# Investigating the Genetic and Molecular Interaction of *α-Synuclein* and *Parkin* in Context to Mitochondrial Dynamics in *Drosophila melanogaster* Model of Parkinson's Disease

**THESIS**

Submitted in partial fulfilment

of the requirements for the degree of

**DOCTOR OF PHILOSOPHY**


**By**

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**BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE PILANI (RAJASTHAN)**

**2024**

# BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE, PILANI

## CERTIFICATE

This is to certify that the thesis titled **"Investigating the Genetic and Molecular Interaction of *α-Synuclein* and *Parkin* in Context to Mitochondrial Dynamics in *Drosophila melanogaster* Model of Parkinson's Disease"** submitted by **Sonia**, ID No **2018PHXF0001P** for award of Ph.D. of the Institute embodies original work done by her under my supervision.

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# Acknowledgments

My Ph.D. journey has been a remarkable and enriching experience, filled with moments of wonder and challenges. I am deeply grateful to numerous individuals who have supported me throughout this journey. I extend my heartfelt appreciation to all those without whose support completing this work would have been an insurmountable task.

First and foremost, I am grateful to the Lord almighty, who is merciful, gracious, and compassionate, for providing me the patience and capability to proceed and successfully complete my thesis.

I would like to thank my family, who has always been there for me to provide support and motivation from time to time. Without their everlasting support, patience and innumerable blessings, none of this would have been possible. I feel blessed for having parents like my mother Mrs. Rajbala and my father Mr. Sataveer Singh Narwal. I owe a special debt of gratitude to my father, Mr. Sataveer Singh, who made me to dream about this journey. Additionally, my husband, Mr. Bhavik Kalidhar, played an instrumental role in turning this dream into reality through his unwavering love, constant support, immense patience, trust, and encouragement, even though he may not be familiar with the intricacies of scientific research. The unwavering love and support from my sister, Mona Narwal during the challenging moments of my journey, a sentiment that goes beyond words. I would also like to deep gratitude to my grandfather, Sub. Maj. Roopchand Narwal, whose unwavering support propelled me through this PhD journey, he not only backed me in my academic pursuits but also imparted invaluable lessons about life, shaping my understanding and character along the way. His guidance and teachings have been a significant influence on my personal and academic growth, making this journey all the more meaningful. I also feel grateful to have supportive in-laws. Their support has not only just made this journey easy for me but it has also been an inspiration for every girl who wants to pursue her career even after getting married.

I wish to convey my heartfelt appreciation to Prof. Meghana Tare, my supervisor, whose indispensable guidance has brought this thesis to fruition. Her teachings about research and life have far surpassed my expectations, and I am truly indebted for her exceptional patience, unwavering encouragement, continuous support, and constructive criticisms. She has been my steadfast pillar of support and an exceptional mentor throughout my Ph.D. journey. I strongly believe that she has instilled in me a resilient attitude towards life, and I remain committed to continued learning from her.

I want to express my sincere gratitude to my co-supervisor Prof. Amit Singh, University of Dayton, OH, USA, whose guidance, encouragement, and stimulating discussions were indispensable for the successful completion of this thesis. I appreciate all his contributions of time, ideas, and motivation to make my Ph.D. experience productive and learning.

I am thankful to my DAC members, Prof. P.R. Deepa and Prof. Syamantak Majumder Department of Biological Sciences, BITS-Pilani, Pilani, for their constructive evaluation of the research work.

I am thankful to the Vice Chancellor, Directors, Deputy Directors and Deans of Birla Institute of Technology & Science (BITS), Pilani for providing necessary facilities and financial support. I am thankful to Convenor of Departmental Research Committee, Prof. Prabhat N. Jha and HOD, Prof. Rajdeep Chowdhury, Department of Biological Sciences, BITS Pilani, for their timely guidance and support regarding the academic formalities throughout the thesis work. I would also like to acknowledge the faculties of Biological Sciences Department at BITS-Pilani, for providing a critical review of my work during my departmental research forum presentations. I would like to thank non-teaching staff of the Department of Biological Sciences Mr. Naresh, Mr. Ajay, Mr. Subhash, Mr. Kamlesh and AGSRD for their help. I thank BITS-Pilani, and ICMR (Indian Council of Medical Research) for providing me research fellowship.

It was my privilege to have seniors like Dr. Poonam, Dr. Vikram, Dr. Vidushi, Dr. Sandeep, Dr. Vikas, Dr. Shahid Khan, Dr. Tripti, Dr. Nidhi, Dr. Aastha Mittal, Dr. Abhilasha who have guided me and helped me whenever I was stuck. I owe a special heartfelt appreciation to my lab-mate Mr. Shreyas Iyer for being there with me in all ups and downs of my professional or personal life.

I am also grateful to my friends Mr. Raghav, Mr. Anirudha Sahu, Ms. Niyati, Mr. Yash, Mr. Ramakrishnan, Mr. Ashish, Ms. Hansa, Ms. Ankita Daiya, Ms. Harshita, M. Shobham Ms. Mamta who have helped me in innumerable ways. All the doubts they have asked me helped me be a better researcher and a good team player. I cannot thank them enough for all the respect, love, and care they have showered on me. I would always cherish the time that I have spent with them. I would also like to take the opportunity to thank my lab colleagues Mr. Sumit Kumar Mandal, Mr. Shiva Chaudhary, Ms. Sonakshi Puri, Ms. Tripti Joshi and Ms. Shelly who with their helping attitude and fun-filled discussions have always made the time spent in the lab even more cherished. I would also like to thank the thesis student Ms. Arushi rai, who acquainted me with the intricacies of working with flies and assisted in standardizing various experiments in the lab, was consistently prepared to offer support in every possible manner.

The journey of Ph.D. became fun and stimulating with all of them. I may not be able to write all the names here but there were many more well-wishers, relatives and friends whose faith and constant moral support has contributed in a big way in the completion of my thesis work. I shall always be indebted for their help.

**(Sonia)**

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# Abstract

Parkinson's disease (PD) is the second most common age-related neurodegenerative disorder, after Alzheimer disease, affecting 0.8% of the global population with only symptomatic treatments available, to date. It is characterised by decreased levels of dopamine, due to progressive loss of dopaminergic neurons in the *substantia nigra pars compacta (SNpc)* region of the mid brain. This depletion of dopamine gives rise to motor symptoms such as slowness of movement (bradykinesia), muscular rigidity, resting tremor and postural instability. A pathological hallmark of PD is presence of intraneuronal Lewy bodies, comprising the aggregation of incorrectly folded α-synuclein (*SNCA*) protein. Pathological α-synuclein aggregates are majorly present in sporadic PD cases. However, the first gene mutation identified in PD is located in *SNCA*, leading to the autosomal dominant familial form of the disease. Another form of PD is characterized by loss-of-function of an E3-ubiquitin ligase, *parkin*. *Parkin* is the second most common gene associated with familial form PD after *α-synuclein*. Mutations in the *parkin* are the predominant cause of autosomal recessive juvenile Parkinsonism (AR-JP) and accounts for almost 50% of all individuals with recessive and typical early onset PD (~40 years). *Parkin* mutations are responsible for 77% of sporadic cases with juvenile PD onset before 21 years. Mutations in both *α-synuclein* and *parkin* are considered major contributors in PD pathogenesis. Despite extensive research on individual effects of *α-synuclein* and *parkin*, their interactions in dopaminergic neurons of disease progression remain understudied.

At cellular level, an involvement of mitochondrial dysfunction has also been implicated in the pathogenesis of PD. Mutations in *α-synuclein* and *parkin* result in impaired mitochondrial morphology, causing loss of dopaminergic neurons. However, mechanism of *α-synuclein* and loss-of-function *parkin* mutation triggering the defects in mitochondria morphology and how it ultimately causes dopaminergic neuronal death is still unclear.

In this study, we employ *Drosophila melanogaster* (*aka* fruit flies) to investigate the genetic and molecular interactions of *α-synuclein* and *parkin* in the dopaminergic neurons of posterior brain and their effect on mitochondrial morphology in time-dependent manner. By inducing *α-synuclein* overexpression and downregulating *parkin* in different tissues, *Drosophila* has effectively recapitulated major PD phenotypes. These include neurodegeneration, locomotor dysfunction, and decreased lifespan, offering a convenient experimental platform to investigate the genetic and molecular interactions between *α-synuclein* and *parkin.*

In context of their interactions, we found that overexpression of *α-synuclein* along with downregulation of *parkin* causes reduction in number of dopaminergic neuronal clusters in posterior region of adult brain which is manifested as progressive locomotor dysfunction. Overexpression of *α-synuclein* and downregulation of *parkin* collectively results in altered mitochondrial morphology in a cluster specific manner, only in a subset of dopaminergic neurons of the brain. Further, we found that *α-synuclein* overexpression causes transcriptional downregulation of *parkin*. However, this downregulation is not further enhanced upon collective *α-synuclein* overexpression and *parkin* downregulation. This suggests that the interactions of *α-synuclein* and *parkin* may not be additive. Our study thus provides insights into a potential link between *α-synuclein* and *parkin* interactions. These interactions result into altered mitochondrial morphology in cluster specific manner for dopaminergic neurons over a period of time thus unravelling the molecular interactions involved in etiology of Parkinson's Disease.

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# List of abbreviations

PD: Parkinson's Disease

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

6-OHDA: 6-Hydroxy dopamine

PQ: Paraquat

*SNCA*: Alpha- Synuclein

*PARK2:* Parkin

*LRRK2*: Leucine-rich repeat kinase 2

*PINK1*: PTEN-induced kinase 1

*DJ-1*: Daisuke-Junko-1

NAC: Non-amyloid-$\beta$ component

DA: Dopaminergic Neurons

SNARE: Soluble N-Ethylmaleimide-Sensitive Factor Attachment Protein Receptor

RING: Really Interesting New Gene

Mfn1 and 2: Mitofusin-1 and -2

OPA1: Optic Atropy-1

Drp1: Dynamin related protein

Fis1: Fission 1 protein

PGC-1*a*: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PARIS: Parkin interacting substrate

DMC: Dorsomedial clusters

PAL: Paired Anterior Lateral

PAM: Paired Anterior Medial

PPM1/2 /3: Paired Posterior Medial

PPL1/2: Paired Posterior Lateral

# *Chapter-1 Introduction and review of literature*

## 1.1 Parkinson's Disease

Parkinson's Disease (PD) is a neurological disorder that, similar to other neurological conditions, tends to impact individuals more frequently as they age. PD is the second most common age-related neurodegenerative disease after Alzheimer disease, initially described as "*paralysis agitans*" in 1817 by an English surgeon James Parkinson in "An Essay on the Shaking Palsy". Two major neuropathological hallmarks of PD are progressive loss of dopaminergic neurons in *substantia nigra pars compacta* region of mid brain, which affects the dopamine level in the striatum; and, the presence of intraneuronal Lewy bodies which are formed mainly due to aggregation of α-synuclein protein (1,2). Depletion of dopamine level within striatum leads to motor symptoms including slowness of movement (bradykinesia), muscular rigidity, resting tremor and postural instability (3) **(Fig 1.1)**.

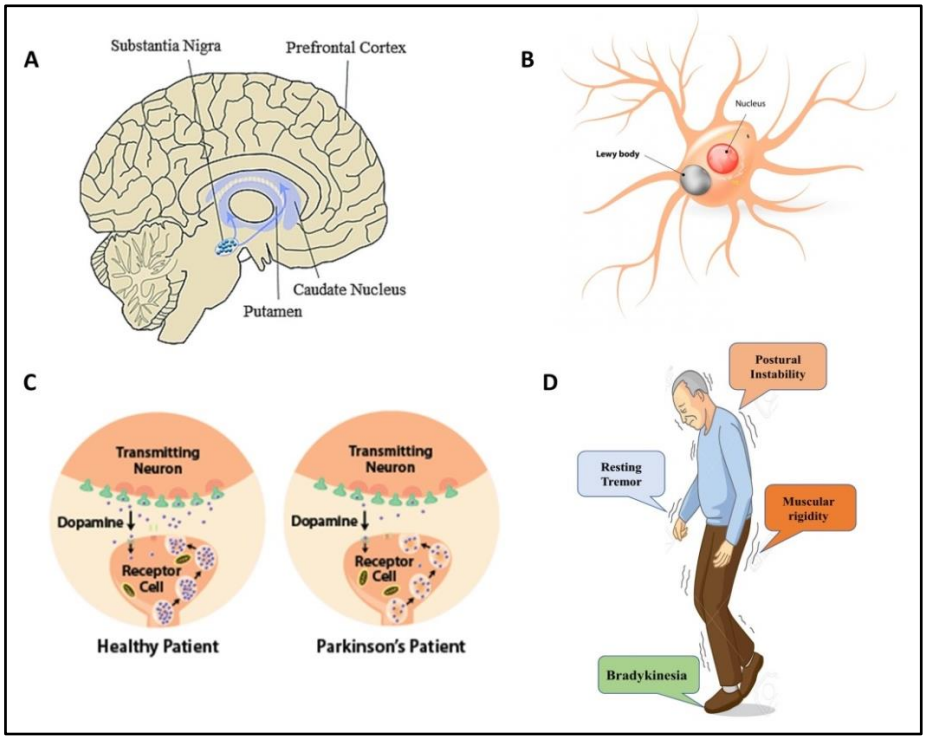



**Figure 1.1. Major hallmarks of Parkinson's Disease.**

PD is also associated with many non-motor symptoms such as rapid eye movement (REM) sleep behaviour disorder (RBD), cognitive effects, autonomic disabilities, and sensory disturbances that contribute to the impairment of patient's quality of life (4,5), although clinical diagnosis of PD relies on the presence of motor symptoms.

The prevalence of PD has doubled in the past 25 years. Global estimates in 2019 showed over 8.5 million individuals with PD. Current estimates suggest that, in 2019, PD resulted in 5.8 million disability adjusted life years (DALYs), an increase of 81% since 2000, and caused 329 000 deaths, an increase of over 100% since 2000. According to WHO Global Burden of disease study, PD affects people with the prevalence of 0.8% of the worldwide population (7 million to 9 million people) among the other neurological disorders and has estimated that 12.9 million people will be affected by PD by 2040 **(Fig 1.2)**. Age-standardized prevalence of PD is higher in men than in women (1.7% for men and 1.2% for the women) at the age of 60 years **(Fig 1.3)** (6,7). In India, prevalence of age-standardized PD has increased from 1990-2019. Notably, increased prevalence of PD is higher in those of above 50 years of age, both in males and females (8).

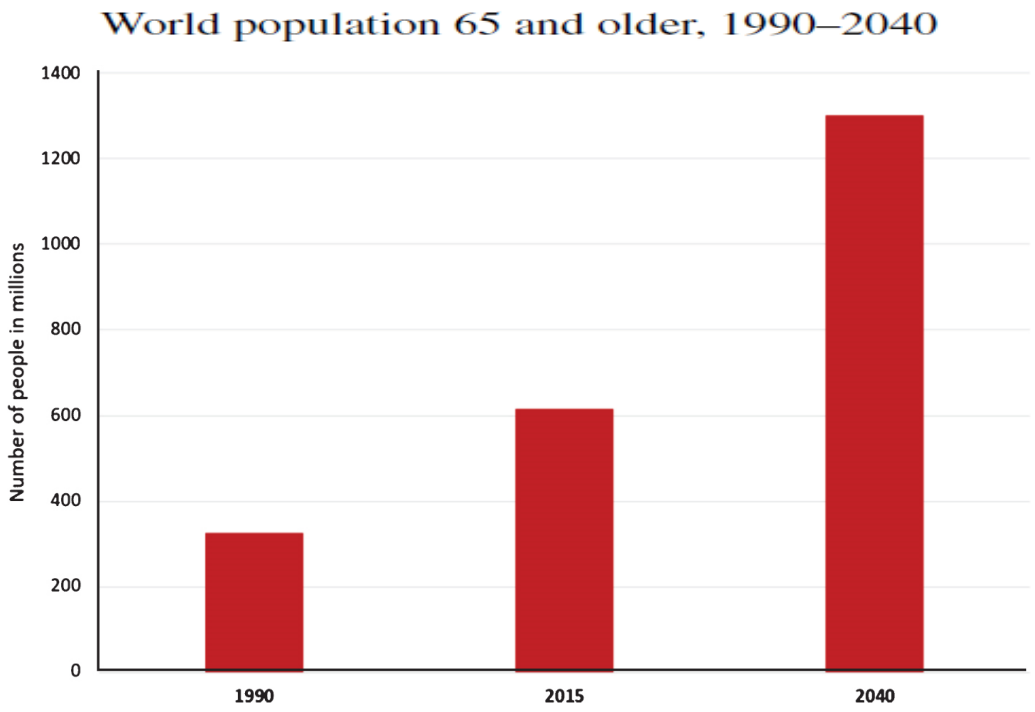



**Figure 1.2 Global burden of PD:** Graph depicting the number of people affecting with PD worldwide. (Feigin VL, et al; Global, regional, and national burden of neurological disorders during 1990–2015: a systematic analysis for the Global Burden of Disease Study 2015. Lancet Neurol. 2017;16 (11):877–97).

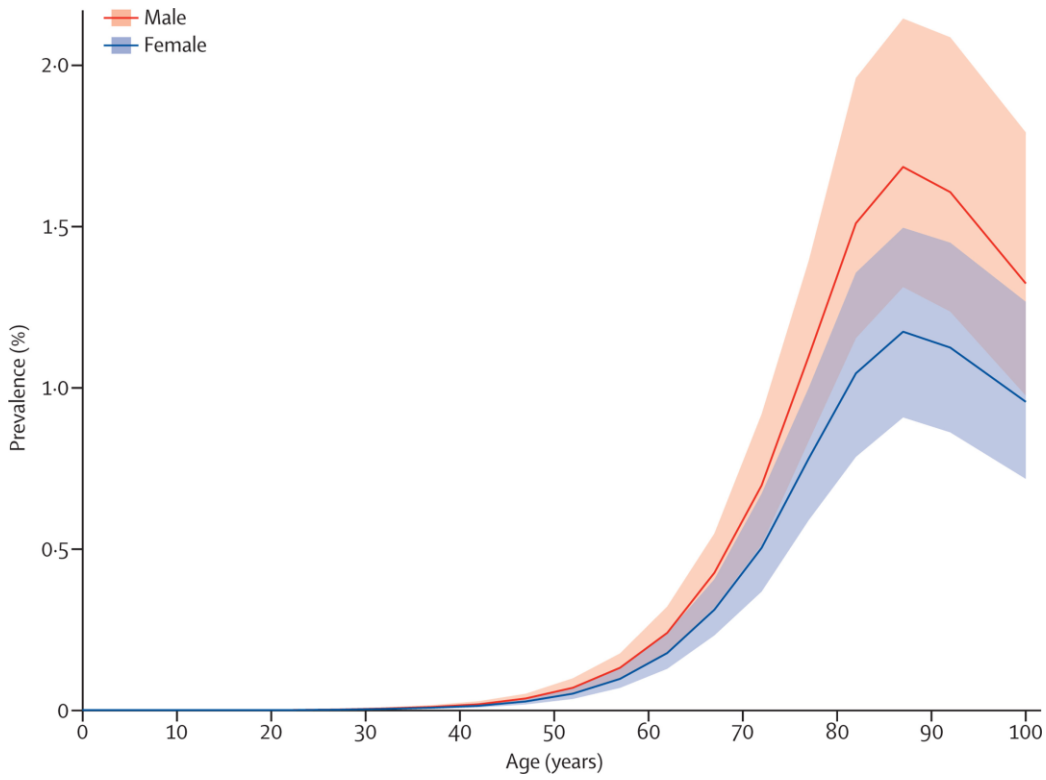



**Figure 1.3 Global prevalence of PD by age and sex:** Graph depicting the prevalence of PD in males and females. (Ray Dorsey E, et al; Global, regional, and national burden of Parkinson's disease, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet Neurol. 2018;17 (11):939–53).

Most of PD cases are sporadic in which environmental factors MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), 6-OHDA (6-hydroxy dopamine), Rotenone, Paraquat (PQ), are involved and cause dopaminergic neuron degeneration (9) . About 10-15% of PD cases are familial (genetic) in nature and have been attributed to single gene mutations (10,11). The genes consisting these mutations include *SNCA* (α-Synuclein), *LRRK2* (Leucine-rich repeat kinase 2), *PARK2* (parkin), *PINK1*(PTEN-induced kinase 1) and *DJ-1* (Daisuke-Junko-1) (12). Among these, *SNCA* and *LRRK2* have been found to be associated with autosomal dominant form of PD, whereas *PARK2*, *PINK1* and *DJ-1* are associated with autosomal recessive form of PD, and may have an early onset of PD (13). However, these genes also play vital role in sporadic PD (12). It is well known that accumulation of wild type and mutant α-Synuclein protein causes loss of DA neurons (14), but the precise function of α-Synuclein in dopaminergic neuron degeneration is not known yet.

Within the genes associated with familial PD, we have focused on *SNCA* and *PARK2*, given substantial roles in the onset and development of Parkinson's Disease.

### 1.1 α-Synuclein:

Human α-synuclein is predominantly present in all over the brain, particularly in the neocortex, hippocampus, substantia nigra (SN), thalamus, and cerebellum regions. It is encoded by *SNCA*, present on chromosome 4, which consists of 6 exons ranging from 42 to 1110bp (15). Because of its localization at presynaptic terminals and in nuclei it derives its name, synuclein, from synapse and nucleus. It was first isolated from neural tissue in Pacific electric ray in 1988 (16) in presynaptic terminal, composed of 140-amino acids and is about 15 kDa in size (16,17). α-Syn is considered major component of Lewy bodies (18). In 1997, Polymeropoulos *et al*. identified first α-synuclein mutation associated with familial PD (A53T) and since then it is identified as an important player in a complex neurodegenerative disease (PD) (11).

#### 1.1.1 Structure of α-synuclein :

α-synuclein is a member of synuclein family which also consist of *β*-synuclein, and *γ*-synuclein (19). It is subdivided into three domains and each responsible for different molecular and biological properties (20). An N-terminal domain (AA 1–65), a non-amyloid-*β* component of plaques (NAC) domain (AA 66–95), and a C-terminal domain (AA 96–140) (**Fig 1.4 A**) (21). N-terminal region contains a highly conserved sequence with 11 amino acid repeats (KTKEGV) that enable α-synuclein to bind the plasma membrane by forming an amphipathic *a*-helix. N-terminal region is reported to have most of the known mutations in α-synuclein that lead to pathologically dysfunctional α-synuclein and hence, emphasizes its importance in familial PD pathology. The central NAC domain (residues 61-95) contains a stretch of 12 amino acids of non-polar side chains, which are hydrophobic in nature and form β-sheet which results in polymerization and aggregation of α-synuclein. The C-terminal domain (residues 96-140) contains negatively charged amino acids. Most of the posttranslational modifications (PTM) occur in this region which have been reported on S129, Y133 and Y136. Among all posttranslational modifications of α-synuclein, S129 was identified as an important modification in α-synuclein for familial as well as sporadic Lewy body disease (22,25,26). C-terminal domain is also involved in $Ca^{2+}$ binding and chaperone-like activity (25). Recently, it has been shown that the binding of $Ca^{2+}$ to the C-terminus of α-synuclein also regulates its binding to synaptic membranes (26).

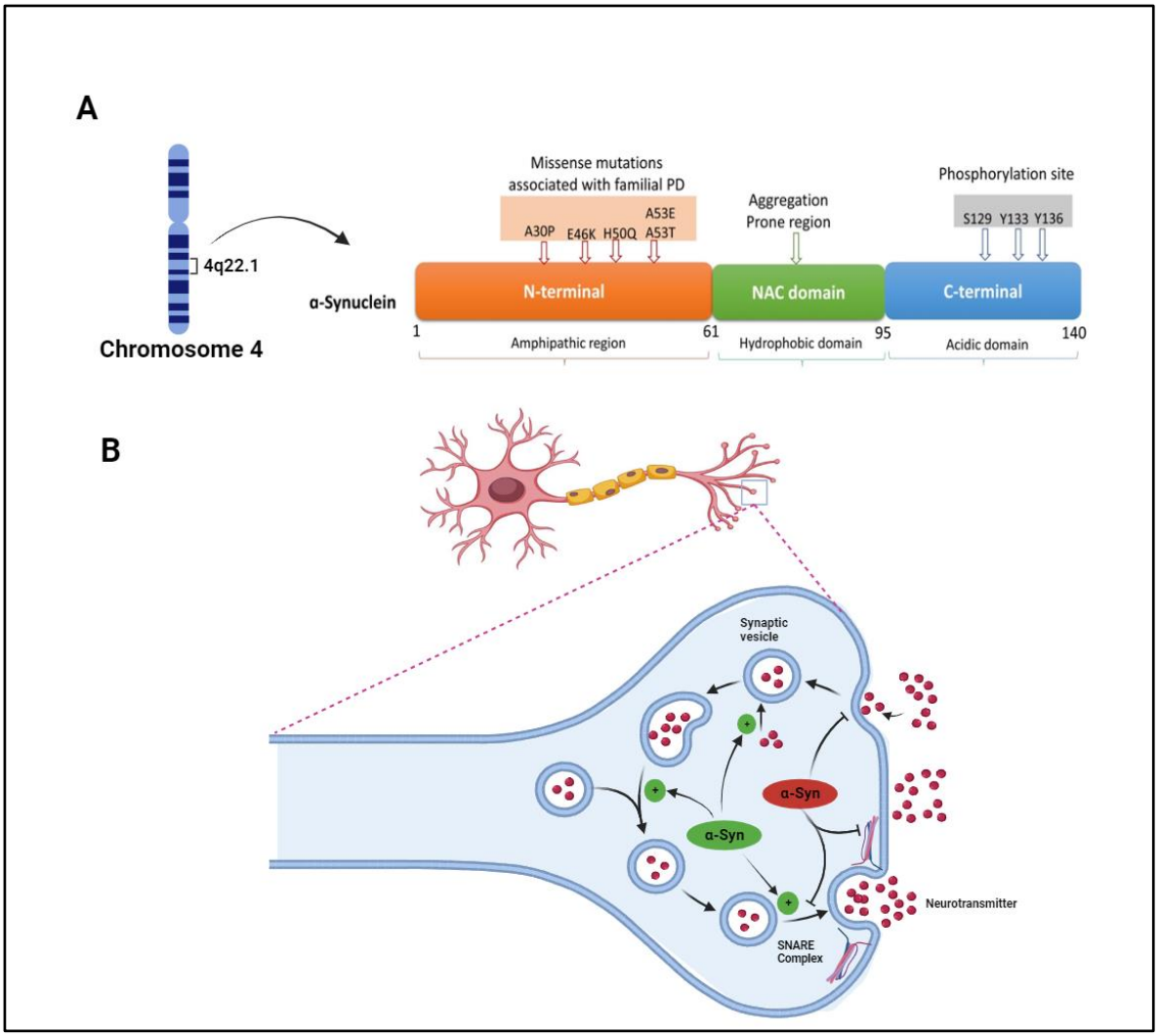



**Figure 1.4. A. Schematic representation of the structure and function of α-synuclein**. The arrows indicate mutations and phosphorylation sites. NAC: Nonamyloid component. **B.** Under physiological conditions, α-synuclein (green) is involved in exocytosis process by promoting SNARE-complex assembly, hence enabling the fusion of intracellular presynaptic vesicles with the presynaptic membrane. Neurotoxic alterations of α-synuclein (red) increase the formation of toxic oligomers and fibrils that disrupt intracellular processes. Figure drawn using BioRender.

### 1.2.2 Function of α-synuclein:

Physiological function of α-synuclein in each subcellular organelle is only partially understood, however numerous physiological functions of α-synuclein are known. In its native form, α-synuclein is present in synaptic terminals, nucleus of neuronal cells (16), mitochondria (27), endoplasmic reticulum (ER) (28), Golgi apparatus (GA) (29), and in the endolysosomal system (30). Different roles of α-synuclein in the regulation of neurotransmitter release, synaptic function and synaptic plasticity has been suggested in *in vivo* and *in vitro* models (33,34). For neurotransmitter release, α-synuclein contributes to the normal functioning of neurotransmitter compartmentalization, storage, and recycling (33) **(Fig 1.4 B**).

Neurotransmitters are secondary messengers which are secreted several times from presynaptic vesicles. For each single neuroanmsmitter release from presynaptic terminal, a cycle of assembly and disassembly of the SNARE (Soluble N-Ethylmaleimide-Sensitive Factor Attachment Protein Receptor) complex is needed to be repeated. SNARE is a complex of proteins involved in membrane fusion. It consists of v-SNAREs (vesicle-associated SNAREs) on the vesicle membrane and t-SNAREs (target membrane-associated SNAREs) on the target membrane. α-Synuclein is directly bound to the SNARE complex (v-SNARE and t-SNARE), and promotes its assembly. This process is made possible through the binding of the N-terminal domain to phospholipids and the C-terminal domain to synaptobrevin-2/vesicle-associated membrane protein 2 (VAMP-2), ultimately facilitating the fusion of intracellular presynaptic vesicles with the presynaptic membrane **(Fig 1.4 B)** (36,37). Unfolded cytosolic α-synuclein monomers bind to presynaptic membranes during the SNARE complex assembly, and form a complex of α-helically folded α-synuclein homomers which lead to neuroprotection in presynaptic terminals (36). Large oligomers of α-synuclein are harmful, and bind to an N-terminal domain of synaptobrevin-2 and prevent assembly of SNARE complex which lead to neurodegeneration (37). Also, α-synuclein oligomers may sequester the v-SNARE using multiple binding sites for t-SNARE on vesicles and inhibit SNARE-mediated vesicle fusion (37). In the context of synaptic function, synapsins, belonging to the cytoplasmic regulatory family of synaptic vesicles (SV), promote the interaction between α-synuclein and synaptic vesicles. This interaction contributes to the facilitation of synaptic vesicle clustering (38). Though, the regulation of neurotransmitter release by α-synuclein is not limited to dopamine transmission (39). Additionally, an elevated expression of α-synuclein has been proposed to disturb calcium homeostasis, rendering dopaminergic neurons susceptible to potential damage (40).

### 1.2.3 α-Synuclein in PD:

α-Synuclein is defined as a 'natively unfolded' monomer; however, it has also been shown that endogenous α-synuclein occurs in large part as a folded tetramer (~58 KDa) with little or no amyloid-like aggregation potential (41). In PD, α-synuclein forms a pathological β-sheet conformation that recruits additional monomers to form oligomers and amyloid fibrils in the neuron soma and in axons called Lewy Bodies and Lewy neurites respectively **(Fig 1.5)** (22,28). These misfolded α-synuclein oligomers and fibrils have been shown to cause impairment in neuronal homeostasis by targeting sub-cellular functions. This impairment arises through various mechanisms, including oxidative and endoplasmic reticulum stress, dysfunction in vesicular trafficking, disturbances in the autophagy-lysosomal pathway, and mitochondrial dysfunction. (42). Pathologic aggregation of α-synuclein has also been shown to be involved in cell-to-cell transmission (**Fig 1.5)** in PD patients as well as in cellular and animal models) (44,45).

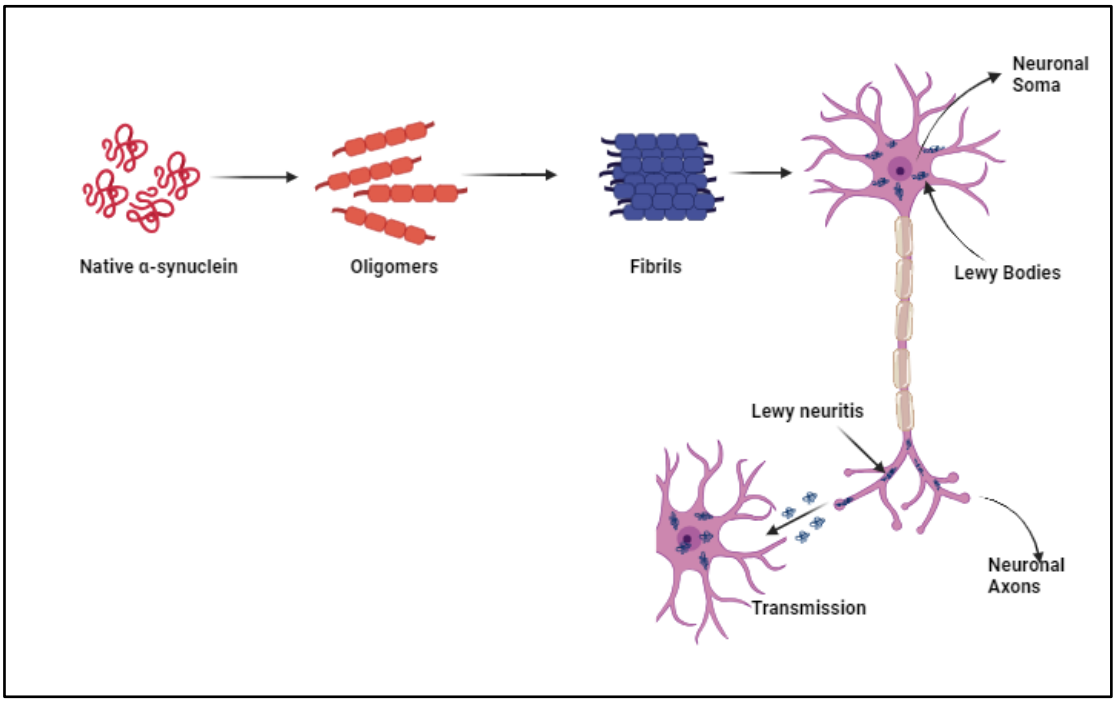



**Figure 1.5**. Schematic illustration of *α*-syn aggregation and transmission.
Source: Kingwell, K. Zeroing in on neurodegenerative α-synuclein. *Nat Rev Drug Discov* (2017).

In 1997, the first link between α-synuclein and PD discovered when a missense mutation (A53T) was identified in *SNCA* (also known as *PARK1*), in a large Italian family. This mutation

was subsequently detected in three unrelated Greek families exhibiting autosomal dominant early-onset PD (11). Subsequent immunostaining studies demonstrated that α-synuclein is a prominent constituent of Lewy bodies (LBs), which are known as a primary pathological hallmark of PD (45). Several missense and point mutations of *SNCA* have linked to early-onset autosomal dominant PD. *SNCA* point mutations include A53T, A30P, E46K, A53E, H50Q, G51D, A18T, and A29S. *SNCA* duplication has been reported to cause late onset of aging and slow progression of neurodegeneration in PD (46,47,50,51,52). In contrast, *SNCA* triplication result into earlier onset of the disease and rapid progression (50, 53). Multiplications of *SNCA* has also been reported in PD (53). Pathologic α-synuclein aggregates are majorly present in sporadic PD cases. Together, *SNCA* mutations and the copy number increase the disease severity from different reports and strongly suggest that α-synuclein is a major contributor in PD.

**1.3 Parkin:**

Parkin is an E3 ubiquitin ligase encoded by *PARK2* gene and is located on the chromosome 6q. The gene locus (PARK2) was discovered in 1997 (54) and thereafter its encoded protein was named parkin (55). It contains 12 exons that are separated by intronic regions and spans more than 1.53 Mb (55). It encodes for a protein of 465 amino acids with molecular weight of 52kDa. It is an evolutionary conserved with orthologs in *Caenorhabditis elegans*, *Drosophila melanogaster*, mouse, rat, and other species (57,58,59,60).

Mutations in the *PARK2* are the predominant cause of autosomal recessive juvenile Parkinsonism (AR-JP) and accounts for almost 50% of all individuals with recessive and typical early onset PD (~ 40 years). *PARK2* mutations are responsible for 77% of sporadic cases with juvenile PD onset (60). The parkin mutations are highly diverse which include rearrangements with deletions of single or multiple exons, duplications or triplications of exons, frameshifts mutations, missense mutations (resulting in the replacement of one amino acid residue by another) and nonsense mutations (resulting in a stop codon), all of them lead to protein loss of function or absence of protein by nonsense mRNA degradation (62,63,64,65). Parkin is widely expressed throughout the brain, and abundant expression of its mRNA has been observed in other tissues such as the heart and skeletal muscles (55).

### 1.3.1 Structure of parkin:

Parkin is a RING (really interesting new gene)-in-Between-RING (RBR) E3 ubiquitin ligase involved in the ubiquitination of various structurally and functionally distinct substrate proteins, including itself (65). It consists of a ubiquitin-like (Ubl) domain at N-terminus that is followed by four cysteine-rich regions and each region binds two $Zn^{2+}$ atoms (66). Three of those regions are the RING domains (RING0, RING1 and RING2). RING1 and RING2 domains are separated by a 51-residue in-between-RING (IBR) domain in the C-terminal region (68,69) (**Fig 1.6**).

The N-terminal Ubl domain is 30% identical in amino acid sequence to ubiquitin and it contains a serine at position 65 that is phosphorylated by PINK1. RING1 and RING2 catalytic domain are the two most important domains for ligase activity; RING1 binds the E2 ubiquitin-conjugating enzyme, where Rcat contains the catalytic site (Cys431) for ubiquitin transfer. Two linkers are present; one is 60 amino acids longer which follows the Ubl domain and contains a short activating element (ACT) that helps in stabilizing the active conformation of parkin. The second linker is repressor-element-of-parkin (REP) between the IBR and Rcat domains and includes a short alpha helical segment which helps in maintaining parkin in an inactive state (66,69).

In absence of PINK1, parkin is present in autoinhibited conformation in the cell cytosol. Parkin is maintained in inactive form by intradomain contacts that block the functional sites required for ubiquitin transfer. The Ubl domain blocks the E2- binding site on RING1 and part of the linker between the IBR and Rcat domain termed the Repressor-element-of-parkin (REP). The RING2 domain is bound to the RING0 domain with the catalytic cysteine inaccessible. Dissociation of these contacts activates parkin by making the active sites available and able to interact with each other (69).

In 12 exons of *PARK2*, more than 100 PD-associated mutations have been identified (67). Mutations in *PARK2* have been found in all the domains of parkin. However, majority of mutations occur in RING-IBR-RING domain, in particular to the first RING domain (RING1), implying essential functional relevance for this domain of the protein.

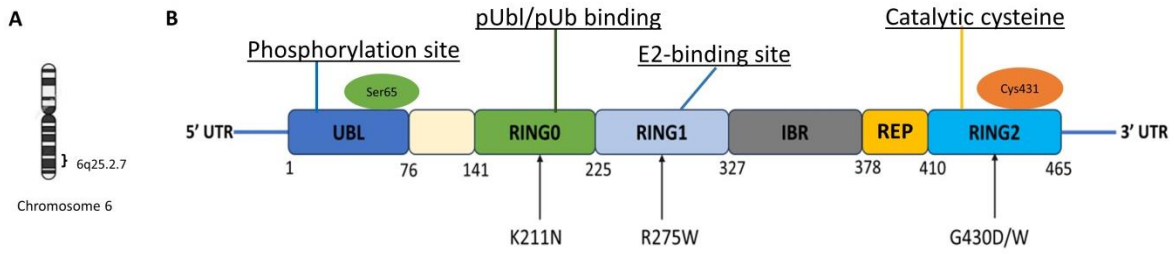



**Figure 1.6. Schematic representation of parkin structure. A.** Presence of *parkin* on chromosome 6. **B.** *Parkin* at transcript level showing different color-coded functional domains and arrows indicate major mutations in PD. Figure drawn using BioRender.

### 1.3.2 Functions of parkin:

Parkin as an E3 ubiquitin ligase, involved in ubiqutination of target substrate for the proteasomal degradation. It mediates both multiple mono-ubiquitination and poly-ubiquitination (through lysines K48 and K63) of its substrates (71,72,73,74). The K48-linked poly-ubiquitinaton targets substrates for proteasomal-dependent degradation (74), whereas the K63-linked poly-ubiquitination regulates substrates through proteasomal-independent lysosomal degradation and target whole organelles for autophagy degradation (71). In proteasomal-dependent degradation process, ubiquitination occurs through the transfer of an ubiquitin molecule from an activated E1 enzyme to the conjugating E2 enzyme, where an E3-ligase catalyses the transfer of the ubiquitin molecule from the E2 enzyme to a protein destined for degradation. Ubiquitin molecules are attached to target proteins via a covalent bond between the glycine at residue 76 (G76) in the C-terminal of ubiquitin and lysine at residue 48 (K48) at the N-terminal of the substrate protein (68) **(Fig. 1.7A**). Under basal conditions, the cytosolic parkin exists in an inactivated form (75). It undergoes substantial conformational rearrangements for its activation through binding of ubiquitin phosphorylated at serine65 by PINK1 (PTEN-induced putative kinase protein 1) (72).

In addition to the function of parkin in the ubiquitin proteasomal system, parkin's E3 ligase activity is involved in diverse aspects of mitochondrial functioning which include; mitochondrial mitophagy, biogenesis, fusion/fission and trafficking (77,78,79). Parkin translocates from the cytosol to dysfunctional mitochondria upon an impaired electrochemical membrane potential leading to mitochondrial depolarization. The dysfunctional mitochondria are removed by the autophagy-mediated 'mitophagy' process (76). Both parkin-dependent and

-independent mitophagy mechanisms are reported in removing damaged mitochondria. In parkin-dependent mitophagy parkin and PINK1 act together in an ubiquitin-dependent mechanism (80,81). When mitochondria are healthy, PINK1 is imported into the mitochondria through the translocase of the outer membrane (TOM) complex to the inner mitochondrial membrane (IMM), mediated by its N-terminal mitochondrial targeting sequence (80,81). Further, it is cleaved by presenilin-associated rhomboid-like protein (PARL), a protease present in the IMM, which leads to fragmentation of PINK1. The fragmented protein is released to the cytosol where it is rapidly ubiquitylated for proteasomal degradation. Therefore, the intracellular levels of PINK1 are low on healthy mitochondria (81). When mitochondria are damaged, PINK1 translocation to mitochondria and processing in IMM is inhibited and results into accumulation of unprocessed PINK1 to outer mitochondrial membrane (OMM) (82). Accumulated PINK1 phosphorylates parkin at serine 65 in the UBL-domain, which increases the ubiquitin chain assembly and hence parkin activity (83). Activated parkin ubiquitinate proteins present on OMM include mitofusin (MFN1/2), voltage-dependent anion-selective channel (VDAC) and mitochondrial fission 1 proteins (FIS1) (74). These ubiquitinated substrates recruit several autophagy receptors (such as optineurin (OPTN) and sequestosome 1 (SQSTM1/p62)) forming autophagosomes, which then fuses with lysosomes and lead to degradation of damaged mitochondria by lysosomal proteases (**Fig 1.7C**) (84).

Mitochondria fusion and fission process is essential to maintain its shape, size and number and critical for organelle distribution and bioenergetics. Mitofusin-1 and -2 (Mfn1 and 2, respectively) on outer mitochondrial membrane and OPA1 (Optic Atropy-1) on inner mitochondrial membrane are responsible for fusion process. Fis1(Fission 1 protein) outer membrane protein and Drp1 (dynamin related protein), the cytoplasmic proteins are responsible for fission process (85). A change in number, distribution may cause cell dysfunction. Parkin has been shown to maintain mitochondria integrity by regulating of fusion and fission process. Many *in-vitro* and *in-vivo* studies have reported that *parkin* promote mitochondrial fission and/or inhibit fusion by negatively regulating Mfn and Opa1 function, and/or positively regulating Drp1 **(Fig 1.7D**) (78).

In mitochondrial biogenesis, parkin regulates the expression of the mitochondrial transcriptional coactivator peroxisome-proliferator-activated receptor gamma coactivator 1-alpha (PGC-1*a*) (86). It degrades the parkin interacting substrate (PARIS), inhibitor of PGC1-α, and leads to activation of the transcription factors nuclear respiratory factor 1 and 2 (NRF1/2), which will switch on mitochondrial biogenesis factors such as mitochondrial transcription factor A (TFAM) (**Fig 1.7B**) (88,89,89).

In mitochondria trafficking, parkin is involved in ubiquitination of the mitochondrial Rho GTPase (Miro) proteins (Miro1/2). Miro is a part of the motor adaptor complex that connects mitochondria to the microtubules and involved in anterograde transport process of mitochondria (89). Both Miro1 and Miro2 are direct substrates of parkin and multi-monoubiquitinated by parkin (90). Upon depolarization of the mitochondria, parkin degrades Miro, that leads to dissociation of the motor adaptor complex from the mitochondrial surface, eventually resulting in arrest of mitochondrial motility (92,93). Hence, parkin induces a decrease in the anterograde transport and a comparative increase in the retrograde transport. These functions of parkin highlight its pivotal role in the ubiquitin-proteasome system as well as its essential function in production and degradation of mitochondria.

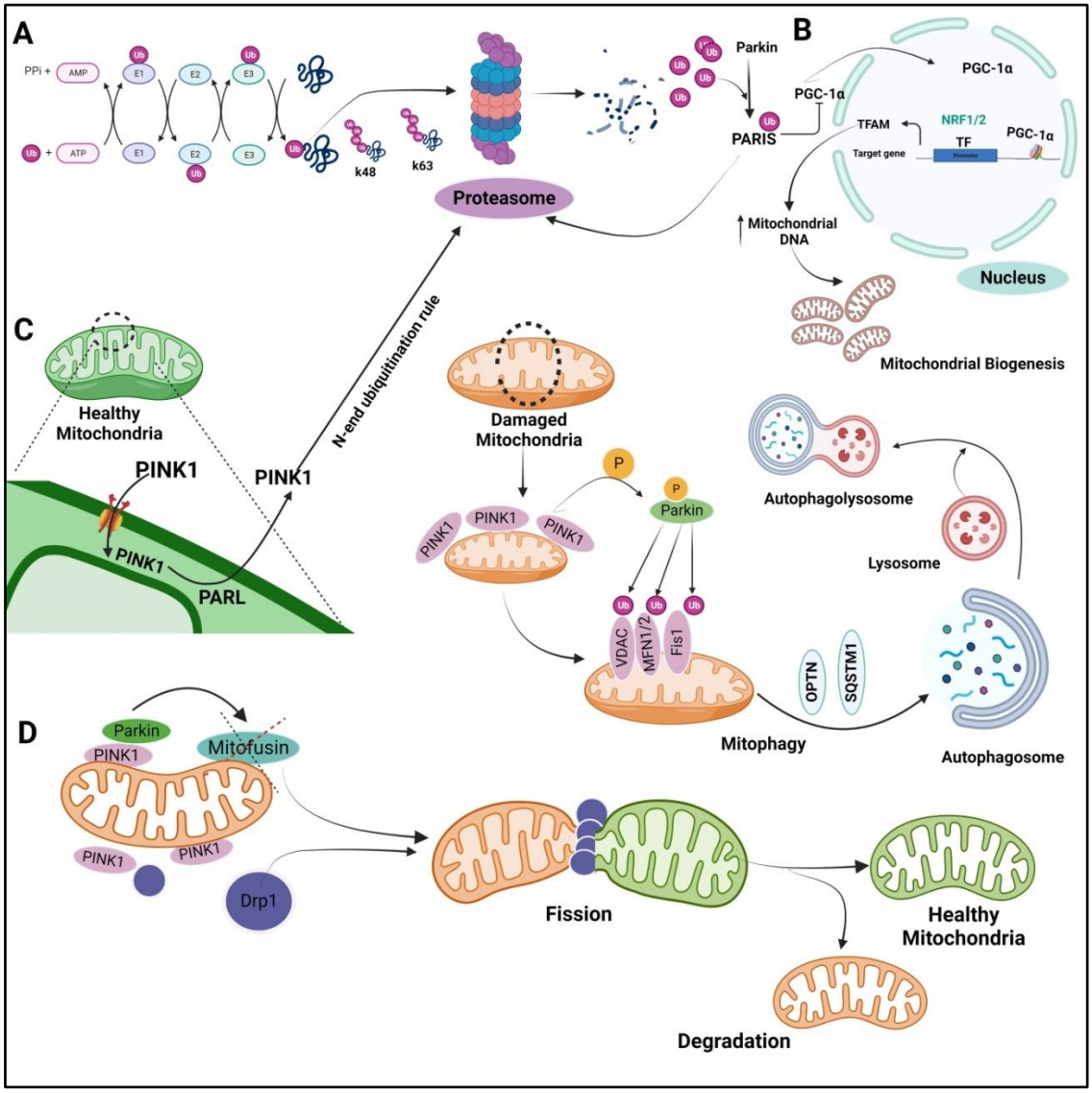


**Figure 1.7. A schematic model depicting the various function of parkin in mitochondrial quality control. A.** Parkin degrades toxic aggregates through the ubiquitin-proteasome system. **B.** Parkin affects mitochondrial biogenesis via the PARIS-PGC1α pathway. **C.** Parkin and PINK1 cooperatively participate in mitophagy to clear damaged mitochondria. **D.** Parkin preserves mitochondrial integrity by regulating mitochondrial fusion and fission process. Figure drawn using BioRender.

### 1.3.3 Parkin in PD:

*Parkin* is the second most common gene associated with familial form of PD after *SNCA* (α-synuclein) (63). To date, 479 *parkin* mutations have been recorded in the Human Gene Mutation Database (HGMD) (93). Among these mutations approximately 350 mutations are reported to be causing PD (93). Clinical features of PD patients with and without *parkin* mutations are very similar (95, 96). There are reports suggesting that usually, Lewy bodies are not detected in brain of PD patients with *parkin* mutations (97-101). However, there are some reports which show LBs presence in *parkin* mutant patients (102- 106). The presence of LBs in *parkin* mutation mediated PD is therefore not certain. The *parkin* mutation carriers are characterized by slow progression, early onset (21–50 years) of the disease, good response to *levodopa* treatment (95, 96, 107). Post-mortem analysis of sporadic PD patients' brains has shown reduced expression of parkin protein along with increased levels of ubiquitinated proteins in striatum region (101). In PD patients, PARIS, has been detected in the striatum and Substantia Nigra region of the brain (88). Considering the role of parkin in autosomal recessive juvenile Parkinsonism (AR-JP), various substrates are identified which are needed to be degraded for proper functioning of dopaminergic neurons. Among these substrates, *CDCrel-1* (cell division control-related protein 1), is predominantly expressed in the nervous system, where it is associated with synaptic vesicles and in neurotransmitter release. Synphilin-1, α-Synuclein–interacting protein, is a component of LBs in PD. Pael-R (Parkin associated endothelin receptor-like receptor), identified as a putative G protein-coupled receptor protein with homology to endothelin receptor type B, is enriched in Lewy bodies and accumulates in dopaminergic neurons of autosomal-recessive PD patients (102).

### 1.2 Functional interaction between α-Synuclein and parkin in PD:

Since both the proteins are involved in progression of PD, investigating the functional interaction between α-synuclein and parkin is crucial for understating how this interaction affects the dopaminergic neurons, There are much evidences that α-synuclein undergoes

extensive post translational modification including phosphorylation, ubiquitination and nitration. Many of these PTMs are present in Lewy bodies which suggest a primary role of α-synuclein in aggregation and neurotoxicity in PD (103). Phosphorylation of *a*-synuclein at serine 129 occurs in conjunction with dopaminergic neuronal cell death in PD (111,112,113). Parkin overexpression causes activation of protein phosphatase 2A (PP2A), which de-phosphorylates *a*-synuclein at Ser 129 and results into attenuation of *a*-synuclein-induced cell death in *in-vivo* model (107). Parkin has also been shown to interact with a novel insoluble glycosylated form of α-synuclein (αSp22) in the human brain, specifically associating with cases of juvenile-onset Parkinsonism featuring mutations in the *parkin* gene. This interaction does not occur with the soluble form of α-synuclein (108). Synphilin-1, α-synuclein–interacting protein and Pael-R (Parkin associated endothelin receptor-like receptor), a putative G protein-coupled receptor protein, are enriched in Lewy bodies and accumulates in dopaminergic neurons of autosomal-recessive PD patients and these are the other substrates for parkin-mediated ubiquitination (109). *Parkin* suppresses DA neuronal death induced by overexpression of α-synuclein and Pael-R in *Drosophila* model (110). WT α-synuclein has been shown to increase PLK2 (Polo-Like-Kinase-2) levels and GSK-3β (Glycogen synthase kinase 3β) activity and which lead to cell death in *in-vivo* model(111). Co-expression of *parkin* in *Drosophila* and rat model has reduced the PLK2 level and increased the PP2A (protein phosphatase-2A) activation leading to attenuation of the cell death (118, 117). Moreover, *a*-synuclein undergoes nitration on four tyrosine residues (Tyr39, Tyr125, Tyr133, and Tyr136) (112). The overexpression of monoamine oxidase B (MAO-B) results in a nine-fold increase in 3-nitrotyrosine at Tyr39 of *a*-synuclein which leads to its oligomerization (113). Parkin suppresses the transcription and expression of MAO-B (114). Parkin has been shown to suppress the neurotoxicity caused by α-synuclein overexpression in an *in-vitro* study and *Drosophila* model of PD (124,125). Thus, there are evidences that *parkin* reduces the neurotoxicity caused by *α-synuclein,* but the exact molecular mechanism remains unexplored.

### 1.5 Role of mitochondria in manifestation of Parkinson's disease

At cellular level, an involvement of mitochondrial dysfunction has been implicated in the pathogenesis of PD. Neurons, due to high metabolic demand; require high-quality of functional mitochondria in order to survive. Mitochondria is an extremely dynamic organelle which undergoes frequent fission and fusion process to maintain its shape, size and number and is critical for organelle distribution and bioenergetics. A change in number, distribution of mitochondria may cause cell dysfunction. The first link between mitochondria and PD has been

originated from the observation that mitochondrial toxin (MPTP) inhibit the respiratory chain complexes and leads to PD-progression (117). Additionally, rotenone and paraquat, two pesticides that inhibit mitochondrial complex I activity, have been reported to cause PD symptoms **(Fig 1.8A**) (118). Clinical studies have also reported the impaired mitochondrial complex I activity in post-mortem SN, striatum, frontal cortex, and cortical brain tissue of patients with PD (128-131). Mitochondrial dysfunction in PD, further supported by the findings from the mutations in autosomal-recessively (*Parkin*, *PTEN-induced kinase 1 (PINK1*), and *DJ-1*) and autosomal-dominantly (*SNCA* and *LRRK2*) inherited genes (**Fig 1.8 B**) (96,51). Patients with mitochondrial *polymerase gamma (POLG*) alteration also exhibit PD symptoms (121). Reduced membrane potential and abnormal morphology of mitochondria were found in the brains of PD patients (122). Mitochondrial defects have also been reported in *in-vitro* and *in-vivo* models (134,135,136). Since, mitochondria are the most important intracellular source of reactive oxygen species (ROS) generation, inhibition of respiratory chain complexes also lead to increased reactive oxygen species production (126).
Extensive studies are being conducted to investigate the mechanisms underpinning the degeneration of dopaminergic neurons induced by α-synuclein. These studies suggest the involvement of multiple pathways, including mitochondrial dysfunction and increased oxidative stress, impaired calcium homeostasis through membrane permeabilization, synaptic dysfunction, impairment of quality control systems, disruption of microtubule dynamics and axonal transport, endoplasmic reticulum/Golgi dysfunction, nucleus malfunction, and microglia activation leading to neuroinflammation (125). Among all these pathways, mitochondrial dysfunction has been considered as the most prime target of α-synuclein induced toxicity which leads to neuronal cell death in both sporadic and familial forms of PD (138,139). Wild-type *α-synuclein* overexpression or mutations have been shown to disrupt mitochondrial function. α-Synuclein has been shown to accumulate in mitochondria and impairing its function via interaction with complex I, complex III and cytochrome c oxidase (Complex IV) (121). Recently, oligomer form of α-synuclein has been shown to interact with mitochondrial ATP synthase (Complex V) and induces toxicity (129). Together, studies suggest that α-synuclein induces deficiency in ETC complexes and it might be a predominant feature of PD pathogenesis, which can in turn lead to the increased level of intracellular ROS (reactive oxygen species) and bioenergetics defects that are frequently observed in both sporadic and familial forms of PD.

There are several lines of evidences which suggest that α-synuclein alters mitochondrial morphology through regulating fusion and fission process (136,141,142). In mitochondrial fusion process Mfn1 and 2 respectively on outer mitochondrial membrane and OPA1 on inner mitochondrial membrane, where in fission process, fis1 (Fission 1 protein) outer membrane protein and drp1, the cytoplasmic proteins are involved (85). It has been reported that wild-type α-synuclein produce fragmented mitochondria and leads to neural degeneration in *in-vitro* and *in-vivo* models (130). In several PD models, the balance between mitochondrial fusion and fission is disrupted and there are studies which report that Drp1 dependent mitochondrial fragmentation plays an important role in mitochondrial abnormalities and cellular dysfunction in Parkinson's Disease (132). α-Synuclein has been shown to directly interact with mitochondrial membrane and produce mitochondrial fragmentation even in absence of Drp1 (133). It has been reported that A53T *α-synuclein* can affect the mitochondrial morphology and reduce Mfn1 and Mfn2 in mice in an age-dependent manner and can modulate mitochondrial dynamics through Mfn1 and Mfn2 dependent mechanism (131). The rat model of PD has also shown that over expression of WT or mutant *α-synuclein* and loss of *PINK1* mutation increased the mitochondrial fission and Golgi fragmentation (134).

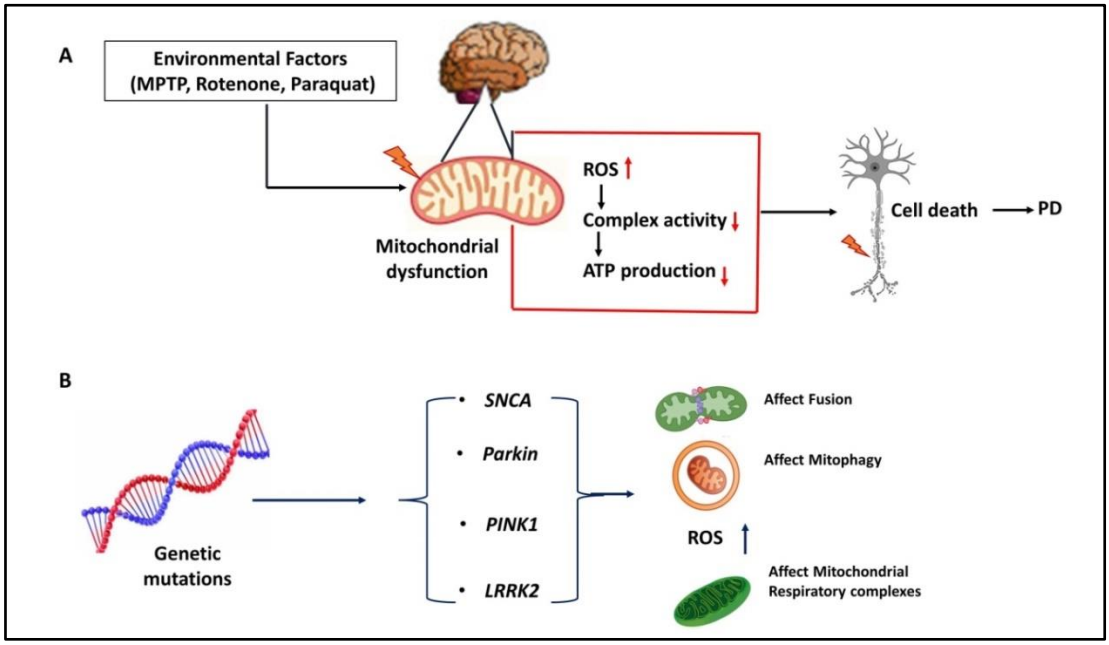



**Figure 1.8. Mitochondria in Parkinson's disease.** A. Environmental toxins causes dysfunctional mitochondria via inhibiting respiratory complexes, increasing ROS (reactive oxygen species) production and reducing ATP production which eventually cases cell death in sporadic form of PD .B. Genetic mutations in *Parkin*, *PINK1*, *SNCA*, *LRRK2* genes cause mitochondrial dysfunctions through affecting fusion, mitophagy, increasing ROS production

and mitochondrial respiratory complexes and lead to the progression of PD. Figure drawn using BioRender.

In *parkin* mutant flies, defective swollen mitochondria with fragmented cristae have been shown in male germline and adult flight muscles tissues which lead to death of flight muscles (146,147). *Parkin* loss-of-function mutation has been shown to promote mitochondrial fission in *Drosophila* (137). In dopaminergic neurons of *parkin* mutant flies, the mitochondria were observed to be swollen (138). Many *in-vitro* and *in-vivo* studies have also reported that *Pink1* and *Parkin* promote mitochondrial fission and/or inhibit fusion by negatively regulating Mfn and Opa1 function, and/or positively regulating Drp1 (143,150,151). In an *in-vitro* study, *parkin* has been shown to interact with the mitochondrial fusion factors, Mfn1 and Mfn2, but not with Drp1 or OPA1 in human cells, where it ubiquitinates the Mfn1 and promotes the turnover of Mfn1 through enhancing its degradation by the proteasome (141). There are reports showing that, inhibition of mitochondrial fission in *α-Synuclein* (A53T) and *PINK1* mutant mouse model attenuated the neurotoxicity.

Thus, there are evidences that show that disrupted mitochondrial dynamics is due to loss of function mutation in *parkin* and overexpression of WT or mutant *α-Synuclein*. However, mechanism of *α-Synuclein* and loss-of-function *parkin* mutation triggering the defects in mitochondria fusion and fission dynamics and how it ultimately causes dopaminergic neuronal death is still unclear.

### 1.6 Apoptosis in PD:

Apoptosis is a programmed cell death and characterized by morphological changes including cell shrinkage, nuclear condensation, and DNA degradation. It is caused by a cascade of events which involves cysteine proteases known as caspases, lead to the cleavage of multiple cellular substrates. Apoptosis mainly consist of two main pathways: Extrinsic and intrinsic pathways. The extrinsic pathway triggered by external ligand molecule and involves death receptors (DRs), whereas intrinsic pathway is mitochondrial-mediated pathway. In extrinsic pathway, initiator caspase-8 and in intrinsic initiator caspase-9 is activated. Both intrinsic and extrinsic pathways are converged onto a common pathway of executioner caspases, involving caspase-3 and caspase-6. It is characterized by the expression of genes, mostly oncogenes that enhance the apoptotic process (i.e., *bax, bcl-x*) and anti-apoptotic genes that inhibit the death process (e.g., *bcl-2, bcl-xL* In PD, apoptosis has been considered main mechanism of the neuronal loss.

In post-mortem and *in-vitro* studies the DNA fragmentation, apoptotic chromatin changes and increased expression of active caspase-3 have identified in dopaminergic neurons of PD patients. In *in-vitro* models of PD, dopaminergic neuronal death is inhibited by overexpression of anti-apoptotic proteins, such as Bcl-2 and caspase inhibitors have also been shown to rescue neurons from death. Increased levels of proapoptotic proteins, such as Bax, have also been observed in post-mortem brain tissue from PD patients (153,154,155). Both α-synuclein and parkin have also been reported to contribute in apoptosis-mediated cell death in PD (145). In sporadic as well as familial form of PD degeneration of dopaminergic neurons through mitochondria-mediated apoptosis has been reported (156,157). Hence, substantial evidences support to the notion that apoptosis is the main mechanism of neuronal death in Parkinson's disease.

### 1.7 Drosophila Model of PD

*Drosophila melanogaster*, *a.k.a.* fruit fly has been widely used as a model organism and emerged as the "Golden Bug" over the past century (182,183). In 1910, Thomas H. Morgan first used *Drosophila* to study genetic inheritance and won the Nobel Prize in Physiology or Medicine in 1933 for formulating the chromosomal theory of inheritance and patterns of inheritance of the white eye pigment in flies (150).

*Drosophila* development and physiology are very similar to complex eukaryotes. Flies are also the simplest model organism and many organs are analogous to those in humans for example, flies have a gut, a beating heart, and clearly defined central and peripheral nervous systems, allowing them to model multisystem phenotypes associated with disease mutations (151). *Drosophila* is a very powerful genetic model system, at the genome level; it is simpler than vertebrate models, with only 4 chromosomes and 13,821 genes. Its genome has relatively limited genetic redundancy: classes of genes with multiple members in humans are often represented by only a single orthologous gene in flies (148). It has shorter generation time (12-15 days) (**Fig 1.9**) (152) and life span (60–80 days). *Drosophila* genome sequencing has shown that 75% of all human disease-related genes have homologs in *Drosophila.* There are many genetics tools available for genetic analysis for e.g. Gal4/UAS, LexA/LexAop, QF/QUAS etc. Gal4/UAS system allows for the expression of genes from any organism in a tissue and time-specific manner. Gal4/UAS system is a transcription activation system co-opted from yeast where Gal4 (the yeast transcriptional activator) binds to UAS (upstream activation sequence), an enhancer and activate the gene transcription. By fusing Gal4 to a tissue or cell-specific

promoter, it is possible to drive the expression or knockdown of genes of interest in specific cell type or tissue (153) (**Fig 1.10**). All these features of *Drosophila melanogaster* make it excellent model system to study the genetic disorders including neurodegenerative diseases (154).

Many cellular and animal models have been used to study PD, among animal models, rodent (mice and rat) are common but these do not always recapitulate PD pathology (155). However, *Drosophila melanogaster* has emerged as an effective model for studying PD (156), due to presence of homologue of human disease genes including PD genes (except *α-Synuclein*)(148), availability of genetic manipulation tools which are easily accessible, ease of culture and cost-effective maintenance.

Though, by expressing human wild-type and mutant forms of *SNCA* (A30P and A53T) have been successfully recapitulated the characteristics of PD in *Drosophila* (157). Aside from *α-Synuclein* overexpression, knockout mutant of *parkin* in *Drosophila*, has also been used to study PD, which has shown reduced lifespan, male sterility, and adverse defects in both flight and climbing abilities (158).

*Drosophila* and human mitochondria show a very high degree of conservation in both genetic architecture and biochemical pathways. The mitochondrial DNA of *Drosophila* has very similar component to human mitochondria DNA. *Drosophila* mtDNA genome at 19,517 bp 3 kb larger than the human mtDNA genome is 16,559 bp, The molecular functions which are associated with mitochondria, such as OXPHOS, mitochondria transport, and biogenesis are highly conserved (159). Mitochondrial dysfunction has also been studied using *parkin* mutant flies (135) and genetic interaction between *parkin* and *pink1* also shown in flies (160).

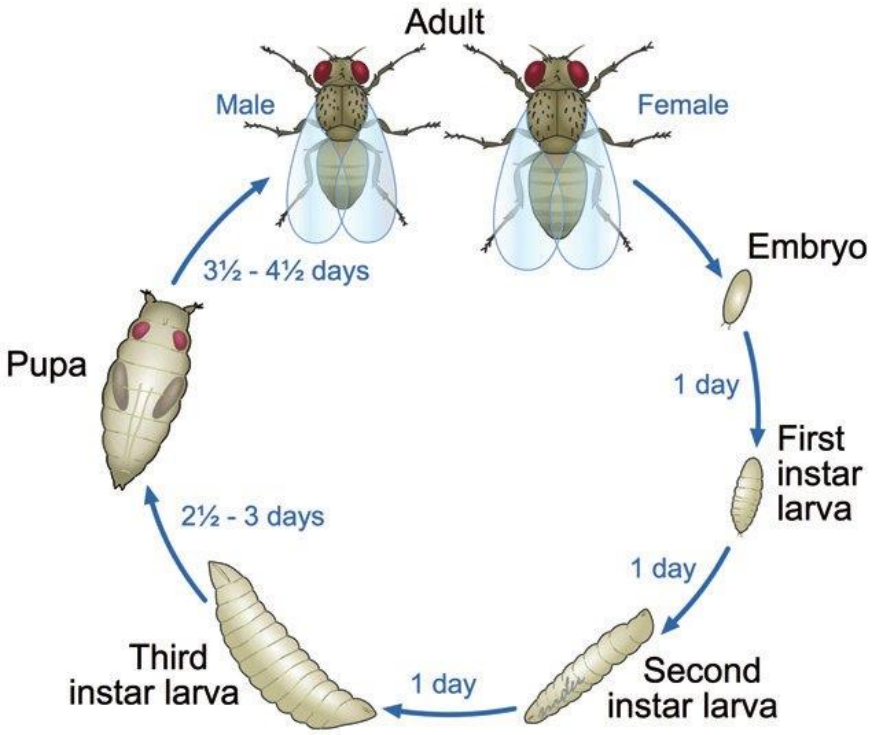



**Figure 1.9 The whole life cycle of *Drosophila***. Diagram showing different development stages: embryo, larva (first instar, second instar and third instar), pupa and adult. Image adapted from (Ong, Cynthia et al. 2015).

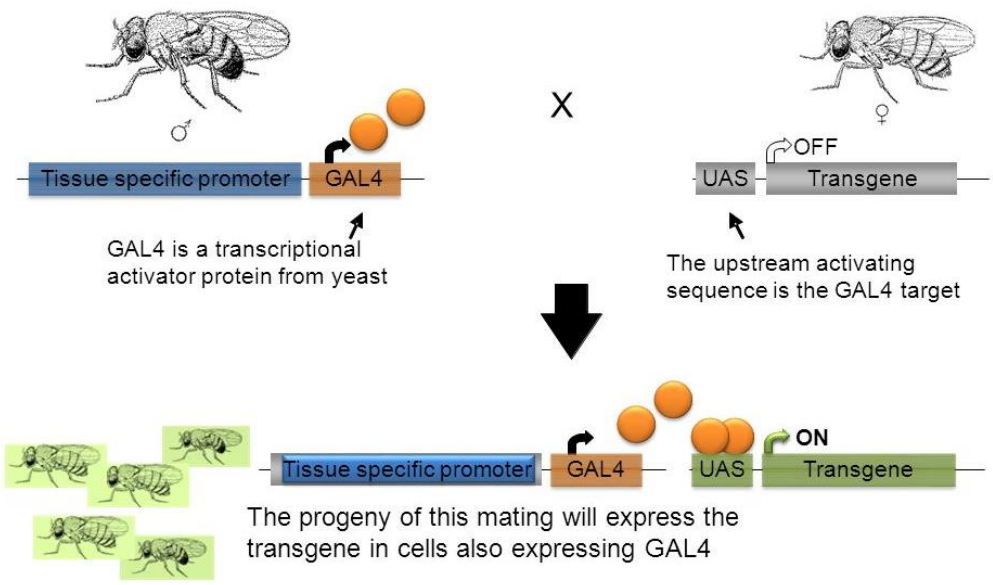
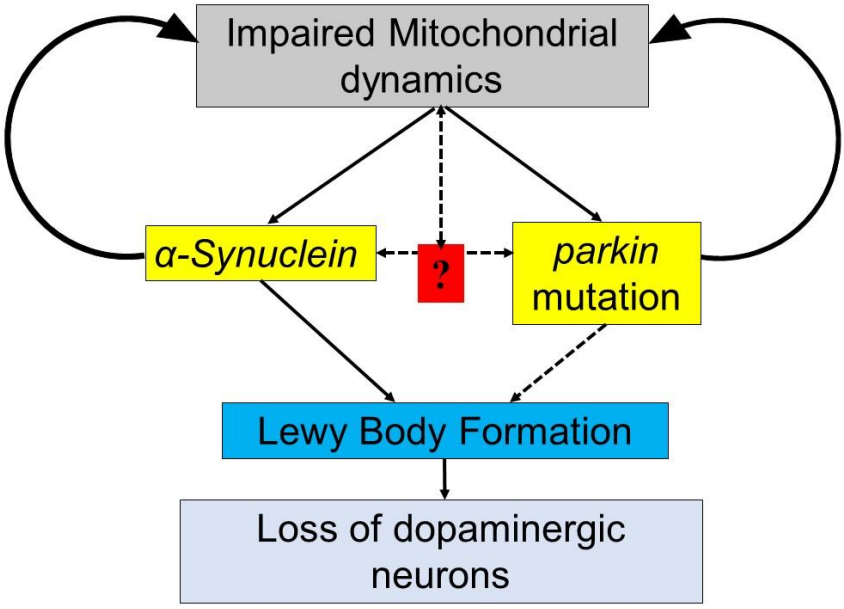



**Figure 1.10**: **Schematic representation of Gal4/UAS system.** Two transgenic fly lines, Gal4-driver and UAS-responder are used in this system. The Gal4-driver fly has a transgene containing the Gal4, the expression of which is under the control of a tissue-specific promoter. The UAS-responder fly has a transgene containing target gene ligated to the UAS promoter, a target of Gal4. In the F 1 progeny of these flies will have target gene expression in promoter specific tissue. Source: https://slideplayer.com/slide/3461435

**1.8 Gaps in research:**

1. Studies have shown the involvement of α-Synuclein (WT or mutant) protein accumulation and loss-of function mutation in *parkin* gene in onset of Parkinson's disease. There are reports indicating that *parkin* reduces neurotoxicity caused by *α-Synuclein*, however molecular and genetic mechanism of this interaction in onset and progression of PD is still unexplored.

2. Although existing research shows that α-Synuclein accumulation and *parkin* mutation lead to impaired mitochondrial dynamics in PD. Since, maintenance of mitochondrial dynamics is required to meet high energy demand within the neurons. However, molecular mechanism of *α-Synuclein* and *parkin* interaction in context of mitochondrial dynamics is yet to be explored.




**1.9 Objectives of research:**

1. To evaluate and optimize the experimental *in-vivo* model of PD in *Drosophila melanogaster*

2. Understanding the genetic and molecular interaction between *α-Synuclein* and *parkin* in *Drosophila* model of PD

3 Characterizing the effects of *α-Synuclein* and *parkin* interactions on mitochondrial dynamics in PD pathogenesis.

# *Chapter-2*

# *Materials & methods*

## 2.1 Materials

### 2.1.1 Fly Stocks

Driver Gal4 lines: GMR-Gal4 and Actin Gal4; Ubi GFP/TM6bTB (a gift from Pfor. Lakhotia's lab, BHU, India), elav-Gal4 (BL-8760), TH-Gal4 or ple-GAL4 (BL-8848) were used to express transgenic lines ubiquitously, in all neurons and in dopaminergic neurons respectively. Transgenic responder lines: UAS-GFP, UAS-Mito-HA-GFP.AP (BL-8442), *UAS-Hsap\SNCA.F*(BL-8146), *UAS-SNCA.J}1/CyO* (BL-51375), *UAS-Parkin*$^{RNAi}$ (BL-37509), *UAS-Parkin*$^{RNAi}$ (BL-31259) and *UAS-park.FLAG.COX-IV*(BL-34746) were used. All fly stocks, genetic crosses and F1 progenies were maintained on standard fly food containing agar, maize powder, yeast, and sugar at 25 °C. GAL4-UAS system was used to obtain desired genotype.

### 2.1.2 Chemicals

HMDS (Hexamethyldisilazane) (#SRL-28437), Acetone (#MERCK- 1.94500.2521), TRIzol method (#INVITROGEN-15596026), Verso cDNA Synthesis Kit (#Thermo Scientific™-AB1453A), 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (#Solis BioDyne-08-24-0000S), 2′,7′-dichlorofluorescin diacetate (DCFDA, # D6883), Triton-X100 (#) RIPA 10X (#MERCK-20-188), Bradford reagent (#) Protease inhibtor cocktail (#) (Nitrocellulose Membrane 0.2 µm (#BIORAD-1620112), 2X Laemelli buffer (#),BSA, Tween-20 (#) Protein ladder (#PUREGENE-PMT2922) were used.

**2.1.3 Instruments:** The major instruments used are enlisted below in Table 2.1.

**Table 2.1 List of major instruments used.**

| Name of instruments | Company |
|---|---|
| Stereomicroscope | OPTIKA |
| Confocal Microscope | Zeiss |
| Scanning Electron Microscope | Thermo fisher scientific |
| Multiskan GO microplate Spectrophotometer | Thermo fisher scientific |
| Cooling Centrifuge | Thermo fisher scientific |
| Vertical/ Horizontal gel electrophoresis unit | Bio-Rad |
| Semi-dry transfer apparatus | Bio-Rad |

| | |
|---|---|
| Real-time PCR | Bio-Rad |
| Thermocycler | Bio-Rad |
| Chemi Doc/Gel Doc | Bio-Rad |
| Concentrator plus | Eppendorf |
| Fluoroskan Ascent | Thermo fisher scientific |

## 2.2 Methods

### 2.2.1 Life span assay

For life span analysis, all F1 progenies of desired genotypes were collected and aged at 25 °C. Flies were transferred to new fresh food vials alternate day without anesthetization and the numbers of dead & surviving flies were recorded each day until the desired age (30 days). Three replicates were carried for each genotype and percentage (%) of survival was calculated. The statistical analysis was done using Gehan-Breslow-Wilcoxon test.

### 2.2.2 Climbing assay

To determine locomotor activity, climbing assays were performed. 10 flies per genotype were transferred into cylindrical glass tube after anesthetization and left for 5-10 min for the revival and acclimatisation at room temperature. Tubes were marked up to 8cm above the bottom of the vial. After acclimatization, gently tapped the flies down to the bottom of vial and the number of flies crossed the 8cm mark were recorded after 10 sec. Three trials were performed, and numbers were then averaged, and the resulting mean was used as the overall value for each single group of flies. For all genotypes 3 replicates were carried out.

### 2.2.3 Quantitative real-time PCR

Total RNA was extracted from 25-30 fly heads using the TRIzol method (Invitrogen) referred from Jove protocol (Jensen, K. et al; Purification of Transcripts and Metabolites from *Drosophila* Heads. *J. Vis. Exp.* (73), e50245, doi: 10.3791/50245 (2013). RNA concentrations were measured with a Nanodrop ND-1000 Spectrophotometer and equal amounts of RNA were reverse transcribed using Verso cDNA Synthesis Kit (AB1453A). QPCR was performed using 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne).

### 2.2.4 Adult Brain Immunohistochemistry

Adult brains of desired genotype were dissected in cold 1X PBS and incubated with fixative solution (4% formaldehyde in 1XPBST (0.1%TritonX-100) for 20 min at room temperature. After three washes with 1XPBST for 10 min each wash, blocking was done using 1% BSA for 1 hr at room temperature. Brains were probed with rabbit anti-TH (#AB152) at 1:1000, mouse anti- synuclein(H3C) (DSHB-S1-890) at 1:200 overnight (12-16) at 4 °C. Following three washes for 10 mins, brains were incubated with goat Anti-Mouse IgG Antibody, Cy3 conjugate (#AP124C) (1:200) and goat anti-Rabbit, Alexa Fluor Plus 555 **(#**A32732) (1:4000) secondary antibodies at room temperature for 2hr. Brains were washed three times for 15 min, then they were mounted between two glass coverslips by using antifade medium on microscope slides. Confocal microscope was used to acquire z-stacks at 1µm intervals with 20×/N.A.0.60 and a 63×/N.A.1.30 oil (for mito-GFP) Plan-Apochromat objective. The numbers of TH-positive neurons were counted manually within each cluster of posterior regions of the brains.

### 2.2.5 Immunoblotting

For western blot analysis, F1 progenies of desired genotype were collected in 1.5ml Eppendorf tubes and snap-frozen in liquid nitrogen. Drosophila heads (~30-50) were decapitated and homogenized in 100ul 1x RIPA buffer (Merck, #20-188) containing 1% protease inhibitor (Sigma, P8340) using sterilized pestle. The homogenates were centrifuged at 10,000 rpm at 4° C for 10min. The supernatant was collected into a new Eppendorf tube and assayed for protein concentration. The protein (80µg) was resolved on 12% SDA-PAGE and then transferred to .2µm nitrocellulose membrane (Bio Rad, #1620112). After blocking the membrane with 3% BSA in TBS-T (.05%Tween-20), membrane was incubated overnight at 4° C with primary antibodies. The primary antibodies used were rabbit anti- Drosophila Parkin (Merck, SAB1300355, 1:500), mouse E7 anti-beta tubulin (DSHB-S1-810, 1:200), mouse ATP synthase beta (Sigma; #A-21352, 1:1000) and rabbit Mitofusin-2 mAb (Cell Signalling #D2D10, 1:1000). Following three washes with TBS-T, membrane was incubated with an appropriate HRP-conjugated secondary antibodies: goat anti-mouse (Thermo Scientific # 31430, 1:1000), mouse anti-Rabbit (GenScript, #A01856, 1:1000) for 2 hr at room temperature and signal was detected using ECL substrate (Bio Rad #1705061). Image analysis and quantification was done using ImageJ software. Western blot was done on the same membranes after stripping between each application of the antibody.

### 2.2.6 Mitochondrial morphology measurement

To assess the mitochondrial morphology in dopaminergic neurons, UAS-Mito-GFP fly strain was used to tag the mitochondria and dopaminergic neurons were stained using anti TH antibody. Z-stack of one PPL1 and one PPM3 DA neuronal cluster per brain was imaged using confocal microscope at 63×/N.A.1.30 oil with 1.5 zoom. A total of 3-4 brains per genotype were scanned. Publically available ImageJ Mito-Morphology Macro created by Dagda et al. (2009) was used to quantify the mitochondria. Average area, circularity and (Area/Perimeter)/minor axis were calculated representing elongation, fragmentation and swelling of mitochondria respectively.

### 2.2.7 Mitochondrial fractionation

Mitochondrial fractionation was done from the heads of desired genotypes using differential centrifugation as described previously (161) with slight modifications. Briefly, ~150 heads of desired genotypes were collected and homogenized in an ice-cold mitochondrial isolation buffer (250 mM sucrose, 10 mM HEPES, 1 mM EDTA and 0.1% fat-free BSA, pH 7.4) using sterilized micro-pestles. Lysate was then centrifuged at 1000g for 5 min at 4 °C. The supernatant was collected and centrifuged at 10,000g for 10 min at 4 °C. The pellets were then resuspended and washed thrice in mitochondrial washing buffer (250 mM sucrose, 10 mM HEPES, and 0.1 mM EDTA, pH 7.4) at 10,000g for 10 min at 4 °C. Finally, the pellet was resuspended in 80µl of 0.1% fat-free BSA suspension buffer with 250 mM sucrose and 10 mM HEPES, pH 7.4.

### 2.2.8 ROS (reactive Oxygen Species) quantification

DCF-DA (2,7-dichlorofluorescein diacetate) assay was used to quantify the ROS as previously described (162) with some modifications. 20 flies of desired genotypes were homogenized in 20mM Tris buffer, pH 7.0. The homogenate was centrifuged at 1600 ×g for 10 minutes at 4°C, and the supernatant was collected for quantification of 2,7-dichlorofluorescein fluorescence. Aliquot of 5µl of supernatant was incubated with 5µM DCFDA at 37 ˚C for 60 min. The fluorescence was monitored at 488nm/530nm excitation/emission using Fluoroskan Ascent.

### 2.2.9 Malondialdehyde (MDA) Quantification

Thiobarbituric acid reactive substances (TBARS) assay was used to assess the oxidative stress by quantifying the levels of malondialdehyde (MDA), a stable by-product of lipid peroxidation

as previously described (163) with some modifications. 30 flies of desired genotypes were homogenized in 1x RIPA buffer (Merck, #20-188) containing 1% protease inhibitor (Sigma, P8340) using sterilized pestle. The homogenates were centrifuged at 10,000 rpm at 4° C for 10min. The supernatant was collected and assayed for protein concentration. Aliquots of supernatant were adjusted to have an equal amount of protein (1 mg/ml) and then 250 µl of 10% trichloroacetic acid (TCA) added. Subsequently, 375 µl of thiobarbituric acid (TBA) added at a concentration of 1% (w/v) under acidic conditions. The resulting solution was kept at 100 ˚C for 15 min, which facilitated the formation of a pink-colored precipitate. The absorbance of this precipitate was measured spectrophotometrically at 530 nm (Multiskan FC, Thermo Scientific, DE). The obtained values were expressed as micromolar concentration (µM) of malondialdehyde per 5 milligram (mg) of protein.

**Table 2.2 List of primary & secondary antibodies used**

| Antibodies | Catalogue No. |
|---|---|
| Rabbit anti-Tyrosine Hydroxylase Antibody | MERCK; #AB-152 |
| Anti- synuclein(H3C) | DSHB; #S1-890 |
| Rabbit anti-Drosophila Parkin (C-term) | Sigma; #SAB1300355 |
| E7 anti-beta-tubulin | DSHB |
| Rabbit Mitofusin-2 mAb | Cell Signalling #D2D10 |
| Mouse ATP synthase beta | Sigma; #A-21352 |
| Goat anti-Mouse IgG Antibody, Cy3 conjugate | Sigma; #AP124C |
| Goat anti-Rabbit, Alexa Fluor Plus 555 | Sigma; #A32732 |
| Goat anti-Mouse IgG (H+L), HRP | Invitrogen; #31430 |
| Mouse Anti-Rabbit IgG Fab (HRP), mAb | GenScript; # A01855-200 |

**Table 2.3. List of primers used for real time PCR.**

| Gene name | Forward primer (5’ to 3’) | Reverse primer (5’ to 3’) |
|---|---|---|
| **RP49** | CCAAGGACTTCATCCGCCACC | GCGGGTGCGCTTGTTCGATCC |
| **Parkin** | ATTTGCCGGTAAGGAACTAAGC | AAGTGGCCGACTGGATTTTCT |

### 2.2.9 Statistical Analysis

GraphPad Prism 8.0.1 was used for statistical analysis and graphical display of the data. Statistical significance is expressed as *p* values which were determined with one-way ANOVA and Two-way ANOVA followed by Tukey's multiple comparison tests as indicated in the figure legends. If p-value was more than 0.05, then the difference was considered not significant (ns); whereas, if p-value was ≤ 0.05 it was considered significant and denoted by symbols *(<.05), ** (<.001), *** (<0.0001), ns-not significant (.12).

# *Chapter-3*

# *Establishment of Drosophila model of Parkinson's disease*

## 3.1 Introduction

More than two centuries ago, clinical syndrome of PD was reported which include selective degeneration of dopaminergic neurons in the *substantia nigra* and locomotor dysfunctions. The locomotor dysfunctions' symptoms include bradykinesia, muscle rigidity, resting tremor, and postural instability (3). The cause of PD is still largely unknown, although involvements of multiple factors such as genetics, environmental agents, and aging have been suggested (3).

The only gold standard for confirming the diagnosis of PD condition is post-mortem. Thus, due to the lack of accessibility of human brain samples, diverse range of experimental models using animals and *in vitro* cultured cells have been generated that could mimic different aspects of PD (195, 196, 197). Cellular-based approach is frequently used because of the ease of manipulation and suitability for large-screen assays (165). Two major approaches: neurotoxin and genetic-based, are used to generate a variety of animal models such as non-human primates (NHP), rodents, zebrafish, *Caenorhabditis* (*C.*) *elegans*, and *Drosophila melanogaster* to understand the pathogenesis and therapeutic development of PD (167).

In neurotoxin-based approaches, 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone, and paraquat are most commonly used to induce the sporadic model of PD (118). In genetic-based approaches, PD-related genes, such as α-synuclein (*SNCA*), Leucine-rich repeat kinase 2 (*LRRK2*), PTEN-Induced Kinase 1 (*PINK1*), parkin (*PRKN*), and protein deglycase (*DJ-1*) are used to induce transgenic models (155). However, each approach has distinct advantages and limitations which determine the applicability of the model for a particular experiment.

In cellular-based models, molecular and cellular function of a gene can be dissected very quickly and robustly using molecular, biochemical, and pharmacologic approaches. Cellular models do offer the advantages of dissecting many insights into the function of the proteins which is implicated in PD. However, interpretation of cellular studies needs to be done cautiously, since they are often prone to artefact and/or misinterpretation (177, 187).

Animal models are valuable tools for studying cellular processes within the context of functional neuronal circuits in unbiased way that can serve as a validation on cellular assays (155). For the further validation findings in transgenic animal models are relied on the use of human post-mortem tissues. Brain post-mortem analysis typically reveals the advanced stages of an illness. However, traces of initial disease pathology often persist in these analyses. These persistence makes post-mortem analysis a benchmark for assessing the ability of animal models to reflect real disease pathogenic processes (155). Animal models can sometimes reveal

valuable insights into the human disease condition that cannot be obtained solely through standard neuropathological evaluations.

Neurotoxins-based animal models of PD generally induce a strong and rapid cell loss in the substantia nigra pars compacta (SNpc), elicit motor symptoms and behavioural changes, but lack the formation of Lewy bodies (169).

On the contrary, genetic-based models can demonstrate variable cell loss, motor symptoms along with α-synuclein pathology, depending on the specific model, by introducing genetic mutations or altering gene expression through transgenic animals or viral transfection (155). Some of the major genes used to generate genetic animal models are *α-synuclein, PINK1 and Parkin*. Overexpressing *WT, A30P* and *A53T* human *α-synuclein* in *Drosophila* recapitulated the major characteristics of PD including age-dependent selective DA neuron loss, Lewy body inclusions, and locomotor deficits (14, 204). *C. elegans* also recapitulated DA neuronal loss but lack the Lewy body formation after overexpressing *WT, A30P* and *A53T* human *α-synuclein* (171). *Drosophila* and *C. elegans* lack a homologous gene for *α-synuclein*. *α-synuclein* transgenic mice have also been generated using different promoters (Mouse Thy1, PrP), however, none of these models represented all the PD characteristics (172). Despite the lack of progressive DA neuronal loss, some of the *α-synuclein* transgenic mice models have functional abnormalities in the nigrostriatal system and are DA responsive (172). In similarity to humans, only *A53T α-synuclein* transgenic mice with mouse prion promoter (mPrP) exhibit α-synuclein pathology including α-synuclein aggregation, fibrils and truncation, α-synuclein phosphorylation and ubiquitination along with progressive age-dependent neurodegeneration (206, 189). *PINK1* and *parkin* mutation in *Drosophila* models exhibit age dependent DA loss, reduced life span and severe climbing defects (147, 149, 207), whereas the mouse models do not completely mimic the major features of PD (208, 209, 210, 211).

Thus, to understand the PD mediated cell death and underlying mechanism, first we have established genetic model of *Drosophila melanogaster* model. *Drosophila* has several advantages including a higher degree of conservation in developmental pathways with mammals including apoptotic pathway (154). Transgenic *Drosophila* has been extensively used as a model system to study several neurodegenerative diseases: Amyotrophic Lateral Sclerosis, Huntington Disease, PolyQ Disease, Alzheimer's Disease (188, 204, 215) and many other developmental disorders (157). This model has also been used to screen for therapeutic effects of various natural compounds (155). Though, *Drosophila* doesn't have *SNCA* homologue, however it recapitulates the major neuropathological features of PD, e.g. Lewy Body formation, locomotor defect, and loss of dopaminergic neurons (216, 217). We therefore

have overexpressed human *WT-SNCA*, A30P and down-regulated *parkin* in different *drosophila* tissues and it has shown the major phenotypes of PD.

## 3.2 Material and methods

### 3.2.1 Fly Stocks

*GMR-Gal4, Actin-Gal4; Ubi GFP/TM6bTB* (a gift from Lakhotia lab, BHU, India), $w^{118;}$ *Elav-Gal4***,** *UAS-Mito GFP/CyO* (BL-8842), *UAS-SNCA (A30P)* (BL-8147), *UAS-SNCA* (BL--51375)**,** *UAS-Park RNAi/ CyO* (BL-37509), *UAS-Park RNAi* (BL-31259) fly lines were used. All the stocks were maintained on standard fly food containing agar, maize powder, yeast, and sugar at 25°C. Using Gal4/UAS system appropriate genetic crosses were carried out to obtain desired genotypes.

### 3.2.4 Survival Assays

For the survival assays, freshly eclosed flies of desired genotypes, were collected. Flies were transferred to new fresh food vials every other day without anesthetization and the number of dead & surviving flies were recorded each day until the desired age (30 days). The statistical analysis was done using Gehan-Breslow-Wilcoxon test.

### 3.2.5 Climbing Assay

To characterize the locomotor dysfunction, climbing assays were performed. Flies were aged up to 30 days. Groups of 10 flies per genotype were transferred into cylindrical glass tube after anesthetization and left for 5-10 min for the revival and acclimatisation at room temperature. Tubes were marked at 8cm above the bottom of the vial. After acclimatization, the flies were gently tapped down to the bottom of vial and the number of flies that crossed the 8cm mark was recorded after 10 sec. Three trials were performed, and numbers were then averaged, and the resulting mean was used as the overall value for each single group of flies. For all genotypes, three replicates were carried out.

### 3.2.6 Quantitative Real-Time PCR

Total RNA was prepared from adult flies or heads using the TRIzol method (Invitrogen) referred from Jove protocol (Jensen, K. et al; Purification of Transcripts and Metabolites from *Drosophila* Heads. *J. Vis. Exp.* (73), e50245, doi: 10.3791/50245 (2013). cDNA was synthesized using Verso cDNA Synthesis Kit (AB1453A). *RP49* was used as reference gene to normalize the *Dronc* amplicon. Quantitative RT-PCR was performed using 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne). Primer Sequences were as follows:

*RP49*: Forward: 5'-CCA AGG ACT TCA TCC GCC ACC-3'

Reverse: 5'- GCG GGT GCG CTT GTT CGA TCC-3'

*Parkin*: Forward: 5'- ATTTGCCGGTAAGGAACTAAGC-3'

Reverse: 5'- AAGTGGCCGACTGGATTTTCT-3'

### 3.2.7 Adult Brain Immunohistochemistry

Adult brains of desired genotype were dissected in cold 1X PBS and incubated with fixative solution (4% formaldehyde in 1xPBST (0.1%TritonX-100) for 20 min at room temperature. After three washes with 1xPBST for 10 min each wash, blocking was done using 1% BSA for 1 hr at room temperature. Brains were probed with mouse anti- synuclein (H3C) (DSHB-S1-890) at 1:200 overnight (12-16) at 4 °C. Following three washes for 10 mins, brains were incubated with goat anti-mouse IgG antibody secondary antibody at room temperature for 2hr. Brains were washed three times for 15 min, then they were mounted between two glass coverslips by using antifade medium on microscope slides. Confocal microscope was used to acquire z-stacks at 1µm intervals with 20×/N.A.0.60 Plan-Apochromat objective.

**3.2.8 Statistical Analysis:** Statistical analysis was performed using GraphPad Prism 8.0.1 software. Data are expressed as mean with SEM of replicates. The *p-value* and statistical significance are mentioned in the legends.

## 3.3. Results

### 3.3.1 *SNCA* overexpression (*WT* and *A30P*) and *parkin* downregulation cause rough eye phenotype

To explore the underlying mechanisms of neurotoxicity in terms of *α-synuclein* and *parkin*, we have established the *in vivo Drosophila* model of PD using Gal4/UAS expression system. Before establishing the model, we have validated the *UAS-SNCA*, *UAS-A30P* and *UAS-parkin*$^{IR}$ (parkin downregulation) transgenes. We overexpressed *GFP* and *SNCA*, *A30P* in dopaminergic neurons using *TH-Gal4* and α-synuclein (*SNCA*, *A30P*) expression was confirmed using H3C antibody for α-synuclein (**Fig3.1**). For *UAS-parkin*$^{IR}$, we downregulated parkin ubiquitously using *Actin-Gal4* and downregulation was confirmed with qRT-PCR (**Fig 3.2**).

To assess the neurotoxicity, we have overexpressed *WT* and *A30P α-synuclein* in *Drosophila* eye using *GMR-Gal4* driver line. The *Drosophila* compound eye is a structured biological system which consists of 800 simple units knows as ommatidia. These ommatidia form a regular hexagonal pattern and this precise organization allows assessing the effect of altered gene expression and mutations in proteins on the external eye morphology. It also allows for the detection of subtle modifications in ommatidia geometry caused by cell degeneration. *Drosophila* eye has also been used in various neurodegenerative diseases (**Fig.3.3**)(204, 218). We have found that overexpression of *WT* and *A30P SNCA* (**Fig.3.4 B & C**) has shown rough eye phenotype compared to control fly's eye (**Fig. 3.4A**). Though, *A30P* phenotype was more robust than *WT-SNCA*. Downregulation of *parkin* has also shown the rough eye phenotype as compared to control. Here we have tested two different lines of *UAS-parkin*$^{IR}$ and both of them have shown rough eye phenotype. *UAS-parkin*$^{IR}$ (BL-31259) (**Fig. 3.4E**) was found to be more robust than *parkin*$^{IR}$ (BL-37509) (**Fig.3.4D**).

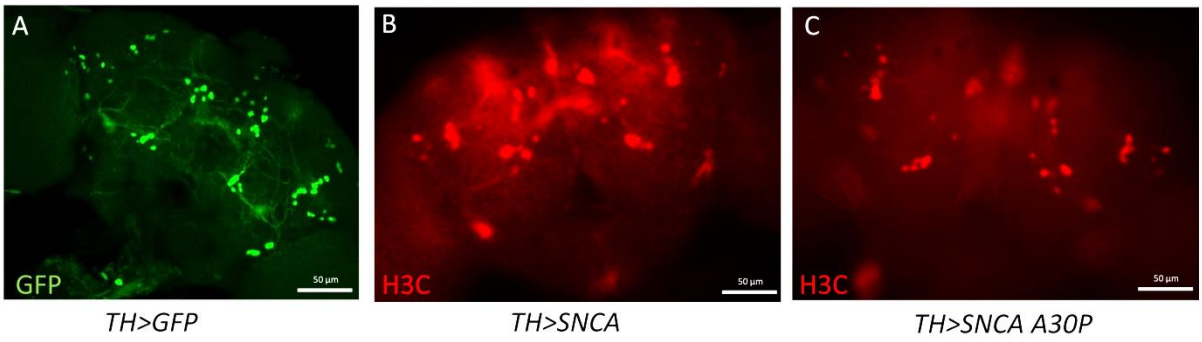



**Figure 3.1. Validation of *UAS-SNCA* transgenes:** Fluorescence micrograph showing GFP (A), *WT-SNCA* (B) and *UAS-SNCA A30P* in dopaminergic neuronal clusters of adult fly brain.

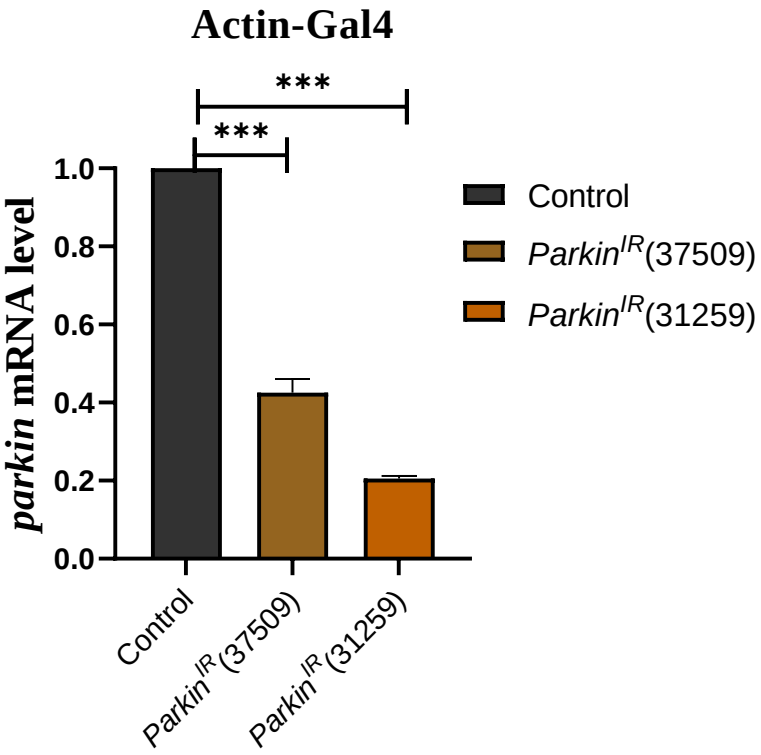
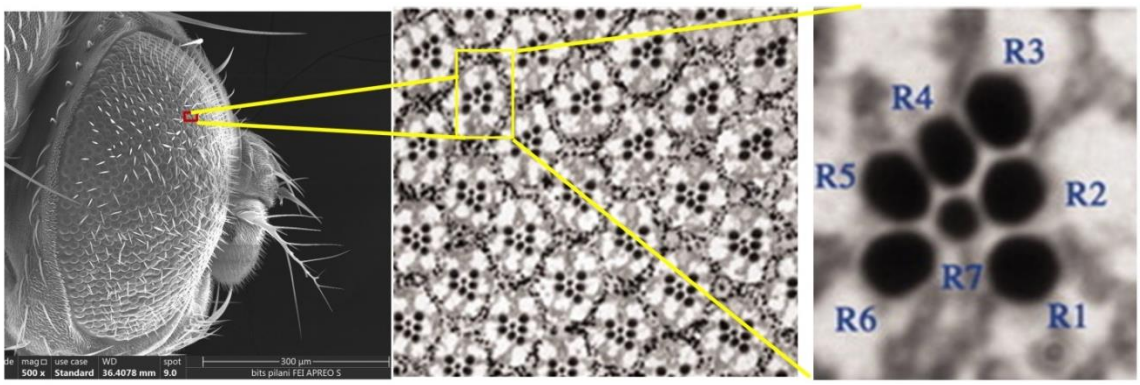



**Figure 3.2. Validation of *UAS-parkin$^{IR}$* transgenes:** Real-time PCR showing decreased mRNA of *parkin*. . One-way Analysis of variance and Tukey's multiple comparisons post-hoc test indicate the following statistically significant differences: * (<.05), ** (.001), *** (<.0001) and ns-not significant, n=3.




Kumar, J.P. (2012), Building an ommatidium one cell at a time. Dev. Dyn., 241: 136-149

**Figure 3.3. Morphology of an adult *Drosophila* eye.**

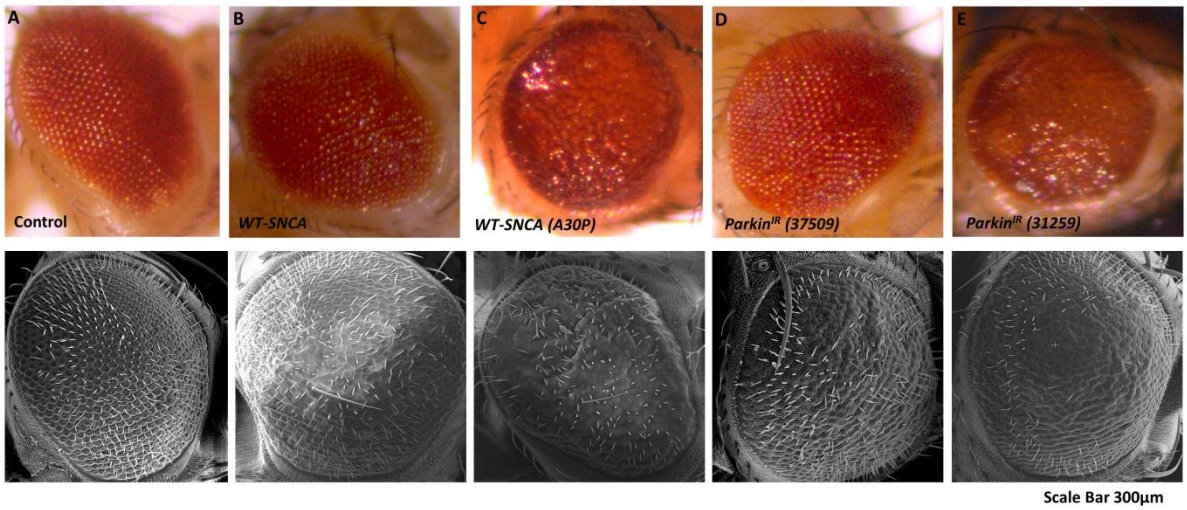
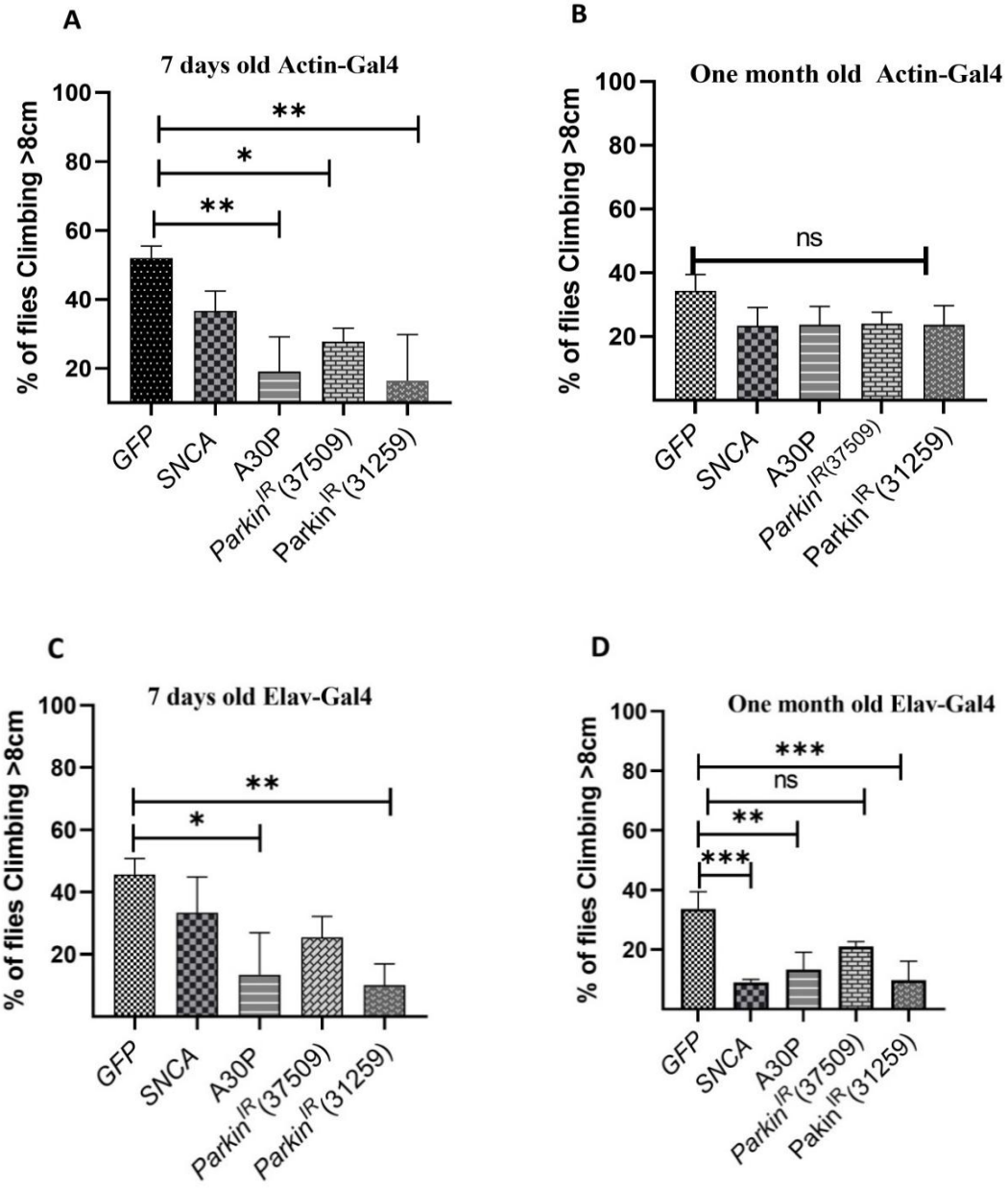



**Figure 3.4. Overexpression of *SNCA* and downregulation of *parkin* cause rough eye phenotype.** Upper panel is showing bright field and lower panel showing Scanning Electron microscopic (SEM) images. Control flies showing normal eye morphology (A). *WT-SNCA* & *A30P* showing rough eye phenotype (B) & (C). *Parkin* downregulation showing rough eye phenotype (D) & (E).

### 3.3.2 *SNCA* overexpression (*WT* and *A30P*) and *parkin* downregulation cause locomotor defects

Since, locomotor dysfunction is considered as one of the major hallmarks of PD; we assessed it through climbing assays. We have overexpressed *WT* and *A30P SNCA* and downregulated *parkin* ubiquitously and pan-neuronally and specifically in dopaminergic neurons using *Actin-Gal4, ELAV-Gal4* and *TH-Gal4* driver lines respectively. We preformed climbing assays at 7-day and one-month time points. We have found that, ubiquitous overexpression *WT* & *A30P SNCA*, and downregulation of *parkin* have shown significantly reduced locomotor ability at 7-

day time point (**Fig.3.5A**). This phenotype was further enhanced at one-month time point (**Fig.3.5B**) as compared to control flies. Overexpression of *WT* & *A30P SNCA* and downregulation of *parkin* pan-neuronally have also shown significantly reduced locomotor ability at 7-day time point (**Fig 3.5C**) and further enhanced at one-month time point (**Fig 3.5D**) as compared to control flies. Also, overexpression of *WT* & *A30P SNCA* and downregulation of *parkin* in dopaminergic neurons have shown significantly reduced locomotor ability at 7-day time point (**Fig 3.5D**) and further enhanced at one-month time point (**Fig 3.5E**) as compared to control flies. On the whole, *SNCA* overexpression (*WT* and *A30P*) and *parkin* downregulation show locomotor dysfunctions with the age in flies.



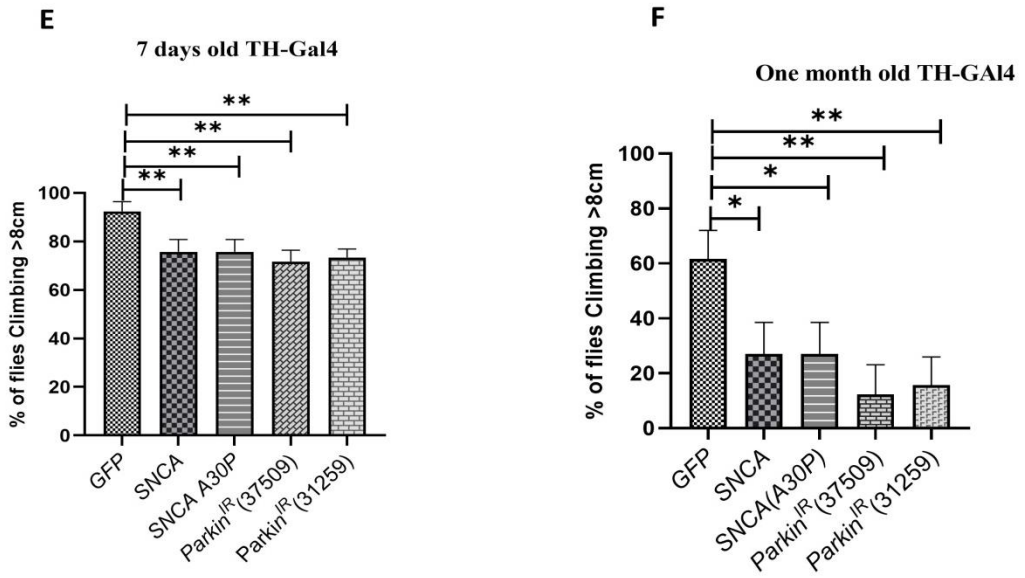



**Figure 3.5 *SNCA* (*WT & A30P*) and *parkin* downregulation affect locomotor ability.** Climbing activity of 7-day-old (A) and one-month-old (B) flies ubiquitously expressing *SNCA* and *parkin* downregulation. Climbing activity of 7-day-old (C) and one-month-old (D) flies pan-neuronally expressing *SNCA* and *parkin* downregulation. Climbing activity of 7-day-old (E) and one-month-old (F) flies expressing *SNCA* and *parkin* downregulation in dopaminergic neurons. One-way Analysis of variance and Tukey's multiple comparisons post-hoc test indicate the following statistically significant differences: * (<.05), ** (<.001) and ns-not significant, n=3.

### 3.3.3 *SNCA* overexpression (*WT* and *A30P*) and *parkin* downregulation cause reduced life span

PD patients have a reduced life span after diagnosis. Mainly, life span has been found to be more reduced in early-onset (at the age of 50 years) as compared to late-onset (at the age of 70 years) PD patients (216, 217). Thus, we have also assessed the viability of the flies till one month. We have found that both *SNCA* overexpression (*WT* & *A30P*) and *parkin* downregulation ubiquitously (**Fig 3.6A**) as well as pan-neuronally (**Fig 3.6B**), have reduced life span of flies as compared to the control flies.

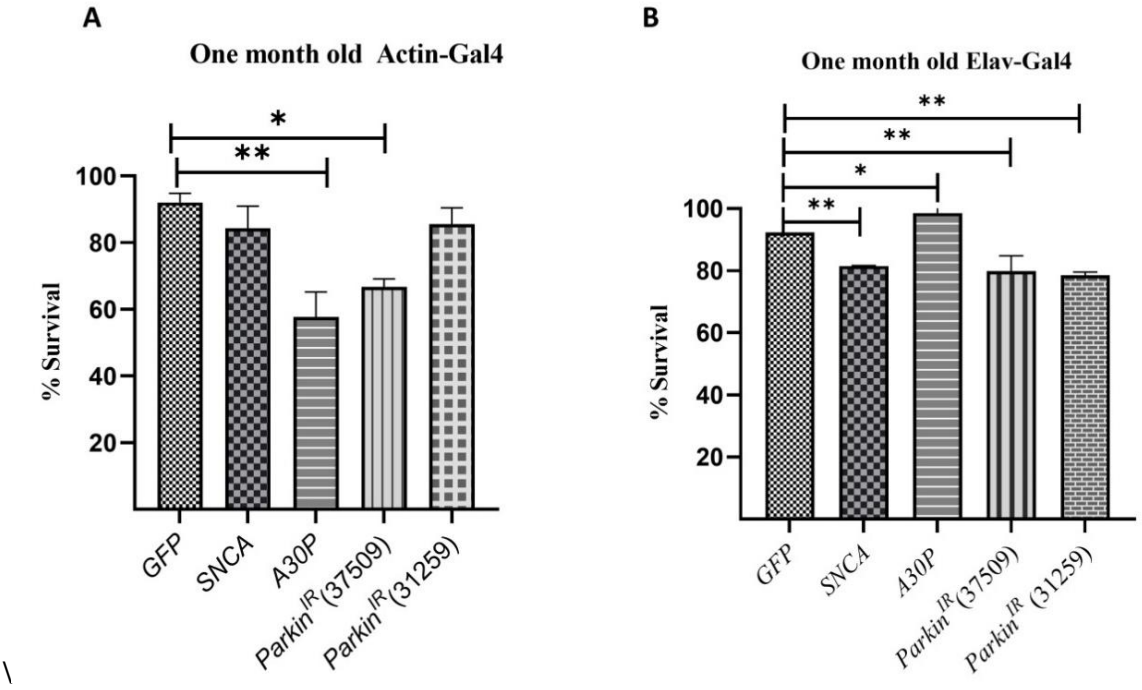



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**Figure 3.6**. **Survival of flies is affected by overexpression of *SNCA* (*WT & A30P*) and downregulation *parkin*** ubiquitously (4A) and pan-neuronally (4B). One-way Analysis of variance and Tukey's multiple comparisons post-hoc test indicate the following statistically significant differences: * (<.05), ** (<.001), *** (<.0001) and ns- not significant. n=3

### 3.4. Discussion

PD, or shaking palsy, results in loss of dopaminergic neurons and causes locomotor defects in the patients. At present, a variety of PD models have been developed to study PD pathogenesis and for therapeutic development, from unicellular eukaryotes to nonhuman primates. Nevertheless, each model comes with its unique strengths and weaknesses, which should be considered when selecting a model system to address a particular problem. *Drosophila* model is employed to examine the distinct phase of biological processes that are linked to the development of the disease. It is known that the likelihood of developing PD significantly rises with advancing age. In this context, the model system that could be most intriguing is *Drosophila*. The limited lifespan of *Drosophila* allows for the examination of the impact of aging on the progression of neurodegenerative processes, a task that proves challenging in models with extended lifespans. Despite the fact of that *Drosophila* lack *α-synuclein* homologue, among the other diseased models, *Drosophila* has been shown to recapitulate the major PD phenotypes including age-progressive neurodegeneration, locomotor dysfunction and Lewy bodies formation (14,192,212). Thus, we have also employed *Drosophila* model to study the underlying molecular mechanism in PD-progression. *α-synuclein* and *parkin*, alteration of both of these genes have been shown to cause PD pathology in *Drosophila* model (155). We have also altered both of these genes in different tissues and observed major PD phenotypes. α-Synuclein *(WT* and *A30P SNCA*) overexpression and *parkin* downregulation in adult eyes have shown neurodegeneration phenotype. Overexpression of both *WT* and *A30P* mutated forms of *SNCA*, and downregulation of *parkin* ubiquitously, all over the neurons and specifically in dopaminergic neurons, have resulted in impaired locomotor function. This effect is observed ubiquitously and across all the neurons and specifically in dopaminergic neurons. *SNCA* (WT and A30P) overexpression and *parkin* downregulation ubiquitously and across all the neurons, have also shown a notable decrease in lifespan. These results align with existing literature (213, 214, 215, 216, 217, 218).

Collectively, neurodegeneration, locomotor dysfunctions and reduced life span in *Drosophila* model due to *SNCA* overexpression and *parkin* downregulation, provide an experimentally convenient platform for exploring the underlying mechanisms of neurodegeneration.

# Chapter-4
# Analysis of α-Syn and Parkin interaction in dopaminergic neurons of Drosophila

## 4.1 Introduction

Next, we wanted to understand genetic and molecular interaction between *SNCA* (α-synuclein) and *parkin* specifically in dopaminergic neurons. Both, *SNCA* and *parkin* are the two major genes involved in both sporadic as well as genetic form of PD (189). *SNCA* encodes an α-synuclein protein and mutations in *SNCA* are associated with autosomal-dominant form of PD (11, 54, 49). Different *in-vitro* and *in-vivo* studies have shown that misfolded α-synuclein aggregation causes neurotoxicity through influencing the neurotransmission, synaptic vesicle exocytosis, recycling as well as endocytosis in the substantia nigra region. Migration of α-synuclein between neurons in prion-like manner to propagate formation of Lewy bodies throughout the substantia nigra have also been suggested (37, 215, 216). *Parkin* encodes an E3 ubiquitin ligase, and is the second most common cause of autosomal recessive early-onset PD (55). *Parkin* loss-of-function causes neurodegeneration with or without forming Lewy bodies in PD patients (99, 97, 102). Studies have reported that overexpression of *parkin* results in reduced neurotoxicity caused by α-synuclein in different models (125, 124, 217). Studies have also reported that *parkin* mutation results in no aggregation of α-synuclein in mice (197, 218). However, very limited *in-vivo* studies have been done to explore the link between *SNCA* and *parkin*.

At cellular level, mitochondria dysfunction has been considered as a major hallmark of PD (195). Studies in different model systems have shown that α-synuclein causes mitochondria fragmentation, membrane potential, complex I deficits and reduced ATP production. In *Drosophila*, elongated as well as fragmented mitochondria have been reported due to *α-synuclein* overexpression (141, 207, 220). Loss-of-function mutation in *parkin* has also shown the mitochondrial pathology demonstrated as mitochondrial elongation, swelling, and cristae disruption in *in-vitro* and *in-vivo* models (151, 146, 182, 147, 221). Although, fused and fragmented mitochondria have also been reported in DA neurons of *parkin* mutant *Drosophila* (198). Moreover, it has been found that mitochondrial fragmentation caused by α-synuclein overexpression can be rescued by co-expression of parkin, PINK1, or DJ-1, indicating that α-synuclein and parkin may function in the same pathway (223, 224). However, limited *in-vivo* studies have been done to test the effect of interaction of *SNCA* and *parkin* together on mitochondrial morphology in Parkinson's disease.

In this report, we have tested the interaction of *parkin* and *SNCA* and their effect on mitochondria in DA neurons using humanized *Drosophila melanogaster* model of PD. *Drosophila* adult fly brain contains approximately 100 DA neurons, which are grouped into

different clusters according to their anatomical position: PAL (paired anterior lateral), PAM (paired anterior medial), PPM1/2 and PPM3 (paired posterior medial), and PPL1 and PPL2 (paired posterior lateral) (225, 226). It has been suggested that each cluster-specific DA neuron project to distinct functional areas of the brain (203), although the function of each of the DA clusters is not completely explored. PPL1 and PPM3 are more explored clusters in case of PD using animal models because these are functionally homologous to mammalian substantia nigra pars compacta region (204). It has been shown that PPL1 is associated largely with memory formation (205) whereas, PPM3 is reported to be a centre for the control of locomotor behaviour (206). In PD models of *Drosophila*, different clusters have been reported to get affected in a context dependent manner.

In this study, we found DA neuronal loss upon genetic alterations is cluster specific, exhibiting altered mitochondrial morphology. Our studies provide leads for cellular and molecular interactions on DA neuronal degeneration during onset and progression of PD.

## 4.2 Material and Methods

### 4.2.1 Fly strains

All fly stocks, genetic crosses and F1 progenies were maintained on standard fly food containing agar, maize powder, yeast, and sugar at 25 °C. GAL4/UAS system was used to obtain desired genotype for the experiments (207)(208). Transgenic *Drosophila* lines: *UAS-GFP* (a kind gift from Prof. S.C Lakhotia's Lab), *UAS-Mito-HA-GFP.AP* (BL-8442), *UAS-Hsap\SNCA.F* (BL-8146), *UAS-SNCA.J}1/CyO* (BL-51375), *UAS-Park*$^{RNAi}$ (BL-37509) were used. Driver Gal4 line *TH-Gal4* or *ple-GAL4* (BL-8848) was used to overexpress or downregulate the responder lines of genes in DA neurons.

### 4.2.2 Climbing assays

To determine locomotor activity, climbing assays were performed (209). Ten flies per genotype were transferred into cylindrical glass tube after anesthetization and left for 5-10 min for the revival and acclimatisation at room temperature. Tubes were marked up to 8cm above the bottom of the vial. After acclimatization, flies were gently tapped down to the bottom of vial and the number of flies crossed the 8cm mark were recorded after 10 sec. Three trials were performed, and numbers were then averaged, and the resulting mean was used as the overall value for each single group of flies. For all genotypes 3 replicates were carried out.

### 4.2.3 Quantitative real-time PCR

Total RNA was extracted from 25-30 fly heads using the TRIzol method (Invitrogen) referred from Jove protocol (Jensen, K. et al; Purification of Transcripts and Metabolites from *Drosophila* Heads. *J. Vis. Exp.* (73), e50245, doi: 10.3791/50245 (2013). RNA concentrations were measured with a Nanodrop ND-1000 Spectrophotometer and equal amounts of RNA were reverse transcribed using Verso cDNA Synthesis Kit (AB1453A). qPCR was performed using 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne*). RP49* was used as the internal control gene. Primer sequences were as follows:
*RP49* Forward: 5'-CCAAGGACTTCATCCGCCACC-3'
Reverse: 5'- GCGGGTGCGCTTGTTCGATCC-3'
*Parkin* Forward: 5'-ATTTGCCGGTAAGGAACTAAG C-3'
Reverse: 5'-AAGTGGCCGACTGGATTTTCT-3'

### 4.2.4 Adult Brain Immunohistochemistry

Brain preparation for confocal microscopy imaging was done as described in Tito et al., 2016. Briefly, adult brains of desired genotype were dissected in cold 1x PBS and incubated with fixative solution (4% formaldehyde in 1xPBST (0.1%TritonX-100) for 20 min at room temperature. After three washes with 1xPBST for 10 min each wash, blocking was done using 1% BSA for 1hr at room temperature. Brains were probed with rabbit anti-TH (#AB152) at 1:1000, overnight (12-16) at 4 °C. Following three washes for 10mins, brains were incubated with Goat anti-Rabbit, Alexa-Fluor Plus 555 (#A32732) (1:4000**)** secondary antibodies at room temperature for 2hr. Brains were washed three times for 15 min, then they were mounted between two glass coverslips by using antifade medium on microscope slides. Confocal microscope was used to acquire z-stacks at 1µm intervals with 20×/N.A.0.60 Plan-Apochromat objective. The number of TH-positive neurons was counted manually within each cluster of posterior regions of the brains.

### 4.2.5 Immunoblotting

Western blot was performed as previously described (211) with some modifications. F1 progenies of desired genotype were collected in 1.5ml eppendorf tubes and snap-frozen in liquid nitrogen. *Drosophila* heads (~30-50) were decapitated and homogenized in 100ul 1x RIPA buffer (Merck, #20-188) containing 1% protease inhibitor (Sigma, P8340) using sterilized pestle. The homogenates were centrifuged at 10,000 rpm at 4° C for 10min. The supernatant was collected and assayed for protein concentration. The protein (80µg) was resolved on 12% SDA-PAGE and then transferred to 0.2µm nitrocellulose membrane (Bio Rad, #1620112). After blocking the membrane with 3% BSA in TBS-T (0.05%Tween-20), membrane was incubated overnight at 4° C with primary antibodies. The primary antibodies used were rabbit anti-Drosophila Parkin (Merck, SAB1300355, 1:500), mouse E7 anti-beta tubulin (DSHB-S1-810, 1:200). Following three washes with TBS-T, membrane was incubated with an appropriate HRP-conjugated secondary antibodies: goat anti-mouse (Thermo Scientific # 31430, 1:1000), mouse anti-Rabbit (GenScript, #A01856, 1:1000) for 2-hr at room temperature and signal was detected using ECL substrate (Bio Rad #1705061). Image analysis and quantification was done using ImageJ software. Western blot was done on the same membranes after stripping between each application of the antibody.

### 4.2.6 Mitochondrial morphology measurement

To assess the mitochondrial morphology in DA neurons, *UAS-MitoGFP* fly strain was used to tag the mitochondria and DA neurons were stained using anti TH antibody. Z-stack of one PPL1 and one PPM3 DA neuronal cluster per brain was imaged using confocal microscope at 63×/N.A.1.30 oil with 1.5 zoom. A total of 3-4 brains per genotype were scanned. We used publicly available ImageJ Mito-Morphology Macro created by (212), to quantify the mitochondria. Average area and circularity were calculated representing elongation and fragmentation of mitochondria respectively.

### 4.2.7 Mitochondrial morphology measurement

To assess the mitochondrial morphology in dopaminergic neurons, UAS-Mito-GFP fly strain was used to tag the mitochondria and dopaminergic neurons were stained using anti TH antibody. Z-stack of one PPL1 and one PPM3 DA neuronal cluster per brain was imaged using confocal microscope at 63×/N.A.1.30 oil with 1.5 zoom. A total of 3-4 brains per genotype were scanned. Publically available ImageJ Mito-Morphology Macro created by Dagda et al. (2009) (Dagda, R.K., Cherra III, S. J, Kulich, S.M., Tandon, A, Chu, Park, D., Chu, C.T. Loss of PINK1 function promotes autophagy through effects on fission in neurons. J.Biol Chem. 284(20):13843-55, 2009.) was used to quantify the mitochondria. Average area, circularity and (Area/Perimeter)/minor axis were calculated representing elongation, fragmentation and swelling of mitochondria respectively.

### 4.2.8 Mitochondrial fractionation

Mitochondrial fractionation was done from the heads of desired genotypes using differential centrifugation as described previously (161) with slight modifications. Briefly, ~150 heads of desired genotypes were collected and homogenized in an ice-cold mitochondrial isolation buffer (250 mM sucrose, 10 mM HEPES, 1 mM EDTA and 0.1% fat-free BSA, pH 7.4) using sterilized micro-pestles. Lysate was then centrifuged at 1000g for 5 min at 4 °C. The supernatant was collected and centrifuged at 10,000g for 10 min at 4 °C. The pellets were then resuspended and washed thrice in mitochondrial washing buffer (250 mM sucrose, 10 mM HEPES, and 0.1 mM EDTA, pH 7.4) at 10,000g for 10 min at 4 °C. Finally, the pellet was resuspended in 80µl of 0.1% fat-free BSA suspension buffer with 250 mM sucrose and 10 mM HEPES, pH 7.4.

### 4.2.9 ROS (reactive Oxygen Species) quantification

DCF-DA (2,7-dichlorofluorescein diacetate) assay was used to quantify the ROS as previously described (162) with some modifications. 20 flies of desired genotypes were homogenized in 20mM Tris buffer, pH 7.0. The homogenate was centrifuged at 1600 ×g for 10 minutes at 4°C, and the supernatant was collected for quantification of 2,7-dichlorofluorescein fluorescence. Aliquot of 5µl of supernatant was incubated with 5µM DCFDA at 37 ˚C for 60 min. The fluorescence was monitored at 488nm/530nm excitation/emission using Fluoroskan Ascent.

### 4.2.10 Malondialdehyde (MDA) Quantification

Thiobarbituric acid reactive substances (TBARS) assay was used to assess the oxidative stress by quantifying the levels of malondialdehyde (MDA), a stable by-product of lipid peroxidation as previously described (163) with some modifications. 30 flies of desired genotypes were homogenized in 1x RIPA buffer (Merck, #20-188) containing 1% protease inhibitor (Sigma, P8340) using sterilized pestle. The homogenates were centrifuged at 10,000 rpm at 4˚ C for 10min. The supernatant was collected and assayed for protein concentration. Aliquots of supernatant were adjusted to have an equal amount of protein (1 mg/ml) and then 250 µl of 10% trichloroacetic acid (TCA) added. Subsequently, 375 µl of thiobarbituric acid (TBA) added at a concentration of 1% (w/v) under acidic conditions. The resulting solution was kept at 100 ˚C for 15 min, which facilitated the formation of a pink-colored precipitate. The absorbance of this precipitate was measured spectrophotometrically at 530 nm (Multiskan FC, Thermo Scientific, DE). The obtained values were expressed as micromolar concentration (µM) of malondialdehyde per 5 milligram (mg) of protein.

### 4.2.10 Statistical analysis

GraphPad Prism 8.0.1 was used for statistical analysis and graphical display of the data. Significance is expressed as *p* values which were determined with one-way ANOVA and Two-way ANOVA followed by Tukey's multiple comparison tests as indicated in the figure legends.

## 4.3 Results

### 4.3.1 *SNCA* overexpression and *parkin d*ownregulation together exhibit locomotor dysfunctions

The α-synuclein induced neurotoxicity (i.e. survival, locomotor defect, and DA neuronal death) is restored by *parkin* in *in-vitro* and *in-vivo* models (213,124, 212,217). Hence, to test whether parkin is involved in α-synuclein mediated PD condition, we created double transgene with RNAi of *parkin* (*parkin*$^{IR}$ here onward) and *UAS-SNCA* and expressed in DA neurons using *TH-Gal4*. Using H3C (DSHB-S1-890) antibody we confirmed the expression of α-synuclein in DA neurons of *UAS-SNCA* (**Fig 4.1A**) and double transgene through imaging via fluorescence microscopy (Fig 4.1B) and downregulation of *parkin* though real-time PCR (**Fig 4.5A and 4.5B, 7-day and 21-day respectively**). We have found that *UAS-SNCA*, *UAS-parkin*$^{IR}$ and *UAS-parkin*$^{IR}$; *UAS-SNCA* expressing flies shown reduced life span as compared to control (**Fig 4.2**).

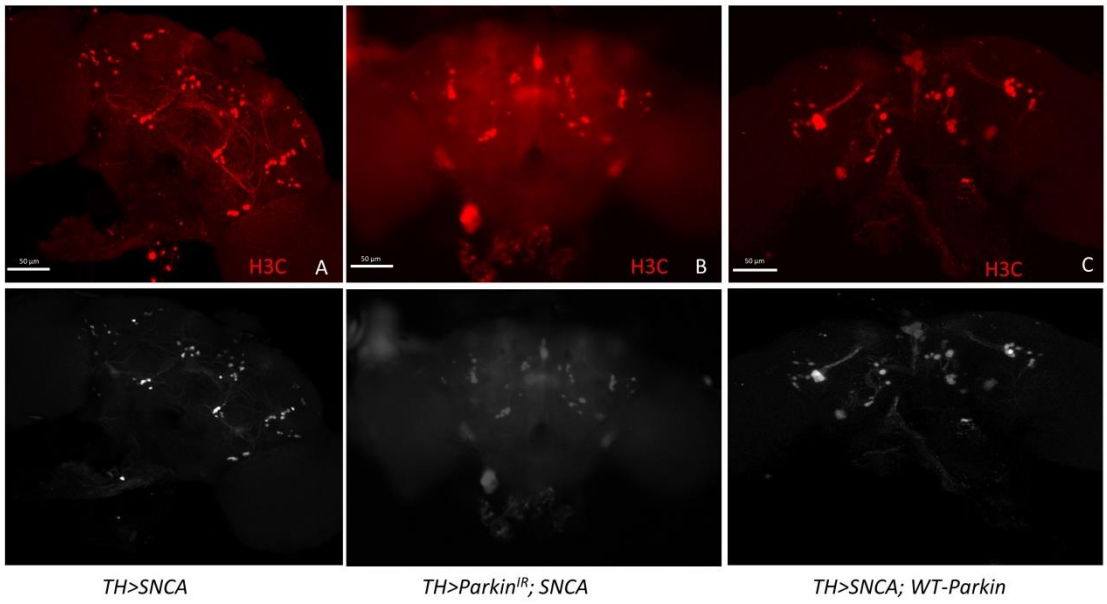



**Figure 4.1. Immunohistochemistry showing α-synuclein expression in DA neurons** of (A) *SNCA* transgene, (B) Double transgene: *parkin* downregulation with *SNCA* (*Parkin*$^{IR}$*; SNCA*) and (C) *wild type-parkin* with *SNCA* (*UAS-SNCA; UAS-parkin*).

However, this reduced life span of *UAS-parkin*$^{IR}$; *UAS-SNCA* expressing flies was at lesser extent with respect to *SNCA* overexpression and *parkin* downregulation alone in age dependent manner. Further, we observed that *UAS-parkin*$^{IR}$; *UAS-SNCA* expressing flies displayed loss of

climbing ability as compared to control (**Fig 4.3**); however, it was also at lesser extent with respect to *parkin* downregulation alone in age dependent manner. Though, both *SNCA* overexpression and *parkin* downregulation independently, displayed loss of climbing ability with the age (3-21 days) as compared to control (*TH>GFP*) (**Fig 4.3**), supporting the existing studies (179,212,215). We also confirmed phenotype of *parkin* downregulation by overexpressing *wild type-parkin* with *SNCA* (*UAS-SNCA; UAS-parkin*). Notably, wild-type *parkin* overexpression with *SNCA* was able to restore the life span (**Fig 4.2**) and climbing ability (**Fig 4.3**). We also confirmed the overexpression of α-synuclein using H3C antibody in *UAS-SNCA; UAS-parkin* transgene through fluorescence microscopy (**Fig 4.1C**). These observations suggest that *SNCA* and *parkin* alteration together do not worsen the locomotor defects.

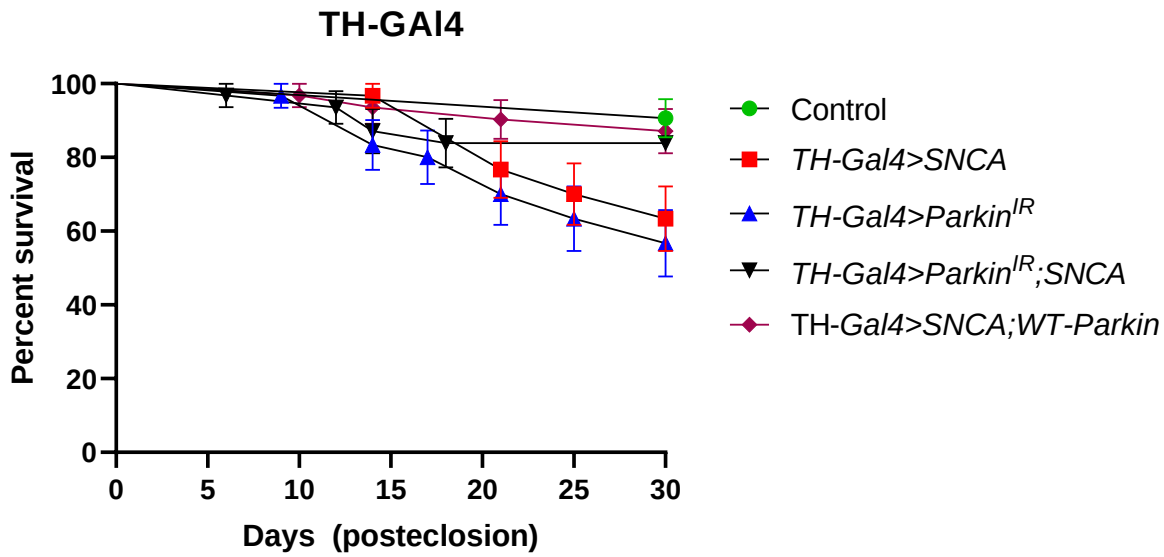



**Figure 4.2. *SNCA overexpression* and *parkin* downregulation (*parkin*$^{IR}$) independently, and together (*parkin*$^{IR}$*; SNCA*) in dopaminergic neurons (DA), exhibit reduced life span**. Survival Curves (Kaplan-Meier) using Gehan-Breslow-Wilcoxon test.

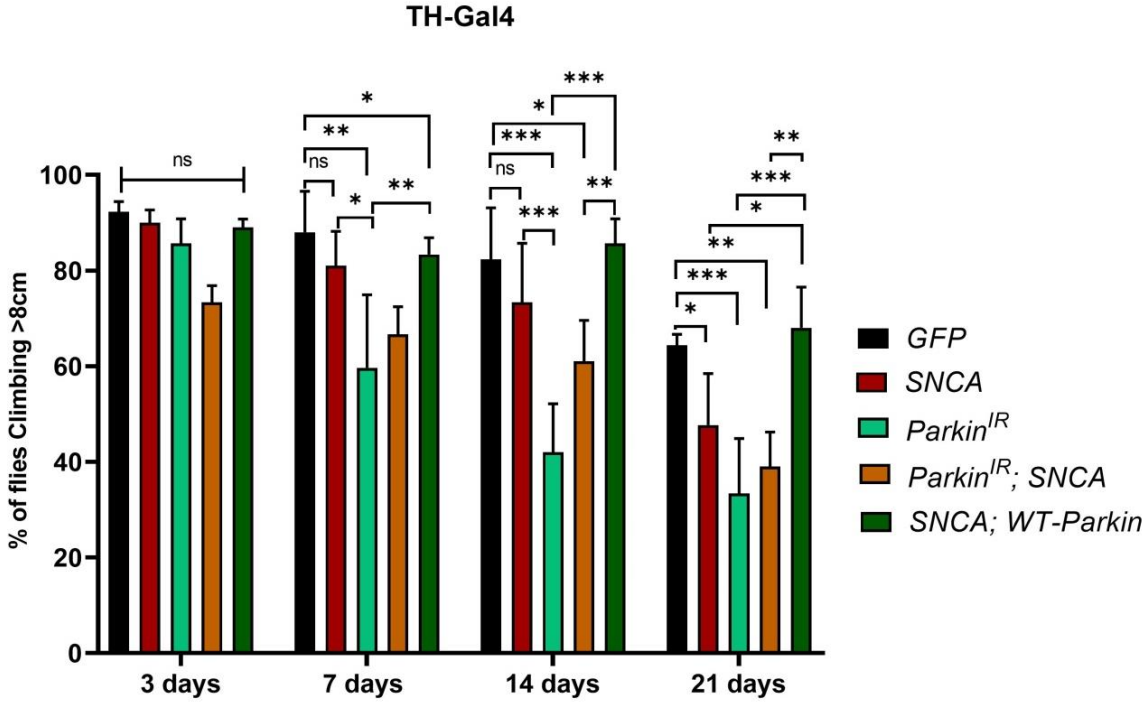



**Figure 4.3. *SNCA overexpression* and *parkin* downregulation (*parkin*$^{IR}$) independently, and together (*parkin*$^{IR}$*; SNCA*) in dopaminergic neurons (DA), exhibit locomotor dysfunctions:** Climbing assay indicates loss of locomotor function with age in flies. A total of 30 (N=30) flies were used per genotype and 10 flies (n=10) were used for the climbing assay. Data is represented as mean with SEM. Statistical analysis were performed using Two-way ANOVA followed by Tukey's multiple comparison test. P value: *(<.05), ** (<.001), *** (<0.0001), ns-not significant.

### 4.3.2 *SNCA* and *parkin* alterations cause dopaminergic neurodegeneration

In PD, cardinal motor symptoms are caused by death of DA neurons in the substantia nigra pars compacta (SNpc). Thus, we examined the DA neuronal clusters in adult fly brain using antibody against tyrosine hydroxylase to investigate whether locomotor defects observed in *SNCA* and *parkin* alteration are due to loss of DA neurons. We have explored posterior protocerebrum DA neuronal clusters: PPM1/2, PPM3, PPL1 and PPL2 (**Fig 4.4A**). To visualize the DA neurons in adult brain, we stained the DA neurons of the *TH-Gal4* driving *GFP* flies with TH antibody (**Fig 4.4B**), hence confirming the activity of *TH-Gal4>UAS-GFP* and TH antibody. In our study, only TH-positive neurons of posterior brain were monitored for DA neuron quantification. We have found substantial reduction in specific DA

neuronal clusters in *UAS-SNCA*, *UAS-parkin$^{IR}$ and UAS-parkin$^{IR}$*; *UAS-SNCA* transgene adult brain with respect to age matched controls (**Fig 4.4**). In 7-day-old adult fly brains, quantification of PPL1, PPM1&2, PPM3 and PPL2 DA neuron clusters, *SNCA* and *parkin$^{IR}$* showed significant reduction in number of DA neurons only in PPL1 and PPM1&2 clusters (**Fig 4.4C-E' & 4.4G**). *UAS-parkin$^{IR}$*; *UAS-SNCA* also showed significant reduction in number of DA neurons in PPL1 and PPM1&2 as compared to control (**Fig 4.4F-F' & 4.4G**). However, number of DA neuronal loss in *UAS-parkin$^{IR}$*; *UAS-SNCA* adult fly brains were less pronounced as compared to *SNCA* and *parkin$^{IR}$* alone (**Fig 4.4D-F' & 4.4G**). In 21-day-old fly brains, DA neurons numbers were further decreased in PPL1 and PPM1 & 2 cluster in similar manner to 7-day-old adult fly brains of *SNCA*, *parkin$^{IR}$* and in *UAS-parkin$^{IR}$*; *UAS-SNCA* (**Fig 4.4H-4K' & 4.4L**). In PPM3 DA neurons clusters, we have observed that numbers of DA neurons were reduced only in *parkin$^{IR}$* adult fly brain (**Fig 4.4G & 4.4L**). In PPL2 DA cluster, we have observed no change in number of DA neurons in *UAS-SNCA*, *UAS-parkin$^{IR}$* and *UAS-parkin$^{IR}$*; *UAS-SNCA* of 7-day-old and 21-day-old adult fly brains. These observations suggest that *SNCA* and *parkin* cause DA clusters specific neuronal loss in posteriors region of adult fly brain.

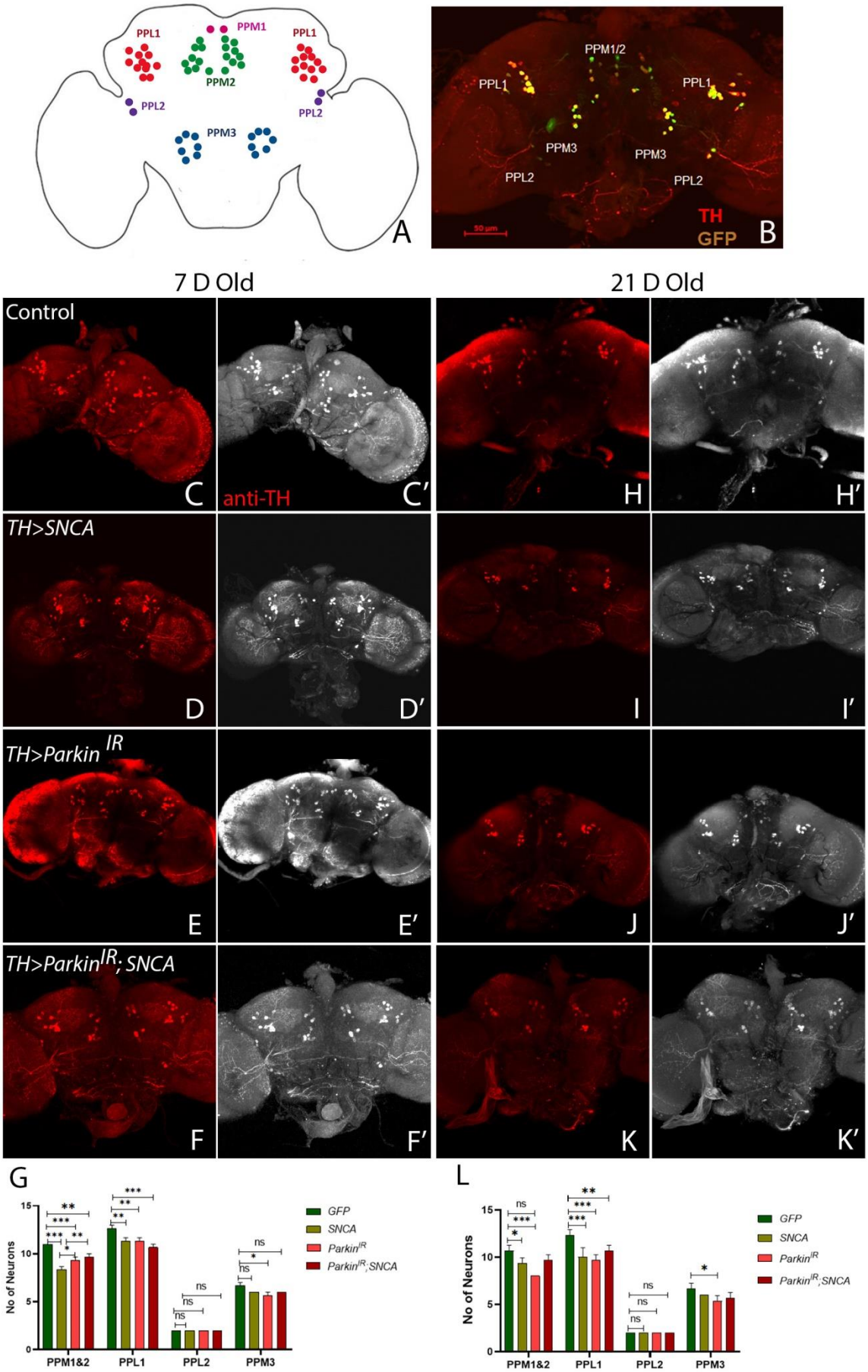

PPL1
PPM1
PPL1
PPM2
PPL2
PPL2
PPM3
A
PPM1/2
PPL1
PPL1
PPM3
PPM3
PPL2
PPL2
TH
GFP
B
7 D Old
21 D Old
Control
C
anti-TH
C′
H
H′
TH>SNCA
D
D′
I
I′
TH>Parkin IR
E
E′
J
J′
TH>ParkinIR; SNCA
F
F′
K
K′
G
No of Neurons
PPM1&2
PPL1
PPL2
PPM3
GFP
SNCA
ParkinIR
ParkinIR;SNCA
L
No of Neurons
PPM1&2
PPL1
PPL2
PPM3
GFP
SNCA
ParkinIR
ParkinIR;SNCA

**Figure 4.4. *SNCA* and *parkin*$^{IR}$ expression independently and together (*parkin*$^{IR}$*; SNCA*) cause DA cluster specific neuronal loss** (A) Schematic representation of DA neuronal clusters (PPL1, PPM1&2, PPM3, PPL2) in posterior region of adult brain. (B) Representative confocal Maximum Intensity Projection (MIP) of WT adult brain stained with GFP (green) and Tyrosine Hydroxylase (TH) (Red) to reveal DA neurons in posterior region. Adult brain of desired genotype were dissected and stained for TH. (C-G) 7-day old adult fly brains show TH stain. (C, C') Control flies, (D, D') *SNCA* overexpression, (E, E') *Parkin*$^{IR}$ expression, (F, F') *Parkin*$^{IR}$*; SNCA* expression, show the TH-stain which is (G) quantified. (H-L) 21-day old adult fly brains flies showing TH stain. (H, H') Control flies, (I, I') *SNCA* overexpression, (J, J') *Parkin*$^{IR}$ expression, (K, K') *Parkin*$^{IR}$*; SNCA* expression show the TH-stain and is (L) quantified. Scale bar 50µm. A total of four adult brains were used (n=4) per genotype. Data is represented as mean with SEM. Statistical analysis were performed using Two-way ANOVA followed by Tukey's multiple comparison test. P value: *(<.05), ** (<.001), *** (<.0001), ns-not significant.

### 4.3.3 *SNCA* affects the expression of *parkin* at transcriptional level but not at translational level in DA neurons

Several *in-vitro* and *in-vivo* studies have shown the reduced neurotoxicity caused by *SNCA* upon *parkin* overexpression suggesting that *parkin* plays a vital role in molecular pathway of PD pathogenesis. It has been reported that mutations in *SNCA* and *parkin* affect DA neuronal loss as well as formation of Lewy bodies (145). However, effect of wild-type *SNCA* on *parkin* is not much explored. Therefore, we tested *parkin* mRNA and protein level of flies with *UAS-parkin*$^{IR}$; *UAS-SNCA, SNCA* overexpression and *parkin* downregulation independently. We found decreased *parkin* mRNA level in *UAS-parkin*$^{IR}$; *UAS-SNCA* flies as compared to control flies; however this reduction in transcript was at a lesser extent compared to *parkin downregulation* and *SNCA* overexpression alone in 7-day adult fly brains (**Fig 4.5A**). Similarly, at 21-day time point, adult fly brains with *UAS-parkin*$^{IR}$; *UAS-SNCA* did not show further decrease in *parkin* mRNA (**Fig 4.5B**). We found this intriguing, since Wilkaniec et al., 2019 have reported the decreased parkin protein level upon α-synuclein oligomerization which induces cell death in *in-vitro* model. In contrast, in our study we have found no significant change in parkin protein level upon *SNCA* overexpression in 7 (**Fig 4.5C and 4.5E**) and 21 days old (**Fig 4.5D and 4.5F**) adult fly brains. However, we observed decreased parkin protein level in flies expressing *UAS-parkin*$^{IR}$; *UAS-SNCA* as compared to control but it was at lesser extent to *parkin* downregulation independently (**Fig 4.5E and 4.5F**). Altogether, these data

suggest that *SNCA* alone has effect on parkin at transcript level. When combined, the effect is not pronounced.

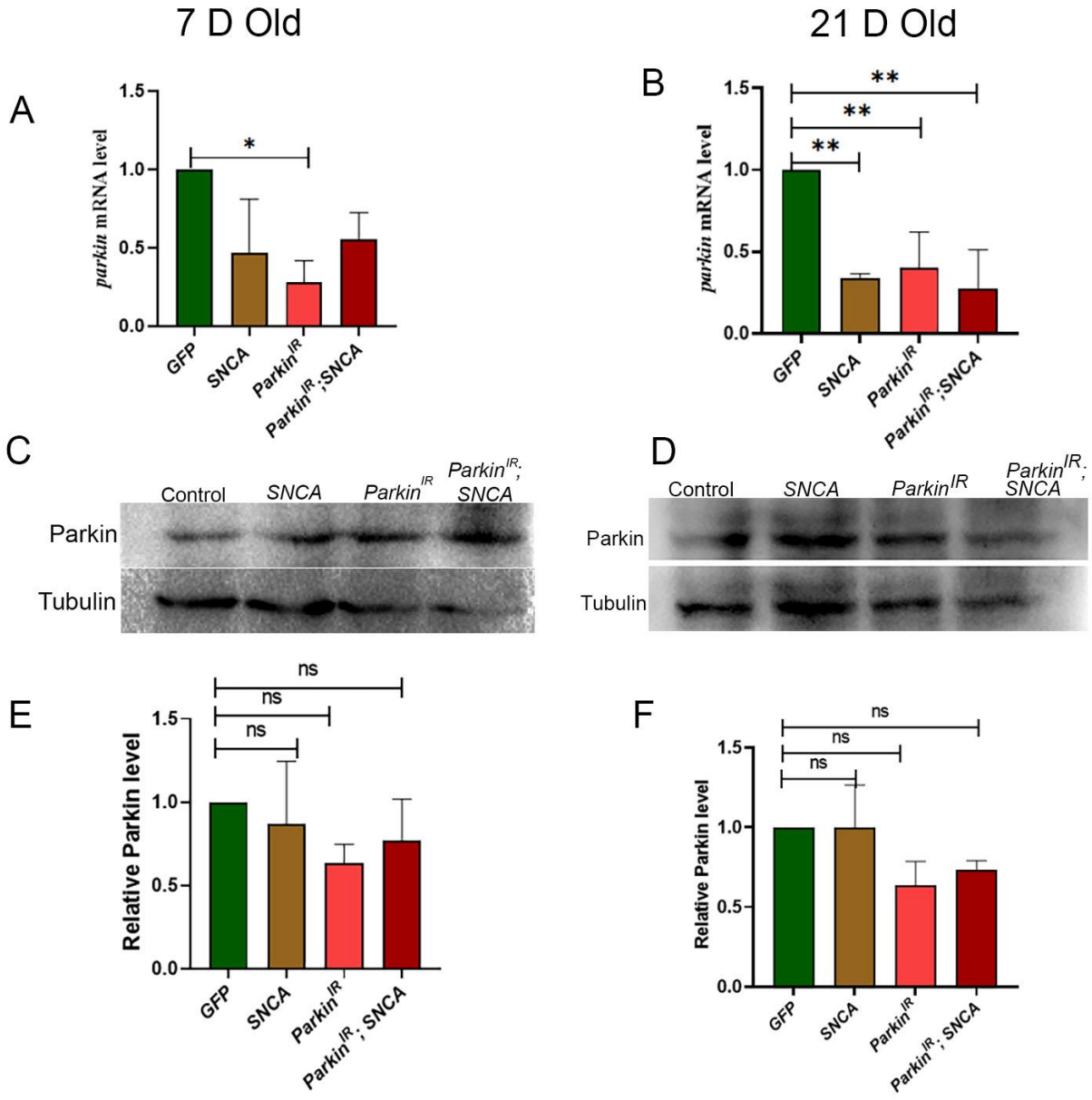



**Figure 4.5. *SNCA* effect the expression of *parkin* at transcriptional level but not translational level.** (A) 7-day old *parkin* mRNA level (B) 21-day old *parkin* mRNA level. (C) Parkin immunoblot and (E) quantification of 7-day-old adult brains normalized to β-tubulin. (D) Parkin immunoblot and (F) quantification of 21-day old adult brains normalized to beta-Tubulin. Data is represented as mean with SEM. Statistical analysis were performed using One-way ANOVA followed by Tukey's multiple comparison test. P value: *(<.05), ** (<.001), ns-not significant.

### 4.3.4 *SNCA* and *parkin* alteration affect mitochondrial morphology independently in PPL1 and PPM3 clusters of adult fly brain

Mitochondria are highly dynamic organelle and maintenance of mitochondrial morphology is essential for survival of the neurons. Therefore, we investigated whether α-synuclein and parkin alteration induced PD phenotypes have any relation with mitochondrial morphology. We have considered only PPL1 and PPM3 DA neuronal clusters for mitochondrial morphology assessment. This is because we have found reduction in number of TH-positive DA neurons in PPL1 clusters of *UAS-parkin*$^{IR}$; *UAS-SNCA* (**Fig 4.6**), *SNCA* overexpression and *parkin* downregulation independently and in PPM3 due to *parkin* downregulation only. We assessed the mitochondrial morphology using *UAS-mitoGFP* in TH-positive neurons. We have found that in PPL1 neuronal clusters, *UAS-parkin*$^{IR}$; *UAS-SNCA* (**Fig 4.6D, D' & 4.6E**) has shown swollen mitochondria as compared to control (*TH>mito-GFP*) (**Fig 4.6A, A'**) in 7-day adult fly brains. Whereas, we observed fragmented mitochondria in 21-day old adult fly brains (**Fig 4.6I, I' & 4.6J**) as compared to control (**Fig 4.6F, F' & 4.6J**). *SNCA* overexpression (**Fig 4.6B, B' & 4.6E**) and *parkin* downregulation (**Fig 4.6C, C'& 4.6E**) independently have shown swollen and/or enlarged mitochondria, which degenerate, in 7-day adult fly brains, as compared to control (**Fig 4.6A, A' & 4.6E**). *SNCA* overexpression (**Fig 4.6G, G' & 4.6J**) and *parkin* downregulation (**Fig 4.6H, H' & 4.6J**) independently have shown further enhanced mitochondrial morphology in 21-day old adult fly brains as compared to control (**Fig 4.6F, F' & 4.6J**).

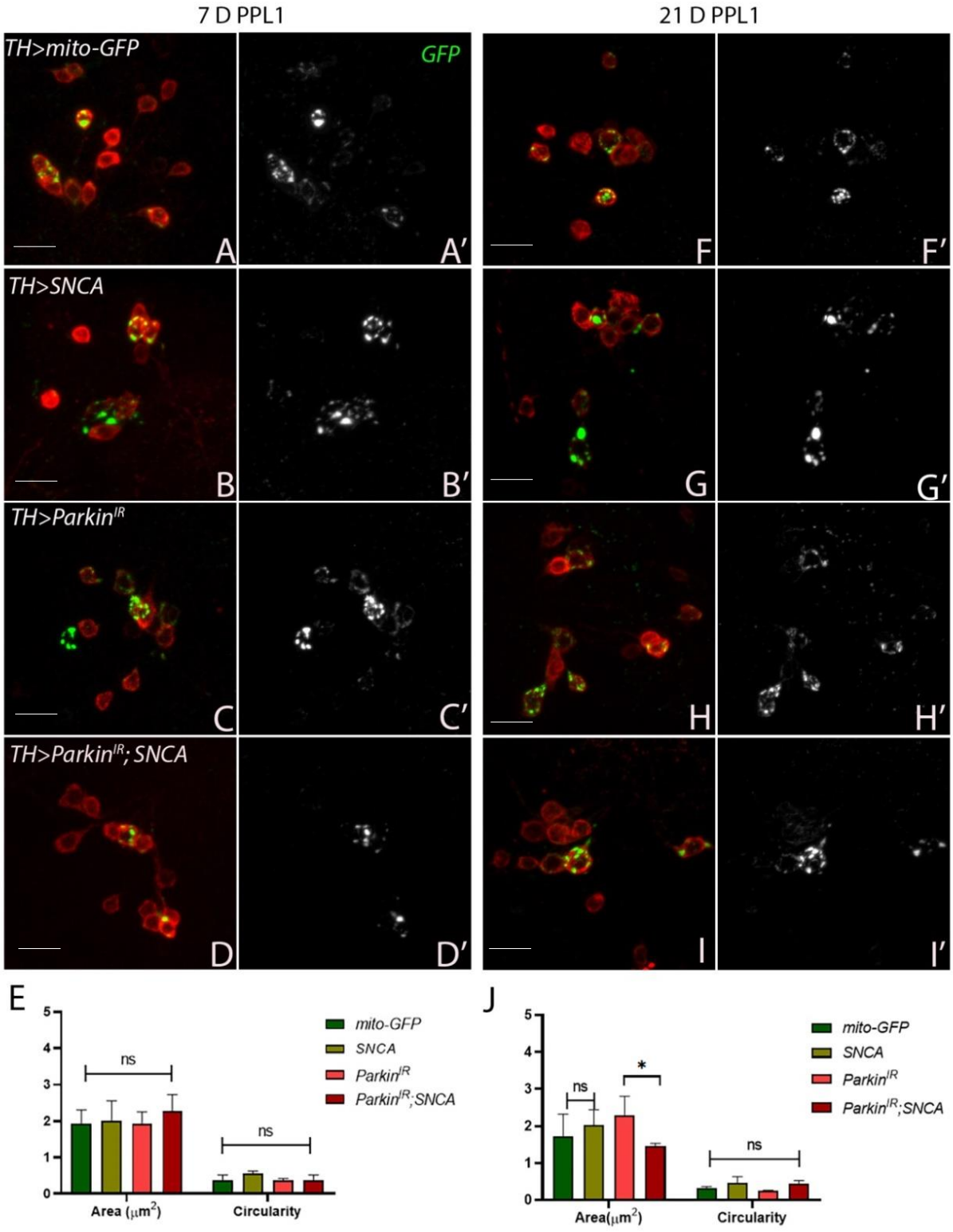
In PPM3 neuronal clusters, *UAS-parkin*$^{IR}$; *UAS-SNCA* (**Fig 4.7D, D' & 4.7E**) and *SNCA* overexpression (**Fig 4.7B, B' & 4.7 E**) have shown fragmented mitochondria in 7-day adult fly brains as compared to control (**Fig 4.7A, A' & E**). In 21-day old adult brains of *UAS-parkin*$^{IR}$; *UAS-SNCA* (**Fig 4.7 I, I' and 4.7J**) and *SNCA* overexpression (**Fig 4.7A, A' & 4.7J**) have also shown fragmented mitochondria as compared control (**Fig 4.7F, F'& 4.7J**) which do not degenerate. The *parkin* downregulation only has shown enlarged and/or swollen mitochondria in PPM3 clusters which degenerate, in 7-day (**Fig 4.7C, C' & 4.7E**) as well as in 21-day old adult fly brains (**Fig 4.7H, H' & 4.7J**) as compared to control (**Fig 4.7A, A' & E**).

In Parkinson's disease models, both *SNCA* and loss-of-function mutations in *parkin,* have been reported to alter mitochondrial morphology through regulating fusion and fission process. Therefore, to understand if there is any role of mitochondrial fission and fusion machinery in determining the mitochondrial morphology under *UAS-SNCA, UAS-parkin*$^{IR}$ and *UAS-*

*parkin*$^{IR}$; *UAS-SNCA* , we tested the protein levels of Mfn2. We observed that *UAS-SNCA* and *UAS-parkin*$^{IR}$ individually have shown significantly increased Mfn2 levels as compared to control flies (**Fig 4.8**). Whereas, *UAS-parkin*$^{IR}$; *UAS-SNCA* (together) were found to be almost comparable to control flies, however this was significantly less than *UAS-SNCA* and *UAS-parkin*$^{IR}$ individually (**Fig 4.8**). Thus, altogether these data suggested that α-synuclein causes mitochondrial morphology defects independent of parkin. To confirm the role of parkin in *SNCA* induced mitochondrial morphology defects, we performed mitochondrial fractionation. This is to quantify the parkin levels in mitochondria and will help us understand its involvement in the mitochondrial morphology alteration. We observed non-significant reduction in mitochondrial localization of parkin (**Fig. 4.9**). Hence, these data suggest the role of parkin being independent to α-synuclein to cause altered mitochondrial morphology in PD progression.

### 4.3.5 Altered mitochondrial and oxidative stress

To understand whether changes in mitochondrial morphology triggered by *UAS-SNCA*, *UAS-parkin*$^{IR}$, and *UAS-parkin*$^{IR}$*; UAS-SNCA* result into oxidative stress, ultimately contributing to locomotor defects and dopamine loss. Both *α-synuclein* aggregation and *parkin* mutations have been reported to be associated with mitochondrial ROS (Reactive Oxygen Species) production that can contribute to oxidative stress in *in-vitro* and *in-vivo* models (218). We have performed DCFDA assay to detect the ROS production in *UAS-SNCA*, *UAS-parkin*$^{IR}$, and *UAS-parkin*$^{IR}$*; UAS-SNCA* (**Fig 4.10A**). We have found no change in ROS level in *UAS-SNCA* as compared to 21 days old control flies. *UAS-parkin*$^{IR}$ has shown significantly increase in ROS level as compared to 21 days old control flies (**Fig 4.10A**). Whereas, *UAS-parkin*$^{IR}$*; UAS-SNCA* flies have shown decreased ROS level as compared to control, though at lesser extent to *UAS-parkin*$^{IR}$. We performed MDA (malondialdehyde) assay**,** one of the biochemical marker for oxidative stress and elevated MDA levels may reflect increased lipid peroxidation and oxidative damage. We have found statistically non-significant increase in MDA level in *UAS-SNCA* and *UAS-parkin*$^{IR}$ flies as compared to control flies, whereas, *UAS-parkin*$^{IR}$*; UAS-SNCA* has shown significant increase in MDA level as compared to 21 days old control flies (**Fig 4.10B**).


7 D PPL1
21 D PPL1
TH>mito-GFP
GFP
A
A′
F
F′
TH>SNCA
B
B′
G
G′
TH>Parkin^IR
C
C′
H
H′
TH>Parkin^IR;SNCA
D
D′
I
I′
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mito-GFP
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Area (μm^2)
Circularity
J
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SNCA
Parkin^IR
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ns
Area(μm^2)
Circularity

**Figure 4.6. *SNCA* overexpression results in swollen mitochondria, *parkin$^{IR}$* expression has shown elongated whereas together (*parkin$^{IR}$; SNCA*) shows fragmented mitochondria in PPL1 DA clusters.** Adult brains of desired genotype expressing the mitochondria targeted green fluorescent protein (mitoGFP) in TH- positive (red) cells. (A-J) 7-day & 21-day old adult fly brains showing mitoGFP in PPL1 cluster. Control brains showing mitoGFP at (A, A') 7-day and (F, F') 21-day. *SNCA* overexpressing flies show mitoGFP (B, B') in 7-day and in (G, G') 21-day. *Parkin$^{IR}$* expressing flies show mitoGFP in (C, C') 7-day and in (H, H') 21-day. *Parkin$^{IR}$; SNCA* expressing flies show mitoGFP in (D, D') 7-day and further enhanced in (I, I') 21-day. (E) Quantification of mitochondria morphology (area & circularity) was done using ImageJ Mito-Morphology Macro. Scale bar 10µm. A total of four adult brains were used (n=4) per genotype. Data is represented as mean with SEM. Statistical analysis were performed using Two-way ANOVA followed by Tukey's multiple comparison test. P value: *(<.05), ns-not significant.

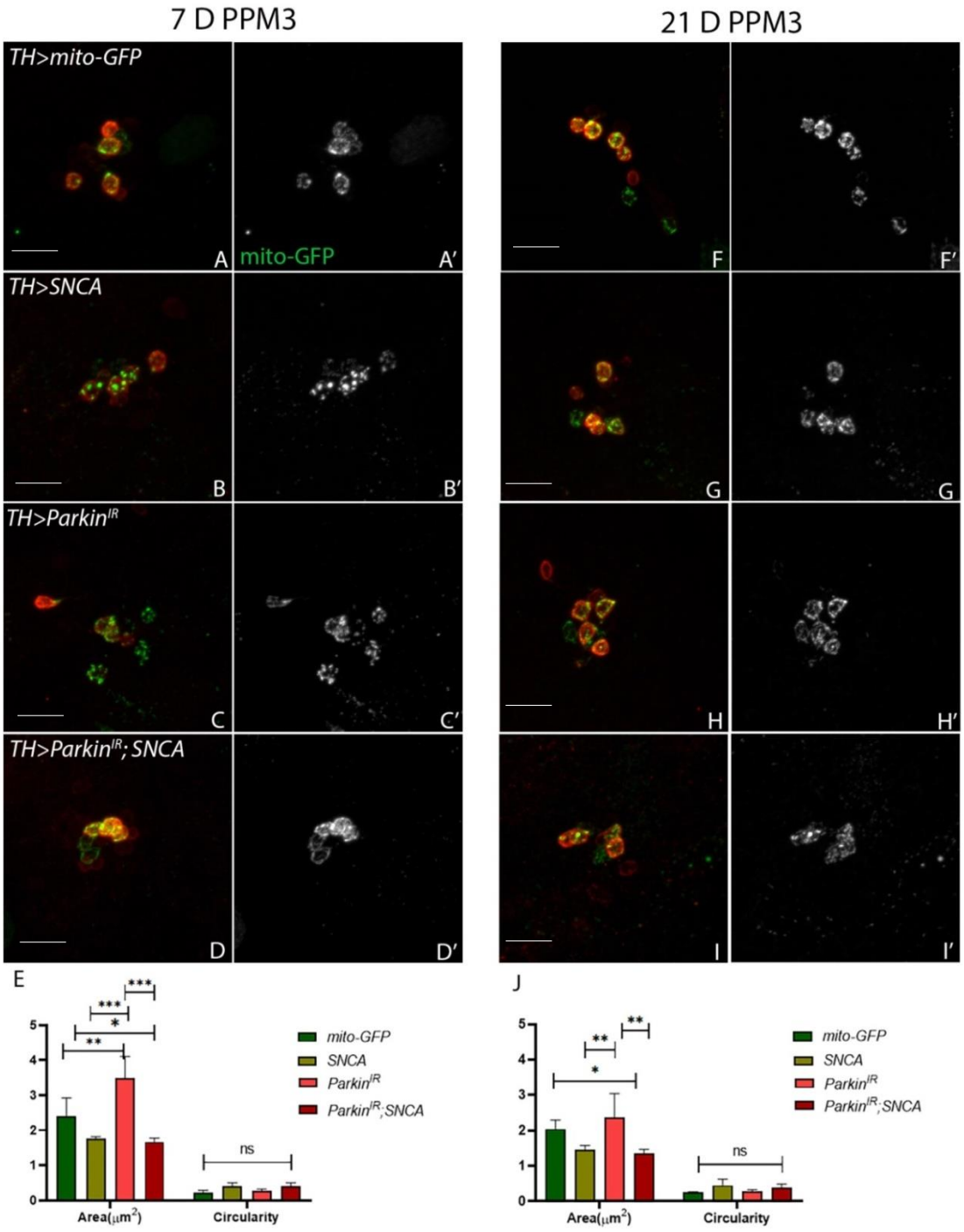


**Figure 4.7. *SNCA* overexpression and *parkin$^{IR}$; SNCA* have shown fragmented mitochondria, whereas *parkin$^{IR}$* expression has shown elongated mitochondria in PPM3 DA clusters.** Adult brains of desired genotype expressing the mitochondria targeted green fluorescent protein (mitoGFP) in TH- positive (red) cells. (A-J) 7-day & 21-day old adult fly brains showing mitoGFP in PPM3 cluster. Control brains showing mitoGFP at (A, A') 7-day

and (F, F') 21-day. *SNCA* overexpressing flies show mitoGFP (B, B') in 7-day and in (G, G') 21-day. *Parkin*$^{IR}$ expressing flies show mitoGFP in (C, C') 7-day and in (H, H') 21-day. *Parkin*$^{IR}$*; SNCA* expressing flies show mitoGFP in (D, D') 7-day and in (I, I') 21-day also. (E) Quantification of mitochondria morphology (area & circularity) was done using ImageJ Mito-Morphology Macro. Scale bar 10µm. A total of four adult brains were used (n=4) per genotype. Data is represented as mean with SEM. Statistical analysis were performed using Two-way ANOVA followed by Tukey's multiple comparison test. P value: *(<.05), ** (<.001), *** (<.0001), ns-not significant.

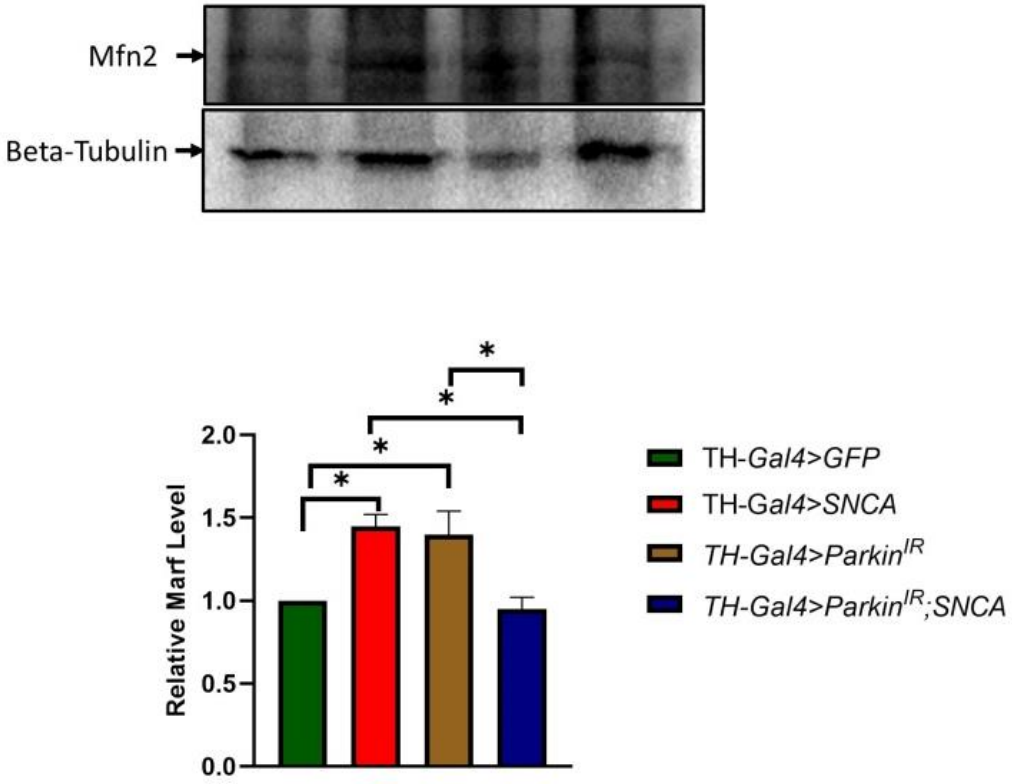


**Figure 4.8. *SNCA* overexpression and *parkin* downregulation regulate Mfn2 protein expression independently.** Data is represented as mean with SEM. Statistical analysis were performed using One-way ANOVA followed by Tukey's multiple comparison test. P value: *(<.05).

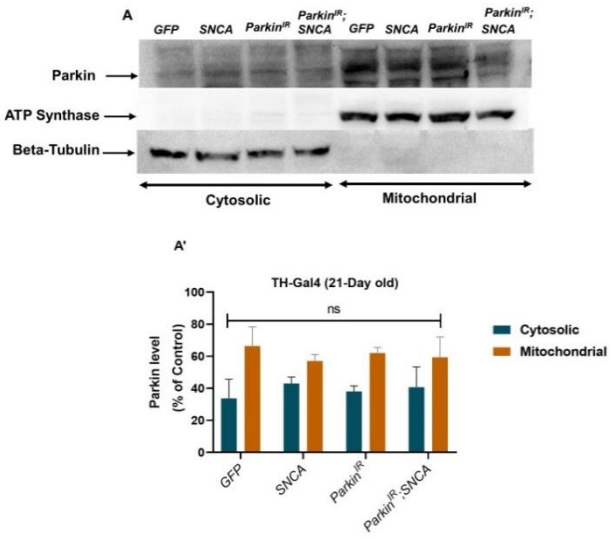


**Figure 4.9. *SNCA*-induced mitochondrial morphology defects is independent of parkin:** Western blot of mitochondria fractionation probed for parkin, ATP synthase and βTubulin (A) and (A') quantification showing reduction in parkin level in mitochondrial and increase in cytosolic fraction. 50 flies (n=50) were used for fractionation. Data is represented as mean with SEM. Statistical analysis were performed using Two-way ANOVA followed by Tukey's multiple comparison test. P value: (ns) not significant.

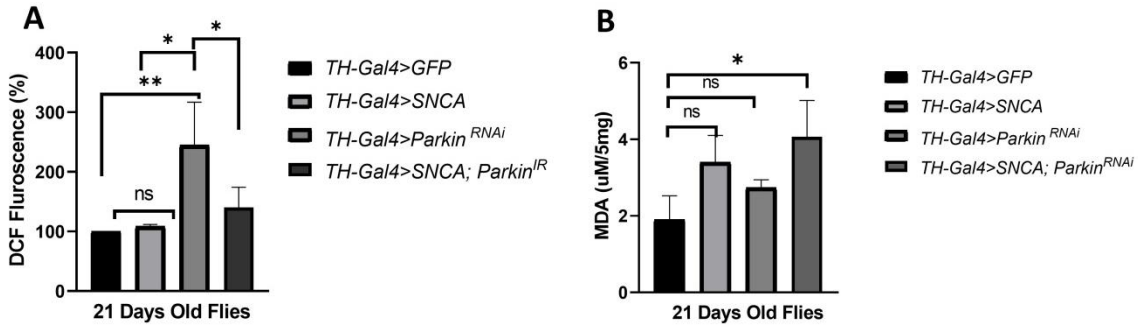


**Figure 4.10. *SNCA* and *parkin* causes oxidative stress at cellular level.** (A) Measurement of spectrophotometric fluorescence intensity of DCF (di-chlorofluorescein) which specifies the enhancement of ROS. (B) Levels of malondialdehyde (MDA) as lipid peroxidation marker. 40 flies (n=40) were used. Data is represented as mean with SEM. One-way ANOVA followed by Tukey's multiple comparison test. P value: *(<.05), ** (<.001), (ns) not significant.

## 4.5 Discussion

Here, we characterize the cluster specific DA neuronal loss associated with interaction of *SNCA* and *parkin*. We have highlighted the effect of *SNCA* overexpression and *parkin* knockdown together in terms of DA neuronal loss in PPL1 and PPM3 clusters of adult fly brain over a time period of 21 days.

*SNCA* and *parkin* mutations have been found to be involved in motor (postural instability, tremor, bradykinesia) and non-motor symptoms (sleep disorders, depression, anxiety, hallucinations) in PD patients. *SNCA* overexpression and *parkin* mutation both have also been shown to cause the age dependent locomotor dysfunction and neurodegeneration in *in-vivo* models (14,218, 215, 207). In our study, *SNCA* overexpression and *parkin* knockdown in DA neurons have also shown the progressive locomotor dysfunction. Interaction of *SNCA* and *parkin* in PPL1 clusters results into progressive locomotor dysfunctions. Although, the effect of genetic alterations are not as pronounced to indicate an additive effect. This is further supported by observed mitochondrial morphology in *UAS-parkin*$^{IR}$; *UAS-SNCA*. With respect to the reports published previously, our work uncovers the relation between *SNCA* and *parkin* in an *in-vivo* system in a novel way by bringing the gene alterations together. This enables us to understand the relationship, between *SNCA* and *parkin* at both transcriptional and translational levels.

Studies have reported that wild-type *SNCA* causes reduction in number of TH-positive neurons in PPM1& 2, PPL1 clusters but not in PPM3 clusters (221 - 224). We have also found that *SNCA* overexpression has reduced number of TH-positive neurons in PPM1&2, PPL1 and not in PPM3, which are consistent with aforementioned reports. Flies with *parkin* mutation have also been shown to cause progressive loss of DA neurons in PPL1 cluster but not in PPM3 (149,215) after 20 days post-eclosion. No loss of neurons has been reported in the dorsomedial clusters (DMC) (also known as PPM) in *parkin* loss of function mutation in adult fly brain after 21 day post-eclosion (135). However, in our study, parkin knockdown alone shows loss of DA neurons in PPM3 along with PPM1&2 and PPL1 clusters in 7 and 21-day old adult fly brains. *SNCA* with *parkin* knockdown (*UAS-parkin*$^{IR}$; *UAS-SNCA*) showed decreased number of DA neurons in PPM1&2, PPL1 clusters as compared to control, though at lesser extent with *SNCA* and *parkin* knockdown independently. Numbers of DA neurons in PPL2 clusters were unaltered in *SNCA* and *parkin* knockdown independently and together, in 7-day as well as in 21-day old fly brains. Hence, these observations suggest that DA neuronal loss was correlating

with locomotor dysfunctions. Since, we did not observe aggravated phenotype in *SNCA* overexpression and *parkin* knockdown together (*UAS-parkin*$^{IR}$; *UAS-SNCA)*; this may suggest that *SNCA* doesn't affect *parkin* directly. Alternatively, this could also mean that *parkin* downregulation is not the only the mechanism involved in *SNCA* induced locomotor dysfunction and neurodegeneration.

Neurons have highly dynamic energy requirements, and hence the intact mitochondrial morphology is an important aspect to preserve the neuronal health. In post-mortem brains of Parkinson's patients, it has been shown that α-synuclein localize to mitochondria and affect mitochondrial homeostasis (223,133, 224, 225). Although, α-synuclein doesn't have an exact mitochondrial targeting sequence, but studies suggest that α-synuclein contains a cryptic mitochondrial targeting sequence in the N-terminus region (221). Recently, it has been reported that N-terminus of α-synuclein plays a role in mitochondrial fragmentation via a DRP1-dependent pathway in *Drosophila* (196). In *Drosophila, C. elegans*, dorsal root ganglia of *Danio rerio* (zebra fish) and in cellular models also, *SNCA* overexpression cause mitochondrial fragmentation (226, 199 227). In our study, *SNCA* overexpression has caused more elongated/or swollen mitochondria in PPL1 clusters, while in PPM3 clusters, it results in more fragmented mitochondria in progressive manner. These results are thus align with the DA neuronal loss in PPL1 clusters but not in PPM3 clusters. This may also be an indication of some other mechanisms involved in rescuing the effect of *SNCA* overexpression and *parkin* knockdown together.

Loss-of-function mutations in *parkin* are the most prevalent cause of recessive form PD (189). Upon mitochondria depolarization, parkin is activated by PINK1 and promotes degradation of Mitofusin 1 and 2 (137, 228, 229) and recruits Drp1 to mitochondria which lead to fission (228). Parkin is also involved in the selective degradation of damaged mitochondria through mitophagy process (229). In tissues of *parkin*-null *Drosophila* mutants, swollen mitochondria have been observed and this suggests that *parkin* may either promote fission or inhibit fusion (147,146). In our study, we performed mitochondrial fractionation to quantify parkin and found non-significant decrease in all three conditions w.r.t. control. This suggests independent and non-coinciding roles of parkin with alpha synuclein in regulating mitochondrial morphology. Reducing *Opa1* or *Mitofusin (MFN2 and MFN1*) has been reported to ameliorate swollen mitochondrial morphology (222,(230)182). Knockdown of *Marf* was shown to reduce abnormal mitochondrial morphology in the muscles of *pink1* and *parkin* mutant flies (231). Conversely, in DA neurons of *parkin* knockout mice, more fragmented mitochondria have been

shown to causes the neuronal loss (197). The presence of *parkin* mutation in causing accumulation of dysfunctional mitochondrial in PD patients has also been established. In our study, *parkin* downregulation caused enlargement of mitochondria in PPL1 and PPM3 clusters in age dependent manner. These results were correlating with the DA neuronal loss in PPL1 and PPM3 clusters.

Several studies have reported that overexpression of *parkin* restores mitochondrial morphology and function caused by *SNCA*, but it is still not clear whether this is through a direct link between *parkin* and *SNCA*, or neuroprotective role of parkin in maintaining mitochondrial dynamics (223, 226,220). In *in-vitro* model, exogenous α-synuclein oligomers or fibrils caused reduction in *parkin* expression and wild-type *parkin* overexpression rescues α-synuclein induced mitochondrial fragmentation (233). However, they have shown that toxic effects of α-synuclein on mitochondria was higher as compared to *parkin* silencing induced mitochondrial dysfunction and suggested that α-synuclein-induced parkin downregulation is not the only mechanism for mitochondrial dysfunction (233). Similarly, in our study, overexpression of *SNCA* with *parkin* downregulation (*UAS-parkin*$^{IR}$; *UAS-SNCA*) shows more fragmented mitochondria in PPL1 as well in PPM3 clusters in age dependent manner, which is just opposite to *SNCA* overexpression and *parkin* downregulation individually. We have found no changes in parkin expression at protein level in *SNCA* overexpressed flies; however, *parkin* transcript was significantly reduced. This warrants that further studies need to be carried out to validate the transcriptional correlation.

There are several line of evidences suggest that both *α-synuclein* and *parkin* mutations cause impaired mitochondrial function which further contribute to the generation of ROS and, consequently, oxidative stress in *in-vitro* and *in-vivo* models (234, 235,134,93,237,137). In our study we did not observe any significant increase in ROS level in *SNCA and SNCA* with *parkin* downregulation flies as compared to control in 21-dyas old flies. *Parkin* downregulation have shown significantly increased ROS level as compared to control flies. *α-synuclein* and *parkin* have shown non-significant increase in MDA as compared to control in 21-days flies. Whereas, *α-synuclein* with *parkin* downregulation has shown significantly increased MDA level as compared to control in 21-days flies, however, this increased MDA level was comparable within *SNCA* with *parkin* downregulation individually. Altogether, these suggest that *SNCA* and *parkin* downregulation independently disrupt cellular homeostasis through regulating some other molecular mechanisms in PD progression.

Current study provides insights into cellular and molecular etiology in case of PD in a time-dependent manner specifically in DA neurons, using overexpression system. Since neuronal mitochondria are highly dynamic, depending on ever-changing metabolic requirements, the morphology changes driving degeneration are limited to only TH positive neurons **(Fig 4.11**). Further work is required to understand if there are any other mechanisms regulating these interactions and if so, how does it affect pathogenesis of Parkinson's disease. Also it would be crucial to understand if mitochondria are affected by other direct or indirect genetic and molecular factors affecting progression of Parkinson's disease.

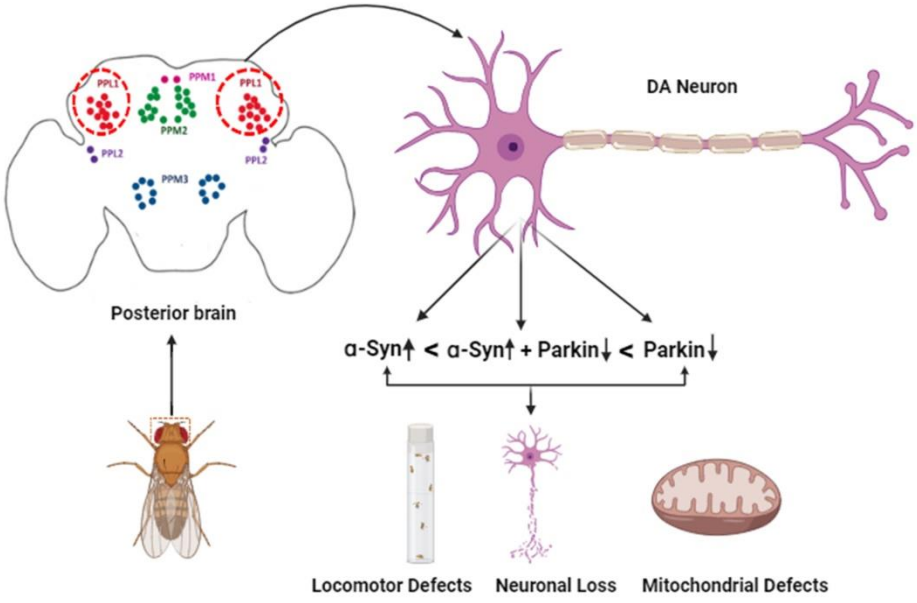


**Figure 4.11.** Schematic representation of effect of α-syn and parkin on specific DA neuronal clusters in adult fly brain. Figure drawn using BioRender.

# Chapter-5
# Conclusions and Future Scope

## 5.1 Conclusions:

1. In this study we have established and characterized the *Drosophila* genetic model of Parkinson's Disease using Gal4/UAS expression system to investigate genetic and molecular interaction between α-synuclein and parkin. Overexpressing α-synuclein and downregulating parkin in various tissues of *Drosophila* successfully replicates the phenotype observed in Parkinson's disease. Observed phenotypes are locomotor defects, reduced life span and Dopaminergic neuronal loss in age-dependent manner. Thus, both α-synuclein overexpression and parkin knockdown could be useful to understand the underlying molecular mechanisms of specific dopaminergic neuronal death in PD. Also, employing the *Drosophila* model for PD may provide valuable insights into the pathogenesis of PD.
2. The present work provides evidence about genetic and molecular interaction between α-synuclein and parkin in specific dopaminergic neuronal clusters. The occurrence of climbing defect, reduced life span and DA neuronal loss phenotypes resulting from genetic manipulation of α-synuclein and parkin implies that α-synuclein and parkin may function independently. Alternatively, it could also indicate that *parkin* downregulation is not the sole mechanism involved in α-synuclein induced locomotor dysfunction and neurodegeneration. α-Synuclein-induced reduction in the level of parkin transcript, without a corresponding decrease in protein expression, further supports the idea of independent functions.
3. In context to mitochondrial morphology, we provide evidence that α-synuclein and parkin cause mitochondrial morphology defect in cluster specific DA neurons. In our study, overexpression of α-synuclein with parkin downregulation (*UAS-parkin*$^{IR}$; *UAS-SNCA*) shows more fragmented mitochondria in PPL1 as well in PPM3 clusters in age dependent manner, which is just opposite to α-synuclein overexpression and parkin downregulation individually. These results are thus aligning with the DA neuronal loss in PPL1 clusters but not in PPM3 clusters. This could suggest the involvement of some other mechanisms aimed at mitigating the effect of α-synuclein overexpression and parkin knockdown together.

## 5.2 Limitations and future Scope of the work:

1. To confirm the role of mitochondrial dynamics (fusion/fission), genetic manipulation of genes involved in mito-fusion (*Marf and OPA1*) and fission (*Drp1*) process with *SNCA* and *Parkin*$^{IR}$ remains to be explored.

2. Exploration of some other mechanisms involved in rescuing the effect of *SNCA* overexpression and *parkin* knockdown together can be further pursued.

3. Using cell isolation techniques from individual DA neuronal clusters of brains will be able to provide more insights at individual neuronal and organelle level, since number of these neurons is limited.

4. To understand the mechanisms involved in regulation at organelle level, more research is required in other animal models, if these interactions are conserved at cellular and molecular level affecting pathogenesis of Parkinson's disease.

5. Additionally, the vulnerability of specific neuronal clusters in *SNCA* and *parkin* is essential to be examined.

6. Limited studies have been done on non-dopaminergic (non-DA) systems that are affected in PD condition. Through investigating non-DA neuronal dysfunction, some of the initial alterations in PD, including olfactory dysfunction, sleep disturbances and gut dysfunctions can be explored.

7. It will be interesting to explore if different sporadic model of PD will exhibit similar cluster specific behavior in terms of mitochondrial dynamics. Further, since sporadic models can be induced by feeding different chemicals, a comparison will provide better insights into underpinnings of genetic and molecular mechanisms of PD, in order to identify therapeutic targets

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# Appendix I

## List of Publications

1. **Narwal, S.,** Singh, A., & Tare, M. Analysis of α-Syn and Parkin interaction in mediating neuronal death in Drosophila model of Parkinson's Disease. *Frontiers in Cellular Neuroscience*, *17*, 1295805. https://doi.org/10.3389/fncel.2023.1295805
2. **Sonia Narwal**, Arushi Rai, Shreyas Iyer, Kirti Tare, Meghana Tare (2023). "Neuroprotective potential of “Ras-Sindoor” on Drosophila model of Parkinson’s disease". Current Science: (Under Review).
3. Rajashree Banerjee, Arushi Rai, Shreyas M. Iyer, **Sonia Narwal**, Meghana Tare. (2022). Animal models in the study of Alzheimer's disease and Parkinson's disease: A historical perspective. ANIMAL MODEL AND EXPERIMENTAL MEDICINE, 1, 27-37.
4. Meghana Tare, Anuradha Venkatakrishnan Chimata, Neha Gogia, **Sonia Narwal**, Prajakta Deshpande, Amit Singh. (2020). An E3 ubiquitin ligase, cullin-4 regulates retinal differentiation in Drosophila eye. GENESIS, 10-11

## Book Chapters

1. Arushi Rai, **Sonia Narwal**, Harsh Kanodia & Meghana Tare. (2020). Eye for an Eye: A Comparative Account on Compound Eye of Drosophila melanogaster with Vertebrate Eye. MOLECULAR GENETICS OF AXIAL PATTERNING, GROWTH AND DISEASE IN DROSOPHILA EYE, 343-357.
2. **Sonia Narwal**, Shreyas Iyer, Arushi Rai, Meghana Tare. (2021). Observing surface topography of Drosophila eye by Scanning Electron Microscopy. Experiments with Drosophila for Biology Courses.441-445
3. **Sonia Narwal**, Shreyas Iyer and Meghana Tare. (2021). Visualizing Actin filaments in Drosophila tissues. Experiments with Drosophila for Biology Courses.293-296

# Appendix II

## Conferences

1. Poster presentation in interdisciplinary conference LSRIEAS-2018 (International Conference on Life Science Research & its Interface with Engineering and Allied Sciences) organized by BITS-Pilani, Pilani campus, Raj.
2. Poster presentation in 5th Asia Pacific Drosophila Research Conference (APDRC5) and Indian Drosophila Research Conference, 2020 organized by IISER Pune.
3. Poster presentation in Indian Drosophila Research Conference (InDRC) 2021, organized by IISER Kolkata.
4. Poster presentation in International Conference on Nutraceuticals and Chronic Diseases conference, 2022 organized by Department of Zoology DU North Campus, Delhi.
5. Poster Presentation in Mini Colloquium on "Excitement in Science: Insights & Perspectives" 3rd Dec 2022, organized by Department of Biological Sciences, BITS Pilani, Pilani campus, Raj.
6. Poser Presentation in 45th AICBC (All India Cell Biology Conference), BHU, Varanasi, organized by Department of Zoology, BHU, Varanasi.
7. Flash-Talk and Poster presentation in 42nd Mahabaleshwar Seminars, Mitochondria and Metabolism, 13th -15th Feb 2023, organized by IISER Pune

# Appendix III

## Biography of Dr. Meghana Tare

Dr. Meghana Tare joined BITS Pilani in July 2017. Before that, she earned her bachelor's degree in Microbiology in 2002 and a master's degree in Biochemistry from Devi Ahilya University, Indore, in 2004. She completed her doctoral diploma in Biology at the University of Dayton, Dayton, OH, in 2013. During her doctoral studies, Dr. Tare investigated molecular and genetic mechanisms involved in organogenesis patterning and neurodegeneration associated with Alzheimer's Disease, utilizing *Drosophila melanogaster* as a model system. She received prestigious awards for her presentations and was recognized as the best researcher during her PhD tenure. In 2012, Dr. Tare was selected to speak at the Annual *Drosophila* Research Conference organized by the Genetics Society of America in Chicago.

Following her Ph.D., Dr. Tare worked as a post-doctoral research fellow in Dr. Andreas Bergmann's Lab in the Department of Molecular Cell and Cancer Biology at the University of Massachusetts Medical School, Worcester, Massachusetts, from 2013 to 2017. Her post-doctoral research focused on understanding the genetic regulation of apoptosis. Dr. Tare has published numerous research articles and book chapters during her Ph.D tenure. After joining BITS Pilani, Dr. Tare has independently authored or collaborated on approximately nine journal articles, two book chapters and four teaching notes.

At BITS Pilani, Dr. Tare's lab is the exclusive facility dedicated to *Drosophila* research on both campus and in the state of Rajasthan. Using *Drosophila melanogaster* (fruit fly) as a model organism, she investigates the genetic and molecular mechanisms associated with the onset and progression of neurodegenerative diseases. Dr. Tare has successfully completed three research projects as a Principal Investigator: Research Initiation Grant (2017-2019), funded by BITS Pilani; an additional Competitive Research Grant for new faculty, funded by BITS Pilani (2019-2021), and Start up Research Grant funded by DST-SERB, India (2019-2022). She has secured a research grant in 2022 from the Indian Council of Medical Research (ICMR), New Delhi, India. Additionally, she serves as the Co-Principal Investigator for a research grant with Prof. Sandhya Marathe (Department of Biological Sciences, BITS, Pilani) and Prof. Vidya Negi (IISER, Mohali) funded by the Department of Biotechnology, New Delhi, India. Currently, Dr. Tare mentors two Ph.D. students and has served as undergraduate and Master's thesis supervisor for several undergraduate and postgraduate students both on and off-campus.

Additionally, Dr. Tare serves on the editorial board of Scientific Reports (Nature Publishing Group) and Frontiers Journal. She is actively engaged as reviewer for PLoS One, Scientific Reports, Frontiers in Molecular Neuroscience, and Environmental Sciences and Pollution Research.

## Biography of Prof. Amit Singh

Prof. Singh is a full-time faculty member at the University of Dayton in Ohio, USA. He earned his B.Sc. from the Government Degree College Nahan, H.P. University, India, and his M.Sc. and Ph.D. from Devi Ahilya University, Indore, India. Following a brief tenure as a Research Associate in Transgenics of Silkworm, *Bombyx mori*, at the Indian Institute of Sciences (IISc.), Bangalore, India, Prof. Singh pursued post-doctoral research in the field of eye development using the *Drosophila melanogaster* model system at Academia Sinica, Taiwan.

In 2002, Prof. Singh transitioned to Baylor College of Medicine, Houston, Texas, to further his work on *Drosophila* eye development and was promoted to an instructor (non-tenure track faculty) position in 2004. He joined the University of Dayton as a tenure-track assistant professor in 2007, progressing through the ranks to become a full professor in 2018. Prof. Singh has a substantial publication record, including three books with Springer publishers, eight book chapters, and approximately 91 journal articles.

The primary focus of Prof. Singh's lab is to investigate the genetic basis of early eye patterning and growth, with a specific emphasis on axial patterning. Additionally, the lab aims to understand the mechanisms of complex neuropathological diseases, such as Alzheimer's Disease (AD). Under Prof. Singh's mentorship, ten Ph.D. students have graduated, and he has mentored two post-doctoral researchers. At present, he is engaged in supervising four Ph.D. students and mentoring three post-doctoral researchers.

Prof. Singh has been a recipient of several prestigious and highly competitive funding awards. These awards include funding from agencies such as NIH (R15 and RO1, twice), NSF Leader Consortium Mini-Grant Advance, Ohio Cancer Research Associates, Knights Templar Eye Foundation (KTEF), Fight for Sight Foundation, Retina Research Foundation Additionally, he has been a recipient of extremely prestigious Schuellein Endowment Fund and STEM Catalyst Initiative University of Dayton; along with start-up and seed research funds from University of Dayton.

Prof. Singh is actively engaged in various professional activities, including membership in organizations such as the Genetics Society of America (GSA), Ohio Miami Valley Society of Neuroscience (OMVSfN), and the Council of Undergraduate Research (CUR). He is an honorary member of the Theta Kappa Chapter of the University of Dayton's Beta Beta Beta

Honor Society. Prof. Singh is also part of the mentor network of the American Society of Human Genetics (ASHG) GENA project.

Furthermore, Prof. Singh holds editorial roles in several reputable journals, including Journal PLoS ONE, Scientific Reports, BMC Genetics, Peer J, and Frontiers in Cell and Developmental Biology. He is also an editorial board member for Frontiers in Genetics, Developmental Dynamics, Journal of Biological Sciences, Journal of Cell Science & Therapy, and Current Research in Neuroscience. Prof. Singh is a member of the Sigma Xi Honors Society.

Prof. Singh is also reviewer on several research grant panels including Neurodevelopment, Synaptic Plasticity and Neurodegeneration Fellowship (F03A) Study Section (x4 times), European Research Council (ERC), Arizona Alzheimer's Disease Core Center Pilot grants, National Science Foundation (NSF) for Major Research Instrumentation (MRI) grant review panel, National Science Foundation (NSF) for GFRP grant review panel, NIH Biology of Vision (BVS) study section, Parkinson's UK, American Heart Association, Alzheimer's Association (AZA), Kentucky Science and Engineering Foundation R&D Excellence Award.

Further, Prof. Singh is actively engaged as a reviewer for PLoS Biology, PLoS ONE, PLoS Genetics, eLife, Development, Biology Open, Disease Model and Mechanisms, Developmental Biology, Developmental Dynamics, Mechanism of Development, Genesis, Genetics, Gene, Organogenesis, International J. Developmental Biology, Journal of Neuroscience, European Journal of Cell Biology, Human Molecular Genetics, Cell Death & Disease, JOVE, Cancer Letters, FEBS, FEBS Letter, Frontiers in Cell and Developmental Biology, Frontiers in Molecular Neuroscience, Frontiers in Neuroscience, Neural Regeneration and Research, Cell Biology Insights, PNAS, Fly, Alzheimer's and Dementia, Journal of Alzheimer's Disease, Biophysical Journal, Brain, Technotome, Journal of STEM Education.

# Appendix IV

## Biography of Candidate

Sonia is currently pursuing her Ph.D. in the Department of Biological Sciences at BITS-Pilani, Pilani, Rajasthan, having joined in 2018 under the guidance of Prof. Meghana Tare. Prior to her Ph.D., she joined in Prof. Pankaj Seth's lab at the National Brain Research Institute (NBRC), Manesar, Gurgaon, and contributed in the project "Role of Zika Viral E Protein in Neural stem cell" from (Nov 2017- July 2018). During this period, she gained valuable experience in cell culture and various molecular techniques. She also co-authored a publication from his project (Bhagat, R. et. al; 2018).

Sonia holds a B.Tech degree in Biotechnology from Sobhasaria Group of Institutions, Sikar, Rajasthan (2013), and an M.Tech in Biotechnology from Banasthali Vidyapeeth University, Bansathali, Rajasthan (2016). As part of her B.Tech degree, she worked on the project "HLA typing in HIV Patients" at the Department of Molecular Biology and Transplant Immunology, Indraprastha Apollo Hospitals, Sarita Vihar, New Delhi. For her M.Tech degree, she engaged in a project titled "Study on selection of promising herbals against *Mycoplasma pneumoniae* by using *in-silico* bioprospection and molecular docking approach" at the Institute of Nuclear Medicine and Allied Sciences (INMAS), DRDO, Timarpur, New Delhi, under the supervision of Dr. Raman Chawla (Oct 2015- Aug 2016). This project provided her with experience in utilizing various bioinformatics tools. Post her M.Tech, she worked as a Product Specialist at Geno-Biosciences Pvt. Ltd., Noida, (March 2017-Aug 2017).

Throughout her Ph.D. tenure, Sonia has made significant contributions to projects, demonstrating meticulous planning and execution of experiments. She has developed a profound understanding of *Drosophila* genetics, cellular, and molecular sciences. Additionally, she actively participated in cutting-edge research projects, collaborated in grant writing with her supervisor, and mentored undergraduate students in the lab. Sonia has also taken on teaching responsibilities, instructing courses on "Biological Laboratory" and "Laboratory Techniques" for graduate and master's students. During her Ph.D. tenure, Sonia has published four Journal articles and three book chapters, independently and in collaboration. She has been awarded a highly competitive and prestigious Senior Research Fellowship from ICMR (Indian Council of Medical Research). She actively participated in national and international conferences and was awarded a travel grant to attend the 45$^{th}$ All India Cell Biology Conference at Banaras Hindu University, Varanasi.

# Appendix V

frontiers | Frontiers in Cellular Neuroscience

TYPE Original Research
PUBLISHED 04 January 2024
DOI 10.3389/fncel.2023.1295805

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RECEIVED 17 September 2023
ACCEPTED 01 December 2023
PUBLISHED 04 January 2024

CITATION
Narwal S, Singh A and Tare M (2024) Analysis of α-syn and *parkin* interaction in mediating neuronal death in *Drosophila* model of Parkinson's disease.
*Front. Cell. Neurosci.* 17:1295805.
doi: 10.3389/fncel.2023.1295805

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# Analysis of α-syn and *parkin* interaction in mediating neuronal death in *Drosophila* model of Parkinson's disease

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One of the hallmarks of Parkinson's Disease (PD) is aggregation of incorrectly folded α-synuclein (*SNCA*) protein resulting in selective death of dopaminergic neurons. Another form of PD is characterized by the loss-of-function of an E3-ubiquitin ligase, *parkin*. Mutations in *SNCA* and *parkin* result in impaired mitochondrial morphology, causing loss of dopaminergic neurons. Despite extensive research on the individual effects of *SNCA* and *parkin*, their interactions in dopaminergic neurons remain understudied. Here we employ *Drosophila* model to study the effect of collective overexpression of *SNCA* along with the downregulation of *parkin* in the dopaminergic neurons of the posterior brain. We found that overexpression of *SNCA* along with downregulation of *parkin* causes a reduction in the number of dopaminergic neuronal clusters in the posterior region of the adult brain, which is manifested as progressive locomotor dysfunction. Overexpression of *SNCA* and downregulation of *parkin* collectively results in altered mitochondrial morphology in a cluster-specific manner, only in a subset of dopaminergic neurons of the brain. Further, we found that *SNCA* overexpression causes transcriptional downregulation of *parkin*. However, this downregulation is not further enhanced upon collective *SNCA* overexpression and *parkin* downregulation. This suggests that the interactions of *SNCA* and *parkin* may not be additive. Our study thus provides insights into a potential link between *α-synuclein* and *parkin* interactions. These interactions result in altered mitochondrial morphology in a cluster-specific manner for dopaminergic neurons over a time, thus unraveling the molecular interactions involved in the etiology of Parkinson's Disease.

KEYWORDS

**α-synuclein, *parkin*, mitochondrial morphology, dopaminergic neurons, Parkinson's disease, tyrosine hydroxylase, *Drosophila melanogaster***