PD samples. Further, the A53E α-SYN could be amplified from 1 out of 11(approximately 9%) control samples compared to 6 out of 15 (approximately 40%). However, the mutant S129Y could be detected in 1 sample only from both the control and PD cohort (fig 16a,b). As expected, the wild-type α-SYN corresponding to each of the mutants could be successfully amplified from all the control and the PD samples (fig 16a, 17e). Although not quantitative, densitometric analysis of all the three mutants relative to the WT α-SYN indicated that the mutant species was present in the human samples between 5-10% (data not shown). Sequencing of the amplified cDNA and the genomic DNA from the same region of the same patient sample showed that the C→A mutation was present only in the RNA (cDNA) and not in the genomic DNA, thus indicating the mutant specie generation during transcription (fig 17f).

Taken together, this figure shows that TM-generated mutant mRNA α-SYN species could be detected in both control and PD post-mortem samples. However, for
the three TM mutants analysed in this study, the overall occurrence of the mutants were relatively higher in PD compared to controls. This relatively higher generation of mutant α-SYN in PD was in line with the high accumulation of 8-oxodG in them.

3.2.3 Increased aggregation of S42Y α-SYN compared to WT α-SYN

We reported earlier in Basu et. al. 2015 that S42Y α-SYN had the highest aggregation score amongst the predicted forty-three mutants of α-SYN as analysed by the aggregation software TANGO. In the last section, we showed that the mutants S42Y, A53E and S129Y α-SYN mRNA could be detected from human post-mortem PD and control brain samples. In this section we investigated the aggregation potential of S42Y α-SYN compared to WT α-SYN using cell-based and recombinant protein experiments.

To assess the aggregation potential of S42Y α-SYN, we over expressed WT and S42Y containing expression vectors in HEK293T α-SYN knock out cells (fig 18a). As expected, we observed a significantly higher aggregated fraction for S42Y α-SYN compared to the WT α-SYN as detected by anti-α-SYN antibody (lane 2 and lane 3 of the 2nd panel) with no or little change in the soluble fraction of α-SYN (lane 2 and lane 3 of the 1st panel) (fig 18b). This increase in S42Y α-SYN aggregation was also supported by the increased phosphorylated α-SYN as detected by the anti-phosphoserine 129 antibody in the aggregated fraction (lane 2 and lane 3 of the 3rd panel) (fig 18b). We transfected the backbone vector (empty) without the α-SYN as a negative control to mock the events of transfection and aggregation. The biochemical data of increased aggregated fraction of S42Y α-SYN was corroborated faithfully using cell based
immunostaining, where it was observed qualitatively that perinuclear punctated aggregates (as shown with the arrow) were relatively more in the S42Y over expression condition compared to the WT condition (fig 18c). Quantitation of the puncta like structures after proteinase-k treatment (to degrade soluble α-SYN) revealed significantly higher number of aggregates in the S42Y over expression condition when compared to the WT α-SYN (fig 18d). Analysis of aggregation assays from recombinant WT and S42Y α-SYN also indicated that S42Y α-SYN started losing soluble protein faster compared to WT α-SYN and also formed significantly higher Thioflavin-t positive aggregates at the end of aggregation period (fig 18e,f).

Taken together these results suggested that S42Y α-SYN had higher potential to aggregate compared to its WT counterpart as predicted by the software TANGO.

3.2.4 Accelerated aggregation of WT α-SYN in the presence of relatively lower amounts of S42Y α-SYN

To explore the contribution of S42Y α-SYN on aggregation of WT α-SYN protein, we developed a bidirectional cell-based and recombinant protein based approach. In accordance with the densitometric analysis of the mutant (S42Y) relative to the WT parental protein at the mRNA level which showed the mutant to be present no more than 5-10% relative to the WT α-SYN, we investigated the effect of 100 times lesser S42Y on the WT α-SYN (WT:S42Y=1:0.01). In order to investigate the aggregation of α-SYN when 1% S42Y (shown as 1: 0.01) was mixed with 99% WT α-SYN, we first titrated various ratios of S42Y α-SYN relative to the WT α-SYN (100%, 50%, 5% and
1%), to confirm that, in the soluble fractions of both the WT and the S42Y were maintaining the ratio in which they were over-expressed (fig 23g).

Once confirmed, we next investigated the aggregation status of WT parental protein in the presence of 1% S42Y α-SYN. Analysis of the SDS soluble (aggregated) fraction showed that 1% S42Y α-SYN can increase the aggregation of the WT protein when compared to the WT α-SYN only (fig 19a). Empty backbone vector was mixed with the WT α-SYN in the same ratio to mimic the transfection of S42Y α-SYN. Since WT α-SYN can aggregate on its own, we see a dosage effect on aggregation between WT 50% and WT 100 % (top panel lane 1 and 2). Similarly, this dosage effect was also observed for the S42Y 50% and 1% (top panel lane 3 and 4) (fig 19a). This increase in aggregation status of WT α-SYN on spiking with 1% S42Y α-SYN was also confirmed by the dramatic increase of phosphorylated α-SYN as detected by anti-phosphoserine129 α-SYN antibody when compared to the WT α-SYN conditions only (panel 2, compare lane 1 and 2 with lane 3 and 4) (fig 19a). The increased aggregation of WT α-SYN in the presence of 1% S42Y was also supported by split-luciferase complementation assay which showed significantly increased luciferase activity (as a measure of aggregation) in the presence of 1% S42Y α-SYN over a period of 72 hours when compared to WT α-SYN only condition in HEK293T α-SYN knock out cells (fig 19b).

Next, we analyzed recombinant WT and S42Y α-SYN in order to further confirm our observation from the cell based assays (fig 19c). In line with the cell based assay to see the effect of 1% S42Y α-SYN on the aggregation of WT α-SYN, we aggregated the WT α-SYN with or without S42Y α-SYN by shaking at 250 rpm at 37°C. On evaluating
the loss of soluble protein over a period of 8 days from both the conditions, we observed that the S42Y spiked WT α-SYN started losing soluble protein from the 2nd day onwards (approximately 60% of the soluble protein lost) compared to the WT only which still retained 95% of the soluble protein on 2nd day (fig 19e). However, on the 8th day we observed that both the conditions had similar percentage of soluble protein remaining (approximately 25%) (fig 19d). This data indicated that presence of S42Y α-SYN even in a relatively lower percentage compared to the WT can dramatically accelerate the aggregation process. The loss of soluble α-SYN of the same samples monitored over a period of 8 days was supported by the corresponding increase in the thioflavin-T binding, which showed increased thioflavin-T reactivity in the presence of 1% S42Y indicating formation of more aggregated β-sheet structures (fig 19e). The dynamics of structural changes upon addition of 1% S42Y α-SYN were monitored by circular dichroism (CD). CD minima at 222 and 208 nm indicated alpha-helix and a single minimum around 216 nm indicated β-sheet structure. The far-UV CD signal of WT+1%S42Y α-SYN increased with time and displayed a well-defined minimum around 216 nm from day 2 against day 4 for the WT only condition, indicating formation of β-sheet structure faster (indicated by arrow) (fig 19f). Time-dependent changes in ellipticities at 208, 216, and 222 nm showed stronger β-sheet signal at 216 nm for S42Y spiked condition (fig. 19e). In order to probe the aggregated species formed, we investigated the WT and 1% S42Y containing WT samples through Atomic Force Microscopy (AFM) (figure 20). Corroborating with the CD spectroscopy, analysis of AFM data showed that WT α-SYN aggregates were morphologically very different from the 1% S42Y containing WT α-SYN, which again varied from the fibrils formed by the
mutant only. Earlier reports demonstrated aggregated α-SYN to form a variety of β-sheet containing fibrillar structure. Fibrils formed by the WT α-SYN appeared longer in height (approximately 14nm) with median width of 30nm, whereas for the 1% S42Y containing WT α-SYN fibrils appeared to have smaller heights (approximately 10-11nm) and median width of 40nm compared to the WT only and also annular structures which had similarity with oligomers or annular protofibrils. The fibrils formed by the mutant only were much consistent with the measurements of matured fibrils indicating that S42Y formed matured fibrils, whereas the 1 %S42Y containing WT α-SYN had initiated the formation the matured fibrils. The aggregated structures formed by the WT α-SYN were more consistent with large protofilaments and not mature fibrils.

Overall this figure investigated the contribution of S42Y in aggregation of the WT α-SYN protein. S42Y α-SYN when present in 100 times lesser proportion compared to the WT α-SYN accelerated the kinetics of aggregation of the WT parental protein as seen biochemically and through recombinant protein study. The data represented and analysed in this figure, illustrated the effect of TM generated mutant on aggregation of WT α-SYN.

3.2.5 Detection of S42Y α-SYN positive Lewy bodies from the substantia nigra region of Parkinsonian brains

In the earlier results, we showed that increased accumulation of 8-oxodG in the genomic DNA of SNpc from post-mortem PD patients generated novel mRNA variants of α-SYN through TM event, which in turn can accelerate the aggregation process involving the WT parental protein. In this section, we will investigate the presence of
protein species of S42Y α-SYN as one of the constituents of mature Lewy bodies from the SNpc region of PD patient post-mortem samples using our newly developed rabbit anti-S42Y α-SYN antibody.

Firstly, we validated the specificity of the newly developed rabbit anti-S42Y α-SYN antibody to make sure it detected only S42Y α-SYN and not WT α-SYN protein using cell based and recombinant protein based experiments (fig 23). The final goal of this study was to investigate the presence of mature Lewy bodies and Lewy neurites from human post-mortem PD samples containing S42Y α-SYN. We analyzed three serial sections from five post-mortem PD SN tissue sections with Hematoxylin & Eosin staining (H&E), ubiquitin staining and S42Y α-SYN staining to detect Lewy bodies containing the mutant S42Y α-SYN. Interestingly, after scanning the entire sections for each patient, we could detect two S42Y positive Lewy bodies from one PD sample and one positive Lewy body from a second PD sample that exactly overlapped with the H&E staining (Lewy bodies are eosinophilic in nature and thus stain with eosin) and ubiquitin staining (fig 21a, 22b). As expected, there were several matured Lewy bodies that could be picked up with the H&E and Ubiquitin staining for each of the five PD sections (see the ubiquitin and H&E stained 10X magnification panel from the first patient sample).

Although expected, we had to be sure that the low frequency of S42Y positive mature Lewy bodies were not an artifact of the staining technique. In order to do so, we tested our newly developed antibody in collaboration with Dr. Denise Dickson on a different cohort of PD samples using a different antigen retrieval technique (using EDTA and proteinase-K) and stained the SN tissues with the exact same concentration of the anti-S42Y antibody (fig 22a). Interestingly, only a few Lewy bodies could only be
detected this time also, indicating that the low frequency of S42Y positive Lewy bodies. The low abundance of S42Y positive Lewy bodies and neurites from PD samples was further fortified with staining of SN sections from patients with Dementia with Lewy bodies where two patient samples had S42Y positive Lewy bodies (out of 5 stained sections) which overlapped exactly with the ubiquitin staining (fig 22c).

We further investigated mid-brain and cortical sections for the presence of S42Y positive aggregated α-SYN from a rotenone-based rat PD model developed in the laboratory of Dr. Timothy Greenamyre. We blindly stained rat cortical and mid-brain tissue sections with anti-S42Y α-SYN from three groups of animal which we identified correctly based on increased frequency of S42Y positive aggregated structures. The groups were controls/vehicle treated, rotenone exposed (age 6 months) and rotenone exposed (age 9 months) (fig 21b). Increased detection of the S42Y positive aggregates in the rotenone based rat model, indicated that S42Y α-SYN correlates with the progression of the disease.

Overall, from this figure we concluded that S42Y α-SYN positive Lewy bodies and Lewy neurites formed under PD like condition as seen from the immunostaining data. However, the staining of the PD samples, DLB samples and also the sections from rat based PD model clearly indicated the low abundance of S42Y α-SYN positive Lewy bodies.
3.3 Discussion

In the present study we identified 8-oxodG-mediated transcriptional mutagenesis (TM) as a novel mechanism promoting α-SYN aggregation in sporadic PD by generation of aggregation prone mutant variants of S42Y α-SYN. Increase in oxidative stress have always been implicated in the aggregation of α-SYN. However, there were no direct links that established the causal relationship between increased oxidative stress and aggregation of the protein as seen in sporadic PD. Through this study, we present data showing that increased DNA damage in the form of 8-oxodG can lead to generation of novel mutants of α-SYN through transcriptional mutagenesis that promote aggregation.

Consistent with previous reports, we observed in our post-mortem samples that the level of 8-oxodG accumulation was significantly increased in the genomic DNA of PD patients compared to their age-matched controls. There are previous reports showing increased OGG1 expression under PD like condition in different regions of the brain. We also observed no significant change in the OGG1 mRNA level or activity in the SN of PD and control samples, although an increasing trend in OGG1 activity was observed in PD. A plausible explanation to support the increasing trend in OGG1 activity and expression (as shown by previous studies) could be that of a compensatory mechanism triggered by the cell to cope with the increasing oxidative stress and 8-oxodG accumulation (figure 15). Therefore, the increase in 8-oxodG levels in PD can be attributed completely to increase in oxidative stress that build up in the dopaminergic neurons and not to any deficit in repair machinery as we might have expected. In ageing condition, the OGG1 activity significantly correlates with the 8-oxodG level when there is moderate buildup of oxidative stress. But, when the oxidative stress gets increased
dramatically in PD, the cells repair machinery tries to cope up to repair the associated damage but eventually fails, resulting in huge accumulation of 8-oxodG. Thus, our finding implicates OGG1 activity as an effect of increased 8-oxodG rather than a cause for it.

The distribution of 8-oxodG accumulation in the nuclear genome has been shown to be uneven, although reports suggest that regions with a high frequency of recombination and single nucleotide polymorphisms (SNPs) were preferentially located within chromosomal regions with a high density of 8-oxodG\textsuperscript{236}. However, there were no reports supporting the distribution of 8-oxodG with a bias towards specific gene encoded in the genomic DNA. Therefore it can be assumed that an event like transcriptional mutagenesis can affect a multitude of functional genes that produce functional proteins in living cells. We modeled our analysis of TM event and its contribution with respect to α-SYN and PD because, structural studies of α-SYN have implicated the sensitivity of the molecule towards amino-acid changes which makes it more aggregation prone thereby affecting the aggregation status of parental WT protein\textsuperscript{109-111}. Although not a quantitative analysis, but our data from figure 17 e clearly shows that the WT α-SYN was relatively much higher in number as a molecule compared to the mutants, which fitted very well to this idea of seed based aggregation\textsuperscript{237}. This observation also supported the fact that detected mutants were not spontaneously present in the genome, wherein amplification would have been biased only for the mutant or of equal intensity for both the mutant and the WT. We believe that an event like TM can be of immense relevance to other neurodegenerative diseases like
Alzheimer’s disease and Huntington’s disease which too involve neuronal death mediated by protein aggregation.

Previous studies have shown significant accumulation of 8-oxodG in ageing brain which gets further increased under conditions like PD or AD\textsuperscript{235,238} (figure 15a). An event like TM can also explain in parts why ageing in itself pose as an indispensable risk factor underlying neurodegeneration. In our data shown in figure 16a, we had also observed the presence of TM-generated mutants S42Y, A53E and S129Y in our cohort of control samples, although the overall distribution of these three mutants were comparably more in case of PD. This finding was in line with previous reports which showed the presence of α-SYN positive aggregates even in older control brains, although the rate of incidence was significantly lower (8.3%) compared to PD (100%)\textsuperscript{239}. Moreover, based on our findings in this study we strongly believe that distribution and analysis of all the aggregation prone predicted TM-generated mutants of α-SYN (out of a total of forty-three) will definitely correlate with the progression and pathogenesis of PD to draw a clear line of demarcation between control and disease-risked individuals. In this study we have focused mainly on the generation of aggregation prone mutants like S42Y α-SYN (highest aggregation score predicted by TANGO), A53E (known familial mutation) and S129Y (S129 is known to phosphorylate and cause aggregation). However, it is also likely that TM can generate α-SYN which may have lower aggregation potential compared to the WT protein. Since our results have shown that S42Y α-SYN can trigger a snow ball like effect on the WT α-SYN aggregation, we believe that presence of TM-generated mutants having lower
aggregation potential will have no overall effect on WT α-SYN aggregation when present together with a higher aggregation prone mutant like S42Y α-SYN.

The major goal of this study was to investigate the contribution of TM-generated mutants like S42Y α-SYN on the aggregation of the WT parental protein when present in a relatively lower amount. Since our method for detection of the TM-generated mutants involved amplification steps, we do not have an exact estimation of the relative quantitation of the mutants with respect to the WT α-SYN. However, the biochemical contribution of hypothetical 1% S42Y on 99% WT (0.01:1) was based on an approximate densitometry of 15 PD samples, which showed the mutant to be present in no more than 5-10% with respect to the WT α-SYN (data not shown). Moreover, cell-based biochemical experiments were performed by controlling the ratio of transfection of the parental WT and mutants S42Y α-SYN plasmids, which under ideal condition would never reflect the intake of plasmids by cells maintaining the ratio (figure 3 b and c). Although we observed the soluble fraction of the WT and S42Y α-SYN to maintain the ratio of transfection to certain degree (figure 19a), to further strengthen our observation on aggregation we performed recombinant protein based experiments, where a stringent control over the ratio of the two species was possible (figure 19c-f).

As previous studies have reported, aggregation of α-SYN is a dynamic process involving formation of different intermediate conformations. In a similar line, the AFM analysis of WT α-SYN versus 1%S42Y containing WT α-SYN also showed vivid structural differences in the aggregates formed at the end of 8 days of incubation (figure 20). The analysis of AFM data showed that only the S42Y α-SYN formed mostly matured fibrils, whereas the 1% S42Y seeded WT started forming what appear to be
premature fibrils with a combination of oligomers. However, the WT only condition formed what appear to be large amorphous aggregated structures having more resemblance to protofilaments. Interestingly, this difference in α-SYN aggregates formed was also partially reflected through the CD data, which showed formation of different β-sheet structures by variation of the peak depth at 220nm from day 5 onwards in the S42Y seeded condition. This trend was not observed in the case of WT α-SYN aggregates which showed a consistent increase in peak depth at 220nm (figure 19g). Despite this fact, we could successfully capture the accelerated transition of α-SYN seeded with 1%S42Y from its random coil to β-sheet structure which was consistent with the other aggregation assays (figure 19d-g).

Finally, consistent with our expectation, we detected matured Lewy bodies and neurites from the post-mortem SN tissue section of PD samples though immunostaining with anti-S42Y antibody. We were not very surprised to detect only a couple of Lewy bodies from a few patient samples, since we expected TM to be a rare phenomenon (figure 21,22). However, we strongly believe analysis of other aggregation prone TM generated mutants in PD samples with their respective antibodies will lead to an increased staining pattern of Lewy bodies. Since, the goal of this experiment was to detect S42Y positive Lewy bodies and neurites from human post-mortem PD samples, we did not quantify the staining of the S42Y positive Lewy bodies from PD with age-matched controls. Based on previous reports which showed the occurrence of Lewy bodies in PD to be significantly high compared to age-matched controls, we stained SN tissues from PD only to maximize our chances of getting a S42Y α-SYN positive Lewy body. Moreover, we failed to biochemically detect S42Y α-SYN from total protein lysates
of post-mortem PD and control brains using immunoblotting with our newly developed antibody. This may be attributed to the low representation of the specie in the pool of α-SYN. We have also seen that S42Y α-SYN has tremendously high potential to aggregate in comparison to the WT protein, which may result in speedy aggregation of the specie to form a component of Lewy body like structure in cell.

Although this study lay down concrete evidence to support the TM hypothesis and its contribution to idiopathic PD, it definitely had a few limitations in terms of assessing the overall distribution of the entire population of TM-generated mutants from a cohort of PD samples. This piece of information will definitely help in a better understanding of the pathogenesis of the disease with respect to α-SYN pathology. This can also give the researchers an idea about any specific region of SNCA gene that is more prone to 8-oxodG accumulation and TM event. For example, occurrence of S42Y α-SYN at mRNA level is relatively higher compared to the other two TM mutations shown in this study (for both control and PD cohort combined) especially in comparison to S129Y. Taken together, this study has shown 8-oxodG-mediated TM event to be a novel mechanism which leads to enhanced aggregation of α-SYN by generation of new mutant species. We report for the first time the existence of a new mutant α-SYN (S42Y) and its increased potential towards aggregation. We strongly foresee this mechanism and generation of novel aggregation prone mutants of α-SYN as a tool for diagnosis of PD (to serve as a biomarker). Moreover, analysis of TM event with respect to PD have the potential to open new avenues in the field of DLB, AD and other neurodegenerative disorders that involve protein aggregation mediated neurotoxicity.
3.4 Materials and methods

3.4.1 Plasmids and primers:

Full-length (α-SYN) (WT, A53T, A53E and S42Y) was cloned into the newly introduced Xba1 and Not1 site of pAAV-IRES-hrGFP vector system (Agilent technologies #240075) with the modified N-terminal 3XFlag. The sequence of each construct was confirmed by sequencing. The primers that were used for generating the mutagenesis sites: S42Y (F’ – TATGTAGGCTACAAAACCAAGGA, R’ – TCCTTGGTTTTGTAGCCTACATA); A53T(F’– GTGCATGGTGTGACAACAGTGGCT,R’–AGCCACTGTTGTCACACCACCATGCAC); A53E(F’–GTGCATGGTGTGAAAACAGTGGCT; R’ – AGCCACTGTTTCCACACCACCATGCAC). For the recombinant protein generation of WT and S42Y α-SYN, the WT α-SYN cloned into pT7-7 vector was purchased from Addgene (Plasmid #36046) and the site directed mutagenesis reaction was done using the primers described above and quick-change site directed mutagenesis kit (Agilent technologies #210515) as per the instruction given in the manual. The constructs for the split luciferase based complementation assay system containing full-length α-SYN was a kind gift from Dr. Nicklaus McFarland (University of Florida, Gainesville)\textsuperscript{240}. The amplification of OGG1 expression was done using primers (F’ – TGGAGTGGTTACTAGCGGA; R’ – GGATGAGCCGAGTCCAAAAA) against the OGG1 sequence and normalized by amplification of an endogenous control gene, β-actin (F’ – GGAGTGCCTGTGGCATCCACG, R’- CTAGAAGCATTGCGGTGGA).
The lists of primers used for RNaseH2 PCR (RH- primers) have been listed in the RNaseH2 PCR section and as a table.

3.4.2 Cell culture and treatment paradigm:

HEK293T (LVX) α-SYN (SNCA) knockout cells were maintained in DMEM (Dulbecco’s Modified Eagle Medium) supplemented with heat-inactivated 10% fetal bovine serum and 1% Penicillin/Streptomycin (Gibco, 10000U/mL). Cells were maintained at 37°C in humidified incubators with 5% CO2 and passaged following trypsinization with 0.25% Trypsin/0.53 mM EDTA (ThermoFisher Scientific).

Transfections for all experiments were performed using X-Fect Polymer (CloneTech # 631317) according to manufacturer’s instruction.

For the validation of RNaseH2 PCR, 1ug of full length S42Y α-SYN (as described in the plasmid section) was transfected into HEK293T (LVX) α-SYN (SNCA) knockout cells. 48 hours post-transfection, cells were harvested followed by extraction of RNA and cDNA (Complementary DNA) synthesis.

The aggregation potential for each mutant was determined following the treatment condition already published \(^{188}\) with slight modification. For the cell based biochemical analysis of aggregation, HEK293T (LVX) α-SYN (SNCA) knock out cells were either transfected with 5ug of full-length WT, S42Y or WT:S42Y (100:1). Following transfection, cells were treated with 300uM of FeCl\(_2\) (Sigma Aldrich # 372870) for 96 hours. Fresh media supplemented with 300uM of FeCl\(_2\) was replenished after 48 hours. On the last day before further processing, cells were treated with 5uM of Mg132 for 6 hours.
For the bioluminescence complementation assay with 1 ug of α-SYN-hGLuc1 (S1) and α-SYN-hGLuc2 (S2) constructs, the cells were transfected with X-fect polymer.

### 3.4.3 Generation of α-SYN knockout HEK293T cell line using CRISPR/Cas9:

HEK293T (LVX) α-SYN (SNCA) knockout cells were generated by transfecting a cocktail of SNCA specific sgRNA using Horizon’s free CRISPR guide program (Horizon discovery Ltd., UK). The sequence of sgRNA’s targeted towards the SNCA gene were 5′-AACAGGGTGTGCAGAAGCA-3′, 5′-AGGAGGGAGTTGTGGCTGCT-3′, 5′-TTGAAAGTCCTTTTATGAAT-3′. The backbone vector containing the sgRNA had a fluorescent dasher-GFP, which was used for fluorescence activated cell sorting (BD biosciences/Aria). The sorted GFP containing cells were genotyped using the primers F’–ACACCCCGAGTGGCAGAAGCA and R’–CTGGAAAAAGCAACAGTTCG. Homozygous knockout of α-SYN protein was confirmed by immunoblotting with specific antibody.

### 3.4.4 Human post-mortem brain samples:

Frozen post-mortem brain tissues (SN region) of 9 control (C3-C11) and 10 Parkinsonian (P1-P10) samples were acquired from NIH Neurobiobank consortium. Age ranged from 73 to 83 years and post-mortem interval (PMI) varied from 6.7 hours to 15 hours (average 11.67 hours) in PD cases. Similarly, the age of the controls ranged from 54 years to 89 years with an average of 73.53 years. PMI for the controls varied from 10 hours to 30.25 hours (average 24.02 hours). Two control (C1-C2) and five PD brain samples (UK1-UK5) were procured from UK Brain bank. Age and post-mortem index
information for those subjects were not available to us. In addition, FFPE (formalin fixed paraffin embedded) serial sections of 5 Parkinson’s disease (PD) and 2 Dementia with Lewy bodies (DLB) samples were procured from the Columbia University Brain bank and Mayo Clinic, Jacksonville, Florida.

3.4.5 Analysis of 8-oxodG levels in human post-mortem brain samples:

The 8-oxodG level analysis from the frozen human post-mortem human brain samples were performed in collaboration with Dr. Fernando Cardozo-Pelaez (University of Montana) following published protocol\textsuperscript{241}. Briefly, analysis of the levels of oxo8dG, expressed as the ratio of 8-oxodG/ 2-deoxyguanosine (2-dG) was performed as follows. The purified DNA was prepared for HPLC analysis by resolving it into deoxynucleoside components. The DNA was digested with nuclease P1 and treated further with alkaline phosphatase. The deoxynucleoside preparation was then ready for HPLC analysis. The amount of 8-oxodG and 2-dG was calculated by comparing the peak area of 8-oxodG and 2-dG obtained from the enzymatic hydrolysate of the DNA sample to a calibration curve for both compounds. Levels of 8-oxodG in the samples were expressed relative to the content of 2-deoxyguanosine (2-dG), for example, the molar ratio of 8-oxodG/deoxyguanosine (fmol 8-oxodG/nmol of 2-dG).

3.4.6 Analysis of OGG1 mRNA level and activity in human post-mortem brain samples:

Analysis of OGG1 mRNA level was analyzed using semi-quantitative reverse transcriptase PCR (RT-PCR). Briefly, RNA was extracted from the human post-mortem brain samples (SN region) using TRIzol Reagent according to manufacturer protocols.
Complementary DNA (cDNA) was generated by conversion of 1 µg total RNA using amfiRivert cDNA Synthesis Platinum Master Mix according to manufacturer’s protocol (GenDEPOT; cat no. R5600-50). cDNA was diluted 1:1 with nuclease free water before PCR with primers listed above.

OGG1 activity from SN region of human post-mortem brain samples were performed in accordance with the published protocol\(^{242}\). Briefly, tissue was dissected on ice and placed under liquid nitrogen, and DNA glycosylases were extracted from tissue following homogenization with buffer containing 20 mM of Tris (pH 8.0), 1 mM of EDTA, 1 mM of dithiothreitol, 0.5 mM of spermine, 0.5 mM of spermidine, 50% glycerol, and protease inhibitors. Homogenates were rocked for 30 min after the addition of a 1/10 vol/vol of 2.5 M of KCl and spun at 14,000 rpm for 30 min. The supernatant was aliquoted and stored at −70°C until the time of assay. Protein levels were determined using the BCA method.

20picomoles of synthetic probe containing oxo8dG (Trevigen, Gaithersburg, MD, USA) was labeled with P-32 at the 5’ end using polynucleotide T4 kinase (Boehringer Mannheim, Germany). For the nicking reaction, protein extract (30 µg) was mixed with 20 µl of a reaction mixture containing 0.5 M of N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid], 0.1 M of EDTA, 5 mM of dithiothreitol, 400 mM of KCl, purified BSA, and labeled probe (approximately 2000 cpm).

**3.4.7 RNaseH2 PCR for detection of TM mutants:**

Primers for the RNaseH2 PCR (RH-primers) were designed following manufacturer’s instruction (Integrated DNA Technologies) to detect the TM-generated
mutants of \( \alpha \)-SYN. For each of the mutant primer designed, we designed a WT primer (with WT sequence at the position of the mutation) in order to get a relative estimation of the mutant with respect to the WT. The reverse primer used for amplifying the product of the RNaseH2 PCR for S42Y and A53E was \( R \) - TCCACAGGCATATCTTCCAGAAT, whereas the reverse primer used for amplifying the S129Y product was \( R \) - TAAAAACTTTTGAGAAATGTCATGACTGGG.

PCR amplification of the mutant mRNA species (S42Y, A53E and S129Y) was done from cDNA synthesized with 1ug of total RNA extracted from human post-mortem control and PD samples. For each PCR reaction from cDNA, 120mU of the RNaseH2 enzyme was used in combination with 10 picomoles of the forward and the reverse primers. The cycling condition was optimized at 57\( ^\circ \)C for forty cycles. For each PCR reaction from plasmids, 0.5mU of the RNaseH2 enzyme was used along with 1 picomole of the forward and the reverse primers.

**3.4.8 Sequencing of the amplified product from the cDNA and the genomic DNA:**

The detected mutant of \( \alpha \)-SYN from the human post-mortem PD brain sample was gel extracted and sequenced after cloning it into pGEMT-Easy vector system I (Promega # A1360). The similar region of the genomic DNA was amplified using the forward primer F–ATTCGACGACAGTGTGGTGAAG and reverse primer R– TCCACAGGCATATCTTCCAGAAT and cloned into pGEMT-Easy vector system I. Five colonies from each of these cloned product were sequence verified to confirm the presence of mutation in the cDNA but not in the genomic DNA.
3.4.9 Western blotting:

Immunoblotting for analysis of monomeric denatured α-SYN protein was done by extracting protein using RIPA (Radio Immuno Precipitation buffer) buffer (PBS, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% PMSF, 100 ng/ml protease inhibitor, dH2O) and 40 μg protein were ran on 10% denaturing SDS gel and transferred to PVDF membrane (Millipore cat no. IPFL00010). Following the transfer process, the membrane was blocked with 5% Non-fat milk in TBS-T. Specific protein bands were detected using rabbit anti-α-SYN antibodies at 1:1000, unless otherwise indicated (BD Transduction Laboratories, cat no. 610786).

For analysis of the aggregated fraction of α-SYN in cell-based experiments, published protocol was used with slight modifications\textsuperscript{243}. Briefly, detergent solubility was performed by adding triton X-100 to total cell lysates (final concentration 1% in TBS containing protease inhibitor cocktail) and rotating for one-hour at 4°C followed by centrifugation (16000 x g, 60 min, 4°C). The supernatant was designated as triton X-100 soluble fraction and the pellet was redissolved in 2% SDS in TBS and sonicated for 30 seconds (2s each with 2s interval till the pellet dissolved). This fraction was designated as the triton X-100 insoluble fraction. Additional washing of the triton X-100 insoluble pellet was done with ice cold TBS by rotating in 4°C for at least three times. Protein concentration was determined using a Lowry protein assay. 20-40 ug of each cell lysate was loaded onto 10% SDS gels for Western blot analysis. SDS-PAGE was performed with SDS-containing running and sample loading buffer according to standard procedures.
For analysis of the recombinant protein, 2μg each of the recombinant protein were run on 10% SDS containing gel following standard procedures. Antibodies were used at a concentration of 1:5000.

Specific protein bands for α-SYN were detected using rabbit/mouse anti-α-SYN antibody (Santa Cruz #SC-7011-R and BD transduction lab #610786). To detect S42Y α-SYN, rabbit polyclonal anti-S42Y α-SYN antibody was developed from Abcam. Phosphorylated α-SYN at 129 was detected using specific rabbit anti-ps129 α-SYN antibody (Santa Cruz # sc135638). The different α-SYN protein levels were normalized against b-actin using mouse anti-b-actin (Sigma # A2228).

3.4.10 Expression and purification of recombinant WT and mutant α-SYN:

Recombinant WT and S42Y α-SYN were generated and purified as described by Volles and Lansbury with slight modifications\(^\text{244}\). Briefly, BL21DE3 were transformed with WT or S42Y α-SYN in pT7-7 vector and induced with 1mM IPTG for 24 hours. The cells were lysed with a lysis buffer containing 1mM EDTA, 50mM TRIS, 150mM NaCl pH 8.0 and then the exact protocol as published was followed to purify the recombinant protein\(^\text{245}\).

Purified α-SYN was filtered to remove any amorphous particulate matter and then diluted to a concentration of 1mg/ml in 500μL total volume with Phosphate-buffered saline (PBS) containing 0.05% sodium azide in 1.5ml paraffin-sealed microcentrifuge tubes. In the monomeric S42Y spiked condition, 0.01mg/ml of S42Y was added to 1mg/ml of WT α-SYN in PBS. The tubes were left to incubate at 37°C and a small aliquot (20μl) of the protein was analyzed every 24 hours for the aggregating α-SYN.
3.4.11 Aggregation assays:

Loss of soluble α-SYN was measured by $A_{280}$ of the soluble material every day following centrifugation at 20,000 x g for 30 minutes at room temperature. The same sample was analysed for binding of the fluorescent dye Thioflavin-T (20uM) and also Circular dichroism analysis spectra.

In CD experiments, 50 μM final concentration of the aggregated protein was analyzed using a J-810 spectropolarimeter (Jasco, Tokyo, Japan) (Ref). To improve the signal-to-noise ratio, the spectra were smoothed using a 13-point Savitzky-Golay linear least squares algorithm embedded in the Igor Pro 5.03 software. The CD spectra represented in the figure averages five measurements of each sample.

For the AFM analysis of the aggregated species, samples were drop casted on the surface of freshly cleaved and ultra-sonicated mica. Samples were incubated for 15-20 minutes and washed thoroughly. Next, sample was air dried and scanned immediately using AFM (NT-MDT). In particular, tapping mode was used to scan the samples. The tapping mode settings were as follows: 0.5 Hz scan rate at a scan size with a resolution of 256 x 256 data points per scan. Super sharp diamond-like carbon (DLC) tips (NSG10_DLC; NT-MDT) having force constant 11.5N/m, and radii of curvature 1-3 nm was used.

3.4.12 Immunohistochemistry:

Slides containing paraffin embedded serial sections of substantia nigra region from human post-mortem PD brain samples were stained for the presence of Lewy bodies. Hematoxylin & Eosin (H&E) staining was done following standard available
protocols. For the immunostaining of the tissue sections, we followed the published protocol in Cristovao et al\textsuperscript{194} with slight modification. Briefly, the FFPE (formalin fixed paraffin embedded) sections were first deparaffinized with xylene followed by antigen retrieval in sodium citrate buffer at pH 5.5 and 100°C. Following the antigen retrieval permeabilization, blocking and washing steps were done following standard procedures. The primary antibodies used for incubation at 4°C overnight were mouse anti – Ubiquitin (1:250) (Chemicon #MAB1510) and rabbit anti- S42Y α-SYN (1:250). The secondary antibodies were biotinyalted anti- mouse and anti- rabbit IgG (1:500). The staining procedure was performed as indicated by the manufacturer of the Vectastain ABC kit and the reaction product was visualized using 3,3’- diaminobenzidine (DAB) reagent in 0.1M PB.

For detection of anti-S42Y α-SYN positively stained Lewy bodies in the SN region, tissue sections were analyzed very carefully at 10X and 40X magnification using (equipment name/Leica) for overlapping immunostained Lewy bodies.

### 3.4.13 Immunocytochemistry and proteinase-K resistant staining:

For immunostaining and proteinase-K resistant staining of α-SYN aggregates, cells after undergoing the aggregation protocol (described above) were fixed in 4%-paraformaldehyde (PFA) for 20 minutes and permeabilized with 0.1% Triton X-100 in PBS for 10 minutes. Blocking was performed by incubation with 10% donkey serum in PBS containing 0.1% tween-20 for 1 hour at room temperature. The cells were then incubated with mouse anti-α-SYN (1:500) (BD transduction #610786). After washing, the cells were incubated with the appropriate anti- mouse antibody conjugated to Alexa
Fluor 546 (1:1000). For nuclear visualization, coverslips were incubated with 2uM Hoechst for 15 minutes. For analysis of α-SYN staining, punctated structures were enumerated from at least 5-10 fields.

The proteinase-K treatment paradigm for α-SYN aggregation was followed according to the published protocol by Cristovao et. al\textsuperscript{194}. Briefly, 10µg/mL of Proteinase-K (PK) was treated to the cells for 30 minutes at 37°C. PK was then inactivated with 3M guanidine-thiocyanate in 10mM Tris-HCl solution for 10 minutes. Between each step the cells were gently washed with PBS for three times. The dilution of anti α-SYN antibody and the secondary antibody was maintained as described above. For quantification of the α-SYN PK-resistant aggregates, punctated aggregate like structures were counted from 5-8 fields under each of the experimental conditions from three coverslips.

**3.4.14 Split complementation assay:** Split complementation assay with the different mutants was performed following the published protocol\textsuperscript{240}. Briefly, equal numbers of HEK293T α-SYN knock out cells were seeded in 24-well plate format in triplicates. For each well, 500 nanograms of the individual α-SYN containing split luciferase constructs S1+S2) was transfected along with 10 nanograms of the WT or mutant α-SYN constructs (1:0.01). At each time point, the media was assayed for luciferase activity using BioLux® Gaussia Luciferase Assay Kit (NEB# E3300S).

**3.4.15 SimPULL and TIRF microscopy:** Flow chambers were constructed with coverslips and microscope slides passivated with mPEG and biotin-PEG. 40 µL of 0.2 mg/mL Neutravidin in PBS was introduced into the chamber, incubated for 5 minutes and washed with 100 µL of 0.1 mg/mL BSA in PBS (wash buffer). Similarly, 40 µL of 2.5
μg/mL biotin-conjugated anti-mouse IgG and 40 μL of 2.5 μg/mL mouse monoclonal α-SYN antibody were introduced. Appropriately diluted recombinant proteins, 20nM of S42Y only, total 20nM of WT: S42Y (10:1) and total 20nM WT: S42Y (50:1), were injected into the chamber and incubated for 20 minutes. Unbound components were washed out, then 40 μL of 2.5 μg/mL rabbit polyclonal S42Y α-SYN antibody and 40 μL of 0.5 μg/mL Alexa 647-labeled anti-rabbit IgG was added sequentially. With an imaging buffer, the chamber was imaged at 10 areas per sample under the TIRF microscope and the number of fluorescent spots was counted and analyzed.

3.4.16 Statistical analysis: Statistical analysis was done using t-test and one-way Anova using Graph pad prism 3.0. To get statistically meaningful data, all experiments were performed in technical triplicates. The times change in the NanoLuc luciferase activities in treated groups were calculated by normalizing the value with respective control for each experiment.
3.5 Figures
Figure 15 Increased 8-oxodG accumulation in the genomic DNA of Parkinsonian patient sample compared to age-matched controls.

(A) Analysis of genomic DNA from the substantia nigra of PD (n=8) post-mortem brain samples showed significant increase in 8-oxodG level compared to age-matched control samples (n=9) (p<0.0004). (B) Semi-quantitative RT-PCR showed no significant change of 8-oxodG DNA glycosylase (OGG1) in the PD (n=9) condition compared to control subjects (n=9) at the mRNA level. (C) Similarly, analysis of OGG1 activity to cleave 8-oxodG containing oligo showed no significant difference in activity between the PD (n=7) and control subjects (n=9). (D) Control samples showed a significant correlation between 8-oxodG accumulation and OGG1 activity (p=0.01; $R^2=0.6356$) while PD samples showed no significant correlation between the two (p=0.06; $R^2=0.4747$) (E). (F) Gel picture showing the OGG1 activity of the control and PD samples in terms of cleaving the oligo containing 8-oxodG.
(A)

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(B)

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<tr>
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<td>Parkinson’s cohort</td>
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Figure 16 Detection of TM generated mutant α-SYN mRNA in the human post-mortem PD versus control brain samples.

(A) Distribution of the S42Y, A53E and S129Y α-SYN in 11 control samples and 15 PD samples. “+” indicates the presence of the mutant mRNA specie in each sample, while “-“indicates the mutant being absent. “+” in the column for WT indicates the amplification of WT α-SYN using all three WT primers corresponding to their respective mutants. In control cohort, 3/11, 1/11 and 1/11 samples amplified S42Y, A53E and S129 α-SYN respectively. In PD cohort, 8/15, 6/15 and 1/15 samples amplified S42Y, A53E and S129Y α-SYN respectively. Sample ID’s are indicated in the first column as C1 through C11, UK1-UK5 and P1 through P10. Age information is provided for the samples C3-C11 and P1-P10. Age information could not be attained for C1-C2 and UK1-UK5. (B) Quantitative representation of the S42Y, A53E and S129Y α-SYN TM mutants in human samples from control and PD cohort.
### (A)

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<td>WT / corresponding to S129Y</td>
<td>CAATGAGGGCTATGAAATGCTTrC/3SpC3∥/3SpC3/TG</td>
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### (B)

![RNAseH2 assay](image)

#### WT a-SYN

### (C)

![cDNA assay](image)

#### S42Y a-SYN cDNA

#### WT a-SYN cDNA
Figure 17 Validation of RNaseH2 PCR.

(A) Sequence of generation-2 RH-primers designed to detect the TM mutants of α-SYN, namely S42Y, A53E and S129Y α-SYN. All the mutants have a corresponding WT primer designed to detect the WT α-SYN from the samples. At the position of mutation, primers have a RNA base to form a heteroduplex with the template DNA, and blocked groups at the end that can be cleaved off to amplify the product. (B) No amplification of the WT α-SYN seen in the absence of RNaseH2 enzyme (top right panel). (C) HEK293 α-SYN knock out cells transfected with expression vectors of S42Y α-SYN (left panel) and WT α-SYN (right panel) respectively and detected with S42Y specific RH-primer. Increasing amounts (0.5ul to 5 ul) of cDNA from 1ug total RNA amplified with S42Y RH-
primer only detects the S42Y α-SYN and not the WT α-SYN, thereby showing the specificity of the RNaseH2 PCR. (D) Validation of S42Y RNaseH2 PCR with plasmid based amplification directly, shows the specificity of the PCR reaction towards the S42Y containing plasmid and not the A53T containing α-SYN plasmid (top left panel). (E) Amplification excerpts of S42Y α-SYN and corresponding WT α-SYN from the human post-mortem PD (PD 60/UK1 and PD 106/UK5) and control samples (C22 or C1) using primers to detect S42Y α-SYN and WT α-SYN mRNA (bottom panel). (F) Sequence analysis of amplified region from cDNA and genomic DNA of PD106/UK5 shows the presence of S42Y mutation (C→A) in the cDNA (or mRNA) but not in the genomic DNA.
(A) 293 KO  293 WT
   a-SYN
   b-actin

(B) Empty  WT a-SYN  S42Y a-SYN
   a-SYN (Soluble)
   a-SYN (Aggregate)
   ps129
   a-SYN (Aggregate)
   b-actin

(C) anti a-SYN (1:500)
   WT a-SYN
   S42Y a-SYN
Figure 18 Increased aggregation potential of S42Y α-SYN over the WT α-SYN.

(A) Generation of the HEK293 α-SYN knock out cell line. Western blot showing complete knock out of α-SYN from HEK293 cells as detected by anti-α-SYN antibody (1:1000). Beta-actin is used as an endogenous loading control (B) HEK293 α-SYN knock out cells transfected with WT α-SYN or S42Y α-SYN were cellular fractionated into soluble (1st panel) and aggregated fraction(2nd panel). Western blot of the soluble and aggregated fraction demonstrates an increased aggregation of S42Y α-SYN in the aggregation fraction over the WT α-SYN when probed with anti-α-SYN and anti-phospho-serine 129 α-SYN (1:1000). (C) Immunostaining of HEK293 α-SYN knock out cell with anti-α-SYN antibody (1:500) shows increased punctated aggregate like structures, shown with the arrow. (D) Proteinase-K treatment also shows significant increase in the punctated α-SYN perinuclear aggregates in the S42Y α-SYN over expressed condition compared to the WT α-SYN condition (p<0.005). (E) Measurement of loss of soluble protein at A280, shows S42Y α-SYN accelerated loss of soluble protein compared to the WT only condition. (F) Thioflavin-T measurement at 483nm shows increased Thioflavin-t reactive structures formed by S42Y α-SYN upon aggregation for 8 days.
Figure 19 Increased aggregation of WT α-SYN protein in the presence of 100 times less S42Y α-SYN.

(A) Western blot analysis of aggregated fraction (SDS-soluble fraction) shows enhanced aggregation of WT α-SYN containing 1% S42Y compared to WT α-SYN condition only (top panel). anti- \textit{phospho-serine} 129 α-SYN (ps129 α-SYN) (1:1000) shows similar result for the WT α-SYN containing 1% S42Y (middle panel). (B) Split-complementation assay performed in HEK293 α-SYN knock out cells showed increased luciferase activity for 1% S42Y containing WT-α-SYN compared to WT α-SYN only condition (red) over a period of 72 hours. S2 only (blue) is a negative control where no luciferase activity is observed. (C) Western blot of recombinant WT α-SYN and S42Y α-SYN as detected by anti-α-SYN antibody (1:5000). (D) Recombinant WT α-SYN and WT α-SYN containing
1% S42Y incubated at 37°C for 8 days and then subjected to aggregation analysis. Loss of soluble α-SYN occurs quickly in the 1% S42Y spiked condition (Red line) compared to WT α-SYN only condition (E) Reactivity with β-sheet binding fluorescent dye thioflavin-t, shows increased affinity of the 1%S 42Y α-SYN containing WT α-SYN compared to the WT only. (F) Circular dichroism (CD) spectra showing accelerated shift of the 1% S42Y containing WT α-SYN from random coil to β-sheet structure (day 2) as compared to the WT α-SYN only (day 4).
Figure 20 Atomic force microscopy (AFM) of the different α-SYN species.

AFM image showing morphologically different WT α-SYN aggregates compared to the 1% S42Y containing WT α-SYN which again differed from the S42Y α-SYN only. Fibrils from the WT α-SYN appeared longer in height (approximately 14nm) with median width
of 30nm, whereas for the 1% S42Y containing WT α-SYN fibrils appeared to have smaller heights (approximately 10-11nm) and median width of 40nm compared to the WT only.
Figure 21 Detection of S42Y α-SYN positive Lewy bodies from the substantia nigra region of Parkinsonian brains.

(A) Immunostaining showing the S42Y α-SYN positive Lewy body with anti-S42Y α-SYN antibody (1:250) from two PD post-mortem brain section which overlapped exactly with anti-Ubiquitin staining (1:250) and Hematoxylin & Eosin staining under 10X and 40X magnification. (B) Immunostaining of rotenone treated rat tissues of two age groups (6 months and 9 months) with anti S42Y α-SYN antibody (1:250), showed the presence of S42Y positive aggregated structure in the cortical and mid-brain section under 40X magnification.
Figure 22 Additional detection of Lewy bodies in PD and DLB brain samples from substantia nigra region.

(A) Immunostaining showing the S42Y α-SYN positive Lewy body and Lewy neurites with anti-S42Y α-SYN antibody (1:250) from PD post-mortem brain section 40X magnification. (B) Immunostaining showing the S42Y α-SYN positive Lewy body with anti-S42Y α-SYN antibody (1:250) of the second PD post-mortem brain section which overlapped exactly with anti-Ubiquitin staining (1:250) and Hematoxylin & Eosin staining under 10X and 40X magnification. (C) Immunostaining of serial sections of Dementia with Lewy body samples (DLB) with anti-Ubiquitin (1:250) and anti S42Y α-SYN (1:250) antibodies, showed the presence of S42Y α-SYN positive Lewy body with no background staining.
Figure 23 Validation of rabbit anti-S42Y α-SYN antibody.

(A) Western blot of recombinant WT and S42Y α-SYN with first and second bleed of the anti-S42Y antibody extracted from four different animals (ID: K281, 282, 283, 284) showed K284 to be the most effective in differentiating the S42Y α-SYN from the WT α-SYN. (B) Purified polyclonal anti-S42Y antibody (1:5000) detects only recombinant GST tagged and untagged S42Y α-SYN, without detecting the WT α-SYN. (C) Western blot image showing specificity of the anti-S42Y antibody towards S42Y α-SYN when titrated with increasing amounts of S42Y and WT protein (0, 10ng, 100ng, 1000ng and 10,000ng). (D) Western blot image showing specificity of the anti-S42Y α-SYN antibody towards aggregated S42Y α-SYN only and no affinity towards any other aggregated conformation of WT α-SYN (left panel) which could be detected by the anti α-SYN antibody (1:5000). (E,F) Single molecule pull down (SIMPULL) based fluorescence microscopy with anti-S42Y α-SYN antibody shows the specificity of the antibody towards recombinant S42Y α-SYN when titrated with WT α-SYN in various ratios (F) Western blot image showing the expression of WT α-SYN and S42Y α-SYN over expressed in HEK293 α-SYN knock out cell detected by rabbit anti-S42Y α-SYN (1:2500) and mouse anti-α-SYN (1:2500) antibodies in the soluble fraction. S42Y α-SYN shows decreasing protein expression from 100% through 1% (as transfected) detected by anti-S42Y antibody, while no change is seen at the level of total α-SYN as detected by anti-α-SYN antibody.
CHAPTER 4: CONCLUSION

Parkinson’s disease is described as an age-related neurodegenerative disorder characterized by cytoplasmic accumulation of proteinaceous aggregates called Lewy bodies and Lewy neurites\(^5\). Amongst all the important genes involved in the pathogenesis of PD, alpha-synuclein (\(\alpha\)-SYN) encoded by the gene SNCA is a key component of the Lewy bodies. As reviewed earlier, majority of idiopathic PD cases are a late-onset form which indicate that apart from genetic mutations in the alpha-synuclein gene (SNCA), extent of aggregation depend on critical factor like transcription of the gene also. Several studies have shown the importance of transcriptional regulation of SNCA especially in determining the severity of the idiopathic form of PD. To successfully develop drugs and therapies that act on the cause of the disease, the mechanisms underlying \(\alpha\)-SYN aggregation must be understood at the molecular level. In this body of work, a bidirectional approach has been adopted to contribute to PD pathogenesis, by designing a tool to monitor endogenous transcription of SNCA and implicated a novel mechanism called transcriptional mutagenesis in causing accelerated \(\alpha\)-SYN aggregation.

Chapter 1 introduces the history, pathological features and important genetic landmark studies associated with the pathogenesis of PD. Moreover, this chapter also lays the foundation to test the novel hypothesis of transcriptional mutagenesis as a potential cause of \(\alpha\)-SYN aggregation as seen in sporadic PD. It gives an insight into the severity of the oxidative stress and its associated damage in the form of 8-oxodG accumulation in the genomic DNA of the dopaminergic neurons of PD patients.
Chapter 2 describes the generation and validation of a novel tool using CRISPR-Cas9 genome editing technique that can efficiently monitor SNCA transcription under different treatment conditions known to up-regulate transcription as seen in Parkinson’s disease (Figure 6). Nanoluc is a small reporter of 19KDa size which is 150-fold more stable than the other commercially available luciferase reporters, thus making it an ideal candidate for tagging genes. The 293T-SNCA-3’NL cells were validated by the linear increase in the Nanoluc activity with the cell number as well as the detection of the fusion Nanoluc tagged α-SYN (Figure 7). The transcriptional changes in SNCA were brought about using some known epigenetic modulators like 5-azacytidine (5-azaC), sodium butyrate and a toxin like dopamine at known concentrations (Figure 8). It was observed with Nanoluc activity that 5-azaC can increase the transcription of SNCA by 3.68 times, whereas sodium butyrate and dopamine treatment can increase the transcription by 1.5 and 1.31 times respectively. We validated the increase in Nanoluc activity to corroborate with the increase in mRNA using semi-quantitative RT-PCR for all the three drugs. For 5-azaC treatment, the methylation status of the 23 CpG sites in the SNCA-intron1 by bisulfite sequencing was evaluated. As expected, a decrease in methylation of intron1 CpG correlated with an increase in transcript levels as measured by the Nanoluc activity (Figure 8). Interestingly, the superiority of the endogenous reporter system over the conventional exogenous system was established by the surprisingly spurious results that were observed after treatment with the epigenetic modifiers (Fig 9). No change in the luciferase activity was expected, because the plasmid-based system lacks the epigenetic environment that can be modulated by the selected drugs. The 293T-SNCA-3’NL cell was developed as a heterozygous cell line.
expressing wild-type (WT) endogenous α-SYN also. The Nanoluc tagged fusion protein was detected at the correct expected size of 34 KDa with the WT α-SYN at 15KDa (Figure 13, 14). It was observed that increase in transcription of the Nanoluc tagged allele and the WT allele was comparable which showed an increasing trend in both proteins (Figure 14). Overall this tool can provide a new diagnostic platform for drug development and testing of compounds believed to regulate SNCA in the cell in an inexpensive and precise way. The newly emerging endogenous reporter system represents a significant paradigm shift in the study of gene regulation and may provide new and exciting opportunities for both basic and translational research. The strategy adopted allowed insertion of a reporter directly into the targeted genome, enabling investigation of endogenous gene regulation while keeping the epigenetic structure intact. This particular feature is extremely relevant in studying SNCA expression, as this gene has been shown to get extensively regulated by its epigenetic structure. Similar strategy can be applied to tag important regulatory genes to study their transcription implicated in other disease states.

Nanoluc luciferase in combination with Halo-tag with has been strongly utilized to develop bioluminescence resonance energy transfer (BRET) to study protein-protein interaction owing to its increased bright nature, stability and small size. This cell line can be further genome edited with the Halo-tag based BRET technology to study α-SYN aggregation endogenously.

From designing a tool to monitor SNCA transcription, the next chapter (Chapter 3) of the thesis explores the contribution of another transcription associated novel phenomenon known as “Transcriptional mutagenesis” in the pathogenesis of PD.
Transcriptional mutagenesis has been primarily described as a DNA-damage (8-oxodG)-mediated mutagenesis event that occur during transcription. The relevance of TM as a contributor to PD pathogenesis had extremely strong rationale because of the increased oxidative stress in the dopaminergic neurons during the diseased condition. Moreover, neurons being post-mitotic cells depend largely on faithful transcriptional to maintain physiological function. In addition to this, several reports had shown the sensitivity of α-SYN to amino acid changes that can make the molecule more prone to aggregation.

The effect of 8-oxodG-mediated TM event has been attributed to C→A transversion mutation in transcripts. In line with this, forty-three positions of α-SYN were predicted on the coding region that could be mutated through TM event (figure 3). Amongst these forty-three, three positions of mutation (S42Y, A53E and S129Y) were evaluated, all of which had strong relevance to aggregation of α-SYN as seen in sporadic PD. In silico analysis of α-SYN aggregation showed Serine 42 tyrosine (S42Y) to have the highest aggregation potential score as predicted by the software TANGO. Previous reports have shown A53E s-SYN to be a familial mutant causing PD. Moreover in PD, phosphorylation at serine 129th position has been consistently implicated in aggregation of α-SYN, for which the generation of S129Y α-SYN through TM was evaluated.

To fortify the foundation of the hypothesis proposed in this chapter, analysis of 8-oxodG in the genomic DNA of substantia nigra showed significant increase in PD cohort when compared to the age-matched control. Interestingly, no change in the expression or activity of the 8-oxodG DNA glycosylase (OGG1) was observed between the same
PD and control subjects. In fact, contrary to the expected outcome, no significant correlation existed between the OGG1 activity and the accumulation of 8-oxodG in the PD patient samples. However, this was contradicted by the significant correlation of OGG1 activity and 8-oxodG accumulation in the control samples (figure 15). Consistent with the accumulation of 8-oxodG in the genomic DNA of PD patients, a higher distribution of the TM mutants (S42Y, A53E and S129Y) could be seen in the mRNA pool of PD patients compared to the age-matched controls. It was also observed that the relative intensity of the PCR product for the mutants were visibly lower compared to the WT α-SYN, indicating the pool of TM mutants of α-SYN were outnumbered by the WT α-SYN. This relatively lower amount of TM-mutant compared to the WT protein was expected (unlike familial mutations) (figure 16).

In order to evaluate the effect of the TM-generated mutants of α-SYN on the WT parental protein aggregation, S42Y was selected as a candidate since it was predicted to have the highest potential for aggregation. Biochemical analysis of aggregation showed the newly discovered S42Y α-SYN to have significantly higher aggregation potential compared to their WT parental counterparts (figure 18). However, to make the hypothesis physiologically relevant, it was observed through biochemical cell based experiments as well as recombinant protein assays that S42Y α-SYN even in a small percentage like 1% when mixed with WT-α-SYN can significantly accelerate the overall aggregation compared to the WT α-SYN condition only (figure 19). This observation strongly suggested that small population of TM-generated mutants can have a big impact on the aggregation of α-SYN as seen in sporadic PD. Due to the high aggregation potential of S42Y α-SYN, it was expected to be a part of mature Lewy
bodies and Lewy neurites. Accordingly, immunohistochemical analysis of post-mortem PD samples, showed matured Lewy bodies and neurites containing S42Y α-SYN positive aggregates as detected by the newly developed anti-S42Y α-SYN antibody (figure 21). Overall, this study strongly implicated TM event as a contributor to the aggregation of α-SYN and PD pathogenesis.

However, like any other novel hypothesis, contribution of TM-species to pathogenesis is also in its initial phase of discovery. It is important to understand the overall load of TM-generated mutant α-SYN in PD versus control samples. The scope of this thesis limits the study to a more simplified form to show the occurrence of the TM event and its contribution. This study is the first of its kind to show TM as an important mediator of PD pathogenesis. Importantly, this study partly answers the ever enigmatic contribution of ageing as a risk factor underlying all neurodegenerative diseases. Although in this study, PD and α-SYN have been focused, the concept of TM-mediated mutant protein generation and its contribution to disease phenotype can be extrapolated to a variety of protein aggregation diseases like Alzheimer’s disease, Multiple sclerosis and Huntington’s disease to name a few.

Although this study focused on the aggregation aspect of an important PD-related protein, transient expression of other mutant proteins via TM could play a very crucial role in regulating degenerative process like apoptosis by having direct effect on the signaling cascade that governs it. Overall, it can be well understood that few existing studies only have begun to shed light on the process of TM and its potential impact in causing disease. However, the extent to which TM occurs in various mammalian cells would be immense importance to understand the relevance of this novel mechanism in
causing disease. In future studies, utilizing a strong reporter tool like Nanoluc to monitor transcriptional mutagenesis event in various cells types will be of immense significance to understand the overall contribution of this mechanism in the etiology of various diseases. These observations together with the results described in this thesis strongly indicate the potential of TM event in the etiology of several disease including the neurodegenerative processes like PD and AD.
Announcing the Final Examination of Mr. Sambuddha Basu for the degree of Doctor of Philosophy in Biomedical Sciences

Dissertation Title: “IMPLICATION OF ALPHA-SYNUCLEIN TRANSCRIPTIONAL REGULATION AND MUTAGENESIS IN THE PATHOGENESIS OF SPORADIC PARKINSON’S DISEASE”

Date: April 25th, 2017    Time: 1:00 PM    Location: BBS, 103 (Live) and BMS, 136 (Simulcast)

ABSTRACT

Parkinson’s disease (PD) is a late-onset neurodegenerative disorder characterized by selective loss of dopaminergic (DA) neurons from the substantia nigra (SN) of the mid-brain. PD is classically associated with cytoplasmic inclusion of aggregated proteins called Lewy bodies. Alpha-synuclein (α-SYN) coded by the gene SNCA, is a major component of Lewy bodies. PD is broadly categorized into familial form (5%) that occurs due to known genetic aberrations like mutation, gene duplication/triplication and the sporadic form (95%) with unknown etiology often like environmental factors. Aggregation of α-SYN into Lewy body is a common pathologic hallmark seen in both and aggregated α-SYN in turn is strongly implicated by the transcriptional upregulation of the gene as seen in both familial forms as well as idiopathic forms. Although, the familial and the idiopathic form of PD are different in their respective cause of occurrence, aggregation of α-SYN into Lewy body is a common pathologic hallmark seen in both.

To monitor the endogenous transcription of SNCA, we developed a stable cell line in which α-SYN has been tagged with Nanoluc luciferase reporter using CRISPR/Cas9-mediated genome editing. Nanoluc is a small stable reporter of 19KDa size and 150 fold brighter compared to firefly and Renilla luciferase, thus making it a very good candidate for endogenous monitoring of gene regulations. Successful in-frame integration of the Nanoluc at 3’end of the gene was demonstrated by the fusion α-SYN protein containing the Nanoluc. This allowed efficient monitoring of α-SYN transcription by keeping its native epigenetic landscape unperturbed which was otherwise difficult using exogenous luciferase reporter systems. The Nanoluc activity monitored by a simple two-step assay faithfully reflected the endogenous deregulation of SNCA following treatment with different drugs including epigenetic modulators and dopamine which were already known to up-regulate SNCA transcription. Interestingly, use of exogenous promoter-assay (firefly luciferase assay) failed to reproduce the similar outcomes. In fact, exogenous reporter system showed contradictory results in terms of the SNCA regulation which arose from spurious effects of the drug on the reporter system. To our knowledge, this is the first report showing endogenous monitoring of SNCA transcription, thus making it an efficient drug screening tool that can be very effectively used for therapeutic intervention in PD.

In the next study, we investigated the effect of oxidative DNA damage in the form of 8-Oxo-2’-deoxyguanosine (8-oxodG, oxidized guanine) on aggregation of α-SYN through a novel phenomenon called transcriptional mutagenesis (TM). It is already known that 8-oxodG is repaired by a specific component of the base excision repair machinery of the cell called 8-oxodG-DNA glycosylase 1 (OGG1). If left unrepaird, 8-oxodG can lead to misincorporation of adenine instead of cytosine (C\rightarrow A transversion) in the synthesized mRNA during transcription for post-mitotic cells like neurons. TM event can generate novel mutant variants of any functional protein. α-SYN, which is implicated very strongly in the pathogenesis of PD, has been shown to become aggregation prone by specific point mutation. We used SNCA as a model gene and predicted the generation of forty-three different positions that can be mutated by the TM event. We investigated the generation of three possible TM mutants from the SN of post-mortem PD and age-matched control brain cohorts based on their potential to aggregate as
predicted by an aggregation prediction software TANGO. The three mutants were Serine42Tyrosine (S42Y), Alanine53Glutamate (A53E) and Serine129Tyrosine (S129Y). We confirmed the presence of all the three mutant \( \alpha \)-SYN (S42Y, A53E and S129Y) in \( \alpha \)-SYN mRNA from the SN tissue using a very sensitive PCR-based detection technique. As expected, overall distribution of the three mutants was more in the PD cohort compared to the age-matched controls. Although we could detect the presence of S42Y, A53E and S129Y \( \alpha \)-SYN in the cohort of PD patients, we focused to analyse the contribution of S42Y towards the aggregation of wild-type (WT) \( \alpha \)-SYN parental protein based on its higher potential to aggregate. By using cell-based biochemical and recombinant protein assays, we saw that S42Y \( \alpha \)-SYN can accelerate the aggregation process involving the WT protein even when present in significantly lower proportion (100 times less compared to the WT). Importantly, we developed antibody to specifically detect the S42Y \( \alpha \)-SYN in human PD cohort. Immunohistochemical analysis of serial post-mortem PD brain sections with, anti-ubiquitin and anti-S42Y \( \alpha \)-SYN together with Hematoxylin & Eosin (H&E) staining showed Lewy bodies that stained positively with S42Y \( \alpha \)-SYN. To our knowledge, this is the first report about TM-related mutations of \( \alpha \)-SYN in Parkinson’s disease and their role in the pathogenesis.

In this dissertation, we describe the designing and functioning of a novel tool to monitor real-time SNCA transcription in Human Embryonic Kidney (HEK) 293T cells. In the next part, we shed light into a novel transcriptional deregulation phenomenon called transcriptional mutagenesis, which leads to accelerated aggregation of \( \alpha \)-SYN as seen in sporadic PD. In brief, the focus of this work is to highlight the importance of transcriptional regulation of SNCA gene, through development of a tool and a mechanism affecting the fidelity of transcription under pathologic condition.

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