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Effect of co-expression of alpha-synuclein and leucine-rich repeat kinase 2 on the hallmark features of Parkinson's disease in *Drosophila melanogaster*

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Alpha-synuclein (SNCA) and leucine-rich repeat kinase 2 (LRRK2) are involved in multiple pathways important for cellular function, implying potential interplay. It is thus important to study the combined effect of SNCA and LRRK2 on the cellular processes involved in the pathophysiology of PD. Mutations in the genes encoding the two proteins have been linked to an autosomal dominant form of Parkinson's disease (PD). This study will help in unearthing underlying mechanisms that might be driven by both proteins working in a synergistic manner. We, hereby developed a model in *Drosophila melanogaster* by co-expressing SNCA and LRRK2 in all neurons, glia, and dopaminergic neurons to determine the interplay between SNCA and LRRK2 which will help us to better understand the pathophysiology of PD. We expressed either individually or co-expressed human SNCA, human wild-type LRRK2, and human mutated form of LRRK2 named LRRK2- G2019S in all neurons, glia, and dopaminergic neurons and evaluated for differences in life span, locomotion, and dopaminergic cell death. The individual expression of SNCA, LRRK2, and LRRK2-G2019S in all neurons, glia, and dopaminergic neurons was shown to cause selective loss of dopaminergic neurons, locomotor dysfunction, and early mortality. Co-expression of SNCA with wild-type and mutant LRRK2 in the same set of cells caused a more severe form of fly mortality, locomotor dysfunction, and dopaminergic neuronal death than an individual expression of the proteins. This study indicated that expression of both SNCA and LRRK2 exhibited a synergistic effect, and both proteins might be involved in molecular pathways leading to PD-associated neurodegeneration. The *Drosophila* model recapitulated the key features of PD and might take us one step closer to understanding the disease and serve as a therapeutic screen for PD-associated interventions.

Keywords: Dopaminergic neurons, GAL4-UAS system, Genetic synergism, Glia

Parkinson's disease (PD) is one of the progressively debilitating neurodegenerative disorders. It is characterized by motor dysfunction, degeneration of dopaminergic (DA) neurons of the substantia nigra pars compacta, and the presence of intra-neuronal protein aggregates called Lewy bodies and Lewy neurites[1]. This disease continues to progress with time and exacts a substantial burden on the patients, families of patients, and caregivers. The major issue with this disease is that it is easily missed, and a wrong diagnosis is very common[2]. There is currently no cure for PD though several treatments are available to alleviate the symptoms and maintain the quality of life to an extent[3].

The disease is so complex to understand that the series of molecular events ultimately resulting in PD have still not been discovered since the time of scientific description provided by James Parkinson in 1817[4]. Its cause has been ascribed to environmental and genetic factors. The disease's overall etiology is multifactorial, involving various genes, environmental exposure, gene-environmental interactions, and their combined effect on the aging brain[1].

Most PD cases are sporadic (idiopathic) in nature. However, the discovery of monogenic, hereditable forms of the disease has been crucial in understanding the cellular mechanisms underlying the pathophysiology of PD[5]. Several mutated genes lead to a familial (hereditable) form of PD, many of which might not have been discovered. The recessive form of PD caused by mutations in parkin, phosphatase and tensin homolog-induced putative kinase-1 (*PINK-1)* and deglycase-1 (*DJ-1)* genes lacks the clinical and pathological features underlying sporadic PD, especially the characteristic Lewy bodies (LBs) in the surviving neurons[6]. Mutations in the second set of genes encoding alpha-synuclein (*SNCA*) and leucine-rich repeat kinase 2 (*LRRK2*) result

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in a dominantly inherited form of the disease exhibiting LB pathology and is almost indistinguishable from the sporadic form of the disease. The SNCA and LRRK2 proteins are involved in important cellular functions such as synaptic vesicle trafficking, lysosomal autophagic pathway, microtubule-based transport, neurotransmitter release, mitochondrial integrity, and inflammation implying that they may share certain common pathways[7-14].

Drosophila melanogaster (fruit fly) is an effective model invertebrate organism to study genetic and neurodegenerative disorders due to the easy manipulation and the availability of several experimental techniques and tools. It is amenable to genetic modifications such as insertion, deletion, and overexpression of genes[15]. It shows several morphological and functional similarities to vertebrates with respect to the nervous system, and it has been found that 77% of disease-causing genes in humans have orthologs in fruit flies[16].

Through our research using *Drosophila melanogaster,* we wanted to bring to focus the effect of co-expression of *SNCA* (full length) and *LRRK2* (full length and mutant form LRRK2-G2019S) on cells of the nervous system and particularly DA neurons and glia. The specific loss of DA neurons observed in the brains of PD patients and the role played by glia in neuroinflammation and neurodegeneration made them important targets of study[17]. Our research will develop a model to decipher the cellular pathways of PD and understand the interplay and the role of SNCA and LRRK2 in the pathophysiology of PD. We expect our model to serve as an effective tool to identify new targets and screen therapeutic interventions that can help halt disease progression.

Materials and Methods

Drosophila stocks

*UAS-LRRK2*and*UAS-LRRK2-G2019S* transgenic flies were gifted by Dr. Patrik Verstreken from VIB Center for the Biology of Disease and KU Leuven, Center for Human Genetics, and Leuven Institute for Neuroscience and Disease, Leuven, Belgium. *Elav*-GAL4 (on the 1st chromosome)and thedouble balancers*(FM7i//Y; Tfd//CyOGFP*; +, +//Y; *If // CyO*; *MKRS // TM6, Tb)*were provided as a gift by Ravikant Yadav, Dr. Indira Paddibhatla, and Dr. G. B. Madhu Babufrom the Department of Biotechnology, School of Life Sciences, University of Hyderabad, Hyderabad, India. *UAS-SNCA* stock was procured from the Bloomington Drosophila Stock center, Indiana University, USA. *Elav-GAL4* (on the 2nd chromosome) and *TH-GAL4* were gifted by Diana Rodrigues and Dr. Gaiti Hasan, respectively, from National Centre for Biological Sciences (NCBS), Bangalore, India. *Repo-GAL4, UAS-GFP,* and other supporting balancer stocks were gifted by Dr. Richa Rikhy and Dr. Girish Ratnaparkhi from the Indian Institute for Scientific Education and Research (IISER), Pune, India. Details of fly stocks can be found in section 1 of supplementary experimental procedures. *GAL4* stocks were validated for their ability to activate the expression of target genes downstream of *UAS* in the correct subsets of cells. The validation procedures are detailed in section 2.4 of supplementary experimental procedures.

Validation of GAL4 fly stocks:

Green fluorescent protein (GFP) was used as the reporter gene to validate *elav-GAL4* and*repo-GAL4* lines. *TH-GAL4* fly line was validated by NCBS before dispatch.

Validation of elav-Gal4 stock

Adult male flies carrying the *elav-GAL4* gene were crossed with virgin females carrying *UAS-GFP* gene (as outlined in cross 1 of supplemental experimental procedures) and the setup was kept at 29ºC. The F1 wandering 3rd instar larvae were picked up from the food medium and observed under a fluorescence microscope through FITC filter. The translucent larvae, whose brains exhibited green fluorescence were separated from the larvae in which no fluorescence was observed and kept in separate food vials to allow them to grow into adults.

Validation of repo-Gal4 stock

Adult male flies carrying the *repo-GAL4* gene were crossed with virgin females carrying *UAS-GFP* gene (as outlined in cross 2 of supplemental experimental procedures) and the setup was kept at 29ºC. The F1 3rd instar larvae were picked up from the food medium and observed under a fluorescence microscope using FITC filter. Phenotypically different larvae, normal and tubby (length shorter than normal) were separated and kept in separate food vials to allow them to grow into adults.

Generation of fly lines individually expressing SNCA, LRRK2, and LRRK2-G2019Sin cell subtypes

Flies individually expressing SNCA, LRRK2,and LRRK2-G2019Sin all neurons, glia, and

dopaminergic neurons were generated with the help of the *GAL4-UAS* system by setting up a series of crosses as outlined in section 2.2crosses 3-11 of supplemental experimental procedures.This system takes advantage of the yeast *GAL4* transcription factor, which specifically binds to an upstream activating sequence (*UAS*) linked to transgenes of interest. Thus, *UAS*-linked genes can be expressed in specific cell types under the control of a given promotor (*promotor-GAL4*). The system expressed the target genes and the associated proteins in cells of interest. Male flies individually harboring the *elav*, *repo,* and *THGAL4* transcription factors were allowed to mate with female flies individually containing the *SNCA, LRRK2,* and*LRRK2-G2019S* target genes at 25°C[18].

Generation of fly lines co-expressing SNCA with LRRK2 and LRRK2-G2019Sin cell subtypes

Flies co-expressing SNCAwithLRRK2 and SNCA with LRRK2-G2019S in all neurons, glia, and dopaminergic neuronswere generated with the help of the *GAL4-UAS* system by setting up a series of crosses as outlined in section 2.3 crosses 12-30 in supplemental experimental procedures.

Validation of co-expression of SNCA and LRRK2by GAL4 drivers in experimental flies

The co-expression of SNCA and LRRK2 and SNCA and LRRK2-G2019S in all neurons, glia, and specifically dopaminergic neuronswas confirmed by western blotting (procedure described in detail in section 2.5 of supplemental experimental methods).

Survival analysis

Cohorts of 60 flies from each genotype were monitored for survival[19]. Freshly eclosed experimental flies were transferred to fresh food every few days, and their mortality was scored daily. The control flies used were *elav-GAL4*, *repo-GAL4*, and *TH-GAL4*.

Climbing assay

The locomotor ability of flies of each genotype was evaluated using a climbing assay known as the negative geotaxis assay[20,21]. Cohorts of 60 flies per sex of each genotype were selected for the experiment. The flies were age-matched and chosen randomly. Of the 60 flies, 10 flies per sex were transferred to a glass vial (25 cm in length and 2 cm internal diameter) and allowed to acclimatize for a minute. The flies were gently tapped to the bottom of the vial, and those crossing a 10 cm mark within 18 seconds were counted. The experiment was repeated for 60 flies and performed every week starting from week 1 till the time of their death. Ten trials were conducted for each time point, and the average of the trials was calculated and expressed in the form of the percentage of climbing activity of the flies. The control flies used were *elav-GAL4,repo-GAL4*, and *TH-GAL4*.

Immunostaining and dopaminergic cell counting

Fluorescent immunostaining was performed on whole-mount dissected adult brains of flies of each genotype of 5 weeks of age. Five flies per genotype were used for immunostaining.

Dissected brains were transferred to a 2% paraformaldehyde solution prepared in PBS pH 7.4 in 0.5 mL microcentrifuge tubes and kept for fixation for 55 min at 29ºC. The fixed brains were washed 4 times for 20 min each at RT with PAT buffer (1% bovine serum albumin and 0.5% Triton-X 100 in PBS). The washed brains were blocked for an h with a combination of PAT buffer and 3% normal donkey serum (blocking solution). After blocking, the brains were incubated with rabbit anti-tyrosine hydroxylase (TH) antibody (1:200) prepared in blocking solution for approximately 21 hs at 4ºC and then transferred to RT for an h. Once the incubation period was over, the brains were washed 4 times for 20 min each at RT with PAT buffer and incubated with donkey anti-rabbit Alexa fluor 594 secondary antibody (1:500) prepared in blocking solution for approximately 22 hs at 4ºC. The brains were washed 4 times for 20 min each with PAT buffer. One of the washing steps included staining with 4′,6-diamidino-2-phenylindole(DAPI). All steps were carried out by placing the microcentrifuge tubes containing the brains on a nutator[22].

Two coverslips were arranged on a microscope slide to form a bridge around the brains. The coverslips were sealed in place with clear nail paint. A P-200 pipette tip was cut at the end and used to transfer the brains from the 0.5 mL microcentrifuge tubes to the glass slide between the two sealed coverslips. The brains were aligned properly with the help of forceps for ease of imaging. SlowFade™ Gold Antifade mountant was added over the brains using a pipette. A no. 1 coverslip was placed on the bridge to seal the brains effectively and sealed with clear nail paint[23].

Confocal microscopy of whole-mount adult brains was carried out using the Leica DMi8 microscope with an SP8 scanner. Images were taken with a slice

thickness of 4 µm, and Z stacks were taken of all samples. The anti-TH labeled cells were counted through each Z stack using Fiji software. The number of cells for PPL1, PPL2, PPM1/2, and PPM3 DA neuronal clusters was recorded for 5 brains per genotype[24].

Evaluation of protein expression by western blotting

Sample preparation

Adult fly heads of flies of all genotypes of 5 weeks of age were homogenized in 400 µl RIPA buffer in the presence of 4 µl protease cocktail inhibitor and were incubated on ice for 10 min. The samples were centrifuged for 15 min at 14,000×g at 4°C. The supernatant was aspirated and placed in a fresh tube kept on ice discarding the pellet. Protein quantification was performed using the BCA method as per the manufacturer's protocol (Pierce BCA protein assay kit, catalog no.: 23225). Determining the quantity of protein to be loaded an equal volume of 2X Laemmeli sample buffer was added to the sample and heated at 95°C for 6 min to denature the sample. The denatured sample was aliquoted and stored at −80°C for future use.

Statistical analysis

Kaplan-Meier method was used to analyze survival data, and p values were calculated by two-way analysis of variance (ANOVA) followed by *posthoc*Bonferroni test for negative geotaxis assay and number of tyrosine hydroxylase positive neurons per cluster. Prism version 7.0 was used for the analysis.

Results

Validation of *GAL4* fly lines

Validation of elav-Gal4 stock

Adults obtained from larvae exhibiting fluorescence had straight wings and the ones obtained from larvae not exhibiting fluorescence had curled wings. The phenotypes matched the genotypes as outlined in cross 1 of section 2.1 of supplementary experimental procedures. Figures 1A and 1B exhibit a larva exhibiting fluorescence and a larva not showing any fluorescence.

Validation of repo-Gal4 stock

Normal-sized larvae (Fig. 2A) exhibited fluorescence and tubby larvae (Fig. 2B) did not exhibit fluorescence. The phenotypes matched the genotypes as outlined in cross 2 of section 2.1 of supplementary experimental procedures. The fluorescence and lack thereupon was observed in the larval brain as shown in (Fig. 3A & B).

Fly lines generated on individual expression ofSNCA, LRRK2, and LRRK2-G2019Sin cell subtypes:

F1 progeny of the following genotypes were generated in the process:

1. *UAS-wild type SNCA// elav-GAL4*
2. *UAS-wild type LRRK2// elav-GAL4*
3. *UAS-wild type G2019S LRRK2// elav-GAL4*
4. *UAS-wild type SNCA//+; repo-GAL4//+*
5. *UAS-wild type LRRK2//+; repo-GAL4//+*
6. *UAS-wild type G2019S LRRK2//+; repo-GAL4//+*
7. *UAS-wild type SNCA//+; TH-GAL4//+*
8. *UAS-wild type LRRK2//+; TH-GAL4//+*
9. *UAS-wild type G2019S LRRK2//+; TH-GAL4//+*

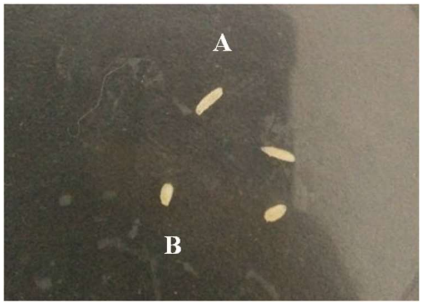

Fig. 2 — (A) Normal sized F1 larvae carrying both the GFP and *repo* genes and exhibit fluorescence; (B) Dwarf sized F1 larvae (tubby) larvae carrying only GFP but no *repo*-GAL4 do not exhibit fluorescence

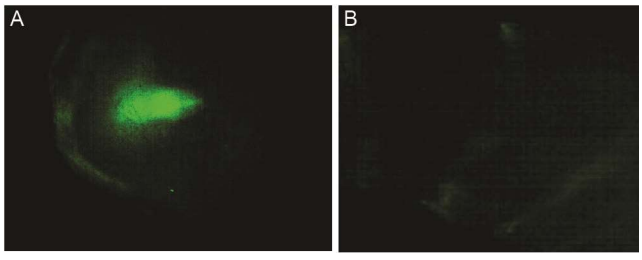

Fig. 1 — (A) Brain of a 3rd instar larva exhibiting green fluorescence due to expression of GFP in its neurons by *elav*-GAL4, and (B) Brain of a 3rd instar larva could not be observed due to lack of GFP expression

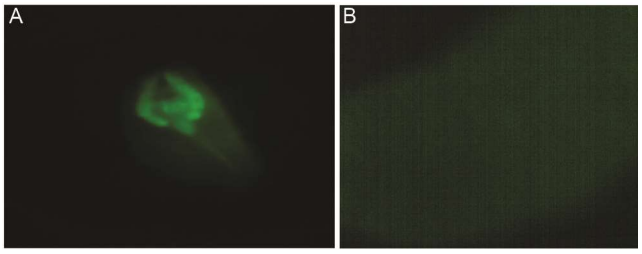

Fig. 3 — (A) Brain of a 3rd instar larva exhibiting green fluorescence due to expression of GFP in its glia by *repo*-GAL4; and (B) Brain of a 3rd instar larva could not be observed due to lack of GFP expression

Fly lines generated on co-expression of SNCA with LRRK2 and G2019S in cell subtypes:

F1 progeny of the following genotypes were developed in the process:

1. *elav-GAL4*; *UAS-wild type SNCA//UAS-wild type LRRK2*
2. *elav-GAL4*; *UAS-wild type SNCA//UAS-wild type LRRK2 G2019S*
3. *UAS-wild type SNCA//UAS-wild type LRRK2; repo-GAL4/+*
4. *UAS-wild type SNCA//UAS-wild type LRRK2 G2019S; repo-GAL4/+*
5. *UAS-wild type SNCA//UAS-wild type LRRK2; TH-GAL4/+*
6. *UAS-wild type SNCA//UAS-wild type LRRK2 G2019S; TH-GAL4/+*

Survival analysis

Expression of SNCA, LRRK2, and LRRK2-G2019S in all neurons of flies cause early mortality

Individual expression of SNCA, LRRK2, and LRRK2-G2019S and co-expression of SNCA with LRRK2 and LRRK2-G2019S in a pan-neuronal manner were found to affect fly viability, causing premature mortality compared to control flies, as shown in (Fig. 4A & B). The ages at which 50% of transgenic SNCA, LRRK2, and LRRK2-G2019S flies survived were 40, 43, and 45 days, respectively. The age at which 50% of transgenic flies co-expressing SNCA and LRRK2 in neurons survived was found to be 40 days. 50% of flies co-expressing SNCA and LRRK2-G2019S in neurons survived for 35 to 37 days with slight gender-based differences.

Expression of SNCA, LRRK2, and LRRK2-G2019S in glia of flies cause early mortality

SNCA, LRRK2, and LRRK2-G2019S expressed individually and in combination (SNCA with LRRK2 and LRRK2-G2019S) in glial cells of flies with the help of *repo-GAL4* driver caused increased mortality compared to control flies, as shown in (Fig. 4C & D). The ages at which 50% of the flies expressing SNCA, LRRK2, and LRRK2-G2019S survived were 46, 47, and 45 days, respectively.50% of female and male

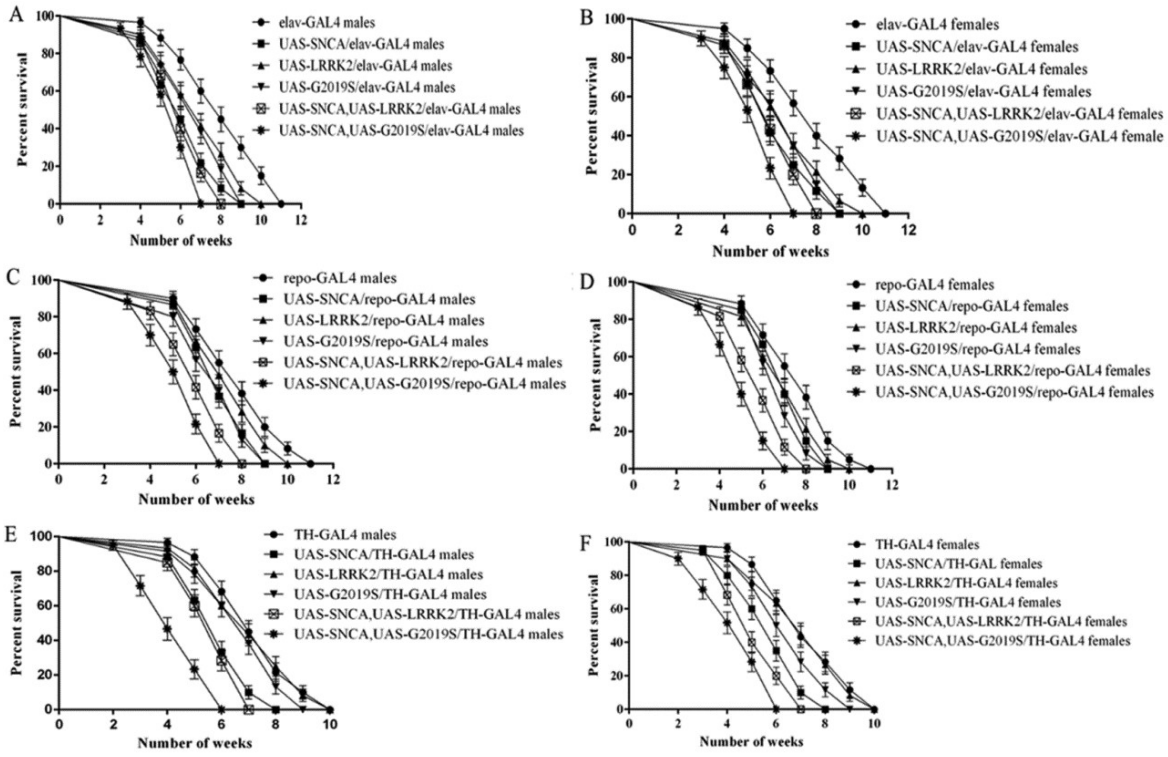

Fig. 4 — Expression of SNCA, LRRK2 and LRRK2-G2019S in all neurons, glia and dopaminergic neurons of flies cause early mortality. (A) Survival curves of male flies individually and co-expressing SNCA, LRRK2, and LRRK2-G2019S in all neurons; (B) Survival curves of female flies individually and co-expressing SNCA, LRRK2, and LRRK2-G2019S in all neurons; (C) Survival curves of male flies individually and co-expressing SNCA, LRRK2, and LRRK2-G2019S in glia; (D) Survival curves of female flies individually and co-expressing SNCA, LRRK2, and LRRK2-G2019S in glia; (E) Survival curves of male flies individually and co-expressing SNCA, LRRK2, and LRRK2-G2019S in dopaminergic neurons; and (F) Survival curves of female flies individually and co-expressing SNCA, LRRK2, and LRRK2-G2019S in dopaminergic neurons

flies co-expressing SNCA and LRRK2 survived for 37 and 42 days, respectively. The ages at which 50% of the female and male flies expressing SNCA and LRRK2-G2019S in glia were found to be 33 and 35 days, respectively.

Expression of SNCA, LRRK2, and LRRK2-G2019S in DA neurons of flies cause early mortality

Individual expression of SNCA, LRRK2, and LRRK2-G2019S and co-expression of SNCA with LRRK2 and LRRK2-G2019S in DA neurons of flies by *TH-GAL4*were found to reduce their life span compared to control flies, as shown in (Fig. 4E & F). The ages at which 50% of the flies expressing SNCA and LRRK2 survived were found to be 38 and 47 days. 50% of female and male flies expressing LRRK2-G2019S survived for 42 and 45 days, respectively. The ages at which 50% of female and male flies co-expressing SNCA and LRRK2 survived were 32 and 37 days, respectively. Twenty-nine days was the age at which 50% of flies co-expressing SNCA and LRRK2-G2019S were found to survive.

Climbing assay (assessing impairment in locomotion of flies)

Expression of SNCA, LRRK2, and LRRK2-G2019S in all neurons of flies cause locomotor impairment

Flies individually and co-expressing SNCA, LRRK2, and LRRK2-G2019S in all neurons exhibited a significant impairment in locomotion as compared to control flies, as shown in (Fig. 5A & B). The climbing activity of flies expressing SNCA was reduced to 50% at approximately 4 weeks compared to about 6.5 weeks for control flies. Flies expressing LRRK2 and LRRK2- G2019S reduced climbing activity to 50% at approximately 4.5 weeks compared to approximately 6.5 weeks for control flies. No significant difference in locomotion was observed between male and female flies. Flies co-expressing

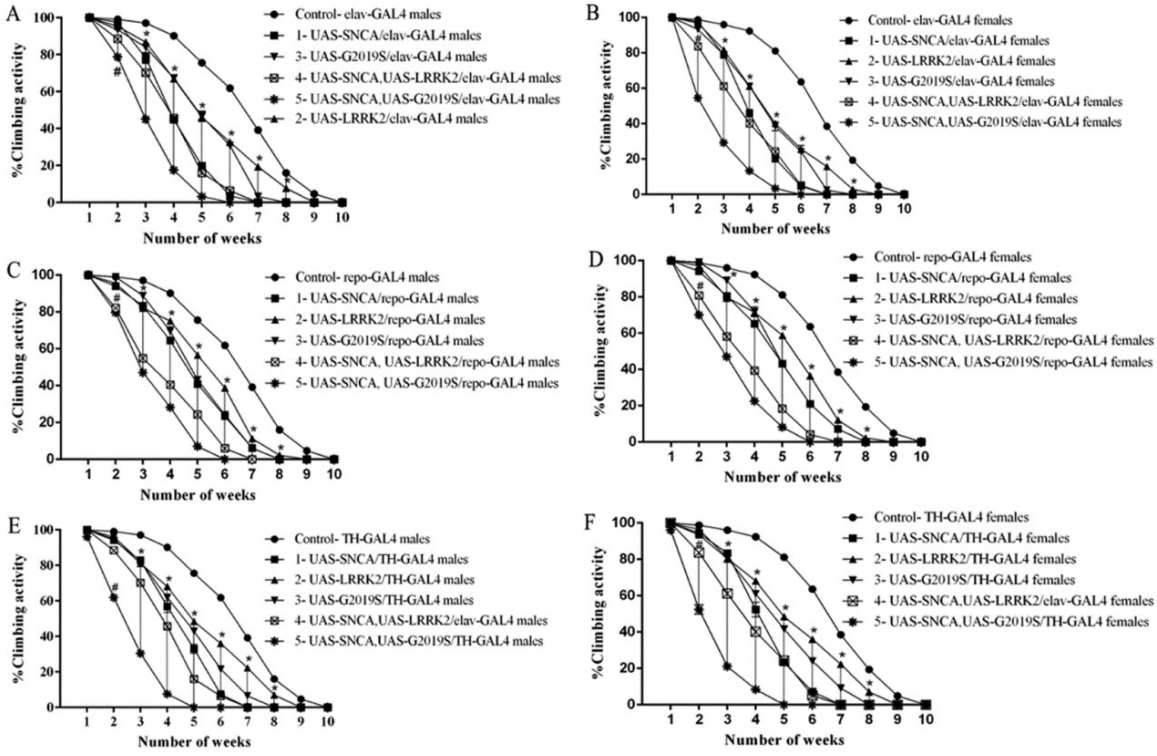

Fig. 5 — Expression of SNCA, LRRK2 and LRRK2-G2019S in all neurons, glia and dopaminergic neurons of flies cause locomotor impairment. (A) Male flies individually and co-expressing SNCA, LRRK2, and LRRK2 G2019S in all neurons subjected to climbing assays; (B) Female flies individually and co-expressing SNCA, LRRK2, and LRRK2 G2019S in all neurons subjected to climbing assays; (C) Male flies individually and co-expressing SNCA, LRRK2, and LRRK2 G2019S in glia subjected to climbing assays; (D) Female flies individually and co-expressing SNCA, LRRK2, and LRRK2 G2019S in glia subjected to climbing assays; (E) Male flies individually and co-expressing SNCA, LRRK2, and LRRK2 G2019S in dopaminergic neurons subjected to climbing assays; and (F) Female flies individually and co-expressing SNCA, LRRK2, and LRRK2 G2019S in dopaminergic neurons subjected to climbing assays; Statistically significant differences between control and experimental flies indicated by *, $P< 0.0001$

* indicates statistical significance for all fly genotypes numbered 1 to 5

# indicates statistical significance for fly genotypes numbered 4 and 5

SNCA and LRRK2 in neurons reduced climbing activity by 50% at approximately 3.5 weeks compared to 6.5 weeks shown by control flies. Female and male flies co-expressing SNCA and LRRK2-G2019S in neurons exhibited a 50% reduction in climbing ability at approximately 2 and 3 weeks, respectively, as compared to 6.5 weeks shown by control flies.

Expression of SNCA, LRRK2, and LRRK2-G2019S in glia of flies cause locomotor impairment

Flies individually and co-expressing SNCA, LRRK2, and LRRK2-G2019S in glia exhibited a significant impairment in locomotion compared to control flies, as shown in (Fig. 5C & D). The climbing activity of flies expressing *SNCA* was reduced to 50% at approximately 4.5 weeks compared to more than 6.5 weeks for control flies. Flies expressing LRRK2 and LRRK2-G2019S reduced climbing activity to 50% at approximately 5 weeks compared to more than 6.5 weeks for control flies. No significant difference in locomotion was observed between male and female flies.The climbing activity of flies co-expressing SNCA and LRRK2 in glia was reduced to approximately 3.5 weeks compared to more than 6.5 weeks shown by control flies. Flies co-expressing SNCA and LRRK2-G2019S exhibited a 50% reduction in climbing ability at about 3 weeks, respectively, compared to more than 6.5 weeks exhibited by control flies.

Expression of SNCA, LRRK2, and LRRK2-G2019S in DA neurons cause locomotor impairment

The climbing activity of flies expressing SNCA was reduced to approximately 50% at 4.5 weeks compared to 6.5 weeks for control flies shown in (Fig. 5E & F). Flies expressing LRRK2 and LRRK2-G2019S reduced climbing activity to approximately 50% at 5 weeks compared to 6.5 weeks for control flies. No significant difference in locomotion was observed between male and female flies. Flies co-expressing SNCA and LRRK2-G2019S in DA neurons exhibited a 50% reduction in climbing ability at approximately 2 weeks, respectively, compared to more than 6.5 weeks exhibited by control flies.

Immunostaining and counting of tyrosine hydroxylase (TH) positive neurons

Expression of SNCA, LRRK2, and LRRK2-G2019S in all neurons of flies cause DA neuronal death

The number of TH-positive neurons by anti-TH staining in brains of *elav-GAL4* control flies at 5 weeks of age has been shown in (Fig. 6). Flies individually and co-expressing SNCA, LRRK2, and LRRK2-G2019S in a pan-neuronal manner exhibited a significant decrease in the number of TH-positive neurons at 5 weeks of age as compared to control flies. There was statistically significant DA neuronal loss in PPL1, PPM1/2, and PPM3 clusters, which were the 3 clusters examined. The cells in the PPM3 cluster were completely lost in all 5 sets of experimental flies by 5 weeks of age. A significant difference was observed in the number of TH positive neurons in all the 3 clusters between flies individually expressing SNCA, LRRK2, and LRRK2-G2019S and flies co-expressing SNCA with LRRK2 and LRRK2-G2019S in all neurons.

Expression of SNCA, LRRK2, and LRRK2-G2019S in glia of flies cause DA neuronal death

The number of TH-positive neurons as monitored by anti-TH staining in brains of *repo-GAL4* control flies at

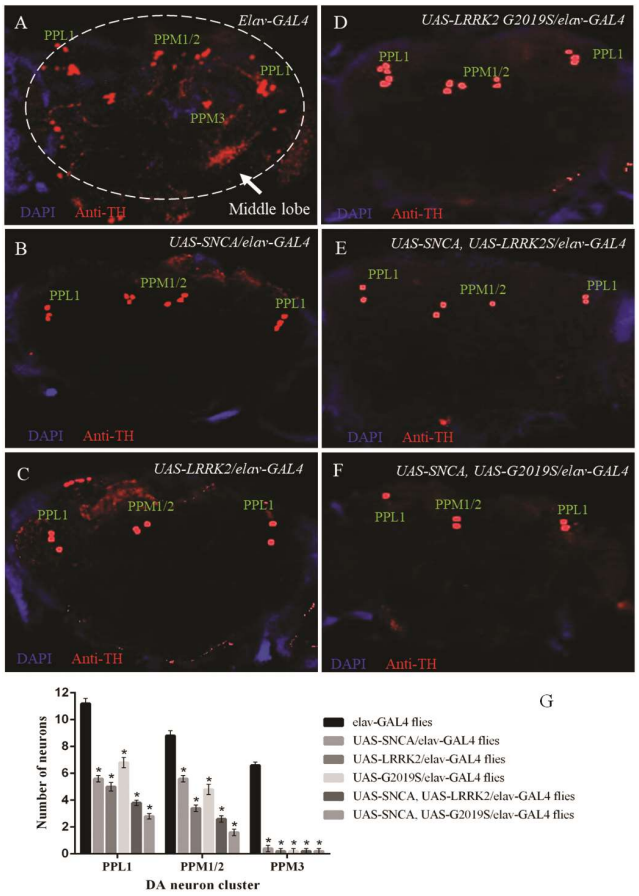

Fig. 6 — Individual and co-expression of SNCA, LRRK2, and G2019S in all neurons by *elav-GAL4* driver induced loss of TH-positive DA neurons. (A-F) Representative images of anti-TH immunostaining of DA clusters of 5-week-old flies of the indicated control and experimental flies, and (G) Average number of TH-positive neurons per DA cluster in 5-week-old flies of the indicated control and experimental flies. Statistically significant differences between control and experimental flies marked by *, $P < 0.0001$

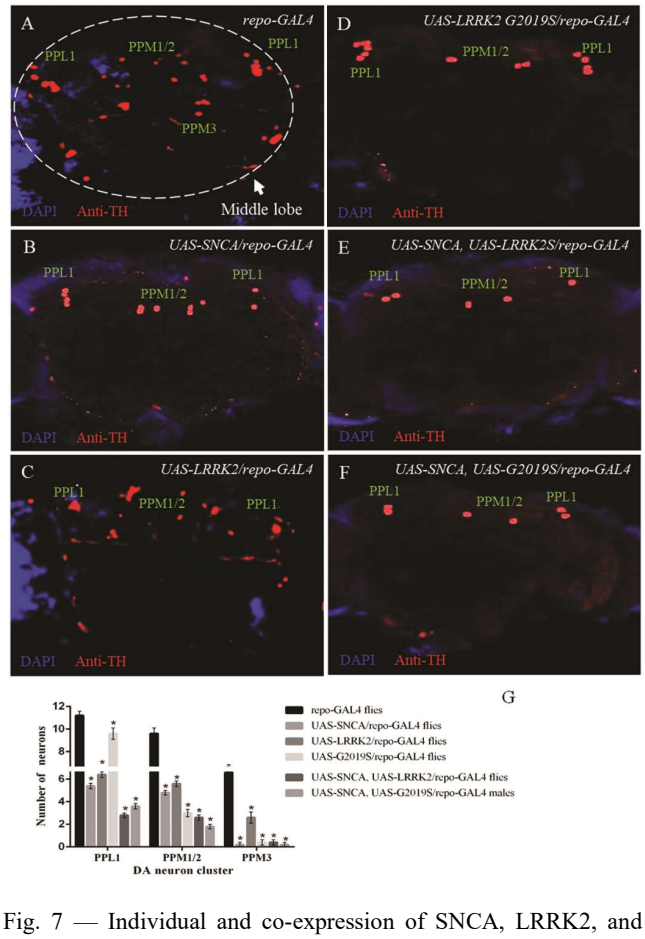

Fig. 7 — Individual and co-expression of SNCA, LRRK2, and G2019S in all neurons by *repo-GAL4* driver induced loss of TH-positive DA neurons. (A-F) Representative images of anti-TH immunostaining of DA clusters of 5-week-old flies of the indicated control and experimental flies, and (G) Average number of TH-positive neurons per DA cluster in 5-week-old flies of the indicated control and experimental flies. Statistically significant differences between control and experimental flies marked by *, $P< 0.0001$

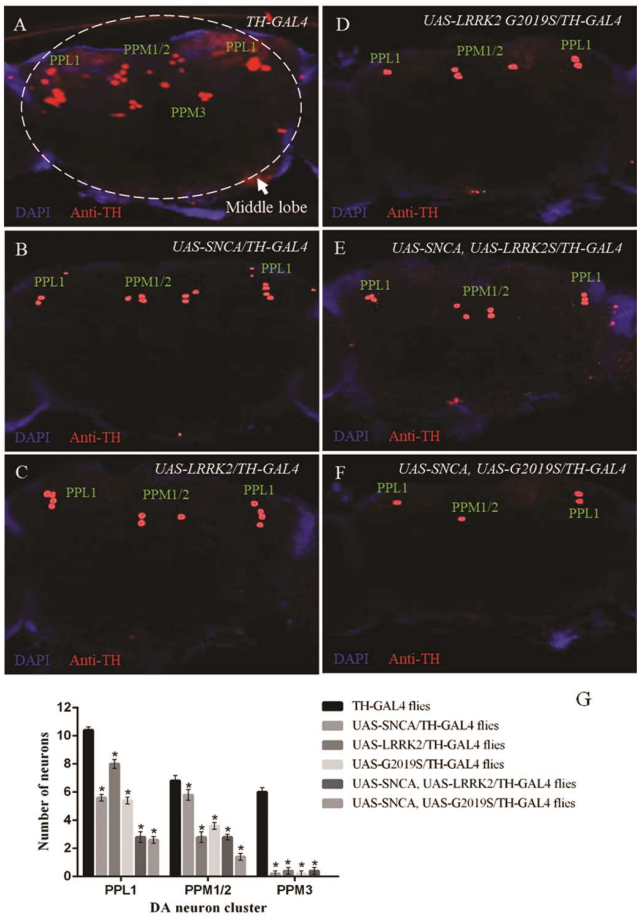

Fig. 8 — Individual and co-expression of SNCA, LRRK2, and G2019S in all neurons by *TH-GAL4* driver induced loss of TH-positive DA neurons. (A-F) Representative images of anti-TH immunostaining of DA clusters of 5-week-old flies of the indicated control and experimental flies, (G) Average number of TH-positive neurons per DA cluster in 5-week-old flies of the indicated control and experimental flies. Statistically significant differences between control and experimental flies marked by *, $P< 0.0001$

5 weeks of age has been shown in (Fig. 7). Flies individually and co-expressing SNCA, LRRK2, and LRRK2-G2019S in glia at 5 weeks of age exhibited a significant reduction in anti-TH staining compared to control flies. A significant decrease in the number of TH-positive DA neurons was recorded in the PPL1, PPM1/2, and PPM3 clusters of experimental flies. A significant difference was observed in the number of TH-positive neurons in all the 3 clusters between flies individually expressing SNCA, LRRK2, and LRRK2-G2019S and flies co-expressing SNCA with LRRK2 and LRRK2-G2019S in glia.

Expression of SNCA, LRRK2, and LRRK2-G2019S in DA neurons of flies cause DA neuronal death

The number of TH-positive neurons of *TH-GAL4* control flies at 5 weeks of age has been shown in (Fig. 8). Individual and co-expression of SNCA, LRRK2, and LRRK2-G2019S, specifically in DA neurons of flies, resulted in a significant reduction in the number of TH positive neurons at 5 weeks of age in comparison to control flies. A marked decrease in TH-positive neurons was observed in flies co-expressing SNCA with LRRK2 and LRRK2-G2019S. The PPL1, PPM1/2, and PPM3 clusters decreased in their neuronal numbers in flies expressing SNCA, LRRK2, and LRRK2-G2019S in TH-positive neurons. A significant difference was observed in the number of TH positive neurons in all the 3 clusters between flies individually expressing SNCA, LRRK2, and LRRK2-G2019S and flies co-expressing SNCA with LRRK2 and LRRK2-G2019S in DA neurons.

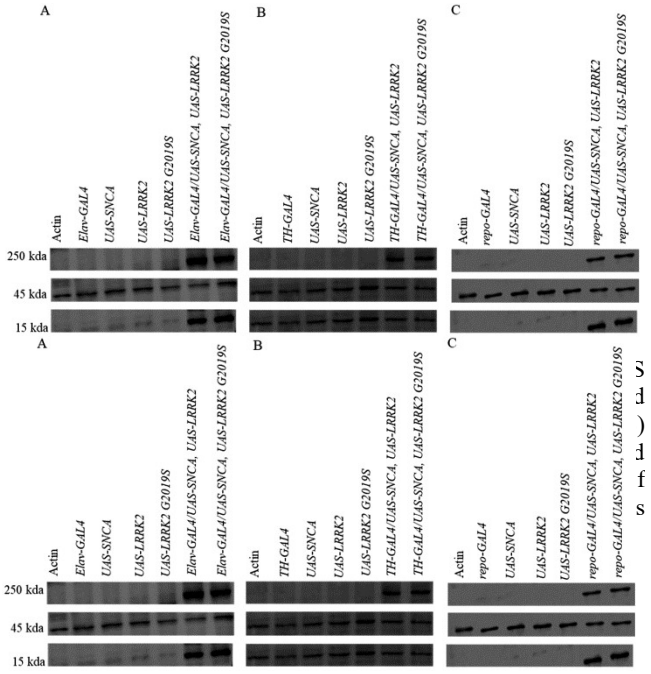

Fig. 9 — SNCA co-expressed with LRRK2 and LRRK2-G2019S in the fly brain. (A) Western blot bands of SNCA co-expressed with LRRK2 and LRRK2-G2019S proteins in all neurons; (B) Western blot bands of SNCA co-expressed with LRRK2 and LRRK2-G2019S proteins in glia; and (C) Western blot bands of SNCA co-expressed with LRRK2 and LRRK2-G2019S proteins in all dopaminergic neurons

Expression of SNCA, LRRK2, and LRRK2-G2019S proteins in the fly brain under the influence of cell-specific transcription factors

Flies individually expressing SNCA, LRRK2, and LRRK2-G2019S in all neurons, glia, and DA neurons exhibited single protein bands as shown in (Fig. 9A-C), respectively. However, flies co-expressing SNCA with LRRK2 and LRRK2-G2019S in all neurons, glia, and DA neurons exhibited protein bands of both the proteins as shown in (Fig. 9A-C), respectively.

Discussion

Mutations in *SNCA* and *LRRK2* lead to typical PD-like neuropathological features such as Lewy body formation, degeneration of DA neurons, and motor dysfunction[6]. These mutations have been associated with clinical progression and phenotypic exhibition similar to the idiopathic late-onset form of the disease. LRRK2 seems to play a central role in the pathophysiology of PD as it interacts with many proteins associated with neurodegeneration[7]. Mutations in LRRK2 resulting in loss or gain of function may influence mechanisms involving SNCA by affecting various cellular pathways resulting in its accumulation. This forms one of the major hypotheses postulated by PD researchers in identifying mechanisms involved in the pathogenesis of PD.

Several studies have indicated potential pathophysiological interplay between the two proteins[25-27]. Our research involved generating transgenic fruit flies over expressing human wild-type *SNCA* with wild-type and mutated forms of *LRRK2* in neurons and glia to investigate whether SNCA and LRRK2 act synergistically or influence each other's cellular functions. We also wanted to develop a model which would mimic the pathophysiology of the disease similar to idiopathic PD. The research aimed at observing the effect of co-expression of SNCA and LRRK2 on two hallmark features of PD: motor dysfunction assessed *via* negative geotaxis assay and DA neuronal degeneration evaluated via loss of TH positive neurons in the fly brain. We also wanted to determine whether expressing the two proteins in the glia affects the neurodegenerative process in the same way as the expression in DA neurons or in a pan-neuronal manner.

Fruit flies expressing wild SNCA and LRRK2 (wild and mutant) exhibited early mortality, locomotor impairment, and loss of DA neurons, similar to results published by Liu *et al*[28] and Feany *et al* [29]. It was observed that flies individually expressing SNCA and mutant form of LRRK2 (LRRK2-G2019S) in all the neurons exhibited a higher mortality rate compared to flies expressing wild-type LRRK2. Co-expression of SNCA with LRRK2 and LRRK2-G2019S in a pan-neuronal manner further reduced the number of weeks showing 100% mortality. The survival curves of flies individually and co-expressing the target genes were found to be significantly different, implying that co-expression of SNCA with either wild-type or mutant forms of LRRK2 had a profound and toxic impact on the life span of flies as compared to individual expression of the target genes in all the neurons.

SNCA, LRRK2, and LRRK2-G2019S expressed in glia also reduced the life span of flies and the results were found to be comparable to when the genes were expressed in neurons. Also, the survival curves of flies individually and co-expressing SNCA, LRRK2, and LRRK2-G2019S were significantly different. This result opened an interesting avenue since the expression of PD-associated genes in the glia resulted in the manifestation of all the hallmark features observed on expression of the same genes in neurons. This phenomenon may be explained by applying the prion theory of propagation, which suggested that

proteins prone to misfolding, in our case SNCA, induced a similar misfolding in neighboring protein molecules and propagated in tissues via cell-to-cell contact[30] [31]. This indicates a possibility that the proteins expressed in neurons might propagate to the neighboring glial cells and vice versa. Another way of explaining this phenomenon may be by relating it to the Braak theory, which demonstrated that PD involved multiple cell types and resulted from changes arising from a few susceptible neurons. It was shown by Braak that the pathology developed in the anterior olfactory nucleus observed in the autopsied brains of PD patients and continued right upto the neocortex affecting multiple cell types[32].

On comparing the life span of flies individually expressing SNCA and flies co-expressing SNCA with wild-type LRRK2, it was found that both fly genotypes showed 100% mortality at similar time points. This might be due to overexpression of SNCA in flies expressing it individually compared to flies co-expressing it with LRRK2, which might have happened due to differences in the external environment of the flies or the functioning of the *GAL4-UAS* system. It can also be hypothesized that LRRK2 might not have a significant effect on the cellular effects of SNCA when co-expressed specifically in DA neurons, although 50% of flies survived for 38 days when they were individually expressing SNCA and LRRK2, and they survived for 32 days when they were co-expressing the two genes. The difference in the days survived may not seem significant, but considering of lieu with the life span of fruit flies, it can be regarded as significant enough. Moreover, flies co-expressing SNCA and LRRK2-G2019S in DA neurons exhibited a much-reduced life span reinforcing that both LRRK2 and SNCA affect cellular mechanisms causing PD.

Locomotion was grossly preserved in young control and experimental flies till 2 weeks. As the control flies aged, they were no longer able to climb rapidly in the vial but instead made short abortive climbs and fell back to the bottom of the vial. However, the performance of experimental flies over time declined more rapidly than that of the control flies. The climbing pattern of transgenic flies individually and co-expressing SNCA with LRRK2 and LRRK2-G2019S in all neurons, glia, and DA neurons started significantly deteriorating after 2 weeks.

The deterioration was most pronounced in flies co-expressing SNCA and LRRK2-G2019S, specifically in DA neurons. This statistically significant change may reflect enhanced cellular toxicity of the mutant form of G2019S which the expression of SNCA may exacerbate.

Compared to individual expression, the progressive, accelerated decline in climbing ability in experimental flies co-expressing SNCA with either LRRK2 or LRRK2-G2019S demonstrated a severe functional deficit produced by their expression in the nervous system. This reiterated that the expression of SNCA and LRRK2 appears to be co-regulated, and both seem to have a deteriorating synergistic effect on the nervous system resulting in systematic degeneration.

The severity of phenotype in terms of locomotor dysfunction in flies expressing the target genes in different cell types was similar to data published by Feany *et al* and Liu *et al*. A part of the result deviated from the data published by Liu *et al*., 2007 in which they reported that the expression of LRRK2 in a pan-neuronal manner caused a less severe phenotype in flies than the expression of LRRK2 in DA neurons.

The beginning of the manifestation of symptoms in PD patients is associated with loss of 50-60% of DA neurons[33]. Our results showed that 5-week-old fly brains of all sets of fly genotypes immunostained exhibited more than 60% loss of DA neurons (TH-positive neurons) in all 3 clusters. The DA neuronal death seen in flies co-expressing SNCA with either LRRK2 or LRRK2-G2019S was more significant than individual gene expression through all cell types. The PPM3 cluster failed to exist in flies of all genotypes at 5 weeks. This data indicates that SNCA, LRRK2, and LRRK2-G2019S either caused loss of DA neurons or hampered TH expression, both of which would ultimately result in loss of DA function. Moreover, all flies exhibited parallel kinetics in locomotor dysfunction and DA neuronal loss, indicating a causal relationship between the two abnormalities.

Loss of TH positive neurons in an expression dependent manner can be correlated with the immunoblot (WB) analysis in the fly brain extracts showing comparable levels of expression of SNCA and LRRK2 proteins of flies of all genotypes at 5 weeks of age.

The decrease in life span accompanied by locomotor impairment and loss of DA neurons due to the expression of SNCA, LRRK2, and LRRK2-G2019S in glia may be due to a well-established fact that PD affects the functions and integrity of glial cells, which may result in loss of protective effect of

glial cells leading to a toxic gain of function accelerating the neurodegenerative process[17]. It has also been reported that glial SNCA causes neurodegeneration, which corroborates our data[34].

At comparable protein expression levels, LRRK2-G2019S was found to cause a more severe parkinsonism-like phenotype than wild-type LRRK2. This result is in agreement with a study wherein G2019S mutation increased the kinase activity of LRRK2, thus causing a gain-of-function in the process resulting in toxicity[35].

Our study demonstrated the development of a gain-of-function model expressing two major genes associated with PD. The limitation of our study is that there might be overexpression of LRRK2 proteins relative to the endogenous expression of a fly homolog of *LRRK2* or might have caused, in general, higher expression of proteins that might not be found at a clinical level. It is noteworthy that this model could provide us data beyond any doubt that there exists a connection, a co-regulation between SNCA and LRRK2 function, which is important in the pathophysiology of PD. Our investigations also revealed that expression of SNCA and LRRK2 in different cell types of the nervous system led to a common and specific form of neurotoxicity, thus adding to the weightage placed on theories such as prion propagation[30] and Braak theory[36] of disease progression. The finding of this study needs to be extended to vertebrate systems and can serve as the basis for understanding the underlying molecular pathways of the disease. The model can further be utilized to build up a DNA array and analyze how the co-expression of SNCA and LRRK2 affects expression of other proteins. This step will get us closer to deciphering the various proteins involved in the pathophysiology of PD, thereby facilitating the discovery process for PD drugs. This model can be directly used for screening small molecules that rescue a PD-associated phenotype or can be subjected to genetic screens to identify modifiers of the said phenotype representing new potential targets for the drugs. Many studies have already been carried out by expressing single PD-associated genes in flies and using them for testing bioactive molecules[37 38]. Here the traditional animal models such as rodents become a poor choice for a primary screen since such screens examine the efficacy of tens of thousands of molecules and reduce them to selective molecules with suitable therapeutic efficacy and minimum toxicity[39]. Thus, as developed in our laboratory, double transgenic flies will serve a dual purpose of acting as an effective PD model and primary drug screening tool.

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Conflict of interest

All authors declare no conflict of interest.

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