A GENETIC SCREEN TO DECIPHER DOPAMINERGIC NEUROPROTECTIVE GENES IN A *DROSOPHILA* MODEL

by

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DECLARATION

I, **Ms. Abuno Thepa,** hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form basis for the award of any previous degree to me or to the best of my knowledge or to anybody else, and that the thesis has not been submitted by me for any research degree in any other university.

This is being submitted to the Nagaland University for the degree of Doctor of Philosophy in Zoology.

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CERTIFICATE

This is to certify that the thesis entitled "**A Genetic Screen to Decipher Dopaminergic Neuroprotective Genes in a *Drosophila* Model**" is a record of original research work done by **Ms. Abuno Thepa**. She is a registered research scholar bearing **Regd. No. Ph.D/ZOO/00129** of the Department and has fulfilled all the requirements of Ph.D. regulations of Nagaland University for the submission of the thesis. The work is original and neither the thesis nor any part of it has been submitted elsewhere for the award of any degree or disctinctions. The thesis is therefore, forwarded for adjudication and consideration for the award of degree of **Doctor of Philosophy in Zoology** under Nagaland University.

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Dedicated to:

My family members, for their blessings, prayers, and unconditional support throughout the years of my research work

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ABBREVIATIONS

Aats-met	:	methionyl-tRNA synthetase
aats-val	:	Valyl-tRNA Synthetase
AD	:	Alzheimer's disease
ANOVA	:	analysis of variance
anti-TH	:	anti-tyrosine hydroxylase
APOE	:	Apolipoprotein ε
APP	:	Amyloid precursor protein
Aux	:	Auxilin
BDNF	:	Brain-Derived neurotrophic Factor
BDSC	:	Bloomington Deficiency Stock Center
bgm	:	Bubble gum
BST1	:	Bone marrow stromal cell antigen
cac	:	Cacophony
cDNA	:	Complementary DNA
Clk	:	Clock
CNS	:	Central nervous system
COMT	:	Catechol-o-methyltransferase
comt	:	Comatose
Crag	:	Calmodulin-binding protein related to a Rab3 GDP/GTP exchange protein
CRISPR	:	Clustered regularly interspaced short palindromic repeat
CS	:	Canton-S
CSF	:	Cerebrospinal fluid
Cyc	:	Cycle
DA	:	Dopamine
DAergic	:	Dopaminergic
DAT	:	Dopamine Transporter
DBH	:	Dopamine β Hydroxylase
Dbt	:	Double time
DCTN1 p150	:	Dynactin 1, p150 subunit
Ddc	:	*Dopa decarboxylase*

Df	:	deficiency
dFOXO	:	*Drosophila FOXO*
DNA	:	Deoxyribonucleic acid
dNrd1	:	Nardilysin
DOPAC	:	3,4-Dihydroxyphenylacetic acid
DOPAC	:	DOPA-Dihydroxy-phenylalanine
drd	:	Dropdead
drn1	:	defense repressor1
dsarm	:	*Drosophila* sterile alpha and Armadillo motif
DSBs	:	DNA double-strand breaks
dsDNA	:	Double stranded DNA
dTOR	:	*Drosophila* target of rapamycin
Dysc	:	Dyschronic
eas	:	Easily shocked
ECD	:	Electro chemical detector
EMS	:	Ethyl Methane Sulfonate
ena	:	Enabled
EOPD	:	Early onset Parkinson disease
ERG	:	Electroretinograms
FGF-20	:	Fibroblast growth Factor 20
FGS	:	Forward genetic screens
fh	:	Frataxin
FI	:	Fluorescence intensity
flyGrAM	:	fly Group Activity Monitor
fly-VRL	:	fly vertically rotating arena for locomotion
FOXO	:	Forkhead box O
FXN	:	Frataxin
GABA	:	Gamma-aminobutyric acid
GAK	:	Cyclin G associated kinase
GBA	:	Glucocerebrosidase
GFP	:	Green fluorescent protein
GSK-3β	:	Glycogen Synthase kinase 3β
GWAS	:	Genome-wide association studies
HD	:	Huntington's disease

hiw	:	Highwire
HLA	:	Human Leukocyte antigen
HPLC	:	High performance liquid chromatography
HR	:	Homologous recombination
HSC70/HSPA8	:	Heat shock cognate 71 kDa
HVA	:	Homovanillic acid
IGF	:	insulin-like growth factor
LB	:	Lewy body
L-DOPA	:	Levodopa
LN	:	Lewy neuritis
loe	:	Löchrig/AMPKγ
lrpprc2	:	Leucine-rich pentatricopeptide repeat containing 2
LRRK2	:	Leucine-rich repeat kinase 2
MAO-B	:	Monoamine Oxidase B
MAPT	:	Microtubule-associated protein tau
marf	:	Mitochondrial assembly regulatory factor
Mfn2	:	Mitofusin
MIP	:	Maximum intensity projection
MnSOD	:	Manganese superoxide dismutase
MPTP	:	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	:	Messenger ribonucleic acid
mTOR	:	Mechanistic/mammalian target of rapamycin
NADA	:	N-acetyl dopamine
NBAD	:	N-β-alanyl dopamine
ND23	:	NADH dehydrogenase ubiquinone 23 kDa Subunit
NDD	:	Neurodegenerative disease
NGA	:	Negative geotaxis assay
NGS	:	Normal Goat Serum
NHEJ	:	Non-homologous end joining
NHP	:	Non-Human Primate
NIRF	:	Near-infrared fluorescence imaging
nmnat	:	Nicotinamide mononucleotide adenylyl transferase
NPLP2	:	Neuropeptide like protein 2
Nrf2	:	Nuclear factor erythroid 2–related factor 2

NUCKS	:	Nuclear Casein Kinase and Cyclin dependent kinase substrate 1
Ogdh	:	Oxoglutarate dehydrogenase
PAL	:	Protocerebral anterior lateral
PAM	:	Protocerebral anterior medial
PAM	:	Protospacer adjacent motif
para	:	paralytic
PARK2	:	Parkin
PBS	:	Phosphate-buffered Saline
PBST	:	PBS containing TX-100
PCL	:	Proximal centriole-like structure
PD	:	Parkinson's Disease
Pdha	:	Pyruvate dehydrogenase E1 alpha subunit
PFA	:	Paraformaldehyde
PINK1	:	PTEN Induced Kinase 1
pir	:	Pirouette
Poc1	:	Proteome of centrioles 1
POLG	:	Polymerase gene
PPL	:	Protocerebral posterior lateral
PPM	:	Protocerebral posterior medial
PQ	:	Paraquat
Ras	:	Renin-angiotensin system
RGS	:	Reverse genetic screens
RNA	:	Ribonucleic acid
Sb	:	Stubble
Scox	:	Synthesis of cytochrome c oxidase
SDS-PAGE	:	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
ses B	:	Stress-sensitive B
shi	:	Shibire
sicily	:	severe impairment of CI with lengthened youth
SLC6A3	:	Solute Carrier Family 6 member 3
SN	:	Substantia Nigra
SNCA	:	α-synuclein

SNpc	:	*substantia nigra pars compacta*
SOD	:	Superoxide dismutase
SP	:	sex peptide
Stj	:	Straightjacket
sws	:	Swiss cheese
TALEN	:	Transcription activator-like effector nuclease
TALEs	:	Transcription activator-like effectors
Tb	:	Tubby
TCA	:	Trichloro acetic acid
TH	:	Tyrosine hydroxylase
tko	:	Technical knockout
tpi	:	Triose phosphate Isomerase
TRITC	:	Tetra methylrhodamine
Tsp68C	:	Tetra spanin 68C
TX-100	:	Triton X-100
UAS	:	Upstream Activation Sequence
Vps26	:	Vacuolar protein sorting 26
Vps35	:	Vacuolar protein sorting 35
VUM	:	Ventral unpaired medial
wcy	:	Wacky
WES	:	Whole Exome Sequencing
WGS	:	Whole Genome Sequencing
ZFN	:	Zinc Finger Nuclease

CHAPTER 1

Review of Literature

1.1. Introduction

'Genetic screens' are used to create and screen a mutant population to find genes associated with a specific phenotype. This process leads to several follow-up experiments that examine the function of specific genes or entire pathways in a given disease process (Hartwell et al., 2008). Mutations in about 200 genes have been associated with neurodegenerative diseases (NDDs) and are distinguished by a gradual loss of neuronal function and structure (OMIM; SysID Database). The increased prevalence of NDDs in recent years presents a risk to human health (Parenti et al., 2020). These are characterized by several pathological features, such as aberrant protein aggregation, proteasomal impairment, oxidative stress, and mitochondrial dysfunction (Cooke et al., 2022; Fawcett, 2020). NDDs like Alzheimer's disease (AD) and Parkinson's disease (PD) have both familial and sporadic forms, while Huntington's disease (HD) is exclusively genetic (Ruffini et al., 2020). Approximately 95% of Parkinsonism cases are estimated to have a sporadic component (Zhu et al., 2024). The complex interaction between genetics and environment, on top of slow and sustained neuronal dysfunction brought on by aging (Pang et al., 2019; Coppede, 2012), is what causes sporadic PD in people over 60 years of age. Present therapies for PD aim to control symptoms through dopamine (DA) replacement, yet they offer variable efficacy and can result in notable adverse effects (Kumaran and Cookson, 2015). Genetic analysis has identified mutations that cause familial forms of PD, shedding light on potential disease processes. Yet, applying these discoveries to the prevalent sporadic cases remains uncertain (Kumaran and Cookson, 2015). Genome-wide association studies (GWAS), have identified several loci that are significantly linked with PD and are thought to be potential risk factors, providing insights into the genetic influence in sporadic PD (Kia et al., 2021). The discovery of the PD risk locus SREBF1 through genome-wide RNAi screens and its role in mitophagy

highlights the need to understand risk factors for sporadic PD, which account for 95% of cases (Ivatt et al., 2014). These findings highlight the substantial genetic component of sporadic PD, with each locus contributing modestly to the overall disease risk. While the functional effector genes at many loci remain uncertain, it is hypothesized that each locus may involve one or two genes sharing common biological functions (Kumaran and Cookson, 2015). These studies underscore the importance of unbiased genetic screens in identifying genetic risks for idiopathic PD. Conducting such comprehensive screens can offer new insights into the molecular mechanisms of dopaminergic (DAergic) degeneration in sporadic PD.

1.2. Importance of *Drosophila* model in understanding human neurodegeneration

Neurodegenerative (ND) phenotypes are accurately modelled in various organisms that include worms (Wang et al., 2017), mice (Kreiner, 2018; Kitada et al., 2007) and flies (Deal and Yamamoto, 2019; Mandya et al., 2019; Mc Gurk et al., 2015; Jaiswal et al., 2012). Ever since Thomas Hunt Morgan chose to investigate the chromosomal theory of inheritance in *Drosophila melanogaster*, it has been one of the most frequently used model organisms by geneticists (Morgan, 1910). This is because *Drosophila* has channelled new research opportunities to study the pathophysiology of NDDs and their underlying molecular and cellular pathology (Rahul and Siddique, 2022; Suzuki et al., 2022; Varga et al., 2014). It serves as an effective model system in biomedical research due to its fully sequenced genome, brief lifespan (10-12 days reared at 25ºC), large number of progenies, and genetic techniques to maneuver its gene expression, reduced genetic redundancy, existence of orthologs or homologs of human disease genes, molecular biology tools for manipulating the genome and generating mutants, as well as performing loss and gain of function studies (Ayajuddin et al., 2018). Notably, the fundamental structural and functional features of the nervous system are also highly

similar and conserved including, synaptic proteins, ion channels, and neurotransmitter systems such as DA, acetylcholine, glutamate, and gamma-aminobutyric acid (GABA) (O'Kane, 2011; Hon et al., 2006; Yoshihara et al., 2000).

With a significant amount of DAergic neurons and a fully developed central nervous system (CNS) (Crews, 2019; White et al., 2010), the fly has efficiently distinct behaviours that are conserved across several strains making it a very cost-effective and efficient model (Lessing and Bonini, 2009). Despite striking physiological differences between the fly and humans, genetic manipulations enable the induction and emulation of human PD pathology in the fly. Furthermore, genetic approaches including the observation of age-dependent DAergic neuronal degeneration and the easy detection of Lewy bodies (LB) and Lewy neuritis (LN), which are clinical features of PD are accomplished using transgenic flies (Feany and Bender, 2000). *Drosophila* PD model primarily exhibits two key phenotypes: degeneration of DAergic neurons with age and motor impairment. Distinct clusters of DAergic neurons are found throughout the fly brain responsible for its most varied behaviours. Hence, *Drosophila* demonstrates intricate motor phenomenon such as climbing, lying, movement, and conditioning to fear. Therefore, age-dependent loss of DAergic neurons sporadically induce PD condition showing DAergic neuronal dysfunction, and subsequent phenotypes are easily characterized which can be related to humans (Feany and Bender, 2000).

In addition to enhancing the comprehension of fundamental biology, *Drosophila* has been successfully used to study several human diseases and provide significant insights into the development and treatment of major NDDs, including PD.

1.3. Art and Design of Genetic Screens

NDDs and neuronal maintenance have significantly benefited from studying genetic screens. Genetic screens have been developed employing a number of model organisms which include yeast, flies, mice, and worms with mutations in the genes that cause familial cases of neurodegeneration (as described in **Figure 1.1**). The modelling of several NDDs in *Drosophila* (includes AD, PD), and eventually genetic modifiers of ND phenotypes, are crucial in determining underlying molecular causes of neurodegeneration and the cellular roles of genes linked to the disease (Mandya et al., 2019).

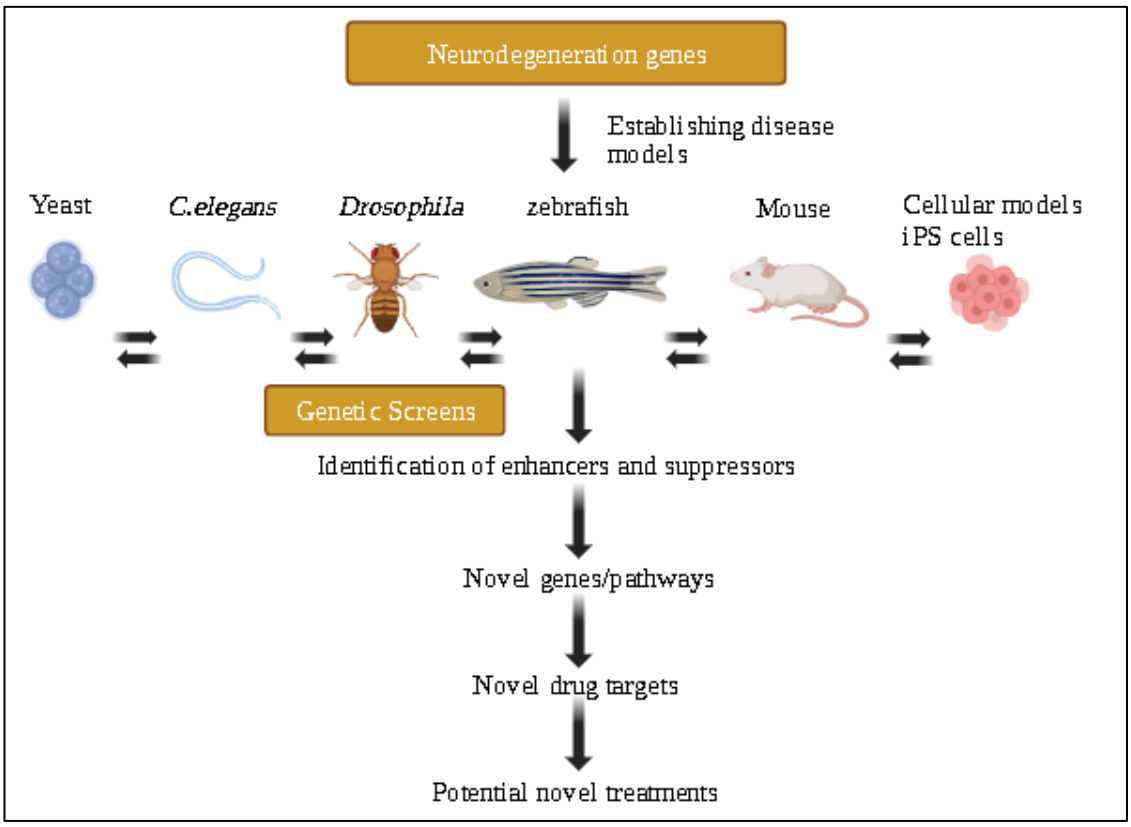


Figure 1.1: Figure depicts the importance of various model organisms used in genetic screens to understand disease pathogenesis and novel therapeutic targets (Image created with Biorender.com).

Genetic screens operate on the premise that alterations in observed phenotypic traits primarily stem from the genetic modifications introduced into the screen. These screens can be categorized as forward and reverse genetic screens, depending on their initiation

point, as described in **Figure 1.2**. Forward genetics, or phenotype-based, screens initiate with a mutant phenotype and aim to pinpoint the gene(s) underlying a specific phenotype of interest. In contrast, reverse genetic screens begin with the disruption of known genes and aim to identify the underlying phenotypic effects of gene(s) in a model organism. Both approaches are useful in understanding gene function and its effect on the characteristics of an organism.

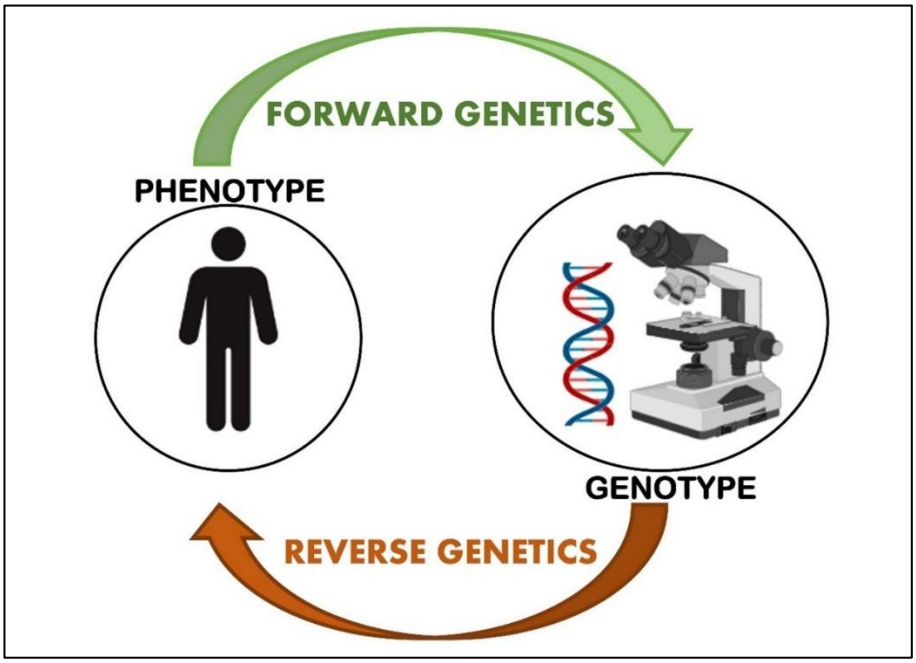


Figure 1.2: Flowchart of Forward and Reverse genetic screens (Image created with Biorender.com).

1.4. Genetic screening approaches in *Drosophila*

The capability of forward genetic screens and conducting large-scale genetic screens is primarily attributed to identifying mutations influencing specific biological processes in *Drosophila melanogaster*. It all changed with Christiane Nüsslein-Volhard and Eric Wieschaus's publication in Nature paper, which won the Nobel Prize for mutations affecting embryo patterning (Volhard and Wieschaus, 1980). This work was revolutionary because it was one of the first mutagenesis screens attempted in any

multicellular organism to search for phenotypes in the embryo rather than the adult, allowing them to identify null or strong mutations in most of the vital patterning genes that are used throughout development (Shi, 2024; Volhard and Wieschaus, 1980). Various approaches which are used to study neurodegeneration genes in *Drosophila* are described as:

1.4.1. Forward genetic screens (FGS)

i) Ems (Ethyl Methane Sulfonate) Mutagenesis Screens:

Inducing Mutations:

Initiating a genetic screen involves deciding how the genome will be disrupted in the hopes that genes that affect the target process will be among the randomly mutated genes. EMS Mutagenesis includes the use of chemical mutagens *viz*., ethyl methane sulfonate/ionizing radiation like X-rays to induce mutations ranging in size from single-base pair modifications to larger chromosomal alterations (St. Johnston, 2002). This process begins by mutating male flies by using radiation/chemicals and mating them with females that have chromosome balancers. EMS treatment is simple to apply and induces frequent random point mutations that saturate the entire genome. As point mutations lead to the loss of gene function, this approach can uncover the role of genes vital for the organism's survival. Despite the availability of new whole-genome sequencing techniques, EMS mutagenesis remains popular (Blumenstiel et al., 2009). The pioneering work on embryonic patterning identified hundreds of new loci famous for their conserved roles in development such as *hedgehog, engrailed, snail,* and *bazooka* (Wieschaus et al., 1984; Nusslein-Volhard et al., 1984). EMS alleles are also available for a large proportion of critical genes that can mutate to give clear phenotypes (St. Johnston, 2002). About one base change is induced by EMS using the conventional protocol for every 400 kb of fly, with G/C to A/T base pair transitions, accounting for 80% of these changes (Winkler et

al., 2005; Blumenstiel et al., 2009). Therefore, amino acid substitutions or non-sense mutations account for harmful mutations in the majority, producing a variety of mutants with varied degrees of severity. While, missense mutations frequently result in weaker or more specialized phenotypes (hypomorphs) and enable the identification of the crucial functional domains of the altered protein, nonsense mutations eliminate gene function (amorphs). The majority of early mutagenesis experiments examine for mutations in a specific area of the genome or for alleles of known genetic loci. Small forward genetic screens were carried out in the 1960s and 1970s by several groups to screen mutants exhibiting particular traits, such as meiotic abnormalities, female sterility, circadian rhythms, or aberrant behaviours (McAlpine et al., 2019; Konopka and Benzer, 1971). A generalized scheme for the Ems screen is demonstrated in the following **Figure 1.3**.

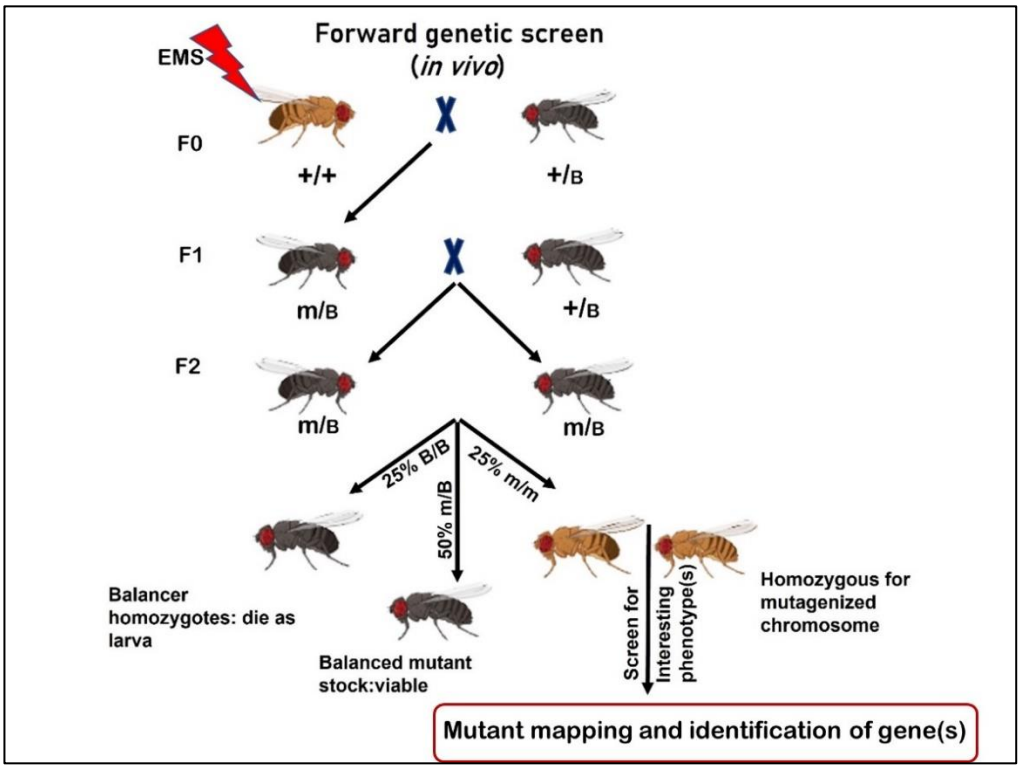


Figure 1.3: Schematic cross scheme for Ems screens. To screen for a specific chromosome (designated as B), male flies exposed to EMS-induced mutagenesis are bred to unmated females carrying a balancer marker. Every male of the F1 progeny inherits a chromosome that has been mutagenized and contains a unique array of mutations. Individual F1 males are backcrossed to balancer stock. Crossing occurs between F2 males and females that have the similar mutated chromosomes. F3 is homozygous for the mutated chromosome in 25% of cases, heterozygotes

(Balanced mutant stock) in 50% of cases which survives, and balancer homozygotes in 25% of cases, which dies (lethal). After which, these were examined for any intriguing phenotype(s), and the gene(s) is/are mapped and identified (Image created with Biorender.com).

ii) P-transposable Element Screens

P-element insert-based mutagenesis screens are specific to fly genetics. Rubin and Spradling developed *P-elements* for transgenesis to restore wild-type function to *rosy* mutant flies in 1982. In order to accomplish this, *Drosophila* embryos were injected with a *P-element* carrying a functional *rosy* gene, and then extracted rescued flies from the offspring of the injected individuals. *P-elements* are segments of foreign DNA that have undergone genetic modification. They physically alter genes, which causes disruption in gene activity and consist of a transposable element and a gene that codes for the transposase enzyme, which catalyzes transposition within the genome by acting on 31 bp inversion repeats at the ends of *P-element*. A large collection of mutants has been generated to examine gene function, and specific genomic regions inserted with *P-elemen*t are mapped and sequenced (Hummel and Klambt, 2008; Bellen et al., 2004). To observe gene expression patterns, *P-elements* are altered to encode beta-galactosidase {P(lacW or P(PZ)} or GFP {P(GawP)} and UAS sequences that could enhance gene transcription close to the insertion site of P(EP) and P(XP) *P-elements* (Rockman and Wolf, 2011). 19,000 *P-element* and *PiggyBac* element insertion strains have been developed that can be used to make molecularly specified deletions throughout the fly genome as a result of efforts to increase the utility of *Drosophila* as a genetic screening tool (Parks et al., 2004; Thibault et al., 2004). These are also used as a germline transformation vector that utterly transformed *Drosophila* genetics. Despite their haphazard insertions, large sets of mobilized *P-elements* have preferred particular genes.

In 1993, Brand and Perrimon inserted flippase recombination targets inside *P-elements* to facilitate exact chromosomal excisions (**Figure 1.4**).

Many engineered constructs including selectable markers, like neomycin resistance or eye color markers, have been created to aid in insertion site mapping. Initiatives such as the Gene Disruption Project, which employ *P-elements* to alter every gene in the *Drosophila* genome, encounter challenges in their endeavour to achieve complete genome coverage, such as gene preferences and insertion bias (Ryder et al., 2003; Spradling et al., 1995). These advancements have improved the genome editing of *Drosophila*, allowing for more accurate gene modification and beneficial research.

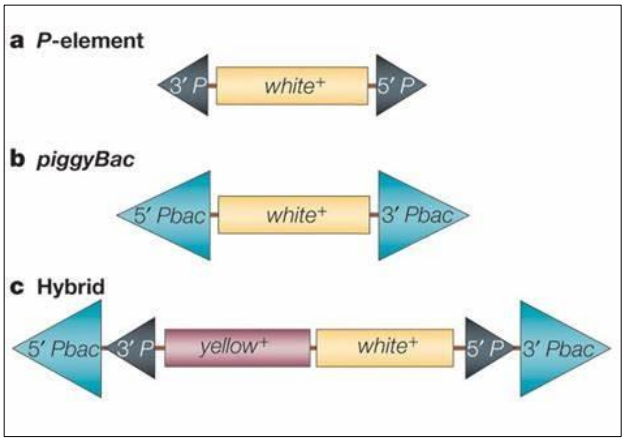


Figure 1.4: *P-transposons* element-mediated screens. The elements utilized in the gene-disruption research are *P-element* (a) transposon of *PiggyBac* (b) and the 5′ and 3′ terminals found in both transposable elements are necessary for effective transposition. *PiggyBac* elements contain a 5′ *PiggyBac* transposase (5′*Pbac*) and a 3′ *PiggyBac* transposase (3′*Pbac*), further encodes 3′ P-transposase (3′ P) and a 5′ P-transposase (5′ P) site by p-elements. The benefits of both *P-element* and *PiggyBac* transposon combined to form a hybrid element (c) making it feasible to screen for imprecise unidirectional deletions (Adapted from Venken and Bellen, 2005).

iii) Molecularly-defined Genomic Deficiencies / FLP/FRT Screens

"Deficiency", a study by Calvin Bridges from 1917, describes the deletion of several genes from a contiguous region of the *Drosophila* genome. The Bloomington Deficiency stock Center (BDSC) has assembled a set of deficiencies known as the "deficiency kit" to facilitate investigations by providing the best possible genome coverage in the fewest

animals. The first chromosomal deletion was identified in *D. melanogaster* and it contains the best genomic deletion coverage and subdivision of any animal. Exelixis and DrosDel are the groups that have produced large sets of genetic deficits that are specified molecularly (Ryder et al., 2007; Thibault et al., 2004). Earlier *Drosophila* deletions resulted from traditional methods include use of chemical mutagens or radiation to induce haphazard chromosomal breakage. Nonetheless, FLP-mediated recombination between FRT-bearing transposon insertions is considered as the most often used technique for producing deletions. In deletion screens, this effective approach enables single-nucleotide resolution. Flies that carried *P-elements* containing FRT sites were generated and identified by FLP recombinase. Deficiencies were created using FLP recombinase-mediated excision of genomic DNA flanked by two neighboring trans-P elements (**Figure 1.5**) (Cook et al., 2012).

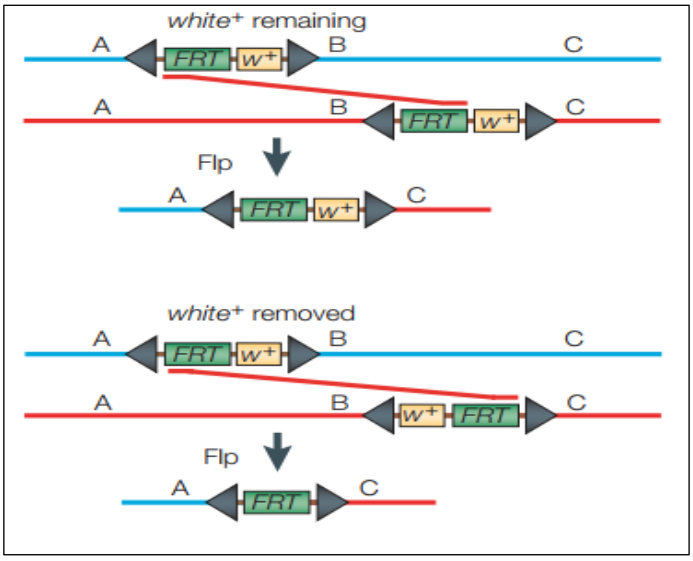


Figure 1.5: FLP-FRT system. Through FLP-FRT recombination, specified deletions are created using two transposons *P-elements/PiggyBacs*, that have FRT sites on the same chromosome pointing in the same direction. When a stable source of FLP expression is present, both transposons are delivered in *trans*. The elimination of the sequence that is in between the two FRT sites is catalyzed by FLP. Excision eliminates either one or both of the w^+ markers, depending on which way they are oriented (Adapted from Venken and Bellen, 2005).

Originally, Exelixis Inc. used Inverse PCR to map the insertion sites and generated a library of approximately 20,000 insertions, each containing an FRT site and subsequently selected an insertion collection to generate 519 molecularly defined deletions, spanning the genome by 56,000% with an average size of 140 kb (Parks et al., 2004). About 3,300 molecularly defined products were identified by the DrosDel project (Ryder et al., 2004), and 426 deletions of approximately 400 kb in size were generated (Russell and Roote, 2012) to achieve about 80% genome coverage. These deletions facilitate the mapping of mutations, and isolation of novel genes that are identified through enhancer or suppressor screens. PCR or sequencing using primers unique to the genome provides confirmation of the deletion ends (Venken and Bellen, 2005). After which progenies are screened for the residual element by detection of the resulting hybrid element using paired genome-specific primers (Cook et al., 2012).

1.4.2. Reverse genetic screens (RGS)

i) GAL4/UAS System Screens

A popular method for expressing transgenes in different tissues and developmental stages in *Drosophila* is the GAL4/UAS system. It is based on the yeast transcriptional activator Gal4, which initiates transcription by binding to the Upstream Activation Sequence (UAS) (Sharma et al., 2019). Genes downstream of a UAS cassette are effectively activated by Gal4, resulting in a modular and inducible expression system (Brand and Perrimon, 1993). Since the UAS-transgene is inactive until crossed with an appropriate Gal4 driver line, this approach might also allow the expression of malignant transgenes. Researchers have developed many Gal4-expression lines by hybridizing with specific gene regulatory elements or using enhancer trapping screens due to the versatility of UAS vectors like PUAST and UASp. Overall, the GAL4/UAS system is a versatile tool for gene manipulation which is expressed in *Drosophila* to study gene function in a controlled

manner. Furthermore, constructing UAS lines with desired cDNA gene is cloned downstream of UAS and a promoter sequence followed by its inoculation into the germline by *P-element-mediated* transformation. The absence of GAL4 protein in an organism do not allow it to undergo transcription process hence the transgenes are not transcribed. Thus, GAL4 driver line crossed to UAS-cDNA flies results in tissue-specific ectopic expression of the transgene. Consequently, this ensures that the transgene could be expressed in F1 generation cells expressing GAL4 protein (**Figure 1.6**).

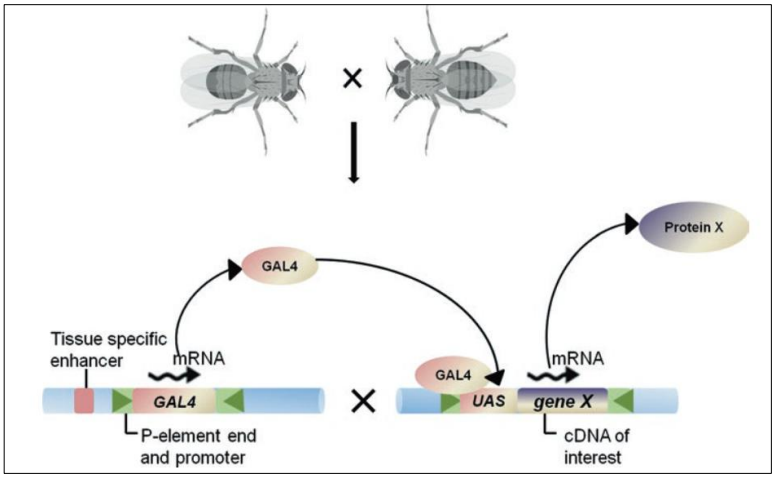


Figure 1.6: GAL4/UAS system. The GAL4 protein is expressed by the enhancer construct in tissues controlled by adjacent enhancers. The UAS promoter controls the interesting cDNA that is present in the UAS construct followed by Tissue-specific ectopic protein expression (Adapted from Sharma et al., 2019).

ii) RNA Interference (RNAi) Screens

Andrew Fire and Craig C. Mello jointly received the 2006 Nobel Prize in Physiology or Medicine for their work on RNA interference (RNAi) in *C. elegans*. Since then, the functional significance of RNA in regulation has been highlighted. sRNA molecules (miRNA and siRNA) are small RNA molecules that modulates gene expression or translation by selectively targeting particular mRNA molecules. These technologies have evolved as a reliable means of gene suppression (**Figure 1.7**). This, along with *Drosophila's* well-known *GAL4*/UAS system, decreases mRNA levels from such a gene, thereby eliminating its function (Qiao et al., 2018; Kennerdell and Carthew, 2000). In

other words, these changes in the transcriptional pattern of the genes are not permanent but, on the other hand, had off-target effects at first.

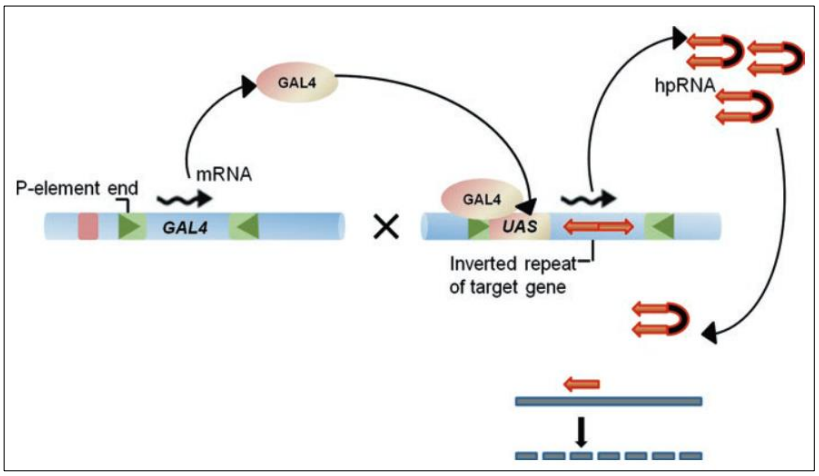


Figure 1.7: RNA interference. The UAS-IR construct incorporates a transgene including an inverted repeat (IR) of the target gene regulated by UAS, which is responsive to GAL4; the dsRNA of the target gene is produced in a tissue-specific manner, facilitating silencing of the target gene (Adapted from Sharma et al., 2019).

Furthermore, these RNAi lines were inferior at knocking down RNA to null levels. GAL4 protein expression is enhanced by an enhancer construct having neighbouring enhancers while UAS construct has target cDNA regulated by UAS promoter. Ectopic protein expression is tissue-specific (Ni et al., 2008). At the Vienna *Drosophila* Research Center and the *Drosophila* RNAi Screening Center, large RNAi stocks from libraries are available to screen most protein-coding genes. Determining the functions of individual genes in cellular and developmental processes from embryonic to adult life stages can be accomplished by knocking down a gene in a well-studied GAL4 line using dsRNA hairpin constructs specific to that gene (Dietzl et al., 2007).

iii) ZFN, TALEN, and CRISPR/Cas9-Based Genome Editing Screens

Sequencing of nucleases in DNA has been purposedly done in model organisms such as *Drosophila* (Bibikova et al., 2002), because these site-specific nucleases are programmatic and can cause DNA double-strand breaks (DSBs), which can result in exogenous replacement of null alleles or non-homologous end joining (NHEJ) and

homologous recombination (HR) at specific loci (Liu et al., 2012). This can lead to frame-shift changes in Zinc Finger Nuclease (ZFN) (Bibikova et al., 2002). Transcription Activator-like Effector Nuclease (TALEN) (Liu et al., 2012), and CRISPR (Clustered regularly interspaced short) palindromic repeat/CRISPR associated protein 9) (Bassett et al., 2013). *Drosophila* biologists are particularly interested in CRISPR/Cas9 because it significantly reduces the time and cost required.

ZFN and TALEN are synthetic chimeric enzymes with two domains: one domain contains a DNA-binding complex for DNA binding and a Fok I nuclease domain, for DNA cleavage (**Figure 1.8**) because DNA cleavage activity alone shows no bias for any particular sequence. ZFN and TALEN, by their specificity for DNA-binding sites can be manipulated to recognize specific sequences in the target gene. TALEN recognizes a base pair from transcription activator-like effectors (TALEs) by DNA-binding domain. Multiple zinc fingers or TALE repeats can be combined to improve gene targeting specificity to generate a single long DNA recognition sequence. The variation in ZFN-induced gene targeting in *Drosophila* is estimated to be substantial, such as 1–10% of the *ry, coil, pask* genes at multiple sites (Carroll et al., 2006; 2010; Beumer et al., 2006). The ZFN system facilitated researchers' aspect of using genes of interest; however, in *Drosophila*, Zebrafish and *C. elegans* (Chen et al., 2013; Wood et al., 2011), the activity of TALEN is higher than that of ZFN. TALE provides better sequencing than ZFN as it targets a single nucleotide. Furthermore, the distance between its DNA binding sites is longer and less flexible in ZFN as compared to TALEN. The specificity of the ZFN is based upon the relationship between the zinc finger site and target site; therefore, ZFN is specifically designed for each targeted purpose. ZFN binding to small sites increases the probability of off-target cleavage, which can lead to increased intracellular DSBs and lead to cell death (Pattanayak et al., 2011). Although TALEN is generally more efficient than

ZFN, it is thought that TALEN does not release its ends immediately after excision and may interfere with the initiation of DNA repair (Lin et al., 2014).

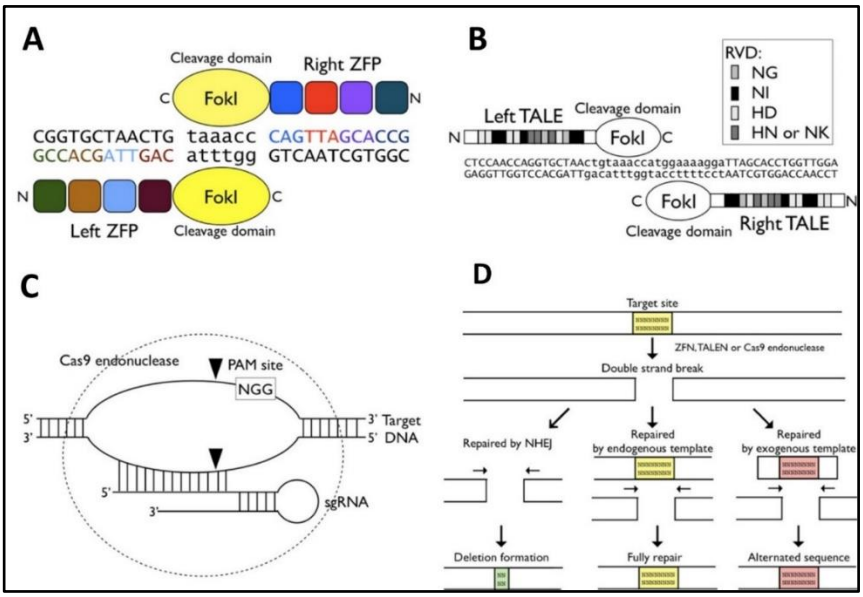


Figure 1.8: ZFN, TALEN, and CRISPR/Cas9 site-specific endonucleases. **(A)** Zinc-finger protein (ZFP) coupled to Fok1 endonuclease makes up ZFN. The arrangement of its α-helix sequence determines the zinc finger protein's selectivity for nucleotide binding. Therefore, using endonuclease, a few ZFPs are joined to form a ZFP chain, which produces high specificity and enables Fok1 to cleave DNA precisely at the target spot. **(B)** Fok1 endonuclease, which encodes DNA cleavage is located at the C-region of TALE. The almost identical repeats found in the central region of TALE mediate binding to specific target sites in the genome. Each of these repeats specifically binds to one base of the target DNA through two amino acids named repeat variable di-residues including NG, NI, HD and HN (or NK) for identifying one of the four distinct nucleotides respectively. **(C)** The Cas9 complex is a sequence-specific endonuclease that combines with sgRNA, and this complex cleaves the 20-bp target sequence with two guanines (NGG) ends, named the PAM region. Cleavage happens upstream of PAM region. **(D)** ZFN, TALEN, or Cas9 endonuclease first induces the DSB, which can be repaired by one of three methods. Random deletions would happen at the location when NHEJ repaired it (left). The sequence would be completely repaired if the endogenous template found within the genome repaired (middle). After repair, if an exogenous changed template is inserted, the sequence is regarded as gene editing (right) (Adapted from Lin et al., 2014).

In conclusion, the last four nucleotides of the sgRNA before the Protospacer adjacent motif (PAM) site, strand selection, GC content, and the distance between the cleavage site and translation start site all affect more efficient Cas9 modification for all target tissues. Developing these technologies in flies has developed highly efficient mechanism

for generating a complete null mutant. First, the specific targeting of ZFN and TALEN genes requires protein engineering.

Cas9 became well-known for the *Drosophila* gene due to following reasons: First, Cas9 can recognize genes with 20-bp sgRNA variants, and the NGG sequence is widely distributed in the fly genome. Second, there are several options where experiments are carried out to introduce the Cas9 system into flies:(a) *In-vitro* transfection of plasmid vector or transcript RNA encoding sgRNA and Cas9 (b) Cas9 transgenic flies fed sgRNA plasmid DNA, resulting in significantly increased editing efficiency and productivity (c) Cas9- mutated gene sgRNA complex, with increased stability and efficiency (Gratz et al., 2014; Kondo and Ueda, 2013). Third, adding selection markers, such as P[acman], helps speed up the search. Sequences (3xP3-RFP/3xP3-DsRed or loxP sequences) are flanked by homologous arms of about 1 kb in length at both ends on the donor plasmid, and Cas9-based knock-ins showed increased success compared to Ends-In methods (Gao et al., 2008). Cas9 can induce rapid transformation up to 88% of transfected cells, additionally, deletion mutations from pre-existing *P- element* insertions can be generated as an alternative approach to Cas9 for genetic modifications (Gratz et al., 2014). However, one issue that needs to be addressed is the off-target consequences of using Cas9-based methods. Unless there is a change in the PAM domain, the Cas9 target binding ability is not affected by one to three base pair mismatches in the target sequence. This is in sharp contrast to TALEN, which is only 1-2 bp mismatch tolerance (Mali et al., 2013). The mammalian genome has revealed additional information that is not a Cas9 target, although TALEN and Cas9 both bear some similarity in the target sequence and have been shown to interfere with high levels of sgRNA shown to impair Cas9 targeting. The development of TALENs and CRISPR/Cas9 genome editing tools has dramatically increased the ability to edit multiple genomes and the introduction of modification

techniques in flies has led to efficient strategies for generating complete loss-of-function/fully functional mutants (Sharma et al., 2019). Despite remarkable advances in gene targeting technologies, ZFN and TALEN still require unique designs for each gene, making it associated with systemic gene disruption. To target DNA, Small guide RNAs (sgRNAs) are used in CRISPR/Cas9 as such complications associated with site-specific gene manipulation are vastly reduced. Hence, CRISPR/Cas9 is the most extensively used method in genome modification. This tool originated as part of the prokaryotic innate immune system (Cong et al., 2013) and uses sgRNA to identify target regions. To recognize Cas9, a 20-bp sgRNA requires a standing NGG sequence called the protospacer adjacent motif (PAM) site (Mali et al., 2013; Cong et al., 2013) (**Figure 1.8**).

The availability of several tools and techniques for genetic manipulation discussed above allows establishment of fly models to understand and/decipher the pathogenic and molecular mechanisms underlying these diseases.

1.5. *Drosophila* genes and their association to human neurological diseases identified through forward genetic screens

Identifying several ND mutants discovered using FGS has prompted several investigations and encouraged numerous studies in gaining mechanistic insights that contribute to neurodegeneration. **Table 1.1** gives a detailed information on several genes discovered through primary FGS for various ND phenotypes, providing valuable insights into our understanding of ND. FGS implemented to detect neuronal loss/deterioration in neuronal function have successfully isolated a set of ND mutants (Singhal and Jaiswal, 2018). Follow-up mechanistic investigations and subsequent mapping of ND mutant genes have identified numerous novel genes crucial for preserving neuronal function and have also furthered our knowledge of ND (Mandya et al., 2019; Jaiswal et al., 2018). In addition to FGS, RGS and modifier screens are widely used models to study NDD in flies.

In all of these genetic screen experiments, the gene homolog associated with NDD in humans is deliberately mutated in flies to investigate the molecular function of gene and determine its interactors. Modifier screens are strategies involving search for additional genes that can either inhibit/enhance the characteristic phenotype exhibited by the mutant of interest. All these investigations (**Table 1.1** & **Table 1.2**) have played a crucial role in examining the molecular mechanisms responsible for NDD. Flies have also been employed for functional screening of genetic variants in a human gene detected through whole genome or exome sequencing (WGS/WES) from NDD patients further providing a glimpse into the disease pathophysiology of the disease (Marcogliese et al., 2018).

Numerous factors affect neuron's survival and state of health, as such, ND can result from varied factors, such as harmful gain-of-function mutations, aberrant activation of stress-induced pathways, and failure of cellular defense mechanisms (Hussain et al., 2018; Jellinger, 2010). To discover the use of flies in isolating genes related to human ND, the first ND mutant, *drop-dead* (*drd*) was identified due to its shortened lifespan, impaired phototransduction, cerebral vacuolization and motor abnormalities upon aging through an EMS genetic screen (Buchanan and Benzer,1993; Hotta and Benzer,1972; Hotta and Benzer, 1969). Another histology-based FGS examined a set of third chromosomal insertion lines, and identified (*löchrig*) *loe* mutant that exhibited vacuolization of the adult brain as well as reduced amyloid precursor protein (APP) processing and increased cholesterol ester levels (Tschäpe et al., 2002). Amyloid-β peptides are canonically produced by APP proteolysis, and the pathophysiology of AD has been associated with the aggregation of these peptides (Müller et al., 2017). Electroretinogram (ERG) screen is another more efficient and straightforward way to screen ND mutants to examine for defects in photoreceptor activity assessed through recording ERG records at several stage of the fly's life (Yamamoto et al., 2014; Stowers et al., 2002; Hotta and Benzer, 1969).

Table 1.1: Neuroprotective *Drosophila* genes and /or its association to human neurological diseases identified through *Drosophila* forward genetic screens (*EMS* and *P- element* screens).

Gene	Human homolog/ orthologs	Biological function	Nature of screens	References
Dropdead (drd)	_	*drd* codes an integral membrane protein involved in female fertility, longevity, neuroprotection, proper function of the gut and respiratory tracheae, regulation of growth and body size	EMS mutagenesis screens	Buchanan and Benzer, 1993
Spongecake	–	*Spongecake* correlates to neuronal brain degeneration causing death of neurons involved in determination of lifespan	EMS mutagenesis screens	Min and Benzer, 1997
Eggroll	–	*eggroll* is involved in degeneration, premature death and is involved in determination of lifespan	EMS mutagenesis screens	Min and Benzer, 1997
Bubblegum (bgm)	ACSBG1, ACSBG2	*bgm* is an acyl-CoA synthetase gene that plays a role in fatty acid, lipid metabolism, activation of metabolic pathways in vertebrates, neuron cellular homeostasis and positive regulation of circadian sleep/wake cycle	P-element insertion Mutagenesis screens	Min and Benzer, 1999
Comatose (comt)	NSF	*Comt* codes for N-ethylmaleimide-Sensitive Factor 1 protein is crucial for maintenance of neurotransmitter release through disassembly/rearrangement of plasma membrane SNARE complexes following synaptic vesicle fusion, enables ATP hydrolysis activity essential for Golgi to lysosome transport, chemical synaptic transmission	EMS mutagenesis screens	Suzuki et al., 1971; Siddiqi and Benzer, 1976
Shibire (shi)	DNM1, DNM2, DNM3	*shi* codes in actin and microtubule binding activity, involved in epithelial cell migration, learning/memory and synaptic vesicle cycle including mitotic spindle, plasma membrane and synapse and is part of sperm individualization complex active in neuromuscular junction	EMS mutagenesis screens	Suzuki et al., 1971; Siddiqi and Benzer, 1976
Pirouette (pir)	–	*pir* is involved in age-dependent circling behaviour and correlates to degeneration in the brain	EMS mutagenesis screens	Eberl et al., 1997
Easilyshocked (eas)	ETNK1, ETNK2	*eas* codes for the protein Ethanolamine Kinase and is involved in phospholipid biosynthesis, normal functioning of existing neural circuits protects from seizure sensitivity through cell-type-specific rescue, plays in excitatory rather than inhibitory neural transmission, promotes proper phospholipid metabolism in normal brain function	EMS mutagenesis screens	Suzuki et al., 1971; Grigliatti et al., 1973
Technical knockout (tko)	MRPS12	*tko* codes for mitochondrial ribosomal protein S12 family and is involved in the response to hypoxia, mechanosensory, courtship behaviour, multicellular	P-element insertion mutagenesis screens	Ganetzky and Wu, 1982; Royden et al., 1987

		organismal process, response to stimulus, abiotic stimulus, reproductive process and external stimulus		
stress-sensitive B (sesB)	MT-ATP6	ADP: ATP antiporter that mediates import of ADP into the mitochondrial matrix for ATP synthesis and export of ATP out to fuel the cell, homeostatic process, regulates membrane potential, trans-synaptic signalling, neuron cellular homeostasis and determination of adult lifespan	P-element insertion mutagenesis screens	Homyk and Sheppard, 1977; Celotto et al., 2006
Swiss cheese(sws)	PNPLA6, PNPLA7	*sws* codes for transmembrane protein that hydrolyzes phosphatidylcholine and binds /inhibit the C3 catalytic subunit of protein kinase A, neuronal and glial regeneration and apoptosis	EMS mutagenesis screens	Kretzschmar et al., 1997
Löchrig/AMPKγ (loe)	PRKAG2	*loe* is involved in cholesterol homeostasis and amyloid precursor protein-like (APPL) processing, neuroprotection, behavioural deficits, and early death	P-element insertion mutagenesis screens	Tschäpe et al., 2002
Triose phosphate Isomerase(tpi)	TPI1	*tpi* codes a soluble metabolic protein dimer that functions in glycolysis catalysing the isomerization between dihydroxyacetone phosphate and glyceraldehyde 3-phosphate essential for effective ATP production cellular process, multicellular organismal process, glucose homeostasis including estimation of adult lifespan, glucose homeostasis and glycolytic process.	EMS mutagenesis screens	Palladino et al., 2002; Gnerer et al., 2006
Nicotinamide mononucleotide adenylyltransferase (nmnat)	NMNAT1, NMNAT2, NMNAT3	*Nmnat* codes for crucial enzyme NAD salvage pathway, facilitates the last stage of NAD production, stress-response protein acts as a chaperone for neuronal maintenance and protection, photoreceptor cell maintenance, inhibition of neuromuscular synaptic transmission, biosynthetic process and maintenance of dendritic spine	EMS mutagenesis screens	Zhai et al., 2006; Zhai et al., 2008
Enabled (ena)	ENAH	*ena* codes for *Drosophila* member functions as a processive actin polymerase, promoting actin addition in epithelial morphogenesis, CNS pathfinding, embryonic development, and regulation of plasma membrane bounded cell projection assembly	EMS mutagenesis screens, GFP-FLP/FRT screens	Rezával et al., 2008
defenserepressor1 (drn1)	MYLIP	*drn1* is involved in neuroinflammation, age dependent neuropathology linked to Imd pathway activation, increased expression of AMP (antimicrobial peptide) genes, and lifespan determination	P-element insertion Mutagenesis screens	Cao et al., 2013
(Drosophila sterile alpha and Armadillo motif) dsarm/Ect4	SARM1	*dSarm* exhibit crucial role in initiating the self-destructive pathway of axons following injury through a signalling pathway involving *dSarm/Sarm1,* its loss cause motor deficits and degeneration of neurons	EMS mutagenesis screens	Neukomm et al., 2014; Sur et al., 2018
Highwire(hiw)	MYCBP2	*hiw* codes Atypical E3 ubiquitin-protein ligase specifically mediates ubiquitination of threonine and serine residues on target proteins, needed in the presynaptic motoneuron to downregulate the levels of (Wallerian degeneration Slow) *wnd* and	EMS mutagenesis screens	Neukomm et al., 2014

		restrain synaptic terminal growth, involved in the regulation of synaptic assembly at the neuromuscular junction		
paralytic (para)	SCN8A, SCN2A	*Para* codes for an α-subunit of voltage-gated sodium channels generating sodium-dependent action potentials, involved in locomotor activity and fly seizure disorders	EMS mutagenesis screens	Suzuki et al., 1971; Siddiqi and Benzer 1976; Yamamoto et al., 2014
dankle2	ANKLE2	*dankle2* codes for an important protein involved in proper development of the third instar larval CNS whose loss causes a small brain phenotype, defects in proliferation and excessive apoptosis	EMS mutagenesis screens	Yamamoto et al., 2014
Valyl-tRNA Synthetase (aats-val)	VARS	*Valyl-tRNA synthetase* codes for valine-tRNA ligase activity, involved in *valyl-tRNA* aminoacylation protein translation and viability of photoreceptors	EMS mutagenesis screens, GFP-FLP/FRT screens	Huang et al., 2015
porin	VDAC1, VDAC2, VDAC3	Porin codes for beta-barrel channel protein in mitochondria, responsible for ion and metabolite translocation, mitophagy, multicellular organismal reproduction, and viability of photoreceptors	EMS mutagenesis screens, GFP-FLP/FRT screens	Huang et al., 2015
Synthesis of cytochrome c oxidase (Scox)	SOC1	*Scox* codes for protein involved in cytochrome complex assembly and regulation of ATP biosynthesis, viability of photoreceptors	EMS mutagenesis screens, GFP-FLP/FRT screens	Huang et al., 2015
NADH dehydrogenase (ubiquinone) 23 kDa Subunit (ND23)	NDUFS8	ND23 is a key component that is involved in mitochondrial ATP generation linked electron transport and the transfer of electrons from NADH to ubiquinone	EMS mutagenesis screens	Loewen and Ganetzky, 2018
Brat	TRIM3	*Brat* codes for tumor suppressor that regulates proliferation in the brain, asymmetric cell division of neural stem cells(neuroblasts) and play role in larval development	EMS mutagenesis screens	Bello et al., 2006; Betschinger et al., 2006; Lee et al., 2006; Loewen et al., 2018
Mitochondrial assembly regulatory factor (marf)	MFN1 MFN2	*marf* codes GTPase in the dynamin-family involves in mitochondrial membrane tethering, fusion, and neuroprotection-neurodegeneration mediated through mitochondrial dysfunction, elevated ROS levels, glial-lipid droplet accumulation	EMS mutagenesis screens	Zhang et al., 2013; Yamamoto et al., 2014
methionyl-tRNA synthetase (Aats-met)	MARS2	*Aats-met* codes for methionine-tRNA ligase, involves in methionyl-tRNA aminoacylation, mitochondrial biogenesis, neuronal function and survival in *Drosophila* photoreceptor neuron, determination of lifespan, and cell proliferation in epithelial tissues.	EMS mutagenesis screens	Thiffault et al., 2006; Bayat et al., 2012
severe impairment of CI with lengthened youth (sicily)	NDUFAF6	*sicily* is involved in the assembly of mitochondrial NADH: ubiquinone oxidoreductase complex (Complex I), protein stabilization, biosynthetic process and mitochondrial respiratory chain complex I assembly, neuroprotection	EMS mutagenesis screens	Zhang et al. 2013; Yamamoto et al., 2014

(Calmodulin-binding protein related to a Rab3 GDP/GTP exchange protein (Crag)	DENND4A DENND4B DENND4C	*Crag* encodes a guanine exchange factor for the products of Rab10 and Rab11, involved in extracellular structure organization, regulation of signalling, cellular component assembly, positive regulation of Golgi to plasma membrane protein transport, cellular component organization or biogenesis, neuroprotection-neurodegeneration mediated through disrupted Rh1 homeostasis creating activity-dependent cellular stress	EMS mutagenesis screens	Xiong B et al., 2012; Haelterman et al., 2014; Yamamoto et al., 2014
Leucine-rich pentatricopeptide repeat containing 2 (lrpprc2)	LRPPRC	*lrpprc2* is involved in regulation of rhodopsin mediated signalling pathway, regulation of oxidative phosphorylation, mitochondrial mRNA stability, mitochondrial transcription, regulation and translation	EMS mutagenesis screens	Yamamoto et al., 2014; Jaiswal et al., 2015
Pyruvate dehydrogenase E1 alpha subunit (Pdha)	PDHA1 PDHA2	biosynthesis of acetyl-CoA from pyruvate, glucose metabolism, oxidative phosphorylation, and the TCA cycle in *Drosophila* mitochondria	EMS mutagenesis screens	Yamamoto et al., 2014; Jaiswal et al., 2015
Vacuolar protein sorting 26 (Vps26)	VPS26A VPS26B	*vps26* is a subunit of the retromer complex and enables cargo receptor activity, endosome to plasma membrane protein transport, regulation of rhodopsin mediated signalling pathway involved in regulation of Wnt signalling pathway	EMS mutagenesis screens	Haft et al., 2000; Yamamoto et al., 2014; Wang et al., 2014
Vps35	VPS35	endosomal transport, vesicle-mediated transport to the plasma membrane, regulation of protein stability and secretion, retrograde sorting and recycling of transmembrane cargo proteins from endosomes to the plasma membrane and trans-Golgi network	EMS mutagenesis screens	Liu et al., 2014; Wang et al., 2014
Cacophony (cac)	CACNA1B, CACNA1E	*cac* encodes the primary structural subunit of a voltage-gated calcium channel involved in evoked neurotransmitter release at neuromuscular synapses, contributes to male courtship behaviour and wide range of neurophysiological processes	EMS mutagenesis screens	Heisenberg and Böhl, 1979; Yamamoto et al., 2014
Straightjacket (stj)	CACNA2D3 CACNA2D4	encode the pore forming subunit and accessory subunit of the *Drosophila* VGCC Transport, localization, neuron projection development, sensory perception of pain and trans-synaptic signalling	EMS mutagenesis screens	Ly et al., 2008; Dickman et al., 2008
Frataxin (fh)	FXN	encodes an evolutionarily conserved mitochondrial protein required for iron-sulfur cluster assembly, enables iron chaperone activity, involved processes including determination of adult lifespan, positive regulation of ecdysteroid biosynthetic process, and positive regulation of oxidative phosphorylation	EMS mutagenesis screens	Haelterman et al., 2014; Yamamoto et al., 2014
Wacky (wcy)	WAC	Negative regulation of autophagy, regulation of autophagy, positive regulation of TORC1 signalling, habituation, neuroprotection mediated increased autophagy due to hypoactive mTORC1	EMS mutagenesis screens	Haelterman et al., 2014; Yamamoto et al., 2014; David-Morrison et al., 2016

Nardilysin (dNrd1)	NRD1	*dNrd1* codes for a protein involved in chaperone-mediated protein folding and proteolysis	EMS mutagenesis screens	Yamamoto et al., 2014; Yoon et al., 2017
Oxoglutarate dehydrogenase (Ogdh)	OGDH OGDHL	enable oxoglutarate dehydrogenase (Succinyl-transferring) activity, involved in intracellular amino acid and tricarboxylic acid cycle homeostasis, neuroprotection mediated through decreased autophagy due to hyperactive mTORC1	EMS mutagenesis screens	Gruntenko et al., 1998; Chin et al., 2014; Yamamoto et al., 2014

Table 1.2: A summary of *Drosophila* deficiency/deletion screens.

Genotype	**Age/Sex**	**Nature of genetic screens**	**Phenotype(s) scored**	**Findings**	**Reference**
w1118, 2L deficiency lines	7 days/male	Deletion screens	Dilated cardiomyopathy	Identified a new Notch ligand weary *(wry)* linked to cardiomyopathy phenotype, introduction of the which restores the normal function	Kim Il-Man et al., 2010
w1118, X chromosome deficiency lines	1 week/ female	Deletion screens	Dilated cardiomyopathy	Disruption of the gene CG3226, orthologue of mammalian Siah-Interacting protein linked to cause cardiomyopathy phenotype, reducing levels of Armadillo produces a small heart in adult *Drosophila*	Casad et al., 2012
Canton S, 149 genomic deficiency lines (1,2,3)	1,3,5 days/male & female	Deletion screens	Reduced and enhanced changes in MnSOD transcript levels, paraquat sensitivity of the deletion genotypes	Identified one enhancer that improves the survival of flies exposed to PQ stress, and four candidate suppressors showed reduced survival of flies exposed to PQ	Paul and Duttaroy, 2003
DSK001 ,439 DrosDel isogenic deficiency strains	embryos, male and female adults	Genome-wide deletion screens	Quantitative trait loci (QTL) associated with thermal resistance	19 QTL for heat resistance, with 16 partially overlapping with previously known QTL and 3 novel QTL on the 2nd and 3rd chromosome	Takahashi et al., 2011
2R Chromosomal deficiency lines	2-4 hr aged ~24 h homozygous embryos	Deletion screens	Defects in amnioserosa cell shapes, canthus formation, and tissue dynamics	jelly belly, shot, tum, even-skipped as dorsal closure genes	Mortensen et al., 2018

2L Chromosomal deficiency lines	4,24hr/ homozygous embryos	Deletion screens	Defects in cell shape, canthus formation, and tissue dynamics	pimples, odd-skipped, paired, sloppy paired1 as dorsal closure genes	Fogerson et al., 2020
Canton S, 149 X deficiencies chromosome, 87% 2nd chromosome, 70% 3rd chromosome	0-4 days/male and female	Deletion screens	Alteration in MnSOD expression positively linked with paraquat sensitivity to deletion genotypes	*Df*(2R)017 significantly upregulated MnSOD mRNA by 1.7-fold as enhancers. *Df*(1)ct-J4, *Df*(2L)BSC4, *Df*(3L)66CG28 and *Df*(3R)Scr, down-regulated MnSOD expression as suppressors	Paul and Duttaroy, 2003

Study by Yamamoto et al. (2014) in large F3 adult genetic mosaic screens on an isogenic *yw FRT19A* X-chromosome deficiencies which consists of 6000 EMS-induced lethal mutants led to the discovery of 165 genes involved in neuronal function and development. Although 92% of the genes found in this screen have human homologs, 30% of those homologs were previously linked to neurological disorders which also includes NDDs in human. Furthermore, few lines that were screened from this study exhibited mutations in fly homologs of *MFN2*, *CACNA1A, LRPPRC* and *C8ORF38* through their observed phenotype as described in **Table 1.1** and these were also found to cause NDDs in humans. *Mfn2* gene causes Charcot-Marie-Tooth type 2A in humans (Kijima et al., 2005).

Studies also reported that paraquat (PQ) exposure leads to oxidative stress, motor deficit and depleted *Mfn2* expression in the health phase of fly thoraces (de Oliveira Souza et al., 2017). *DfOXO*/FOXO signalling is considered crucial in regulating apoptosis, helps manage oxidative stress response and influence cell survival thereby providing neuroprotection and in *Drosophila* and *in-vitro* neuronal cells respectively (Keshavarz et al., 2016; Moskalev et al., 2012; Van der Vos and Coffer, 2011; Kojima et al., 1999). Additionally, it regulates *Mfn2* and mediates mitochondrial complementation and quality control, thereby conferring neuroprotection (de Oliveira Souza et al., 2017; Zhao et al., 2017). Also, *Mfn2* expression levels were inhibited in *Drosophila* PD brain during health phase and further feeding with curcumin rescued diminished *Mfn2* level by nearly 2-fold (Das, 2022). *SNpc* of PD patients shows diminished *Mfn2* translate level when compared to age-matched controls (Zhao et al., 2017). All this evidence also shows that downregulation of *Mfn2* is related to the onset of PD. An ERG-based screen also isolated *dNrd1(Drosophila nardilysin1)* mutant whose loss resulted in accumulation of α-ketoglutarate dehydrogenase (OGDH), a tricarboxylic acid cycle enzyme which converts α-ketoglutarate to succinyl-CoA and in turn triggers mammalian target of rapamycin

complex 1 *(mTORC1)* signalling cascade, thereby suppressing cell autophagy (Yoon et al., 2017). Further, rapamycin suppresses the ND phenotype in *dNrd1* mutants. However, in increased autophagy, rapamycin aids in preventing accumulated toxic alpha-synuclein and protect death of DAergic neurons. Activity of *mTORC1* has been implicated as a potential therapeutic value for treating PD where either excessive or inadequate *mTOR* activity may be lethal to DAergic neurons (Webb et al., 2003). Further studies identified *ubiquilin* gene *(ubqn)* which codes for a ubiquitin-binding protein, with its human homolog (UBQLN2, UBQLN4) is also implicated in mitochondrial signalling mechanism involved in Amyotrophic lateral sclerosis (ALS) (Teyssou et al., 2017). Şentürk et al. (2019) reported that *ubqn* mutant flies exhibit both mitochondrial accumulation and gradual loss of neurons and glia cells. Also, *ubqn* mutants exhibit suppressed TOR activity yet decreased autophagic flux, contrary to expectations where earlier it showed suppressed TOR activity and increased autophagy (Lin et al., 2015) which proved their attempt to explain this puzzling behaviour, a different function for *ubqn* involved in lysosomal function. These studies clarified the role of autophagy and cell organelle clearance in conferring neuroprotection and cellular quality regulation. Axons from synaptic region gradually degenerate to the cell body in a condition known as "dying back" phenomenon /axonal loss, an early disease of aging, although molecular mechanisms involved in axonal retraction during aging are unknown (Kanaan et al., 2018; Salvadores et al., 2017). *dSarm/Ect4* gene identified through EMS-screen plays a critical role in initiating the self-destructive pathway of axons after injury involving *Sarm/Sarm1* (Neukomm et al., 2014). Studies have shown that early *Sarm1* induction plays a role in triggering loss of DAergic neurons in rotenone-exposed flies, thereby inducing motor deficits and even neuronal death (Sur et al., 2018; Neukomm et al., 2014). Increased exposure of rotenone to

brain milieu of *w1118* flies increases induction of *Eiger*, which in turn causes DAergic neurons to express more of the *NADase Ect4* which results in ND phenotype. *VPS35* gene identified through EMS screen, is the third autosomal-dominant gene associated with PD. It is involved in endosomal transport, vesicle-mediated transport from endosomes to the plasma membrane and enables cargo receptor activity.

Studies have shown that all *VPS35* mutations in PD cause mitochondrial fragmentation and neuronal death, linking *VPS35* to mitochondrial homeostasis; suggesting a mechanism, which possibly may be due to abnormal trafficking of the autophagy protein ATG9A (Zavodszky et al., 2014; Tsika et al., 2014). *VPS35* natural function is bound to the PARK2 gene, *parkin*, which regulates endosomal activity based on its interaction with *VPS35* (Song et al., 2016).Further, loss of *VPS26* gene (another subunit of the retromer complex) also displayed synaptic dysfunction and is shown to link with late-onset AD (Muhammad et al., 2008). *FXN* mutations causes Friedreich's ataxia (Bradley et al., 2000), while *Dfh* fly homolog of *FXN* identified through ERG-based screen cause ROS-independent ND. These mutants exhibit abnormal mitochondrial accumulation, increased formation of iron, reduced levels of ATP and reduced electron chain activity but no enhancement of oxidative stress. Decreasing the dietary iron uptake actively and downregulation of sphingolipid synthesis/knockdown of *Pdk1* or *Mef2*, suppresses degenerative photoreceptors phenotype in *fh* mutant (Chen et al., 2016). All of this demonstrates unbiased genetic screens as a reliable genetic tool for isolating mutations in genes necessary for neuronal survival.

1.6. Genome wide modifier screens to identify neuroprotective/neurodegenerative genes

‘Neurodegeneration’ refers to the gradual loss of neurons with subsequent repercussions on cognition, motor function, and other brain activities affecting millions worldwide, with

numbers suffering from NDDs in human, anticipated to increase as population ages at an alarming rate (Wakhloo, 2022). Numerous genetic studies, starting from traditional family linkage studies to genome-wide association studies (GWAS), studies of the genetic effects on endophenotypes within each disease, have revealed a plethora of loci, and in certain cases, specific genetic variants, that confer varying degrees of predisposition to a particular disease (Pan et al., 2023; Lavoy et al., 2018). In total, GWAS alone has discovered approximately 200 distinct loci associated with NDDs (OMIM, SysID Database). Genetic modifiers are defined as genes that change the expression of other "target" genes (Rahit et al., 2020). Genetic modifier loci that impact the penetrance, severity, or other clinically significant aspects of diseases brought on by uncommon mutations in target genes have historically been the focus of genetic modifier research (Génin, 2008). These screens help to identify dominant enhancers/suppressors of phenotype which are caused by manipulation of the gene of interest. In particular, dopaminergic pathway genes are associated with the etiology of PD and are thought to affect cognitive ability. PD is presumed to have resulted from complex interactions among gene-gene and gene-environmental factors. Apart from candidate genes, several susceptible factors or disease-modifying loci have been implicated in disease development and also with age at onset, sex, severity, and disease progression. The polymorphisms in MAPT (Microtubule Associated protein tau), SNCA (Synuclein Alpha), NUCKS (Nuclear Casein Kinase and Cyclin-dependent kinase substrate 1), HLA (Human Leukocyte antigen) region, GAK (Cyclin G associated kinase), BST1 (Bone marrow stromal cell antigen 1) have been linked with PD as reported in GWAS (Pankratz et al., 2009; Satake et al., 2009; Simon-Sanchez et al., 2009). Additionally, the modifier genes, APOE (Apolipoprotein ε), FGF-20 (Fibroblast growth Factor 20), GSK-3β (Glycogen Synthase kinase 3β), BDNF (Brain-Derived neurotrophic Factor) and DAergic

pathway genes like, DBH (Dopamine β Hydroxylase), DRD2,3, (D_2 dopamine receptor), COMT (Catechol-O-Methyl Transferase), SLC6A3 (Solute Carrier Family 6 member 3) or DAT (Dopamine Transporter) gene, MAO-B (Monoamine Oxidase B) were reported to be linked with PD (Das et al., 2012; Bialecka et al., 2008; Hong et al., 2003; Pankratz et al., 2006). The susceptible genes like MAPT, GSK-3β, CDK5R1, GSTT1, FGF-20, DBH, APOE, and BDNF were previously linked with PD in the East Indian cohort (Ghosh et al., 2019; Sadhukhan et al., 2018; Das et al., 2009). Additionally, a genome-wide modifier screen in a *Drosophila LRRK2 G2019S* model identified 177 genes which includes the top 19 genes that cause a broad range of variation in its phenotype linked to neural projection outgrowth/degeneration. Genes like *Pros, pbl, ct,* and *CG33506* were shown to selectively alter age-related dopamine neuron loss and relative locomotor impairment in several DGRP lines (Lavoy et al., 2018). Furthermore, these studies demonstrates that even among individuals who possess the same deleterious mutation, the manifestation of the disease may vary across multiple individuals.

Studies through genome-wide modifier screens have also identified previously two known PD interacting genes such as *opa1* and *drp1* thereby leading to the identification of two unknown PD-interacting genes *viz., debra, Pi3K21B,* and *b4GalNAcTA* using 2nd and 3rd chromosomal deficiency lines, transgenic and *P-element* lines. The phenotypes observed in these screens consistent with the PD phenotype resulted in abnormal wing posture, reduced life span, and reduced fertility rate (Fernandez and Rao, 2011). Simultaneously, modifier screens using 199 deficiency which consists of 6300 genes were screened for modifiers of Aß-peptide neurotoxicity in the AD brain. Six deficiency lines encompassing 52 deleted *Drosophila* genes were found to show reduced climbing phenotype in aged flies, and further validation with *RNAi* lines identified 2 genes as strong candidates with its human orthologs *HPD,* and *PRCC*; *HPD* with intraneuronal Aß-42

peptide modifiers of AD which further give insights to investigate into the pathogenesis of sporadic AD model (Belfiori-Carrasco et al., 2017). In addition to the gene-environment interaction, this background genetic variation may play a significant role in driving disease variability. Therefore, numerous backgrounds are also crucial to be investigated to correlate with genotype-phenotype link that establishes the expressivity of a pathogenic mutation. **Table 1.3** lists out few of the modifier screens studied using deficiency lines background for PD and AD phenotypes.

Table 1.3: Lists of modifier screens studied using deficiency lines background for PD and AD phenotypes.

Modifiers screens using Deletion lines background	**Model system**	**Nature of genetic screens**	**Phenotype(s) scored**	**Findings**	**Reference**
PD	4-6 days F1 male and female *Drosophila* (*UAS-Pink1-RNAi, UAS-park RNAi*,P-element insertions, >200 2nd & 3rd Chromosomal deficiency lines	Genome-wide modifier screens, *RNAi*-mediated inhibition screens	Abnormal Wing Posture, reduced life span and reduced fertility rate	Identified two known PD-interacting genes *opa1* and *drp1*, and three novel PD-interacting genes *debra*, *Pi3K21B* and *b4GalNAcTA*	Fernandes and Rao, 2011
	7- and 28-days male *Drosophila*, *Oregon*-R,270 chromosomal deficiency lines, ddc>A53T UAS - transgenic lines	Genome-wide modifier screens, *RNAi*-mediated inhibition screens,	Reduced climbing ability, lethality, reduced viability, more sensitive to OS, low DA levels and DA neurons	Identified mitochondrial chaperone protein TRAP1 as a novel modifier of the toxicity induced by [A53T] α Synuclein. hTRAP1 overexpression rescued locomotor defect, loss of DA in fly heads and loss of DA neurons	Butler Erin K et al., 2012
	4 ,11 & 18 days old *Drosophila* Oregon-R, UAS genetic models, *Df*(3L)ED4606 deficiency line	*RNAi* mediated inhibition screens, Kyna feeding (5mg/ml)	Reduced climbing ability, Age- dependant progressive loss of DA neurons, reduced lifespan, elevated levels of ROS	Scarlet is neuroprotective in a PD model Dopaminergic neuron-specific expression of Scarlet rescues the phenotype	Cunningham Patrick C et al., 2018
AD	10-12 days male *Drosophila* Canton S, transposable element, 2nd Chromosomal deficiency lines, UAS-transgenic lines	*RNAi*-mediated inhibition screens	Reduced climbing ability, progressive loss of adult brain neuropil in conjunction with massive brain Overgrowth	*Brat* gene as a neuroprotective gene	Gevedon Olivia et al., 2019; Loewen C et al., 2018

	5 & 18 days Male *Drosophila* UAS transgenic lines ,199 lines 2nd, 3rd & 4th Chromosomal deficiency lines	Genome-wide modifier screens, *RNAi*-mediated inhibition screens,	Reduced climbing ability at 18 days post-eclosion, neuronal loss, accumulation of non-fibrillar species of Ab42, increased vacuolization in flies expressing Ab42	CG11796 and CG17249 were validated and identified as potential modifiers of Aβ42 toxicity Six lines with deletion of 52 *Drosophila genes* with human orthologs, significantly modified Aβ42 neurotoxicity in 18-day-old flies	Belfiori-Carrasco et al., 2017
Others	3-5day old male *Drosophila* Canton S, 121 large chromosomal deficiency lines (2nd & 3rd), UAS-transgenic lines	Genome-wide modifier screens, *RNAi*-mediated inhibition screens,	Loss of Myc, either by mutation or neuro-specific knockdown induces male-male courtship behaviour and elevates DA levels.	Identified 23 deficiencies as suppressors and 12 deficiencies as enhancers,86 deficiencies showed no effect. DOPA decarboxylase (Ddc) overexpression enhanced DA level, whereas loss of Ddc downstream target of Myc suppresses loss of Myc-induced elevated DA level	Pan Yu et al., 2022
	w; *SMN73Ao FRT2A/TM3, Ser female, y w hsFLP*; *ovoD1 FRT2A/TM3 Ser male, SMN73Ao,* 128 chromosome deficiency lines	Genome-wide modifier screens	Reduced survival motor neuron protein levels and depressed adult viability	*SMN* is involved in Oogenesis, *gurken* and *oskar* mRNAs contribute to the embryonic death	Aquilina and Cauchi, 2018; Chang et al., 2008

1.7. Review of Deletion screens in the *Drosophila* model

Screening of deletion mutations is called deficiencies (*Df*s)/deletion. There are two ways in which chromosomal deletions can aid in genetic analysis experiments. Firstly, it does not correct loss-of-function mutation in genes that are found in the deletion chromosomal region. The use of deletion is to map mutations to particular chromosomal regions, screen for new mutations in closely related gene sets, and evaluate the allelic strengths of new mutations. Secondly, mutant traits may be enhanced or suppressed by heterozygous deletions. The phenotype brought on by aberrant expression of a different gene engaged in the same biological process can be changed by reducing the copy number of one gene (Cook et al., 2014; Roote and Russell, 2012). Reduced gene dosage is harmful to *Drosophila melanogaster*, as it is to many other eukaryotes. Various literature search in the PubMed database was surveyed in search of Deficiency/deletion screens in the *Drosophila* fly model, and using phrases such as, "*Drosophila* and Parkinson's disease" retrieved 1249 articles **(Figure 1.9 A**), "*Drosophila* AND Genetic screens" retrieved 6103 articles (**Figure 1.9 B**), and "Genetic Screens AND Parkinson's Disease" retrieved 160 articles (**Figure 1.9 C**).

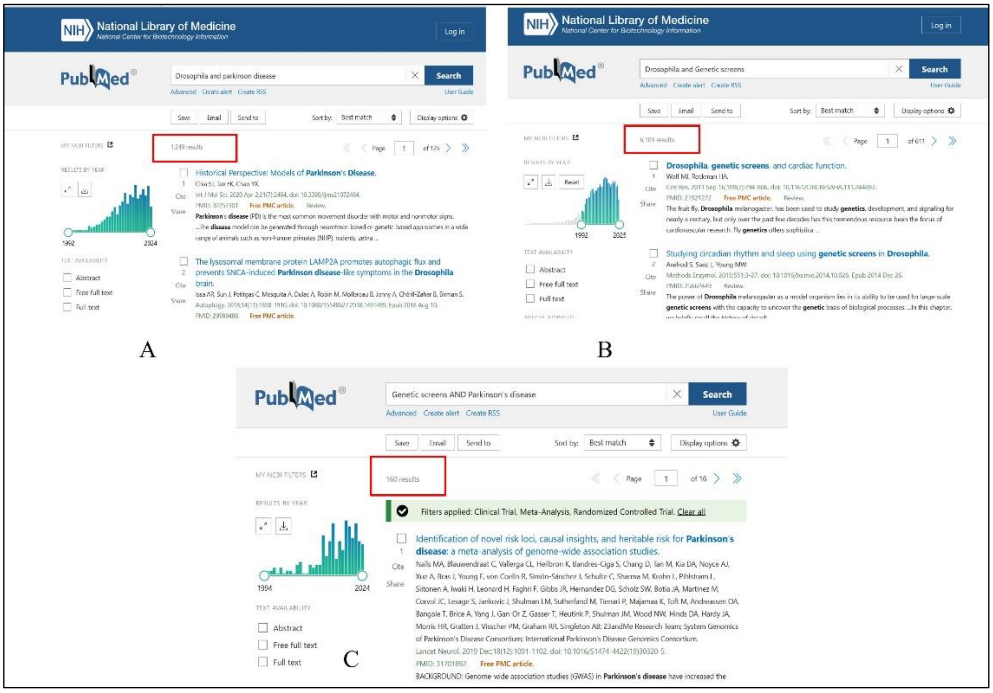


Figure 1.9: Snapshots depict search results in the PubMed network database. The search phrase "*Drosophila* and Parkinson's disease" retrieves 1249 articles (**A**). The search phrase "*Drosophila* AND Genetic screens" retrieves 6103 articles (**B**). The search phrase "Genetic Screens AND Parkinson's Disease" retrieves 160 articles (**C**).

However, a few of the deletion screens studies (also listed in **Table 1.2**) which were found reviewed from PubMed sources under the phrase 'Genetic screens AND *Drosophila*' were reviewed and studies performed show a screening of various phenotypes in flies as mentioned:

1.7.1. *Cardiomyopathy*

Drosophila has been used as a model for dilated cardiomyopathy described as enlarged end-diastolic and end-systolic dimensions along with decreased fractional shortening of the heart (Souidi and Jagla, 2021). Deletion screens using genome-deficiency lines are investigated to study the fly's genetic circulatory system of heart development and disease using optical coherence tomography in adult flies. A genetic deletion screen on chromosome 2L genomic deficiency lines in awake adult *Drosophila* was performed by Kim et al. (2010) to phenotype cardiac function. This screen identified a haplo insufficient

region on deletion mutant, *Df*(2L)Exel7007, characterized by dilated cardiomyopathy. Further, based on multiple complementary approaches, using transposon insertion mutants and transgenic rescue lines identified *weary* as a candidate gene causing its impaired phenotype whose over-expression of the gene resulted in normal heart function in awake *Drosophila*. Further, they also identified *weary* as a new notch ligand that is critical in maintaining normal cardiac function (Kim et al.,2010). Similar deficiency screen studies were performed in the X chromosome line and identified *Df*(1) Exel6240 having cardiomyopathy phenotype where deletion of an uncharacterized gene CG3226, caused the phenotype. This gene was confirmed to have homologs linked to the mammalian Siah-interacting protein, which is an adaptor protein involved in β-catenin/Armadillo signalling in the adult fly. Fly's heart function was measured and observed that alterations in this Armadillo signalling caused changes in the size and function of the adult heart during development (Casad et al., 2012). Through this approach, accurate identification of cardiac phenotype and function was achieved and also clues linked to its evolutionarily conserved molecular entities that target the disease were discovered. Flies with 3L chromosome deficiencies were also observed for abnormal cardiac phenotype, and deficiency mutants encompassing the rho3 locus showed enlarged cardiac phenotype mediated by the rho3-EGFR signalling pathway, as rho3 is found to be crucial for normal adult cardiac function (Yu et al., 2010).

1.7.2. *Mitochondrial integrity*

Mitophagy is a quality control mechanism that facilitates the targeted elimination of defective mitochondria. An effective *Drosophila* genetic screen with deficiencies for glossy-eye flies was identified and 23 complementation groups were compared with previously identified mutants (*tend*, *pdsw*, *mRpL4*, *mRpL17*, and *CoVa*) and it was observed to encode mitochondrial protein where new mutations that were mapped were

found to be involved in mitochondrial function/protein synthesis as they displayed similar symptoms as mutants derived from their previous screens (Liao et al., 2006; Mandal et al., 2005). These mutants further showed appropriate neuronal differentiation, and proper early cell G1-S cell cycle during 3rd instar larva stage and exhibited reduced mitochondrial function, brought on by its absence in one of the individual components, resulting in an enforcement of the G1-S checkpoint cell cycle (Mandal et al., 2005). This approach proved the fact that systematic screen can encode genes directly linked to mitochondrial function, paving the way for identifying and improving critical cellular functions. Furthermore, a genome-wide screen results in heterozygous deletions covering approximately 70% of the euchromatin also identified catalytic subunit of mtDNA polymerase gene (POLG), *tam* as a nuclear modifier affecting mtDNA. It was found that reducing the dose of *tam* led to the elimination of defective mitochondrial genomes indicating that targeting specific nuclear genes can potentially block the propagation of pathogenic mitochondrial mutations (Chiang et al., 2019).

1.7.3. *Embryonic Phenotypes, Orphan Receptor Ligands, and Dorsal Closure*

Screening of embryos homozygous for more than 700 distinct deficiency mutations for orphan receptor ligands, embryonic phenotypes, and genes affecting protein localization in *Drosophila* allowed observation of novel axon guidance phenotypes in the neuromuscular, CNS, and quantitative estimation of the number of potential genes involved in controlling the guidance of particular motor axon branches in a few of the deficiencies (Wright et al., 2010; Fox and Zinn, 2005). Dorsal closure in *Drosophila* is a highly robust mechanism that coordinates conserved gene expression patterns and signaling cascades to regulate the movements and changes in cell shape. It is a well-characterized stage in *Drosophila* embryogenesis and a paradigm for cell sheet morphogenesis. Dorsal closure deficiencies that collectively deletes 98.5% of genes on

the 2R chromosomal arm were screened unambiguously and homozygous embryos for each deficiency were photographed across time to determine the duration of dorsal closure. Embryos homozygous for 47 deficiencies for diverse deficits in closure were phenotypically observed as defects in tissue movement and cell shapes (Mortensen et al., 2018). Additionally, similar screens were performed on the 2L chromosomal arm, identifying 87.2% of genes in embryos homozygous for 49 deficiency defects related to dorsal closure. This supported the involvement of *paired*, *sloppy-paired*, *pimples*, and *odd-skipped* as novel dorsal closure genes on the 2L arm (Fogerson et al., 2020).

1.7.4. *Life span*

The discovery of genetic changes that potentially prolong lifespan using model organisms has changed the field of gerontology. Single gene mutations in the insulin and insulin-like growth factor (IGF) signalling (IIS) pathways can extend the life span of worms, flies, and mice, suggesting that the mechanisms involved have been conserved throughout evolution (Ziv, 2011). The *SOD* genes of *Drosophila* are essential for a healthy lifespan and tolerance to oxidative stress, and are therefore studied concerning aging mechanisms. Manganese superoxide dismutase (*MnSOD*) lengthens lifespan through mitochondrial and cellular redox balance and induces unfolded protein response in the mitochondria (Tower, 2015). Genome-wide screens in the *Drosophila* genome identified defects that impact *MnSOD* mRNA expression levels, hence affecting MnSOD gene transcription (Paul and Duttaroy, 2003). Similar phenotypes were observed in mice, where knockout of *MnSOD* resulted in abnormalities in brain development, heart development, behaviour, atypical muscle fatigue, and abnormal liver lipid metabolism (Bhaskaran et al., 2023; Wallace, 2001; Lebovitz et al., 1996). A genome-wide deficiency screen of 439 deficient strains, encompassing 65.6% of the *Drosophila melanogaster* genome, was conducted to identify quantitative trait loci (QTL) associated with thermal resistance. This study found

three new QTL for heat resistance and narrowed the large QTL discovered by Norry et al. (2004) to 16 significantly smaller candidate regions (Takahashi et al., 2011). Also, further mutant screens for a gene that enhanced life span in *Drosophila melanogaster* showed 30% enhancement in its average lifespan with improved resilience to heat, stress, starvation and dietary component paraquat, which produces free radicals (Lin et al., 1998).

Thus, observable phenotypes through deletion screening can provide further clues in understanding their biological functions, understanding epistasis where the activity of one gene may depend on other and gene redundancy in *Drosophila* where certain genes can compensate for the absence of others. Additionally, by systematically deleting different regions of the genome, a researcher can also map which chromosomal regions may be responsible for specific traits, phenotypes and functions.

1.8. Necessity of *Drosophila* deletion screens to decipher DAergic neuroprotective genes

DAergic neuron's nigrostriatal pathways are more vulnerable to late-onset dysfunction due to changes in DA metabolism, uptake, and synthesis with aging (Surmeier, 2018; Collier et al., 2017). PD being a late-onset disease with incurable and ensuing effects, there is a lot to understand about its etiology, progression, and the underlying genes responsible for the disease. Therefore, using the art and design of genetic screens, one can also understand the mechanisms underlying its susceptibility. If the gene of interest to be mutated is viable in early developmental stages of flies, a climbing assay is conducted as the primary screen for phenotypic markers of diminished brain functioning, such as low climbing mobility (Gevedon et al., 2019). Subsequently, secondary screening which includes histological analysis /quantification of TH proteins of brain tissue are performed in order to verify the neuroprotective function of the gene by scoring

neurodegenerative phenotypes. Furthermore, gene mapping procedures which include meiotic and deficiency mapping relying on these assays are followed by DNA sequencing to search for possible nucleotide base changes in the mutation of interest. Therefore, in the context of DAergic neurons, deletion screens may help to identify genes whose loss/absence may lead to neuronal degeneration or susceptibility, thereby highlighting their protective roles allowing one to screen large genomic regions efficiently and identify specific genes that contribute to DAergic neuron integrity. These screens may uncover both unknown and novel genes involved in neuroprotection/neurodegeneration, providing a comprehensive understanding of the genetic landscape influencing DAergic neuron survival (Davis et al., 2021). If any neuroprotective genes are identified, subsequent studies are carried out to define the molecular mechanisms and signaling pathways they regulate. Understanding these pathways may further help and/or provide clues in identifying potential therapeutic targets and strategies for neuroprotection in humans. Furthermore, deletion screens in *Drosophila* can model human NDDs in general by imitating gene loss-of-function observed in patients and also test for potential interventions in a controlled environment.

Therefore, keeping the above views in mind, literature search in ‘Pubmed’ source was surveyed with phrase ‘Chromosomal Deletion screens AND Parkinson’s Disease’ and results retrieved ‘No results found’ as depicted in **Figure 1.10**.

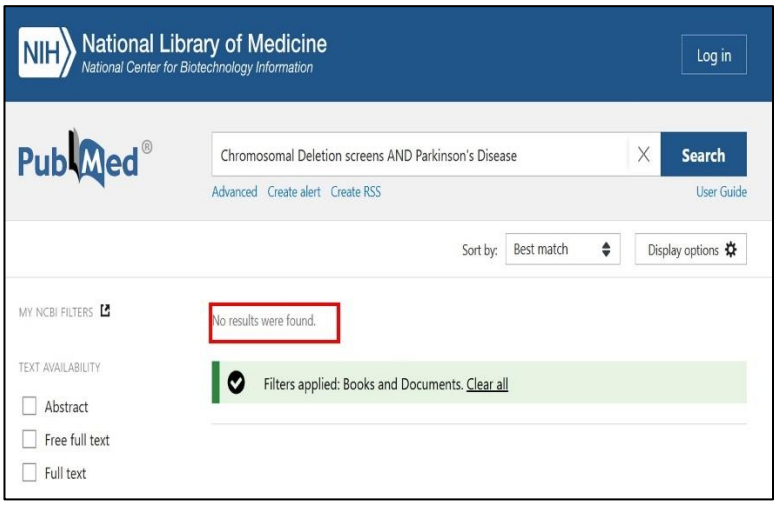


Figure 1.10: Snapshot depicts search results in the PubMed network database. The search phrase 'Chromosomal Deletion screens AND Parkinson's Disease' retrieves zero results found; suggesting the necessity, importance and opportunity of chromosomal deletion screens to understand the extent of brain DAergic neurodegeneration and/or neuroprotection in a deletion mutant of the PD model.

Studies showed that no large systematic deficiency /deletion screens were performed thus far, to directly probe neurodegeneration in PD condition owing to an opportunity to screen a large number of deficiencies in this direction. Hence, taking advantage of deletion screens with the use of deficiency lines, one may also be able to figure out chromosomal regions that is critical for the phenotype(s)that a researcher is looking for, the mobility defects linked to dopaminergic neurodegeneration which is one of characteristic feature linked with NDDs such as PD.

Notably, behaviour is very critical in understanding and identifying any phenotypical variation in biology, here more or so related to neurodegeneration which also serves as one of the primary markers to begin any primary screening assays in experimental research. Fly tends to climb towards the light/against gravity *i.e.*, positively phototactic/negatively geotactic. Based on the phenotype of the fly that climbs towards the light, climbing mobility is assessed by Negative geotaxis assay (NGA).

1.9. Conclusion

Despite performing various research studies thus far with varied genetic screening techniques, there remains a gap in the existing knowledge of understanding chromosomal deletion role in studying DAergic neurodegeneration in the Idiopathic PD model. Specifically, further exploration of 3rd chromosome deletion lines in *Drosophila* could shed some light on the mechanisms underlying PD. Therefore, by taking advantage of the 3rd chromosome deficiency lines, the following objectives were carried forward:

1. Primary screening of deletion lines for mobility defects using Negative Geotaxis Assay (NGA).
2. Characterization of *Df* 8097 line for DAergic neurodegeneration using whole brain TH-Immunostaining.
3. Characterization of brain DA metabolism in *Df* 8097 using High-Performance Liquid Chromatography (HPLC-ECD).

CHAPTER 2

Materials and Methods

2.1. Fly stock and husbandry

The flies used in this experiment were Canton S (CS), *Drosophila* stocks (3rd Chromosome deficiency lines). Deficiency lines were procured from BDSC (Bloomington Drosophila Fly Stock Center, Indiana University, USA) (http://flybase.bio.indiana.edu). Lines containing the deletions were maintained as heterozygotes against an appropriate balancer chromosome. All analyses were performed in these stock-defined states, including balancers. Flies were maintained at 22ºC ± 2ºC temperature with humidity of 60% at 12:12 hrs light and dark conditions and fed on food media containing (Sucrose, Agar-Agar, Yeast, and Propionic acid) (Phom et al., 2014; Luckinbill et al., 1984), under standard laboratory condition.

The following *Drosophila* stocks (3rd Chromosome deficiency lines) used in the study are depicted in **Table 2.1.**

Table 2.1: Lists of 3rd Chromosome deficiency lines tested in the study.

GENOTYPE	STOCKS	Observed bkpts R6 Seq or Cyto Bands	Estd. Cyto Locs
Df(3L)Exel6085	7564	3L:548528;3L:749303	61C3;61C9
Df(3L)ED4196	8050	3L:639583;3L:1478937	61C7;62A2
Df(3L)BSC800	27372	3L:1628101;3L:1647451	62A9;62A9
Df(3L)Aprt-32	5411	3L:1668654-1795328;3L:2554847-2587456	62A10-62B4;62E6-62E7
Df(3L)ED4287	8096	3L:1795442;3L:2551761	62B4;62E5
Df(3L)BSC23	6755	3L:2589553-2591159;3L:3193242	62E7;63B11
Df(3L)Exel6092	7571	3L:2821245;3L:3047162	62F5;63A3
Df(3L)BSC671	26523	3L:2982129;3L:3193143	63A2;63B11
Df(3L)BSC672	26524	3L:3081311;3L:3206906	63A7;63B12
Df(3L)ED4293	8058	3L:3226338;3L:3250564	63C1;63C1
Df(3L)BSC371	24395	3L:4868210;3L:5634506	64C1;64E1
Df(3L)BSC410	24914	3L:5770673;3L:6490185	64E7;65B3
Df(3L)BSC27	6867	3L:6942786;3L:7156003	65D3;65E5
Df(3L)BSC815	27576	3L:8263064;3L:8506640	66C3;66D4
Df(3L)BSC389	24413	3L:8422185;3L:8589597	66C12;66D8
Df(3L)BSC816	27577	3L:8639081;3L:8745362	66D9;66D12
Df(3L)BSC673	26525	3L:9763614;3L:10180958	67C7;67D10
Df(3L)ED4470	8068	3L:11096989;3L:11833230	68A6;68E1
Df(3L)ED4486	8072	3L:12514419;3L:13032485	69C4;69F6
Df(3L)BSC12	6457	3L:13044488;3L:13227765	69F6;70A3

Df(3L)ED4502	8097	3L:13227765;3L:13993551	70A3;70C10
Df(3L)ED4543	8073	3L:13935225;3L:14758040	70C6;70F4
Df(3L)ED4674	8098	3L:16661284;3L:17049418	73B5;73E5
Df(3L)BSC414	24918	3L:16969873;3L:17476126	73E1;74C3
Df(3L)Exel6132	7611	3L:17421582;3L:17533027	74B2;74D2
Df(3L)BSC419	24923	3L:21224932;3L:21604778	78C2;78D8
Df(3L)ED230	8089	3L:22134651;3L:22834371	79C2;80A4
Df(3L)ED5017	8102	3L:22835497;3L:22998301	80A4;80C2
Df(3L)1-16	7002	3L:23689640-24074180;3L:25798441-27136525	80F9-h47; h50-h52
Df(3L)6B-29+*Df*(3R)6B-29	2596	3L:24977118-25115180;3L:27967244-281102 27	h53R; h58
Df(3R)10-65	2597	3R:3461351;3R:4208469	81F;81F
Df(3R)ED5147	8967	3R:5087120;3R:5367804	82E7;83A1
Df(3R)ED5156	8965	3R:5264933;3R:5458852	82F8;83A4
Df(3R)Tpl10	1990	3R:5551168-5758997;3R:6896253-7086599	83B1-83C2;84A6-84B2
Df(3R)ED5177	8103	3R:5600629;3R:5624095	83B4;83B6
Df(3R)BSC47	7443	3R:5632351;3R:5861298	83B7;83C6
Df(3R)BSC633	25724	3R:7080388;3R:7123376	84B2;84C3
Df(3R)Mlp84B[y-37]	8644	3R:7122971;3R:7124547-7127971	84C3;84C3-84C4
Df(3R)BSC507	25011	3R:9259246;3R:9394580	85D6;85D15
Df(3R)BSC476	24980	3R:9417673;3R:9554982	85D16;85D24
Df(3R)Exel6264	7731	3R:9550705;3R:9704950	85D24;85E5
Df(3R)Exel6154	7633	3R:9793365;3R:9928791	85E9;85F1
Df(3R)Exel6155	7634	3R:9928791;3R:10089458	85F1;85F10
Df(3R)ED5514	8957	3R:10884998;3R:11569253	86C7;86E11
Df(3R)ED10642	9482	3R:16453757;3R:16625271	89B17;89D5
Df(3R)ED5644	9090	3R:14017903;3R:14625709	88A4;88C9
Df(3R)ED5664	24137	3R:14697309;3R:15228849	88D1;88E3
Df(3R)BSC741	26839	3R:15265187-15265191;3R:15340663	88E8;88F1
Df(3R)BSC43	7413	3R:20762299;3R:21112186	92F13;93B13
Df(3R)BSC819	27580	3R:20851577;3R:21060603	93A2;93B8
Df(3R)Exel6272	7739	3R:20957703;3R:21112334	93A4;93B13
Df(3R)ED6096	8684	3R:22587681;3R:23221969	94B5;94E7
Df(3R)slo3	6367	3R:22961926-23021279;3R:24753510-24753849	94D5-94D10;96A19
Df(3R)BSC619	25694	3R:23061559;3R:23346416	94D10;94E13
Df(3R)BSC461	24965	3R:25003409;3R:25292912-25292997	96B15;96D1
Df(3R)Exel6201	7680	3R:25137839;3R:25196998	96C2;96C4
Df(3R)Exel6202	7681	3R:25292997;3R:25515673	96D1;96E2
Df(3R)BSC321	24909	3R:25637827;3R:25680519	96E6;96E9
Df(3R)BSC497	25001	3R:27165617;3R:27905585	97E6;98B5
Df(3R)L127	3547	3R:29468941-29565244;3R:30387804-30459521	99B1-99B3; 99F2-99F5
Df(3R)BSC547	25075	3R:29621303;3R:29821399	99B5;99C2
Df(3R)BSC620	25695	3R:29877018;3R:30034890	99C5;99D3
Df(3R)BSC502	25006	3R:30060875;3R:30134759	99D3;99D8
Df(3R)BSC504	2500	3R:30428067;3R:30687263	99F4;100A2

Df(3R)ED6346	24142	3R:30783562;3R:31048884	100A5;100B1
Df(3R)ED50003	24516	3R:31985757;3R:31985757	[100E1-100E1]; [100E3-100E3]

2.2. Deficiency lines

Deficiency lines (stocks maintained as a heterozygote in the presence of 3rd chromosome balancer marker, stubble (Sb) was collected and aged for 10 days. Flies tested carried the heterozygous deletion and were of the genotype '*Df*/Sb'. In all the cases, CS flies serve as controls (deletion lines were backcrossed with CS for six generations) (Boynton and Tully, 1992). Alternatively, to decipher gene-environment interaction, 10-day old flies (both control and deletion lines as explained above) were exposed to neurotoxicant PQ and then mobility performance was assessed.

Male deletion mutant homozygote (if viable)/non-balancer flies (*Df/Df*) were collected and aged for 10 days. For mutant homozygote, along with other control CS, the heterozygote mutant line *per se* also serves as control. Most homozygous deletions are lethal; therefore, deletion mutant stocks are maintained as heterozygote in the presence of a balancer chromosome. Moreover, to understand if the mobility phenotype in deletion mutant is due to the balancer chromosome or the deleted regions, we tested deletion mutants in the context of CS control genetic background. Both stocks retained the same phenotype even in the absence of balancer chromosomes for mobility phenotype in the fly.

2.3. Preparation of media

Materials: Sucrose (Cat. No. 1947139) procured from Sisco Research Laboratory (SRL, Mumbai, India), market-available sugar-tolerant dry yeast (Angel, instant dry yeast), agar-agar (Cat. No. GRM666) procured from Hi-Media (Thane, India) and Propionic acid (Cat. No. 8006050-500-1730) procured from MERCK (Rahway, USA).

Methods: The media was prepared following the protocols of Phom et al. (2014). The components, which included 1.5 grams of agar-agar, 16 grams of yeast, and 27 grams of sucrose, were dissolved in double-distilled water for a few minutes. On an induction cooker, the mixture was brought to a boil for 12 minutes, stirring occasionally every 4 minutes. 1.78 mL of propionic acid was added after the medium has been cooked. The vials were then filled with the media.

2.4. Collection and aging of adult male files

Materials: Diethyl ether (MERCK, Cat: 1.00923.0521), glass bottle, vials containing media, glass plate, brush, and stereo zoom microscope were used for fly collection.

Methods: Flies in the propagation vials were tapped and transferred into a glass bottle. It was mildly anesthetized using few drops of diethyl ether, and the bottle was tapped gently. Once flies were anesthetized, they were transferred to a glass plate to separate males and females. A total of 25 male flies were transferred to a fresh vial containing media. Care was taken to avoid flies sticking to the media while switching them into fresh media vials every third day which was aged for ten days. Only male 10-day-old flies were employed in the present study.

2.5. Preparation of paraquat stock

Materials: Sucrose (Cat. No. 1947139) procured from Sisco Research Laboratory (SRL, Mumbai, India), Methyl viologen dichloride hydrate/Paraquat (PQ) (Cat. No. 856177) procured from Sigma Aldrich (St. Louis, MO, USA) were used for preparing the paraquat stock.

Method: Ten mM PQ stock was prepared by dissolving the required amount of PQ in 5% sucrose solution. Whatman filter paper no.1 disc cut in a form of disk placed in a 30×100mm glass vial was used as a feeding medium for the experiment.

2.6. Fly treatment

A volume of 275µL of 5% sucrose and 275µL of treated solution (10mM PQ) prepared was pipetted upon a filter disk placed on a 30×100mm glass vial and fed to the control and treated flies, respectively. Twenty-five flies were transferred on each vial for the treatment. Male flies (8-10 days old) were treated with freshly prepared 5% sucrose (Control) and 10mM PQ solution (Treated) for 3 hrs, 4 hrs, 5 hrs, 6 hrs. Male flies were used in all experiments.

2.7. Longevity assay

A minimum of 100 adult male flies consisting of 4 vials were placed at 22 ± 2ºC with humidity of 60% and 12 h:12 h light and dark cycle. Flies were moved to a fresh medium vial on each 4th day. Their mortality over time was recorded. Longevity curves were obtained from independent trials with a minimum of 100 flies per experiment until all the flies were recorded dead.

2.8. Screen strategy

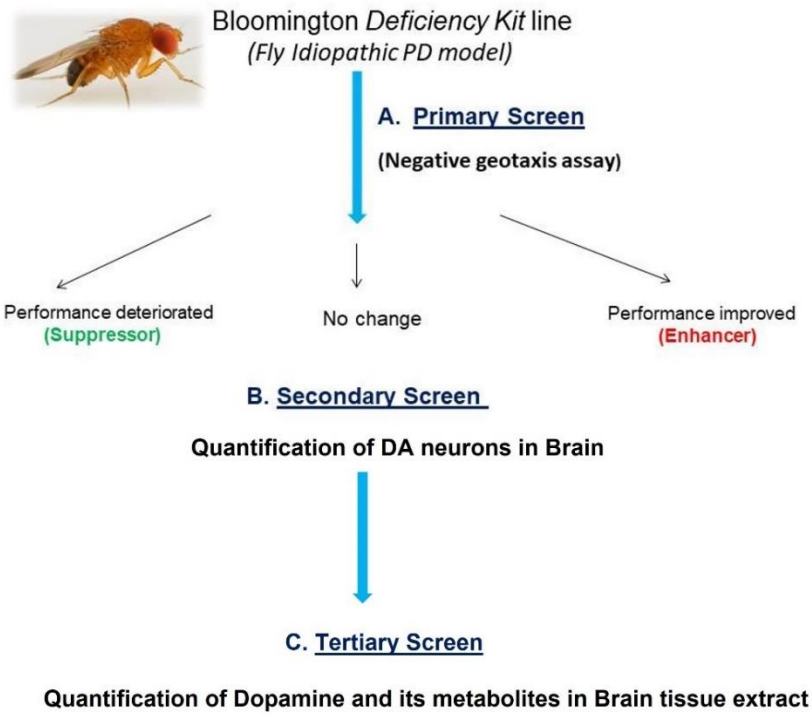


Figure 2.1: Flow chart depicts the genetic screen strategy involving Primary screen (Negative geotaxis assay) **A**. Secondary screen (For quantification of DA neurons in Brain) **B**. and Tertiary

screen (For quantification of Dopamine and its metabolites in Brain tissue extract) **C**. to understand mobility defects associated with dopaminergic neurodegeneration/neuroprotection in *Drosophila* PD model.

2.8.1. Balancing Deficiency Lines

In *Drosophila melanogaster*, balancers are multiply inverted and rearranged chromosomes employed for various functions, including preserving harmful alleles in the stock, maintaining the linkages between alleles, and preventing the recovery of recombinant chromosomes. Most balancers have dominant, obvious markers that make their inheritance simple to trace in crosses and recessive lethal/sterile mutations that keep the flies from becoming homozygous in stock. In *Drosophila melanogaster,* balancers are available for all chromosomes except the small fourth chromosome (which does not usually undergo exchange) and the Y chromosome (Miller et al., 2019; 2018). For a balancer chromosome to be viable, it should have a heterozygosity genotype. These chromosomes carry unique genetic 'markers' along with them to be readily observable phenotypically under a microscope, hence helping to identify mutations of interest. Accurate genetic crosses are enabled by visible 'marker' mutations that allow the selection of offspring that inherited one version or the other of a chromosome (Miller et al., 2016). In this study, 3rd chromosome balancer , known as '*TM3/TM6B'*, plays a role in stabilizing the third chromosome. *TM3* and *TM6* are chromosomal balancers and the deficiency was obtained over a balancer chromosome containing the multiple inversion '*TM3,Sb'* codes for *Sb* (Stubble) which has stubbly, short bristles carries a mutant */*TM3,Sb* (**Figure 2.2 A**) and */*TM6,Tb* codes *Tb* (Tubby), a dominant allele coding for short, fat larvae and pupae (**Figure 2.2 B**) gene as dominant markers. Flies with the *TM3, Sb* balancer exhibit shortened (or stubbly) hairs on their back, visible under a microscope, while those with *TM6B,Tb* balancer display short and stout bodies, which serves as an effective larval and pupal markers (Greenspan, 1997).

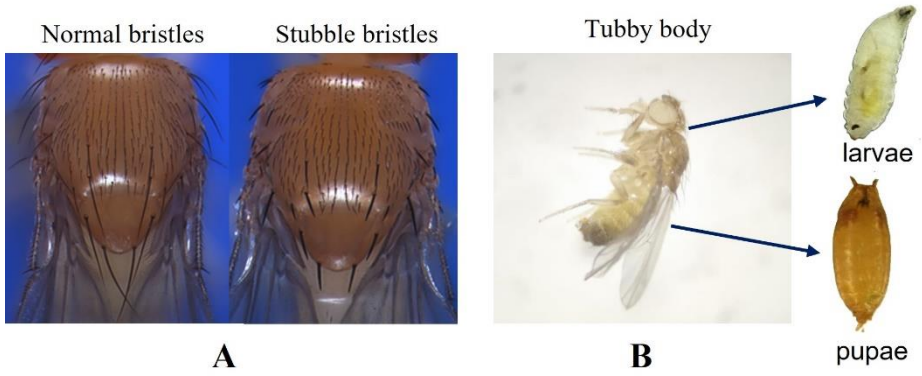


Figure 2.2: Phenotypes associated with 3rd chromosome balancer carrying stocks as Stubble '*Sb*' with short stubbly hairs on its thorax (**A**) and Tubby '*Tb*' (**B**) with short stout body (have stout larvae and pupae which also serve as markers).

Figure 2.3 represents generalized scheme to balance a deletion stock using 3rd chromosome deficiency line balancer *(TM3/TM6B).*

Scheme to balance a line:

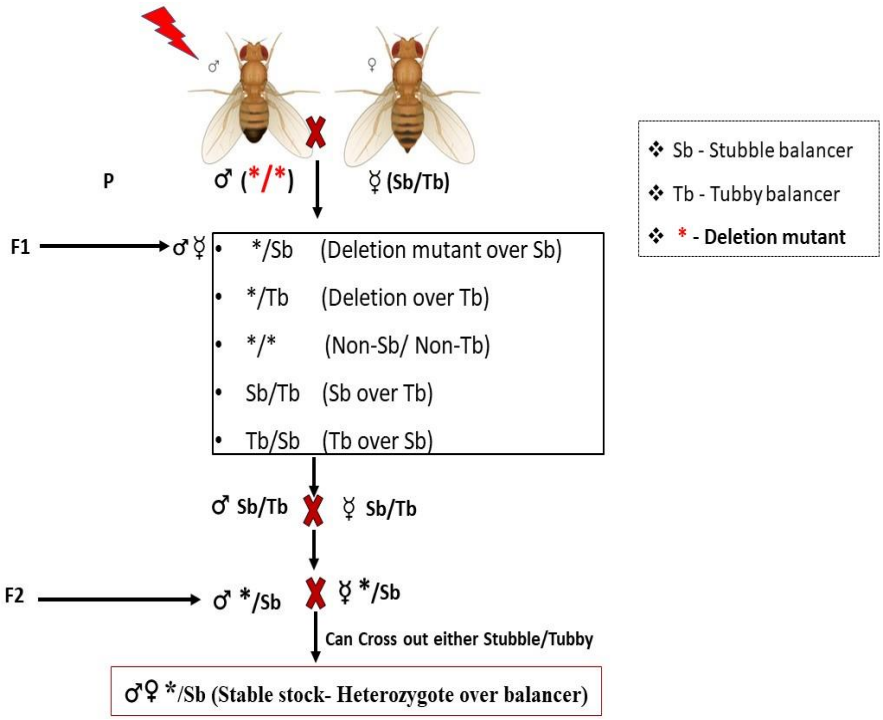


Figure 2.3: Generalized scheme to balance 3rd Chromosome deficiency line. Parental generation with male (♂) deletion line (mutagenized line with red mark) and female (♀) with appropriate balancer line (TM3/TM6) tagged with balancers/markers are crossed to each other. Parental flies are removed and the following types of progeny produced in the F1 generation are observed and represented in the scheme above. Sb/Tb (♂♀) from the F1 generation are crossed to each other and the progeny produced in the F2 generation; here, either */Sb or */Tb (♂♀) is crossed out and the final stable stock to be maintained are produced in F3 generation either as (*/Sb) deletion over

stubble balancer/(*/Tb) deletion over Tubby balancer. (***** represents- deletion mutant; Sb- Stubble balancer marker; Tb-Tubby balancer marker; */*-non-Sb/non-Tb) (Image created with Biorender.com).

2.9. Negative geotaxis assay

Materials: Glass vials, plastic tubes with cap, 10 mL graduated glass/plastic pipettes, stopwatch, mouth aspirator, sponge pad (at least 2.5 cm height) and test tube stand were used for negative geotaxis assay.

Method: Negative geotaxis assay (NGA), as described by Phom et al. (2021) and Botella et al. (2004) is a climbing assay-based scoring of mobility phenotype which serves as a primary screening strategy to screen lines for mobility phenotypes. An individual fly was placed into the plastic tube and allowed to acclimate for 2 minutes. The flies were tapped to the bottom of the tube, and the height it climbed in 12 seconds was recorded. A minimum of 15 flies were scored for each group and was repeated thrice for each fly. The same procedure was followed for all the flies, and at least 12-15 flies were used for each treatment group (**Figure 2.4**).

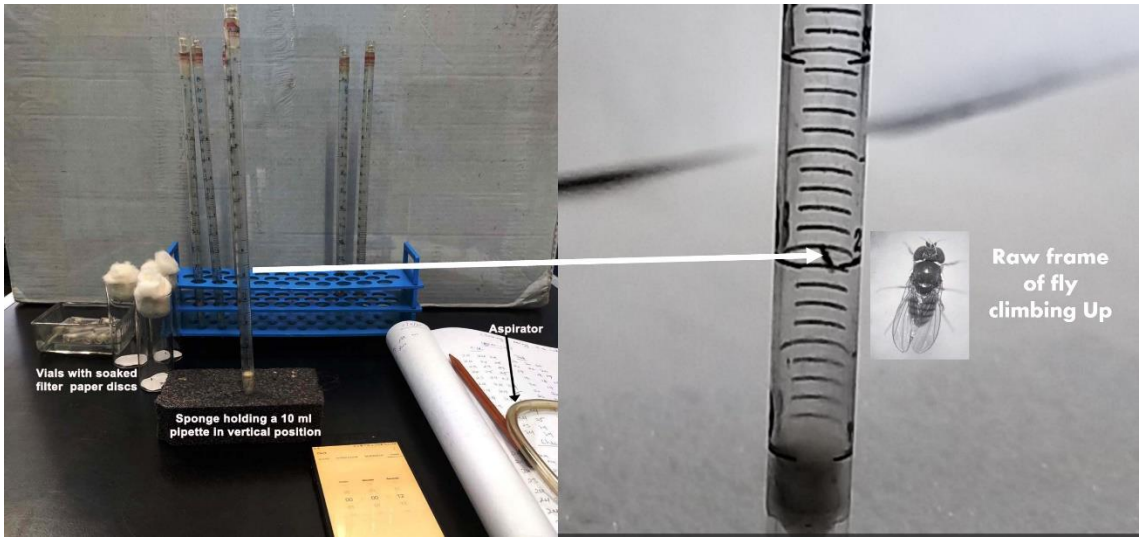


Figure 2.4: Experimental set-up for negative geotaxis assay (Adapted from Phom et al., 2014; 2021).

2.10. Characterization of neurotoxicant treatment window at which fly does not exhibit motor dysfunctions

For the characterization of the neurotoxicant treatment window, I decided to examine the earliest time point in the lifetime of a fly at which mobility defects first become observable in the sporadic PD model of *Drosophila* following previously established protocol in our laboratory by Koza et al. (2023), which developed a neurotoxicant exposure window at which fly does not exhibit mobility defects tested by NGA. Accordingly, I exposed control CS male flies to different time points at 3 hrs, 4 hrs, 5 hrs, and 6 hrs (**Figure 2.5**) and subjected to a negative geotaxis assay. PQ-induced motor dysfunction was evaluated through its mobility efficiency. Here, Flies showed no difference in climbing speed at 4 hrs of PQ exposure as compared to control flies which were subjected to 5% sucrose solution. Further exposure *i.e.*, 5 hrs onward, the fly started showing mobility defects as compared to control (**Figure 2.5**). However, there was no significant difference in the motor ability between control and treated flies up to 4 hrs treatment, providing an opportunity to understand the susceptibility levels of loss-of-function mutations in deletion mutants. Therefore, observation at a 4 hrs time point was chosen for all assays of deletion fly lines.

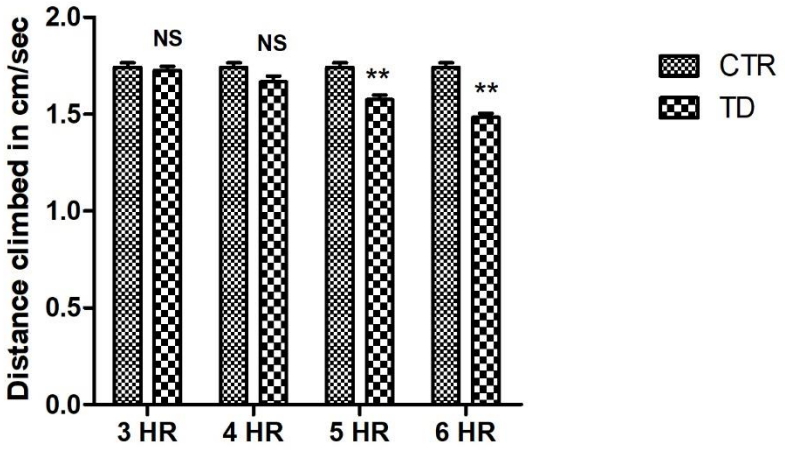


Figure 2.5: Characterization of neurotoxicant exposure window at which fly does not exhibit motor dysfunctions as determined by negative geotaxis assay. *Drosophila* male (8-10 days

old) was exposed to 10 mM paraquat and 5% sucrose at 3hrs, 4 hrs, 5 hrs, and 6 hrs time points. NGA was performed to assess the climbing mobility of the fly. Fly started showing motor defects from 5 hrs treatment onwards. However, there was no significant difference in the motor ability between control and treated flies up to 4 hrs treatment, providing an opportunity to understand susceptibility levels of loss-of-function mutation in deletion mutants. For summative analysis, a T-test was performed; (** signifies P<0.01; NS: Not significant).

2.11. Quantification of dopaminergic neurons and TH fluorescence intensity using fluorescence microscopy

2.11.1. TH Immunostaining

Sterilized 1.5 mL centrifuge tubes (Tarsons, WB, India, catalog number: 500010), Parafilm™ wrapping film (Bemis, WI, USA, catalog number: PM996), Conical flask (Borosil, Mumbai, India, catalog number: 5100), Magnetic stirrer bar #8 mm × 40 mm (Tarsons, WB, India, catalog number: 4113), SPINNOT™ digital magnetic stirrer hotplate (Tarsons, WB, India, catalog number: 6090), Sterilized micro tips (Tarsons, WB, India, catalog number: 521010), Freshwrapp aluminum foil 9-11 µm (Hindalco, Maharashtra, India, catalog number: HV2241), Glass plate (Suwimut, USA, catalog number: B08FRB2NTM), Fingernail polish (Faces Canada, Mumbai, India, catalog number: CC4403), Glass spacer (Borosil, Mumbai, India, catalog number: 9115S01), Microscopy slides #76 mm × 26 mm (ReliGlas, Haryana, India, catalog number: 7101), Gold-seal coverslips (22 mm^2) (Electron Microscopy Sciences, PA, USA, catalog number: 63765-01), Whatman™ filter paper (GE Healthcare, Buckinghamshire, UK, catalog number: 1001917), Paraformaldehyde (PFA) pH 7.4 (Sigma-Aldrich, St. Louis, MO, USA, catalog number: I58127), Phosphate buffered saline (PBS) pH 7.4 (HiMedia, Maharashtra, India, catalog number: ML023), Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA, catalog number: T8787), Normal goat serum (NGS) (Vector Labs, CA, USA, catalog number: S1000), Rabbit anti-tyrosine hydroxylase (anti-TH) polyclonal primary ab (Millipore, MA, USA, catalog number: Ab152), Goat anti-rabbit IgG H&L (TRITC-labeled) polyclonal secondary ab (Abcam, MA, USA, catalog number: Ab6718),

VECTASHIELD® mounting medium (Vector Labs, CA, USA, catalog number: H1000), Fly head capsule handling items e.g., needles #31 G × 6 mm (Tentabe BD, Punjab, India, catalog number: 324902).Dissecting fine forceps (EMS, PA, USA, catalog number: 78620-4B), Brush (TEYUP, Delhi, India, model number: SR-1013), Delicate task Kim wipers (KIMTECH™, GA, USA, catalog number: 370080), Micropipette *i.e.*, 1,000 µL, 50 µL, 10 µL, 2 µL (Gilson, WI, USA, catalog number: 30040), Frost-free refrigerator (Whirlpool, MI, USA, model number: FF26 4S), pH/mV meter (Hanna Instruments, RI, USA, model: HI2211-02), -20ºC ES Series refrigerator (Thermo Scientific, MA, USA, model: 50616100444443250), -80ºC ultra-low temperature freezer (New Brunswick Innova, Hamburg, Germany, model: U101-86), Stereo zoom microscope (Carl Zeiss, Jena, Germany, model: Stemi 305), Stereo zoom microscope (Leica, Wetzlar, Germany, model: E24), Fume hood (BIOMATRIX, Telangana, India), BOD incubator (Percival, IA, USA, model: DR-36VL), Test tube rotator (Tarsons, Rotospin, WB, India, catalog number: 3070) and disk for 24 × 1.5 mL tube (Tarsons, WB, India, catalog number: 3071), Axio Imager M2 fluorescence microscope fitted with 100W Mercury lamp (Carl Zeiss, Jena, Germany, catalog number: 430004-9902-000), AxioCam ICm1 monochromatic camera (Carl Zeiss, Jena, Germany, catalog number: 426553-9901-000), ZEN 2012 SP2 blue edition, version 2.0.14283.302 (Carl Zeiss, Jena, Germany), Microsoft Office Excel Worksheet 2007 (Microsoft Inc., WA, USA).

Recipes:

1. 4% PFA solution (50 mL)

PFA 2 g
1X PBS 50 mL

a. Add PFA in 1X PBS in a conical flask, cover it with parafilm, and shake it thoroughly for 10 min.

b. Transfer the flask with a magnetic stirrer on the hotplate for heating/boiling with a temperature ranging from 80ºC to 110ºC with moderate stirring at 150 rpm.

c. Keep the flask on the hotplate until the cloudy solution becomes transparent.

d. After this, switch 'off' the hotplate and continuously stir for 15 min. Allow the solution to cool down, aliquot it in a 1.5 mL centrifuge tube and store it at -80ºC.

Critical: Do not store the solution for more than a week.

Caution: PFA is a potential carcinogen; hence, the whole process should be done under a fume hood. Wear proper hand gloves and lab coat during handling and preparation of PFA solution.

2. 0.1% PBST (phosphate buffered saline and Triton X-100) (50 mL)

10X PBS 5 mL
Autoclaved enzyme-free water 45 mL
Triton X-100 50 µL

a. Add 5 mL of 10× PBS in 45 mL of autoclaved enzyme-free water.

b. Mix 50 µL of Triton X-100 and vortex it for 10 seconds. The solution can be stored at room temperature for one week.

3. 0.5% PBST (50 mL)

10X PBS 5 mL
Autoclaved enzyme-free water 45 mL
Triton X-100 250 µL

a. Add 5 mL of 10X PBS in 45 mL of autoclaved enzyme-free water.
b. Mix 250 µL of Triton X-100 and vortex it for 10 sec. The solution can be stored at room temperature for one week.

4. 5% NGS blocking buffer solution (1 mL)

NGS 50 µL
0.5% PBST 950 µL

a. Add 50 µL of NGS in 950 µL of 0.5% PBST and mix it properly by vortexing for 10 secs. The solution can be stored at room temperature for 1-2 hrs.

5. Anti-TH polyclonal primary ab solution

Anti-TH polyclonal primary ab 5 µL
5% NGS blocking buffer 1,245 µL

a. Take 1,245 µL of 5% NGS blocking buffer and add 5 µL of anti-TH polyclonal primary ab (1:250 dilution). Mix it gently by inverting the tube slowly and place it on the ice until used.

6. TRITC-labeled polyclonal secondary ab solution

TRITC-labeled polyclonal secondary ab 5 µL
5% NGS blocking buffer 1,245 µL

a. Take 1,245 µL of 5% NGS and add 5 µL of TRITC-labeled polyclonal secondary ab (1:250 dilution). Mix it gently by inverting the tube slowly and store it on ice until used.

2.11.2. Characterization of DAergic neurodegeneration

The following four steps were taken into consideration to comprehend neurodegeneration in the fly model of sporadic PD:

A) Anti-TH immunostaining of the whole *Drosophila* brain.

B) Image acquisition.

C) Quantification of DAergic neurons.

D) Quantification of neurodegeneration through quantification of fluorescence intensity (FI) of DAergic neurons.

A) Anti-TH Immunostaining in the whole *Drosophila* brain

The *Drosophila* brain was immuno-stained for fluorescence microscopy (Carl Zeiss, Axio Imager M2 with ZEN software, Germany) according to the protocol of Chaurasia et al. (2024); Ayajuddin et al. (2023); Koza et al. (2023). Elaborately, Anti–TH Immunostaining procedures were carried out as follows:

Methods:

1. The whole fly head tissue were fixed in 4% paraformaldehyde (PFA; pH 7.4) containing 0.5% Triton X-100 (TX-100) for 2 hrs through mixing using a test tube rotator with constant velocity (10 rpm) at room temperature (RT).

2. PFA was then removed after 2 hrs of fixation by washing the fly brains with PBS that contains 0.1% TX-100 (0.1% PBST) three times after every 15 minutes at RT.

3. Dissection of brains were carried out in PBS (pH 7.4) under a stereo zoom microscope using fine forceps and needles to remove the head capsule and connecting tissues at RT.

4. Brains were then washed with 0.1% PBST for 5 times after every 15 minutes at RT.

5. Brains were blocked with 5% NGS in PBS containing 0.5% TX-100 (0.5% PBST) for 120 minutes at RT.

6. Further, it was incubated/probed with primary anti-TH polyclonal antibody in the dilution of **1:250** for 72 hrs at 4ºC through mixing using a test tube rotator at constant velocity (10 rpm).

7. The excess primary antibodies were washed off using 0.1% PBST for 5 times after every 15 minutes at RT.

8. Brains were then incubated with a TRITC (Tetramethyl rhodamine) labelled polyclonal secondary antibody in the dilution of **1:250** for 24 hrs in the dark (**Note**: Cover centrifuge tube containing brains with aluminium foil) by thorough mixing with a test tube rotator at a constant velocity (10 rpm) at RT.

9. Further, to eliminate excess polyclonal secondary antibodies, the brains were washed with 0.1% PBST for 5 times after every 15 minutes at RT.

10. The brains were mounted in VECTASHIELD® mounting medium and then topped with cover glass (Electron Microscopy Sciences). **Note:** Glass spacers were placed around the VECTASHIELD® mounting medium to protect brains from being crushed by a coverslip.

Critical: Brains were scanned in a dorsoventral orientation.

11. Clear fingernail polish was used to seal the edges.

12. The samples were prepared for image acquisition.

Precautions and Recommendations

1. During fixation, brains were thoroughly mixed using a circular rotator (Rotospin from Tarsons, India Cat: 3070) at a constant speed of 10 RPM.

3. Circular rotator was used for proper incubation/mixing of primary and secondary antibodies to the brain samples.

4. To prevent brains from being crushed, care was taken by fixing glass spacers on its edges while mounting the brain with a cover slip.

5. To prevent the samples from being drying out, the edges were carefully sealed with nail polish.

6. Simultaneously, acquisition of images was done on the same day to avoid bleaching.

B) Image Acquisition

ZEN 2012 SP2 software of fluorescence microscope equipped with a 100W Mercury lamp was used to capture brain images. Steps for acquisition of *Drosophila* brain Image for quantification of DAergic neurons and fluorescence Intensity (FI) using fluorescence microscope (Axio Imager 2, Carl Zeiss) with *ZEN 2012 SP2* software illustrates from **Figure 2.6** to **Figure 2.16**.

Methods:

1. At 40x objective lens of fluorescence microscope, prepared/stained brains were viewed/observed (**Figure 2.6**).

2. Images were scanned and captured using a monochromatic camera with a Rhodamine fluorescence filter (**Figure 2.6**).

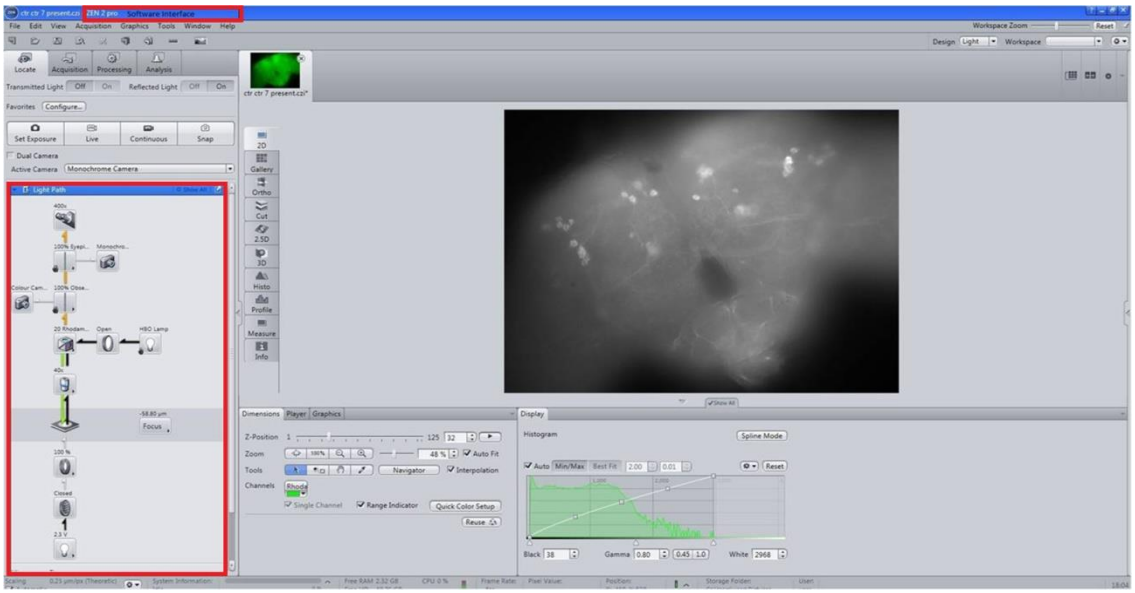


Figure 2.6: Scanning of the whole brain of *Drosophila*. Scan the anti-TH immunostained *Drosophila* brain using Carl Zeiss, Axio Imager M2 (40x objective lens) with ZEN 2012 SP2 software that interactively controls image acquisition, image processing, and analysis of the images.

3. A red dot test was performed in the control brain in the acquisition panel (select range indicator from *Dimensions* and set exposure from *Acquisition parameter*) for visibility of DAergic neurons and to assess the signal saturation during the image acquisition. Allow same exposure time for all brain samples (**Figure 2.7**).

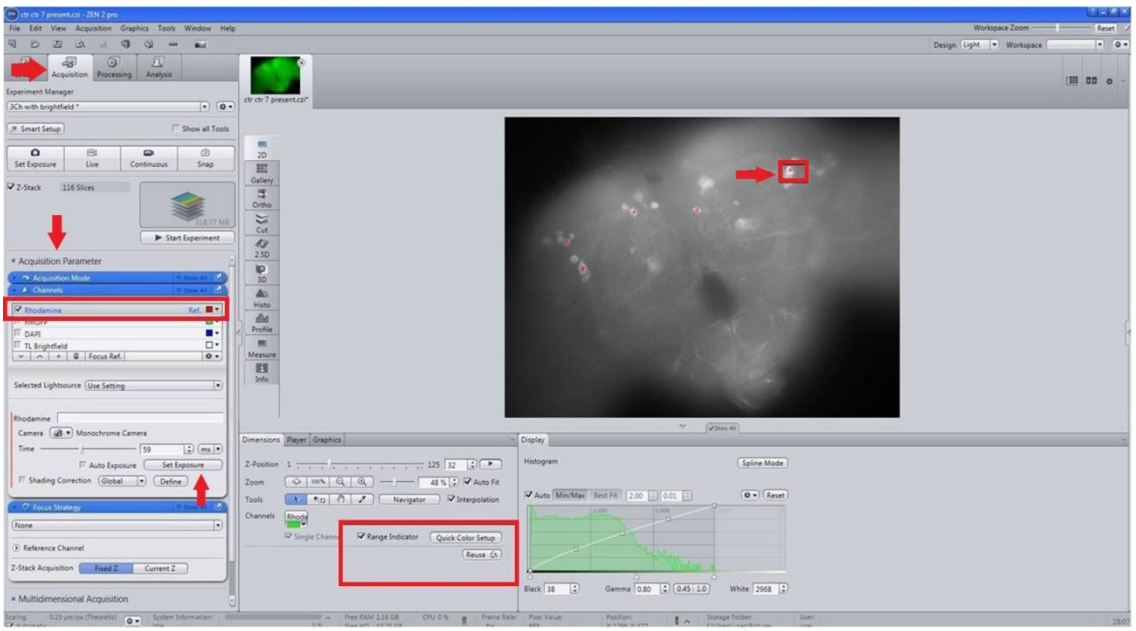


Figure 2.7: Image acquisition and performing the red dot test. For image acquisition, select a monochromatic camera with a Rhodamine filter. Perform a red dot test for visibility of

dopaminergic (DAergic) neurons and assessing of its saturation point using a single brain, incorporating the same exposure time for other samples.

4. Then, Z-stack programming was performed with constant interval of 1.08 µm for each image (**Figure 2.8**).

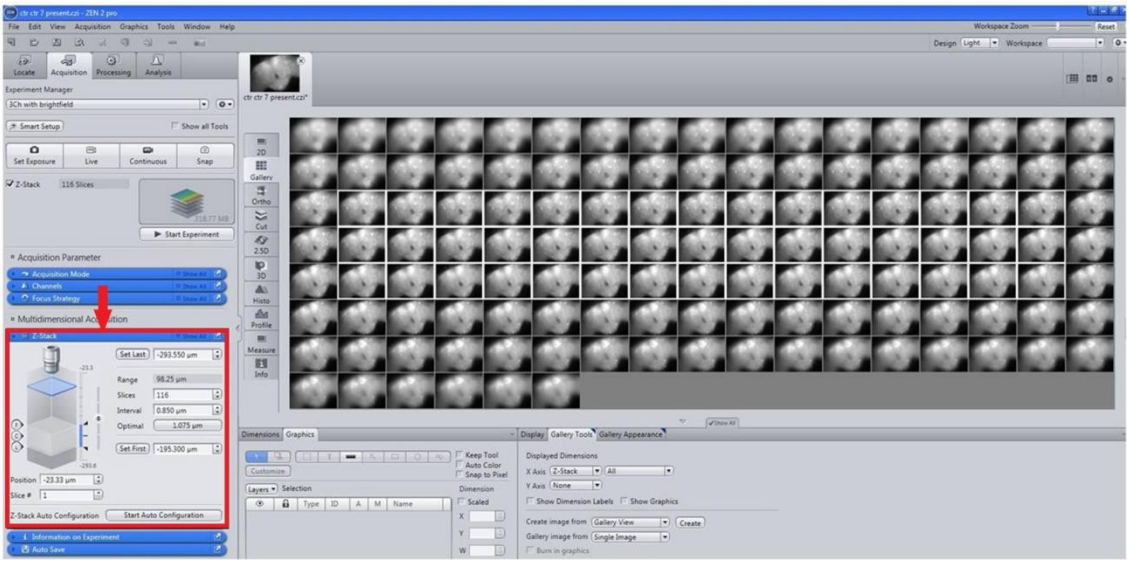


Figure 2.8: Selection of images and Z-Stacking.

5. For processing/generation of image in 2D, on the method column apply *Ortho* and *Maximum intensity projection (MIP)* from *Ortho display* with *X–Y Plane* (**Figure 2.9**).

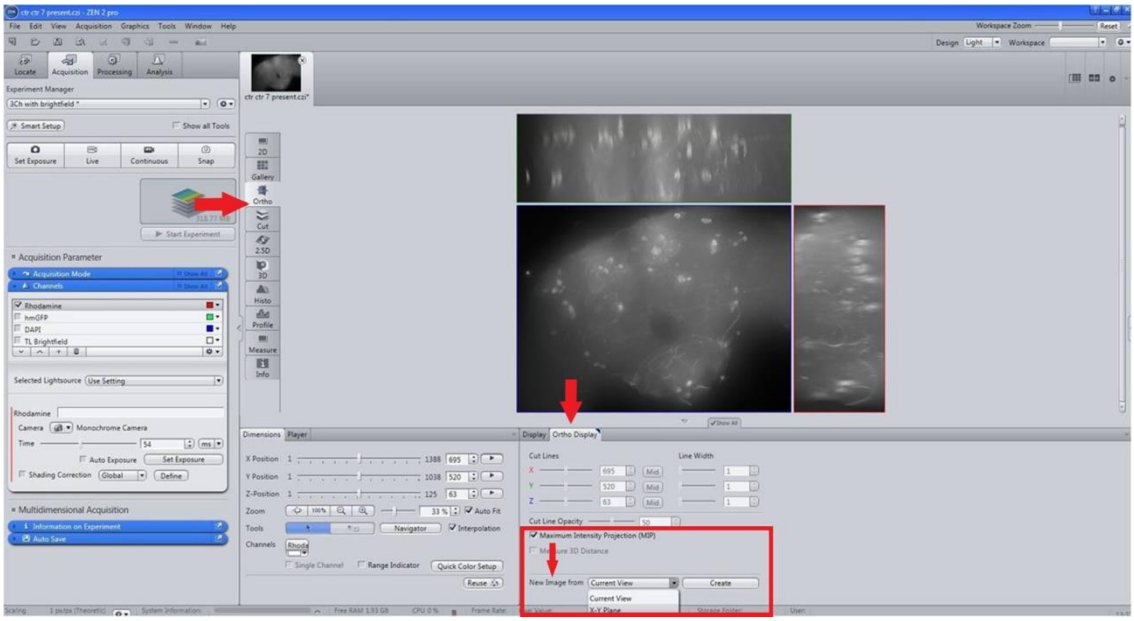


Figure 2.9: Creation of 2D image. For creating a 2D merged image, on the *Method* column, select *Maximum intensity projection (MIP)* with *X-Y Plane.*

6. The 2D image of the brain was exported in.jpg format for presentation (**Figure 2.10**).

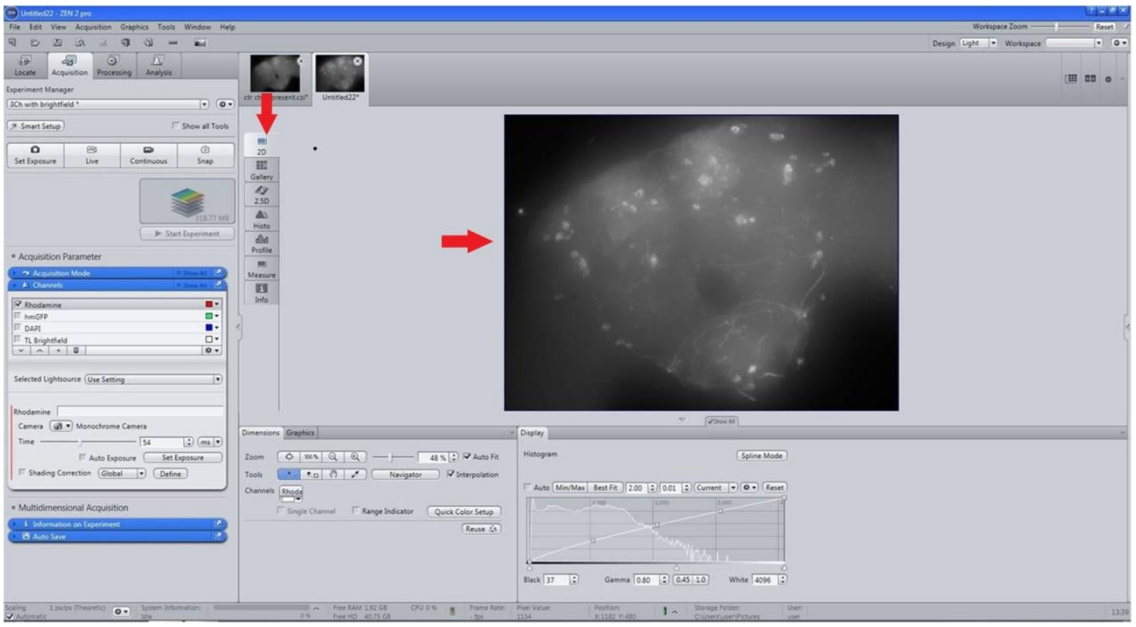


Figure 2.10: Export of 2D brain image to the required format.

Precautions and Recommendations:

1. Special attention/care was taken during image acquisition for the same orientation of the brains.

2. The red dot test was carried out carefully.

3. The same setting was incorporated for all the brain images.

4. Care was taken to ensure that all the DAergic neurons were covered and scanned while performing the Z-stack programming.

After the images were acquired through Z-stack programming, the subsequent steps were taken into consideration:

C) Quantification of the total number of DAergic neurons

Over the last five years, numerous *Drosophila* models have been reported elucidating the mechanisms of PD development, progression, and rescue strategies (Ayajuddin et al., 2022; Akinade et al., 2022; Navarro et al., 2014; Phom et al., 2014; Whitworth, 2011). The ground-breaking findings by Feany and Bender (2000), prompted the excitement surrounding this model that demonstrated the age-associated loss of DAergic neurons in the α-synuclein-mediated *Drosophila* PD model that was similar to human PD. The

DAergic neuronal system and its placement in the *Drosophila* brain were described using DA and anti-TH immunoreactivity (Budnik and White, 1988; Nässel and Elekes, 1992). These studies led to the characterization of individual clusters, which were named according to their anatomical position in the brain (Monastirioti, 1999). The details of neurons' anatomical location and numbers are presented in **Table 2.2** whereas the summarisation of variations in the loss of DAergic neurons in *Drosophila* models of PD is presented in **Table 2.3**.

Table 2.2. briefs the anatomical location and number of DAergic neurons in the *Drosophila* brain, arranged in each hemisphere in different clusters. There are total 280 DAergic neurons in the *Drosophila* brain. While the majority of these clusters can be quantified, the PAM cluster cannot not be counted/quantified using fluorescence microscopy. (Modified from Nässel and Elekes, 1992).

Clusters	**Abbreviated as**	**Number**	**Location**	**Remark**
Protocerebral anterior medial	PAM	~100	Medial tips of and areas posterior to horizontal lobes	Not countable
Protocerebral anterior lateral	PAL	4-5	Optic tubercle, superior posterior slope, ventral medial protocerebrum	countable
Protocerebral posterior medial	PPM1	1-2	Ventrally along midline	countable, too close and usually clubbed together as PPM1/2
	PPM2	7-8	Subesophageal ganglion, ventral medial protocerebrum	
	PPM3	5-6	Central complex	countable
Protocerebral posterior lateral	PPL1	11-12	Mushroom bodies and vicinity, superior arch	countable
	PPL2	6	Calyx, lateral horn, posterior superior lateral protocerebrum, Lobula	countable
Ventral unpaired medial	VUM	3	Lower subesophageal	Easily countable
Protocerebral posterior deutocerebrum	PPD	0-1	Posterior slope	Too low or absent
Protocerebral posterior dorsomedial	PPM4	0-1	Central complex	Too low or absent
Protocerebral posterior lateral	PPL3	0-1	Superior posterior slope, dorsal edge of the lateral horn	Too low or absent
	PPL4	0-1		
	PPL5	0-1		

Table 2.3. Summarisation of variations in the loss of DAergic neurons in *Drosophila* models of PD (both genetic and sporadic) from different laboratories. (Yes: DAergic neuronal loss in individual clusters and/or total DA neuronal number; No: No DAergic neuronal loss in individual clusters and/or total DA neuronal number).

Method	**Paraffin section / light microscopy**		**Whole-mount / confocal microscopy**		**Reference(s)**
Cluster/ Model	PPL1	PPM1/2	PPL1	PPM1/2	
α-Syn	No	Yes	-	-	Feany and Bender, 2000
	Yes	Yes	-	-	Auluck et al., 2002
	-	Yes	-	-	Auluck and Bonini, 2002
	-	Yes	No	No	Auluck et al., 2005
	-	Yes	-	-	Chen and Feany, 2005
	-	-	-	No	Pesah et al., 2005
Parkin	No	No	-	-	Greene et al., 2003
	-	-	-	No	Pesah et al., 2004
	-	No	-	-	Yang et al., 2003
	-	-	Yes	No	Whitworth et al., 2005
	-	No	-	-	Cha et al., 2005
DJ-1α	-	-	No	No	Menzies et al., 2005
	-	-	No	No	Meulener et al., 2005
	-	Yes	-	-	Yang et al., 2005
DJ-1β	-	-	No	No	Meulener et al., 2005
	-	-	No	No	Park et al., 2005
Rotenone	PPL1	PPM1/2	PPL1	PPM1/2	
50 µM	-	-	Yes	Yes	Wang et al., 2007
250 µM	-	-	Yes	No	Lawal et al., 2010
250 µM	-	-	Yes	Yes	Coulom and Birman, 2004
500 µM	-	-	Yes	Yes	Coulom and Birman, 2004
500 µM	-	-	No	No	Meulener et al., 2005
500 µM	-	-	No	No	Navarro et al., 2014
10 µM 500 µM			No	No	Ayajuddin et al., 2022
Paraquat	PPL1	PPM1/2	PPL1	PPM1/2	
100 µM	-	-	No	No	Meulener et al., 2005
10 mM	-	-	Yes	No	Lawal et al., 2010
10mM			Yes	Yes	Inamdar et al., 2012
10mM			Yes	Yes	Shukla et al., 2014
10mM			No	No	Ayajuddin et al., 2023
20mM			Yes	Yes	Shukla et al., 2014
20 mM	-	-	Yes	Yes	Chaudhuri et al., 2007
20mM	-	-	No	No	Navarro et al., 2014
5mM			Yes	Yes	Chaouhan et al., 2022
5mM			Yes	Yes	Maitra et al., 2019; 2021
1mM			Yes	Yes	Ortega-Arellano et al., 2017

The quantification of the DAergic neurons was followed by articulating these steps:

Methods:

1. Clusters were identified from Z-stack images/scans and obtained through Z-stack programming with constant intervals (**Figure 2.10**).

2. The image was enlarged to reveal the cell body/structure (**Figure 2.11**).

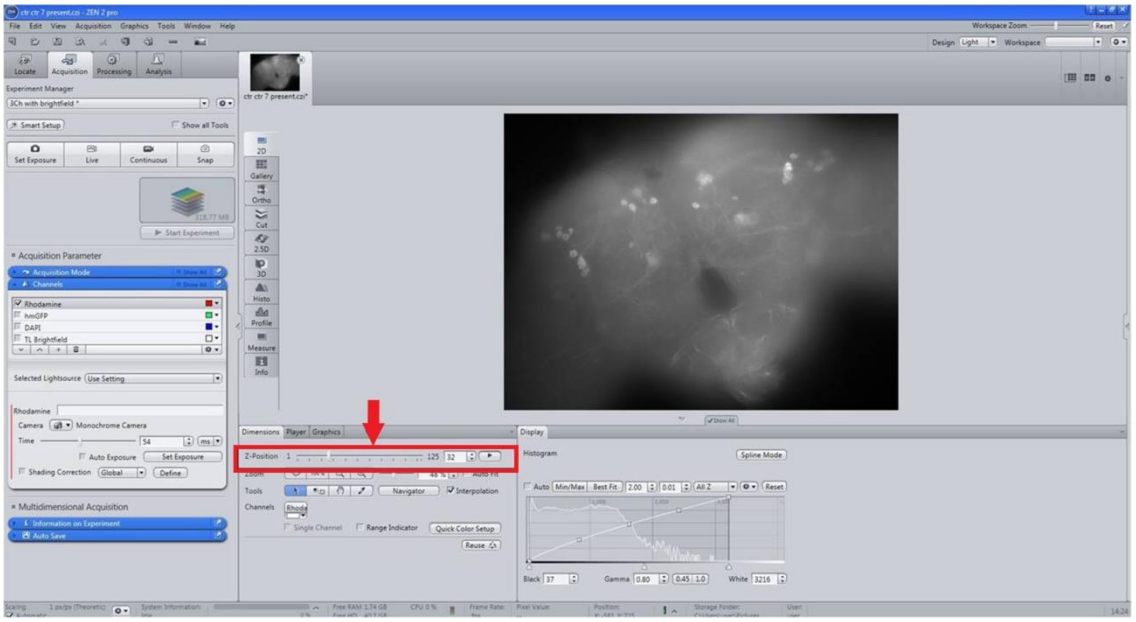


Figure 2.11: Quantification of dopaminergic (DAergic) neuronal number and fluorescence intensity (FI). For the quantification of DAergic neuronal number and FI, select 3D images/scans of Z-Stack with brain regions; PAL, PPL1, PPL2, PPM1/2, and PPM3 (PAL: Protocerebral anterior lateral; PPL: Protocerebral posterior lateral; PPM: Protocerebral posterior medial).

3. The number of DAergic neurons in each cluster was determined/counted in an unbiased manner.

4. For each group of treatments, a minimum of 5 to 6 brains were quantified.

D. Quantification of the fluorescence intensity of secondary antibodies to characterize neurodegeneration.

The loss of DAergic neurons was observed differently depending on the method adopted (**Table 2.3**). However, there are two methods widely used to quantify the DAergic neurodegeneration, *viz*., immunostaining of the fly brain using anti-tyrosine hydroxylase (anti-TH) antibody and subsequently with secondary antibody and by tagging DAergic neurons with green fluorescent protein (GFP) using a TH-Gal4 driver line. The TH-Gal4

driven decrease in the fluorescence signal intensity of the GFP reporter correlates with the state known as "neuronal dysfunction" (Navarro et al., 2014), which denotes decrease in TH and DAergic degeneration. Hence, by taking advantage of the anti-tyrosine hydroxylase (anti-TH) antibody immunostaining method (Chaurasia et al., 2024; Ayajuddin et al., 2023) Here, I tried to understand the extent of DAergic neurodegeneration in deletion mutant brain by measuring the FI of the fluorescently labeled secondary antibody targeted against the primary antibody (anti-TH) using *ZEN 2012 SP2* software from Carl Zeiss, Germany. *ZEN 2012 SP2*, Carl Zeiss software is a single user and license must be acquired to utilize the imaging system to interactively control image acquisition, image processing, and analysis fluorescence microscope. The protocol for quantification of the FI is described below.

Methods:

1. Regions of the fly brain's PAL, PPL1, PPL2, PPM1/2, PPM3, and VUM (quantifiable DA neuronal clusters) were chosen from 3D scan images (**Figure 2.11**).

2. The brain images were enlarged to see the clear neurites (**Figure 2.12**).

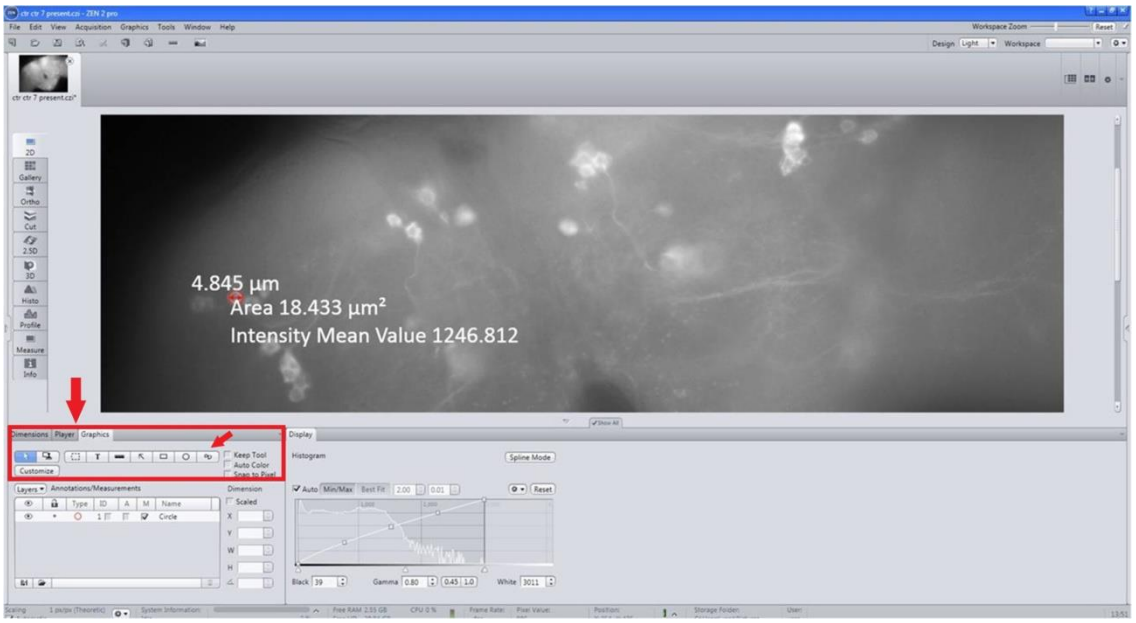


Figure 2.12: Details of the quantification of the fluorescence intensity (FI). Enlarge the images to see clear neurites, select appropriate tools, *draw spline contour* from graphics and draw a line around the neuron, and display intensity mean value and area.

3. The appropriate graphics tools' *draw spline contour'* was selected, and a line was drawn to encircle the neuron, giving intensity mean and area (**Figure 2.12**).

4. *More measurement options* were selected, and the *intensity sum* was chosen by right-clicking inside the neuron (**Figure 2.13**).

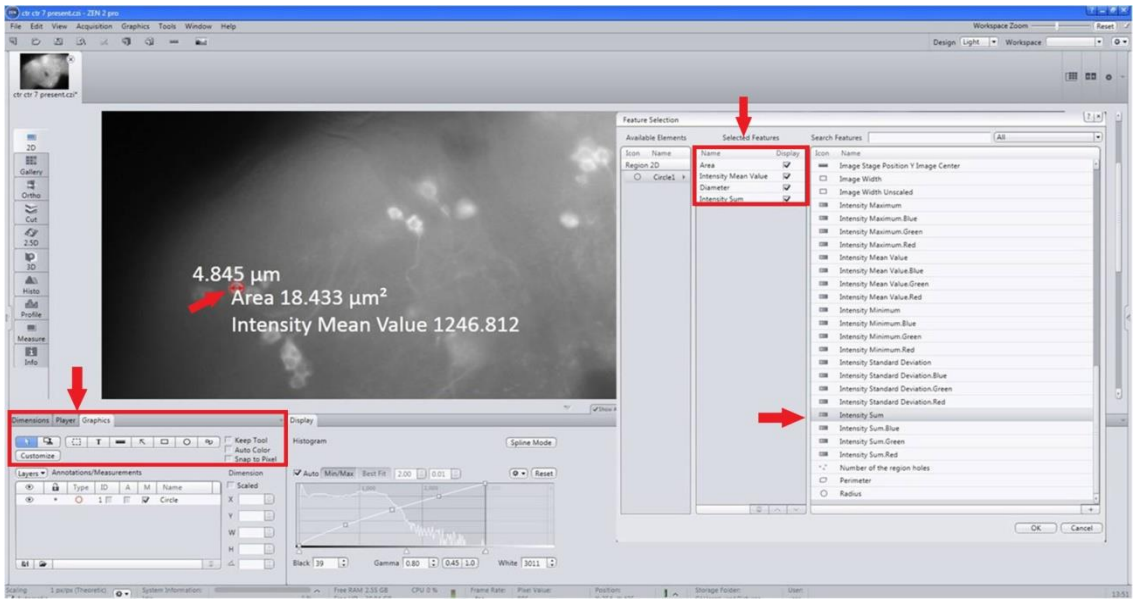


Figure 2.13: Measurement of fluorescence intensity (FI) sum. Select *intensity sum* by opting for *more measurement options* (software provides the pixel value upon right-clicking on the neuron).

5. *List, view all*, and *create document* were selected from the *measurement* tab on the left side of the panel (**Figure 2.14**).

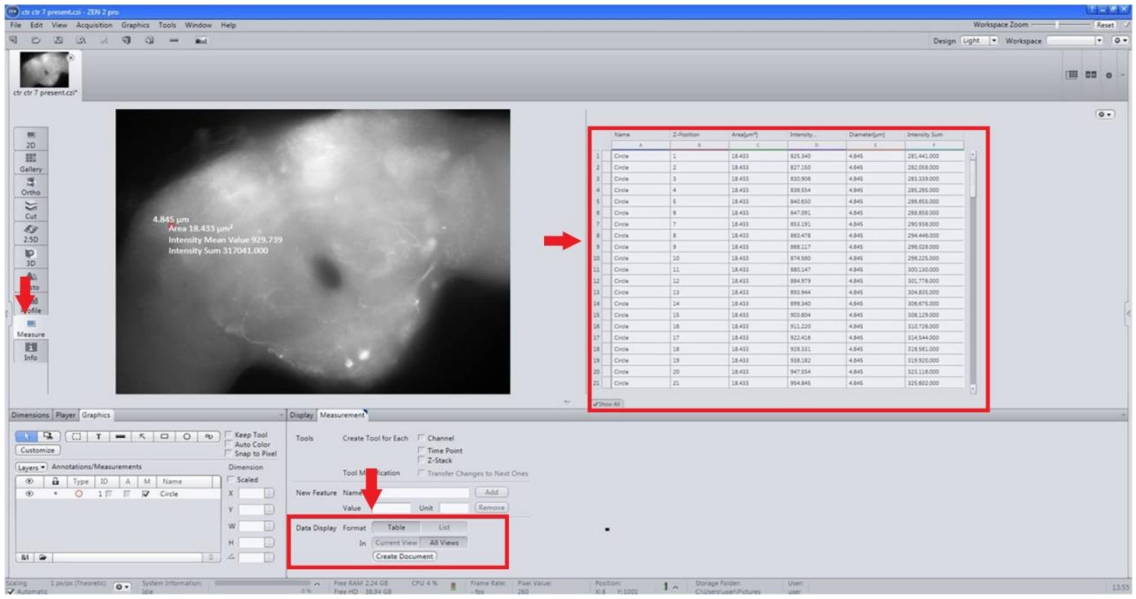


Figure 2.14: Fluorescence intensity (FI) compilation. From the *measurement* option select *list*, *All views*, and *create document*.

6. The area and FI sum were recorded for each scan of a neuron in .xml format (**Figure 2.15**).

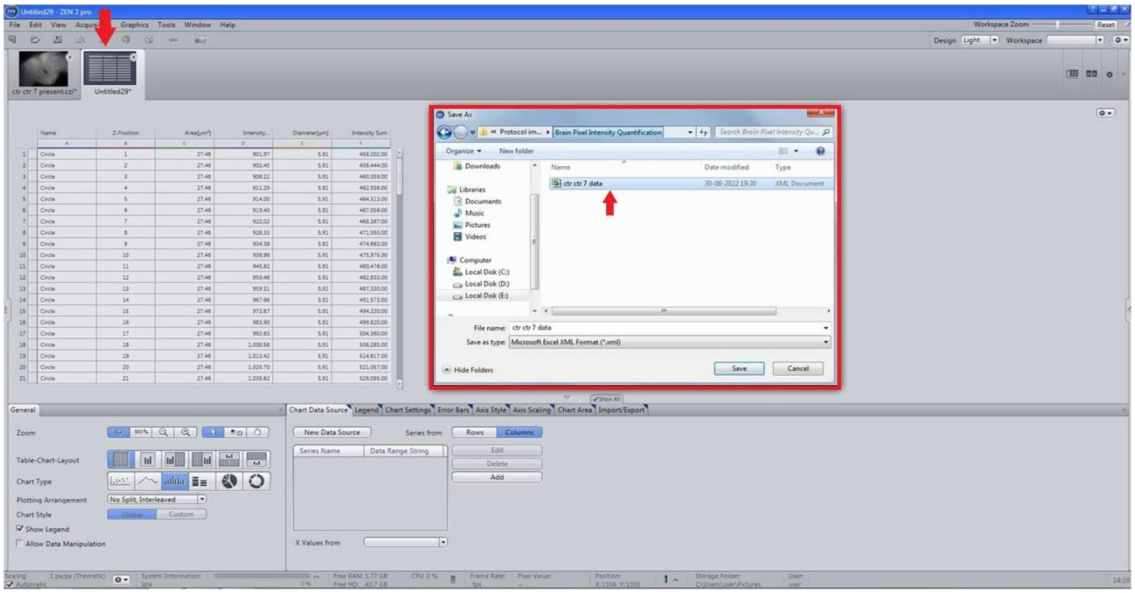


Figure 2.15: Measuring the FI sum for each scan of a neuron in .xml format.

7. For quantification of FI of a single neuron, a total of eleven scans with an interval of 1.08 µm for each scan, *i.e.*, cumulative of 11.88 µm width was considered (**Figure 2.16**).

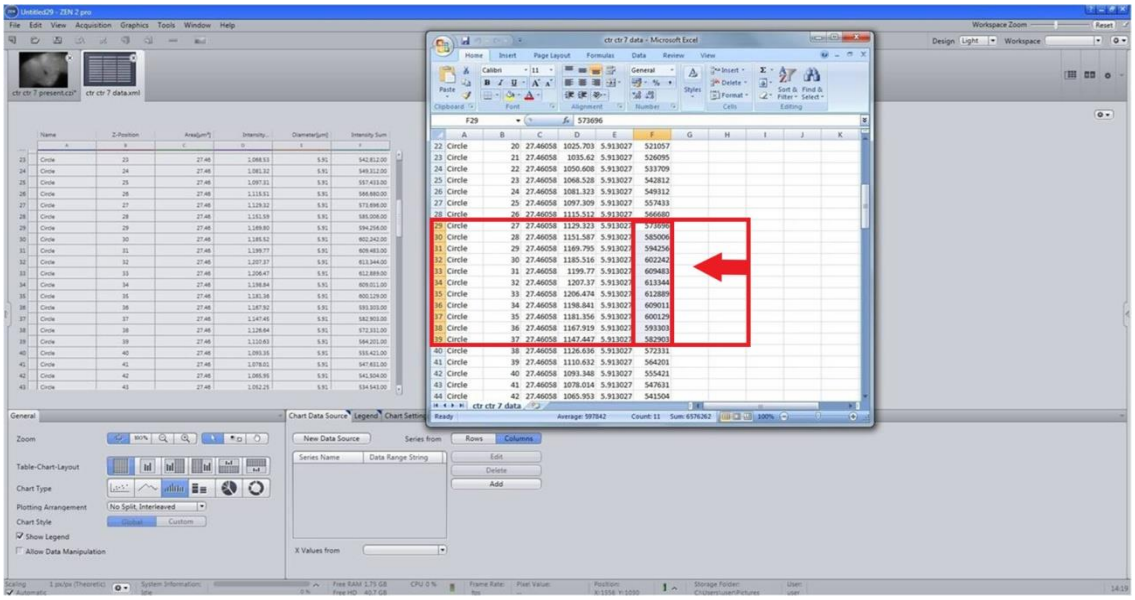


Figure 2.16: Compilation of fluorescence intensity (FI) of a single neuron and all the neurons of a cluster. For the characterization of FI of a single neuron, a total of 11 scans with an interval of 1.08 µm for each scan (cumulative 11.88 µm width) was considered. Take the average and find the standard error. Follow the same method/step(s) for all the dopaminergic (DAergic) neurons. The intensity sum of all the neurons in a specific cluster gives the total FI of that particular region (cluster-wise). The total FI is the sum of the FI of all the neurons belonging to all the DAergic neuronal clusters.

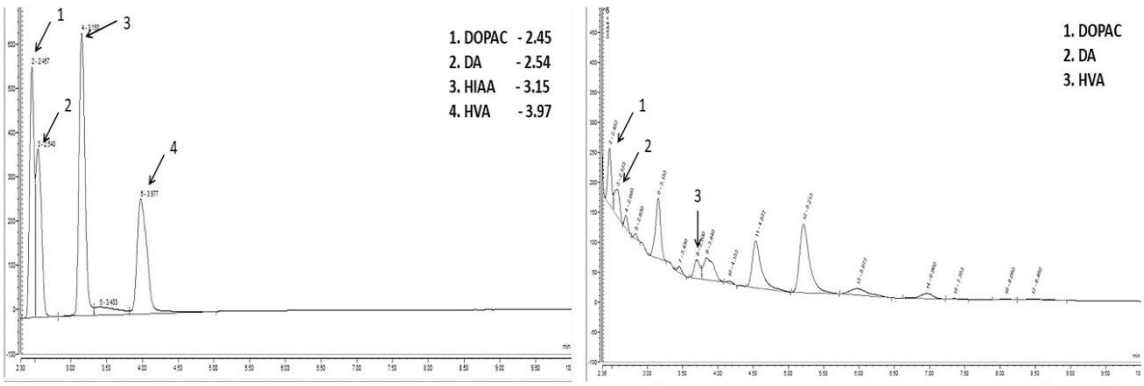
8. The intensity sum of all the neurons in a cluster gives the total fluorescence intensity (FI) of that particular region (cluster-wise).

9. Total FI is the sum of the FIs of all the neurons belonging to all the DAergic neuronal clusters.

2.16. Quantification of dopamine and its metabolites using HPLC

HPLC-ECD is the most steadfast process to measure the level of catecholamines in a model system. For catechol-modified proteins, SDS-Page (Rees et al., 2007) and the protein pull-down assay (Plotegher et al., 2017; Liu et al., 2014) are used for quantification. The nIRF scanning is also used to detect and quantify the o-quinones and other modified proteins in cells and tissues (Jinsmaa et al., 2018; Burbulla et al., 2017). All other techniques except for HPLC-ECD are less sensitive, thus making HPLC-ECD the best option for the quantification of catecholamines. The advantages of HPLC-ECD lie in its time efficiency, accurate detection of brain-specific catecholamines, and higher flexibility to modify for detection of other related catecholamines (Allen et al., 2017).

To understand the biological importance of DA metabolism in the *Drosophila* model of PD, I, tried to quantify the levels of DA and its metabolites (DOPAC and HVA) using the HPLC-ECD (Thermo-scientific, Dionex Ultimate 3000RS) equipment. Standard DA and metabolites were quantified to provide a precise retention time and area with which samples were compared to quantify catecholamines in the tissue samples (**Figure 2.17**). DA and its metabolites were quantified following the protocols of Ayajuddin et al. (2023; 2021).



(A) (B)

Figure 2.17: Characterization of retention time of standard DOPAC, DA and HVA (**A**), and brain-specific DA and its metabolites levels (**B**). Chromatogram of the standard catecholamines gives a particular RT comparing with which the catecholamines in the fly brain sample is analyzed.

Materials for catecholamine quantification

Dopamine (DA, Sigma-Aldrich, Cat: H8502); 3,4-Dihydroxyphenylacetic acid (DOPAC; Sigma-Aldrich, St. Louis, USA, Cat: 11569); Homovanillic acid (HVA; Sigma-Aldrich, St. Louis, USA, Cat: 69673); Phosphate-buffered saline (PBS; HiMedia, Thane, India, Cat: ML-023); Trichloro Acetic Acid (TCA; SRL, Mumbai, India, Cat: 204842); MDTM mobile phase (Thermo-scientific, Waltham, USA, Cat: 701332); HPLC grade water (JT Baker, Radnor Township, USA, Cat: 4218-03); Acetonitrile (JT Baker, Radnor Township, USA, Cat: 9017-03); Methanol (JT Baker, Radnor Township, USA, Cat: 9093-68) were used for quantification of DA and its metabolites using HPLC-ECD 3000 RS system (Thermo-scientific Dionex Ultimate 3000).

Miscellaneous: Sterilized Eppendorf tubes, Pipette (cleaned with 70% ethanol before and after use), Sterilized pipette tips, Nanodrop® 2000c Spectrophotometer (Thermo-Scientific, Waltham, USA).

Flies tissue sample

Fly heads were used for brain-specific catecholamine quantification. After 4 hrs of exposure, flies were immediately frozen. For each treatment group, 15 fly heads were

decapitated. To avoid thawing of tissue and degradation of biomolecules, the heads of frozen flies were decapitated on top of an ice tray having a chilled metal sheet. Dissection equipment were cleaned with 70% ethanol to avoid contamination.

A. Preparation of standard DA, DOPAC, and HVA

Preparation of standards

Standards were prepared by dissolving 2 mg of commercially available catecholamines in 2 mL PBS. It was further diluted to get varying concentrations as mentioned in **Table 2.4**. For loading the standard, 200 ng/µL of the concentration was used.

Table 2.4. Table showing preparation of multiple concentrations of standard catecholamines Catecholamine concentration that is to be loaded on the HPLC system was mixed with 5% TCA (the 5%TCA should be centrifuged at 6000 rpm for 10 minutes at 4ºC prior to application to remove any undissolved solute particles) in a 1:1 ratio.

Standard	PBS	Concentration	Stock Name
2 mg	2 mL	1000 µg/mL	S
100 µL of **S**	900 µL	100 µg/mL	S1
100 µL of **S1**	900 µL	10 µg/ mL	S2
100 µL of **S2**	900 µL	1000 ng/ mL	S3
200 µL of **S3**	800 µL	200 ng/ mL	S4
150 µL of **S3**	850 µL	150 ng/ mL	S5
100 µL of **S3**	900 µL	100 ng/ mL	S6

Sample preparation

1. 15 heads of adult flies were collected in 300 µL of 1X PBS (prepared in HPLC grade water).

2. It was then homogenized and subjected to sonication for 20 secs at intervals of 5 secs with a 30% amplitude (always kept on ice during the process).

3. The samples were centrifuged for 10 minutes at 6000 rpm at 4ºC.

4. The supernatant was collected.

5. 200 µL of the supernatant was removed (the remaining were set aside for protein

quantification), and unto it, 200 µL of 5% TCA was added.

6. It was then centrifuged for two times at 5000 rpm for 10 min each at 4ºC.

7. The supernatant was then collected for further downstream assay.

Precautions

1. The tissues were homogenized and sonicated on ice (to avoid heat generation and prevent degradation).

2. In order to avoid any degradation of the molecules of tissue extract and standard catecholamines, both solutions were kept on ice in between procedures.

3. All the reagents were made in HPLC grade or Milli-Q water to prevent any contaminating molecules to come in contact that could create a false positive peak in the chromatogram.

4. Fresh pipette tips were used to prepare serial dilutions of the standard and to transfer tissue extract.

B) Quantification of protein

The Bradford technique was used to measure the concentration of protein. 2 mg/mL of BSA was dissolved in PBS to make BSA stock. A working concentration of 0.2 µg/µL was prepared by dissolving the 100 µL of stock solution in 900 µL PBS. The serial dilution was carried out accordingly as mentioned in **Table 2.5**.

Table 2.5. Table showing the preparation of serial dilutions using standard BSA.

BSA (µg/mL)	Working solution (µL)	PBS (µL)	Bradford (µL)
0.5	2.5	497.5	500
1	5	495	500
1.5	7.5	492.5	500
2	10	490	500
2.5	12.5	487.5	500
3	15	485	500
3.5	17.5	482.5	500

NanoDrop 2000C (Thermo-Scientific, Waltham, USA) was used to read the absorbance at 595 nm after 5 min of room temperature incubation to produce a standard graph. 3 µL of the pure tissue extract was used to measure the concentration of protein. Therefore, the µg/mL of total protein concentration that was obtained during the assay was derived from 3 µL of extract which was combined with PBS and Bradford reagent. Hence, to get the actual protein concentration per µL of the tissue extract, the total µg of protein was divided by 3 µL.

C) Setting up the HPLC system

Solvent Reagents Required

Load the solvent tubing ports of the HPLC-ECD system with the following reagents

1. 100% HPLC grade Methanol.

2. 80% Acetonitrile (Prepared in HPLC-grade water).

3. 20% Acetonitrile (Prepared in HPLC-grade water).

4. MDTM Mobile phase.

The following "Preloading instructions" were followed for solvent reagents.

Preloading instructions for solvent reagents

"Preloading of the solvent reagents" refers to the process of placing solvent reagent bottles on the HPLC solvent rack and attaching the respective tubings to the bottle. "Preloading Instructions" mentioned below is a manual for handling the solvent reagents and their containers while preparing solutions, placing the containers on the solvent rack, and attaching them to the tubing ports of the HPLC platform.

1. All the reagent bottles were sufficiently filled (minimum 350 mL in each).

2. While filling, the bottle was tilted and the reagents were poured slowly to prevent bubble formation.

3. Mobile phase was filtered with 0.22-micron filter paper and then poured onto the respective reagent bottle as mentioned in point 2 (Miscibility of the components of the mobile phase is a concern while running through the column. Even the ready-made mobile phases may contain undissolved salt residues and suspended particles due to minute-level coagulation of organic components of the mobile phase. Filtration using a 0.22-micron nylon membrane ensures separation of such residues which could otherwise choke the C18 column fitted with the HPLC-ECD system).

4. All the reagent bottles were sonicated in a bath sonicator at an ultrasonic frequency of 40 kHz for 15 min at RT before being connected to the HPLC system.

System/ Column cleaning

Columns and electrodes of the detectors may contain tissue debris from the previous HPLC experiment performed. Further, to avoid any fungal growth, the components of the HPLC platform such as column, ECD and tubing were filled and stored in 100% methanol after completion of an experiment. Therefore, it is crucial to clean the system when it is turned on with the flow of the mobile phase to remove any left-over tissue debris and methanol ensuring the removal of any air bubble during when the HPLC platform was kept idle for a long time between experiments. The following steps were used for system and column cleaning:

1. The system was cleaned by purging (each solvent port is put into a high flow rate from the pump to outside the system) from all the ports for 5 min each to remove any trapped air bubbles.

2. The purge knob was then closed to direct the flow from the pump to column. To start cleaning the column after purging, 100% flow with 20% acetonitrile was enabled in the system for 30 minutes at a flow rate of 0.5 mL/min.

3. A 100% mobile phase was made to flow through the column at the same flow rate for another 30 min after which the mobile phase may be recycled (drainage pipe outlet from the column will be wiped with a lint-free tissue soaked in the mobile phase and will be inserted back into the mobile phase container bottle).

Setting up the HPLC parameters

Detection of catecholamine through ECD is best when the oxidation potential is within the range of 340 mV (Yang and Beal, 2011). In our laboratory, it is discovered that the catecholamines are most effectively detected with the DIONEX ULTIMATE ECD 3000 system, utilizing a reduction and oxidation potential range of -175 mV and 225 mV respectively. The reduction potential provides an identical state for all the catecholamines inside the HPLC platform irrespective of their physiological redox state in the tissue. The excitation of all concerned catecholamines inside the HPLC platform is regulated by the optimum oxidation potential, and within the range of this oxidation potential, the concerned catecholamines may be detected. The following parameters were set for efficient detection and analysis of catecholamines.

Oxidation potential	:	+225 mV
Reduction potential	:	-175 mV
Omni cell	:	±500 mV (For noise reduction)
Gain range	:	1 µA
Data collection rate	:	5 Hz
Detection Filter	:	2.0 (for all cells)
Column temperature	:	Room temperature
Auto sampler temperature	:	4ºC
Flow rate	:	0.5 mL/min

ECD priming

1. Once the mobile phase is switched to recycle mode, the ECD was primed.

2. After setting up the required parameters for ECD, the system was kept on acquisition mode for at least 2 hrs to monitor the state of the baseline.

3. The two lines (denoted as ECDRS 1 & ECDRS 2) were checked to run in parallel denoting equilibrium while fluctuation was denoted by non-parallel line over time.

4. The baseline was regarded to be stabilized if the drifting was less than 2nA/hour.

D) Standard and Sample loading

20 µL of Standards were injected followed by 50 µL of the sample was loaded for analysis. Standard, DOPAC, DA and HVA showed an optimal peak at 20 µL injection of 200 ng/mL concentrations.

Critical: The same PBS was used to prepare samples as well as to dissolve standard metabolites. A minimum of 300 µL of standard and tissue extract was kept in the vial for injection.

E) Analysis

1. The chromatogram obtained using the ECDRS2 channel was employed for analysis.

2. To inhibit noisy or false peak integration to the chromatogram, the "Inhibit Integration range" function was applied to the whole solvent front section of both the standard and sample chromatograms.

3. The chromatogram obtained from ECDRS2 channel was used and the sample chromatogram was superimposed with the standard chromatogram.

4. Upon comparing the two chromatograms, one may determine the peaks of the specific catecholamine present in the sample. Factors such as retention time, and the behaviour of the peaks with respect to other catecholamines are to be considered. To precisely pinpoint the DA, DOPAC and HVA peak in the sample, 10 µL of the composite standard was mixed and the sample was run again in HPLC. The peaks that spiked according to the detection sequence were identified as the monoamines of interest.

5. If the peaks were co-eluted *i.e.*, peak shoulders are joined together, then the peaks were split into two peaks with the user interface.

6. Software tools such as the automated tool, delimiter tool, peak tool, baseline tool, etc. were used to increase the accuracy of the peak area.

7. After the peak was accurately determined, one could further process for quantitative analysis.

8. In order to quantitate catecholamine levels in tissue extract, the peak area of the sample catecholamine's was normalized to the standard.

F) Calculation of concentration of catecholamines in the sample with example

i. The concentration of the standard catecholamines: DA (DA_{Std}), DOPAC ($DOPAC_{Std}$) and HVA (HVA_{Std}) used in the HPLC assay was 200 ng/mL each.

ii. Injection volume of all standard catecholamine to the HPLC column was I_{Std} = 20 µL

iii. Peak area for a catecholamine was obtained from standard and sample chromatograms (**Figure 2.18**).

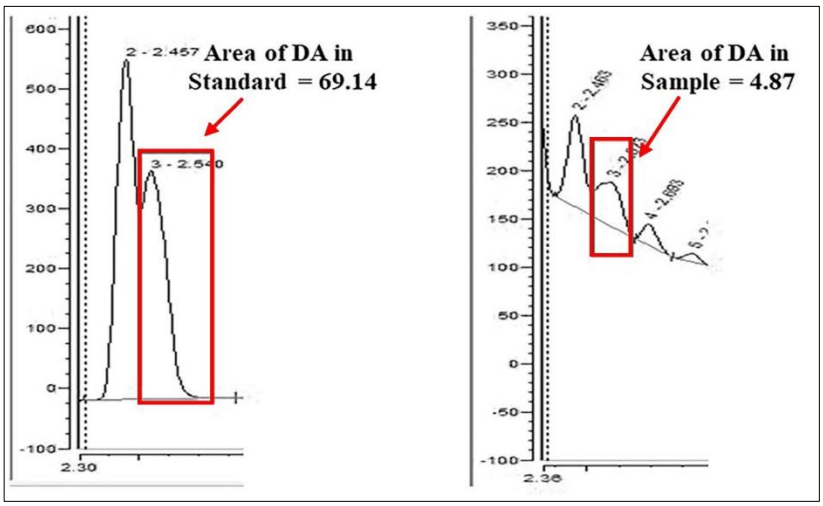


Figure 2.18: Image of chromatogram showing the area of the standard and sample.

iv. Area of the peak of the standard catecholamines (DA, DOPAC and HVA) in the chromatogram was A_{DA_Std} = 69.14, A_{DOPAC_Std} = 81.59 and A_{HVA_Std} = 101.57

v. Injection volume of tissue extract to the column was I_{Samp} = 50 µL

vi. Area of the peak of catecholamines (DA, DOPAC and HVA) in the tissue sample chromatogram was A_{DA_Samp} = 4.87, A_{DOPAC_Samp} 18.38= and A_{HVA_Samp} = 0.83

vii. Suppose, a particular tissue extracts from an experimental group used for HPLC assay, that was quantified beforehand for total protein was TP_{Samp} = 0.1 µg/ µL

viii. The following steps were followed for calculating the actual amount of the catecholamines in tissue extract (**Table 2.6**).

Table 2.6: Steps for calculation of the amount of catecholamines for a single fly brain.

Calculation Steps	**Metabolites**		
	DA	DOPAC	HVA
Step I: Concentration of standard catecholamines in 20 µl	DA_{Std} X I_{Std}/1000 i.e. (200 X 20)/1000 = 4 ng	$DOPAC_{Std}$ X I_{Std}/1000 i.e. (200 X 20)/1000 = 4 ng	HVA_{Std} X I_{Std}/1000 i.e. (200 X 20)/1000 = 4 ng
Step II: Concentration of catecholamines in brain tissue extract	(A_{DA_Samp} X 4)/ A_{DA_Std} i.e. (4.87 X 4)/69.14 = 0.28 ng	(A_{DOPAC_Samp} X 4)/ A_{DOPAC_Std} i.e. (18.38X 4)/81.59 = 0.90 ng	(A_{HVA_Samp} X 4)/ A_{HVA_Std} i.e. (0.83 X 4)/101.57 = 0.03 ng
Step III: Determining the total protein in 50 µl that was injected into column	(TP_{Samp} X I_{Samp}) i.e. (50 X 0.2) = 10µg	(TP_{Samp} X I_{Samp}) i.e. (50 X 0.2) = 10 µg	(TP_{Samp} X I_{Samp}) i.e. (50 X 0.2) = 10 µg
Step IV: Determining the catecholamine in total protein that was injected and normalizing in 1 mg of total protein	[0.28 /10] = 0.028 ng	[0.90 /10] = 0.09 ng	[0.03 / 10] = 0.003 ng
Step V: Determining the actual amount of catecholamine as injected tissue extract solution had brain tissue extract + TCA in a 1:1 ratio	0.028 X 1000/(2X15) = 0.93 pg/brain	0.09 X 1000/(2 X15) = 3 pg/brain	0.003 X 1000/(2 X15) = 0.1 pg/brain

2.12. Statistical analysis

Graphs were created using Graph Pad Prism 5.0 software (Graph Pad Inc., San Diego, CA, USA). Statistical analysis was completed, and results were expressed as the mean ± standard error of the mean (SEM). For negative geotaxis assay analysis, a one-way analysis of variance (ANOVA) followed by Tukey Post-Test was carried out to draw significance. For the longevity assay analysis, survival significance curves were drawn

using log-rank (Mantel-Cox test). A Two-way ANOVA followed by Bonferroni post-test and one-way ANOVA followed by Newman-Keuls multiple comparison test was carried out to draw significance for DAergic neuronal number and TH-protein synthesis quantification. For HPLC analysis, Statistical significance was determined using a two-tailed unpaired t-test for data from two experimental groups. P value < 0.05 was considered significant.

CHAPTER 3

Primary screening of deletion lines for mobility defects using Negative Geotaxis Assay (NGA)

3.1. Introduction

Developing a suitable animal model is important for understanding the pathophysiology of late-onset NDDs such as PD. Understanding the multitude of behaviours and characterizing different aspects more or so related to neurodegeneration is very critical to understand phenotypic variation in any model organism. Developing a PD model should exhibit pathophysiological features associated with PD such as gradual loss of movement mobility, a decreasing life expectancy, and age-dependent neurodegeneration at a life stage during which the disease onsets in humans (Khan et al., 2023). Various assays are used to measure the effects of genetic mutation and/or environmental conditions on *Drosophila* climbing behaviour (Madabattula et al., 2015). Flies are positively phototactic and negatively geotactic in nature. Therefore, taking advantage of this stereotyped behaviour, one can assess its mobility defects. A simple and reliable assay such as Negative geotaxis assay, is needed for primary screening of a large number of genotypes/drugs affecting its mobility of flies in a short period of time. Several research studies have also characterized degeneration of the DAergic neurons with the help of the negative geotaxis behaviour in *Drosophila* (Ayajuddin et al., 2023; 2022, Botella et al., 2008; Chaudhuri et al.,2007; Chen and Feany, 2005). With the help of this assay, mobility deficits may be assessed to identify mobility phenotype that is linked to the early disease onset.

3.2. Pathophysiology of PD

The main neuropathological characteristics of PD is the selective loss of dopaminergic (DAergic) neurons in the *substantia nigra pars compacta* (SNpc), which causes motor cortical excitation and inhibition. Tremor, stiffness, bradykinesia, and postural immobility are associated with the loss of DAergic neurons (Ni and Ernst, 2023). These neurons are essential for proper muscle and coordination function. Even though the

degeneration of neurons starts in the dopaminergic neurons of the SNpc, non-DAergic neurons also experience degeneration in the later stages of PD (Hornykiewicz and Kish, 1987). The axons of DAergic neurons that extend towards the putamen and caudate nuclei of the striatum make up the nigrostriatal pathway. Depletion of striatal DA is caused by the degenerating DAergic neurons. Decreased nigrostriatal input causes an increase in inhibitory output from Globus Pallidus internal to the thalamus and indirectly to the cortex, which represses the initiation of movements and eventually results in motor deficits (Rodriguez et al., 2009). Both the loss of nigrostriatal cells and the reduction in striatal dopamine levels are linked to the severity of bradykinesia (López-Aguirre et al., 2023). Most PD cases are either sporadic or idiopathic, meaning their cause is unknown. Nonetheless, there is mounting evidence that hereditary and environmental factors both play a significant role.

3.3. Assays performed to characterize locomotion in animal PD models

Animal models allow researchers to recapitulate some of the clinical manifestations of the diseases in humans hence giving an optimal opportunity to better understand the mechanism involved in the disease progression. Though model animals share several homologous molecular and cellular processes with humans there exist limitations too. Therefore, by investigating these processes and behaviours in animal models, one can gain an understanding of the basic biology underlying them and apply this knowledge to figure out how diseases occur and find corrective measures.

3.3.1. *Rodent Models*

Mice/Rats

Commonly used animal models in PD are mice and rats. Most of these models have been developed with the use of neurotoxins and also genetic models through genomic manipulations.

Rotational behaviour

Rotational behaviour (Ungerstedt, 1971) is the classical phenomenon observed in hemi-parkinsonian rat/mice model to quantify the degree of lesion in the PD model (Longo et al., 2017). Rotation behaviours are spontaneous but most of the studies induced rotations by injection of amphetamine/apomorphine which caused rotation due to the unbalanced DAergic activity stimulated by these reagents in the striatum (Suzuki et al., 2015). Male Wistar rats of this kind exhibit consistent rotational behaviour with affected dopamine transporter-positive neurites and striatal TH-positive loss (Penttinen et al. 2016).

Open Field test

The behavioural trials in this test revealed a significant motor deficit in elderly rats. The impaired locomotor and exploratory behaviours in the open field test, are observed by the decreased number of crossings, immobility, and reduced supported and unsupported rearing activities in elderly rats, indicating motor functional impairment (Acquarone et al., 2015). Notably, unsupported rearing in the open field is regarded as a significant indicator of the emotional condition of the animal, in addition to its motor activity (Sturman et al., 2018). Earlier studies have highlighted the similarity between the motor deficits seen in elderly rats and those shown in people affected by PD (Barata-Antunes et al., 2020), suggesting that the aging process may underlie the development of PD pathology. Through this test, BALB/c mice with MPTP administration also showed induced motor impairment along with loss of DAergic neurons and dysregulated astroglial cells in the nigrostriatal pathway (Liu et al., 2019).

Rotarod test assay

The integrity of the motor system was evaluated using the rotarod test. Animals are positioned on a rotating rod that progressively increases in speed. To evaluate the motor coordination and posture of all rats, their movements on the rod were captured on video

(Rosa Avila-Costa et al., 2023; Razgado-Hernandez et al., 2015). After intraperitoneal administration of 10, 20, and 30 mg of MPTP/kg 4 times to males C57BL/6 N mice, impairment in motor performance and nigrostriatal neurotoxic PD mechanisms including DA, tyrosine hydroxylase (TH), and protein carbonylation through this assay was observed (Hwang et al., 2019). Similar observations on MPTP-treated mice compared to their controls after 6th day of MPTP administration exhibits impaired motor behaviour and decreased TH-positive signals in the substantia nigra, globus pallidus, caudate-putamen, and subthalamic nucleus, limbic regions (amygdala and hypothalamus) with increased expressions of TH signal intensity (Roostalu et al., 2019). Conversely, studies on elderly Wistar albino rats do not show substantial alterations in DA levels. Nevertheless, some revealed a significant decrease in DA levels in the striatum. The observed disparities indicate that the susceptibility of the dopaminergic neuronal system may be a cause of aging influenced by hereditary factors (Longo et al., 2017; Branch et al., 2016).

3.3.2. *Zebra Fish*

Swimming behaviour test

It is employed in the zebrafish model to study an array of cognitive processes such as learning, memory, anxiety, fear, perception, social skills, and even sleep patterns (Costa et al., 2020). Abnormal swimming behaviour and decreased swimming speed model-related bradykinesia-like PD symptoms are observed in the neurotoxin-induced PD model (Robea et al., 2020). The locomotor activity in zebra fish was markedly altered after the i/p injection of MPTP neurotoxin. Specifically, the alteration was already detected by 24 hrs after MPTP injection and remained altered 96hrs after the injection (Razali et al., 2021). MPTP administration to both larval and adult zebra fish exhibits reduced swimming reflexes (Kalyn et al., 2019), following administration, suppression of

monoamine oxidase B or the dopamine transporter reduces the neuronal degeneration that occurs after the injection of MPTP (Lam et al., 2005).

Light-Dark test

A movement pattern modulated by the alternation of light and darkness condition in zebrafish exhibit anxiety-like behaviour. Zebrafish movement is enhanced by light to dark transition, but reduced by dark to light transition. Rotenone-exposed fish exhibit a decline in DA levels in the brain and reduced Th expression, following a decrease in swimming time similar to the decline in physical movement seen in PD patients, also exhibit increased duration in the light and a prolonged delay in entering the dark component (Wang et al., 2017). These fishes also showed reduced affinity for amino acids, suggesting a potential impairment of olfactory function, a prevalent symptom of PD (Ruan et al., 2012). Initially lacking extensive research including some other species, zebrafish, being a relatively young model organism, is rapidly gaining popularity as a model due to the emergence of novel genome editing technologies and the advancement of imaging tools.

3.3.3. *Drosophila model*

Locomotion in adult flies has been studied in a multitude of fields, including circadian rhythm, phototaxis, geotaxis, courtship, space research, and intra-population variability. *Drosophila* also executes complex behaviour such as mating, conditioning to fear, aggression, learning, and motor behaviours such as flying, walking and climbing which are affected by the PD onset and progression like in humans (Stein, 2023). This multitude of behaviour is very much helpful to characterize different features of PD. Pioneering work by Carpenter observed that flies are innately positively phototactic, show negatively geotactic behaviour in enclosed space and mechanical agitation has a kinetic effect upon flies and induces locomotion (Carpenter, 1905). The two classical approaches for

Drosophila geotaxis (movement in response to gravity) originated with the understanding of the genetic basis of its behaviour.

The first method known as the **'Hirschian'** approach by Hirsch and colleagues in 1962 examined the impacts of naturally occurring variation while the second was the **'Benzerian'** approach in 1967 which focused on the effects of induced mutations. These two approaches are complementary in that they address subtly different questions. Investigations of naturally existing variation identify the specific group of genes that undergo segregation in nature, whereas mutagenesis investigations establish the genes necessary to generate the behaviour.

Counter-current assay

Benzer and colleagues developed the assay, with mechanical agitation being an intricate part of this assay (Benzer, 1967) (**Figure 3.1**). Flies tend to accommodate to a constant environment but are much more responsive to light when startled. Hundred flies are laid at the tube in a horizontal position and allowed several minutes to acclimate under the conditions to be used.

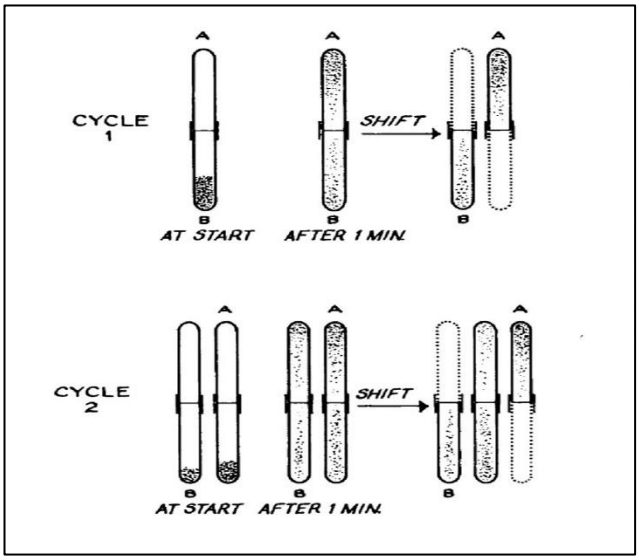


Figure 3.1: Counter current procedure for fractionating the *Drosophila* population. Here, the flies are positioned in double-tube Pyrex vials. To initiate, the flies are brought to one proximity, bottom B and now either remain in the B part or migrate toward top A. After a time, A is displaced to join a new mate while being replaced by a fresh tube. Thus, all the flies that moved into A are transferred. Proceeding with the second cycle, all the flies are simultaneously brought to the B

end, and the procedure is repeated. By staggering the starts, the partitioning times are maintained constantly for all tubes (Image adapted from Benzer, 1967).

Using this apparatus in a screen for mutants, one of the first neurodegenerative mutant to be identified was *drd*, for deficient in rapid phototaxis and histologically further showed a widespread vacuolization in the brain neuropil. A study by Konopka and Benzer (1971), confirmed the presence of a rhythm of locomotor activity in *Drosophila* previously recorded by Frank and Zimmermann (1969).

***Negative geotaxis assay* (NGA)**

NGA was developed wherein the principle lies in introducing a known number of flies in a sterile polystyrene vial vertically set on a single-vial shaker. The entire apparatus was lit by a fluorescent tube kept vertically to provide the same illumination throughout the length of the vial. The shaker delivers a 3-sec mechanical stimulation rotating the vial at an angular speed of 3.85 rad/s (temperature room was 23±2ºC, relative humidity not controlled) which led fly to the bottom of the vial and maximal height it attained in 20 secs at the top of vial after cessation of shaking was assessed (Le Bourg and Lints, 1992; Ganetzky and Flanagan, 1978). Feany and Bender (2000), using the same method first observed the pan-neuronal expression of the PD-linked human α-synuclein gene that accelerates progressive loss of startle-induced negative geotaxis behaviour in *Drosophila*. They also proved that α-synuclein is expressed in different cell types and used this functional read-out to investigate the neuroprotective effect of the *Nrf2* pathway in this *Drosophila* model of PD. Botella et al. (2004) observed age-dependent reduced locomotor activity in *sniffer* mutant through this method. Similarly, in another study age-dependent reduced locomotor climbing ability was observed in *α*-syn A30P PD fly model (Shaltiel-Karyo et al., 2012).

Drosophila Island Assay

An efficient high-throughput and low-cost screening assay described by Schmidt et al. (2012) for evaluating different *Drosophila* locomotor phenotypes. Taking advantage of fly's innate behaviour and traditional fly climbing assays, mobility defects were assessed over the years. However, these assays are time-consuming as behaviour was scored by keeping track of the flies visually and scored manually for a minimum distance climbed in a fixed window time. Therefore, over the years, these assays are used successfully in large-scale screenings to identify genes with glia-specific functions, in evaluation of *Drosophila* models of intellectual immobility, and evaluation of fly motor behaviour (Volkenhoff et al., 2015).

Schmidt et al. (2012) performed Glial cell-specific *RNAi* screen for *khc* mutants and observed axonal swellings, and paralysis in a total of 5273 UAS-dsRNA elements and observed 15% crosses of glial-specific silencing showed lethality, 0.3% were semi-lethal and 2.3% of the tested genes demonstrate locomotion defects, remaining 82.4% of the crosses observed no defects (**Figure 3.2**).

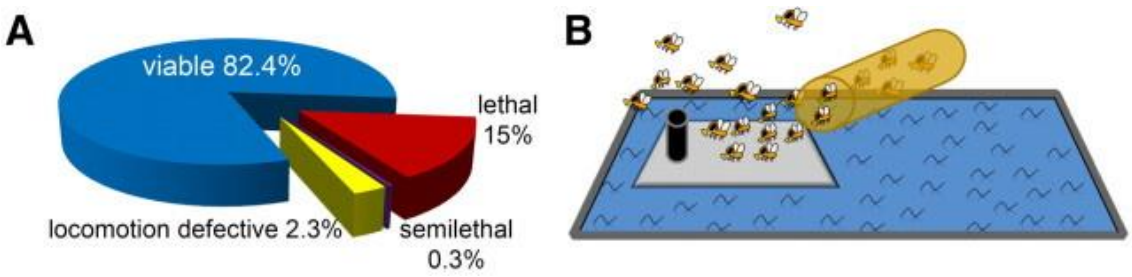


Figure 3.2: *Drosophila* Island Assay. A glial cell-specific RNAi screen performed for glial functions of *kinesin* for its locomotor defect (A). Schematic *Drosophila* Island assay (B) (Image adapted from Schmidt et al., 2012).

This experiment involves a 25×35 cm^2 watery soap bath with soap specifically designed to kill flies that comes in contact with water. Adult flies are centrally positioned inside the soap bath in 10×10 cm^2 island. Assessment of adult mobility was assayed based on

the duration required for the flies to disappear, its flying capabilities, and levels of hyper- or hypoactivity. Flies exhibiting atypical movement were subjected to screening thrice in a double-blind fashion. Post-videotaping flies that stayed on the platform were manually tallied on the computer screen, and three independent experiments were conducted. (Eidhof et al., 2017). This assay provides quicker and more efficient screens for locomotion defects. Nonetheless, it is generally observed that healthy young fly strains fly away immediately when introduced onto the platform, whereas older flies with locomotor deficits remain longer on the platform and eventually jump or fall off the platform. Despite these limitations, this assay provides a very accurate measure of locomotor behaviour.

Fly vertically rotating arena for locomotion (fly-VRL)

This assay illustrates refined behavioural apparatus for fly climbing behaviour with minimal human intervention which does not cause mechanical agitation and is automated for behaviour and data analysis. This setup is based on the vertical rotation of the behaviour cassette which is uniformly illuminated from the back with the help of a custom-built infrared light source (**Figure 3.3**).

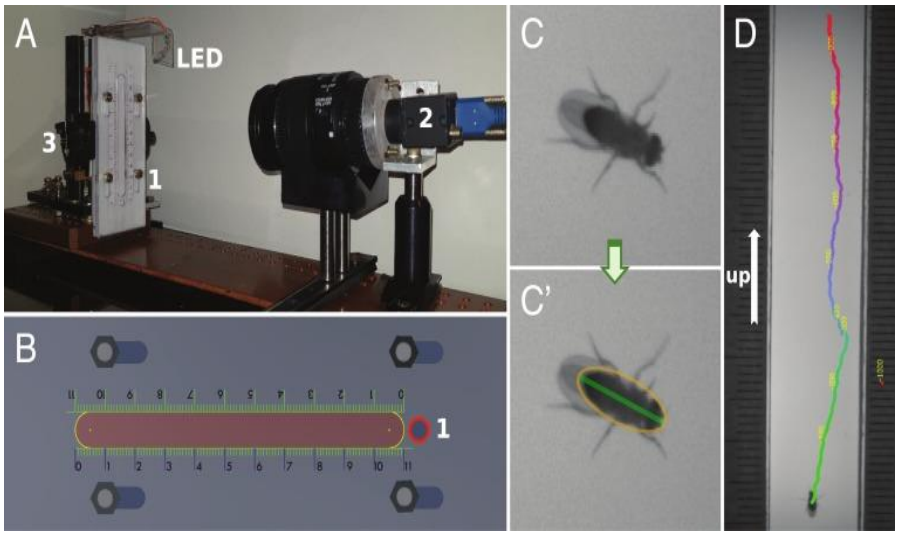


Figure 3.3: Arduino-controlled servo motor-based mechanism fly climbing setup. Actual photo of the fly climbing experiment setup, which includes a UV- LED light, a climbing cassette (1) a

camera (2), and a cassette rotating mechanism (3). Schematic view of where hole 1 represents the entrance of the experimental fly into the climbing arena (B). Top view of wild-type *Drosophila* during one of the documented climbings (C). Figure (C′) depicts an example of segmenting the same fly inside the same video frame; the green line shows the length of the fly's body, and the orange oval represents the automatically identified body contour. Raw image of a fly ascending in the arena overlaid with its trajectory after an ad hoc computation by FlyConTra software (D). The fly's location throughout the track is shown by the green-to-red color map, where green and red denote the fly's beginning and ending positions, respectively. The numbers in yellow indicate the frame number about FlyConTra's detection of the fly's location in the track at that specific moment in time. (Image adapted from Aggarwal et al. (2019).

The behaviour of *LRRK*^{ex1}/+ mutant and the effect of heterozygote *park*^{25}/+ in trans-heterozygous state were investigated and significant locomotor impairment was observed in a heterozygote park^{25}/+ PD model (Aggarwal et al., 2019). Simultaneously, they observed locomotor abnormalities in a homozygous pro-prioceptory mutation in Trp-γ1 flies linked to impact fine motor coordination in *Drosophila*. This assay offers economical and cost-effective instrument for accurately assessing the locomotor behaviours in both wild-type and mutant flies, thereby uncovering even the most subtle motor abnormalities in flies (Aggarwal et al., 2018).

Fly Group Activity Monitor (FlyGrAM)

It is an open-source software platform developed by Scaplen et al. (2019). It involves an open-source software platform with a cost-effective video-based behavioural apparatus that allows for real-time quantification of group locomotor activity in *Drosophila*. These automated monitoring methods allows a researcher to study complex behaviours through high-throughput behavioural screening. The platform provides a reliable real-time quantification using open-source software and a user-friendly interface for data quantification and analysis. Characterizing ethanol-induced locomotor activity in a dose-dependent manner along with effects of thermal and optogenetic manipulation of ellipsoid body neurons is crucial for ethanol-induced locomotor activity (Scaplen et al., 2019). Modest amounts of ethanol elicit an initial startle and gradual increase in group activity

whereas excessive amounts of ethanol prolonged group activity followed by sedation. Ellipsoid body ring neurons were inactivated by thermogenetic, resulting in decreased group activity. This activity aims to investigate the impact of heat and optogenetic manipulation on ellipsoid neurons crucial for motor activity (Nuñez et al., 2023).

Gradually, semi-automated methods developed, using mechanical apparatus to tap the fly and video recording to record their motion, hence enhancing precision and minimizing inconsistency. Recent progress includes the use of fully automated systems that include mechanical stimulation, high-throughput configurations, and software for monitoring and evaluating fly dynamics. Therefore, an automated system with less human involvement for assessing behaviour would be optimal for obtaining reliable and uniform results. Furthermore, the automation of the measurement equipment and the computerization of data processing would enhance the reliability of the output and reduce the need for extensive human tracking and timing of the flies. Accurate identification of small alterations in behaviour is crucial for comprehending the presentation of locomotor disorders.

3.4. Importance of Heterozygote and Homozygote mutation

Gene dosage refers to the alteration in the number of copies of a gene caused by chromosomal changes, such as duplications or deletions. This alteration might result in an imbalance in the production of protein and imbalance that significantly contributes to NDDs (Lupski, 2022). Defects in genes crucial for development are often homozygous lethal, so it is important to identify heterozygote mutations as well. A heterozygote mutation is defined by the presence of two distinct alleles for a certain gene, one being the normal allele and the other being a mutant allele. While a single copy of the gene in a mutant heterozygote state is sufficient to manifest a phenotype or symptom associated with a disease, a homozygote must possess both copies of the defective gene in order to

exhibit an abnormality of the disease. Identification of common risk alleles for typical sporadic PD has been achieved using genome-wide SNPs (Lu et al., 2021). This implies that the use of large-scale homozygosity mapping might reveal novel genes in seemingly outbred people with autosomal recessive disorder, as well as provide an estimate of the prevalence of recessive loci within a certain disease group. The presence of homozygous mutation often leads to a more pronounced manifestation of the illness. Many individuals with these mutations experience the onset of PD symptoms at an earlier stage in life compared to those with heterozygous mutation or sporadic cases. Specifically, genes such as *PARK2 (parkin), PARK7 (DJ-1),* and *PINK1 (PARK6)* often exhibit an autosomal recessive inheritance pattern, resulting in the manifestation of the illness throughout early stages of life. These findings were made by detecting mutations in consanguineous families through homozygosity mapping and positional cloning. The results indicate that individuals with Early onset Parkinson's Disease (EOPD) have a higher degree of genomic homozygosity compared to those without the disease. This suggests that prolonged periods of homozygosity may contribute to the development of the disease (Lubbe et al., 2020). Haplozygosity mapping has also shown mutations in *ATP13A2, PLA2G6, FBXO7,* and *SPG11* that result in a similar condition known as pallido-pyramidal early-onset parkinsonism (Shen et al., 2018). Five percent of cases with early onset PD have mutations in recognized autosomal recessive genes. No less than 20 genes are linked to familial PD, and over 20 genetic risk loci have been identified in PD GWAS (Funayama et al., 2023). The most noted genes include α-synuclein (*SNCA*), glucocerebrosidase (*GBA*), parkin (*PARK2*), Pten-induced kinase 1 (*PINK1*), microtubule-associated protein tau (MAPT), and leucine-rich repeat kinase 2 (*LRRK2*). Mutations in the *LRRK2* gene have been identified as hereditary risk factors for both familial and sporadic types of PD including features such as DAergic neuronal cell death,

decreased DA neurotransmission, abnormalities in protein synthesis and degradation, inflammatory reactions, and oxidative damage, which have been linked to the increased kinase activity of *LRRK2* pathogenic mutations (Jeong and Lee, 2020). Understanding familial forms of PD linked to autosomal dominant and recessive mutations may also help in knowing the importance of genetic basis of sporadic PD.

Therefore, in this Chapter, all the climbing mobility of the flies was assessed through a Negative geotaxis assay as described by Botella et al. (2004) and Phom et al. (2021); method described in **Chapter 2** (**section 2.9**). This method is important in evaluating mobility phenotypes during the early stages of disease onset, here linked to neurodegeneration. Here, I aimed to screen all deficiency lines to decipher mobility phenotypes, and based on the performance of the fly's climbing mobility, the phenotype was characterized and established in heterozygote and homozygote condition *viz.,* a) mutant *per se* and b) with PQ interaction.

Furthermore, to understand gene-environment interaction in these mutants, Deficiency lines are screened before the onset of mobility defect *i.e.*, at 4 hrs to observe and understand the susceptibility levels of mutant both in *per se* and with PQ interaction. This is done to understand the importance and influence of chromosomal deletion screens on 3rd chromosomal deficiency mutants to screen mobility phenotypes (if any) and characterize the nature of neurodegeneration behind the mobility phenotype. As behaviour is critical to understand the phenotypic variation underlying neurodegeneration, observing first its mobility defect remains crucial.

3.5. Results

3.5.1. Balancing of 3rd chromosome deletion mutants

Deletion mutants were balanced using appropriate balancer stocks following the scheme as mentioned in **Chapter 2** (**Figure 2.3**).

3.5.2. Primary screening of Heterozygote and Homozygote mutation to characterize mobility defects though NGA

To understand the influence of chromosomal deletions on the climbing mobility of the loss-of-function mutation under heterozygote (one-copy) and homozygote (two-copy) mutation *per se* and upon subsequent interaction with PQ, NGA was employed on all deletion lines. Out of the 66 deletion lines,11 lines were homozygous viable. **Figure 3.4**. shows a pictorial representation of 3L chromosome arm on the left pane and the 3R chromosome arm on the right pane where all 66 deletion lines are positioned and pointed with arrows on their respective chromosome regions. Climbing mobility for all 66 deletion lines were assayed though NGA and observed results are summarized (with labelled abbreviations) **Table 3.1** analyzed and characterize according to heterozygote and homozygote phenotype condition.

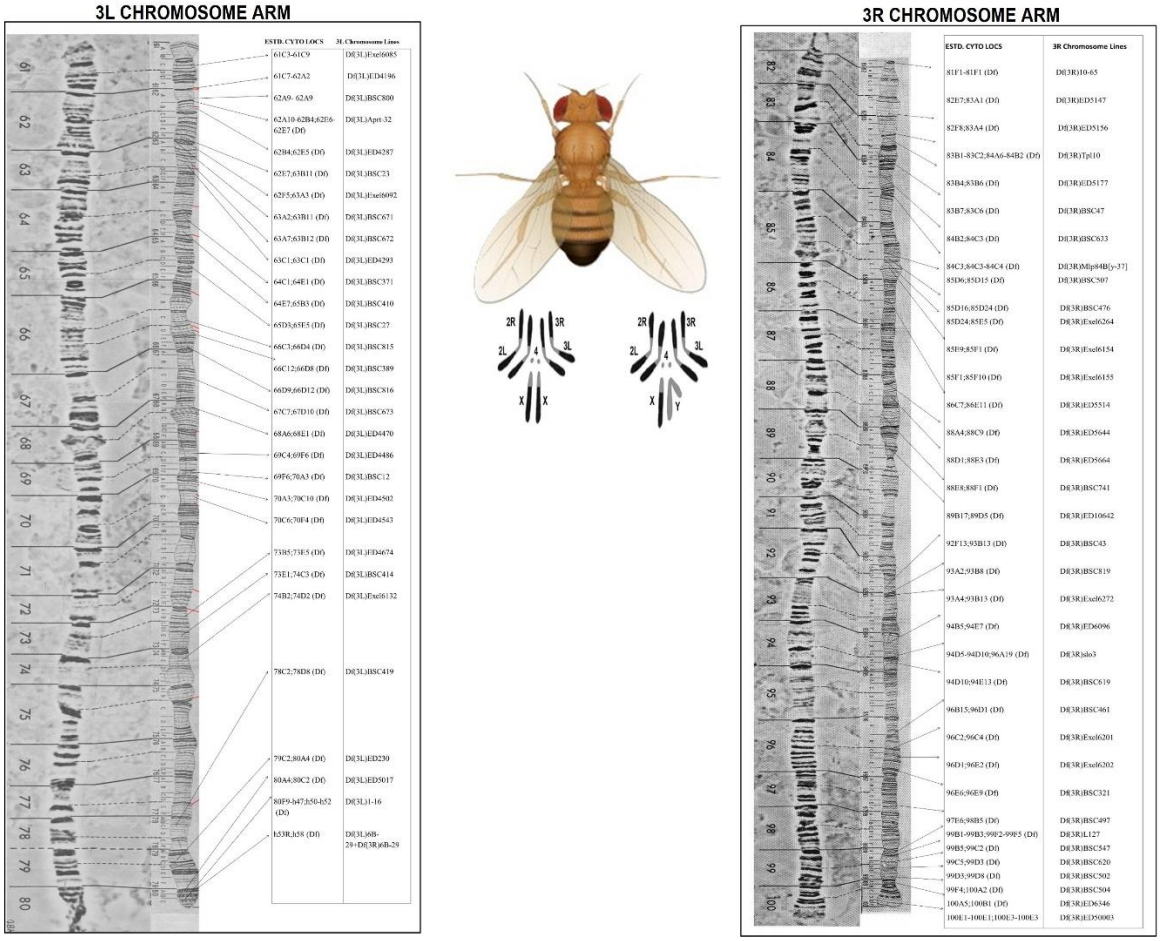


Figure 3.4: Cartoon presents *Drosophila melanogaster* chromosome arm showing 3L arm on the left pane and 3R arm on the right pane where all 66 deletion lines were assayed to characterize mobility defects for neurodegeneration using NGA (labeled with stock names; detailed description described in Chapter 2 (Table 2.1). All lines are placed vertically with arrows pointed towards their respective position on the chromosome arm region on the left side pane and right side pane. (3L & 3R Karyotype image adapted from Flybase).

Table 3.1. Summary of primary screening of deletion mutants in heterozygote and homozygote mutation *Per se* and with PQ interaction as tested by NGA.

3rd Chromosome lines	Control	Homozygote		Heterozygote		Homoviable
7564	X	UT	TD	UT	TD	-
8050	X	-	-	X	X	NV
27372	X	-	-	X	X	NV
5411	X	-	-	X	X	NV
8096	X	-	-	X	X	NV
6755	X	-	-	X	X	NV
7571	X	-	-	X	X	NV
26523	X	✓ *	✓ **	X	X	V
26524	X	-	-	X	X	NV

8058	X	-	-	X	✓ *	NV
24395	X	-	-	X	X	NV
24914	X	✓ *	✓ **	✓ *	✓ **	V
6867	X	✓ *	✓ **	X	X	V
27576	X	-	-	X	X	NV
24413	X	-	-	X	X	NV
27577	X	-	-	✓ *	✓ **	NV
26525	X	-	-	✓ *	✓ **	NV
8068	X	-	-	X	X	NV
8072	X	-	-	X	X	NV
6457	X	X	✓ **	X	X	V
8097	X	X	X	X	X	NV
8073	X	-	-	✓ *	✓ **	NV
8098	X	-	-	X	✓ *	NV
24918	X	-	-	X	X	NV
7611	X	X	✓ *	X	X	V
24923	X	-	-	X	X	NV
8089	X	✓ *	✓ **	X	✓ *	V
8102	X	✓ *	✓ **	X	✓ *	V
7002	X	-	-	X	X	NV
2596	X	-	-	X	X	NV
2597	X	-	-	X	X	NV
8967	X	-	-	X	X	NV
8965	X	-	-	X	X	NV
1990	X	-	-	X	X	NV
8103	X	-	-	X	X	NV
7443	X	-	-	X	X	NV
25724	X	-	-	X	X	NV
8644	X	✓ *	✓ **	X	X	V
25011	X	-	-	X	X	NV
24980	X	-	-	X	X	NV
7731	X	✓ *	✓ **	X	X	V
7633	X	-	-	X	X	NV
7634	X	-	-	✓ *	✓ **	NV
8957	X	-	-	X	X	NV
9482	X	-	-	X	X	NV
9090	X	-	-	X	✓ *	NV
24137	X	-	-	✓ *	✓ **	NV
26839	X	-	-	X	X	NV
7413	X	-	-	X	X	NV
27580	X	-	-	X	X	NV
7739	X	-	-	X	X	NV
8684	X	-	-	X	X	NV
6367	X	-	-	X	X	NV
25694	X	-	-	X	X	NV
24965	X	-	-	X	X	NV
7680	X	-	-	X	X	NV
7681	X	-	-	X	X	NV

24909	X	-	-	X	X	NV
25001	X	X	X	✓ *	✓ **	NV
3547	X	✓ *	✓ **	X	✓ *	V
25075	X	X	X	X	✓ *	NV
25695	X	X	✓ **	X	X	V
25006	X	-	-	X	✓ *	NV
25008	X	-	-	X	✓ *	NV
24142	X	-	-	✓ *	✓ **	NV
24516	X	-	-	X	X	NV

[Here, **X**-No mobility defect; **UT**- Untreated; **TD**-Treated; **NV**-Not Viable (Homozygous flies are absent)**;** **V**-Viable (Homozygous flies are present)]

✓ * - Heterozygote Untreated mobility defect

✓ ** - Heterozygote Treated enhanced mobility defect

✓ * - Homozygote Untreated mobility defect

✓ ** - Homozygote Treated enhanced mobility defect

3.5.3. Screening heterozygotes *per se* and with neurotoxicant (PQ) interaction for mobility defects

In healthy brain condition, control wild-type flies CTR UTD did not exhibit any mobility defect in its motor performance *per se* and with PQ interaction condition at 4 hrs window time. Therefore, to understand whether having one-copy of the mutant allele affect its climbing mobility, NGA was performed in heterozygotes *per se* and in interaction with PQ and compared with healthy control flies CTR UTD on the 10^{th} day. Results showed reduced mobility defects *per se* in 7633/+ UTD, 8097/+ UTD, 9090/+ UTD, 24909/+ UTD, 25008/+ UTD, 24413/+ UTD, 27577/+ UTD) as described in **Table 3.1** & **Figure 3.5** approximately by 13-30% (**p<0.001) compared to control flies CTR UTD. Further upon subsequent interaction to PQ, showed enhanced mobility defect in (7633/+ TD, 8097/+ TD, 9090/+ TD, 24909/+ TD, 25008/+ TD, 24413/+ TD, 27577/+ TD) approximately by 30-60% (***p<0.0001) as compared to their controls. Statistical analysis was performed and graphs were plotted accordingly.

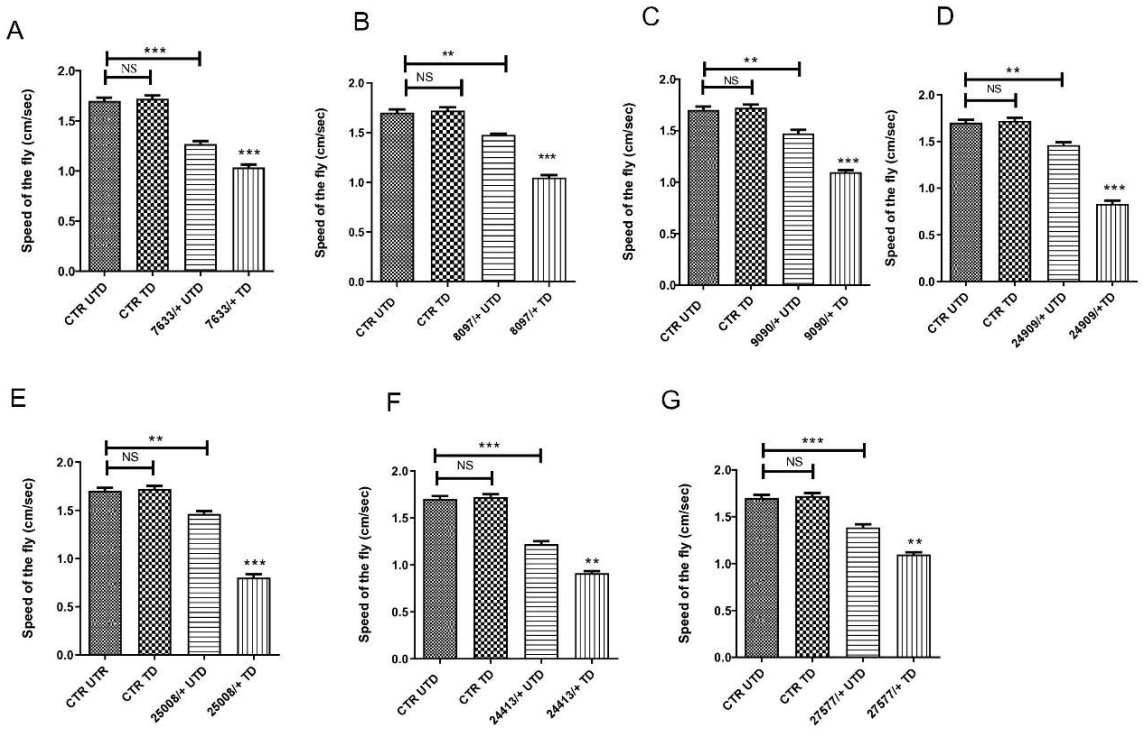


Figure 3.5: Negative geotaxis assay of one-copy mutation lines showed reduced climbing mobility in both *per se* and with PQ interaction in 10-day old flies. Flies were treated as per reference in Chapter 2 (section 2.5). Control wild-type flies at 4-hrs exposure does not show any mobility defect in untreated CTR UTD and treated CTR TD conditions in all 8 groups. Mobility defect is seen in all deletion lines *perse* and with PQ interaction, mobility defects were significantly enhanced as shown above (A, B, C, D, E, F, G) suggesting gene-environment interaction. (For summative analysis, one-way ANOVA was performed followed by Tukey post-test) Here, CTR TD compared to CTR UTD, deletion line *per se* (*Df*/+ UTD) tagged with stock number) compared to CTR UTD and deletion line (*Df*/+TD) tagged with stock number compared to deletion *per se* (*Df*/+ UTD) & CTR UTD; **p<0.001; ***p<0.0001; NS: No significance).

3.5.4. Screening homozygotes *per se* and with neurotoxicant (PQ) interaction for mobility defects

In healthy brain condition, control wild-type flies CTR UTD did not show any variation in climbing mobility both *per se* as and with PQ interaction at 4 hrs window time. To determine whether having two copies of the mutant allele could affect climbing behaviour *per se*, untreated homozygote mutants were compared to untreated control flies CTR UTD and heterozygote mutant in the climbing assay on the 10th day. Here following observations are observed in **Figure 3.6**. Flies showed mobility defect in climbing

mobility under two-copy mutation *per se* in 24395/24395 UTD approximately by 35% (***p<0.0001) compared to controls 24395/+UTD, & CTR UTD, further upon subsequent interaction with PQ (24395/24395 TD) mobility defect was enhanced by approximately 13% (*p<0.05) compared to 24395/24395 UTD & CTR UTD (**Figure 3.6 A** & **Table 3.1**). Consequently, climbing mobility was also reduced for one-copy mutation of this line 24395/+ UTD *per se*, and the climbing phenotype was aggravated *per se* and in PQ interaction condition. Simultaneously, other mutant in its two-copy mutation *per se* 7571/7571 UTD, 24914/24914 UTD, 24918/24918 UTD, 24980/24980 UTD, 25075 UTD /25075 UTD and 25724/25724 UTD in **Figure 3.6** experienced similar phenotype as 24395/24395 UTD line (*p<0.5; **p<0.001; ***p<0.0001) in both untreated and treated condition compared to their controls. However, in its one-copy mutation, no alteration in climbing mobility in *per se* and in PQ interaction was observed (**Table 3.1** & **Figure 3.6**), indicating that phenotypes that were not observed in heterozygote condition could show up in homozygote condition. In another condition, few lines under two-copy mutation did not experience any deficit in climbing mobility *per se* such as in line 8072/8072 UTD [**Figure 3.6 (H)**], while, with PQ interaction a significant deficit in its climbing mobility was observed. However, no mobility defect was observed in its one-copy mutation in both *per se* and in PQ interaction.

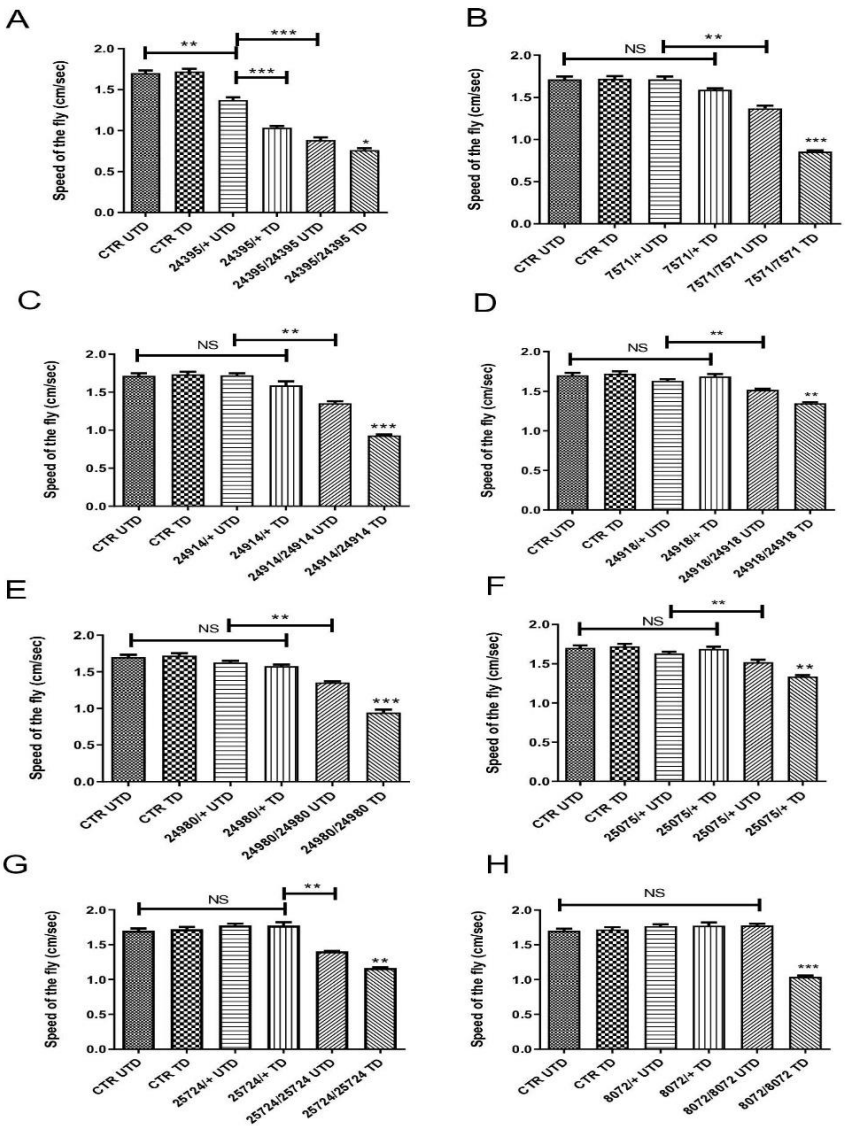


Figure 3.6: Negative geotaxis assay of two-copy mutation lines showed reduced climbing mobility in *per se* and with PQ interaction in 10-day old flies. Flies were treated as per reference in Chapter 2 (section 2.5). Control flies at 4-hr exposure do not show any mobility defect in untreated CTR UTD and treated CTR TD condition in all the 8 groups. Two-copy mutation lines resulted in mobility defects in *per se* as well as in PQ interaction condition (A, B, C, D, E, F, G), whereas (8072/8072) line in Figure G showed no variation in climbing mobility in *per se* however observed reduced climbing mobility in PQ interaction condition. (For summative analysis, one-way ANOVA was performed followed by Tukey post-test; Here, CTR TD compared to CTR UTD, two-copy deletion line *per se* (UTD tagged with line number) was compared to CTR UTD and one-copy deletion line *per se* (UTD tagged with line number) as its immediate control. Two-copy PQ interaction deletion line compared to its deletion line *per se*, one-copy PQ interaction deletion line & CTR UTD; **p<0.001; ***p<0.0001; NS-Not significant).

This condition may indicate a gene-environment interaction in which a few lines that under normal circumstances result in a phenotype can occasionally be "unmasked" or

triggered upon subsequent external insults, causing previously silent mutations to result in a visible or harmful phenotype. In such a case, the external environment plays an important role in revealing the effects of a mutation.

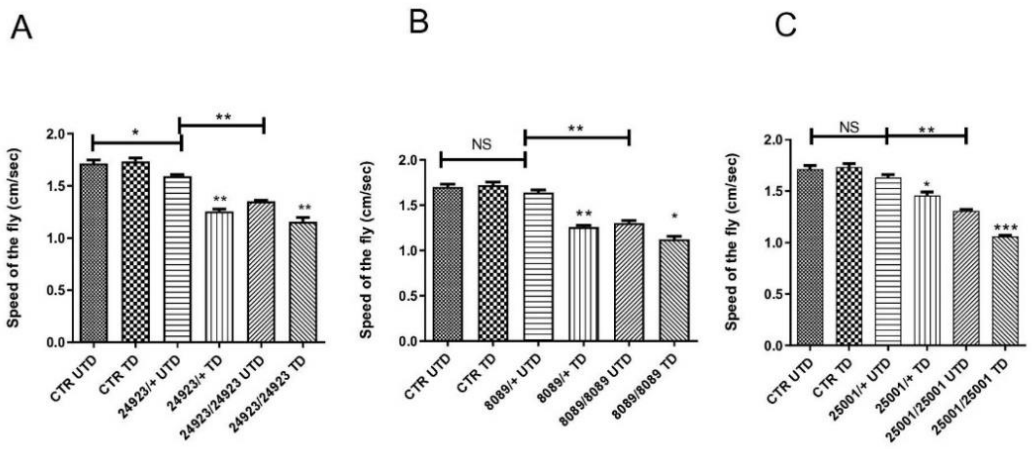


Figure 3.7: Negative geotaxis assay of two-copy mutation lines showed reduced climbing mobility in *per se* and with PQ interaction in 10-day old flies. Flies are treated as per reference in Chapter 2 (2.5). Control flies at 4-hr exposure do not show any mobility defect in untreated CTR UTD and treated CTR TD condition in the above groups. Two-copy mutation lines resulted in mobility defects in *per se* as and in PQ interaction condition. (For summative analysis, one-way ANOVA was performed followed by Tukey post-test; Here, CTR TD compared to CTR UTD, one-copy PQ induced-PD deletion (*Df*/+ TD tagged with line number) was compared to CTR UTD and its deletion *per se* (*Df*/+ UTD tagged with line number); two-copy PQ induced-PD deletion (*Df*/*Df* TD tagged with line number) was compared to its deletion one-copy PQ induced-PD deletion (*Df*/+TD tagged with line number) & CTR UTD; **p<0.001; ***p<0.0001; NS-Not significant). For summative analysis, one-way ANOVA was performed followed by a Tukey post-test.

Here, 24923/24923 UTD, 8089/8089 UTD & 25001/25001 UTD (**Figure 3.7 A, B, C**), in two copy mutation both *per se* and in PQ interaction showed reduced climbing mobility compared to their controls, whereas its one copy mutation *per se* do not show any mobility defect, however, with PQ interaction exhibits reduced mobility defect (*p<0.05; **p<0.001) compared to their controls. When compared between the groups, *i.e*., two copy PQ interaction and one copy PQ interaction the phenotype was aggravated more in induced-PD homozygote line and showed highly significant variation in 25001/25001

TD; **Figure 3.7 C** (***p<0.0001) and 24923/24923 TD, 8089/8089 TD (**p<0.0001); *p<0.01) (**Figure 3.7 A** & **B**).

Based on the above results on the performance of its climbing phenotype, out of 66 lines, I screened 7 lines that exhibited mobility defects *per se* in heterozygote condition. Further, I wanted to see the susceptibility levels of loss-of-function of these mutants in interaction with PQ. Therefore, flies were exposed to 4 hrs of PQ exposure, and results showed that mutant lines that exhibited mobility defect *per se* also showed enhanced mobility defect at this time point. Hence, among the 7 lines screened *viz.*, (*Df* 7633, *Df* 8097, *Df* 9090, *Df* 24909, *Df* 25008, *Df* 24413, *Df* 27577) (**Figure 3.5 A, B, C, D, E, F, G**).

Df 8097 deletion mutant was selected as it exhibited acute phenotype in its climbing mobility *per se* approximately by 14% (**p< 0.01) and further aggravated by 29% (***p< 0.001) with PQ interaction as compared to its control. Moreover, the chromosomal deletion region of this mutant encompasses some of the genes (information from the fly base) that are involved in motor protein synthesis in regulating protein aggregation linked to axonal transport, neuronal transport, neurogenesis, axonal transport of mitochondrion, cell fate determination, evoked neurotransmitter secretion linked to locomotor and lifespan determination. Hence, *Df* 8097 deletion mutant (**Figure 3.5 B**) was picked up to further characterize the nature of neurodegeneration behind the observed mobility variation.

3.5.5. Survivability of *Df* 8097 deletion mutant

The maximum life span of *Drosophila melanogaster* includes 120 days with a median life span of 95 days in regular culture medium under favourable environmental conditions (Phom et al., 2014). The adult health span of a fly is distinct at a point of time when no natural deaths occur. The control fly and deletion mutant survival curves show that CTR UTD flies have a maximum life span of 120 days and a median life span of 91 days. For

Df 8097, the maximum life span is 110 days, and the median life span is 79 days in 8097/+UTD flies raised in regular culture media under favourable environmental conditions. Results shows variation of 12% (*p<0.01) between the group which may suggests, subtle difference in their survival (**Figure 3.8**).

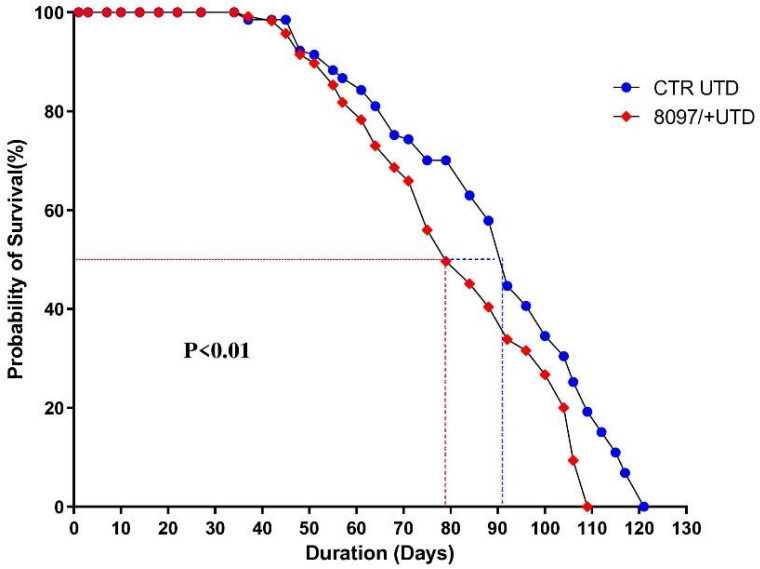


Figure 3.8: Survivability of male 8097/+ UTD deletion mutant flies reared on regular culture medium. Mortality was recorded till all death of flies occurred. Adult Control flies survived for approximately 120 days in media and deletion mutant for approximately 110 days and survival percentages were plotted accordingly. Survival curves revealed a slight variation between both control and deletion mutant *per se* (log-rank [Mantel–Cox test, *p < 0.01]).

3.5.6. Discussion

Drosophila has been widely employed as a model organism for more than two decades. Owing to its diverse genetic toolbox, it has proven as a valuable model organism for understanding the genetics of PD. Over ten years of research on *Drosophila* models of PD has contributed to our understanding of the interplay between many genetic variables, including *parkin* and *PINK1*, in this disease (Aggarwal et al., 2019). Fly climbing experiments are commonly employed to investigate locomotor behavioural traits for these genes. Even though certain locomotor symptoms are easily measured by these straightforward experiments for locomotor defects in *Drosophila* mutants, identifying

minute behavioural alterations is also crucial to comprehend how locomotor problems occur. Profound locomotor impairment has always been one of the foremost readouts in NDDs such as PD (Chaudhuri et al., 2007). Epidemiological studies demonstrate a relationship between exposure to environmental toxins such as PQ and the onset of PD which is considered an additive factor of sporadic PD, and accounts for over 90% of PD cases (See et al., 2024). Climbing mobility was also used as a parameter in several studies as it is a well-established model to investigate PQ effect on motor ability and lifespan (Bonilla-Ramirez et al., 2013; Chaudhuri et al., 2007). A PQ-induced fly model of sporadic PD developed in our laboratory by Phom et al. (2014) revealed that 90% of the flies could reach the top of the column in 12 secs under normal conditions, but PQ-induced flies were unable to do so. PQ-treated *Drosophila* exhibits bradykinesia and resting tremors, which are also characteristic features of PD seen in human patients. Earlier studies from our laboratory observed marked decline in the climbing speed of the flies approximately by 33% after 24 hrs exposure to the neurotoxicant PQ in a *Drosophila* sporadic PD model (Ayajuddin et al., 2023; Phom et al., 2014). Here, few flies attempted to climb the walls, however, failed to maintain its grip and fell down to the bottom of tube, whereas others displayed overactive/restless behaviour as evident by rapid wing flipping.

Climbing behaviour in many PD-related fly mutant lines, including park^{25}/+ and Lrrk^{ex1}/+ mutants *per se*, was investigated in addition to wild type flies and compared with control PINK1^{RV} flies. Although mutations in *parkin* and *PINK1* are known to be autosomal recessive, genes heterozygous for these mutations are thought to increase risk for early onset PD (Corti et al., 2011). These two genes are also implicated in the fly model of PD. Climbing mobility for heterozygous flies *park25/+*, and *Lrrk^{ex1}*/+mutants *per se* showed locomotor defects, in addition to it, *park25*/*Lrrk^{ex1}* in trans-heterozygous, climbing

mobility were also compared to its heterozygote mutants. *park25/Lrrk*^{ex1} flies showed enhanced mobility defects mimicking features of observable bradykinesia as seen in PD patients. Aberrant Locomotor defects in a homozygous pro-prioceptory mutation (*Trp-γ1*) were also observed as compared to heterozygote as this were known to affect fine motor control in *Drosophila* (Aggarwal et al., 2019; 2018). Understanding these familial forms of PD linked to autosomal dominant and recessive mutations may also help in knowing the importance of the genetic basis of sporadic PD (Day and Mullin, 2021).

Simultaneously, taking advantage of deletion screens, few deficiency lines have been studied in fly's genetic circulatory system of heart development and disease, embryonic phenotypes, orphan receptor ligands, dorsal closure, and mitochondrial integrity using various assays. In earlier studies, Kim et al. (2010) used genetic deletion screens to observe for cardiomyopathy phenotype in cardiac function using chromosome 2L deficiency lines in awake adult *Drosophila*. The deletion mutant exhibited dilated cardiomyopathy, and identified *Weary* as a potential gene responsible for the impaired phenotype. When overexpressed, awake *Drosophila* displayed normal cardiac function expressing its function as a new notch ligand. Through this approach, the cardiac phenotype and function were accurately identified. Additionally, clues about the disease-targeting molecular entities that have evolved to preserve their evolutionary history were discovered.

In the present study, from primary deletion screens of 3rd chromosomal deficiency lines, assays were performed on heterozygote and homozygote conditions and are summarized as (tabulated in **Table 3.1)**.

A) Heterozygote mutants exhibit mobility defect *per se* and upon subsequent interaction with neurotoxicant, its climbing mobility was further aggravated (**Figure 3.1 A, B, C, D, E, F, G**) as compared to their controls.

B) Homozygote mutant exhibits mobility defect *per se* and further aggravated with PQ interaction. Simultaneously, its condition in heterozygote exhibits mobility defect per se and with PQ interaction (**Figure 3.3 A**) as compared to their controls.

C) Homozygote mutant exhibits mobility defect *per se* and further aggravated with PQ interaction. However, its condition in heterozygote did not show any variation in climbing mobility *per se* and with PQ interaction (**Figure 3.3 B, C, D, E, F, G**) as compared to their controls.

D) Homozygote mutant exhibits mobility defect *per se* and further aggravated with PQ interaction. Its condition in heterozygote did not show any variation in climbing mobility *per se*; however, its mobility was reduced with PQ interaction (**Figure 3.4 A, B, C**) as compared to their controls.

The possible reasons for the above results may possibly due to-

i) Certain mutations may remain silent or not readily evident in their phenotype. Although these mutations may be present in either a heterozygote or homozygote state, they do not cause any noticeable effects in normal condition.

ii) However, under certain environmental stressors, these previously silent mutations can be unmasked/become active as the natural capacity of the body to compensate for the mutation may be overpowered, therefore exposing the inherent genetic susceptibility. Instances as in individuals with a mutation in mitochondrial DNA may initially be compensated by healthy mitochondria, however, exposure to environmental toxins like pesticides may trigger the onset of Parkinson's-like symptoms, as the mitochondrial function becomes increasingly compromised suggesting gene-environment interaction (Cabezudo et al., 2020; Patrick et al., 2019).

iii) Environmental insults may lead to phenotype variability wherein, either the severity of symptoms and/or the timing of onset can differ widely even among individuals with the same mutation. The observed variability also underscores the significance of external variables in altering the expression of a mutation.

Results demonstrate that wildtype flies at this window time do not exhibit any mobility defect, However, climbing mobility of mutant *per se* in both conditions *viz.*, heterozygote and homozygote was affected relative to features as seen in PQ-induced flies. Upon subsequent interaction to PQ, mobility phenotype was aggravated as is consistent to features observed in *Drosophila* sporadic PD model.

3.6. Conclusion

In this chapter using deficiency lines, primary screening was performed in all 66 deletion lines, wherein 11 lines were homozygotes and accordingly phenotypes were characterized. In the heterozygote condition, I screened 7 lines (**Figure 3.5**) that exhibited mobility defects. Further, to understand gene-environment interaction, these lines were screened with PQ interaction, and results showed enhanced mobility defects in all these lines. Therefore, out of these lines, I selected *Df* 8097 (**Figure 3.5 B**) mutant as it showed acute phenotype in its climbing mobility both *per se* and with PQ interaction, and also genes encompassing these regions show some critical functions linked to its impaired mobility. Hence, in Chapter 4, I wanted to further characterize the *Df* 8097 mutant and understand the health of the deletion mutant, whether the observed mobility defect is either due to degeneration of DAergic neurons and/or altered brain dopamine metabolism in mutant *per se* and under neurotoxicant interaction (gene-environment interaction).

(Contributions: Animal treatments: Abuno Thepa; climbing assay: Abuno Thepa, Nukshimenla Jamir)

CHAPTER 4

Characterization of brain dopaminergic neurodegeneration and dopamine metabolism in *Df* 8097 deletion mutant

4.1. Introduction

Multiple behaviours such as locomotion (Pendleton et al., 2002; Phom et al., 2021; Ayajuddin et al., 2023), sleep and arousal (Liu et al., 2013; Ueno et al., 2012), learning and memory (Waddell, 2010; Liu et al., 2012; Berry et al., 2012), circadian rhythm (Allada et al., 2010; Cirelli et al., 2008), courtship behaviour (Koza et al., 2023; 2021; Shaltiel-Karyo et al., 2012; Villela et al., 2008) flight orientation and attention decision making (van Swinderen, 2011; Zhang et al., 2007) are involved in *Drosophila* brain dopaminergic (DAergic) system. Tyrosine Hydroxylase (TH) activates the first and rate-limiting step in catecholamine biosynthesis and is highly conserved among evolutionarily diverged species. The amino acid, tyrosine is converted into L-DOPA *via* the rate-limiting enzyme, TH (Budnik and White, 1987). Further, L-DOPA is converted into Dopamine (DA) *via* the enzyme *Dopa decarboxylase* (*Ddc*) (Livingstone and Tempel, 1983), which serves as an important enzyme for the biosynthesis of serotonin. In mammals, DA is degraded either *via* oxidation or methylation through the enzymes, Monoamine oxidase (MAO) and Catechol-O-methyltransferase (COMT), respectively (Meiser et al., 2013) (**Figure 4.1**).

TH is expressed by the *ple* gene and is located on the left arm of chromosome 3 in *Drosophila.* The loss of *ple* gene resulted in embryonic lethality (Neckameyer and White, 1993). Reduction of TH or *Ddc* levels also caused pale pigmentation and reduction of dopamine levels (Samantha et al., 2023; True et al., 1999; Livingstone and Tempel, 1983; Budnik and White, 1987). The lethality in the *ple* mutants causing the specific developmental mechanism was not exactly known, but it could be prevented by feeding L-DOPA (Budnik and White, 1987). Riemensperger et al. (2011) rescued TH-null lethality by restoring the hypoderm-specific, but not by CNS-specific expression of TH. Also feeding of L-DOPA and carbidopa to TH-deficient flies showed improvement in the

levels of DA in the brain (Cichewicz et al., 2017). TH-deficient flies, consequently DA-deficient are observed to have hypoactivity, decreased alertness, longer sleep duration, a lack of desire for sucrose, decreased alertness, defective olfactory, aversive learning, and locomotor impairments which tend to aggravate more and worsen with age (Cichewicz et al., 2017; Riemensperger et al., 2011).

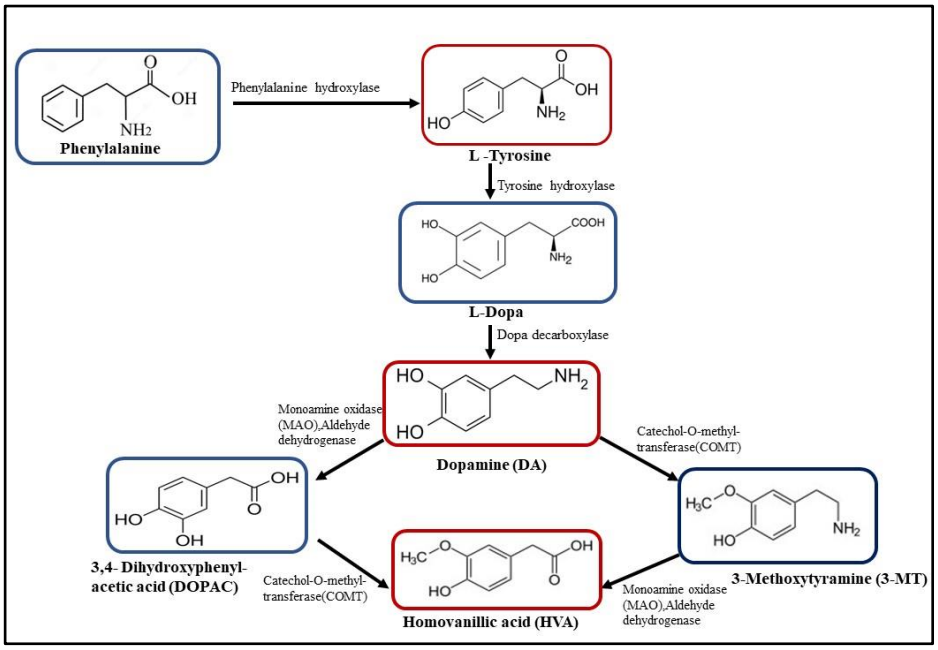


Figure 4.1: Synthesis and metabolism of DA in mammalian brain. Released dopamine (DA) can be absorbed by the presynaptic DA transporter (DAT) and transported back to the cytoplasm, where it is converted into 3,4-dihydroxyphenylacetic acid (DOPAC) by the enzyme monoamine oxidase (MAO) or packed into storage vesicles. The extracellular left in the synaptic gap can be methylated to 3-methoxytyramine (3-MT) by the enzyme catechol-o-methyltransferase (COMT). Monoamine oxidase (MAO) can then carry this chemical, (3-MT) back into the cytoplasm where it can undergo further oxidation to produce homovanillic acid (HVA). DOPAC in the cytoplasm may leak out to the extracellular space and be methylated to HVA by COMT (DOPA-Dihydroxy-phenylalanine)

The *Drosophila* models of PD are characteristic of certain pathophysiological features of human PD; such as locomotor defects, DAergic neurodegeneration, reduced brain dopamine (DA) (Ayajuddin et al., 2023; Chaouhan et al., 2022; Shukla et al., 2014; Chaudhuri et al., 2007; Feany and Bender, 2000). Early loss of TH activity followed by a decline in TH protein is considered one of the factors contributing to DA deficit and

phenotypic manifestation in PD, DOPA-responsive dystonia, and/or infantile parkinsonism in mammals (Blanchard-Fillion et al., 2001; Nagatsu et al., 1990).

The misexpression of human α-Synuclein in the CNS of *Drosophila* causes degeneration of DAergic neurons, disturbance of eye-ommatidial, development of filamentous aggregates that are structurally similar to LB inclusions, and progressive age-dependent locomotor dysfunction similar to the clinical manifestations in human PD (Feany and Bender, 2000). The first *Drosophila* PD model was developed by Feany and Bender (2000) and since then many laboratories started employing the same animal model to study the effects of mutations or over-expression of genes involved in PD. Time and dose-dependent DAergic neurodegeneration followed by variations in neuronal appearance, such as the aggregation of cell bodies into circular shapes, fragmentation, and eventually the selective loss of subsets of DAergic neurons from the particular cluster are observed with PQ-mediated fly PD model (Chaouhan et al., 2022; Maitra et al., 2021; 2019; Chaudhuri et al., 2007; Song et al., 2017; Lawal et al., 2010).

Drosophila approximately has 280 DAergic neurons per brain. These DAergic neurons are distributed among eight clusters per hemisphere, each consisting of four to thirteen individual neurons except for the PAM (Protocerebral anterior median) cluster that has nearly 100 neurons per hemisphere (Mao and Davis, 2009; Nässel and Elekes, 1992). However, the quantifiable DAergic neurons in the whole fly brain are PAL (4-5 neurons), PPL1 (11-12 neurons), PPL2 (6-7 neurons), PPM1/2 (8-9 neurons), PPM3 (5-6 neurons) and VUM (3 neurons) (PAL-Protocerebral anterior lateral; PPL- Protocerebral posterior lateral; PPM- Protocerebral posterior medial) that can be tagged with primary anti-TH antibody (Chaurasia et al., 2024; Ayajuddin et al., 2023; Koza et al., 2023; Navarro et al., 2014).

DA is a monoamine neurotransmitter that is generated by DAergic neurons in the ventral tegmental region of the substantia nigra (SN) in the midbrain. (Kaufling, 2019). Mammals have five DA receptors called G-protein coupled receptors which exist throughout the body and play a role in the activities of both the peripheral and CNS (Gurevich et al., 2016). DA was first detected in 1951, in the brains of different animals including humans (Raab and Gigee, 1951). Experiments conducted in the late 1950s were instrumental in establishing DA's distinct roles both inside and outside the brain. Additionally, it was shown that L-DOPA, when administered to animals, could transform into DA (Holzer and Hornykiewicz, 1959; Hornykiewicz, 1958). The connection between PD and DA insufficiency was a significant finding. Several laboratories took part in finding brain structures linked to the striatum as the key cause for PD; whereas others demonstrated that the SN dopamine deficiency was the cause for the striatal depletion of DA. L-DOPA was first suggested as a treatment for PD shortly after these early findings (Hornykiewicz, 1963). Later, after recognizing the lack of DA in certain brain regions as the main cause of motor symptoms and realizing that DA cannot pass across the blood-brain barrier, they considered utilizing L-DOPA, as its precursor as it readily crosses the blood-brain barrier effectively transforming L-DOPA into DA in the brain (Franco et al., 2021; Olanow et al., 2004).

Two enzymes are required for DA synthesis: L-tyrosine hydroxylase, which is used as a marker of DA-producing cells/neurons, and L-3,4 dihydroxyphenylalanine (L-DOPA) decarboxylase. The breakdown pathway of DA involves two enzymatic processes facilitated by MAO and COMT. DA is synthesized, processed, or enclosed into vesicles prior to its transportation into the synapses. L-DOPA is synthesized from tyrosine by TH and then converted into DA by dopa-decarboxylase (DDC). Excess DA is taken by the presynaptic neuron and MAO can then catabolize DA to produce DOPAC. Further,

DOPAC can be transported to astrocytes, a specialized glial cell for further catabolism into HVA through COMT activity (Chakrabarti and Bisaglia, 2023; Winner et al., 2017) (**Figure 4.1**). This procedure results in the formation of homovanillic acid (HVA), a final product in the DA metabolism.

DA dynamics and signalling in *Drosophila* and mammals are almost identical, but there exist a few key differences in their metabolism. In invertebrates, the main mechanisms by which dopamine is broken down are believed to be β-alanylation through the β-alanyl amine synthase (expressed by the *ebony* gene in *Drosophila*) and acetylation through the acetyltransferase (encoded by the *speck* gene in *Drosophila*) (Yamamoto and Seto, 2014). Nevertheless, insects also facilitate the conversion of DA into melanin to provide the appropriate cuticle color and structure. Genes associated with dopamine metabolism are indeed responsible for alterations in cuticle color (Samantha et al., 2023; Spana et al., 2020; True et al., 1999).Conversely,glial cells in the brain of the fly enzymatically degrade DA to generate NBAD and NADA, while dopaminergic neurons re-metabolize NBAD into DA. Notably, *Drosophila* lacks an orthologue for the COMT and human MAO genes. Furthermore, apart from NBAD and NADA, the fly's brain has been discovered to include DA oxidative products such as DOPAC and HVA (Freeman et al., 2012; Wakabayashi-Ito et al., 2011; Chaudhuri et al., 2007; Zhang et al., 2005). This insight gives clues about the existence of metabolic pathways and components that resemble the mammalian system in the fly brain (Yamamoto and Seto, 2014).

According to reports, DAergic neurons gradually diminish at a rate of roughly 4% as people age normally (Fearnley and Lees, 1991).Compared to age-matched controls, PD patients experience a faster decline in DAergic neurons, with a terminal reduction of 70% and a 40–50% decrease in striatal DAergic neurons (Carola et al., 2021; Cheng et al.,

2010). Thus, in the case of late-onset NDD as PD, it is plausible that the rapid death of DAergic neurons will also lead to a progressive reduction in the level of DA.

Over the years, an extensive genetic toolkit has been generated in the fruit fly, making it a great system for dissecting dopaminergic neural circuitry and signalling involved in many behavioural contexts (Frighetto et al., 2022; Xie et al., 2018; Jenett et al., 2012). In addition to the fly's contribution to understanding dopaminergic circuitry, *Drosophila* has also been used to identify and investigate the cellular functions of PD genetic risk factors like *PINK1* and *PRKN* (*i.e.*, *Pink1* and *parkin* in the fruit fly) (Clark et al., 2006). An RNAi-based cuticle pigmentation screen performed by Deal et al. (2023) also observed pale pigmentation phenotype in TH knockdown flies, which also showed reduced head dopamine levels by ~60% and brain DA levels by ~32% changes in dopamine metabolism. Further, downstream of dopamine synthesis also caused pigmentation phenotypes that do not correlate either with head or brain dopamine level differences in adult flies. Their screen studies also revealed that 78% of genes with human homologs were linked to human disease with some type of neurological and/or neurodevelopmental phenotype. These striking findings underscore the applicability and significance of using animal models to study NDD-PD in humans.

In this Chapter, I aim to characterize the *Df* 8097 deletion mutant to understand the nature of neurodegeneration; and whether the observed mobility defect is either due to DA variation/subsequent dopamine metabolism variation in mutant *per se* and with PQ interaction, if any; to understand the health of the deletion mutant brain through fluorescence microscopy which will further be confirmed by quantifying brain-specific DA and its metabolites through the HPLC method. The following observations are undertaken in this study:

A) Quantification of DAergic neuronal number.

B) Quantification of the level of tyrosine hydroxylase (TH) protein in DAergic neurons through quantification of the fluorescence intensity (FI) of secondary antibodies which targets the primary antibody anti-tyrosine hydroxylase (anti-TH).

C) Quantification of brain-specific DA and its metabolites DOPAC (3,4-dihydroxyphenyl acetic acid) and HVA (Homovanillic acid) levels.

D) Quantification of DA turnover rate.

4.2. Results

4.2.1. Anti-TH immunostaining of whole-brain *Df* 8097 deletion mutant indicates that there is no loss in the number of DAergic neurons but exhibit diminished Tyrosine Hydroxylase (TH) synthesis in *per se* and with PQ interaction.

The adult *Drosophila* brain consists of six quantifiable DAergic neuronal clusters in each brain hemisphere (**Figure 4.2 A**) (Chaurasia et al., 2024; Ayajuddin et al., 2023; 2022; Whitworth et al., 2006). The number of DAergic neurons in PAL, PPL1, PPL2, PPM1/2, PPM3, and VUM are 4-5, 11-12, 6/7, 8/9, 5-6, and 3, respectively. To understand the extent of DAergic neuronal dysfunction in *per se* and with PQ induced deletion mutant fly compared to the control fly, brains were dissected and immunostained for TH (the rate-limiting enzyme in the synthesis of dopamine) (**Figure 4.2 A**). Employing fluorescently labeled secondary antibodies directed against the primary antibody which labels DA synthesizing TH, it was possible to count the number of DAergic neurons (**Figure 4.2 B**).

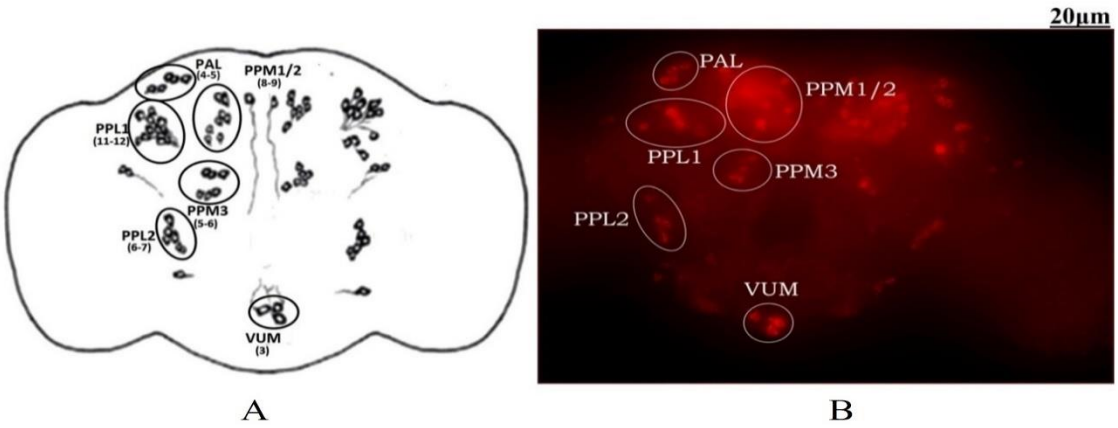


Figure 4.2: Quantifiable dopaminergic neuronal clusters in whole brain of *Drosophila*: Cartoon of *Drosophila melanogaster* brain illustrating the position of quantifiable DAergic neurons (**A**) and image of whole-brain mount of *Drosophila* captured by ZEN software of Carl Zeiss Fluorescence Microscope using fluorescently labeled secondary antibody targeted against the primary anti-TH antibody (**B**). There are around 141 dopaminergic neurons (including ~ 100 neurons of the PAM cluster which cannot be quantified) arranged in different clusters in each hemisphere. The scale bar of the brain image in the panel is 20 µm. (Adapted from Chaurasia et al., 2024; Ayajuddin et al., 2023). [PAL: Proto-cerebral Anterior Lateral; PAM: Proto-cerebral Anterior Medial; PPL: Proto-cerebral Posterior Lateral; PPM: Proto-cerebral Posterior Medial; VUM: Ventral Unpaired Medial].

Figure 4.3 A, show images of the various experimental groups in the *Drosophila* brain. The anti-TH antibody immunostaining reveals that there is no discernible cluster-wise neuronal number difference in all the clusters of all treatment groups (**Figure 4.3 B**) as compared to their controls. Further, the total neuronal number in the whole fly brains of all different groups did not vary as compared to their control groups (**Figure 4.3 C**). The FI of the DAergic neurons was quantified further to examine if there was any difference/reduction in the quantity of TH protein synthesis (a secondary antibody that is fluorescently labeled tags the primary antibody anti-TH). Upon quantifying the fluorescence intensity of DA neurons of control wildtype flies at 4 hrs PQ treatment, results revealed that there is no alteration in the fluorescence intensity in all neuronal clusters between CTR UTD and CTR TD group (**Figure 4.3 D**), which confirms that there is no change in the levels of the rate-limiting enzyme of DA synthesis. In deletion mutant 8097/+ UTD *per se*, the FI of the fly brain(s) DAergic neurons belonging to PAL, PPL1,

PPL2, and PPM3 were decreased in few clusters observed as 27% (***p<0.001), 12% (*p<0.05), 23% (***p<0.001), and 26% (***p<0.001) and no difference is observed in PPM1/2 cluster respectively (**Figure 4.3 D**) as compared to CTR UTD. Simultaneously, in 8097/+ TD brain, FI of the DAergic neurons belonging to PAL, PPL1, PPL2, PPM1/2, and PPM3 clusters is further reduced by approximately 20% (***p<0.001), 23% (***p<0.001), 28% (***p<0.001), 20% (***p<0.001), and 25% (***p<0.001) respectively (**Figure 4.3 D**) as compared to CTR UTD. Also, 8097/+ TD compared to its another control CTR TD brain, observed a significant reduction in PAL, PPL1, PPL2, PPM1/2, and PPM3 clusters approximately by 16% (***p< 0.01), 18% (***p< 0.001), 20% (***p< 0.001), 20% (***p< 0.001),12% (**p< 0.01), 18% (***p< 0.001) (**Figure 4.3 D**). Further, FI of the fly brain(s) DAergic neurons of all clusters between 8097/+ TD and its immediate control 8097/+UTD showed variation in PPL1 and PPM1/2 with a statistical difference(*p<0.05), whereas other clusters such as, PAL, PPL2, PPM3 showed no significant variation.

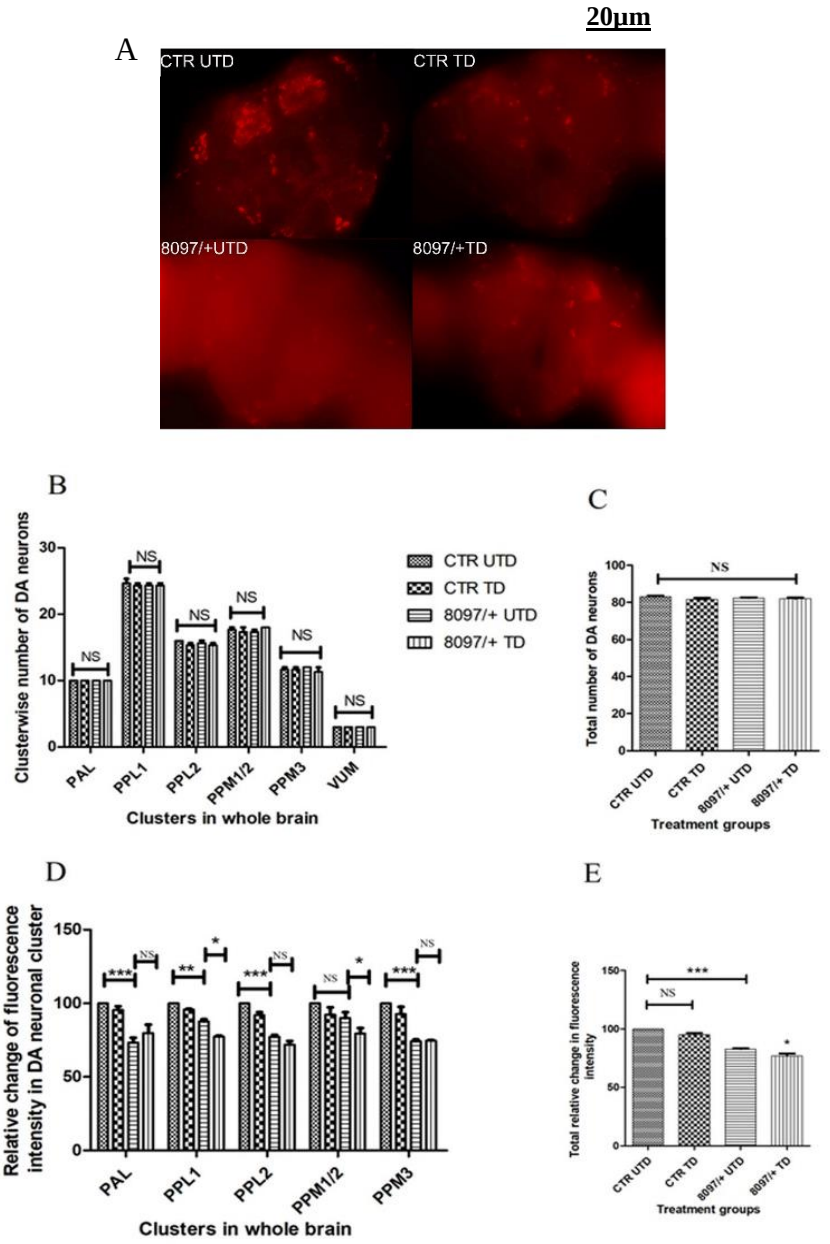


Figure 4.3: Characterization of DAergic neurodegeneration in the whole fly brain of Control and *Df* 8097 deletion line (**A**) through anti-TH antibody, immunostaining reveals that there is no loss in the number of DA neurons *per se* in the clusters (**B**), and *in toto* (**C**) However, quantification of fluorescence intensity of fluorescently labeled secondary antibody that targets the anti-TH primary antibody reveals that there is a significant change in the level of TH protein in the 8097/+ UTD and 8097/+ TD group clusters (**D**) and *in toto* (**E**) compared with the control group. The significance was drawn by analysing a minimum of three to five brains using One-way ANOVA followed by "Newman-Keuls Multiple Comparison Test'' and two-way ANOVA followed by "Bonferroni post-test". (*p<0.05; **p<0.01; ***p<0.001 compared with the control group; NS-Not significant). The scale bar of all the images in panel (**A**) is 20 µm. Represented images are "merged" Z-stacking images; however, the quantification of DAergic neuronal number and fluorescence intensity is performed in 3D Z-stack images. (CTR UTD- Control *per se;* CTR TD-Control treated with PQ; 8097 UTD/+- deletion mutant *per se*; 8097/+ TD - deletion mutant treated with PQ. (Here, CTR TD compared to control CTR UTD; 8097/+UTD compared to

control CTR UTD, for 8097/+TD, along with control CTR UTD, deletion mutant *per se* 8097/+ UTD, CTR TD serve as control).

Similarly, the group-wise merged FI of all the quantifiable DAergic neurons (five DA neuronal clusters) of the whole brain mount between CTR UTD and CTR TD exhibited no variations. Group-wise merged FI of all the quantifiable DAergic neurons (five DA neuronal clusters) of the 8097/+ UTD *per se* exhibited a reduction of 17% (*** $p<0.001$) as compared to CTR UTD, whereas, 8097/+ TD brain exhibited a significant reduction of 23% (***$p<0.001$) compared to control CTR UTD. Also, 8097/+ TD compared to CTR TD exhibits a reduction of 18% (***$p<0.001$). Group-wise merged FI between 8097/+ UTD and 8097 TD groups were also compared and a difference (* $p<0.01$) was observed (**Figure 4.3 E**).

Results revealed that deletion mutant fly *per se* has a significant reduction in the level of TH enzyme (Diminished level of TH synthesis), which was further reduced upon treatment with neurotoxicant PQ. Here it is observed that deletion mutant in healthy brain *per se* reveals diminished FI as is consistent with the results observed in PQ interaction deletion mutant brain. The diminished level of TH protein synthesis directly correlates with FI. These results also correlate with the findings of Navarro et al. (2014) and Ayajuddin et al. (2023) in a sporadic PD model, wherein, it showed a reduction in the FI of GFP reporter protein rather than actual neuronal cell death, which indicates that while TH protein synthesis level is decreased, DA neuronal structure (cell body) is not degenerated (no loss in the number of neurons) suggesting “neuronal dysfunction’’. The findings of the current study also corroborate with the results of the negative geotaxis assay in deletion mutant, wherein showed a decrement in the climbing mobility in healthy mutant *per se* as well as with PQ interaction.

4.2.2. HPLC- ECD data revealed there is a variation in DA or/and its metabolites in 8097 deletion mutant

To understand if there is any alteration in DA metabolism, quantification of brain-specific DA and its metabolites (DOPAC and HVA) was performed using the HPLC method. Standard DA and its metabolites were quantified as described in **Chapter 2** (**section 2.12**) to provide a precise retention time and area with which samples were compared to quantify catecholamines in the tissue samples. DA and its metabolites were quantified following the protocols of Ayajuddin et al. (2023;2021). Using the standard and sample chromatogram obtained from the HPLC-ECD unit as described in (**Figure 4.4**), the concentration of brain DA and its metabolites (DOPAC and HVA) was measured to understand DA metabolism.

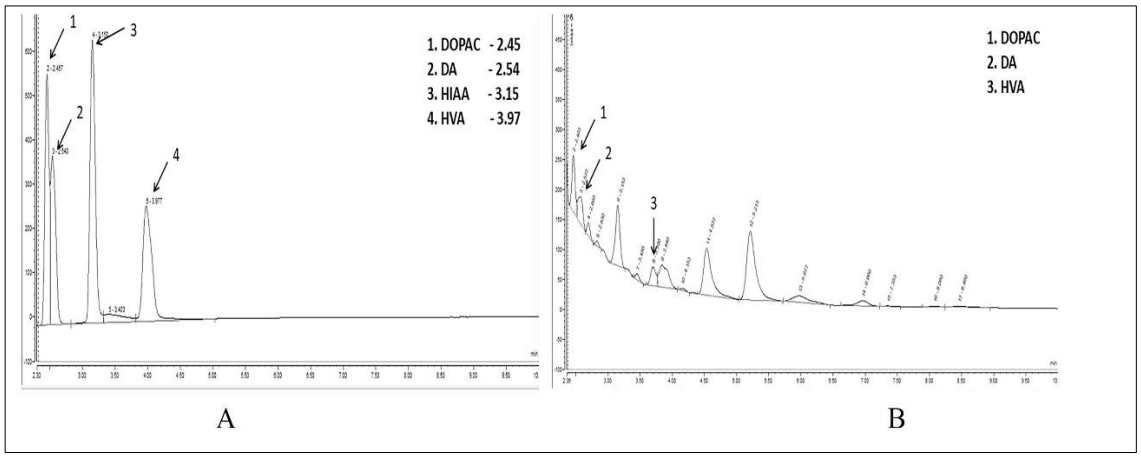


Figure 4.4: Characterization of retention time of standard DOPAC, DA, and HVA (**A**) and brain-specific DA and its metabolites levels (**B**) Chromatogram of the standard catecholamines gives a particular retention time compared with which the catecholamines in the fly brain sample is analyzed.

Figure 4.5. show images on the Quantification of Dopamine (DA) and its metabolites 3,4-Dihydroxyphenyl acetic acid (DOPAC) and Homovanillic acid (HVA) of the various experimental groups in the *Drosophila* brain. Results demonstrate that, in the control fly brain CTR UTD at 4 hrs PQ treatment, there is no alteration in the level of the DA

however its immediate metabolite *i.e.*, DOPAC level is slightly enhanced as described in (**Figure 4.5 B**). Another metabolite is HVA, the final product of DA metabolism which works through the MAO/COMT analogous pathway in the *Drosophila* brain wherein DA and DOPAC are degraded to HVA (Wichit et al., 2021; Yamamoto and Seto, 2014). The HVA levels were analyzed and no alteration in their levels was observed between CTR UTD & CTR TD.

Simultaneously, mutant *per se* 8097/+ UTD brain were also compared with the control CTR UTD brain. In 8097/+ UTD *per se* brain, no alteration in the level of the DA and DOPAC was observed when compared to CTR UTD brain. However, the HVA level was enhanced by 29% (***$p<0.001$) (**Figure 4.5 C**) when compared to the CTR UTD. Higher synthesis of DA downstream either DOPAC /HVA in the 8097/+ UTD brain may also suggests that these monoamines play a role in the onset and progression of disease such as PD as they are considered endogenous neurotoxins. Whereas in the 8097/+ TD brain, the DA level was reduced by 23% (***$p<0.001$) but no changes in DOPAC and HVA level (**Figure 4.5 A**) was observed when compared to controls (**Figure 4.5 B**).

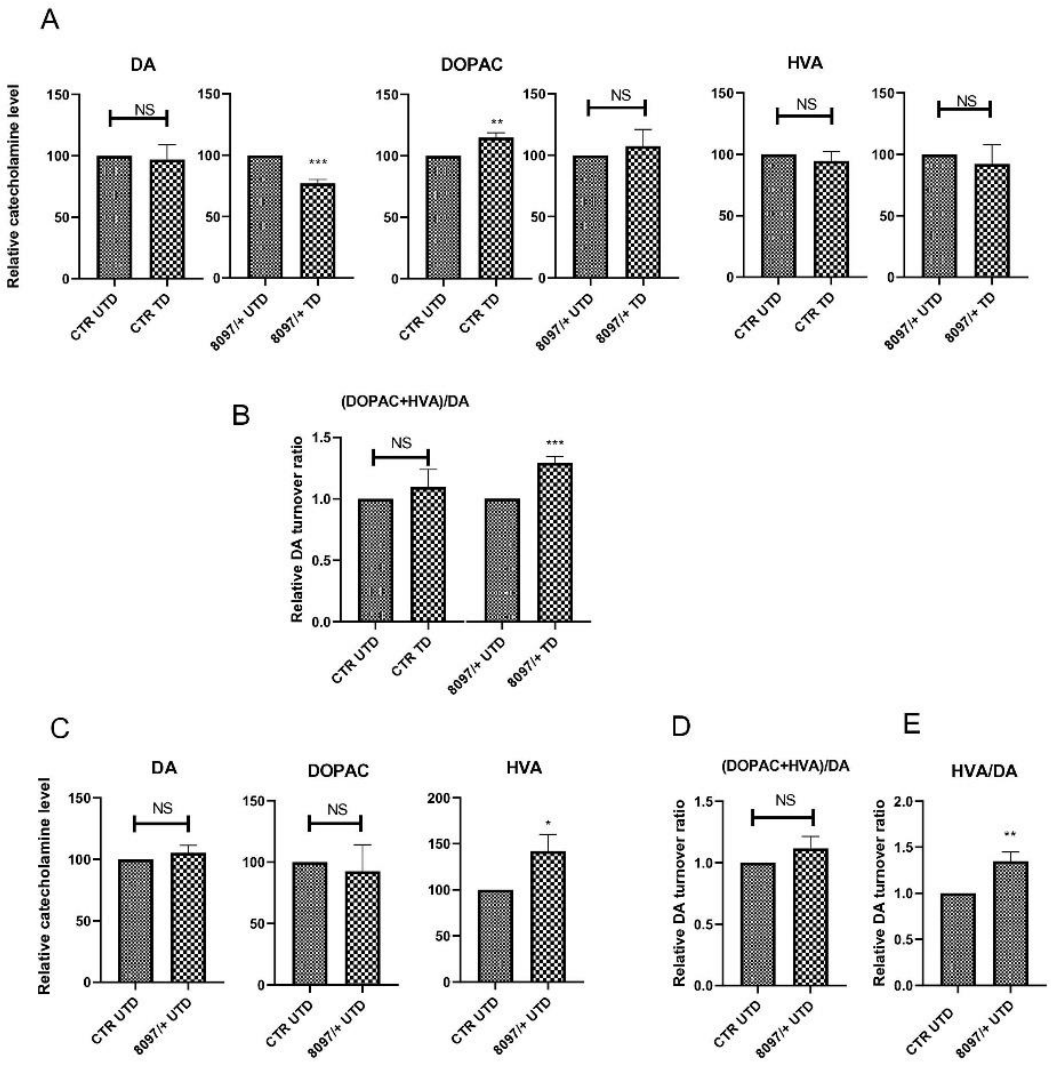


Figure 4.5: Quantification of Dopamine (DA) and its metabolites-3,4-Dihydroxyphenyl acetic acid (DOPAC) and Homovanillic acid (HVA) using High-Performance Liquid Chromatography (HPLC). The level of DA and HVA is not depleted in the CTR UTD but the DOPAC level was slightly enhanced upon neurotoxicant exposure at 4 hrs exposure window (**A**). No changes in DA turnover ratio were observed in the CTR TD compared to 8097/+ UTD (**B**) suggesting DA is not affected at 4 hrs exposure window. Also, 8097/+ UTD *perse* does not show any changes in DA and DOPAC (**C**). However, the HVA level was enhanced in 8097/+ UTD when compared CTR UTD (**C**). No changes in cumulative DA turnover ratio were observed in 8097/+ UTD *per se* when compared to CTR UTD (**D**). However, significant turnover in the HVA/DA ratio is observed due to enhanced HVA level, which may be due to a higher degradation of HVA to DA although the DA level remains unchanged (**E**). On the other hand, 8097/+ TD showed diminished DA level but no changes in DOPAC and HVA (**A**), however increase in DA turnover ratio was observed when compared to CTR UTD, CTR TD & 8097/+ UTD (**B**). (Statistical analysis was performed using an unpaired *t*-test (compared to the control) (*p<0.05; **p<0.01; ***p<0.001; NS: Not significant- compared to CTR & PQ treated group) (CTR UTD- Control *per se;* CTR TD- Control treated with PQ; 8097 UTD/+- deletion mutant *per se*; 8097 TD/+ - deletion mutant treated with PQ. (Here, CTR TD compared to control CTR UTD; 8097/+ UTD compared to control CTR UTD, for 8097/+ TD, along with control CTR UTD, deletion mutant *per se* 8097/+ UTD, CTR TD serve as control).

4.2.3. Differential regulation of DA oxidative turnover ratio between control and PD groups

The alteration in the levels of DA, DOPAC, and HVA pools in the PQ interaction condition was further investigated to understand DA catabolism and turnover. Dopamine turnover is determined as the ratio between dopamine metabolites and dopamine *per se*. This ratio is calculated using the formula [(DOPAC+HVA)/DA]. It was observed that there is no alteration in the DA turnover ratio of the wild-type control fly since no alteration in DA level was observed (**Figure 4.5 B**). Further, in 8097/+UTD *per se* brain, no alteration in DA level was seen, hence no cumulative DA turnover ratio was observed (**Figure 4.5 D**). However, the HVA/DA turnover ratio was also analyzed to see the degradation of the HVA level since an increment in the level of HVA in mutant *per se* brain was observed (**Figure 4.5 E**). A higher HVA/DA ratio approximately by 25% (**p<0.01) as compared to CTR UTD was observed which may suggest higher degradation of HVA to DA or increased dopamine metabolism. On the other hand, in the 8097/+TD brain, its cumulative DA turnover ratio was higher by 35%(***p<0.001) as compared to their respective control (**Figure 4.5 B**), suggesting depletion in DA level is higher in the PQ-treated condition.

4.3. Discussion

Fly models of PD also exhibit mobility defects, loss of DAergic neurons, and diminished brain DA levels in PD conditions (Ayajuddin et al., 2023; 2022; Chaudhuri et al., 2007; Feany and Bender, 2000). Therefore, employing fluorescence microscopy, firstly, DAergic neurons were quantified in the entire fly brain before characterizing DA "neuronal dysfunction". Furthermore, to see the extent of "neuronal dysfunction" if any, the FI of fluorescently labelled secondary antibody, that targets the primary anti-TH antibody was measured. The fluorescent FI is directly proportional to the TH protein

abundance and synthesis. This was performed to decipher the extent of DAergic neurodegeneration/dysfunction in *per se* and with PQ interaction condition.

In the present study, it was observed that there is no variation/loss in the number of DAergic neurons between the control and mutant brains (**Figure 4.3 B** & **C**). This observation is in line with earlier findings from other studies (Koza et al., 2023; Ayajuddin et al., 2022; Navarro et al., 2014; Menzies et al., 2005). The above studies are attributed in light of the "dying back" phenomenon which states that neurodegeneration starts from the axonal terminus (Wong et al., 2019). This was further approved through findings that showed degeneration of striatal terminals followed by its cell bodies in SNpc of MPTP-induced Parkinsonism monkeys and protection of these terminals prevented loss of DA neurons (Wu et al., 2003). The "dying back" of DAergic neurons is also the reason behind the non-complementation of time-tested L-DOPA supplementation therapy by the DAergic neuronal terminals where massive L-DOPA supplementation led to irregular uptake and activation of DA receptors due to axonal degeneration which led to dyskinesia and toxicity from the plasma L-DOPA (Nakmodde et al., 2023). Previously, our laboratory has also demonstrated no loss of DAergic neuronal number; however, a significant reduction in the level of TH synthesis with PQ interaction was observed in a sporadic fly model of PD (Ayajuddin et al., 2023). Similar, results were also observed in a mitochondrial complex-I inhibition rotenone-mediated fly model of PD from our laboratory (Ayajuddin et al., 2022). Feany and Bender (2000), initially demonstrated the *Drosophila* model of PD by expressing normal and mutant forms of *α-syn* and observed adult-onset loss of DAergic neurons. Loss-of-function mutation flies in PD-associated genes like *PARKIN*, and *PINK1,* observed only two to four neurons from specific DAergic neuronal clusters (PPM1/2 or PPL1) that were degenerated (Kim et al., 2012; Trinh et al., 2010; 2008; Whitworth et al., 2005). A genome-wide screen in 201 DGRP lines observed

nine top associated genes containing SNPs associated with the loss of DA neurons and further validation of *RNAi* knockdown, mutagenesis, and behavioural testing of these nine genes found neurodegeneration in the PPL1 and PPM1/2 clusters which was accompanied with a decline in locomotor function in selected genes (Davis et al., 2022; 2021). Loss of function mutation in *scarlet* flies showed degenerative loss of DAergic neurons in the PPL1 cluster, with locomotor defects, reduced lifespan, and further overexpression of the *scarlet* transgene rescued neuronal loss in the mutant flies (Cunningham et al., 2018).

Apart from the genetic models, several studies also demonstrated toxin-induced PD models such as PQ-based models which led to significant DAergic neuronal loss in PPM and PPL1 cluster upon exposure to 5 mM PQ at 12 and 48 hrs (Chaouhan et al., 2022; Maitra et al., 2021; 2019; Soares et al., 2017). Similar to α-synuclein toxicity, decreased *Aux* expression in a fly model also revealed a change in the number of neurons in the PPM1/2 cluster. Moreover, flies with decreased *Aux* expression were found to be susceptible to PQ and α-synuclein overexpression suggesting genetic and environmental interaction influencing the DAergic neurodegeneration in the late health stage phase of flies (Song et al., 2017). Therefore, there exists a disparity between previous results and the present state of research on the depletion of DAergic neurons in the *Drosophila* model. Previously, this issue has been thoroughly investigated in several fly models in both inherited and sporadic forms of PD, and it has been shown that there is no structural degeneration of DAergic neurons. Specifically, neurons exhibited a reduced production of GFP (GFP reporter) and TH proteins correlated with reduced functional integrity of neurons (Ayajuddin et al., 2022; Das, 2022; Navarro et al., 2014).

Here in my study, control flies treated with PQ at 4 hrs exposure alone did not cause any reduction in FI of different clusters (**Figure 4.3 D**) as well as in group-wise merged FI

(**Figure 4.3 E**). The results correlate with the observation of Koza et al. (2023) in *Oregon K* flies at this window time. Further, in deletion mutant *per se* significant reduction in FI in different clusters such as PAL, PPL1, PPL2, and PPM3 is observed as compared to control wild-type flies (**Figure 4.3 D**) whereas, more reduction in FI of different clusters is also observed in all clusters (**Figure 4.3 D**) in PQ-induced deletion mutant as compared to deletion mutant *per se* which may suggest that genes expressed in these DAergic neurons may have roles in determining susceptibility when exposed to neurotoxicant PQ. Simultaneously, the levels of FI of all clusters between the PQ interaction deletion mutant and deletion mutant *per se* (its immediate control) were also compared and it was observed that significant reduction in FI was observed in PPL1 and PPM1/2 clusters of both groups (**Figure 4.3 D**). This observation also correlates to several studies which have demonstrated in fly PD models that the extent of DAergic neurodegeneration also varies among different DA neuronal clusters which also proves that degeneration can also be cluster-specific (Chaouhan et al., 2022; Maitra et al., 2021; Wang et al., 2007; Chaudhuri et al., 2007; Coulom and Birman, 2004). Additionally, analysis was done by quantifying merged-wise FI of all the DAergic neurons in the fly brains of all the groups (**Figure 4.3 E**). The observations followed similar results as seen in the cluster-wise pattern of groups compared to their controls, whereas comparison between deletion mutant *per se* and PQ interaction deletion mutant shows a statistical significance of (*$p<0.05$) approximately. As ‘neuronal dysfunction’ could be the underlying cause of early and late-onset PD model, quantifying the TH signals would help understand early neurodegeneration in the sporadic model condition. Therefore, it is our observation that the current window time may give an opportunity to also observe and further study the extent of screening and validation in finding the genes behind the neurodegeneration/protection through available fly genetic tools.

Reduction in brain dopamine levels is a key feature of PD in both human and animal models of PD (Ayajuddin et al., 2023; Phom et al., 2014; Goldstein et al., 2011). Similarly, mice exposed to MPTP showed nigral cell damage which followed a substantial decrease in striatal dopamine (Di Monte et al., 2000; Chan et al., 1997). In fly models, feeding 10 mM or 20 mM PQ to adult young flies belonging to CS and white eye strains {*y W1118, Df(1)w,y*}for 24 hrs showed depletion in DA level in the adult brain (Inamdar et al., 2012; Chaudhuri et al., 2007).

Therefore, to further understand the implication of diminished TH synthesis in wild-type and deletion mutant brain in my study, the DA levels were quantified (**Figure 4.5 A**). The result demonstrates that wildtype-type flies treated with PQ at 4 hrs exposure alone did not cause any alteration in DA levels and its metabolites (DOPAC and HVA), and no variation in TH protein levels which correlates with the study of Koza et al. (2023), which explains the absence of variation may lie with the potential existence of cell type-specific genetic differences in dopamine DA and its metabolites, suggesting wild-type fly is not vulnerable to stressors at this window time.

Hence, taking this into account I aimed to screen the susceptibility levels of loss-of-function mutation at this window time in both conditions. Here, in our observation, it is revealed that deletion mutant *per se*, reveals no alteration in the levels of DA as compared to control wild-type flies (**Figure 4.5 C**). Although TH protein levels are diminished, DA levels remain relatively unchanged. However, when metabolite levels were analyzed, the DOPAC level remained unaltered but an increment in the HVA level was observed which suggests high degradation/natural oxidative stress generation of HVA in the dopamine metabolism pathway suggesting upregulated DA turnover. The above results also suggests that HVA may possibly be synthesized from DA through an alternate route *i.e.*, DA>3-MT>HVA (**Figure 4.6**), and may not be possibly through the DA>DOPAC>HVA

pathway since the DOPAC level remains unaltered in this condition. Studies have reported that CSF's HVA levels were also increased in patients with mild PD symptoms. This increase in DA metabolites was found to directly correlate with motor impairment. Furthermore, the upregulation of HVA is also considered to be a possible bio-marker for the depletion of DAergic neurons in mild PD patients. Hence, investigation of dopamine metabolism may also provide valuable understanding of the biochemical changes behind the DAergic system in this direction (Stefani et al., 2017).

Ossowska et al. (2006) observed acute dose of PQ did not alter levels of DA and DOPAC. However, it transiently enhances dopamine metabolism as shown by increase in HVA levels and HVA/DA ratio within 12 hrs after PQ administration. These findings indicate that the rate of dopamine turnover was elevated immediately after the injection of paraquat but returned to normal condition within 24 hrs. Wichit et al. (2021) performed clinical studies on PD patients and measured HVA levels in urine and DA levels in plasma wherein, they observed that DA had a negative relationship with disease duration whereas the HVA/DA turnover ratio was significantly higher when compared to control groups. Similarly, they have hypothesized that compensatory mechanism may have occurred in which neurons that survive damage may prevent dopamine depletion in the striatum and could be able to restore neurotransmitter tissue levels (McCormack et al., 2002).

Furthermore, to understand the gene-environment interaction of the deletion mutant brain with PQ interaction, the DA levels were compared to their respective controls. Decreased DA levels and enhanced DA turnover ratio was observed, however, DOPAC and HVA levels remained unaltered, suggesting that the deletion mutant may be susceptible to PQ and associated brain neurotransmitter imbalance may be attributed to certain genes deleted encompassing the regions of this chromosomal deletion mutant.

Caudle et al. (2007) highlighted important aspects of the DA system's response to neurodegeneration in the *VMAT2 LO* mouse model of PD. The study found that while dopamine levels declined, DOPAC and HVA levels remained unchanged, and the dopamine turnover ratio was elevated. These findings may indicate that the system may counteract dopamine loss by increasing its production and metabolism. However, a compensatory increase in activity at the remaining nerve terminals may initially help to stabilize the levels of neurotransmitters but may heighten its activity causing extra strain on the remaining terminals and leading to depletion of DA and its metabolite (Caudle et al., 2007; Lee et al., 2000). Further, exogenous insults such as neurotoxicant exposure impair the compensatory mechanism eventually leading to the DA metabolism pathway, enhancing degradation, depletion of DA level, enhanced DA turnover, and ultimately neuronal dysfunction/degeneration.

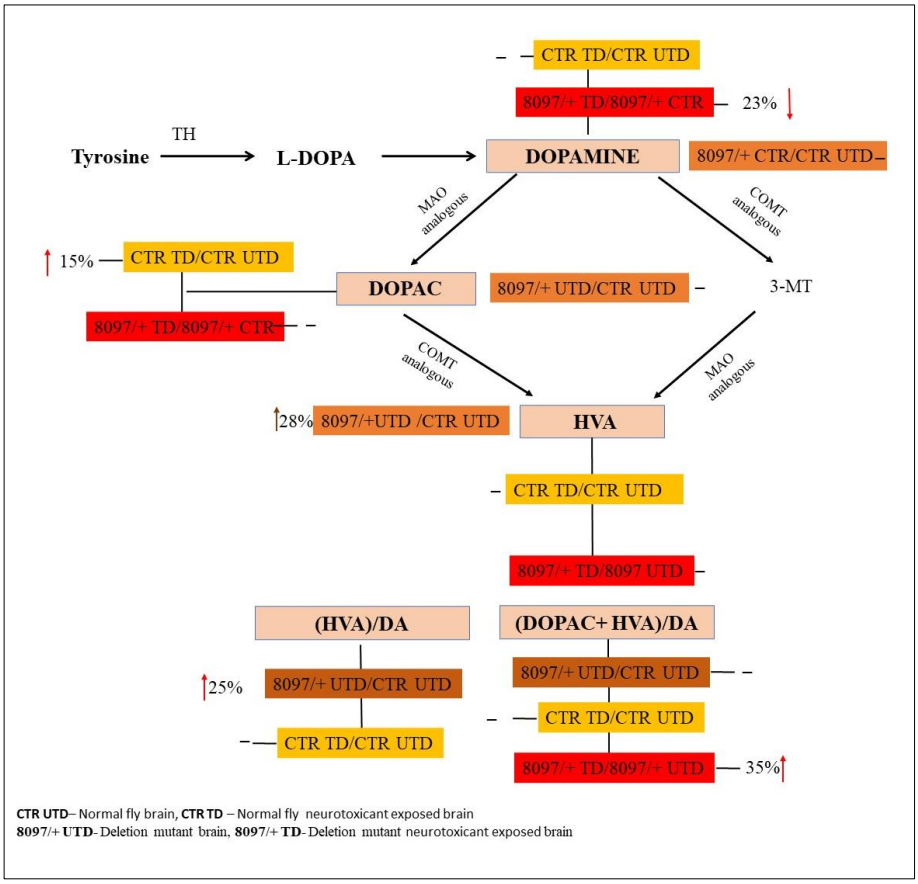


Figure 4.6: Schematic representation of DA metabolism in deletion mutant brain *per se* and PQ-interaction deletion mutant brain. In deletion mutant *per se* brain, DA levels remain unaltered whereas HVA level is increased. The relatively higher HVA level shows further upregulation of DA turnover ratio i.e., HVA/DA ratio which may suggest, higher DA oxidation and natural OS generation of DA to HVA in the untreated condition whereas, with PQ interaction, higher depletion of DA level and upregulated cumulative DA turnover ratio is observed which suggests the entirety of the insight from the current study that PQ interaction not only contributes to DA depletion but also enhances DA oxidative breakdown to the downstream catabolites.

These results corroborate with results from our lab studies of the *Drosophila* sporadic PD model wherein using neurotoxicant PQ and rotenone for a longer duration causes PD-associated motor impairments such as impaired climbing mobility, depleted brain DA and TH protein levels (Ayajuddin et al., 2022; Das, 2022; Phom et al., 2014), which further gives more insights into the *Drosophila* deletion mutant PD model that is observed to be more vulnerable *per se* and its effect enhanced under neurotoxicant stress.

4.4. Conclusion

Insights from the current study demonstrate that the *Df* 8097 deletion mutant *per se* in untreated condition exhibits diminished TH protein levels and unaltered brain DA levels. However, it exhibits altered DA metabolism as demonstrated through upregulated DA turnover, through HVA/DA turnover ratio suggesting degradation may possibly be *via* the HVA>3-MT>DA pathway (**Figure 4.6**) as the DOPAC levels remained unchanged. Further, upon subsequent interaction with neurotoxicant PQ, it showed depleted levels of DA suggesting gene-environment interaction which further demonstrates enhanced DA turnover ratio suggesting acute/further DAergic degeneration in the *Df* 8097 deletion mutant, which may suggest that *Df* 8097 mutant is vulnerable to the stressor and may attribute to certain genes encompassing the deleted region. Therefore, by looking into the chromosomal genomic regions of this deficiency mutant, it may be possible to logic out the involvement of genes in the pathway (such as *JNK*, and mitochondrial dynamics pathways) involved in DAergic neuronal physiology and DA metabolic pathway. However, using the power of fly genetics with the help of further studies their involvement needs to be confirmed experimentally.

(Contributions: 1. Animal treatments and climbing assay: Abuno Thepa; Processing of brain tissues: Rahul Chaurasia and Lemzila Rudithongru; 2. Scanning and analysis: Rahul Chaurasia and Lemzila Rudithongru); 3. HPLC experiment and analysis: Abhik Das and Mohammad Ayajuddin)

SUMMARY

Genetic mutations play a significant role in the development of various neurodegenerative diseases (NDDs) by disrupting molecular and cellular networks necessary for brain development altering gene expression, signal transduction, synaptic function protein homeostasis, and neuronal migration. These changes impair brain connectivity, synaptic plasticity, and cortical organization, ultimately contributing to the phenotypes seen in various NDDs such as Parkinson's disease (PD). Understanding NDDs and neuronal maintenance through *Drosophila* genetic screens has been invaluable throughout the history of fly genetics. PD, being a late-onset disease with incurable and ensuing effects, there is much more to understand about its aetiology, progression, and the underlying genes responsible for the disease. Dopaminergic (DAergic) neuron's nigrostriatal pathways are more susceptible to late-onset dysfunction due to age-dependent changes in DA metabolism, its uptake, and synthesis (Surmeier, 2018; Collier et al., 2017). Therefore, by adhering to the art and design of forward genetic screens, one can identify responsible genetic players and also understand the mechanisms underlying their susceptibility. Therefore, in the context of DAergic neurons, deletion screens may help to identify interacting cytological regions and/or genes whose loss/absence may lead to neuronal degeneration or susceptibility, thereby highlighting their protective roles and allowing one to screen large genomic regions efficiently and pinpointing specific genes that contribute to DAergic neuron integrity. These screens may uncover both unknown and novel genes involved in neuroprotection/neurodegeneration providing a comprehensive understanding of the genetic landscape behind the DAergic neuronal integrity.

Simultaneously, various research studies have demonstrated a close relationship between exposure to environmental toxins such as paraquat (PQ) and the onset of PD, considering it as an additive factor of sporadic PD, which includes more than 90% of PD cases.

Understanding the familial forms of PD linked to autosomal dominant and recessive mutations may also help in knowing the importance of the genetic basis behind sporadic PD.

Several deletion screens have been performed to understand various phenotypes linked to *Drosophila* cardiomyopathy, embryonic phenotypes, orphan receptor ligands, dorsal closure, mitochondrial integrity, and life span; however, no large systematic deletion screens to directly probe neurodegeneration in deletion mutants *per se* and idiopathic PD condition was performed providing an opportunity to screen a large number of deficiencies in this direction. Therefore, taking advantage of the chromosomal deficiency lines, primary screening for mobility defects through Negative Geotaxis Assay (NGA) was performed under two conditions *viz.*, a) mutant *per se* and b) with PQ interaction.

Therefore, keeping the above views in mind, In Chapter 3, primary screens were performed in all 66 deletion lines using NGA to decipher mobility phenotypes, and based on the performance of the fly's climbing mobility, its phenotype was characterized and established in heterozygote and homozygote condition *per se*. Out of 66 lines, I scored 7 lines that exhibited mobility defects. Further, I wanted to see the susceptibility levels of loss-of-function of these mutants in interaction with PQ. Therefore, flies were exposed to 4 hrs of PQ exposure following our previously established protocol in our laboratory wherein wildtype flies at this window time did not exhibit any mobility defects. Results showed that mutant lines which exhibited mobility defect *per se*, its phenotype was aggravated upon exposure to neurotoxicant PQ. Hence, among the 7 lines screened, *Df* 8097 deletion mutant was selected as it exhibited acute phenotype in its climbing mobility and moreover, the chromosomal deletion region of this mutant encompasses some of the genes that are involved in motor protein synthesis in regulating protein aggregation linked

to axonal transport, neuronal transport, neurogenesis, axonal transport of mitochondrion, cell fate determination, evoked neurotransmitter secretion linked to locomotor and lifespan determination.

Further, in Chapter 4, I tried to characterize the *Df* 8097 deletion mutant to see the nature of neurodegeneration and understand whether the observed mobility defect is either due to degeneration of DAergic neurons and/or altered brain dopamine metabolism in mutant *per se* and under neurotoxicant interaction (gene-environment interaction), if any; to understand the health of deletion mutant brain. With this objective in mind, further characterization of brain-specific phenotypes relating to PD such as neuronal dysfunction, brain DA, DOPAC, HVA levels, and dopamine turnover rate was investigated *per se* and with PQ interaction brain. The result demonstrates that wild-type flies treated with PQ at 4 hrs exposure alone did not cause any alteration in TH protein levels, DA levels, and its metabolites (DOPAC and HVA) suggesting wild-type fly is not vulnerable to stressors at this window time. However, it is revealed that deletion mutant *per se,* exhibit diminished TH protein levels, but no variation in DA level. However, when metabolite levels were analyzed, an increment in HVA level was observed which suggests high degradation and/or natural oxidative stress generation of HVA in the dopamine metabolism pathway suggesting upregulated DA turnover. These results suggest the possibility that HVA may be more likely synthesized from DA through an alternate route *i.e.*, DA>3-MT>HVA, and may not be through the DA>DOPAC>HVA pathway. Furthermore, the upregulation of HVA is also considered to be a possible bio-marker for the depletion of DAergic neurons in mild PD patients. Furthermore, with PQ interaction, it was observed that TH protein levels were diminished with decreased DA levels and enhanced DA turnover ratio, suggesting that the deletion mutant may be more susceptible to PQ and associated brain

neurotransmitter imbalance may be attributed to certain genes deleted encompassing the regions of this chromosomal deletion mutant. A compensatory increase in activity at the remaining nerve terminals may initially help to stabilize the levels of neurotransmitters but may heighten its activity causing extra strain on the remaining terminals and leading to depletion of DA and its metabolite. However, further, exogenous insults such as neurotoxicant exposure impair the compensatory mechanism eventually leading to the DA metabolism pathway, enhancing degradation, depletion of DA level, enhanced DA turnover, and ultimately neuronal dysfunction/degeneration.

The above results of my study also corroborate with results from our lab studies of the *Drosophila* sporadic PD model wherein using neurotoxicant PQ and rotenone for a longer duration causes PD-associated motor impairments such as impairment in the climbing ability, depleted brain DA and TH protein levels (Ayajuddin et al., 2022; Das, 2022; Phom et al., 2014), which further gives more insights into the *Drosophila* deletion mutant PD model that is observed to be more vulnerable *per se* and its effect enhanced under neurotoxicant stress.

Therefore, using chromosomal deficiency lines, one can look into the chromosomal genomic regions of these mutants, and it may be possible to further provide clues into the involvement of molecular players/genes in certain pathways involved in DAergic neuronal physiology and DA metabolic pathways through the power of fly genetics.

This approach will not only enhance the understanding of PD etiology and progression but will also provide a powerful platform for discovering therapeutic targets aimed at preserving DAergic neuron function.

SUPPLEMENTARY INFORMATION

ANNEXURE I

Chapter 4: Characterization of brain dopaminergic neurodegeneration and dopamine metabolism in *Df* 8097 deletion mutant

I. Figure 4.3 B: (Cluster wise DA neuronal number in whole fly brain of control and experimental groups)

Neuronal cluster		CTR UTD		CTR TD			8097 UTD			8097 TD		
PAL	10	10	10	10	10	10	10	10	10	10	10	10
PPL1	24	26	24	24	25	24	24	24	25	24	25	24
PPL2	16	16	16	16	15	15	16	16	15	16	15	15
PPM1/2	18	18	17	18	16	18	18	17	17	18	18	18
PPM3	12	11	12	12	12	11	12	12	12	10	12	12
VUM	3	3	3	3	3	3	3	3	3	3	3	3

II. Figure 4.3 C: (Total DA neuronal number in whole fly brain of control and experimental groups)

CTR UTD	CTR TD	8097 UTD	8097 TD
83	83	83	81
84	81	82	83
82	81	82	82

III. Figure 4.3 D: (Cluster wise FI of DA neurons in whole fly brain of control and experimental groups)

Neuronal cluster	CTR UTD			CTR TD			8097 UTD		8097 TD			
PAL	100	100	100	99.75321	91.0088	95.50967	78.81524	67.98655	73.56023	69.95432	89.81752	79.59365
PPL1	100	100	100	96.84544	94.47089	95.73801	85.11272	90.40211	87.57957	78.51036	76.29063	77.47513
PPL2	100	100	100	88.81796	95.34591	91.86154	75.32772	79.44242	77.24615	67.84972	76.35899	71.81708
PPM1/2	100	100	100	100.7757	84.16811	92.48819	96.88876	83.12025	89.98837	73.23632	85.80479	79.53528
PPM3	100	100	100	101.4133	84.16811	92.48819	76.29446	72.29548	74.22482	75.45634	73.84428	74.62203

IV. Figure 4.3 E: (Total FI of DA neurons in whole fly brain of control and experimental groups)

CTR UTD	CTR TD	8097 UTD	8097 TD
100	97.32746	84.25152	73.82078
100	92.6606	81.1058	80.1459
100	95.06577	82.72701	76.88612

V. Figure 4.5 A: (DA, DOPAC and HVA amount in fly brain of control and experimental groups)

	CTR UTD			CTR TD			8097 UTD			8097 TD		
DOPAC	0.69	2.891	1.250	0.82	3.319	1.390	0.72	1.955	1.310	0.68	2.374	1.400
DA	1.024	0.904	1.100	1.137	0.811	0.990	1.081	1.006	1.090	0.795	0.796	0.860
HVA	0.544	0.102245	0.230	0.485	0.105654	0.210	0.805	0.16	0.280	0.798	0.118391	0.290

conversion to relative scale

	CTR UTD			CTR TD			8097 UTD			8097 TD		
DOPAC	100	100	100	118.8185	114.7991	111.2	100	100	100	94.17936	121.4198	106.8702
DA	100	100	100	111.1007	89.72914	90	100	100	100	94.17936	121.4198	106.8702
HVA	100	100	100	89.07446	103.3337	91.30435	100	100	100	94.17936	121.4198	106.8702

VI. Figure 4.5 B, D, E: (DA turnover ratio in fly brain of control and experimental groups)

		CTR UTD	CTR TD	8097 UTD	8097 TD
(DOPAC+HVA)/DA		2	1.871212	2	2.625607
		2	2.431014	2	2.46873
		2	2.250048	2	2.667226
	scale in 1	1	0.935606	1	1.312804
		1	1.215507	1	1.234365
		1	1.125024	1	1.333613

	CTR UTD	8097 UTD
HVA/DA	1	1.401778
	1	1.43404
	1	1.22856

ANNEXURE II

Table shows 83 deleted genes spanning the *Df* 8097-deletion mutant on the 3L chromosome arm (70A3-70C10 cytological position) referred from FlyBase (https://flybase.org/).

Abp1	*asRNA:CR45886*	*asRNA:CR46266*	*bru3*	*caps*	*CG10089*	*CG10116*	*CG10140*	*CG10154*	*CG10171*
CG10222	*CG10710*	*CG10713*	*CG10725*	*CG10738*	*CG13737*	*CG13738*	*CG14105*	*CG14106*	*CG14107*
CG14109	*CG14110*	*CG14111*	*CG14113*	*CG17362*	*CG8750*	*CG8757*	*CG8833*	*CG9040*	*cmb*
CG17364	*CG17687*	*CG32119*	*CG32121*	*CG32137*	*CG33263*	*CG42481*	*CG43147*	*CG43184*	*CG8745*
DCTN1-p150	*dysc*	*flr*	*Hml*	*Hsc70-1*	*ImpL1*	*JMJD7*	*Liprin-β*	*lncRNA:CR43146*	*lncRNA:CR43911*
lncRNA:CR43912	*lncRNA:CR43913*	*lncRNA:CR44555*	*lncRNA:CR44557*	*lncRNA:CR44558*	*lncRNA:CR44559*	*lncRNA:CR45120*	*lncRNA:CR45178*	*lncRNA:CR45253*	*lncRNA:CR45254*
lncRNA:CR45255	*lncRNA:CR45752*	*lncRNA:CR45753*	*lncRNA:CR45825*	*Meics*	*mir-289*	*Nplp2*	*Nxf3*	*Poc1*	*Rgl*
sens	*Sfp70A4*	*SNCF*	*SP*	*Spt20*	*ssp2*	*stv*	*Tgi*	*tRNA:Asp-GTC-1-10*	*tRNA:Val-AAC-2-3*
tRNA:Val-AAC-2-4	*Tsp68C*	*Vps36*							

Supplementary information

Table shows deleted genes with biological functions and Human orthologs/homologs spanning the *Df* 8097 deletion mutant of 3L arm (70A3-70C10 cytological position) (FlyBase-https://flybase.org/).

Gene Name	**Known Biological Function**	**Human Orthologs/Homologs**	**References**
DCTN1-p150 (Dynactin)	Motor protein involved in axonal transport, neuronal transport, neurogenesis, axon extension, axonal transport of mitochondrion, cell fate determination, evoked neurotransmitter secretion, Dynactin mutant flies have decreased mortality and exhibit progressive loss of motor function.	6	Tsuboi et al., 2021; Charng et al., 2014; Lorenzo et al., 2010
Caps (Capricious)	Axon guidance, motor neuron axon guidance photoreceptor cell axon guidance, synapse assembly	24	Abrell and Jäckle, 2001
Stv (Starvin)	Regulates life-span, age-dependent locomotor behaviours, p38Kb regulates age-dependent protein aggregation through interaction with *stv*, role in regulating age-dependent protein aggregation, chaperone pathway, co-chaperone for BAG3 and the Heat shock cognate 71 kDa (HSC70)/HSPA8 ATPase in the autophagic clearance of proteins.	3	Ryan et al., 2021; Brooks et al., 2020
Hsc70-1 (Heat shock cognate-1)	Chaperone cofactor-dependent proper protein folding, spermatocyte growth progression through the insulin/TOR pathway in *Drosophila*	20	Azuma et al., 2021
BicDR (Bicaudal D related)	Bicaudal D adaptor proteins link dynein motors to specific cargos binding, Golgi to secretory granule transport, neuron projection development, and vesicle transport along microtubule	2	Reck-Peterson et al., 2018; Hoogenraad and Akhmanova, 2016
Vps36 (Vacuolar protein sorting 36)	Component of the endosomal sorting complex required for transport ESCRT, involved in the formation of neuroblasts, Vps36 regulates Smo trafficking in the Hedgehog signalling pathway in *Drosophila*, ensuring proper cell signalling by controlling the degradation and localization of Smo.	3	Anding et al., 2018; Woodfield et al., 2013
Liprin-β	Protein homodimerization activity, axon target recognition, regulation of synapse assembly at neuromuscular junction.	7	Astigarraga et al., 2010
*SNCF (*SoxNeuro Co-Factor)	Neuroblasts development, central nervous system, lifespan determination	-	Bonneaud et al., 2013; Hall et al., 2019
*Hml (*Hemolectin)	Immune response defective, lifespan determination	4	Hall et al., 2019

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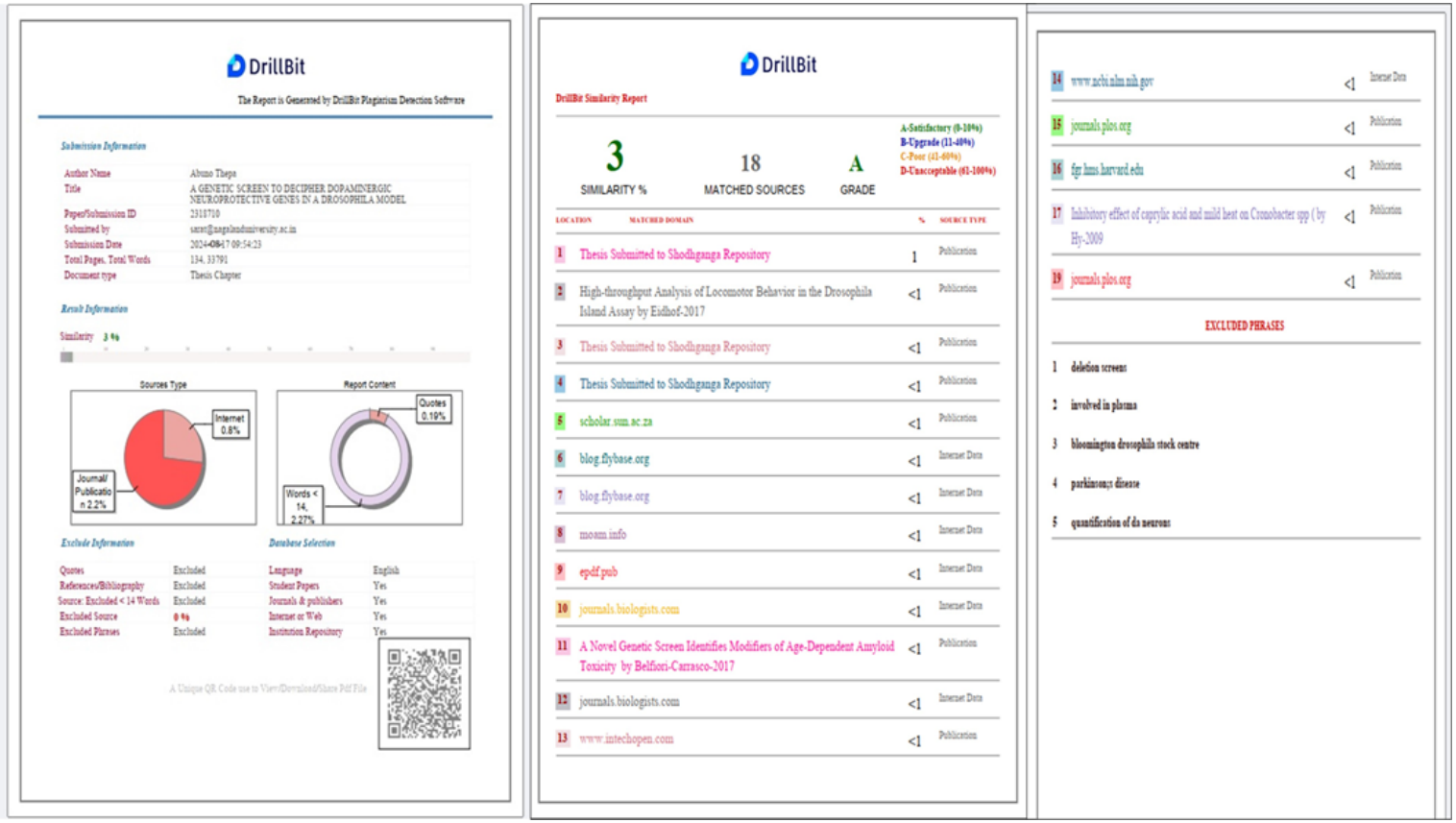

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EXCLUDED PHRASES

1 deletion screen
2 involved in plasma
3 bloomington drosophila stock centre
4 parkinson;s disease
5 quantification of da neurons

CONFERENCES/SEMINARS/WORKSHOPS

International

- **Oral presentation** at Abhayapuri College in association with Department of Zoology, Guwahati University and Aaranyak, Assam**:** *In* 3-day virtual International Conference on Impacts & Consequences of Environmental Degradation on Animal Health and Human Wellbeing on 2nd-4th September, 2021.
  Topic*: Drosophila* Model Based Screening Strategies to Decipher Genetic Basis of Dopaminergic Neurodegeneration: Relevance to Human Health.

- **Poster presentation** at IIT Guwahati, Assam, India: *In* 4th International Conference on Nutraceuticals and Chronic Diseases on 23rd-25th September 2019
  Topic: Fly Genetic Deletion lines to Study Dopaminergic Neurodegeneration: Smart tools to screen Neuroprotective Nutraceuticals.

National

- **Oral presentation** at Department of Zoology, Nagaland University, Lumami, Nagaland, India. *In* National Conference on Contemporary Excitement in New Biology on 30th-31st October 2018
  Topic: Search for Dopaminergic Neuroprotective genes: Clues from Deficiency Screen in fly Model.
- **Oral presentation** at Nagaland University, Lumami, Nagaland, India. In National Seminar on Climate Change and Sustainable Development with Special Focus on North East India on 17th-18th May 2017
  Topic: Looking into the Genome and Identifying the Putative Targets for Neuroprotection using *Drosophila* Model.

Workshop

- Attended Online Workshop on "DNA Bar-Coding: A Challenge to Linnaeus Classification" organized by CytoGene Research & Development, Lucknow on 22nd-23rd May 2021.
- Attended International Course entitled "Anti-Inflammatory Life Style for Prevention and Treatment of Cancer and Neurodegeneration: Facts and Fiction"

organized by MHRD-GIAN (Global Initiative of Academic Networks), Nagaland University held on 1st-5th October 2019.

- Attended National Seminar on "Climate Change and Sustainable Development: With Special Focus on Northeast India" organized by UGC sponsored at Nagaland University, Lumami held on 17th-18th May 2017.

Fellowship Availed

- Awarded and availed **DBT- Junior research fellowship** (12th December 2016-31st October 2018) from **Department of Biotechnology, Government of India, New Delhi.**
- Awarded and availed **Non-NET fellowship** (1st November 2018- 23rd August 2021) from University Grant Commission (UGC) through **Nagaland University**.

- Awarded and availed **Nagaland state Research Fellowship** (29th October 2020-30th October 2023) from **Government of Nagaland, India.**

Conferences/seminars/workshops

Three Day Virtual International Conference on

"Impacts & Consequences of Environmental Degradation on Animal Health and Human Wellbeing"

Aaranyak

Certificate

This is to certify that **Abuno Thepa** of Nagaland University (Central), Lumami, Nagaland participated and presented her research paper titled **"Drosophila Model Based Screening Strategies To Decipher Genetic Basis Of Dopaminergic Neurodegeneration: Relevance to Human Health "** in the three day virtual International Conference on **"Impacts & Consequences of Environmental Degradation on Animal Health and Human Wellbeing"** held on 2nd to 4th September, 2021, organized by Abhayapuri College in association with Department of Zoology, Gauhati University and Aaranyak, Assam.

We wish him/her all success in life.

Prof. Jogen Chandra Kalita (Chief Adviser) Head, Dept. of Zoology, GU

Dr Sadananda Nath (President) Principal, Abhayapuri College

Dr Pori Devi (Convener) Asst. Prof., Abhayapuri College

ONLINE WORKSHOP

DNA Bar-Coding:
A Challenge to Linnaeus Classification

CytoGene
Inventing Thoughts

Certificate

This is to certify that Prof./Dr./Mrs./Miss./Mr.

Abuno Thepa

from Nagaland University, Lumami (Nagaland)

has actively participated in the Online Workshop on

"DNA Bar-Coding: A Challenge to Linnaeus Classification"

on 22nd & 23rd of May, 2021, Organized by CytoGene Research & Development, Lucknow.

Dr. Umashankar Director R&D CytoGene Research & Development, Lucknow

Er. Sujeet Kr. Singh Director CytoGene Research & Development, Lucknow

Celebrating 25 Glorious Years of IIT GUWAHATI

INTERNATIONAL CONFERENCE ON "NUTRACEUTICALS AND CHRONIC DISEASES" INCD 2019

Certificate

OF PARTICIPATION

This is to certify that Abuno Thepa has participated at the Fourth International Conference on "Nutraceuticals and Chronic Diseases" held at Indian Institute of Technology Guwahati, Assam, India from 23rd -25th September, 2019.

Prof. Bharat B. Aggarwal, Chief Patron, INCD 2019 | Prof. Oommen V. Oommen, President, Society for Nutraceuticals and Chronic Diseases | Prof. Ajaikumar B. Kunnumakkara, Organizing Secretary, INCD 2019 | Prof. Latha Rangan, Chairperson, INCD 2019

Celebrating 25 Glorious Years of IIT GUWAHATI

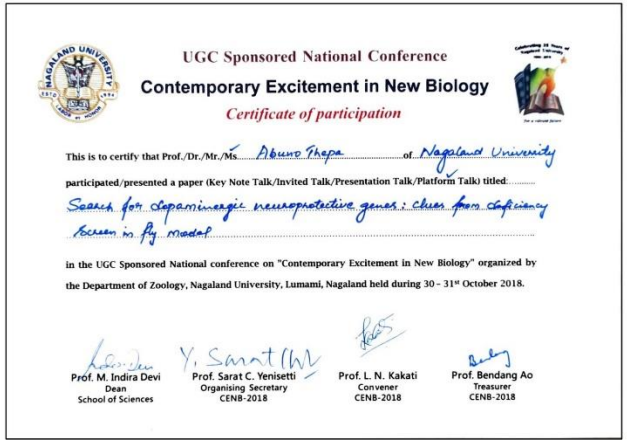
INTERNATIONAL CONFERENCE ON "NUTRACEUTICALS AND CHRONIC DISEASES" INCD 2019

Certificate

OF APPRECIATION

This is to certify that Abuno Thepa has presented a poster entitled Fly genetic deletion lines to study dopaminergic neurodegeneration : Smart tools to Decipher at the Fourth International Conference on Nutraceuticals and Chronic Diseases" held at Indian Institute of Technology Guwahati, Assam, India from 23rd -25th September, 2019.

Prof. Bharat B. Aggarwal, Chief Patron, INCD 2019 | Prof. Oommen V. Oommen, President, Society for Nutraceuticals and Chronic Diseases | Prof. Ajaikumar B. Kunnumakkara, Organizing Secretary, INCD 2019 | Prof. Latha Rangan, Chairperson, INCD 2019



UGC Sponsored National Conference

Contemporary Excitement in New Biology

Certificate of participation

This is to certify that Prof./Dr./Mr./Ms. Abuno Thepa of Nagaland University participated/presented a paper (Key Note Talk/Invited Talk/Presentation Talk/Platform Talk) titled:

Search for dopaminergic neuroprotective genes : clues from deficiency screen in fly model

in the UGC Sponsored National conference on "Contemporary Excitement in New Biology" organized by the Department of Zoology, Nagaland University, Lumami, Nagaland held during 30 - 31st October 2018.

Prof. M. Indira Devi, Dean, School of Sciences | Prof. Sarat C. Yenisetti, Organising Secretary, CENB-2018 | Prof. L. N. Kakati, Convener, CENB-2018 | Prof. Bendang Ao, Treasurer, CENB-2018

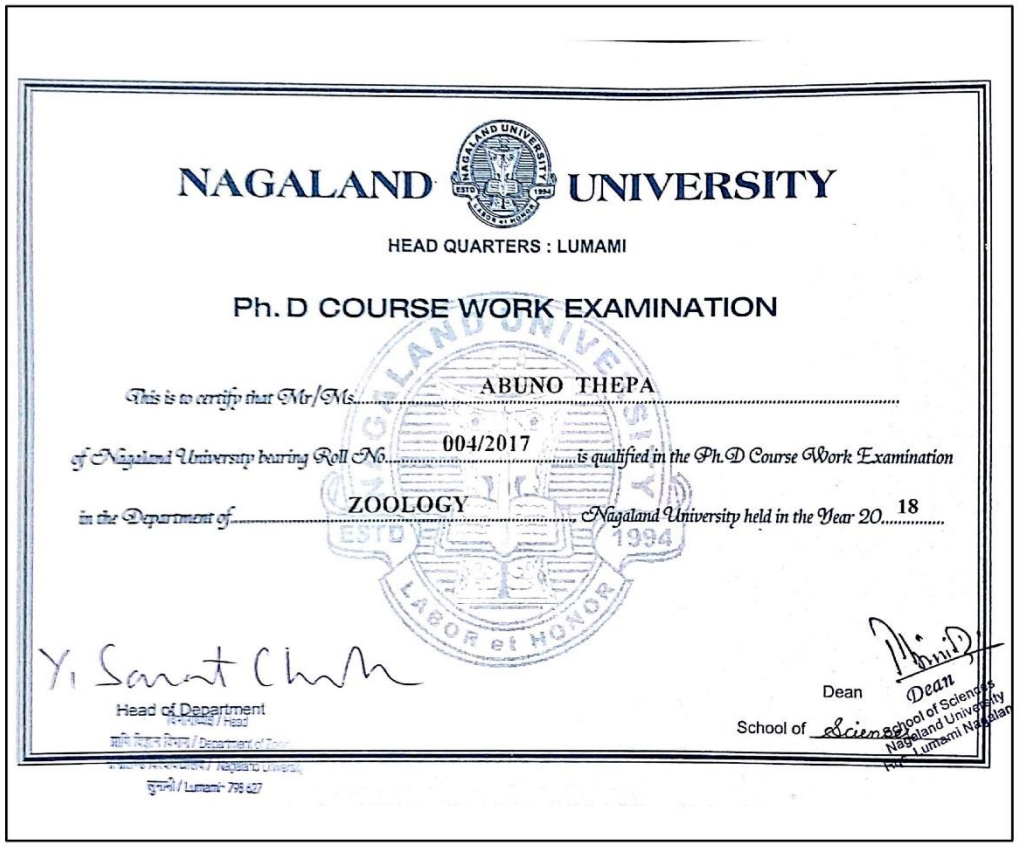
Certificate

This is to certify that Prof/Dr/Mr/Ms. Abuno Thepa has presented a paper entitled Looking into the Genome and Identifying the Putative Targets for Neuro-protection using Drosophila Model in the two day National Seminar on Climate Change and Sustainable Development with Special focus on North East India organised by NUTA, Nagaland University, held on 17th-18th May, 2017.

Organising Secretary NSCCSD 2017

Convener NSCCSD 2017

Course work certificate



NAGALAND UNIVERSITY

HEAD QUARTERS : LUMAMI

Ph. D COURSE WORK EXAMINATION

This is to certify that Mr/Ms. ABUNO THEPA

of Nagaland University bearing Roll No. 004/2017 is qualified in the Ph.D Course Work Examination

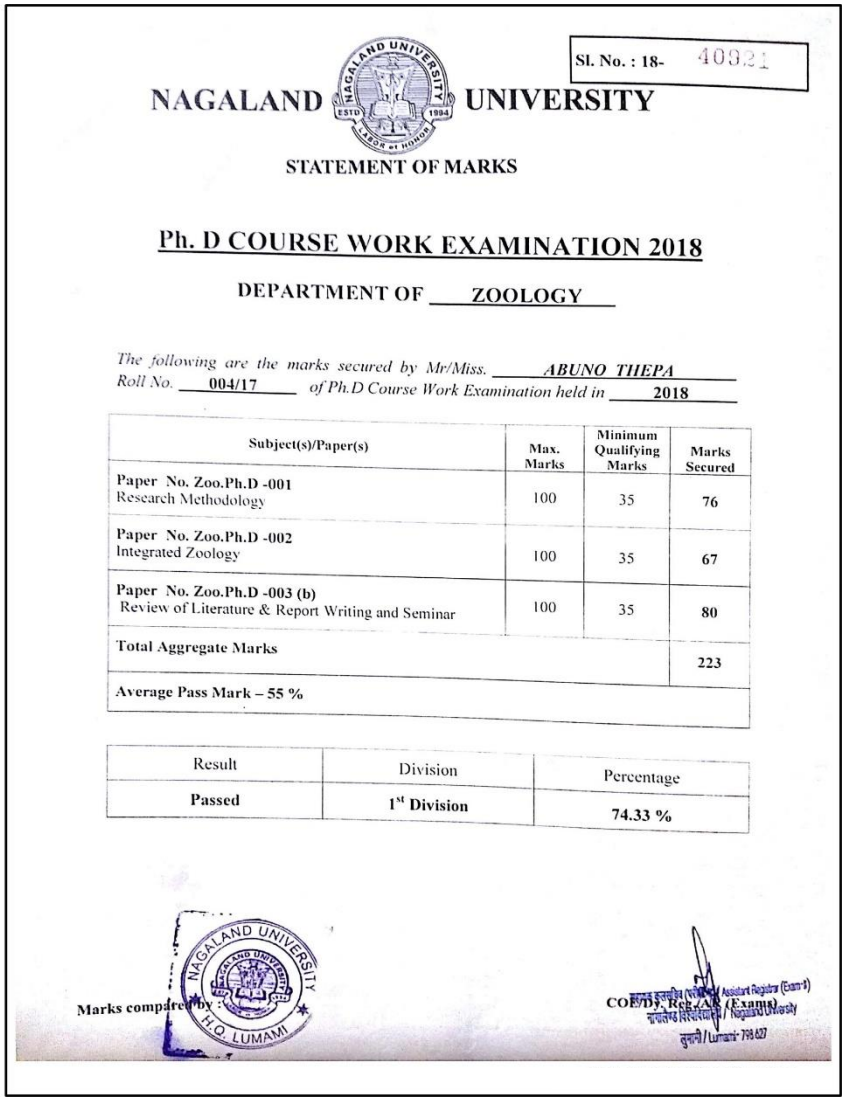
in the Department of ZOOLOGY Nagaland University held in the Year 20 18

Head of Department

Dean

School of Science

Dean
School of Sciences
Nagaland University
Lumami Nagaland



Sl. No. : 18- 40921

NAGALAND UNIVERSITY

STATEMENT OF MARKS

Ph. D COURSE WORK EXAMINATION 2018

DEPARTMENT OF ZOOLOGY

The following are the marks secured by Mr/Miss. ABUNO THEPA
Roll No. 004/17 *of Ph.D Course Work Examination held in* 2018

Subject(s)/Paper(s)	Max. Marks	Minimum Qualifying Marks	Marks Secured
Paper No. Zoo.Ph.D -001 Research Methodology	100	35	76
Paper No. Zoo.Ph.D -002 Integrated Zoology	100	35	67
Paper No. Zoo.Ph.D -003 (b) Review of Literature & Report Writing and Seminar	100	35	80
Total Aggregate Marks			223
Average Pass Mark – 55 %			

Result	Division	Percentage
Passed	1st Division	74.33 %

Marks compared by :

NAGALAND UNIVERSITY H.Q. LUMAMI

COE/Dy. Reg./A.R. (Exams)

PUBLICATIONS

Research Papers

- Ayajuddin M, Phom L, Koza Z, Modi P, Das A, Chaurasia R, **Thepa A**, Jamir N, Neikha K and Yenisetti S.C. (2022). Adult Health and Transition Stage specific Rotenone Mediated *Drosophila* Model of Parkinson's Disease:Impact on Late-onset Neurodegenerative Disease Models. *Frontiers in Moecular Neuroscience* 15:896183.doi:10.3389/fnmol.2022.896183.

Research Methodologies

- Modi P, **Thepa A**, Jamir N, Das A and Yenisetti S.C. (2021). Extraction and Processing of Fly Brain Proteins for Western Blotting; In Experiments with *Drosophila for Biology Courses* (Eds: Lakhotia S.C. and Ranganath H.A. Indian Academy of Sciences), pp 381-388. ISBN: 978-81-950664-2-1.

- Neikha K, Jamir N, **Thepa A**, Walling B, Das A and Yenisetti S.C. (2021). Use of Paper Microscope (Foldscope) for Class Room Teaching of Genetics; In *Experiments with Drosophila for Biology Courses* (Eds: Lakhotia S.C. and Ranganath H.A. Indian Academy of Sciences), pp 97-102. ISBN: 978- 81-950664-2-1.

Book Chapters

- Ayajuddin M, Das A, Phom L, Modi P, Chaurasia R, Koza Z, **Thepa A**, Jamir N, Singh P.R., Sentinungla, Lal P and Yenisetti S.C. (2018). Parkinson's Disease: Insights from *Drosophila* model; In *Drosophila melanogaster*: Model for Recent Advances in Genetics and Therapeutics. Eds: Perveen FK. Intech ,London, UK, pp 157- 192.ISBN:78-953-51-3854-9. doi:10.5772/66545.

- Neikha K, Walling B, **Thepa A** , Jamir N and Yenisetti S.C. (2020) .Utility of paper microscope (Foldscope) in biomedical research. Current status of research in biosciences. 193-200. Eds: Joshi PC, Joshi N, Reshman Yasmin, Mansotra DK (Today and Tomorrow publishers, New Delhi, India). ISBN 10:81- 7019-661-5.

- Walling B , Neikha K, **Thepa Abuno**, Jamir N and Yenisetti S.C. (2020). Utility of paper microscope (Foldscope) in classroom teaching of genetics 397-403. Eds:

Joshi PC, Joshi N, Reshman Yasmin, Mansotra DK (Today and Tomorrow publishers, New Delhi, India). ISBN 10:81-7019-661-5.

- Pukhrambam R S , **Thepa A**, Jamir N, Phom L, Ayajuddin M and Yenisetti S.C. (2017). Parkinson's disease and therapeutic strategies. *International Journal of Neurology and Neurosurgery* 19(2): 172-186. ISSN 0975- 0223.