STUDIES ON THE ROLE OF DEVELOPMENTAL NEUROTOXICITY ON DOPAMINERGIC NEURODEGENERATION IN *DROSOPHILA* MODEL

by

NUKSHIMENLA JAMIR

Registration No.: **PhD/ZOO/00130**

Submitted to

NAGALAND UNIVERSITY

In Partial Fulfilment of the Requirements for Award of the Degree of

DOCTOR OF PHILOSOPHY IN ZOOLOGY

DEPARTMENT OF ZOOLOGY

SCHOOL OF SCIENCES

NAGALAND UNIVERSITY

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NAGALAND UNIVERSITY
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DECLARATION

I, **Ms. Nukshimenla Jamir**, 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 and 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 Nagaland University for the degree of Doctor of Philosophy in Zoology.

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CERTIFICATE

This is to certify that the thesis entitled "**Studies on the Role of Developmental Neurotoxicity on Dopaminergic Neurodegeneration in *Drosophila* Model**" is a record of original research work done by **Ms. Nukshimenla Jamir**. She is a registered research scholar bearing **Regd. No. PhD/ZOO/00130** 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 other degree or distinctions. 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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Nukshimenla Jamir

(03/10/2024, Nagaland)

Dedicated to:

My late father, whose patience, resilience, constant support, love, and blessings have guided me throughout this journey.

His memory and inspiration continue to drive me every day.

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ABBREVIATIONS

6-OHDA	:	6-Hydroxydopamine
AADC	:	Aromatic Amino Acid Decarboxylase
AD	:	Alzheimer's Disease
ADHD	:	Attention-Deficit Hyperactivity Disorder
ALDH	:	Aldehyde Dehydrogenase
ANOVA	:	Analysis of Variance
ASD	:	Autism Spectrum Disorder
ATP	:	Adenosine Triphosphate
BBB	:	Blood-Brain Barrier
BCB	:	Blood-Cerebrospinal Fluid Barrier
BDNF	:	Brain-Derived Neurotrophic Factor
BSA	:	Bovine Serum Albumin
CERAD	:	Consortium to Establish a Registry for Alzheimer’s Disease
CNS	:	Central Nervous System
COMT	:	Catechol-O-Methyltransferase
CSF	:	Cerebrospinal Fluid
Cu	:	Curcumin
DA	:	Dopamine
DAergic	:	Dopaminergic
DAT	:	Dopamine Transporter
DBS	:	Deep Brain Stimulation
DDC	:	Dopa-Decarboxylase
dFoxO	:	*Drosophila* Forkhead Box O
DMP	:	Dimethylphosphate
DMSO	:	Dimethylsulfoxide
DNA	:	Deoxyribonucleic Acid
DNT	:	Developmental Neurotoxicity
DOHaD	:	Developmental Origins of Health and Disease
DOPA	:	Dihydroxyphenylalanine
DOPAC	:	Dihydroxyphenylacetic Acid
DOPAL	:	3,4-Dihydroxyphenylacetaldehyde
dTor	:	*Drosophila* Target of Rapamycin

ECD	:	Electrochemical Detection
EcR	:	Ecdysone Receptor
ETC	:	Electron Transport Chain
ETPB	:	Ethyl Paraben
FI	:	Fluorescence Intensity
FOAD	:	Fetal Origins of Adult Disease
FSIQ	:	Full-Scale Intelligence Quotient
GFP	:	Green Fluorescent Protein
GPCRs	:	G Protein-Coupled Receptors
HP	:	Health Phase
HPLC	:	High-Performance Liquid Chromatography
Hsp	:	Heat-Shock Protein
HVA	:	Homovanillic Acid
InR	:	Insulin Receptor
IQ	:	Intelligence Quotient
JNK	:	C-jun N-Terminal Kinase
L-DOPA	:	Levodopa
MAO	:	Monoamine Oxidase
MDA	:	Malondialdehyde
MPP	:	1-Methyl-4-Phenylpyridinium
MPTP	:	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mTOR	:	Mechanistic Target of Rapamycin
NADA	:	N-Acetyl Dopamine
NADH	:	Nicotinamide Adenine Dinucleotide
NBAD	:	N-β-Alanyl Dopamine
NDD	:	Neurodegenerative Disease
NDF	:	Neuronal Dysfunction
NF-κB	:	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NGS	:	Normal Goat Serum
Nrf2	:	Nuclear Factor Erythroid 2–Related Factor 2
OS	:	Oxidative Stress
PAL	:	Protocerebral Anterior Lateral
PAM	:	Protocerebral Anterior Median
PBS	:	Phosphate-Buffered Saline

PBST	:	Phosphate-Buffered Saline with Tween 20
PD	:	Parkinson Disease
PFA	:	Paraformaldehyde
PI3K	:	Phosphatidylinositol 3-kinase
PNS	:	Peripheral Nervous System
PPAR	:	Peroxisome Proliferator-Activated Receptor
PPD	:	Protocerebral Posterior Deutocerebrum
PPL	:	Protocerebral Posterior Lateral
PPM	:	Protocerebral Posterior Medial
PQ	:	Paraquat
ROS	:	Reactive Oxygen Species
ROT	:	Rotenone
RT	:	Retention Time
SEM	:	Standard Error of the Mean
SNCA	:	α-Synuclein
SNpc	:	*Substantia Nigra Pars Compacta*
SOD	:	Superoxide Dismutase
TCA	:	Trichloro Acetic Acid
TH	:	Tyrosine Hydroxylase
TNF-α	:	Tumor Necrosis Factor-alpha
TP	:	Transition Phase
TRITC	:	Tetramethylrhodamine
VM	:	Ventral Mesencephalon
VMAT	:	Vesicular Monoamine Transporter
VUM	:	Ventral Unpaired Median
Wnt	:	Wingless-Related Integration Site

CONTENTS

Chapter 4

CHAPTER 1

REVIEW OF LITERATURE

1.1. Introduction

The developing nervous system exhibits a precarious sensitivity to its surroundings, where even the most minuscule neurotoxic exposures can pivot the course of neurological outcomes for a lifetime. Teratologists and reproductive toxicologists recognize that certain substances may cause delayed teratogenic effects within the central nervous system (CNS), leading to the concept of "developmental neurotoxicity" (DNT) (Aoyama et al., 2015). Over four decades ago, researchers discovered that, besides common morphological problems that occur at birth, CNS teratogens like methylmercury and polychlorinated biphenyls can also lead to neurological dysfunctions such as sensory, motor, and cognitive impairments—an area of study initially termed 'behavioral teratology' (Henck and Morford, 2010). This recognition has prompted agencies such as the U.S. Environmental Protection Agency to develop definitions of DNT that highlight the potential for functional and structural damage to the developing nervous system from exposure to DNTs during prenatal and breastfeeding periods (Wallenborn et al., 2024).

Several essential cellular and molecular processes are essential to develop and operate the CNS and peripheral nervous system (PNS). Neurogenesis, migration, axon guidance, synaptogenesis, myelination, phenotypic specification of neurons (such as, glutamatergic or dopaminergic neurons), expression and maturation of receptors and ion channels to facilitate synaptogenesis, neuronal network formation, and brain segmentation are some of these processes (Scott-Solomon et al., 2021). Unfortunately, there is a scarcity of well-documented pathways that demonstrate how chemicals can potentially disrupt these processes (Bal-Price and Meek, 2017).

Though neurotoxicity has been defined as "any adverse effect on the chemistry, structure or function of the nervous system during development or at adulthood, provoked by chemical or physical influences" (Costa, 1998), DNT specifically confronts the issue that

the human brain, in its developmental stages, exhibits greater vulnerability to chemical exposure compared to adulthood (Mundy et al., 2015; Grandjean and Landrigan, 2006, 2014). Consequently, most DNT research is devoted to developing strategies for predicting how chemicals may affect the developing embryo or fetus. In recent years, DNT has attracted significant attention due to the strong link between exposure and the rising incidence of neuropsychiatric and neurological disorders, such as schizophrenia, attention-deficit hyperactivity disorder (ADHD), autism spectrum disorder, Parkinson's disease (PD), and Alzheimer's disease (AD) (Grandjean and Landrigan, 2014). Neurodegenerative diseases (NDDs) are typically diagnosed between ages 40 and 60, but underlying pathological changes start much earlier, with neuronal circuits losing connectivity and cell(s) death before clinical symptoms appear. This early loss of connectivity signifies the beginning of NDDs, where protective mechanisms fail due to molecular imbalances, potentially originating from insults during early childhood or embryonic development, highlighting the long latency between disease onset and clinical diagnosis (Shabani and Hassan, 2023). Despite the growing recognition of these early origins, a comprehensive framework to explicitly explore the intricate link between brain development and neurodegeneration is currently lacking, underscoring the necessity for further research in this field.

Understanding the complex link between early life exposures and subsequent health outcomes involves several fundamental concepts. Beginning with Barker's seminal work on the Fetal Origins of Adult Disease (FOAD) (Barker, 2007), which investigated the prenatal origins of diseases such as coronary artery disease, the framework eventually expanded into the Developmental Origins of Health and Disease (DOHaD), focusing on the profound effects of maternal diet, smoking, and toxins on fetal development, exposing individuals to a variety of health risks later in life (Tarantal and Berglund, 2014; Hanson,

2013; Gillman, 2005). Lahiri et al. (2009) put forward a "Latent Early-life Associated Regulation" (LEARn) model that enlarges both hypotheses by focusing specifically on biochemical and molecular pathways that address the 'black box' of most idiopathic sporadic disorders. They characterized environmental exposures as "hits", and later, a second hit occurs in those organisms that would lead to disease. This delay in manifestation supports Reuhl's concept of "silent damage" and stresses the period when toxicity is clinically undetected (Reuhl, 1991).

Meanwhile, the researchers published in The Lancet Neurology on a "global, silent epidemic of neurodevelopmental toxicity," stressing the insidious nature of neurotoxicity that may only become apparent after years of exposure (Grandjean and Landrigan, 2014). Lucio G. Costa's concept of the "event threshold" in PD illustrates this phenomenon, in which symptoms show after significant loss of dopaminergic (DAergic) neurons and gradually worsen with age (Costa, 2017). Given the complexities of environmental impacts, the exposome concept, first proposed by Wild, in the context of cancer epidemiology (Wild, 2005), has gained broad acceptance and took over the previous approach of viewing environmental influences as a whole rather than discrete components. This comprehensive approach integrates biological fingerprints, predisposing factors, external impacts, and biological changes, emphasizing the temporal characteristics of chronic diseases like Parkinson's and Alzheimer's, which evolve silently over many years (Lefèvre-Arbogast et al., 2024). Understanding the long-term impacts of environmental exposures, especially during crucial periods such as childhood, emphasizes the significance of addressing geographic exposures across the lifespan.

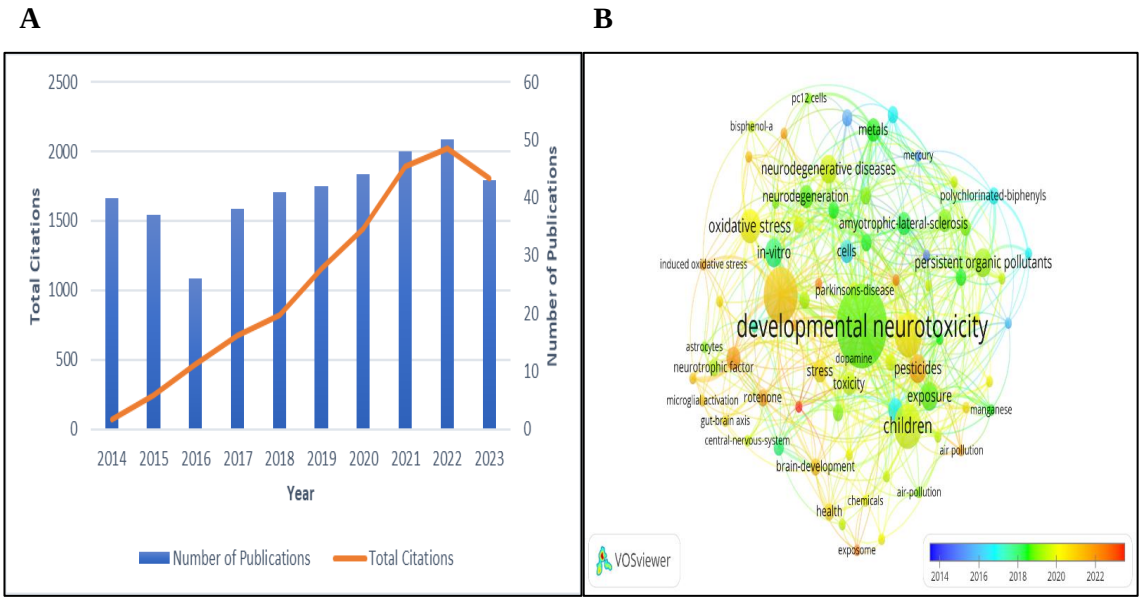


Figure 1.1. Year of publications and total citations (A), and top author's keywords (B) upon searching from Web of Science (WoS) using the terms "Developmental neurotoxicity," AND "neurological disorder," AND "environmental pollutants" OR "neurotoxicants" between 2014 and 2024.

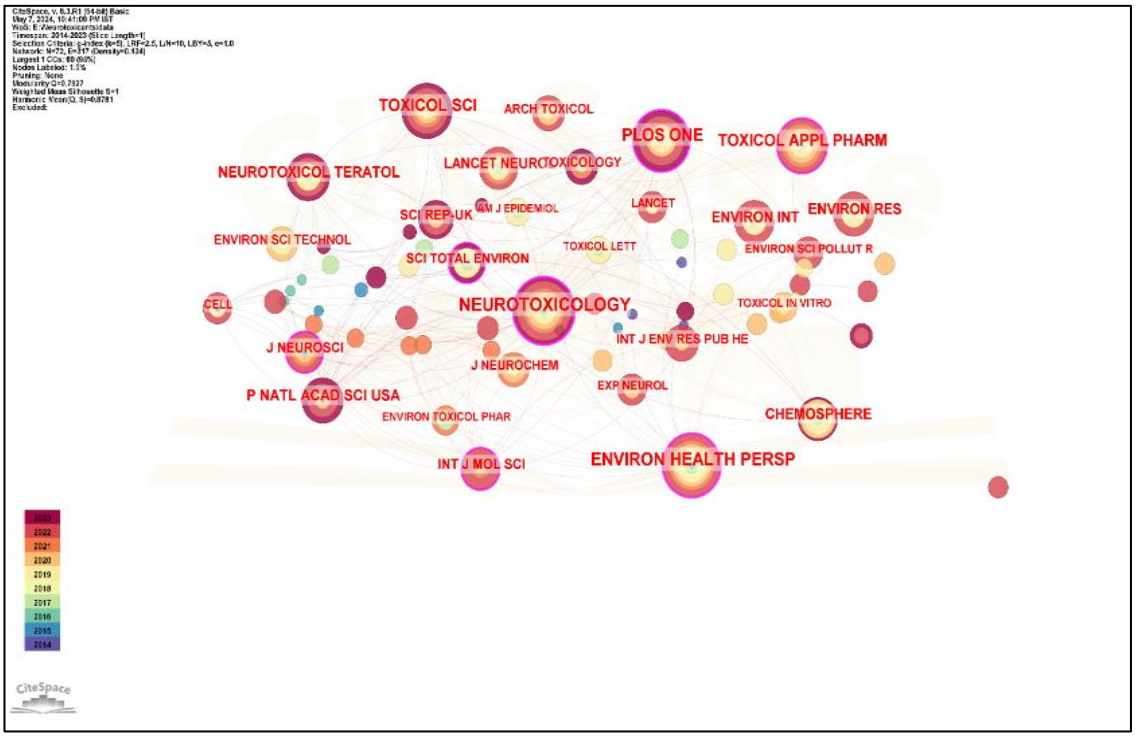


Figure 1.2. Journal network visualization upon searching from WoS using the term "Developmental neurotoxicity," AND "neurological disorder," AND "environmental pollutants" OR "neurotoxicants" between 2014 and 2024.

Bibliometric information can be extracted from various databases, but the Web of Science (WoS) stands out for its comprehensive and reliable coverage, encompassing millions of studies from over 12,000 high-impact journals worldwide (Wang et al., 2024; Tywabi-Ngeva et al., 2022). In this review, a title search was conducted on the WoS database on May 8, 2024 using keywords "Developmental neurotoxicity," AND "neurological disorder," AND "environmental pollutants" OR "neurotoxicants," with a total of 409 publications from the past decade (2014 to 2024) analyzed bibliometrically using Vosviewer 1.6.18, and CiteSpace. The data reveals a significant increase in publications related to environmental neurotoxicants, with a 25% rise in publications and peak citation counts in 2022 (**Figure 1.1 A**), indicating a growing global interest in this field. Notable peaks in citations in specific years, such as from 2014 to 2020, suggest that studies from these periods had substantial impacts on subsequent research. Key research themes identified include "developmental neurotoxicity," "oxidative stress," and neurodegenerative diseases like "Alzheimer's" and "Parkinson's," with a focus on how environmental toxins affect vulnerable populations, particularly children (**Figure 1.1 B**). Prominent journals in this domain include *Toxicological Sciences (TOXICOL SCI)*, *Neurotoxicology*, *Environmental Science & Technology*, *PLOS ONE,* and *Lancet Neurology* (**Figure 1.2**). Using tools such as CiteSpace and VOSviewer, bibliometric analysis is essential for understanding research trends, identifying influential studies, and recognizing emerging research areas. This approach reveals the evolution of scientific knowledge, highlights gaps in the literature, and aids in prioritizing research efforts and fostering collaborations. In the context of environmental neurotoxicants, these studies underscore the increasing importance of understanding how environmental factors contribute to neurological health challenges.

1.2 *Epidemiological studies on developmental neurotoxicity and adult neurodegeneration*

The DOHaD model aims to deviate from the traditional epidemiologic risk factor approach by emphasizing the enduring effects of early-life nutritional scarcity and the resulting long-term developmental consequences. Originating from epidemiological studies linking childhood nutrition to later health, the model has broadened to incorporate intrauterine dietary status and growth (Lacagnina, 2020). The prevalence of neurological conditions is likely to rise with the increasing life expectancy in most countries. About 50 million people are currently affected by dementia (Li et al., 2023), which is estimated to increase to 152.3 million by 2050. Dementia is a significant cause of disability, the need for long-term institutional care, and mortality, and the global cost is US$ 1 trillion (Nichols et al., 2022). AD is the most prevalent form of neurodegeneration and constitutes for 60–70% of all dementia cases, where 20% of women and 10% of men have developed AD (Seshadri and Wolf, 2007). PD is the second most prevalent neurodegenerative disease, following AD in prevalence, a chronic ailment that gradually worsens over time and affects a person's ability to move (Yenisetti et al., 2023). The prevalence of PD is approximately 2% of the population in those above 65 years of age. The burden of PD has more than doubled over the course of 26 years, from 2·5 million patients in 1990 to 6·1 million patients in 2016 (Rocca, 2018). Suppose the doubling of the number of patients diagnosed with PD between 1990 and 2016, as reported, is accurate and validated by additional research. In that case, the trend is expected to continue for 30 years. By approximately 2050, an optimistic projection of doubling the patient population over the subsequent three decades would result in the global patient count surpassing 12 million (Dorsey et al., 2018).

The 1944 Dutch famine was one of the first to demonstrate a link between maternal nutritional status and neurodevelopmental outcomes in offspring, revealing increased rates of CNS congenital abnormalities and emphasizing how inadequate maternal nutrient intake affects both neural cell proliferation in early pregnancy and neural differentiation in later stages of life (Lubrano et al., 2024). Epidemiological research has established a correlation between increased usage of pesticides and NDDs, with prenatal exposure to pesticides associated with various neurodevelopmental disorders. The mode of action varies between substances, but the most common are organophosphates, carbamates, pyrethroids, and neonicotinoids (Martín Reina et al., 2017). For years, there has been a developing amount of evidence that suggests that neurodegenerative diseases, such as PD, are partially man-made (Gunnarsson and Bodin, 2019; Mostafalou and Abdollahi, 2013).

1.2.1 Neurodevelopmental and neurodegenerative risks from environmental exposures: Insights from cohort, systematic reviews, and case-control studies

Cohort Studies

Guilbert et al. (2021) analyzed 416 prenatal maternal urine samples collected during pregnancy and used a chemical mixture weighted for specific compounds, including 2-hydroxy-4-methoxybenzophenone (BP3), triclosan, methylparaben (MEPB), and ethyl paraben (ETPB), and several phthalate metabolites, and investigated their combined effects on child behaviour which was a link to more externalizing behaviors such as hyperactivity, aggressiveness in 2-year-old French children. Waits et al. (2022) studied 76 ADHD diagnoses and 98 controls in 4- to 15-year-old Taiwanese children and reported common associations between a chemical mixture involving two organophosphate pesticide metabolites identified as dimethyl phosphate (DMP) and diethyl phosphate (DEP) and two phthalate metabolites named monoethyl phthalate (MEP) and mono-

benzyl phthalate (MBZP); the correlation demonstrated elevated ADHD symptoms. Notably, these chemicals are prevalent among the general population and show potential complications of thyroid and neurotransmitter functions (Darbre, 2022). These findings demonstrate the relevance of addressing ADHD symptoms while understanding exposure to early childhood chemicals.

A Slovakian study established that exposure to polychlorinated biphenyls (PCBs) is closely related to reduced cognitive and motor development in mothers, where the attributes affect children's development through direct or indirect exposure blocking neurotransmitter function, leading to endocrine disruption or a reduction in thyroid hormones during brain development in utero. The research depicts that early exposure can initiate the onset of diseases later in life, such as AD development, as suggested by the LEARN model (Park et al., 2010). Exposure to endocrine-disrupting compounds during critical developmental periods can affect various organs and biological processes, including brain-derived neurotrophic factor (BDNF) signaling, calcium homeostasis, sexual differentiation, locomotor activity, and parenting behavior during early developmental stages, leading to changes in behavior, physiology, and increased disease risk later in life; even low-level exposure can have lasting cognitive and behavioral impacts (Raja et al., 2022).

A recent cohort study indicated that high levels of bromine in the brain may contribute to AD neuropathology progression as indicated by standardized measures, such as Braak, CERAD, and NIA-Reagan scores. The findings support the prediction that bromine exposure poses a risk to brain health and suggest a potential link between bromine accumulation in the brain and exposure during early prenatal or childhood, which may eventually lead to disease progression of AD neuropathology (Agarwal et al., 2020). In addition, pesticides are also reported in Ecuadorian children, such as pyrethroids and

organophosphates, which cause neurotoxicity where exposure has proven to negatively affect gestation development and increase the risk of AD during later life (Grandjean et al., 2006).

Systematic Reviews

A systematic review conducted by Heng et al. (2022) on the impact of heavy metals on children's neurodevelopment showed an increased risk of disorders such as autism and cognitive impairment in low- and middle-income countries, suggesting that exposure to arsenic at a young age is a concern in a research conducted in countries from Bangladesh, Thailand, China, India, and Mexico. Postnatal lead exposure can lead to cognitive decline, poor language skills, motor delays, and behavioral problems, while maternal lead exposure during pregnancy, especially in the third trimester, can lead to lower IQ scores in young children. Children with higher levels of cadmium in their urine had lower FSIQ (full-scale intelligence quotient) scores, while manganese exposure was associated with decreased intellectual function in children, impairing cognitive abilities (Heng et al., 2022). Findings suggested that metals such as lead and manganese may enhance each other's neurotoxic effects, suggesting that future research should employ mixture-based analytical methods, as documented by research conducted in Taiwan (Lin et al., 2013), where levels of both metals exhibited considerably worse developmental scores than children with high levels of lead alone, which emphasizes the vital need of taking into account different metal exposures and using mixture-based analytical approaches to assess their influence on child health.

Autism spectrum disorders (ASD), a neurodevelopmental disorder, are distinguished by communication challenges and repetitive behaviors. A review of 11 epidemiologic studies, including 5 case-control, 4 cohort, one ecological, and one cross-sectional survey, explored the association of pesticide exposure with the incidence of autism. The results

showed higher maternal urine levels of dimethylthiophosphate (DMTP), dialkylphosphate (DAP), diethylphosphate (DEP), several maternal blood levels of hexachlorocyclohexane (HCH), trans-nonachlor was related to more significant autistic behaviors, among girls. Furthermore, exposure to pesticides, including Dichlorodiphenyldichloroethylene (DDE), organochlorines, organophosphorus, imidacloprid, and chlorpyrifos during the prenatal period increased the risk of autism. This evidence indicates concurrence with the potential relationship between prenatal exposure to pesticides and the occurrence of ASD (Arab and Mostafalou, 2022). Anesti et al. (2023) investigated the influence of prenatal and early life exposure to heavy metals such as arsenic and mercury on neurodevelopment among children aged 3 to 8 years and found numerous associations with cognitive, language, speech, and motor impairment and social responsiveness relevant to ASD.

Case Studies

In epidemiological studies, diagnostic accuracy is a challenge for PD to establish a delayed development of PD in later life while linking up with the early prenatal stage. Studies frequently highlight pesticides as potential risk factors for PD. Nearly three decades ago, Koller et al. (1990) reported that rural living and drinking well water were associated with PD, leading to suspicions about pesticide exposure. Among pesticides, organochlorines are linked with the highest risk, with studies confirming their presence in the brains of PD patients. Further studies found that these substances can reach humans through various channels beyond agriculture, such as in cosmetic products at concentrations higher than those permitted by the European Commission (Adekunle et al., 2018).

According to reports, mercury persists in the brain for far longer than in other organs (Torrey and Simmons, 2023). A case study of a 34-year-old man who experienced tremors

and mercury toxicity symptoms after 18 months of exposure found that mercury remained in his brain for 16 years, primarily in DAergic neurons of the *substantia nigra* (Hargreaves et al., 1988). Further, a 2014 study, as part of an 18-year follow-up of the Nurse Health Study, which included 97,430 nurses, discovered that 425 were diagnosed with PD. While airborne exposure to eight metals failed to attain statistical significance, mercury had a nearly significant positive relationship with PD risk, particularly among those residing in metropolitan areas (Palacios et al., 2014). Methylmercury (MeHg) first came to light in 1865 and subsequently led to a significant poisoning outbreak in Japan in the 1950s, leading to the development of the neurological condition known as Minamata disease. MeHg, present in contaminated seafood, can accumulate in the brain when exposed in early childhood, perhaps leading to the development of PD. Investigation revealed that mercury was detected in neurons and oligodendrocytes inside the brain regions affected by PD in persons who had been exposed to mercury and was found to be linked to the presence of α-synuclein aggregates in Lewy bodies (LB) and neurites (Pamphlett and Bishop, 2022).

Go et al. (2020) conducted a longitudinal study of the Kuakini Honolulu Heart Program cohort on Japanese males brought to Hawaii between 1885 and 1924 for plantation labor, revealing that extended periods of employment substantially elevate the likelihood of developing PD as a result of possible exposure to organochlorine pesticides (OGC). Methylation-based biomarkers and neurotoxic pathways are identified in PD patient's blood and brain tissues, while high OGC levels in PD brains are connected to increased dopamine (DA) and LB pathology, and pathway analyses suggest effects on glial genes and pathways involved in immune control, inflammation, and protein clearance.

The study conducted by Liou and colleagues provided evidence that prolonged exposure to herbicides/pesticides and paraquat in the environment for a period of 20 years was

linked to a significant 4.5- to 6.4-fold increase in the chance of developing PD (Liou et al., 1997).

In a recent study of COVID-19, a probable case of PD was diagnosed in a 45-year-old Jewish man after severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. The patient had hypomimia, moderate cogwheel rigidity in the neck and arm, bradykinesia and an episode of tremor (Cohen et al., 2020).

Various epidemiological studies and evidence have considered early infections and exposures can lead to long-term consequences, incurring a risk to the fetus that would be born with limited protection to DAergic neurons and are more vulnerable to developing PD in later stages of life.

1.3. *Developmental neurotoxicants*

Developmental neurotoxicity (DNT) is rarely considered in chemical risk assessments because mammalian models are ethically contentious, expensive, and resource-intensive. As a result, efforts are underway to create cost-effective and novel alternative methods for DNT testing. DNT assessments have traditionally been undertaken only when there is a prior trigger or in retrospective research because animal testing is expensive, time-consuming, and financially impracticable for screening numerous compounds (Collins et al., 2024).

Reproductive and developmental health is influenced by various factors starting from the prenatal stage, including social, physical, and nutritional environments, as well as exposure to different chemical agents (Olden et al., 2011; Huen et al., 2009). Early-life exposure to neurotoxicants, such as industrial chemicals, insecticides, heavy metals, and anesthetics, can cause neurotoxicity. These substances disrupt cell signaling pathways, cause DNA damage, change gene expression, promote oxidative stress, and affect neural development (Song et al., 2022). Endocrine-disrupting chemicals (EDCs) can also affect

brain development, leading to NDDs like PD (Özel and Rüegg, 2023). Many diseases, including trisomy 21 and alcohol consumption during prenatal development, can impede normal brain development or lead to neurodegenerative conditions later in life, such as brain oxygen deprivation or genetic anomalies (Vilain, 2024). **Table 1.1** summarizes the impact of various environmental toxins on neurodegenerative disorders. Hence, the susceptibility of the developing nervous system to these neurotoxins might cause long-term cellular abnormalities, perhaps contributing to NDDs as people age. Understanding these pathways is critical for creating strategies to reduce developmental neurotoxicity and prevent severe neurological consequences.

Sl.No.	Environmental Toxin	Model Used	Duration/Dose	Effects	References
1.	Chlorpyrifos	Adult male rats (Long Evans)	21 days/3 and 10 mg/kg/day	a) Cortical Acetylcholinesterase (AChE) b) Suppression, hippocampus AChE suppression c) Whole blood ChE reduction d) Transcriptome alterations in genes producing hippocampal neuropeptides such as brain-derived neurotrophic factor (BDNF), cortistatin (CORT), and neuropeptide Y (NPY)	Lee et al. (2016)
2.	Aluminium chloride (AlCl3)/ aluminium lactate (Al (lac)3)	Mice	3 months/ 10 mg	Acetylcholinesterase activation	Zatta et al. (2002)
3.	Lead acetate	Pregnant Wistar female rats	2 weeks/ 15 mg/kg	Production of pro-inflammatory cytokines such as IL-1 and TNF-α in the hippocampus and IL-6 in the forebrain of immature rat brain	Balali-Mood et al. (2021)
4.	Trichloroethylene	Elderly rats	6 weeks/200 mg/kg	a) Nigrostriatal dopaminergic impairment b) Elevated oxidative stress c) Induced endolysosomal impairment d) α-synuclein deposition	De Miranda et al. (2021)
		Aged rats	6 weeks/200 mg/kg	a) Elevated oxidative stress b) Caused endolysosomal dysfunction c) Protein accumulation (α-synuclein) d) Induced LRRK2 kinase activity, which resulted in the selective dopaminergic neurotoxicity	Castro et al. (2020)
5.	Paraquat	Male C57BL/6 mice	3 weeks/i.p. 10 mg/kg once a week	a) Increased no. of falls b) 58% loss of TH+ cells in SNpc c) Increase in microgliosis in SNpc d) No astrogliosis in SNpc e) No change in L-ferritin levels in SNr	Nixon et al. (2018)
		Drosophila melanogaster Oregon K	5 Day, 55 Day/ 10 mM PQ	a) Induced mobility defects b) Depletion of brain dopamine levels	Phom et al. (2014)
6.	Organophosphate pesticide [chlorpyrifos]	Rat (male and female)	6, 9, 12, 15, and 24 months/ 3 and 10 mg/kg/d	a) Chronic microglial dysregulation b) Accelerated neurodegeneration in both males and females	Voorhees et al. (2019)

7.	Inorganic arsenic (iAs)	3xTgAD mouse	6 months/ 0.2 mg/Kg/day	a) Decreased ATP content via the decline of complex-I levels b) Increased ROS production in the hippocampus c) Greater immune-positive responses to amyloid isoforms d) Phosphorylated tau was seen in the frontal cortex and hippocampus	Niño et al. (2019)
8.	PCBs	Rat	14 days/ 2µM, 8µM	a) Altered DA neurochemistry b) DAergic protein downregulation c) Elevated oxidative stress d) Neuronal injury e) The deterioration of both VM and striatal GABA neurons prior to the death of VM DA neurons	Lyng et al. (2007)
9.	Simulated vehicle exhaust exposure (SVEE)	Adult male Sprague Dawley rats	2 weeks (5 h/ day)/ 250 µM	a) Behavioural and cognitive abnormalities b) Elevated oxidative stress c) Decreased antioxidant response d) Mitochondrial impairment	Salvi et al. (2020)
10.	Indoor nanoscale particulate matter (INPM)	3D human organotypic model	24h/ 5,10,20,40µg/ml	a) Aggravated inflammation caused by ROS b) Stimulated abnormal expression of the nuclear transcription factor *Nrf2* following ROS accumulation c) Disruption of γ-glutamate synthase (γ-GCS) and heme oxygenase (HO-1) synthesis d) Exacerbating the antioxidant system's imbalance and thereby influencing BBB bio-function by Keap1-*Nrf2*-*ARE* pathways	Li et al. (2020)
11.	Rotenone	Male C57BL/ 6 mice	8 and 9-week-old/ Oral;30 mg/kg/ week for 4 weeks	a) Motor dysfunction b) Gastrointestinal dysfunction c) Change in fecal microbiota d) Increased TLR2 & inflammatory cytokine expression in the colon e) α synuclein aggregation in the gut f) Dysfunction in gut microbiota might promote rotenone-induced PD	Yang et al. (2018)
			5day, 50 day/ 10, 25, 50, 100, 250, 500, and 1,000 µM	a) Exhibits mobility defects b) inhibited mitochondrial complex I activity c) Dopaminergic neuronal dysfunction (no loss of DAergic neuronal number)	Ayajuddin et al. (2022)

		Drosophila melanogaster Oregon K		d) Reduction in rate-limiting enzyme tyrosine hydroxylase synthesis and e) Alteration in levels of dopamine and its metabolites: DOPAC and HVA.	
12.	Arsenite	Primary astrocyte cultures from rat brain Cultures of primary astrocytes	2 h, 6h/0.1 mM, 0.3 mM, 1 mM 24h/ 0, 2.5, 5, 10, 20, or 30 µM	a) Rapid GSH export stimulation b) MRP1 inhibition prevented arsenite-induced GSH export c) Glycolytic lactate production stimulation a) Increased glutamate-induced astrocytic calcium levels b) Increased levels of D-serine c) γ aminobutyric acid and glycine d) Affected glutamate-induced gliotransmitter release from astrocytes and e) Disturbed neuronal function	Tadepalle et al. (2014) Dringen et al. (2015) Wang et al. (2012)
13.	Triphenyl phosphate (TPP) and diphenyl phosphate (DPP)	Weaned male mice (C57/BL6)	30 days/(0, 50, or 150 mg/kg/day)	a) Thalamus and hippocampus inflammation b) Changes in glutamic acid, N-acetyl CoA metabolites, and organic acid levels c) Interference with amino acid, lipid metabolism, brain transcription, and cell death processes (*FOXO* and *MAPK* signalling pathways) d) Upregulation of Anti-inflammatory cytokines such as *TNF-α* and interleukin-6 (*IL-6*) and downregulation of antioxidant genes such as nuclear factor-E2-related genes	Liu et al. (2020)
14.	Lead (Pb) and Manganese (Mn)	Male and female Sprague Dawley rats	20s, 30s, 60s/day/ Pb (10 µg/ml), Mn (2 mg/ml) or a mixture	Low levels of Pb and Mn produced gender-specific neurological impairments	Betharia and Maher (2012)
15.	Aluminium (Al) and Mercury (Hg)	Human neuronal-glial (HNG) cells	24 h/ 0, 20, 50, 200, 500 or 1000 nM	Large upsurge in pro-inflammatory signalling mechanisms via notable induction of *NF-kB* (p50/ p65) in response to Al and Hg individually or in combination	Alexandrov et al. (2018)
16	PM2.5	SPF male C57BL/6J mice	7 days/ 0, 0.193, 1.93, and 19.3 mg/kg/day	a) Cognitive deficits b) Loss of neurons c) Protein aggregates were detected	Liu et al. (2019)

17.	DDT and DDE	Human neuroblastoma cells	48 h/1µM	Increased amyloid precursor protein levels	Richardson et al. (2014)
18.	Sodium arsenite	lymphocytes	72 h/ 0, 0.1 or 1 µM	Impaired *GLUT1* trafficking and function via calpain dysregulation	Pánico et al. (2019)
19.	Manganese	Children	12, 18, 24, 30, and 36 months / 24.3 µg/L and 21.1 µg/L,	Inferior intelligence quotient (IQ) scores	Roels et al. (2012)
20.	6-OHDA	Adult C57BL/6 mice	3 weeks/2 and 4 µg/µL	a) Loss of DA neurons in SNc & striatum, b) Disturbances in STN activity corresponding to pathophysiological changes of PD c) Reproduce the progressive stage of PD	Park et al. (2018)
21.	MPTP	G2019S-LRRK2 male mice	24 h intervals for 5day/ 2 injections of 2.5 mg/kg	a) Decreased rotarod performance b) Loss of DA neurons in SN c) Loss of DA fibers in striatum d) Increased activation of astrocyte e) G2019S-LRRK2 mutation enhanced MPTP toxicity in mice	Arbez et al. (2020)

Table 1.1: A summary on the impact of environmental toxins on neurodegenerative disorders.

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

In 1982, a byproduct of a meperidine analog known as MPTP was identified as the first neurotoxicant leading to parkinsonism after a small number of drug users in the United States mistakenly injected heroin contaminated with this neurotoxin and developed severe and irreversible PD-like symptoms, known as "frozen addicts," which similarly affected humans and non-human primates within a few days of exposure (Langston et al., 1984; Langston et al., 1983). Once MPTP crosses the blood-brain barrier (BBB), it is converted to its active form, MPP^+, by monoamine oxidase-B (MAO-B) (Mei et al., 2019). MPP^+ specifically targets DAergic neurons through the dopamine transporter (DAT) and inhibits mitochondrial complex I, inducing reactive oxygen species (ROS) and causing neuronal death (Martí et al., 2017). Additionally, this toxin degrades the vesicular monoamine transporter-2 (VMAT-2), leading to DA auto-oxidation and neurotoxicity (Lohr et al., 2015). MPTP is mostly used in non-human primates, including dogs, cats, and mice (Yun et al., 2015), which is also used to investigate the non-motor symptoms of PD, as it affects both the nigrostriatal DAergic system and the peripheral nervous system (Mingazov et al., 2018). Iglesias et al. (2018) conducted a review on the impact of MPTP exposure during fetal development and found variability in *substantia nigra* characteristics across several animal models. The sub-cytotoxic MPTP doses in mouse N2a neuroblastoma cells inhibit axon-like processes growth and reduce DA concentrations in embryonic mouse brains.

Additionally, MPTP administered as a single dose on a specific gestational day can reduce dopamine transporter and tyrosine hydroxylase (TH) mRNA and protein levels, as well as DA content in newborn mice midbrain and striatum, while also reducing the number of TH-positive neurons in the *substantia nigra*. This effect changes with conditions and gestational timing, highlighting the sensitivity of specific brain areas to MPTP-induced

neurotoxicity. One notable shortcoming of this model is its inability to develop Lewy bodies (LBs), which is essential in PD (Blesa and Przedborski, 2014). Additional disadvantages include the resilience of rats to MPTP, the varying sensitivity of different mouse strains to the toxin (Meredith and Rademacher, 2011), and its inability to replicate clinical outcomes for developing PD treatments (Lindholm et al., 2016).

Lead

Lead (Pb) is a widely recognized hazardous metal that has detrimental effects on human health, particularly for cognitive development in children exposed to low quantities. Despite substantial studies into Pb neurotoxicity, our understanding of its transgenerational impacts is limited. Environmental Pb exposure has been strongly linked to a variety of neurological disorders, including autism (Dórea, 2019; Hessabi et al., 2019), AD (Bihaqi, 2019), PD (Nabi and Tabassum, 2022; Ullah et al., 2021), Amyotrophic lateral sclerosis (Farace et al., 2020), and Attention-deficit/Hyperactivity Disorder (Dórea, 2019).

Pb penetrates the brain through the BBB and blood-cerebrospinal fluid barrier (BCB), disturbing their integrity and accumulating in endothelial and choroid plexus cells, causing increased permeability, brain edema, and hemorrhages. It can cross the BBB by passive diffusion, binding to anions, or using divalent metal transporters, and it breaks tight junctions by activating protein kinase C (PKC) and tyrosine kinase *Src*. Pb buildup in astrocytes and oligodendrocytes disrupts cell function and gene expression, causing neurotoxicity, particularly in children (Virgolini and Aschner, 2021).

Extensive research has highlighted the neuropathological routes of Pb toxicity in the developing brain, revealing that it interferes with proteins required for neuronal maturation, migration, and differentiation. This interference causes functional deficits in specific brain areas. Furthermore, Pb exposure decreases synapse formation and repair,

affecting synaptic function and decreasing neural synaptic plasticity (Wang et al., 2022). Moreover, Pb exposure disrupts glial cell development and differentiation, affecting the nervous system's immune function (Li et al., 2021; Wu et al., 2021).

Wu et al. (2024) studied the transgenerational neurotoxic consequences of Pb exposure in *Drosophila* resulting from a previous history of usage in lead gasoline. They discovered that Pb exposure during grandparenting caused aberrant neurobehavior and synaptic dysfunction in their grandchildren's kids, which might be attributed to altered genome methylation. Adult flies had aberrant muscle nerve innervation and a mushroom body β-lobe midline-crossing phenotype, indicating this impact.

Acrylamide

Acrylamide (ACR), a potential neurotoxin produced during food processing through the Maillard reaction between reducing sugars and amino acids, has raised concerns over the past decade due to its neurotoxic effects (Zhao et al., 2022). Initially recognized in the 1950s, large-scale industrial production of ACR led to occupational neurotoxicity in factory workers, characterized by peripheral neuropathy, ataxia, numbness, muscle weakness, cerebellar dysfunction, cognitive impairment, and progression to severe NDDs like Parkinson's and Alzheimer's (Rajeh, 2024). The focus on ACR intensified in the early 2000s when it was detected in high-temperature cooked foods such as coffee, bread, potatoes, and meat, posing a daily exposure risk (Schouten et al., 2020) and was also found to be present in cosmetics, pesticides, dyes, and paper manufacturing sites (Tepe and Çebi, 2019).

ACR exposure disrupts the synaptic vesicle cycle by forming irreversible adducts with nitric oxide acceptors and other neuromodulators, inhibiting proteins such as dopamine transporters, NEM-sensitive factors, and vesicular monoamine transporters, resulting in synaptic toxicity and neuronal death (Zhang et al., 2020).

Studies have shown that ACR causes neurotoxicity, reproductive toxicity, and behavioral abnormalities (Senthilkumar et al., 2020) by interfering with cellular activities and producing mitochondrial malfunction, resulting in diminished energy and neurotransmission (Prasad, 2012). Mice exposed to ACR during gestation showed lower expression of vital neurodevelopmental genes (*Esx1*, *Hand1*, and *Hand2* mRNA), decreased proliferation of placental cells, and increased apoptosis, as demonstrated by alterations in the proteins cleaved-caspase-3, cleaved-caspase-8, Bcl-2, and Bax. This exposure resulted in the collapse of fetal vessels and a significant reduction in labyrinth vessels, which are necessary for nutrient exchange and fetal growth. The study emphasizes that ACR impairs placental development, gene expression, vascular integrity, and cellular processes required for healthy neurodevelopment in offspring (Yu et al., 2019). Li et al. (2016) observed that ACR can cause DAergic neuron degeneration and α-synuclein aggregation, potentially leading to PD.

Paraquat

Paraquat (PQ, 1,1'-dimethyl-4,4'-bipyridinium) is a nonselective quaternary ammonium herbicide produced by Syngenta in the early 1960s and is one of the world's most widely used weed killers. Despite its extensive use, many nations have outlawed PQ due to its lethal toxicity to humans, though its production and export remain controversially tolerated (Bastías-Candia et al., 2019). Acute human exposure to PQ causes severe and irreversible damage to the kidneys, lungs, and liver, resulting in mortality even at low levels. Initially, PQ's potential neurotoxicity was underestimated due to low brain concentrations after acute exposure, as it was assumed that the BBB would prevent PQ entry into the brain, with its presence attributed to the cerebral circulatory system (Widdowson et al., 1996; Naylor et al., 1995; Houzé et al., 1990). However, cases of PQ-induced brain injury were later described (Hughes, 1988; Grant et al., 1980), and research

confirmed that PQ might penetrate the BBB via the neutral amino acid transport pathway rather than by disrupting BBB function (McCormack and Di Monte, 2003; Shimizu et al., 2001). Chronic low-dose exposure to PQ causes more long-term neurodegeneration in the brain than acute exposure (Prasad et al., 2007).

PQ, which is structurally identical to MPP^+, is identified by the DAT and may contribute to the development of PD (Vaccari et al., 2019). PQ crosses the BBB, inducing degeneration of Daergic neurons in *substantia nigra pars compacta* (SNpc), inhibits the complex I of electron transport chain (ETC), and reducing ATP production, leading to neurotoxicity in neurons and glial cells (Briñez-Gallego et al., 2023).

PQ has been demonstrated to cause Parkinsonian symptoms in *Drosophila* in a dose-time-dependent approach (Phom et al., 2014), generate substantial levels of reactive oxygen species (ROS), and selectively reduce DAergic neurons in the *substantia nigra* (Liu et al., 2018; Zhang et al., 2016). Furthermore, Widdowson and his colleagues (1996) found much higher PQ concentrations in the brains of newborn mice than in adults, implying that PQ impairs BBB integrity in neonates. According to environmental studies, low-dose prenatal exposures to hazardous substances might cause dysfunctions and disorders later in life (Barouki et al., 2012). Rodent studies have shown that embryonic exposure to PQ and MB, followed by adult re-challenge with the same pesticides, causes considerable locomotor activity reduction and DAergic loss (Colle et al., 2020). These findings support the hypothesis that early-life environmental insults have long-term consequences.

1.4. *Animal models of developmental neurotoxicity*

Animal studies can complement human studies by identifying similarities in age windows of susceptibility to external environmental stimuli. These models are critical for answering key questions about developmental manipulations and neurobehavioral consequences and examining critical age windows of vulnerability to environmental insults (Heyer and Meredith, 2017). Various animal models have been established to understand the mechanisms of NDDs and evaluate therapeutic strategies. These models range from invertebrate species (such as *Drosophila melanogaster* and *Caenorhabditis elegans*) to vertebrate species, including rodents, such as mice and rats, and non-human primates (Briñez-Gallego et al., 2023), providing essential insights into the pathophysiology of various diseases, including neuronal cell properties, developmental changes, organ function, and the effects of aging and drug use. Although these models have proven useful for studying drug toxicity, sensory function, motor coordination, learning, and memory functions, they often fail to fully replicate the complexity of the human nervous system, limiting their clinical implementation (Tello et al., 2022). To address this issue, researchers have also been using lower model animals such as *Drosophila melanogaster* and *Caenorhabditis elegans* due to the low cost and easy maintenance of these animals; however, it becomes evident that the pathological similarity to humans is limited (Modi et al., 2016). Animal models induced by chemical agents or toxins have evolved significantly since the late 1950s. The development of transgenic animals expressing human mutant genes has provided new insights into disease mechanisms, helpful in studying how exposure to environmental toxins early in life leads to NDDs later in life, emphasizing the importance of developmental sensitivity (Banerjee et al., 2022). Overall, animal models are critical for studying behavioral phenotypes, neuronal morphology, gene expression, and brain networks, helping to

identify new biomarkers and enable *in vivo* testing of innovative therapies, thereby providing valuable insights into the onset and progression of NDDs (Guerreiro and Maciel, 2023).

1.4.1. *Drosophila melanogaster as a model to study developmental neurotoxicity*

In 1910, Thomas Hunt Morgan first established the use of *Drosophila melanogaster* as a research model. Over the next five decades, genetic studies in *Drosophila* led to ground-breaking insights into biological systems, which further advanced genetic understanding and paved the way for breakthrough discoveries (Ong et al., 2015). As a result, *Drosophila* emerged as an indispensable model for studying human diseases and evaluating treatment methods. Modern toxicological research has effectively used the fly, leading to the development of the specialist term known as "Drosophotoxicology" (Rand, 2010).

Exposure of *Drosophila* embryos to methylmercury (MeHg) induces embryonic neural development, including disruptions in neuron localization, neurite outgrowth, and cell migration, which could result in long-term effects in later life (Rand et al., 2009), where disruptions in neurite outgrowth signaling pathways may lead to neuronal migration and differentiation (Stanton-Turcotte et al., 2022; Khodosevich and Monyer, 2010), resulting in NDDs and injuries such as PD and AD, and traumatic brain and spinal cord injury (Lilienberg et al., 2021). Additionally, MeHg exposure causes sexual dysfunction associated with increased oxidative stress and reduced triglyceride levels in both male and female *Drosophila* (Chauhan et al., 2017).

Lead exposure in *Drosophila melanogaster* causes transgenerational developmental neurotoxicity by altering genome methylation, which affects axonal targeting, disrupts neuromuscular junctions (NMJs), and causes structural changes in the brain, resulting in abnormal synaptic transmission and autism-like behavior in adulthood. These neurodevelopmental and behavioral phenotypes are inheritable, persisting in the F3

generation even without direct lead exposure, indicating significant DNA methylation changes in neurodevelopmental genes and implying an epigenetic mechanism for the inheritance of these acquired traits as a result of environmental lead exposure (Wu et al., 2024).

Studies show that exposure of *Drosophila* to acrylamide delays larval growth and causes developmental toxicity by affecting egg chamber morphology and gene expression related to neuronal development, disrupting microtubule organization, and affecting essential egg polarity genes such as *Oskar* and *Gurken*. This exposure also induces oxidative stress-induced neuronal damage, leading to movement disorders and inhibition of cholinergic neurotransmission due to reduced acetylcholinesterase (AChE) levels, thereby highlighting its potential neurotoxicity and impact on neuronal health (Senthilkumar et al., 2020). The sex-linked recessive lethal test has shown that acrylamide has mutagenic effects in the germline, resulting in lower fertility, sterility, and increased death, furthermore, acrylamide treatment in larvae resulted in single and twin spots in adult wings, indicating mutagenicity in somatic cells as well (Tripathy et al., 1991).

Neves et al. (2022) investigated the impact of paraquat (PQ) on *Drosophila melanogaster* and found age-related differences in susceptibility to PQ-induced parkinsonism, with 15-day-old flies more susceptible than 2-day-old flies, showing signs of bradykinesia and reduced survival, emphasizing the need to consider developmental stages in understanding environmental toxins impact on NDDs. Early-life exposure of *Drosophila* to PQ alone decreased the brain's dopamine level, which caused mobility defects comparable to PD symptoms, such as bradykinesia and a resting tremor. This dopamine depletion is essentially linked to the manifestations of PD in flies (Phom et al., 2014).

Studies in female *Drosophila melanogaster* have shown that exposure to rotenone (ROT) causes severe reproductive and developmental disorders. ROT exposure leads to ovarian

abnormalities, including impaired border cell growth and nurse cell misorientation, altered expression of developmental genes, and suppression of KIF-5B motor protein levels; increased caspase activity and apoptosis were also observed. In third-instar larvae, ROT exposure resulted in behavioral defects, developmental delays, and decreased levels of dopamine, suggesting that ROT may contribute to neurodevelopmental disorders (Kumar et al., 2022), with dopamine (DA), a chemical that is biogenic and regulates the nervous system, which has a regulatory role in insects, specifically in the neuronal networks that control movement, might be involved in the observed gradual and permanent decrease in movement (Silva et al., 2020). Additionally, ROT exposure in early health span flies resulted in a notable decrease in DA levels, a significant reduction in mitochondrial complex I activity, and locomotor defects similar to those observed in human PD patients (Ayajuddin et al., 2022).

Toluene exposure resulted in delayed developmental stages, decreased hatching rates, increased larval and pupal durations, and decreased pupal and adult percentages. The presence of toluene significantly affected the length and width of the larvae and pupae, reducing the levels of antioxidant enzymes like catalase, glutathione-S-transferase, and superoxide dismutase (Pb et al., 2020).

ASD patients show abnormal axon pathfinding (McFadden and Minshew, 2013), a phenomenon also observed in *Drosophila* when exposed to bisphenols (BPs), which affect the β lobe of the mushroom body leading to midline crossing defects (Nguyen et al., 2021), where mushroom body in *Drosophila* is crucial for learning, memory, and behaviors such as sleep, aggression, and courtship (Modi et al., 2020). These defects in axon pathfinding, linked to BPA exposure, correlate with learning and memory impairments in *Drosophila*, highlighting the need for further investigation into the relationship between BP exposure and mushroom body β-lobe abnormalities.

Exposure to BPs delayed larval development and altered axonal growth, resulting in aberrant midline crossing of axons in the β lobules of *Drosophila* mushrooms. However, the alternative bisphenols BPE and BPF did not produce significant damage (Wang et al., 2023). Embryonic exposure to bisphenol A (BPA) causes developmental and behavioral problems, including reduces levels of DA and tyrosine hydroxylase (TH) activity due to oxidative damage, mimicking neurodevelopmental diseases such as autism and ADHD. These findings highlight the importance of BPA's impact on DA pathways, as exposure in parental flies suggests a transgenerational effect via epigenetic alterations and a possible relationship to neurological disorders (Musachio et al., 2021).

1.4.2. *Mouse as a model to study developmental neurotoxicity*

Mice have been extensively used in biomedical research since the sixteenth century, with pioneers such as William Harvey and Robert Hooke pioneering the field. By the nineteenth century, scientists were exploring genetic traits, laying the groundwork for Mendelian genetics, and in the early twentieth century, standardized strains such as DBA/2 and C57BL/6 emerged. These improvements have allowed researchers to investigate neuropsychiatric diseases, synaptic impairments, and genetic and environmental interactions in disorders such as autism and schizophrenia, while gene editing tools have enhanced the precision of mouse models in translational neuroscience (Hedrich and Bullock, 2004).

In mice, developmental exposure to chlorpyrifos can cause various neurobehavioral consequences in addition to cognitive abnormalities, with time of exposure being critical. Mice carrying the C57BL6 gene were exposed to low concentrations of chlorpyrifos between GD12 and GD15, resulting in offspring with a delayed onset of the neonatal reflex and early-onset autism-like behavioral characteristics such as repetitive behavior, decreased social interaction, and limited exploration of new objects (Lan et al., 2017).

A recent study observed that early postnatal exposure (PDN 5-19) of Swiss mice to a combination of the herbicide PQ and the fungicide maneb (MB) resulted in a state of sensitization that lacked the hallmarks of toxicity. Despite no observed adverse effects on mortality, motor-related parameters, oxidative stress, or inflammation at a young age (PDN 30), there were reductions in the amount of TH or DAT in the striatum and *substantia nigra*, as well as a reduction in the activity of NADH dehydrogenase (complex I) and complex II. This sensitivity made them more susceptible to re-exposure to the same pesticide mixture throughout adulthood than mice exposed only during postnatal days or adulthood. As a result, the mice that were postnatally exposed exhibited a severe decrease in the amount of TH and DAT in the *substantia nigra* and a deficiency in movement when re-exposed to the pesticide mixture in adulthood implying to the pathogenesis of PD (Colle et al., 2020).

A study found that administering iron to B6D2 mouse pups during the neonatal period (PND10-17) did not result in dopamine depletion or loss of TH^+ DAergic neuron phenotype in the *substantia nigra* at a young adult age (2 months). However, at later ages (12, 16, and 24 months), an increase in neuron loss was observed following exposure to MPTP, a neurotoxin; this suggests that iron exposure in neonatal life increases neurons susceptibility to subsequent neurotoxin exposure during adulthood, potentially increasing the risk of age-related neurodegeneration (Kaur et al., 2007).

Dieldrin exposure during both gestation and lactation caused latent alterations in mice's developing DAergic systems, which became apparent by MPTP exposure in adulthood. This resulted in increased DAT and VMAT2 expression, notably in males, as well as changes to DOPAC levels and regulatory gene expression. Subsequent MPTP exposure revealed an increased risk of PD, as evidenced by striatal DA decreases, increased GFAP

(Glial Fibrillary Acidic Protein) and α-synuclein levels, and an increased DAT: VMAT2 ratio, particularly in males (Richardson et al., 2006).

Thiruchelvam et al. (2002) observed that mice exposed to a combination of PQ and maneb (MB) during the neonatal period had lower locomotor activity at 6 weeks and 6 months old, with a significant decrease observed when PQ and MB were rechallenged at 6.5 months. This rechallenge also resulted in substantial reductions in striatal DA levels, DA turnover, and TH^+ DAergic cell counts in *substantia nigra pars compacta*, indicating ongoing nigrostriatal DA system lesions and increased susceptibility to environmental toxicants in adulthood. In a subsequent study, the same research group observed that MB increased the neurotoxicity of adults exposed to PQ in offspring, this resulted in the loss of nigral neurons of DA and a decrease in the level of DA and its metabolites in comparison to gestational or adult-only pesticide exposure. Notably, mice that are postnatally exposed (PND 5-19) to PQ and MB have a greater propensity to develop adverse effects when rechallenged with the same pesticides in adulthood, this suggests that combinations of pesticides during brain development may lead to long-term changes and increased susceptibility to neurotoxicity in adulthood (Cory-Slechta et al., 2005). Wu et al. studied the possible processes by which lead exposure enhances the progression of AD in mice, C57BL/6J and APP/PS1, through drinking water from a week before mating until the offspring were seven months of age, resulting in elevated blood lead levels, accelerated Aβ1-42 deposition, and aberrant alterations in blood-brain barrier proteins Zonula Occludin-1 and Claudin-5. Moreover, it changed the mRNA and protein expression of low-density lipoprotein receptor, amyloid beta precursor protein, and beta-secretase 1, elevated p-tau expression, and increased astrocyte activation. Aβ1-42 accumulation around cerebral blood vessels was also observed, indicating a possible cerebrovascular pathway for lead-induced AD (Wu et al., 2020).

1.5. *Nutraceutical-mediated prophylactic/therapeutic strategies in neurodegenerative diseases*

Nutraceuticals have been utilized for about 3000 years, as Hippocrates (460-377 B.C.) famously stated, "Let food be thy medicine and medicine be thy food". In the early 1900s, American food makers began incorporating iodine into salt to avoid goiters. In India, nutraceuticals are food components made from herbal or botanical raw materials used to mitigate or cure a wide range of chronic and acute diseases (Jadhav et al., 2024).

Stephen DeFelice coined the term "nutraceutical" in 1989, which combines "nutrition" and "pharmaceutical". Nutraceuticals comprise a wide range of edible items such as plant products, animal-derived vitamins and minerals, prebiotics, and probiotics that improve health (Puri et al., 2022). The Merriam-Webster Online Dictionary (2014) defines nutraceuticals as "a specially treated food, vitamin, mineral, herb, etc., that you eat or drink to improve your health" or "a foodstuff (as a fortified food or dietary supplement) that offers health benefits in addition to its basic nutrition" (Banerjee et al., 2021).

Nutraceuticals are currently a rapidly growing industry segment, showing an estimated compound annual growth rate (CAGR) of 7.5%. The global nutraceutical market is anticipated to surge from $241 billion in 2019 to $373 billion by 2025 (Soni et al., 2024). The fundamental goal of nutraceuticals is to deliver therapeutic benefits while limiting adverse effects. The development of nutraceuticals is a time-consuming and intricate process that involves the isolation of bioactive compounds, rigorous *in vitro* and *in vivo* testing, and clinical trials to demonstrate efficacy, despite this growth (Nwosu and Ubaoji, 2020). Levodopa continues to be the primary treatment for PD; however, long-term use can result in levodopa-induced dyskinesia (LID), which complicates disease management (Blosser et al., 2020; Lane, 2019). Additional therapies, including DA agonists, MAO-B inhibitors, and COMT inhibitors, are implemented to alleviate symptoms of PD (Armstrong and Okun, 2020). An additional technique that has been demonstrated to alter

the progression of the disease in patients with severe motor dysfunctions who are not responsive to medications is deep brain stimulation (DBS) (Armstrong and Okun, 2020). In rat models, chronic electrical stimulation of the subthalamic nucleus was demonstrated to protect DAergic neurons in the SNpc during preclinical investigations (Spieles-Engemann et al., 2010; Harnack et al., 2008). Despite the neuroprotective efficacy of DBS in animal models, a clinical study has demonstrated that it is unable to modify α-synuclein aggregation or DAergic neuronal degeneration in PD patients (Pal et al., 2018). In light of these challenges, there is an immediate requirement for alternative therapies, particularly those that have been shown to be promising in preclinical studies, such as natural products and phytochemicals (Ntetsika et al., 2021).

1.5.1. *Role of antioxidants and nutraceuticals in mitigating Parkinson's disease*

Nutraceuticals, including antioxidants, cell signaling regulators, anti-inflammatory chemicals, anti-apoptotic agents, and mitochondrial homeostatic regulators, have demonstrated the potential to decrease clinical markers in models of PD (Onaolapo et al., 2021). A combination of CoQ10 and creatine has been investigated in an MPTP mouse model of PD. Researchers supplemented mice with a diet containing 2% creatine and 1% CoQ10 one week before MPTP treatment (40 mg/kg body weight daily for 28 days through osmotic pumps). This regimen produced additive neuroprotective effects, including reduced DA depletion in the striatum, preservation of TH neurons in the SNpc, decreased lipid peroxidation, reduced pathological SNCA (α-synuclein) accumulation, and decreased loss of DAergic neurons (Jeong et al., 2018).

Other nutraceuticals, such as resveratrol, quercetin, curcumin, and chicoric acid, have shown significant effects in reducing oxidative stress, boosting antioxidant defenses, improving mitochondrial dynamics, and decreasing inflammation (Soni et al., 2024). For instance, resveratrol, known for its antioxidant properties, exhibits extensive

pharmacological effects, including anti-inflammatory and neuroprotective activities. Oral therapy with resveratrol or a resveratrol liposome (20 mg/kg/day) for 14 days protected DAergic neurons in PD rats, reduced overall ROS levels, and increased nigral tissue antioxidant capacity (Singh et al., 2019).

Protein aggregation, specifically of α-synuclein, causes neurodegeneration in PD (Yenisetti et al., 2023). However, nutraceuticals such as β-amyrin (Wei et al., 2017), chlorogenic acid (He et al., 2023), and lipoic acid-loaded gold nanoparticles (Piersimoni et al., 2020) induce autophagy and decrease protein aggregation. Additionally, phytochemicals like bisabolol (Javed et al., 2020), berberine (Dadgostar et al., 2022), and sesamol (Abu-Elfotuh et al., 2022) prevent excitotoxicity and apoptosis by regulating essential proteins and maintaining calcium balance.

Neurodegeneration in PD is exacerbated by inflammation, while nutraceuticals, such as polyphenols, can decrease levels of cytokines and neuroinflammation by specifically affecting important pathways like NF-κB and TLR4 (Pajares et al., 2020). Luteolin, a polyphenolic substance present in foods such as parsley, celery, and peppers, dramatically reduced ROS production and avoided declines in mitochondrial activity, CAT, and GSH in ROS-insulted primary neurons (Ikram et al., 2020).

PD is defined by an imbalance of neurotransmitters, primarily DA, where studies have indicated that nutraceuticals such as rosinidin (Alghamdi et al., 2023) and AtreMorine (Tancheva et al., 2020) can increase DA levels and provide neuroprotection. Combining PPARs (Peroxisome Proliferator-Activated Receptor), specifically PPAR-γ, with PGC1α improves mitochondrial biogenesis and development, and curcumin enhances the expression of these proteins and improves mitochondrial function (Bernardo et al., 2021).

The gut-brain axis is also a critical factor in the progression of PD, and dietary interventions such as the Mediterranean diet can enhance the composition of gut bacteria

and decrease inflammation (Chu et al., 2021). This emphasizes the potential of nutraceuticals in treating the progression of PD.

S-allyl cysteine (SAC), a sulfur-containing compound derived from garlic, has demonstrated protective actions against oxidative stress induced by MPP^+ in the striatum of C57BL/6J mice. Pretreatment with SAC (125 mg/kg i.p.) daily for 17 days followed by MPP+ administration (0.72 mg/kg i.c.v.) significantly attenuated MPP^+-induced striatal DA depletion (32%), blocked lipid peroxidation, reduced superoxide radical production, and improved locomotion impairment (35%) (Zhong et al., 2019).

Gallic acid and its derivatives have shown potential protective effects against neuronal damage by inhibiting mitochondrial apoptotic signaling molecules and scavenging ROS, including superoxide anions, hydroxyl radicals, and hydrogen peroxide (Shabani et al., 2020). Kaempferol has been demonstrated to protect C57BL/6 mice from MPTP-induced PD by slowing DAergic neuron death, increasing DA and DOPAC levels, and improving DA turnover, resulting in enhanced motor function (Shahpiri et al., 2016). Quercetin mitigates rotenone-induced toxicity by enhancing neuronal density, modulating DA metabolism, and reducing oxidative stress in the striatum (Josiah et al., 2022). Apigenin affects DAergic neurotransmission by reducing α-synuclein expression and increasing DA production through elevation of D2 receptor protein expression and TH (Anusha et al., 2017).

The aqueous extract of *Boerhavia diffusa* root has been demonstrated to repair embryonic toxicity and deficits in fertility, fecundity, lifespan, and antioxidant defense mechanisms caused by toluene in *Drosophila melanogaster* (Pb et al., 2020).

Acanthopanax senticosus extract (ASE), which contains a high polyphenol content, was reported to improve motor coordination in MPTP-induced PD mice (C57/BL6) over 15 days. ASE controls 128 proteins and modulates pathways such as insulin receptor

signaling, *PI3K/AKT* signaling, and *Fcg* receptor-mediated phagocytosis, indicating that it may be effective as an anti-PD dietary supplement (Li et al., 2023).

Given that early life is more vulnerable to neurotoxicity, extensive research and epidemiological studies have been conducted to understand induced neurological diseases and develop protective and therapeutic strategies. Natural compounds such as curcumin and other phenolic compounds can serve as therapeutic interventions due to their antioxidant properties. These substances have been found to improve disorders such as obesity, depression, and other neurological ailments by replenishing depleted brain dopamine. Various toxicity testing procedures should be applied to chemicals before they are marketed.

1.5.2. *Polyphenolic compounds as promising agents in Parkinson's disease neurotoxicity: Role of curcumin*

In 1815, Vogel and Pelletier extracted curcumin, a "yellow coloring material," from the rhizomes of *Curcuma longa* (turmeric). Their following discovery in 1842 identified turmeric as a complex combination from which pure curcumin oil could be efficiently separated. Later, in 1910, Milobedeska and Lampe recognized curcumin's structure as diferuloylmethane, also known as 1,6-heptadiene-3,5-dione-1,7-bis(4-hydroxy-3-methoxyphenyl), and synthesized it three years later (Nebrisi, 2021).

Curcumin's (Cu) neuroprotective benefits in PD have been well-researched in various animal models. For instance, Zbarsky et al. (2005) showed that it protects TH-positive neurons and striatal DA levels against 6-hydroxydopamine (6-OHDA)-induced neurodegeneration in PD mice. Cu's iron-chelating characteristics are also important in lowering oxidative stress and iron buildup in neuronal cells, which is especially

advantageous in diseases such as PD, where iron deposition in the *substantia nigra* contributes to neuronal degeneration (Rainey et al., 2019).

Du et al. (2012) demonstrated Cu's efficiency by reducing iron-positive cells in a 6-OHDA-induced PD model after Cu treatment. Cu combined with desferrioxamine, an iron-chelating agent, has been shown to protect against DAergic neuronal loss in PD models (Lv et al., 2014).

Wnt/β-catenin signaling pathway activation promotes brain neurogenesis and protects against neurodegeneration. Cu has been shown to promote this pathway, increasing cell survival and decreasing neuronal death in oxidative stress-induced neurodegenerative models of PD (Arredondo et al., 2020; Wang et al., 2017).

Cu can act as a stage-specific inducer for sustaining longevity when exposure occurs in subsequent intervals for degenerating neuronal cells (Soh et al., 2013).

Furthermore, Cu intervention has shown potential in alleviating PD symptoms caused by neurotoxicants such as MPTP and rotenone. It inhibits MAO-B activity, maintaining DA and DOPAC levels stable in the brain (Rajeswari and Sabesan, 2008). Cu has also been shown in recent research to protect DAergic neurons by lowering oxidative stress indicators and enhancing gut microbiota in MPTP and rotenone-induced PD models (El-Shamarka et al., 2023; Zhu et al., 2022; Motawi et al., 2020). A randomized, triple-blind, placebo-controlled pilot study demonstrated that the administration of Cu (80 mg/day) to PD patients (n = 30) resulted in diminished scores on the Movement Disorder Society-sponsored revision of the Unified PD Rating Scale Part III, suggesting an enhancement in quality of life (Ghodsi et al., 2022). Hence, the multifaceted neuroprotective properties of Cu emphasize its potential as a nutraceutical agent in preventing the progression of PD, providing promising opportunities for future study and clinical application.

1.6. *Conclusion*

Early life exposure to developmental neurotoxicants has shown neurodegeneration either during adult life stages or prepone the stage of onset of neurodegeneration in multiple disorders, including AD and PD. PD prevalence increases with age, yet pre-clinical studies often use younger animals that may not accurately model age-related neurodegeneration. Older animal models better replicate PD since aging is a crucial factor in the disease. Early detection can cause greater possibilities for protection in later life by understanding the developmental background and the essential process of neurotoxicity during animal development. Previously, in our research, a *Drosophila* model of sporadic PD established that Cu's neuroprotective efficacy is adult life stage-specific (ALSS). Cu was able to restore PD-associated mobility deficits and replenish brain dopamine levels only during the health phase, not the transition period (Ayajuddin et al., 2022; Das, 2022; Phom et al., 2014). This demonstrates a limitation of Cu as a therapeutic compound. No concrete evidence is available for the later life stages, which is an essential paradigm for developing therapeutic approaches for PD.

Drosophila's adult life is divided into three stages: health (no natural death), transition (a slight decline in the mortality curve with 10% death), and senescence (constant decline in the mortality curve from the end of the transition phase to the maximum life span) (Arking et al., 2002). The analysis of these stages in model organisms revealed varied gene expression patterns similar to those observed in equivalent human life stages (Arking, 2009, 2015; Pletcher et al., 2002).

Few investigations have been carried out in experimental animals regarding prenatal exposure to developmental neurotoxins that contribute to the development of PD later in life. It is crucial to have a fundamental understanding of these features in both sporadic and genetic PD model systems. This will help us understand the cellular and molecular

basis for PD, identify therapeutic targets, and develop early-life preventive measures to reduce the burden of neurological diseases like Parkinson's. Understanding the impact of early life insults on the late-life development of PD in *Drosophila* models would aid in determining the relative contributions of genetic and environmental factors to this neurodegenerative disorder.

Generating basic insights in this area would enable us to develop intervention and preventative strategies early in life to dampen or prevent any adverse late-life outcomes. Nutraceutical interventions, such as antioxidants and mitochondrial function enhancers, can be introduced during early life to mitigate the impact of prenatal/early life insults and reduce the risk of neurodegeneration in later life. Further, these interventions will facilitate the identification of therapeutic targets that aid in developing novel therapeutics to reduce the burden of PD. The choice of a specific model during research is a crucial step, and our review highlights the knowledge gaps regarding the effects of prenatal/early life insults and nutraceutical interventions, reinforcing their importance in the design of experiments focused on neuroprotection across the lifespan.

Review of the literature illustrates that Cu fails to confer DAergic neuroprotective efficacy during the adult transition phase, and thus far, no study has been performed to sustain the Cu efficacy during the late phase of adult life, which is critical to use Cu as a therapeutic agent for late-onset NDD such as PD. Hence, the objectives of the present study are as follows:

1. Sustaining the curcumin neuroprotective efficacy during the adult transition phase in the *Drosophila* model of PD- Implications to human health.

2. Prenatal developmental feeding of curcumin rescues paraquat-induced mobility defects in the adult transition stage of a *Drosophila* model.

CHAPTER 2

MATERIALS AND METHODS

2.1. Fly stock

Oregon-K strain of *Drosophila melanogaster* flies was acquired from the National Stock Centre, University of Mysore, Karnataka, India. Only male flies were utilized in the present study.

2.2. Fly husbandry

The *Drosophila* flies were kept in a regulated environment within a Percival DR41VL incubator (USA), maintaining a consistent temperature of 22±2℃ and humidity at 65±5%. The flies were fed with standard culture media (**Section 2.3**). The circadian rhythm was upheld with alternating 12-hour light and dark cycles.

2.3. Preparation of sucrose agar media

Materials: Sucrose (SRL, India, Cat: 84973), Yeast (Angel, instant dry yeast), agar-agar (Himedia, India, Cat: GRM666), and propionic acid (MERCK, Cat: 8006050 500 1730) were used for media preparation.

Methods: The media was prepared following the protocols of Phom et al. (2014).

Dissolved 27 g of sucrose, 16 g of yeast, and 1.5 g of agar-agar in double distilled water and allowed it to soak briefly. The mixture was then boiled in an induction cooker for a total of 12 minutes, stirring every 4 minutes. Following the cooking process, 1.78 mL of propionic acid was incorporated into the media. Finally, the prepared media was poured into vials.

2.4. Preparation of Delcour agar cups

Materials: Agar Agar (Cat. No. GRM666) procured from HiMedia (Thane, India), absolute ethanol (Analytical CS reagent, Jiangshu Province), glacial acetic acid (Cat. No. 61780705001730) procured from MERCK (Rahway, USA), market-available sugar tolerant dry yeast (Angel, instant dry yeast), plastic container (15 cm x 7 cm) for use as egg collection chamber, Delcour cups (2.5 cm diameter and 1.5 cm height), wooden rod (20 cm), fine paintbrush, spatula, needle, stainless surgical blade, adhesive tape, and stereo-binocular microscope.

Methods: **Figure 2.1** illustrates the set-up of the Delcour container. Delcour agar cups were prepared using 3% agar-agar. The agar-agar was added gradually to warm water while stirring constantly to prevent clumping. Subsequently, 2.5 mL of absolute alcohol and 1.5 mL glacial acetic acid were dissolved in the boiling water, and the contents were mixed thoroughly until the agar melted into the solution. The resulting media was poured into the cups with a few seconds between each pour to facilitate faster solidification until a convex surface was formed. After approximately one hour of waiting for solidification, a very thin layer was cut from the media surface using a stainless blade, creating a slightly rough surface for the flies to lay eggs. Quarter sections were then made on the surface of Delcour agar for easier counting of eggs before being introduced into the container containing the flies.

Figure 2.1. Set-up for Delcour container.

2.5. Preparation of yeast paste

Yeast paste was prepared by adding 20% yeast to distilled water in a falcon tube to achieve a thick consistency. The mixture was dissolved until proper fermentation was observed.

2.6. Flies for embryo collection

Young adult flies, approximately 10 days old, were starved for about 4 hours in a container designated for egg collection.

2.7. Experimental set-up for the embryo collection following the Delcour methodology (1969)

A plastic container served as the fly chamber, and a hole was created on the lid for a cotton plug to allow aeration and prevent excessive humidity for the flies (**Figure 2.2**). The cups were attached to a wooden rod in a zig-zag pattern, leaving some space between them and securing them with adhesive tape. Yeast paste was evenly spread with a spatula on the rough surface of the agar cups. The rod, with attached cups, was introduced into the container containing the flies. Gently tapping the container caused the flies to fall off, and the rod was immediately inserted, closing the container's mouth with the palm. A second gentle tap is followed by plugging the container with cotton. Starved flies were then fed on the yeast-agar medium for the required number of hours at the desired temperature, depending on experimental requirements.

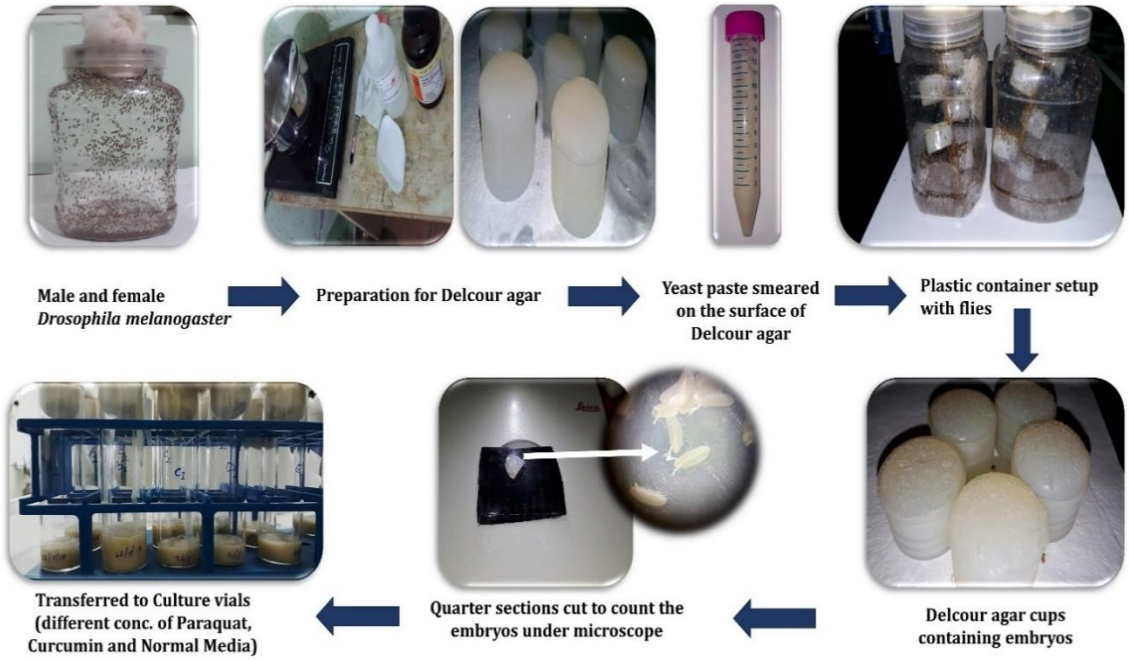


Figure 2.2. Set up for egg collection and culturing of *Drosophila melanogaster* following Delcour method (1969).

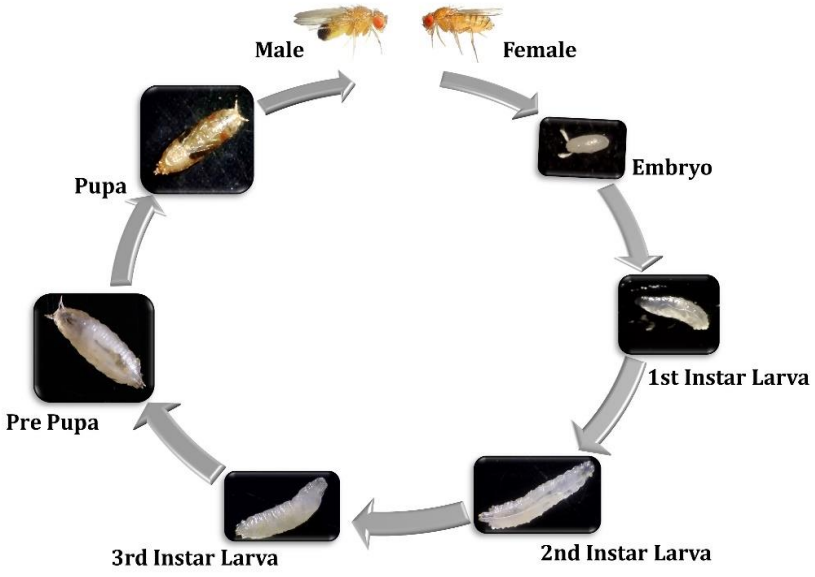


Figure 2.3. *Drosophila melanogaster* life cycle.

2.8. *Drosophila* embryo collection and handling

The following day, gently detach the cups from the wooden rods by tapping off the flies from the container. Delcour recommends discarding the initial batch of eggs obtained during the first hour post-starvation due to the potential variation in developmental stages. This suggestion is based on the understanding that these eggs may exhibit diverse developmental stages. In contrast, eggs laid after a specific preliminary period demonstrate a high degree of uniformity. Now, take a one-quarter section of the agar with a fine needle and transfer it under the microscope against a dark background. Count the required number of embryos using a small paint brush and needle, carefully separating any clustered embryos. Transfer the counted embryos to separate media vials and, add one to two drops of yeast paste to maintain moisture. Flies begin to emerge after ten days.

2.9. Developmental scoring of embryo, pupae and adult of *Drosophila*

In this study, *Drosophila melanogaster* embryos, larvae, and pupae were carefully collected and staged to analyze developmental progression. Embryos were obtained through timed egg laying, and their precise developmental stages were determined using established morphological criteria (Roberts, 1998; Ashburner, 1989). Larvae and pupae were subsequently selected at specific time points following eclosion into flies. Imaging was conducted using a stereo-binocular microscope, and representative images were captured to illustrate the developmental stages of *Drosophila*, as depicted in **Figure 2.3**.

2.10. Collection and aging of fly

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

Methods: The fly from the propagation vial was carefully tapped and transferred into a glass bottle. A few drops of diethyl ether were administered to induce mild anesthesia,

ensuring the flies safety by tapping the bottle gently. Once anesthetized, the fly was delicately relocated onto a glass plate. Under microscopic observation, males and females were carefully separated using a brush. Subsequently, 25 male flies were transferred to a new vial filled with fresh media, with careful attention to prevent sticking to the food media. Aging of the flies was facilitated by transferring them to fresh vials containing media every three days until they were ready for experimentation.

2.11. Preparation of paraquat stock and adult fly exposure

Materials: Methyl viologen dichloride hydrate /Paraquat (PQ) (Cat. No. 856177) and Sucrose (SRL, India, Cat: 84973) were used for preparing the PQ stock.

Methods: To prepare a 10 mM PQ solution, the required amount of PQ was dissolved in a 5% sucrose solution. Whatman filter paper no.1 disc in a 30x100 mm glass vial was preferred as a feeding medium for exposure. A minimum of 100-150 flies were exposed per group for 24 hours, with 25 flies per vial (Phom et al., 2014).

2.12. Preparation of curcumin stock and adult fly feeding protocol

Materials: Agar Agar (Cat. No. GRM666), Curcumin (Cu) (Sigma, Cat: 1386), Dimethylsulfoxide (DMSO) (Sigma, Cat: D8418), and Sucrose (SRL, India, Cat: 84973) were used for feeding the fly.

Methods: A stock solution of Cu was made by dissolving 4 mg in 20 µL of DMSO, resulting in a stock concentration of 543 mM. The desired concentration was further diluted in a solution containing 5% sucrose.

Cu 1 mM was prepared from the original Cu stock of 543 mM. Sucrose- agar media was used to prepare Cu media. The required amount of Cu was added after the media was

cooked and allowed to cool down to room temperature, mixed with 30ml of food media for the final concentrations of 100 µM and 10 µM Cu.

2.13. Preparation of culture media supplemented with curcumin

Counted 100 Delcour eggs were transferred to each vial and allowed to develop in diets consisting of nine varying concentrations of Cu, ranging from 5 µM to 5 mM (5 µM Cu, 10 µM Cu, 25 µM Cu, 50 µM Cu, 100 µM Cu, 500 µM Cu, 1 mM Cu, 2.5 mM Cu, and 5 mM Cu). The working stock was prepared in sucrose-agar media. For preparing concentrations of 500 µM and below diets, the working stock was 1 mM Cu, which was derived from the original 543 mM stock. For the 1 mM and 2.5 mM concentrations, the working stock was 5 mM Cu, also derived from the original 543 mM stock. The 5 mM concentration was prepared directly from the original 543 mM curcumin stock.

Sucrose-agar media was used as the base to prepare the curcumin-infused diets. The required amount of curcumin was added after the media had been cooked and allowed to cool to room temperature. This mixture was then combined with 30 mL of sucrose-agar media to achieve the desired final concentrations. Finally, all media were dispensed into four vials for each experimental group.

2.14. Preparation of culture medium containing neurotoxicant PQ

Counted 100 Delcour eggs were transferred to each vial and allowed to develop in diets consisting of five varying concentrations of PQ, ranging from 1 mM to 7.5 mM (1 mM PQ, 1.5 mM PQ, 2.5 mM PQ, 5 mM PQ, and 7.5 mM PQ). A stock solution of 100 mM PQ was then prepared. Sucrose-agar media was used as the base for preparing the PQ media. The required amount of PQ was added after the media had been cooked and allowed to cool to room temperature. This mixture was then combined with 30 mL of

sucrose-agar media to achieve the desired final concentrations. Finally, all media were dispensed into four vials for each experimental group.

2.15. Preparation of culture medium for developmental PQ-Cu co-feeding regime

A working stock of 100 µM Cu was prepared from the original Cu stock of 543mM for the preparation of 25 µM Cu and 5 µM Cu media *per se*. 1 mM PQ *per se* was prepared from a stock solution of 100 mM PQ. Co-feeding treatments were achieved as follows: for 1 mM PQ + 5 µM Cu, 1.5 mL of 100 µM Cu and 300 µL of 100 mM PQ were added to a final volume of 30 mL media. For 1 mM PQ + 25 µM Cu, 7.5 mL of 100 µM Cu and 300 µL of 100 mM PQ were added to a final volume of 30 mL media. Control groups consisted of sucrose-agar media without PQ or Cu. Finally, all media were dispensed into four vials for each experimental group. Counted 100 Delcour eggs were transferred to each vial and allowed to develop in their respective diets.

2.16. *Drosophila* longevity assay

The lifespan of male flies was evaluated using the methodology described by Phom et al. (2014). Twenty-five flies were placed in each vial, which contained 3 mL of medium, after being newly eclosed and transferred to a fresh culture medium. A census was conducted, and all flies were transferred to a new medium every third day while recording any instances of mortality. Survivorship was observed and reported every 24 hours until all flies died, with a total of 100 flies used at each concentration.

2.17. Assessing of viability of developmental stages to adult fly

Eggs of the same age (± 6 hours) were placed in equal numbers (100 eggs per vial) into normal sucrose-agar media as well as media containing different concentrations of Cu and PQ and were allowed to develop. The newly hatched larvae were continuously fed on these media. The rate of development and viability from egg to pupae and from pupae

to adult were analyzed after all the adult flies emerged. As the pupae began to darken, indicating impending eclosion, the vials were regularly checked for the emergence of adult flies. The number of adult flies that emerged was observed and recorded and only the male flies were transferred to fresh sucrose-agar media for experimental purposes. This process continued until no further eclosion was observed for three to four consecutive days or until all flies had emerged. The total number of flies that emerged from each vial was used to calculate the viability for each treatment group. Subsequently, 100-150 flies from each group were aged appropriately to proceed with stage-specific experiments.

2.18. Experimental feeding regime and exposure protocols in stage-specific *Drosophila* model of sporadic PD

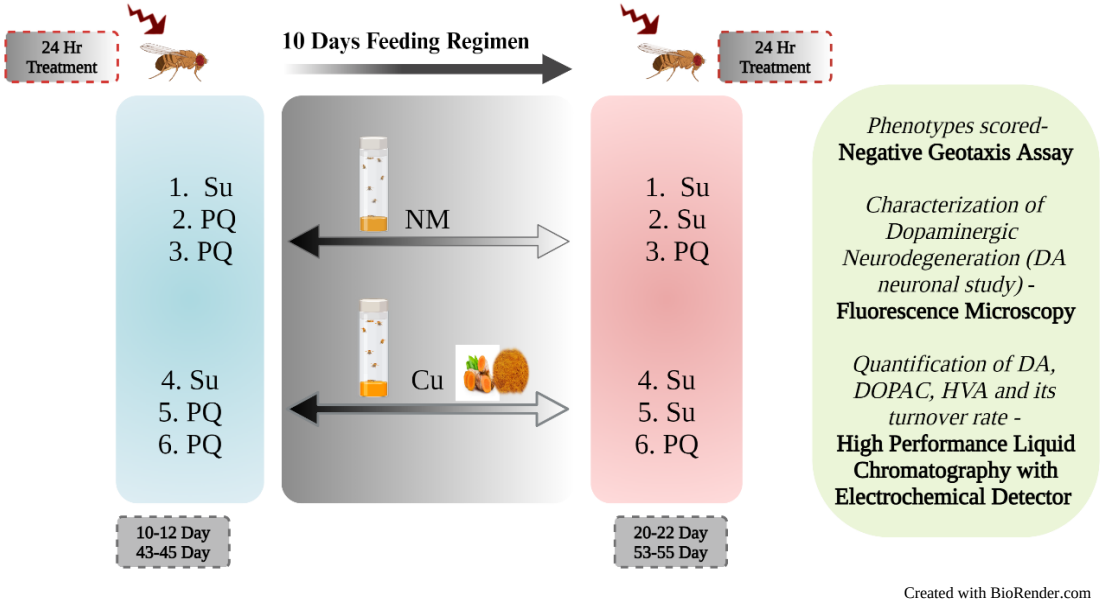


Figure 2.4: *Drosophila* feeding regime. In this experimental design, male *Drosophila* flies (10-12 days and 43-45 days old) were divided into six groups to study the effects of early and late-phase exposures to sucrose (Su) and paraquat (PQ). Initially, flies were exposed to either 5% sucrose or 10 mM PQ for 24 hours on Whatman filter paper, followed by a 10-day feeding on either normal sucrose-agar medium (NM) or 100 µM curcumin (Cu) medium. Flies were then re-challenged with either 5% sucrose or 10 mM PQ for 24 hours on Whatman filter paper at 20-22 days and 53-55 days old. The six experimental groups included various combinations of early and late-phase treatments with sucrose or paraquat, and with or without curcumin. Behavioral tests, dopaminergic neurodegeneration analysis using fluorescence microscopy, and dopamine metabolite quantification via high-performance liquid chromatography were conducted to assess motor symptoms and neurodegenerative changes in the fly model of PD.

Early Health and Early Transition Phase Exposure

Male *Drosophila* flies of 10-12 day and 43-45 day old were divided into six experimental groups. Each group was exposed to 275 µL of 5% sucrose (Su) or 10 mM Paraquat (PQ) for 24 hours on Whatman filter paper. Specifically, each set of three groups consisted of one group exposed to 5% Su and two groups exposed to 10 mM PQ. After this initial exposure, the flies were transferred to different media for 10 days. The first set of three groups (one exposed to sucrose and two exposed to PQ) was switched to a sucrose-agar normal medium (NM), while the second set of three groups (one exposed to sucrose and two exposed to PQ) was switched to a 100 µM Curcumin (Cu) medium.

Re-challenge Exposure at Late Health and Late Transition Phase

Flies previously exposed to 5% Su or 10 mM PQ during the early health and transition periods, and subsequently fed on either NM or Cu medium, were re-challenged at two different ages. At 20-22 days old and again at 53-55 days old, the flies were exposed to 275 µL of 5% sucrose or 10 mM PQ for 24 hours on Whatman filter paper.

This experimental design consisted of six groups, as follows:

i. **Su // NM // Su**: Treated with sucrose during the early health phase and re-challenging at the late health phase, received normal medium for 10 days.
ii. **PQ // NM // Su**: Treated with neurotoxicant during the early health phase and re-challenging with sucrose during the late health phase, received normal medium for 10 days.
iii. **PQ // NM // PQ**: Treated with neurotoxicant during the early health phase and re-challenging at the late health phase, received normal medium for 10 days**.**
iv. **Su // Cu //Su**: Treated with sucrose during the early transition phase and re-challenging at the late transition phase, received curcumin medium for 10 days.

v. **PQ // Cu // Su**: Treated with neurotoxicant during the early transition phase and re-challenge with sucrose during the late transition phase, received curcumin medium for 10 days.

vi. **PQ // Cu // PQ**: Treated with neurotoxicant during both the early transition phase and re-challenge during the late transition phase, received curcumin medium for 10 days.

The flies were first subjected to behavioral tests on days 20-22 and at 53-55 days old (negative geotaxis assay) after the re-challenged exposure to detect potential motor symptoms commonly observed in experimental models of PD. Additionally, DAergic neurodegeneration in the whole fly brain was characterized and quantified through fluorescence microscopy following the protocol described by Chaurasia et al. (2024) and Ayajuddin et al. (2023). The levels of dopamine and its metabolites were quantified using high-performance liquid chromatography as outlined by Ayajuddin et al. (2021, 2023) and Das (2022).

2.19. Experimental design for *Drosophila* exposure on developmental neurotoxicity studies

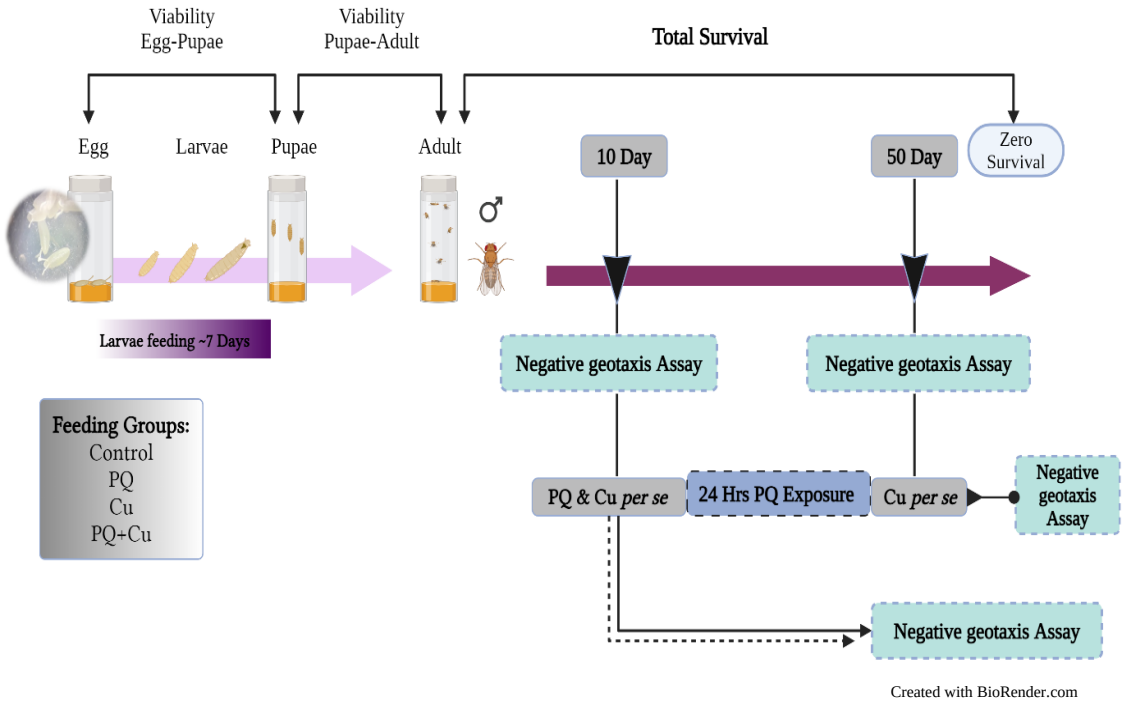


Figure 2.5: Experimental setup for the prenatal feeding regime in *Drosophila*. The figure illustrates *Drosophila* eggs exposed to different treatment groups: Normal Media (NM), Paraquat (PQ), Curcumin (Cu), and PQ+Cu. Egg-to-pupae and pupae-to-adult viability were recorded. After eclosion, adult flies were aged on normal media. Motor function was assessed using negative geotaxis assays during both the health phase (HP) i.e.,10th day, and the transition phase (TP) i.e.,50th day. During HP, the first experiment assessed the motor function of flies that were only prenatally fed. In the second experiment, the Cu and PQ per se groups were exposed to 10 mM PQ for 24 hours on Whatman filter paper, followed by a negative geotaxis assay the next day. In the third experiment, the same Cu and PQ per se groups, initially exposed on their 10th day, were assessed for motor function on their 50th day (TP). Additionally, during the TP, negative geotaxis assays were conducted on the prenatally fed groups to assess motor function. A second experiment was carried out with the Cu per se group, where flies were exposed to 10 mM PQ for 24 hours on Whatman filter paper. Mortality rates were also monitored throughout the experiments.

Following the Delcour method (Delcour, 1969), *Drosophila* eggs were exposed to various treatment groups, including Normal Media (NM) as a control, paraquat (PQ), curcumin (Cu), and a combination of PQ+Cu media. Egg-to-pupae and pupae-to-adult viability were assessed and recorded. After the emergence of the flies, they were fed and aged on normal media throughout their lifespan.

A. During their health phase (HP), at 10 days old, a negative geotaxis assay was conducted to examine the impact of feeding.

   Three separate experiments were conducted during this phase:

   (i) The first involved the negative geotaxis assay performed in the group that received no exposure and was only prenatally fed.

   (ii) The second experiment involved the Cu and PQ *per se* groups, exposed to 10 mM PQ for 24 hours on Whatman filter paper. The negative geotaxis assay was conducted on the following day.

   (iii) In the third experiment, the same Cu and PQ *per se* groups that were initially exposed to 10 mM PQ on their 10th day, a negative geotaxis assay was performed on the 50th day, i,e., the transition phase.

B. Additionally, during the transition phase (TP),

   (i) a negative geotaxis assay was conducted for the prenatally fed groups alone,

   (ii) a second experiment involved the Cu per se group, where flies were exposed to 10 mM PQ for 24 hours on Whatman filter paper. Mortality was assessed throughout the experiments.

2.20. Negative geotaxis assay

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

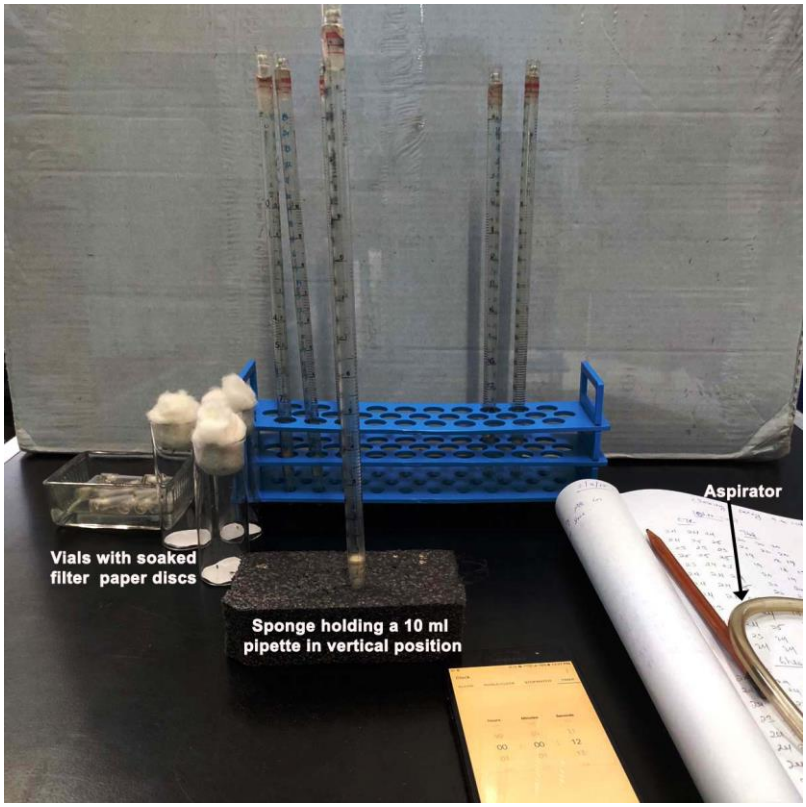


Figure 2.6: Experimental set-up for negative geotaxis assay (adapted from Phom et al., 2014, 2021).

Methods: The climbing ability was evaluated with minimal modifications from Botella et al. (2004). The motor ability of flies was assessed using a negative geotaxis assay. One experimental fly was dropped into a vertically fixed 10 mL pipette, closed with a cap, and acclimatized for 2 minutes. The fly was subsequently tapped to the base, and the distance it climbed in 12 seconds was recorded. This procedure was repeated three times for each fly, with 12-15 flies tested per treatment group (**Figure 2.6**).

2.21. Quantification of dopaminergic neurons and fluorescence intensity using fluorescence microscopy

2.21.1 TH Immunostaining

Sterilized 1.5 mL centrifuge tubes (Tarsons, WB, India, catalog number: 500010), Parafilm^{TM} 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^{TM} 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 (FacesCanada, 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^{TM} 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), -20°C ES Series refrigerator (Thermo Scientific, MA, USA, model: 50616100444443250), -80°C 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

   1× PBS 50 mL

   a. Add PFA in 1× 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 80 °C to 110 °C 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 but keep the stirring for 15 min. Allow the solution to cool down, aliquot it in a 1.5 mL centrifuge tube, and store it at -80 °C.

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 hand gloves and a lab coat during handling and preparation of PFA solution.

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

10× 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)

10× PBS 5 mL

Autoclaved enzyme-free water 45 mL

Triton X-100 250 µL

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

b. Mix 250 µL of Triton X-100 and vortex it for 10 s. 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

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

5. **Anti-TH polyclonal primary ab solution**

   Anti-TH polyclonal primary ab 5 µL

   5% NGS blocking buffer 1,245 µL

   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

   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.

2.21.2 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 immunostained 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 hours through mixing by using a test tube rotator with constant velocity (10 rpm) at room temperature (RT).

2. PFA was then removed after 2 hours 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 was 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. The brains were then washed with 0.1% PBST for 5 times after every 15 minutes at RT.

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

6. Then, the brains were incubated/probed with primary anti-TH polyclonal antibody in the dilution of **1:250** for 72 hours at 4°C through mixing by using a test tube rotator at constant velocity (10 rpm).

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

8. The brains were then incubated with a TRITC (Tetramethylrhodamine) labelled polyclonal secondary antibody in the dilution of **1:250** for 24 hours in the dark (**Critical**: Cover centrifuge tube containing brains with aluminum foil) by thorough mixing with a test tube rotator at a constant velocity (10 rpm) at RT.

9. Again, 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). **Critical:** 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 keeping glass spacers while mounting the brain with a cover slip.

5. To prevent the drying of the samples, the edges were carefully sealed with nail polish.

6. In order to prevent bleaching, image acquisition was carried out on the same day.

B) Image Acquisition

The *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 2.7 to figure 2.17**.

Methods:

1. At a 40x objective lens of fluorescence microscope, prepared/stained brains were viewed/observed (**Figure 2.7**).

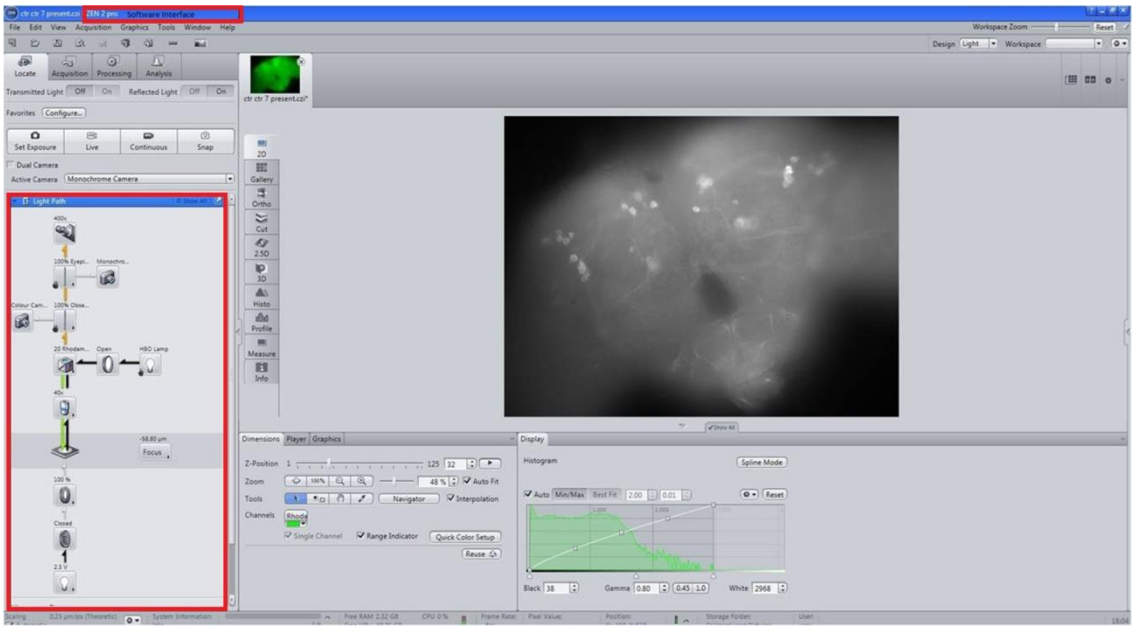


Figure 2.7. Scanning of the whole brain of *Drosophila*. Scan the anti-TH immunostained *Drosophila* brain using Carl Zeiss, Axio Imager M2 (40× objective lens) with ZEN 2012 SP2 software that interactively controls image acquisition, image processing, and analysis of the images.

2. Images were scanned and taken using a monochromatic camera with a Rhodamine fluorescence filter (**Figure 2.8**).

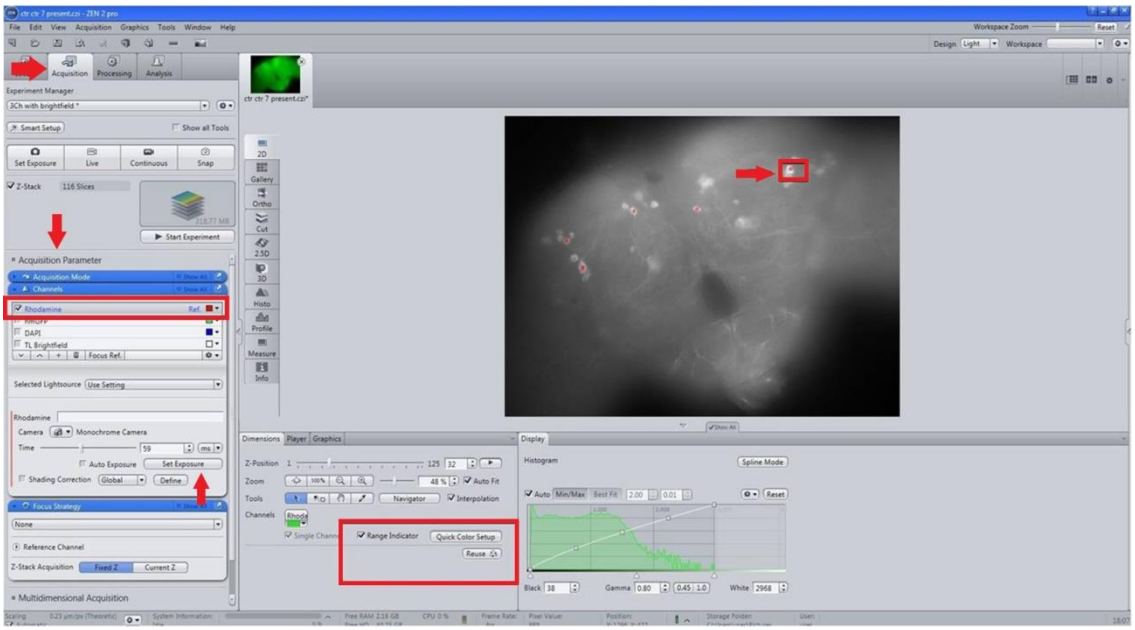


Figure 2.8. Image acquisition and performing the red dot test. For image acquisition, select a monochromatic camera with a Rhodamine filter. Perform a red dot test for visibility of DAergic neurons and assessing saturation using a brain, reusing the same exposure time for other samples.

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. Reuse the same exposure time for all brain samples (**Figure 2.8**).

4. Then, Z-stack programming was performed with constant interval of 1.08 µm for each image (**Figure 2.9**).

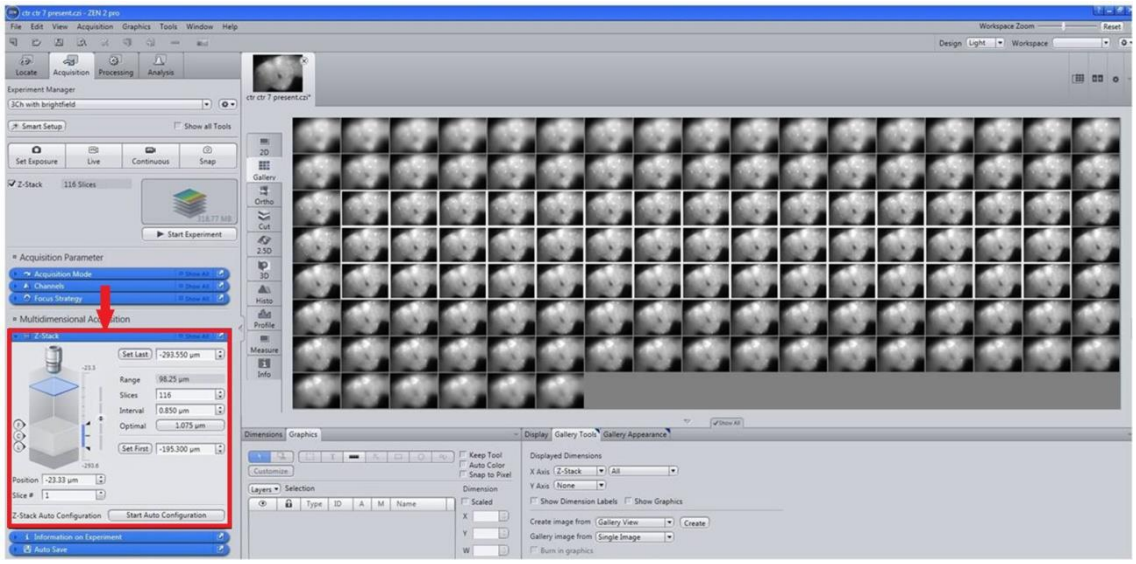


Figure 2.9. Selection of images and Z-Stacking.

5. For image processing/generating in 2D, on the method column apply *Ortho* and *Maximum intensity projection (MIP)* from *Ortho display* with *X–Y Plane* (**Figure 2.10**).

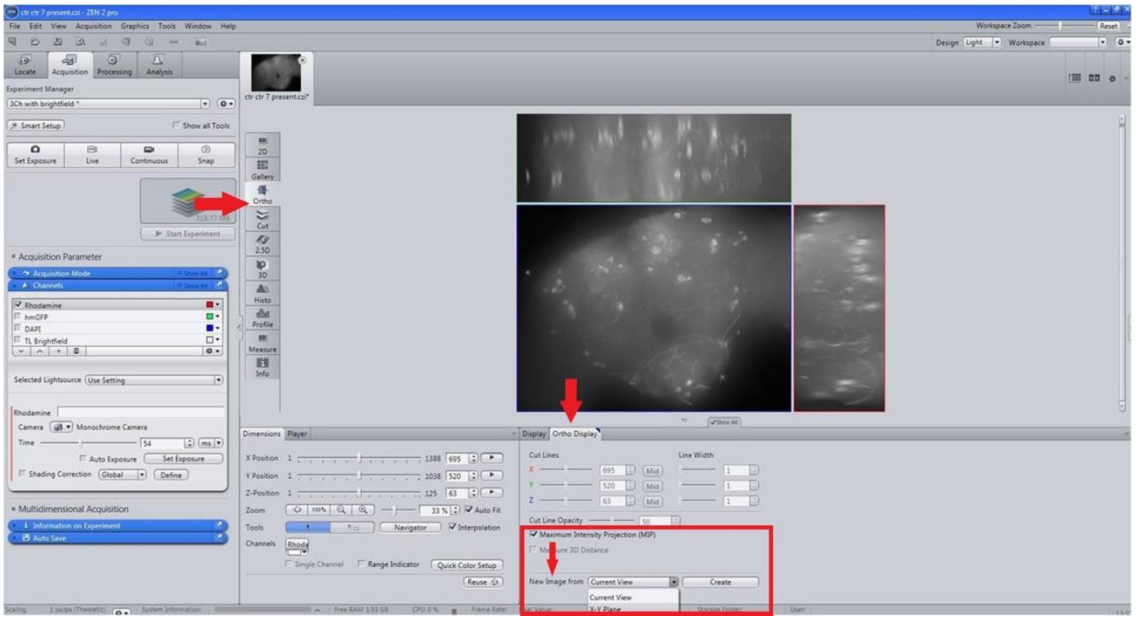


Figure 2.10. Creation of 2D image. For creating a 2D merged image, on the *Method* column, select *Maximum intensity projection (MIP)* with *X-Y Plane.*

6. The 2D image of the brain was exported in.jpg format for presentation (**Figure 2.11**).

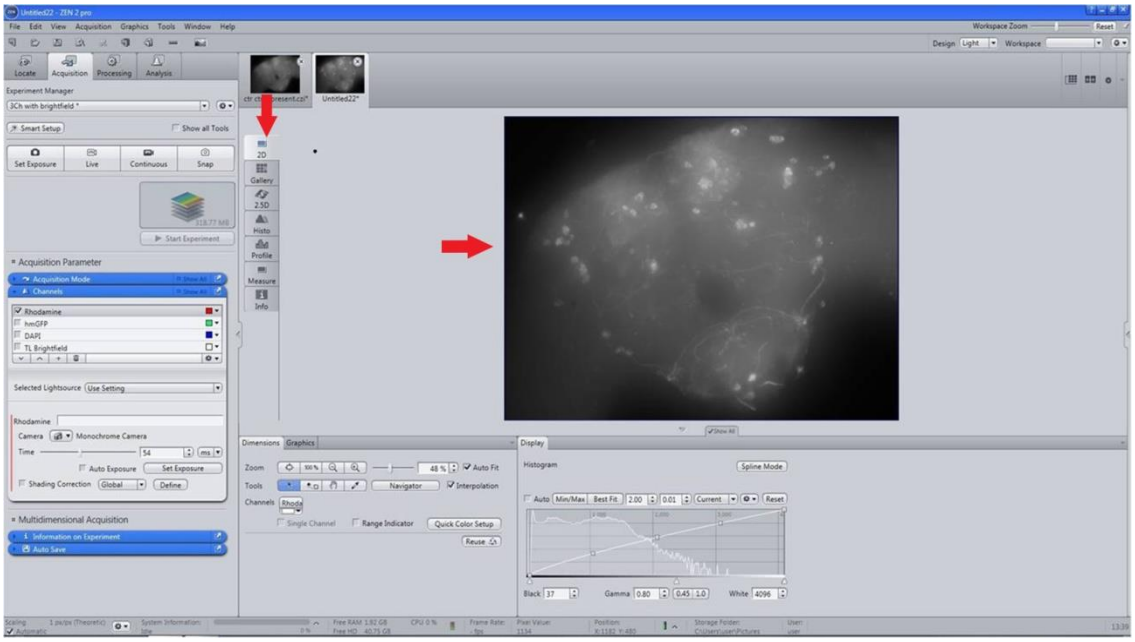


Figure 2.11. Export of 2D brain image to the required format.

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 always reused 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 DAergic neurons.

D) Quantification of the fluorescence intensity (FI) of secondary antibodies to characterise neurodegeneration/ neuroprotection

C) Quantification of the total number of DAergic neurons

In the past one and half a decade, to gain a better understanding, numerous *Drosophila* models have been reported elucidating the mechanisms of PD development, progression, and rescue strategies (Akinade et al., 2022; Ayajuddin 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 were similar to human PD. The DAergic neuronal system and its placements in the *Drosophila* brain were described using DA and anti-TH immunoreactivity (Nässel and Elekes, 1992; Budnik and White, 1988). 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 were presented in **Table 2.1**, and the *Drosophila* brain cartoon (**Figure 3.4A**) depicts the position of DAergic neurons in the fly brain.

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

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

Table 2.2: Summarization 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).

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

Methods:

1. Clusters were identified from the Z-stack images/scans by obtained through Z-stack programming with constant intervals (**Figure 2.12**).

2. The image was enlarged to reveal the cell body/structure (**Figure 2.12**).

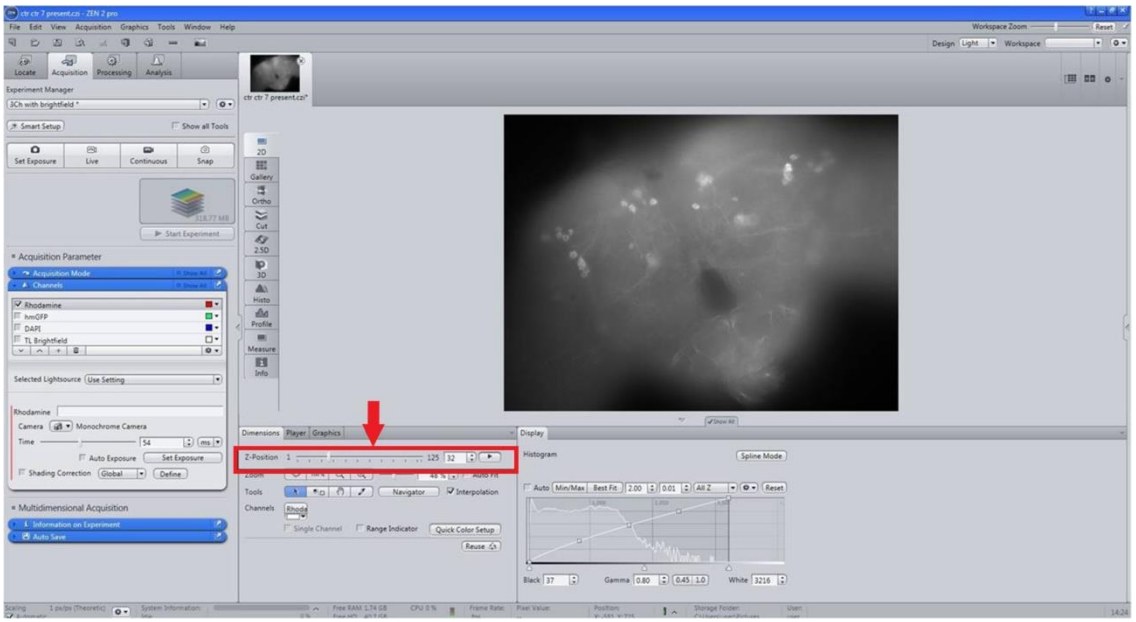


Figure 2.12. Quantification of DAergic neuronal number and fluorescence intensity (FI). For the quantification of DAergic neuronal number and FI, select 3D images/scans of Z-Stack with brain regions; PAL, PPL1, PPL2, PPM1/2, and PPM3 (PAL: Protocerebral anterior lateral; PPL: Protocerebral posterior 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/neuroprotection

The loss of DAergic neurons was observed differently depending on the method adopted (**Table 2.2**). 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 underlies the decrease in TH and denotes 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 attempted to investigate the DAergic neurodegeneration and Cu feeding neuroprotection 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 a 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 2.12**).

2. The brain images were enlarged to see the clear neurites (**Figure 2.13**).

Figure 2.13. 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 2.13**).

4. *More measurement options* were selected, and the *intensity sum* was chosen by right-clicking inside the neuron (**Figure 2.14**).

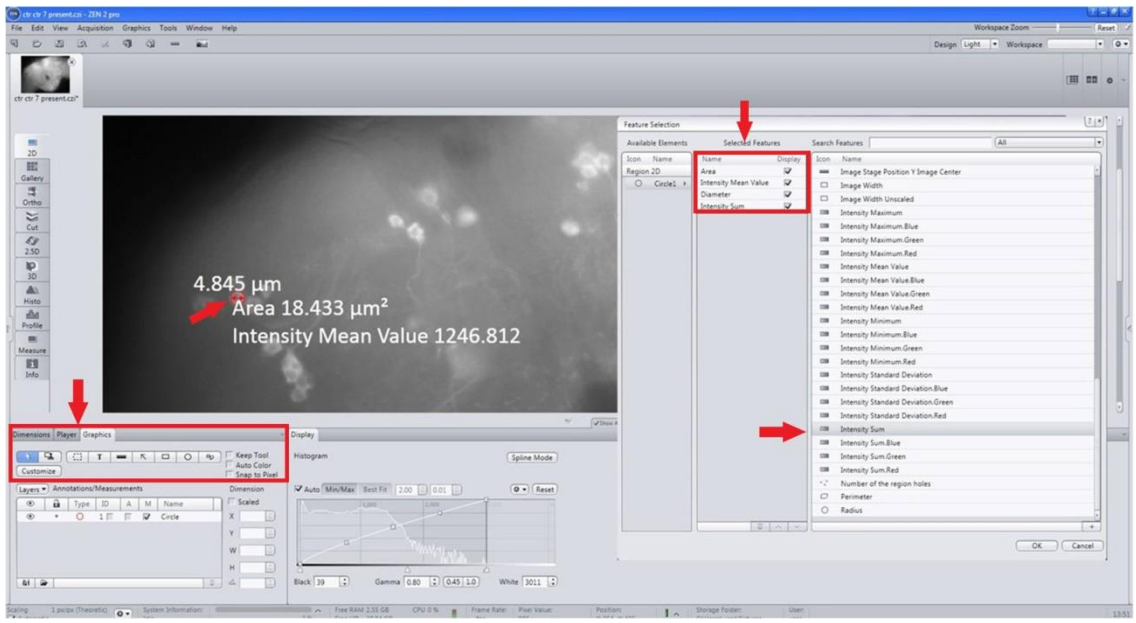


Figure 2.14. Measurement of fluorescence intensity (FI) sum. Select *intensity sum* by opting for *more measurement options* (software provides the pixel value upon right-clicking on the neuron).

5. *List*, *view all*, and *create document* were selected from the *measurement* tab on the left side of the panel (**Figure 2.15**).

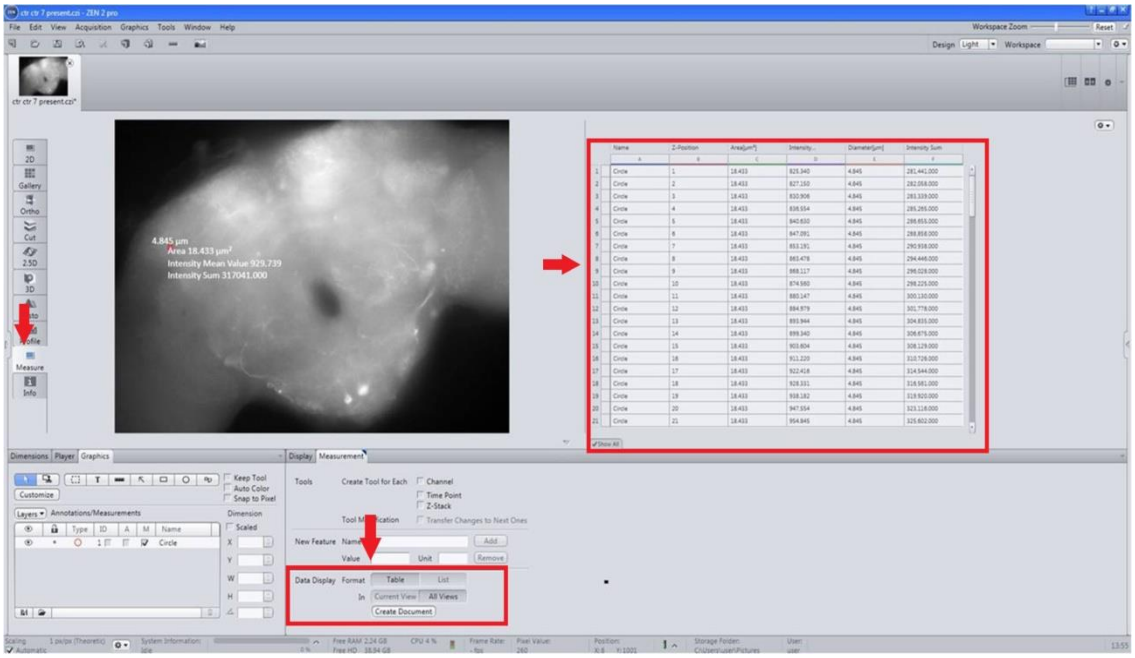


Figure 2.15. Fluorescence intensity (FI) compilation. From the *measurement* option select *list*, *All views*, and *create document*.

6. The area and FI sum were recorded for each scan of a neuron in .xml format (**Figure 2.16**).

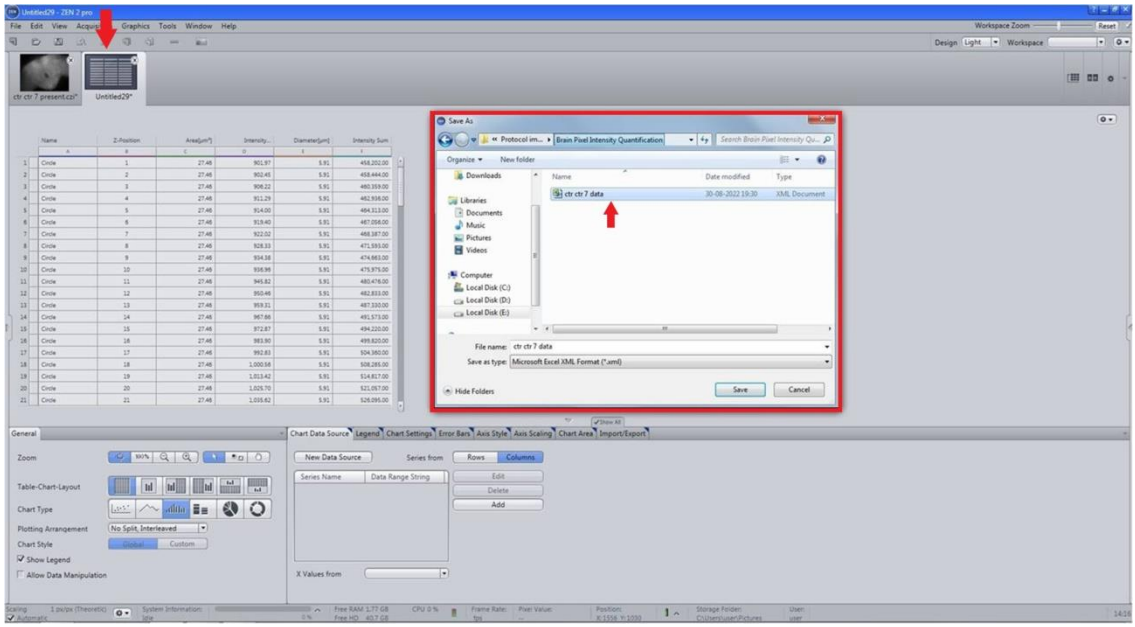


Figure 2.16. Measuring the FI sum for each scan of a neuron in .xml format

7. For quantification of FI of a single neuron, a total of eleven scans with an interval of 1.08 µm for each scan, meaning the cumulative of 11.88 µm width was considered (**Figure 2.17**).

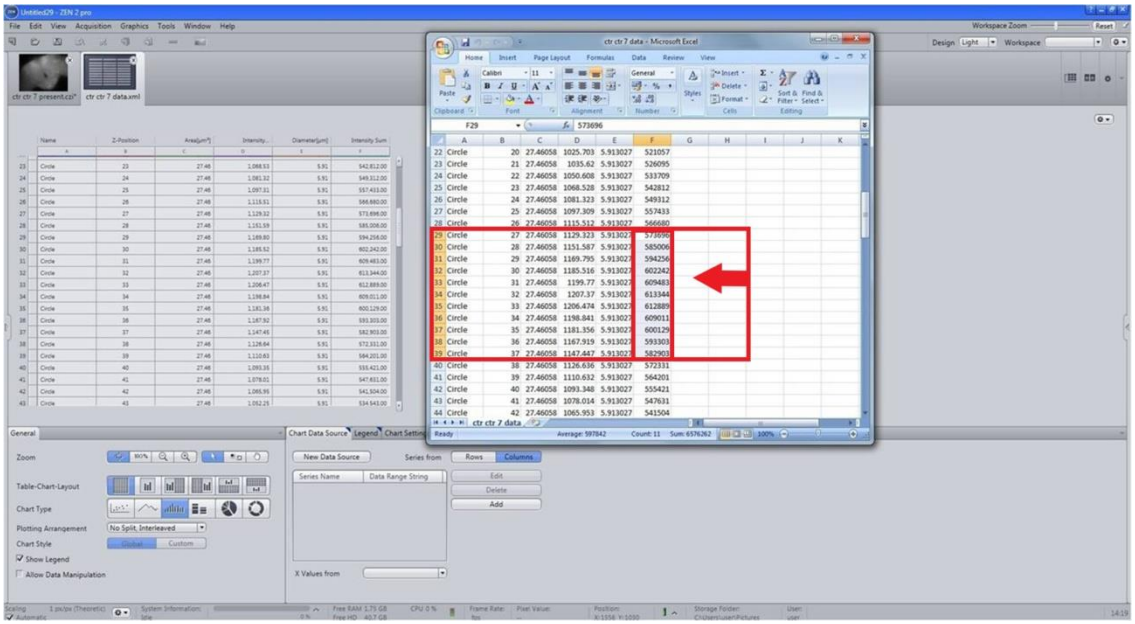


Figure 2.17. Compilation of fluorescence intensity (FI) of a single neuron and all the neurons of a cluster. For the characterization of FI of a single neuron, a total of 11 scans with an interval of 1.08 µm for each scan (cumulative 11.88 µm width) were considered. Take the average and find the standard error. Follow the same method/step(s) for all the 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.

2.22. Quantification of DA and its metabolites using High Performance Liquid Chromatography (HPLC)

In order to comprehend the biological importance of DA metabolism in the *Drosophila* model of PD, I, therefore, attempted to quantify the levels of DA and its metabolites (DOPAC and HVA) using the HPLC-ECD equipment (Ayajuddin et al., 2021, 2023; Das, 2022). Standard DA and metabolites were quantified to provide a precise retention time

and area with which samples were compared in order to quantify catecholamines in the tissue samples. Quantification of DA and metabolites were done following the protocols of Ayajuddin et al. (2021).

Catecholamine quantification

Sterilized 1.5 mL centrifuge tubes (Tarsons, WB, India, catalog number: 500010), Parafilm^{TM} wrapping film (Bemis, WI, USA, catalog number: PM996), Sterilized micro tips (Tarsons, WB, India, catalog number: 521010), Freshwrapp aluminum foil 9–11 μm (Hindalco, Maharashtra, India, catalog number: HV2241), Whatman^{TM} filter paper (GE Healthcare, Buckinghamshire, UK, catalog number: 1001917), Phosphate buffered saline (PBS) pH 7.4 (HiMedia, Maharashtra, India, catalog number: ML023), Dissecting fine forceps (EMS, PA, USA, catalog number: 78620-4B), Brush (TEYUP, Delhi, India, model number: SR-1013), Delicate task Kim wipers (KIMTECH^{TM}, GA, USA, catalog number: 370080), Micropipette i.e., 1,000 μL, 50 μL, 10 μL, 2 μL (Gilson, WI, USA, catalog number: 30040), pH/mV meter (Hanna Instruments, RI, USA, model: HI2211-02), -20°C ES Series refrigerator (Thermo Scientific, MA, USA, model: 50616100444443250), -80°C ultra-low temperature freezer (New Brunswick Innova, Hamburg, Germany, model: U101-86), Stereo zoom microscope (Carl Zeiss, Jena, Germany, model: Stemi 305), Dopamine (DA) (Sigma-Aldrich, St. Louis, MO, USA, catalog number: H8502), 3,4-Dihydroxyphenylacetic acid (DOPAC) (Sigma-Aldrich, St. Louis, MO, USA, catalog number: 11569), Homovanillic acid (HVA) (Sigma-Aldrich, St. Louis, MO, USA, catalog number: 69673), Trichloro Acetic Acid (TCA) (SRL, Maharashtra, India, catalog number: 204842), MDTM mobile phase (Thermo Scientific, Waltham, USA, catalog number: 701332), HPLC grade water (JT Baker, Radnor Township, USA, catalog number: 4218-03), Acetonitrile (JT Baker, Radnor Township, USA, catalog number: 9017-03), Methanol (JT Baker, Radnor Township, USA, catalog

number: 9093-68), HPLC-ECD 3000 RS system (Thermo Scientific, MA, USA, model number: Dionex Ultimate 3000), Nanodrop® 2000c Spectrophotometer (Thermo Scientific, Waltham, USA, model number: ND2000CLAPTOP).

Tissue sample

Fly heads were used for brain-specific catecholamine quantification. After 24 hours of exposure, flies were immediately frozen. For each treatment group of the health and transition phase, 15 fly heads were decapitated. To avoid the 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's were cleaned with 70% ethanol to avoid contamination.

A) Preparation of DA, DOPAC, and HVA

Preparation of standards

To prepare the standards, 2 mg of commercially available catecholamines were dissolved in 2 mL of PBS. Different concentrations were achieved through dilution as shown in **Table 2.3.** For loading the standard, 200 ng/L of the concentration was used.

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

Table 2.3: Preparation of multiple concentrations of standard catecholamines.

The concentration of catecholamine 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 4°C prior to application to remove any undissolved solute particles) in a 1:1 ratio.

Sample preparation

1. 15 heads of adult flies were collected in 300 µL of 1x PBS, prepared in HPLC-grade water.

2. The sample was homogenized and then subjected to sonication at 30% amplitude for 20 seconds, with intervals of 5 seconds, ensuring the sample remained on ice throughout the process.

3. The homogenized sample was centrifuged at 6000 rpm for 10 minutes at 4°C.

4. The supernatant was carefully collected.

5. 200 µL of the supernatant was removed (the remaining were set aside for protein quantification), and to it, 200 µL of the 5% TCA was added.

6. The mixture underwent two additional centrifugations at 5000 rpm for 10 minutes each at 4°C.

7. The final supernatant was collected for downstream analysis.

Precautions

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

2. Both the tissue extract and standard catecholamine solutions were kept on ice between procedures to prevent degradation.

3. All reagents were prepared using HPLC-grade or Milli-Q water to avoid contamination that could lead to false positive peaks in the chromatogram.

4. Fresh pipette tips were used for serial dilutions of the standard and for transferring the tissue extract to prevent cross-contamination.

B) Quantification of protein

The Bradford technique was employed to measure protein concentration. A stock solution of Bovine Serum Albumin (BSA) was prepared at a concentration of 2 mg/mL by dissolving BSA in PBS. To create a working solution, 100 µL of this BSA stock was diluted with 900 µL of PBS, resulting in a final concentration of 0.2 µg/µL. Serial dilutions of the working BSA solution were then performed as outlined in **Table 2.4.**

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

Table 2.4: Preparation of serial dilutions using standard BSA.

Absorbance at 595 nm was measured using the NanoDrop 2000C (Thermo-Scientific, Waltham, USA) after incubating the samples at room temperature for 5 minutes to generate a standard curve. Protein quantification was conducted using 3 µL of the pure tissue extract. The total protein concentration (in µg/mL) obtained from the assay was

based on the 3 µL of tissue extract combined with PBS and Bradford reagent. To determine the actual protein amount per µL, the total protein (in µg) was divided by 3 µL.

C) Setting up the HPLC system

Solvents

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” was followed for solvents

Preloading instructions for solvents

The process of placing solvent reagent bottles on the HPLC solvent rack and connecting the corresponding tubing to the bottles is referred to as "preloading of the solvents." The "Preloading Instructions" provided below serve as a guide for handling the solvents and their containers. These instructions cover the steps for solvent preparation, positioning the bottles on the solvent rack, and securely connecting them to the tubing ports of the HPLC platform.

1. Each reagent bottle was optimally filled (minimum 350 mL in each).
2. To prevent the formation of bubbles, the bottle was slanted during filling.
3. As described in point 2, the mobile phase was poured into the appropriate reagent container only after being filtered using a 0.22-micron filter paper. (This step is crucial because the miscibility of the mobile phase constituents can cause issues when passing through the column due to minute-level coagulation of the organic components. Even ready-made mobile phases may contain undissolved salt residues and suspended particles.

Filtering with a 0.22-micron nylon membrane ensured that these residues, which could otherwise clog the C18 column of the HPLC-ECD system, were removed).

4. Prior to being connected to the HPLC system, all the reagent bottles were sonicated in a bath sonicator for 15 minutes at room temperature using a 40 kHz ultrasonic frequency.

System/ Column cleaning

Tissue debris from prior HPLC experiments may accumulate inside the columns and electrodes of the detectors. To prevent fungal growth, components of the HPLC platform, including the column, ECD, and tubing, were loaded and stored in 100% methanol. It is essential to flush the system with the mobile phase to remove any residual tissue debris and methanol, as well as to ensure that no air bubbles remain after the HPLC platform has been idle for an extended period between experiments. The following procedures were used to clean the system and column:

1. The system was cleaned by purging each solvent port by setting the flow rate to high and directing the flow from the pump to outside the system while keeping the purge valve open. This process was carried out for 5 minutes per port to eliminate any trapped air bubbles.

2. After purging, the purge knob was operated to close the purge valve, redirecting the flow from the pump to the column. To start cleaning the column, a 100% flow with 20% acetonitrile was enabled for 30 minutes at a flow rate of 0.5 mL/min.

3. Following the acetonitrile wash, a 100% flow of the mobile phase was allowed to pass through the column for an additional 30 minutes at the same flow rate. After this, the mobile phase was recycled (Drainage pipe outlet from the column will be wiped with a lint-free tissue soaked in the mobile phase and insert back into the mobile phase container bottle).

Setting up the HPLC parameters

The ideal oxidation potential for catecholamine detection using electrochemical detection (ECD) typically falls within a range of 340 mV (Yang and Beal, 2011). In our laboratory, it has been discovered that catecholamines are most effectively identified using the DIONEX ULTIMATE ECD 3000 system, which operates with a reduction potential of -175 mV and an oxidation potential of 225 mV. The reduction potential within the HPLC platform creates an environment that mimics the *in-vivo* state for all catecholamines. The excitation of these catecholamines within the HPLC system is controlled by the optimal oxidation potential, and within this specific range, the catecholamines of interest can be reliably detected. The following parameters were set for efficient detection and analysis of catecholamines

Reduction potential	:	-175 mV
Oxidation potential	:	+225 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	:	4^0C
Flow rate	:	0.5 mL/min

ECD priming

1. The ECD was primed after switching the mobile phase to recycle mode.

2. The system was kept in acquisition mode for at least 2 hours to monitor the state of the baseline after configuring the necessary ECD parameters.

3. The parallelism of the two lines, representing the signal acquisition of ECDRS 1 and ECDRS 2 electrodes, was checked. The lines should be parallel if the system is properly equilibrated; non-parallel lines indicate fluctuation.

4. If the drifting was less than 2 nA/hour, the baseline was considered to be stabilized.

D) Standard and sample loading

20 µL of standards were injected, followed by 50 µL of samples for analysis. The standards, including DOPAC, DA, and HVA, showed an optimal peak with a 20 µL injection of 200 ng/mL concentration

Note: The same PBS was used both to prepare the samples and to dissolve the standard metabolites. A minimum of 300 µL of the standard and tissue extract was retained in the vial for injection.

E) Analysis

Quantitative analysis was performed using Chromaleon® version 6.8 software provided along with the HPLC system.

1. For analysis, the chromatogram obtained using the ECDRS2 channel was employed.

2. The “Inhibit Integration Range” option was applied to the entire solvent-front area of the standard and sample chromatograms to prevent the integration of noisy or false peaks.

3. The sample chromatogram was superimposed over the standard chromatogram.

4. Specific catecholamine peaks in the sample were identified by comparing them with the standard chromatogram, considering factors such as retention time and peak behavior relative to other catecholamines. To accurately identify DA, DOPAC, and HVA peaks in the sample, 10 µL of a composite standard was prepared, and the sample was run again in the HPLC system. The peaks that spiked in response to the detection sequence have been highlighted as the monoamines of interest.

5. If peaks were co-eluted (i.e., peak shoulders were joined), they were split into two distinct peaks using 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. Once the peaks were precisely determined, additional processing was carried out for quantitative analysis.

8. To quantify catecholamine levels in the tissue extract, the peak area of the sample catecholamine was normalized to the standard.

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

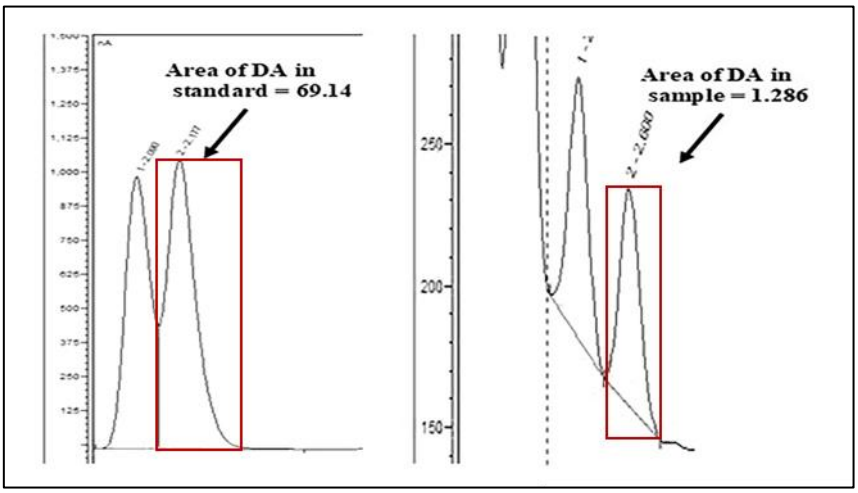


Figure 2.18: Image of chromatogram showing the area of the standard and sample.

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**)

iv. Area of the peak of the standard catecholamines (DA, DOPAC and HVA) in the chromatogram was

$$A_{DA\_Std} = 69.14,\ A_{DOPAC\_Std} = 63.59 \text{ and } A_{HVA\_Std} = 84.63$$

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} = 1.31, A_{DOPAC_Samp} = 4.78 and A_{HVA_Samp} = 1.04.

vii. Suppose, a particular tissue extracts from an experimental group that was used for HPLC assay, was quantified beforehand for total protein which was TP_{Samp} = 0.124 µg/ µL.

viii. The following steps were followed for calculating the actual amount of the catecholamines in tissue extract (**Table 2.5**).

Calculation:

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. (1.31 X 4)/ 69.14 = 0.0758 ng	(A_{DOPAC_Samp} X 4)/ A_{DOPAC_Std} i.e. (4.78 X 4)/ 63.59 = 0.3 ng	(A_{HVA_Samp} X 4)/ A_{HVA_Std} i.e. (1.04 X 4)/ 84.63 = 0.049 ng
Step III: Determining the total protein in 50 µL that was injected into column	(TP_{Samp} X I_{Samp}) i.e. (0.124 X 50) = 6.2 µg	(TP_{Samp} X I_{Samp}) i.e. (0.124 X 50) = 6.2 µg	(TP_{Samp} X I_{Samp}) i.e. (0.124 X 50) = 6.2 µg
Step IV: Determining the catecholamine in 1 µg of protein	[0.0758/6.2] = 0.0122 ng	[0.3/6.2] = 0.0484 ng	[0.049/6.2)] = 0.008 ng
Step V: Determining the actual amount of catecholamine per head as injected tissue extract solution had brain tissue extract + TCA in 1:1 ratio	0.0122 X 1000/ (2 X 15) = 0.41 pg/brain	0.0484 X 1000/ (2 X 15) = 1.61 pg/brain	0.008 X 1000/ (2 X 15) = 0.26 pg/brain

Table 2.5: Steps for calculation of the amount of catecholamines for single fly brain.

2.23. Statistical analysis

Data analysis was performed using GraphPad Prism 5.0 software (GraphPad Inc., San Diego, CA, USA). Results are expressed as the mean ± standard error of the mean (SEM). For datasets involving more than two groups, statistical significance was determined using a one-way analysis of variance (ANOVA) followed by the Newman-Keuls Multiple Comparison Test for fluorescence data, and the Tukey post-hoc test for climbing and HPLC data. A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant. Survival analysis was conducted using the Log-rank [Mantel-Cox] test.

CHAPTER 3

Sustaining the Curcumin Dopaminergic Neuroprotective Efficacy During the Adult Transition Phase in *Drosophila* Model of Parkinson's Disease - Implications to Human Health

3. 1 Introduction

Aging is a multifaceted deterioration encompassing a plethora of degenerative processes in metabolism, behavior, stress resistance, reproductive capability, nervous system, and immune systems, ultimately leading to death (Lee and Min, 2019). Molecular changes are universal across eukaryotes as an organism age, with some specific to taxonomic units. This molecular alteration controls the transcriptional activation of age-related genes, the potential to repair DNA, and programmed cell death in living cells. The combination of these alterations with environmental stress and damage accumulation triggers an exponential rise in morbidity and frailty, which in turn plays a critical role in determining an organism's longevity (Bordet et al., 2021). Researchers are working on developing safe interventions to combat and slow the aging process, which requires a dependable model system for testing. An optimal model system for studying interventions to slow the aging process should have a significant degree of genetic similarity to humans and be amenable to genetic manipulation. A comparative analysis of whole-genome sequencing revealed that over 75% of genes responsible for human diseases have corresponding homologues or orthologues in flies (Contreras and Klämbt, 2023; Reiter et al., 2001). *Drosophila,* like humans, exhibits complex behaviors such as mating, fear conditioning, aggression, learning, and motor skills, including flying, walking, and climbing. These behaviors are controlled by neural circuits that are influenced by the onset and progression of NDDs like Parkinson's disease (PD). In PD models, impairments in motor skills and cognitive functions, which are central to these behaviors, reflect the neurodegenerative processes affecting DAergic neurons, making *Drosophila* a valuable model for studying PD (Lessing and Bonini, 2009).

In *Drosophila melanogaster*, several longevity-associated genes have been identified, such as the insulin receptor (*InR*), methuselah (*mth*), ecdysone receptor (*EcR*), Indy,

superoxide dismutase (*SOD*), catalase, heat shock protein 70 (*hsp70*), peptide methionine sulfoxide reductase (*PMSR*), c-jun N-terminal kinase (*JNK*), *Sir2*, and *p53* (Lee et al., 2010). While ethical and technical difficulties prevent direct manipulation of these genes in humans, researching these genetic pathways in fruit flies provides crucial insights for designing pharmacological therapies that could delay or reduce aging.

According to Arking et al. (2002), there are three adult life stages in *Drosophila*: health, transition, and senescence, which are linked to aging. The health span is characterized by gene expression patterns that optimize tissue function while limiting inflammatory and other detrimental reactions. The transition phase experiences a life cycle that usually lasts 31 to 60 days and shows a survival rate drop from 89% to 81%, with an enhanced early survival rate that significantly increases the average lifespan without affecting the maximum lifespan. The transition phase substantially reduces the cell's regulatory capacity, while a stochastic deterioration in the gene expression marks the senescent phase network (Phom et al., 2014; Arking, 2009; Park et al., 2009; Pletcher et al., 2002). The senescence period lasts 61-120 days and is marked by physiological decline, higher mortality, decreased conception, slower movement, cardiovascular difficulties, abnormal growth, oxidative damage, and cognitive decline (Phom et al., 2014; Arking, 2005; Arking et al., 2002). Investigation of various life stages in model organisms has revealed clear and specific gene expression patterns. According to Soh et al. (2013), genotropic drugs are only effective when their target molecules are present at particular life cycle stages. Since, PD and other adult-onset disorders usually manifest later in life, so it is crucial to have a thorough knowledge of the life-stage effects of toxins and treatments on model organisms.

Drosophila models of PD mimics the distinctive pathophysiological characteristics observed in PD patients, including locomotor impairments, degeneration of DAergic

neurons, and decreased DA levels in the brain (Chaouhan et al., 2022; Shukla et al., 2014; Chaudhuri et al., 2007; Feany and Bender, 2000). Flies with tyrosine hydroxylase (TH) deficiency, and consequently DA deficiency, exhibit hypoactivity, a lack of desire for sucrose, a prolonged sleep duration, decreased alertness, defective olfactory, aversive learning, and locomotor impairments that exacerbate with age (Cichewicz et al., 2017; Riemensperger et al., 2011).

A pathological feature of PD is the progressive loss of DAergic neuron synthesis and decreased DA content in the *substantia nigra pars compacta*, with DA disturbances promoting neurodegeneration in various PD models. DA instability leads to its oxidation and metabolism, producing reactive and toxic by-products such as reactive oxygen species, DA quinones, and 3,4-dihydroxyphenylacetaldehyde, contributing to PD pathophysiology (Latif et al., 2021). In some cases, DA neuron loss is followed by the existence of α-synuclein aggregates in the surviving neurons, known as Lewy bodies (Bloem et al., 2021; Poewe et al., 2017). Although the precise cause of DA neurodegeneration in PD is yet unknown, it is most likely a result of a confluence of environmental and genetic predispositions (Bloem et al., 2021; Panicker et al., 2021). Indeed, several pathways and mechanisms appear to be involved in PD pathogenesis, including misfolded protein aggregate accumulation, mitochondrial dysfunction, increased oxidative stress (OS), energy failure, neuroinflammation, and genetic alterations (Maiti et al., 2017). Intriguingly, recent research has demonstrated that bioenergetic disruptions may be a significant factor in the neuropathology of PD. In particular, the glycolytic rate increased in PD models, indicating a correlation between neuronal mortality, redox homeostasis, cellular bioenergetics, and glucose metabolism (Solana-Manrique et al., 2020; Anandhan et al., 2017). Post-mortem of brains from individuals with PD reveals a depletion of neuromelanin in the *substantia nigra*, which

contains the cell bodies of neurons, as well as a gradual decline in DAergic terminals in the striatum. There is evidence of neuronal dysfunction in the surviving neurons, which manifest as Lewy bodies—aggregated α-synuclein protein inclusions (Bucher et al., 2020).

Drosophila brains have approximately 280 DAergic neurons distributed over eight clusters per hemisphere, with the PAM (PAM - Protocerebral anterior median) cluster being the largest, with about 100 neurons per hemisphere (Mao and Davis, 2009; Nässel and Elekes, 1992). However, the quantifiable DAergic neurons in the entire fly brain are PAL (4-5 neurons) (PAL- Protocerebral anterior lateral), PPL1 (11-12 neurons) (PPL- Protocerebral posterior lateral), PPL2 (6-7 neurons), PPM1/2 (8-9 neurons) (PPM- Protocerebral posterior medial), PPM3 (5-6 neurons), and VUM (3 neurons) (VUM- Ventral unpaired median), which can be tagged with primary anti-TH antibody (Chaurasia et al., 2024; Ayajuddin et al., 2023). Since Feany and Bender (2000) developed the first PD model, it has been frequently utilized to examine gene mutations or overexpression associated with PD. The PQ-mediated fly PD model exhibits time- and dose-dependent DAergic neurodegeneration, as evidenced by changes in neuronal morphology such as cell body aggregation, fragmentation, and selective loss of DAergic neurons (Chaouhan et al., 2022; Maitra et al., 2019, 2021; Song et al., 2017; Shukla et al., 2014; Lawal et al., 2010; Chaudhuri et al., 2007).

DA in *Drosophila* regulates behaviors comparable to those found in mammals, such as locomotion, drug response, circadian rhythms, aggression, attention, reward, and learning and memory. Like humans and other vertebrates, flies generate DA from the amino acid tyrosine by two enzymatic processes (Kasture et al., 2018). The first and most important step is the enzymatic action of tyrosine hydroxylase (TH), encoded by the flies pale (*ple*) gene and transforms tyrosine into L-3,4-dihydroxyphenylalanine (Jürgens et al., 1984)

where TH requires the cofactor BH4, which is synthesized from GTP by GTP cyclohydrolase I (GTPCH) (Jung-Klawitter et al., 2024; Bowling et al., 2008; Krishnakumar et al., 2000). In the second phase, the aromatic amino acid decarboxylase (AADC) produced by the DOPA decarboxylase gene (*Ddc*) converts L-DOPA to DA (Yamamoto and Seto, 2014). DA is synthesized within DAergic neurons in the brains of both humans and flies, but its therapeutic application in NDDs like PD is limited because DA cannot cross the blood-brain barrier (BBB) due to its hydrophilic nature, which prevents effective replenishment in the brain (Cichewicz et al., 2017; Haddad et al., 2017).

There are five DA receptors in humans: the D1-like receptors, which include D1 and D5, and the D2-like D4 receptors (D2, D3, and D4) (Beaulieu et al., 2015). Dop1R1 (dD1 or DUMB) and Dop1R2 (DAMB) are classified as D1-like receptors in

. The only D2-like receptor homolog is Dop2R (DD2R). These receptors exhibit several conserved features common to all rhodopsin-like GPCRs, such as a disulphide bond between cysteines in the first and second extracellular loop (Karam et al., 2020).

The correlation between DA depletion and DAergic neurodegeneration is complex, since impaired DA metabolism and oxidation aggravate reactive oxygen species (ROS) stress, ultimately resulting in neurodegeneration (Masato et al., 2019). Within mammals, DA is produced, stored in vesicles, released into synapses, and subsequently reabsorbed for breakdown into 3,4-dihydroxyphenylacetic acid (DOPAC) by monoamine oxidase (MAO). This DOPAC is then transformed into homovanillic acid (HVA) by catechol-O-methyltransferase (COMT) within astrocytes (Kaur et al., 2020; Meiser et al., 2013). In *Drosophila*, DA metabolism involves unique pathways such as N-acetylation and β-alanylation. Although flies lack genes to generate norepinephrine and epinephrine from DA, DA is necessary for melanin formation (Yamamoto and Seto, 2014) (**Figure 3.1**).

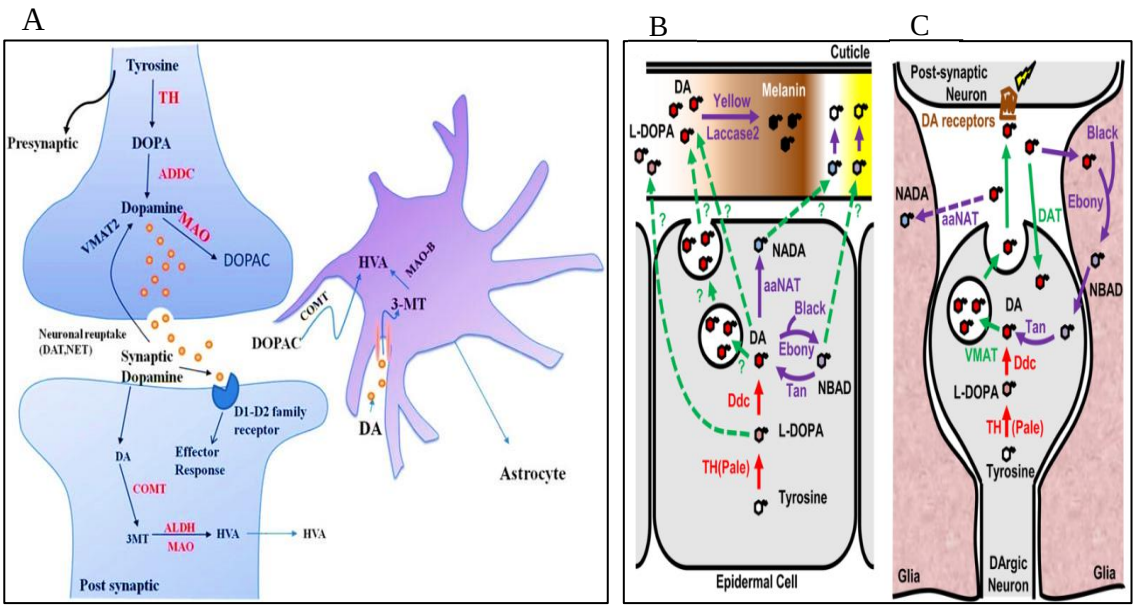


Figure 3.1. Illustrates the process of DA metabolism in the brain of mammals (A), the epidermal tissue, and the brain of flies (B,C). In a presynaptic neuron (blue), DA is synthesized, processed, or packaged into vesicles before being released into synapses. Following its synthesis from tyrosine by the enzyme TH, L-DOPA undergoes conversion to dopamine by the action of aromatic amino acid decarboxylase (ADDC). Monoamine oxidase (MAO) can then convert DA to DOPAC, which is then transported to astrocytes (purple) and converted into HVA by COMT (A; adapted from Kaur et al., 2020). In contrast, insights into DA catabolism in the fly brain and epidermal cell reveal that DA is transformed to NBAD and NADA by the activities of aaNAT, Ebony, Black, Tan (B, C; adapted from Yamamoto and Seto, 2014). DA is transported to the cuticle for pigmentation, whereas NBAD and NADA are transported to the cuticle for hardening (B). The fly brain catabolizes DA through neuronal and glial cells. Glial cells reabsorb excessive dopamine at synapses and convert it into NBAD and NADA through the action of aa-NAT, Black, and Ebony enzymes. NBAD can be carried back into neuronal cells and subsequently transformed back into dopamine by the activity of Tan (C).

In flies, DA is converted to NBAD and NADA, which are implicated in pigmentation and cuticle hardening, with similar metabolic cycles in epidermal cells and the brain (Paxon et al., 2005). Although *Drosophila* lacks orthologues for mammalian MAO and COMT, DA oxidative products such as DOPAC and HVA are found in fly brains, implying similar metabolic pathways (Yamamoto and Seto, 2014; Freeman et al., 2012; Wakabayashi-Ito et al., 2011; Chaudhuri et al., 2007; Zhang et al., 2005). The production of ROS and peroxide can be induced by endogenous neurotoxins, DOPAC and HVA, which in turn contribute to the degeneration of DAergic neurons and emphasize the susceptibility of these neurons to PD pathogenesis (Cao et al., 2021; Zhang et al., 2019).

Various studies in PQ-induced fly and mouse models of sporadic PD have reported that DA depletion in brain tissue is accompanied by increased DA degradation to its metabolites, emphasizing the importance of studying DA catabolism as well as the DA pool in the brain to understand PD pathogenesis (Rudyk et al., 2015; Shukla et al., 2014; Inamdar et al., 2012; Chaudhuri et al., 2007). Post-mortem tissue analysis of PD patients demonstrates impaired VMAT2 expression and function, resulting in inadequate DA sequestration. Consequently, there is a rise in the breakdown of DA inside the cytosol, as indicated by an increase in DA turnover and a decrease in the metabolites DOPAC, HVA, and DOPAL levels. Furthermore, decreased VMAT2 mRNA in circulating platelets indicates a systemic VMAT2 shortage, which contributes to PD etiology (Bucher et al., 2020; Pifl et al., 2014; Goldstein et al., 2013).

Levodopa remains the primary treatment for PD, though long-term use can lead to complications like levodopa-induced dyskinesia, and additional therapies such as dopamine agonists and inhibitors are used to manage symptoms (Blosser et al., 2020; Lane, 2019). While deep brain stimulation (DBS) has shown neuroprotective effects in animal models, it has not been effective in modifying key pathological features in clinical settings, highlighting the need for alternative therapies like natural products and phytochemicals (Armstrong and Okun, 2020). Hence, currently, no medication can cure or postpone the progression of PD, which necessitates exploring more effective alternatives including active compounds from natural products. The pursuit of natural treatments is driven by the side effects of conventional pharmaceuticals, aiming to prevent further suffering for the patient. The majority of PD-causing factors are found in the brain, so any therapeutic medication, such as curcumin, must reach these variables to be effective.

Cu shows promise in slowing PD symptoms by potentially preventing further degradation of DAergic neurons and having a safer profile than current medications in animal models (Xu et al., 2023; Ayajuddin et al., 2022; Das, 2022). Cu, with a molecular weight of 368.38 g/mol, can penetrate the BBB (400 daltons), which generally blocks the absorption of many medications but permits small-molecule compounds under 400 daltons to permeate (Sharifi-Rad et al., 2020).

Cu, also known as diferuloylmethane, is a yellow compound that is lipophilic and phenolic and has very low solubility in water. It belongs to the Zingiberaceae family, the primary active compound in turmeric, and is frequently used as a nutritional supplement and traditional medicine in Asia (El-Saadony et al., 2023). Cu has been used for over 2,500 years in India and China to treat infections, stress, depression, and dermatological conditions. Cu has a number of therapeutic properties, the most notable of which is its antioxidant potential, making it a promising choice for the therapeutic treatment of PD (Kunnumakkara et al., 2023). Cu is essential for preserving telomere stability and preventing shortening, contributing to cellular aging, which is significantly accelerated in PD patients. Cu has been shown in studies to reduce arsenic-induced apoptosis and ROS production by improving telomere structural stability and telomerase activity, emphasizing its potential for preventing telomere-related degeneration in PD (Wang et al., 2020; Jahanbazi Jahan-Abad et al., 2017).

Cu improves cellular health and longevity by activating several critical signaling pathways, such as PI3K/AKT, FOXO, Nrf2/ARE, and mTOR, which work together to reduce oxidative stress, regulate inflammation, and promote the expression of antioxidative and survival genes (Zia et al., 2021; Martins et al., 2016; Mazucanti et al., 2015). Cu reduces oxidative damage by modifying transcription factors such as FOXOs and stabilizing Nrf2, enhances nuclear translocation of protective proteins, and increases

the synthesis of antioxidant enzymes such as HsP70, heme oxygenase-1 (HO-1), and sirtuins, all of which contribute to its powerful anti-aging benefits (Singh et al., 2024; Zia et al., 2021). Furthermore, Cu's inhibition of the mTOR pathway, together with its more significant antioxidant activity when compared to vitamins C and E, positions it as a powerful agent in alleviating age-related cellular damage and increasing longevity in a variety of model species (Nebrisi, 2021; Zia et al., 2021).

Cu has been demonstrated to mitigate oxidative stress and lipid peroxidation in Swiss albino mice with PD that MPP^+ has induced. This is supported by the upregulation of SOD, CAT, and GSH (Rajeswari, 2006). Following PQ exposure, Cu pre-treatment in SH-SY5Y cells resulted in a decrease in ROS levels and an increase in the expression of antioxidant genes *SOD* and *GPx*. Cu's protective effects against nitrosative stress were further demonstrated in studies conducted on SH-SY5Y and PC12 cells transfected with mutant α-synuclein through the NF-κB signaling pathway (Abrahams et al., 2019; Wang et al., 2010).

Cu has been demonstrated to accelerate adult hippocampus neurogenesis by increasing the density of newly generated cells in the brain's dentate gyrus regions (Garodia et al., 2023; Kim et al., 2008). Cu inhibits oxidative stress-induced neurotoxicity in 6-OHDA PD models, enhancing the Wnt/β-catenin pathway, increasing cell viability, and reducing neuronal death. Its neuroprotective actions may also include altering downstream mediators of the Wnt signaling cascade, such as c-Myc and cyclin D1(Ashrafizadeh et al., 2020). Cu's antioxidant properties depend on the methoxy and phenolic groups attached to the benzene rings and the β-diketone moiety in its structure (Nebrisi, 2021; Malik and Mukherjee, 2014). Pre-treatment with Cu is employed as a prophylactic measure against the development of PD, while co-treatment and post-treatment strategies

evaluate its therapeutic potential during and after the advent of the disease. The objective of all of these methods is to utilize Cu as a therapeutic potential for the treatment of PD. The current study uses PQ as a stressor to examine Cu's neuroprotective impact. Several environmental chemicals are potential neurotoxicants in animal models of PD. Of these, many have utilized PQ as a neurotoxin in PD animal models. This is consistent with the idea that PQ represents a major environmental risk factor for PD as it mimics the structural features of MPP^+, an active moiety responsible for inducing parkinsonism (Tamura et al., 2022; Zhang et al., 2016). Studies have demonstrated that PQ can cross the BBB and cause DAergic neurons to degenerate (Paul et al., 2024). PQ accumulates in the subcellular organelles, particularly mitochondria, and blocks the electron transport chain function at the mitochondrial complex I by production of superoxide anions and other redox products, causing PD (Colle et al., 2020; Rani and Mondal, 2020). Exposure to PQ causes many brain abnormalities in PD patients, including DAergic neuronal degeneration in SNpc, increased oxidative stress, and accumulation of α-synuclein aggregates (See et al., 2022). Thus, understanding the association between pesticide exposure and PD development is vital for developing a proper treatment for this NDDs.

Animal models are crucial for studying disease progression and developing therapeutic approaches for NDDs like PD. It is important to develop age-specific models because certain genetic targets of compounds, such as curcumin, may only be active during certain life stages. As such, the compound will be effective in the organism only during those phases when its target molecules are present (Soh et al., 2013), which is an important and interesting paradigm. Our laboratory's research with the *Drosophila* PD model has demonstrated that curcumin exhibits DAergic neuroprotective efficacy exclusively during the healthy phase of adult life. Specifically, curcumin successfully rescues mobility defects and restores brain DA levels during the health phase but fails to do so during the

transition phase when late-onset NDDs, such as PD, typically set in (Ayajuddin et al., 2022; Phom et al., 2014). This highlights a significant limitation in curcumin's therapeutic potential and suggests the involvement of alternative molecular pathways in neurodegeneration/neuroprotection. These findings prompt a re-evaluation of the strategies employed in using young animal models to study late-onset NDDs like PD, underscoring the necessity of life stage-specific models for late-onset NDDs.

Life-stage specific research facilitates the initial identification of markers and the development of timely interventions, making it essential to evaluate the susceptibility and resistance of models to neurotoxicants and the efficacy of drugs across different life stages.

Several studies have hypothesized that PD is associated with early life insults, such as those during an intrauterine or early postnatal development (Colle et al., 2020), and environmental studies have also demonstrated that low-dose developmental exposures to toxic compounds, which did not induce any teratogenic effects or immediate symptoms of toxicity, can result in dysfunctions and diseases that manifest later in life (Barouki et al., 2012). Exposure to PQ and MB during development, followed by re-exposure of mice to the same pesticides, is lethal, as indicated by a considerable reduction in locomotor activity as well as damage within the DAergic system (Barlow et al., 2007; Cory-Slechta et al., 2005; Thiruchelvam et al., 2002). The "silent neurotoxicity" hypothesis suggests that early-life exposure to neurotoxicants may result in an increased susceptibility to environmental factors that are known to trigger neurodegenerative disorders and/or alter the normal aging process in adults (Fan et al., 2011; Fortier et al., 2004). However, little is known about the alterations that occur during brain development that might contribute to increasing neurotoxicity later in life.

Studies have demonstrated that combined exposure to metals, pesticides, or both increases the risk of DAergic neurodegeneration compared to single exposure (Colle et al., 2020; Singh et al., 2007; Mendola et al., 2002; Gorell et al., 1997). Exposure to environmental contaminants during crucial developmental stages accelerates DAergic neurodegeneration when re-exposed in adulthood (Mittra et al., 2020; Tilson, 1998). Zinc and PQ co-exposure disturbs DA homeostasis by decreasing the function of dopamine transporter (DAT), which is involved in DA re-uptake, and vesicular monoamine transporter (VMAT-2), which stores DA in synaptic vesicles (Mittra et al., 2020; Kumar et al., 2010).

Real-life situations necessitate simultaneous or sequential exposure to multiple compounds rather than exposure to a single environmental toxin, as demonstrated by experimental findings showing an increased propensity for neuronal loss following combined exposure to pesticides and/or metals (Mittra et al., 2020). Animal studies reveal that systemic administration of PQ causes DAergic neurodegeneration in the SNpc and motor impairment in mice (Bastías-Candia et al., 2019; Mitra et al., 2011; McCormack et al., 2002).

An overview of the literature shows that Cu fails to confer DAergic neuroprotective efficacy during the adult transition phase (TP), and thus far, no study has been performed to sustain this efficacy during the late phase of adult life, which is critical for using Cu as a therapeutic agent for late-onset NDDs such as PD. The majority of studies utilize young animals to establish models for neurodegenerative disorders that occur later in life, potentially restricting their applicability to human circumstances (Ayajuddin et al., 2022; Maitra et al., 2021; Sur et al., 2018). *Drosophila* and humans exhibit significant variations in gene expression profiles at different life stages, highlighting the need for models that account for age-specific differences (Soh et al., 2013; Pletcher et al., 2002). This is critical

for understanding disease development and therapeutic efficacy, as evident by Cu's life-stage-specific neuroprotective benefits in a *Drosophila* PD model (Phom et al., 2014). Understanding these variations can help to improve therapies and drug development for late-onset NDDs. This study offers pioneering insights into the transition phase, demonstrating how Cu alleviates sporadic PD symptoms and underscores the importance of sustaining its DAergic neuroprotective effects. The study aims to investigate whether Cu feeding can serve as a prophylactic across different life stages beyond the health span and sustain DAergic neuroprotection during the transition phase in a *Drosophila* model of sporadic PD. Additionally, with the re-challenge by PQ, whether Cu intervention could potentially sustain neuroprotection, highlighting the necessity to evaluate the efficacy of Cu throughout various life stages. Furthermore, the study explores whether Cu feeding intervention can sustain neuroprotection upon re-challenge with PQ, highlighting the need to assess its efficacy across various life stages. In the present study, I characterized DAergic neurodegeneration, quantified dopamine and its metabolites, and assessed locomotor function during the health and transition phases of the adult life stages in the *Drosophila* model of Parkinson's disease.

3.2 Results

Previously, studies in our laboratory demonstrated that Cu fails to rescue the mobility defects induced by PQ under co-treatment and pre-treatment regimes during the transition phase of the *Drosophila* model of PD. The efficacy was examined in the transition period (55 days old) using the same feeding strategies as the health phase (Phom, 2018; Phom et al., 2014). Interestingly, all of the Cu doses used in the experiment failed to rescue the climbing deficits in the negative geotaxis assay in both the co-treatment and pre-treatment regimens (**Figure 3.2. A, B**). The findings indicate significant limitations on the treatment efficacy of curcumin in NDD such as PD, where the average age of onset in humans is approximately 60 years. As a result, it is critical to thoroughly evaluate and assess the efficacy of treatment agents at the disease's onset (Phom, 2018; Phom et al., 2014)

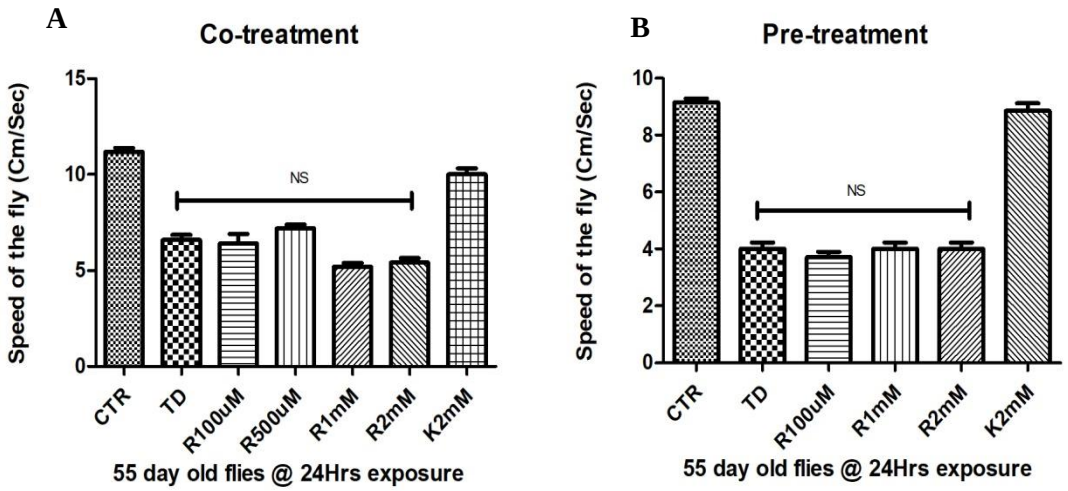


Figure 3.2: Negative geotaxis assay for the co-treatment regime in the transition phase (55-day-old flies). Co-treatment and pre-treatment regime for 55-day-old adult flies for 24hr (A) Co-treatment (B) pre-treatment indicates a different pattern than that of the health phase. All the curcumin concentrations (100µM, 500µM, 1 mM, and 2 mM) fail to rescue the mobility defect induced by paraquat (10 mM). Feeding curcumin alone does not alter the mobility defect (NS- Not Significant). (CTR=control, TD=treated with paraquat, R (rescue)=curcumin+paraquat, K=curcumin) (Adapted from Phom et al., 2014; Phom, 2018).

3.2.1 *10-day Cu feeding regime preceded and followed by PQ treatment rescues mobility defects during the HP of Drosophila*

Following the feeding regimen illustrated in **Figure 2.4**, an environmental toxin-induced *Drosophila* model replicating PD's behavioral and pathological symptoms was utilized as a screening platform to identify phytochemicals with therapeutic potential against herbicide-induced PQ toxicity. Studies have shown that the wild-type *Drosophila* strain, Oregon K, experiences a substantial decrease in survival and a significant impairment in climbing abilities due to PQ exposure (Phom et al., 2014). Adult male flies were employed exclusively in the experiments due to their increased sensitivity to PQ toxicity, leading to neurodegenerative PD phenotypes earlier than female flies. This is in accordance with the fact that the prevalence of PD is higher in male patients (Chaudhuri et al., 2007).

Behavioral parameters related to motor function were evaluated after 10 days of Cu feeding and following the final administration of either sucrose or PQ. After 24 hours of PQ exposure, *Drosophila* exhibited resting tremors and bradykinesia, characteristic clinical symptoms associated with PD in human patients. Few flies attempted to climb the walls but failed to maintain their grip and slipped to the bottom, whereas others displayed overactive or restless behavior, indicated by rapid wing flipping.

Flies exposed to PQ during the early health phase and re-challenged with the same neurotoxicant in the late health phase were the most affected, showing significant deficits in motor performance compared to flies exposed only during the early health phase period. This significant impairment in motor performance was evident in the negative geotaxis assay, where climbing speed decreased by 21% (***$p<0.001$) in PQ-NM-PQ compared to Su-NM-Su. In contrast, movement defects were not apparent when 100 µM Cu was fed to flies exposed to PQ (PQ-Cu-PQ). The speed of the flies significantly

improved by 22% (***p<0.001) compared to PQ-NM-PQ (**Figure 3.3**). Flies fed with Su-NM-Su and Su-Cu-Su showed similar climbing speed, suggesting that Cu feeding *per se* had no negative influence on mobility. Additionally, Cu feeding rescued the mobility defects by 25% (***p<0.001) in flies exposed to PQ alone during their early health phase (PQ-Cu-Su) compared to PQ-NM-PQ (**Figure 3.3**). In summary, Cu significantly ameliorated the mobility defects induced by PQ in *Drosophila* models of PD, when administered during the late health phase following early phase exposure.

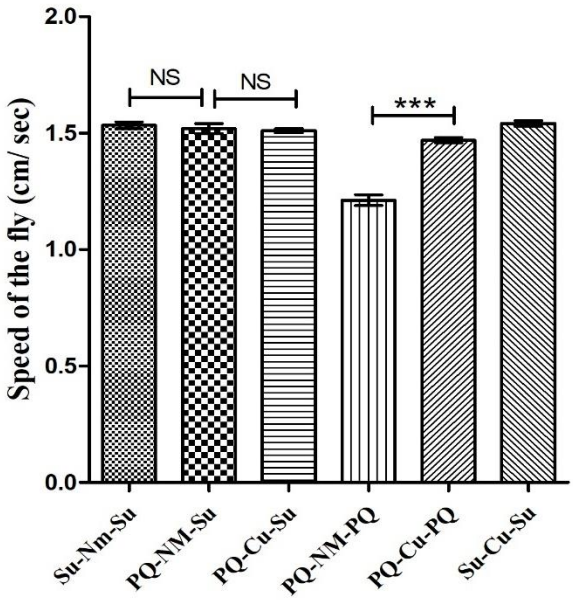


Figure 3.3: The protective effects of Cu against paraquat-induced toxicity and mobility defects was assessed by negative geotaxis assay during the health phase of flies under different feeding conditions. Climbing ability was evaluated on the 20-22nd day for health-phase flies. PQ-NM-PQ led to a significant reduction in climbing ability when compared to Su-NM-Su. This decline was significantly ameliorated when 100 µM Cu was introduced as part of their diet during the health phase. However, Su-Cu-Su did not yield any significant change in their mobility when compared to Su-NM-Su (Su-Sucrose 5%; PQ-Paraquat 10mM; Cu-Curcumin 100 µM; NM-Normal media). Statistical differences were based on one-way ANOVA, with Tukey post hoc test [***p<0.001; NS: Not significant].

3.2.2 Characterization of DAergic neurodegeneration in the whole fly brain using anti-TH antibodies

The brain of the adult *Drosophila* consists of six quantifiable DAergic neuronal clusters in each brain hemisphere (**Figure 3.4 A**) (Chaurasia et al., 2024; Ayajuddin et al., 2023). The quantity 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. Utilizing fluorescently labeled secondary antibodies directed against the primary antibody, which tags DA synthesizing rate-limiting enzyme TH, it was possible to count the number of DAergic neurons (**Figure 3.4 B**).

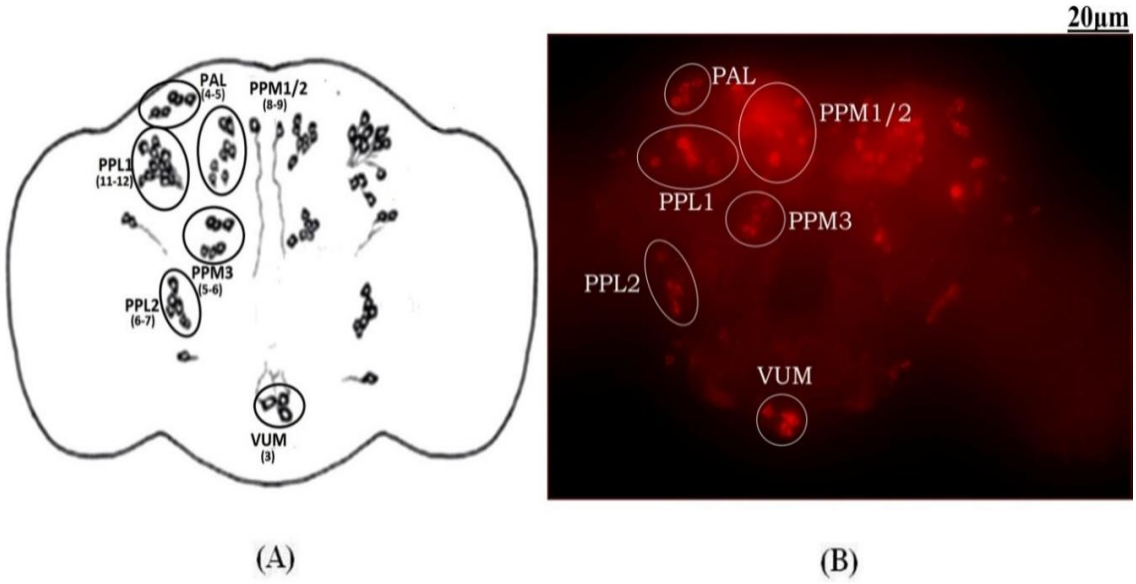


Figure 3.4: (A) Cartoon of *Drosophila* brain showing the position of different clusters of DAergic neurons. The brain of *Drosophila* has around 141 DAergic neurons in each hemisphere which are arranged into different clusters. Some of them are PAL (4-5 neurons), PPL1 (11-12 neurons), PPL2 (6/7 neurons), PPM1/2 (8/9 neurons), PPM3 (5-6 neurons) and VUM (3 neurons). These DAergic neurons are easily countable using a fluorescently labeled secondary antibody against the anti-TH primary antibody. There are other regions like PAM with around 100 neurons which are not easily quantifiable. Scale bar of the brain image is 20µm. (B) Image of whole-brain mount of *Drosophila* captured using ZEN software of Carl Zeiss Fluorescence Microscope (Adapted from Chaurasia et al., 2024; Ayajuddin et al., 2023). [PAM: Protocerebral Anterior Medial; PAL: Protocerebral Anterior Lateral; PPM: Protocerebral Posterior Medial; PPL: Protocerebral Posterior Lateral; VUM: Ventral Unpaired Medial].

3.2.3. *10-day Cu feeding regime preceded and followed by PQ treatment rescues DAergic neurodegeneration during the HP of Drosophila*

It has been demonstrated that PQ causes no loss of DAergic neurons in *Drosophila* PD models but induces neuronal dysfunction (Ayajuddin et al., 2023; Das, 2022). Given that Cu dietary intervention enhanced PQ-induced mobility defects, the impact of Cu on DAergic neurodegeneration in the adult HP of *Drosophila* brain was investigated. To further understand the efficacy of Cu in protecting DAergic neurons, flies fed with Cu were compared to those fed with NM.

Figure 3.5 A shows images of the various experimental groups in the *Drosophila* brain. The findings indicated no significant variation in the number of neurons among different treatment groups throughout the HP (**Figure 3.5 B**). Additionally, there was no significant difference in the total number of neurons in the whole fly brain among various treatment groups (**Figure 3.5 C**). Hence, in the present study, PQ-induced effects did not alter the number of DAergic neurons, either in a cluster-specific manner or when analyzed in a group wise manner. To further investigate, the fluorescence intensity (FI) of DAergic neurons was evaluated to ascertain any possible changes in tyrosine hydroxylase (TH) synthesis, and the results indicated a clear correlation between TH protein synthesis and FI.

Flies exposed to neurotoxicant at a single time frame, i.e., during the early health phase (PQ-NM-Su), did not exhibit any significant variations in their FI of clusters in the whole brain (**Figure 3.5 D**) and also the overall FI (**Figure 3.5 E**) compared to flies solely exposed to sucrose (Su-NM-Su). However, substantial reductions in FI were observed when flies were re-challenged to PQ. The FI of DAergic neurons in the PAL, PPL1, PPL2, PPM1/2, and PPM3 clusters reduced by approximately 30% (**$p<0.01$), 21% (*$p<0.05$), 25% (*$p<0.05$), 27% (*$p<0.05$), and 24% (*$p<0.05$) during the HP in the PD model (PQ-NM-PQ) compared to Su-NM-Su (**Figure 3.5 D**).

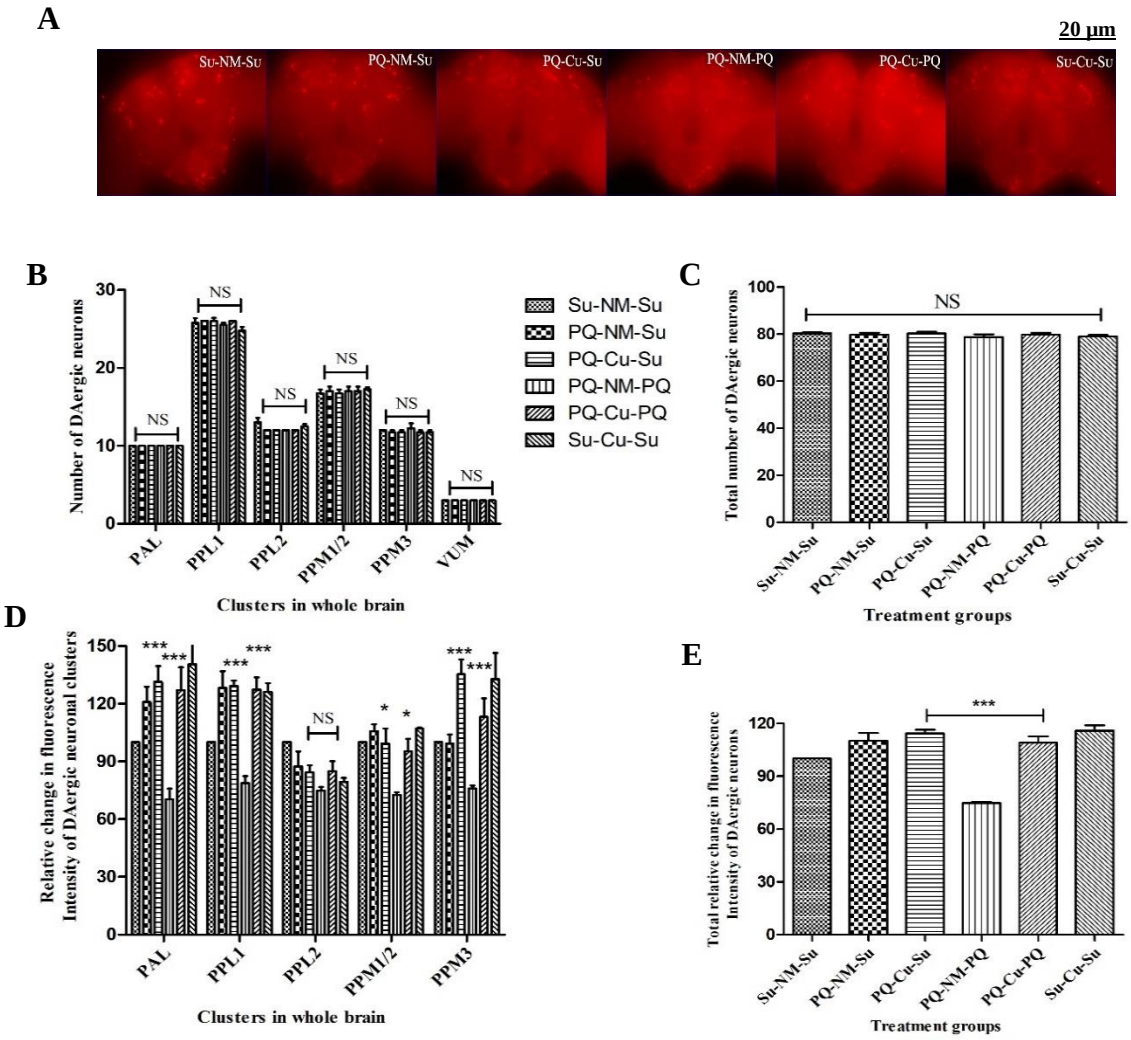


Figure 3.5: Whole-brain immunostaining (A) with anti-TH indicates that, upon treatment, PQ does notresult in a decrease in the number of DAergic neurons (B,C) but rather reduces the synthesis of Tyrosine Hydroxylase (TH) during the health phase (HP) of *Drosophila*. However, PQ leads to "neuronal dysfunction" (NDF), characterized by a decrease in DAergic neuronal fluorescence intensity, proportional to the amount of TH protein. This NDF is rescued by curcumin (Cu) (D,E). The significance was determined by analyzing a minimum of three brains, followed by two-way ANOVA and Bonferroni post-test for cluster-wise analysis. For summative analysis, one-way ANOVA was performed, followed by the Newman-Keuls multiple comparison test (*p<0.05; ***p<0.001 compared with the PQ-NM-PQ treated group; NS- Not significant). The scale bar depicted in all images in panel (A) is 20 µm. The represented images are "merged" Z-stacking images; however, the quantification of DAergic neuronal number and fluorescence intensity was performed on 3D Z-stack images [Su-Sucrose 5%; PQ-Paraquat 10mM; Cu-Curcumin 100 µM; NM-Normal media].

The data indicates that re-challenging the flies with 10 mM PQ (PQ-NM-PQ) considerably decreases the FI, thereby reducing the synthesis of TH protein. The findings imply that early-life pesticide exposure leaves a persistent effect on the DAergic nigrostriatal system, rendering it more susceptible to subsequent exposure to the same

pesticides in later life stages. The results suggest that the number of neurons remains unaffected despite the reduction of TH synthesis, and the structure of the DA neurons (cell body) does not degenerate. The observation correlates with the results of a study conducted by Navarro et al. (2014), in which they measured and demonstrated a reduction in the FI of the GFP reporter protein rather than the actual loss of neurons.

The cluster-wise depletion of FI in flies exposed to PQ twice (PQ-Cu-PQ) was rescued by the 10-day Cu feeding, compared to those fed on NM (PQ-NM-PQ). The FI of DAergic neurons in the PAL, PPL1, PPM1/2, and PPM3 clusters were rescued by approximately 57% (***p<0.001), 49% (***p<0.001), 23% (*p<0.05), and 37% (***p<0.001), respectively, however, no rescue was observed in the PPL2 cluster (**Figure 3.5 D**). In addition, when comparing groups that were exposed to PQ once and then fed with Cu (PQ-Cu-Su) to those that were exposed to PQ twice and fed in NM (PQ-NM-PQ), the DAergic neurons FI in the PAL, PPL1, PPM1/2, and PPM3 clusters were rescued by 61% (***p<0.001), 51% (***p<0.001), 27% (*p<0.05), and 60% (***p<0.001) respectively. This pattern was consistent with the prior outcomes, which showed no rescue in the PPL2 cluster (**Figure 3.5 D**).

Further, the overall FI of all DAergic neurons in the fly brain was combined for further analysis. The findings indicate that re-challenged neurotoxicant exposure resulted in a 25% depletion of the FI (PQ-NM-PQ) (***p<0.001) in comparison to Su-NM-Su, indicating reduced levels of TH protein synthesis. Cu feeding significantly increased the reduced FI by 34% (***p<0.001) in the PQ-Cu-PQ and 40% (***p<0.001) in the PQ-Cu-Su treatment groups (**Figure 3.5 E**) compared to PQ-NM-PQ group, indicating a replenishment of TH level/synthesis. These findings suggest that Cu can potentially reverse the DAergic neurodegeneration/neuronal dysfunction induced by PQ exposure during HP.

3.2.4. Quantification of Brain DA and Metabolite Level with HPLC (ECD)

Utilizing the standard and sample chromatogram obtained from the HPLC-ECD unit I measured the concentration of brain DA and its metabolites (DOPAC and HVA) to understand DA metabolism (**Figure 3.6**).

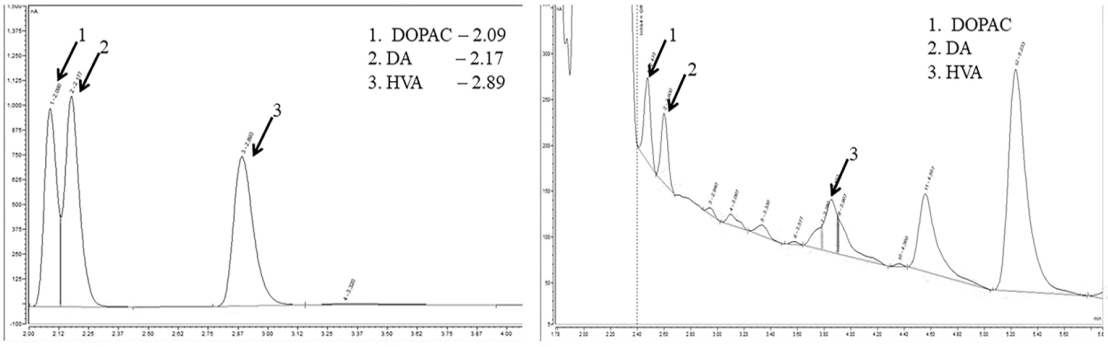


Figure 3.6: Characterization of retention time of standard DOPAC, DA and HVA (A) and brain-specific DA and its metabolites levels (B): Chromatogram of the standard catecholamines gives a particular RT comparing with which the catecholamines in the fly brain sample is analyzed.

3.2.4.1. *10-day Cu feeding regime preceded and followed by PQ treatment rescues DA and its metabolites during the HP of Drosophila*

As Cu feeding protects against PQ-induced neuronal dysfunction and motor deficits, further investigation was conducted to determine whether Cu feeding replenishes DA levels in the context of PQ neurotoxicity. DA level and its metabolites, DOPAC and HVA, were measured using HPLC in the fly brains of feeding regime groups. In the PD brain (PQ-NM-PQ), during HP, the DA level was reduced by 55% (***$p<0.001$) compared to Su-NM-Su (**Figure 3.7**). Quantification of DAergic neurons showed that the FI intensity was significantly reduced upon exposure to PQ, revealing diminished TH synthesis and neuronal dysfunction (**Figure 3.5**). Therefore, reduced TH synthesis is directly correlated to reduced DA level. The immediate metabolite of DA, i.e., DOPAC

level, was also reduced by 43% (***p <0.001) (**Figure 3.7 A**). Decrements in DA and DOPAC levels are also observed in the post-mortem brains of PD patients (Goldstein et al., 2011). HVA is the final product of DA metabolism, wherein the *Drosophila,* through the MAO/COMT analogous pathway, DA and DOPAC can be degraded to HVA (Yamamoto and Seto, 2014; Meiser et al., 2013). Hence, HVA levels were measured, indicating a 49% depletion (***p <0.001) (**Figure 3.7 A**) in the PD brain. However, despite this reduction, no significant statistical differences were observed according to Tukey's post hoc test. DA depletion in the PD brain is much higher compared to DOPAC and HVA.

Flies exposed to a single insult, i.e., during the early health phase (PQ-NM-Su), exhibit significant reductions in their DA level by 55% (***p <0.001) compared to Su-NM-Su (**Figure 3.7 A**). However, quantification of DAergic neurons did not exhibit any significant variations in their FI of clusters in the whole brain or the overall FI compared to flies solely exposed to sucrose (**Figure 3.5**). A significant reduction of 36% (***p <0.001) was also observed in the DOPAC level (**Figure 3.7 A**). HVA levels showed a 33% depletion; however, no significant statistical differences were observed in Tukey's post hoc test compared to Su-NM-Su (**Figure 3.7 A**). Similarly, DA depletion is much higher compared to DOPAC and HVA. This suggests higher DOPAC and HVA synthesis than DA in the PD brain during HP. Higher synthesis of DA downstream DOPAC and HVA in the PD brain suggests that these monoamines might have a role in PD onset and progression as they are considered endogenous neurotoxins.

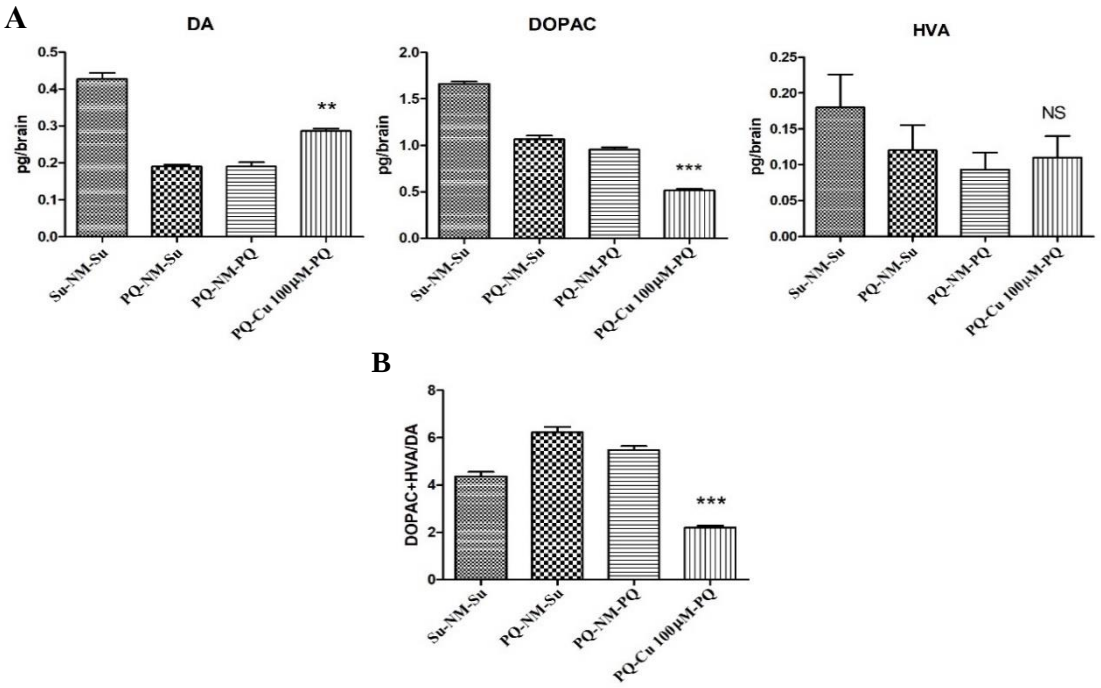


Figure 3.7: Quantifying DA and metabolite levels in fly brain using HPLC-ECD. During the health phase, administering PQ-NM-PQ has significantly decreased brain DA, DOPAC, and HVA levels, compared to Su-NM-Su (A). The DA depletion level is higher than downstream metabolite DOPAC, suggesting an enhanced DA turnover rate (B). Curcumin dietary intervention, PQ-Cu-PQ, during the health phase rescues the diminished DA level; although the intervention is inhibited in DOPAC and HVA levels, it is enough to prevent DA turnover. Significance was drawn by analyzing the data of a minimum of three replicates with one-way ANOVA followed by Tukey Post-Test for each age group. [***p<0.001; **p<0.01; NS: Not significant - compared to PQ-NM-PQ treated group].

10 days of Cu feeding in the HP PD brain (PQ-Cu-PQ) rescued the diminished DA level by 48%. However, DOPAC levels could not be replenished, while HVA levels remained unaltered. This implies that the rescue of DA levels is sustained by the HP-specific neuroprotection of Cu, which may be facilitated by the restoration of TH synthesis. Furthermore, Cu feeding might have selectively prevented DOPAC production or accumulation while maintaining stable HVA levels in the HP PD brain, thus limiting endogenous toxicity. This, in turn, protects the neurons from internal biochemical stressors that can worsen the neurodegenerative process.

Changes in DA, DOPAC, and HVA levels under PD conditions and following Cu intervention were examined to understand DA catabolism and turnover. The DA

degradation/turnover ratio, calculated as [(DOPAC+HVA)/DA], was higher in HP PD brains compared to Su-NM-Su (**Figure 3.7 B**). This suggests that DA depletion in PD may be due to increased degradation of DOPAC, resulting in enhanced oxidative turnover of DA. However, the Cu feeding prevented the DA turnover ratio (***P<0.001), indicating its neuroprotective efficacy. Interestingly, the turnover ratio in the single-insult group (PQ-NM-Su) was comparatively higher than in the two-hit PQ exposures. Despite the significant depletion of DA and DOPAC, unaltered HVA levels suggest that the system responded by increasing dopamine metabolism. This response likely involved a compensatory mechanism that accelerated the depletion of DA and DOPAC to mitigate their loss, thereby maintaining stable fluorescence intensity (FI) clusters and overall FI in the whole brain (**Figure 3.5**), resulting in no significant variations.

3.2.5 *10-day Cu feeding regime preceded and followed by PQ treatment rescues mobility defects during the TP of Drosophila*

Given that Cu feeding protects against PQ-induced motor deficits during HP, further investigation was conducted to determine whether Cu feeding could still rescue the motor deficits, as in our previous lab study, Cu failed to restore the PQ-induced motor defects during the TP (Phom et al., 2014). Flies were re-challenged to the same neurotoxicant during the transition stage from days 43-45 and again on days 53-55.

Behavioral parameters related to motor function were evaluated after 10 days of Cu feeding and following the final administration of either sucrose or PQ. After 24 hours of PQ exposure, *Drosophila* exhibited resting tremors and bradykinesia, characteristic clinical symptoms associated with PD in human patients.

Flies exposed to PQ during the early transition phase and then re-challenged with the same neurotoxicant (PQ-NM-PQ) during the late transition stage showed the most

significant deficits in motor performance compared to flies exposed only during the early transition phase (PQ-NM-Su). This significant impairment in motor performance was evident in the negative geotaxis assay. 10-day Cu feeding preceded and followed by PQ treatment rescued mobility defects, as indicated by a significant 26% (***p<0.001) improvement in climbing speed compared to PQ-NM-PQ (**Figure 3.8**). Flies fed with Su-NM-Su and Su-Cu-Su showed similar speeds, suggesting that Cu feeding *per se* had no negative influence on mobility. Additionally, Cu feeding rescued the mobility defects by 30% (***p<0.001) in flies exposed to PQ only during their early transition phase (PQ-Cu-Su) compared to PQ-NM-PQ (**Figure 3.8**).

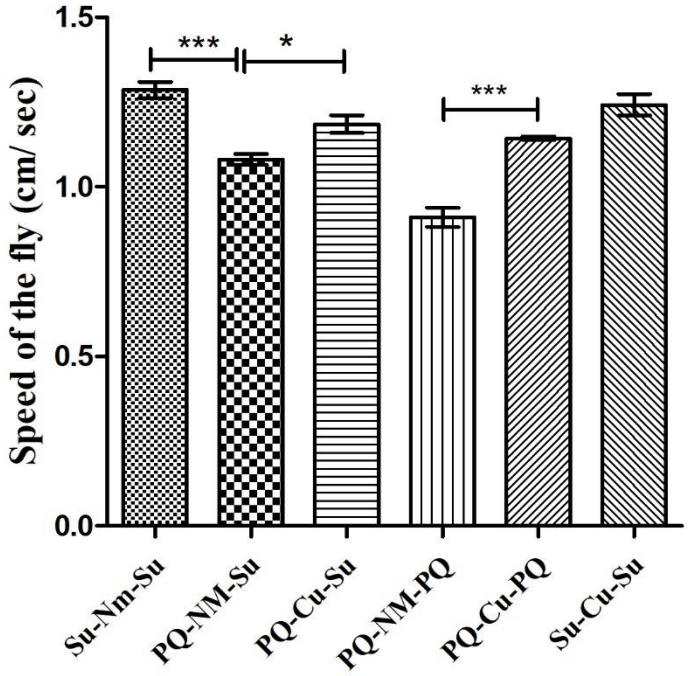


Figure 3.8: The protective effects of Cu against paraquat-induced toxicity and mobility defects was assessed by negative geotaxis assay during the transition phase of flies under different feeding conditions. Climbing ability was evaluated on the 53- 55 day for transition-phase flies. Re-challenged with the same neurotoxicant (PQ-NM-PQ) during the late transition stage showed the most significant deficits in motor performance compared to flies exposed only during the early transition phase (PQ-NM-Su). This decline was significantly ameliorated when 100 µM curcumin (Cu) was introduced as part of their diet. However, feeding the flies with Su-Cu-Su did not impact their mobility compared to Su-NM-Su (Su-Sucrose 5%; PQ-Paraquat 10mM; Cu-Curcumin 100 µM; NM-Normal media). Statistical differences were based on one-way ANOVA, with Tukey post hoc test [***p<0.001; *p<0.05; NS: Not significant].

Interestingly, with the alteration of the feeding regime, Cu significantly ameliorated the mobility defects induced by PQ in *Drosophila* models of PD, especially when administered during the late transition stage following early transition phase exposure, Whereas in our previous lab study, Cu failed to rescue mobility defects during the transition phase, this highlights not only the importance of age-related, time- and dose-dependent factors but also the critical role of the feeding strategy in determining the efficacy of Cu administration.

3.2.6 *10-day Cu feeding regime preceded and followed by PQ treatment rescues DAergic neurodegeneration during the TP of Drosophila*

Given that Cu 10 day feeding regime enhanced PQ-induced mobility defects during TP, the impact of Cu on DAergic neurodegeneration in the TP of the *Drosophila* brain was investigated. **Figure 3.9 A** shows images of the various experimental groups in the *Drosophila* brain. A similar pattern to that observed during the HP was also noted in the TP, with findings indicating no significant variation in the number of neurons among the different treatment groups (**Figure 3.9 B**). Additionally, there was no significant difference in the total number of neurons in the whole fly brain among various treatment groups (**Figure 3.9 C**). To further investigate, the FI of DAergic neurons was evaluated to ascertain any possible changes in TH synthesis, and the results indicated a clear correlation between TH protein synthesis and FI.

Flies exposed to neurotoxicant at a single time frame, i.e., during the early transition phase (PQ-NM-Su), did not exhibit any significant variations in their FI of clusters in the whole brain (**Figure 3.9 D**) and also the overall FI (**Figure 3.9 E**) compared to flies solely exposed to sucrose (Su-NM-Su). However, substantial reductions in FI were observed when flies were re-challenged to PQ. The FI of DAergic neurons in the PAL, PPL1, PPL2, PPM1/2, and PPM3 clusters reduced by approximately 36% (**$p<0.01$), 25% (*$p<0.05$),

36% (**p<0.01), 34% (**p<0.01), and 44% (***p<0.001), respectively, during the TP in the PD model compared to Su-NM-Su (**Figure 3.9 D**).

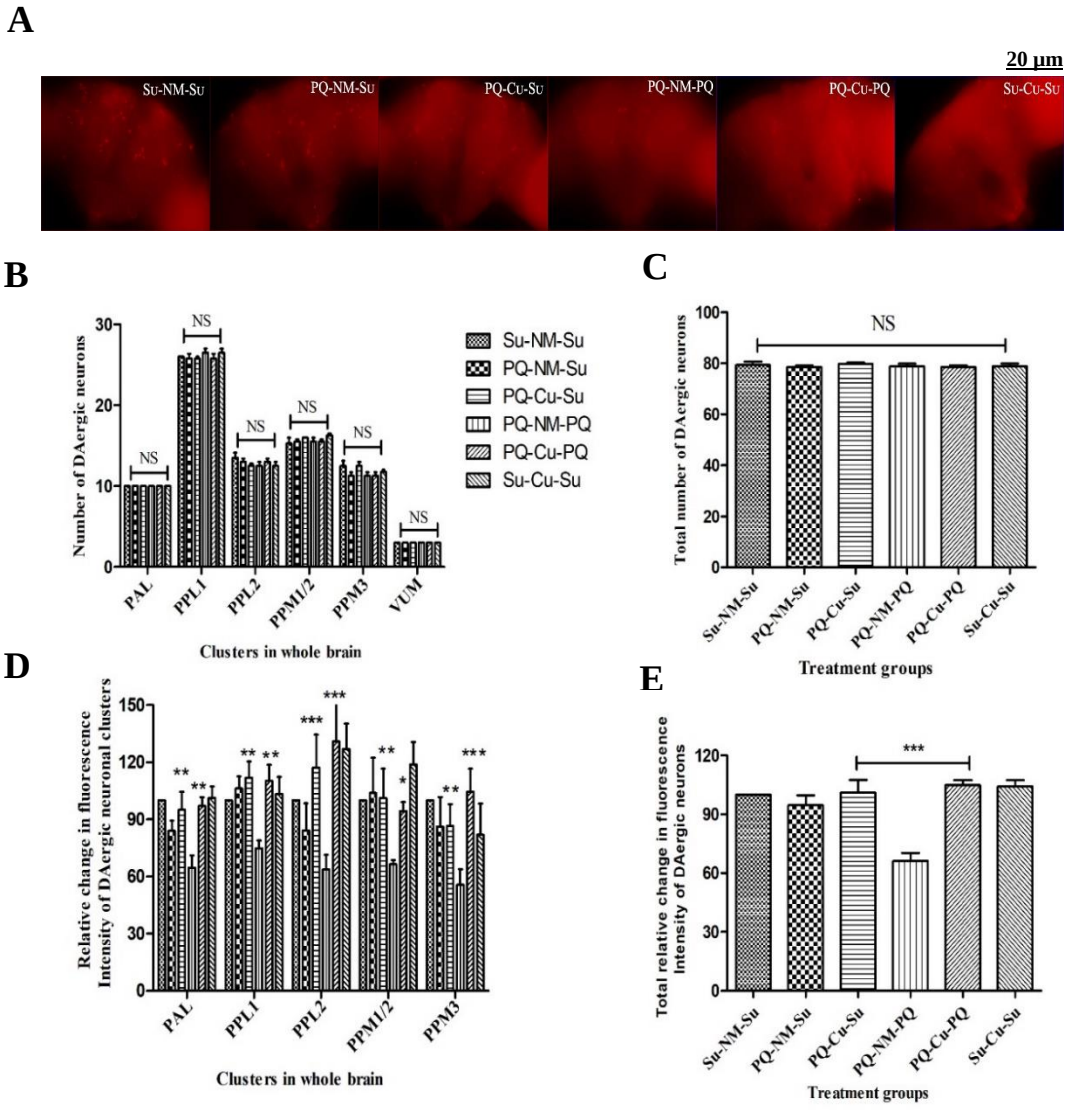


Figure 3.9. Cu feeding rescues PQ-induced DAergic neurodegeneration during TP of *Drosophila*. Whole-brain immunostaining (A) to characterize DAergic neurodegeneration reveals no loss in the number of DAergic neurons upon treatment with paraquat (PQ) during the transition phase (TP) of *Drosophila* (B,C). However, PQ leads to "neuronal dysfunction" (NDF), characterized by a decrease in DAergic neuronal fluorescence intensity, which is proportional to the amount of TH protein. This NDF is rescued by curcumin (Cu) during the TP (D,E). The significance was determined by analyzing a minimum of three brains, followed by two-way ANOVA and Bonferroni post-test for cluster-wise analysis. For summative analysis, one-way ANOVA was performed followed by the Newman-Keuls multiple comparison test (*p<0.05; **p<0.01; ***p<0.001 compared with the PQ-NM-PQ treated group; NS- Not significant). The scale bar depicted in all images in panel (A) is 20 µm. The represented images are "merged" Z-stacking images; however, the quantification of DAergic neuronal number and fluorescence intensity was performed on 3D Z-stack images [Su-Sucrose 5%; PQ-Paraquat 10mM; Cu-Curcumin 100 µM; NM-Normal media].

The data indicates that re-challenging the flies with 10 mM PQ (PQ-NM-PQ) considerably decreases the FI, thereby reducing the synthesis of TH protein. The findings imply that early-life pesticide exposure leaves a persistent effect on the DAergic nigrostriatal system, rendering it more susceptible to subsequent exposure to the same pesticides in later life stages. The results suggest that, despite reduction in TH synthesis, the number of neurons remains unaffected and the structure of the DA neurons (cell bodies) does not degenerate. The observation correlates with the results of a study conducted by Navarro et al. (2014), in which they measured and demonstrated a reduction in the FI of the GFP reporter protein rather than the actual loss of neurons.

DAergic neurons of the flies exposed to PQ twice (PQ-Cu-PQ) demonstrated a significant increase in FI following the Cu feeding regimen compared to the PQ-NM-PQ group. In particular, the FI of neurons in the PAL, PPL1, PPL2, PPM1/2, and PPM3 clusters increased by approximately 33% (**p<0.01), 36% (**p<0.01), 67% (***p<0.001), 28% (*p<0.05), and 49% (***p<0.001), respectively (**Figure 3.9 D**). Further comparisons were done between flies exposed to PQ once and then fed Cu (PQ-Cu-Su) and those exposed to PQ twice but fed NM (PQ-NM-PQ). DAergic neurons were significantly protected in the PAL, PPL1, PPL2, PPM1/2, and PPM3 clusters, with FI rescue rates of 30% (**p<0.01), 37% (**p<0.01), 54% (***p<0.001), 35% (**p<0.01), and 31% (**p<0.01) respectively (**Figure 3.9 D**).

The total FI of all DAergic neurons in the fly brain was measured to consolidate these findings. Exposure to the neurotoxicant resulted in a 34% decrease in FI (PQ-NM-PQ) (**P<0.01) compared to Su-NM-Su, indicating a reduction in TH protein synthesis. Cu intervention increased FI by 39% (***P<0.001) in the PQ-Cu-PQ group and 35% (***P<0.001) in the PQ-Cu-Su group (**Figure 3.9 E**) compared to PQ-NM-PQ. These

findings support Cu's ability to reduce DAergic neurodegeneration and neuronal dysfunction caused by PQ exposure throughout the adult transition period.

3.2.7. *10-day Cu feeding regime preceded and followed by PQ treatment rescues DA and its metabolites during the TP of Drosophila*

Interestingly, as Cu feeding protects against PQ-induced neuronal dysfunction and motor deficits during the TP, further investigation was conducted to determine whether Cu feeding replenishes DA levels in the context of PQ neurotoxicity. DA level and its metabolites, DOPAC and HVA, were measured using HPLC in the fly brains of feeding regime groups.

In the PD brain (PQ-NM-PQ), during TP, depletion of DA by 49% (***$p<0.001$) was observed when compared with Su-NM-Su (**Figure 3.10 A**). Quantification of DAergic neurons showed that the FI intensity was significantly reduced upon re-challenged to PQ, revealing diminished TH synthesis and neuronal dysfunction (**Figure 3.9**). Therefore, reduced TH synthesis is directly correlated to reduced DA level. The immediate metabolite of DA, i.e., DOPAC level, was also reduced in TP of PD brain by 33% (**$p<0.01$) (**Figure 3.10 A**). Decrements in DA and DOPAC levels are also observed in the post-mortem brains of PD patients (Goldstein et al., 2011). HVA is the final product of DA metabolism, wherein the *Drosophila*, through the MAO/COMT analogous pathway, DA and DOPAC can be degraded to HVA (Yamamoto and Seto, 2014; Meiser et al., 2013). Hence, HVA levels was also measured, and it was found that in TP PD brain, the HVA level is depleted by 18%; however, no significant statistical differences were observed in Tukey's post hoc test compared to Su-NM-Su (**Figure 3.10 A**). Similarly, as observed in HP, DA depletion in the TP PD brain is much higher than that of DOPAC and HVA.

Flies exposed to a single insult, i.e., PQ-NM-Su, exhibited significant reductions in DA level by 42% (***p<0.001) compared to Su-NM-Su (**Figure 3.10 A**). However, quantification of DAergic neurons did not exhibit any significant variations in their FI of clusters in the whole brain or the overall sum intensity compared to flies solely exposed to sucrose during TP (**Figure 3.9**). Further, no significant differences were observed in DOPAC and HVA levels when compared to Su-NM-Su. Similarly, DA depletion is much higher compared to DOPAC and HVA.

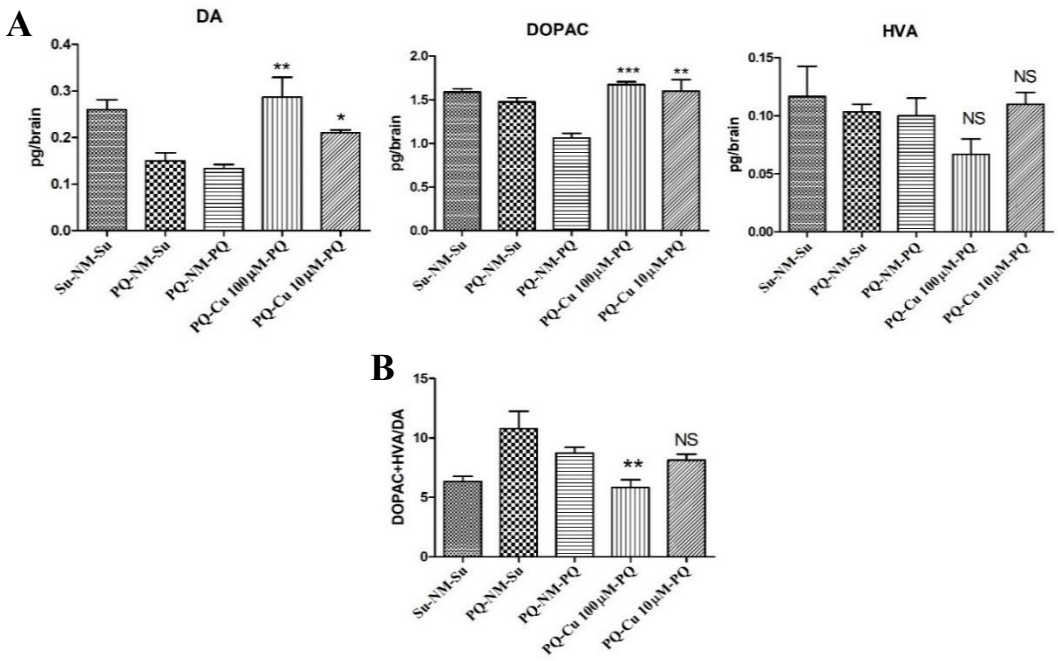


Figure 3.10: Quantification of DA and metabolite levels in fly brain using HPLC-ECD. During the transition phase, feeding the flies with PQ-NM-PQ alone led to a significant reduction in brain DA, DOPAC with no significant change in HVA levels compared to Su-NM-Su (A). The level of DA depletion is higher compared to downstream metabolite DOPAC, suggesting an enhanced DA turnover rate (B). Curcumin intervention, during transition phase rescues the diminished DA and DOPAC level, which is enough to prevent DA turnover. Insights suggest that curcumin is unable to fully mitigate DA depletion and inhibit the DA turnover rate during the transition phase of Parkinson's disease (PD) brain. However, the feeding regimes that were conducted, successfully rescued DA levels even during the transition phase. Significance was drawn by analyzing the data of a minimum of three replicates with one-way ANOVA followed by Tukey Post-Test for each age group. [***p<0.001; **p<0.01; *p<0.05; NS: Not significant - compared to PQ-NM-PQ treated group].

Cu intervention in the TP PD brain (PQ-NM-PQ) was observed to have a pronounced rescue in the depleted DA levels by 113%. Additionally, the DA downstream DOPAC levels, which were depleted in the PD brain, were rescued by 58%, while HVA levels

were unaltered (**Figure 3.10 A**). Given that 100 µM concentration of Cu demonstrated a significant efficacy, a lower concentration of 10 µM was tested to evaluate if it could also be effective against PQ exposure. When PQ was re-challenged in the later stage, it was determined that 10 µM Cu could also rescue DA levels by 56%. DOPAC levels were rescued by 50%, whereas HVA levels remained unchanged, which was observed to have a similar pattern in both concentrations (**Figure 3.10 A**). This shows that the rescue of DA levels is due to Cu's feeding intervention during the HP and TP-specific neuroprotection, which may be aided by the rescue of tyrosine hydroxylase (TH) synthesis, as revealed in the previous section in the study on maintaining DAergic neuroprotection with dietary interventions. Prior research indicates that Cu is ineffective in improving DA depletion during the TP (Das, 2022; Phom et al., 2014). Nevertheless, in the present study, the altered feeding regimens restored DA levels during this phase, illustrating Cu's potential efficacy in neuroprotection during the TP of PD.

The changes in DA, DOPAC, and HVA levels in PD conditions and following Cu feeding intervention were also examined to understand DA catabolism and turnover. The DA degradation/turnover ratio, calculated as [(DOPAC+HVA)/DA], was higher in TP PD brains compared to groups fed with Su-NM-Su (**Figure 3.10 B**). This suggests that DA depletion in PD might be due to increased degradation of DOPAC. Interestingly, the turnover ratio in the single-insult group (PQ-NM-Su) was comparatively higher, with a similar pattern as observed during the HP. Despite the decline in dopamine levels, the unchanged levels of DOPAC and HVA, combined with an elevated dopamine turnover ratio, suggest that the system compensates for DA loss by increasing its production and metabolism. This compensatory mechanism helps maintain stable fluorescence intensity (FI) clusters and overall FI in the brain, resulting in no significant variations. Caudle et al. (2007) demonstrated that the age-dependent loss of striatal DA appears to be the cause

of the DA turnover increase, as the levels of DOPAC and HVA remain unaltered in the aged VMAT2 LO rodents PD model. It is unclear whether or not this observation identifies a potential therapeutic window to halt disease progression; however, it does seem that fundamental neurochemical compensatory alterations can precede nigral dopamine neuron death. Cu dietary intervention prevented the DA turnover ratio (**$p<0.01$), indicating neuroprotective efficacy. However, previous lab study reported that Cu intervention failed to inhibit enhanced DA turnover in the TP PD brain, suggesting its limitation in promoting neuroprotection and preventing DA degradation, resulting in increased DOPAC and HVA synthesis (Das, 2022). In the present study, with a 10 day Cu feeding regime, Cu feeding effectively prevented the high DA turnover ratio during both phases and successfully rescued the diminished DA levels during both HP and TP (**Figure 3.9, 3.10**), highlighting the advantages of prophylactic feeding with this nutraceutical in sustaining or delaying the late-onset of PD.

3.3. Discussion

Curcumin's therapeutic potential is due to its antidepressant, anti-inflammatory, antioxidant, and acetylcholinesterase-inhibiting properties, however, clinical utility has been restricted by its insolubility in aqueous conditions, poor pharmacokinetics, low bioavailability, quick degradation, and inadequate transport across the blood-brain barrier (BBB) (Turer and Sanlier, 2024; Govindaraju et al., 2019). *Drosophila melanogaster* has several benefits for investigating NDDs such as PD owing to its short lifetime and rapid generation time, simple nervous system, and ease of genetic modification possibilities (Yenisetti et al., 2023). Hence, the present study evaluated the ameliorative effects of curcumin-containing diets using a *Drosophila melanogaster* model of paraquat (PQ)-induced neurotoxicity.

PD is a neurodegenerative condition that typically emerges in late life, with the potential for morbidity to be linked to insults that occurred earlier in life. The extent of exposure is challenging to ascertain, and the diagnosis of PD can be challenging (Zuo et al., 2023). Although a single toxic substance has been demonstrated to induce Parkinsonian features in experimental animals, the multiple-hit hypothesis is more applicable in practical human scenarios. Early life exposure increases the incidence of PD later in life, and developmental or early exposure to certain neurotoxins enhances the susceptibility of DAergic neurons upon adulthood re-exposure (Mittra et al., 2020).

In our laboratory, the adult lifespan of *Drosophila* is categorized into three stages: (a) the health phase, divided into early (~5 days) and late (30 days) stages, and (b) the transition phase, spanning from 31 to 60 days, which corresponds to the onset of late-onset neurodegeneration observed in humans (Ayajuddin et al., 2022; Phom et al., 2014).

Previously, our laboratory studies found that Cu's neuroprotective effects are stage-specific, employing the fruit fly model as sporadic PD. Cu has been demonstrated to

enhance mobility and replenish brain dopamine (DA) levels exclusively during the health phase, not the transition period (Ayajuddin et al., 2022; Phom et al., 2014). This underscores a constraint in the therapeutic potential of Cu. These results indicate the presence of alternative molecular pathways in neurodegeneration/neuroprotection and necessitate a reassessment of the use of early-life models to investigate late-onset neurodegenerative disorders such as PD. It also emphasizes the constraints of evaluating the potential neuroprotective efficacy of nutraceuticals solely based on stress marker-based studies.

In the present study, the focus was on sustaining the DAergic neuroprotective efficacy of Cu during the adult transition phase in a fly model of PD. In the 10 day Cu feeding regime, a re-challenge model was established to investigate the effects of early-life PQ exposure on motor neurobehavior in a late-life stage, in which flies were exposed to sucrose or PQ after 10 days of Cu feeding. No effect on the motor function of flies was observed in the group during single pesticide exposure. However, ingestion of PQ upon re-challenge negatively affected mobility, as evidenced by the fly's inability to climb normally, indicating that locomotor activity reductions were also age-dependent, with older flies exhibiting more pronounced reduction (**Figure 3.2**). Cu feeding upon PQ exposure showed recovery of motor deficits, with improved climbing speed in both the health and transition stages, indicating that the feeding regime could sustain the neuroprotective efficacy of Cu even during the adult transition phase.

A comparable age-related increase in neurotoxicity was observed for DAergic markers, particularly for PQ. Previous research has demonstrated substantial correlations between rodents on motor impairments and nigral levels of DAergic markers (Colle et al., 2020; Santos et al., 2017). Fly models of PD demonstrate age-dependent mobility defects, reduced brain dopamine levels, and the loss of DAergic neurons (Chaudhuri et al., 2007;

Feany and Bender, 2000). DAergic neurons in the entire fly brain were quantified using fluorescence microscopy to evaluate "neuronal dysfunction." A secondary antibody's fluorescence intensity (FI) that targeted the primary anti-TH antibody was measured and correlated with TH protein levels and synthesis (Chaurasia et al., 2024; Ayajuddin et al., 2023). This method assessed the potential neuroprotection from Cu dietary intervention and assisted in determining the extent of DAergic neurodegeneration and dysfunction under induced PD conditions.

Normal brain aging can cause nigrostriatal DAergic neurons to degenerate, leading to functional loss in this pathway (Li et al., 2020).Toxic events in later life, such as exposure to neurotoxic chemicals, might hasten degeneration, resulting in earlier onset of PD symptoms (Ayajuddin et al., 2022). Neurotoxic insults that occur during brain development may also result in the loss of DAergic function or neurons. Research implies that developmental insults may result in more severe neurodegenerative effects when the central nervous system is re-challenge later in life. A hypothetical model for DAergic neurotoxicity, which has been previously investigated in rodent models (Colle et al., 2020; Cory-Slechta et al., 2005; Thiruchelvam et al., 2002), demonstrates that developmental exposure to PQ + MB can render the DAergic system more susceptible to subsequent insults. The behavioral and histological outcomes in re-challenged animals are likely the result of additive rather than synergistic toxic events.

Ayajuddin et al. (2022) reviewed the ongoing debate and discrepancies regarding DAergic neuronal degeneration in *Drosophila* PD models. In a *Drosophila* model of PD, Feany and Bender (2000) first demonstrated the adult-onset loss of DAergic neurons. Subsequent studies have reported varying degrees of DAergic cell death in different clusters, contributing to ongoing debates and discrepancies in the field.

Auluck and Bonini, (2002), Auluck et al. (2002) and Yang et al. (2003), stated a 50% loss of DAergic neurons, while studies on flies with mutations in PD-associated genes like *PARKIN* and *PINK1* showed degeneration of only a few neurons in specific clusters (Trinh et al., 2008, 2010; Cha et al., 2005; Whitworth et al., 2005). Conversely, Pesah et al. (2004) found no neuronal loss in the PPM1/2 cluster of *PARKIN* mutants, suggesting selective vulnerability.

In *PINK1* models, discrepancies range from minimal neuron loss in PPL1 (Park et al., 2006) to significant neuronal decrease in RNAi knockdown flies (Wang et al., 2006; Yang et al., 2006). Toxin-induced PD models, such as those using PQ, also show variable results. Exposure to 5 mM PQ for 12-48 hours caused significant DAergic neuronal loss in specific clusters (Chaouhan et al., 2022; Maitra et al., 2021; Soares et al., 2017), while higher PQ concentrations led to selective neuronal loss countered by *HSP70* overexpression (Shukla et al., 2014). Reduced *Aux* expression, mimicking α-synuclein toxicity, affected PPM1/2 clusters and increased susceptibility to PQ and α-synuclein overexpression (Song et al., 2017). Contradictory findings are also present, with some studies reporting no change in neuronal numbers with PQ exposure (Navarro et al., 2014). These discrepancies highlight the complexities and ongoing debates in research on DAergic neuronal loss in *Drosophila* PD models. This suggests that while there may be no structural loss of DAergic neurons, there is often a decrease in GFP levels or FI, indicating reduced TH production in DA neurons (Chaurasia et al., 2024; Ayajuddin et al., 2022, 2023; Koza et al., 2023; Navarro et al., 2014).

Previous research also demonstrated that Cu could restore DAergic system levels and protect DA neuronal malfunction only throughout the HP of the adult lifespan, not the TP (Ayajuddin et al., 2022; Das, 2022). This highlights a limitation in Cu's therapeutic potential. Treatment with L-DOPA (levodopa) can temporarily alleviate PD symptoms

by replacing decreasing natural DA, but it does not halt disease progression. This is because the "dying back" phenomenon, where neurodegeneration starts at the axonal terminus, causes irregular L-DOPA uptake and inconsistent DA receptor activation, leading to dyskinesia and increased plasma L-DOPA toxicity (Watanabe et al., 2024).

In the present study, flies treated with PQ and re-challenged with the same pesticide exhibited a greater degree of neurotoxicity in the DAergic system than those exposed to a single exposure. The significant reduction in FI across different neuron clusters was evidence of this increased susceptibility. However, this reduction in FI could be significantly rescued upon feeding with Cu during both the HP (**Figure 3.5 D**) and the TP (**Figure 3.9 D**), except for the PPL2 cluster during HP, where no significant rescue was observed upon PQ exposure with Cu feeding. The total FI of all DAergic neurons in the fly brains of various experimental groups was also measured (**Figure 3.5 E, 3.9 E**). When these groups were analyzed separately during both the HP and TP, comparable outcomes were observed. Interestingly, the FI did not decrease when flies were exposed to PQ alone during either the HP or TP.

Studies have shown that Cu mitigates MPTP-induced neurotoxicity and locomotor defects in a PD mouse model by suppressing α-synuclein aggregation and reducing oxidative stress (Xia et al., 2016). Cu enhanced locomotor abilities and lifespan while reducing oxidative stress and DA neuron degeneration in *Drosophila* model of PD induced by rotenone and expressing human α-synuclein (Siddique et al., 2014; Liu et al., 2013). In a 6-OHDA rat model, Cu treatment also protected DA neurons in the *substantia nigra* and preserved dopamine levels (Zbarsky et al., 2005).

Our findings correspond with the study of Colle et al. (2020), suggesting that the nigrostriatal DAergic system in rodents undergoes mild neurotoxicity due to PQ + MB exposure during the early postnatal period without resulting in substantial motor

impairment. Nevertheless, postnatally and in adulthood, mice exposed to these pesticides are more susceptible to subsequent adult exposure, as demonstrated by motor deficits and reduced levels of DAergic markers. According to Thiruchelvam et al. (2003), young rodents can initially compensate for neurotoxic damage by increasing TH activity to maintain dopamine levels. Nevertheless, this compensatory capacity will deteriorate as they age, resulting in irreversible and progressive harm to their DAergic system. This also suggests that the absence of *PINK1* or *Parkin* does not influence mitophagy levels in flies during their early life stages, whereas in aging flies, basal mitophagy levels are reduced where compensatory mechanisms initially maintain basic mitophagy levels. Despite this, these mechanisms are insufficient to sustain mitophagy requirements over time, leading to age-dependent decreases in mitophagy levels following the loss of *PINK1* or *Parkin* (Vos and Klein, 2021). Axonal sprouting may be a compensatory mechanism for the DAergic system's regenerative capacity, as shown by increased TH fibers and new synapse formation after neurotoxic lesions (Colle et al., 2020).

Reduced TH protein quantities are associated with decreased FI, which suggests "neuronal dysfunction" in the absence of neuronal cell body loss. This dysfunction may be the underlying cause of PD in both early and late-onset modeling. Therefore, the quantification of TH synthesis enables us to assess the effect of DAergic neuroprotection provided by curcumin intervention and to measure neurodegeneration in the PQ-induced fly model.

Various animal models have been used to illustrate the neuroprotective efficacy of Cu (Setzu et al., 2024; Rathore et al., 2023), where several studies use young PD fly models that are either co-treated or pre-treated during the healthy phase to understand DAergic neurodegeneration better and assess the effectiveness of therapeutic compounds. Small molecules, pharmaceuticals, and nutraceuticals are screened using this method to

ascertain their DAergic neuroprotective efficacy (Maitra et al., 2021; Nguyen et al., 2018; Sur et al., 2018).

Researchers evaluate the neuroprotective efficacy of these molecules by analyzing biochemical markers, such as levels of antioxidant enzymes, brain DA, and its metabolites, as well as behavioral markers, such as mobility defects. Furthermore, cytological markers, including the degeneration of DAergic neurons throughout the entire brain of young animals, are assessed. It is crucial to note that late-onset NDDs, such as PD, usually begin during the transitional period of adulthood. Nevertheless, the efficacy of Cu seems to be stage-specific, with more significant benefits reported in the early stages of PD than in the later periods (Ayajuddin et al., 2022; Das, 2022; Phom et al., 2014).

Research suggests that cerebrospinal fluid (CSF) levels of DA may not reliably indicate central DA deficiency due to influences from adaptive changes compensating for the loss of DA neurons or adaptations from anti-Parkinsonian medication (Goldstein et al., 2012). In contrast, the acidic metabolites homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) are regarded as more reliable indicators of DAergic neuron loss in untreated PD (Andersen et al., 2017). Research on DA metabolism offers insights into the biochemical modifications in the DAergic system linked to PD.

In the present study, neurotoxicant exposure led to the depletion of DA levels in the brain across both phases (**Figure 3.7, 3.10**). In the HP, both the two-hit re-challenge of PQ and PQ ingestion alone consistently showed DA depletion, accompanied by decreased DOPAC levels and no significant reduction in HVA levels. However, the resulting DA turnover was higher in the single exposure PQ-NM-Su group compared to the PQ-NM-PQ (**Figure 3.7 A, B**).

The PQ re-challenge in the TP exhibited a similar pattern, with DA depletion accompanied by a relatively lesser reduction in DOPAC and no significant change in HVA levels, resulting in increased DA turnover (**Figure 3.9 A, B**). However, with a one-time exposure to PQ, DA levels decreased, but DOPAC and HVA levels did not show significant changes in TP.

Studies also demonstrated that chronic PQ decreased DOPAC levels but did not affect HVA levels in the *substantia nigra* of male C57BL/6 mice (McCormack et al., 2002). After administering PQ for four weeks, Ossowska et al. (2006) observed no change in DOPAC levels and an increase in HVA levels, however, DOPAC levels increased after 8 weeks of treatment, while HVA levels remained unaltered. Additionally, an increase in DA turnover was observed after 4 weeks of treatment. Methamphetamine administration, whether as a single dose or multiple doses, resulted in DA depletion and an increase in turnover. The turnover was substantially higher than that caused by multiple doses simultaneously, and these effects were reproduced at 24 hours with a single dose. There was no change in the levels or activity of TH within the first 24 hours after administration despite the similar DA depletion (Pereira et al., 2006).

In humans, the DA, DOPAC, and HVA levels are significantly altered in the putamen, nucleus accumbens, and caudate nucleus of the PD post-mortem brain (Toulorge et al., 2016; Gerlach et al., 1996). DA, DOPAC, and HVA levels are significantly reduced in cerebrospinal fluid of human PD patients (Goldstein, 2021; Andersen et al., 2017). A decrease in DOPAC and HVA in PD patients reflects the loss of DAergic neurons in the striatum and inhibition of MAO-B pathways (McDermott et al., 1995).

Further research is needed to investigate whether changes in DOPAC and DA levels in the fly model are associated with neurological issues in addition to observed PD motor symptoms. It is hypothesized that the increased oxidation and degradation of DA as a

result of neurotoxicant exposure will result in decreased DA levels and increased DOPAC synthesis. It is observed that the degradation of DOPAC to HVA is comparatively reduced during the HP (**Figure 3.7**), and TP (**Figure 3.10**) when they are re-challenged with the PQ exposure. However, there are no significant changes in HVA levels. This suggests a different synthesis route: DA>3-MT>HVA or DA>DOPAC>HVA. The synchronized depletion of DA and DOPAC implies that changes to the DA are immediately reflected in the DOPAC. The DOPAC in cerebrospinal fluid (CSF) is a reliable marker of DA deficiency in human PD. This is because the nigrostriatal region and CSF exhibit a DOPAC deficiency, highlighting the DA deficiency in the central brain.

This study suggests the progression of PD in the aging brain could possibly be influenced by an increase in the oxidative degradation of DA. Although more research into DA metabolism in the late-onset fly model of PD is needed, our findings show that PQ-induced sporadic PD conditions cause DA depletion throughout both the HP and TP and increase DA oxidative breakdown to downstream catabolites. The neurotoxic effects of these DA catabolites and the production of ROS/peroxides during the catabolic process (Watanabe et al., 2024) are likely to contribute to neurodegeneration.

Cu has shown the ability to restore brain DA levels exclusively during the HP but not the TP of the adult lifespan (Ayajuddin et al., 2022; Phom et al., 2014). Additionally, Cu has been shown to restore DA, DOPAC, and HVA levels in neurodegeneration mediated by lipopolysaccharide in young Sprague-Dawley rats (Sharma and Nehru, 2018). Cu restored the depletion of DA and DOPAC induced by MPTP in adult Swiss mice (Rajeswari and Sabesan, 2008). The present study demonstrates that diminished levels of DA upon PQ exposure can be significantly rescued through the remodelled feeding regime with Cu feeding during the HP and, interestingly, the TP of adult flies (**Figure 3.7, 3.10**).

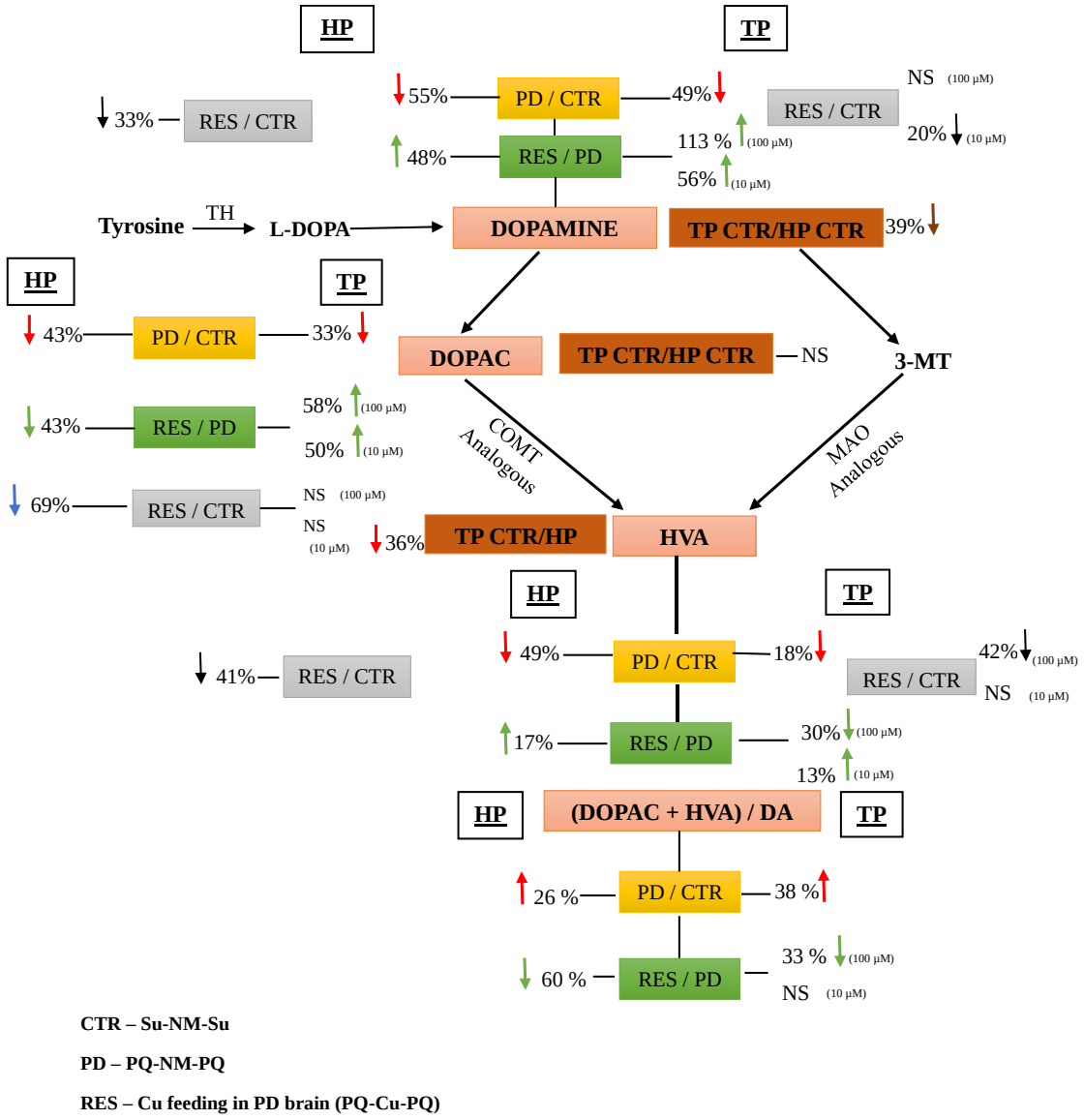


Figure 3.11. Schematic representation of DA metabolism in the PD brain and with Cu interventions: During HP, DA, and DOPAC levels are decreased, while HVA levels, although reduced, did not show significant differences under the PQ-mediated PD condition according to Tukey's post hoc test. The relatively higher depletion of DA in the fly brain under the PD condition in comparison to DOPAC and HVA suggests that DA oxidation is higher. Cu intervention restored the significantly reduced DA level and rescued the altered turnover of DA during HP. In the TP PD brain, DA and DOPAC levels were depleted, but HVA levels, despite being reduced, did not exhibit significant differences according to Tukey's post hoc test. In contrast to DOPAC and HVA, DA depletion was more significant, suggesting that DA turnover was higher. Cu intervention restored the reduced DA level and rescued the altered DA turnover during TP.

Cu demonstrated the ability to restore brain DA levels exclusively during the health phase, not the transition phase of the adult lifespan (Ayajuddin et al., 2022; Phom et al., 2014). This highlights a limitation in Cu's therapeutic potential. Insight on the Cu dietary intervention (PQ-Cu-PQ) suggests that during HP, depleted DA level is rescued;

however, no rescue is observed on DOPAC level, hence, further inhibition and normalization of HVA pool, resulting in lesser DA turnover (**Figure 3.7**). Interestingly, the DA and DOPAC levels have a pronounced rescue in the PD brain, and the HVA level remains unaffected during TP. Cu dietary intervention successfully inhibits the DA turnover in the TP PD brain (**Figure 3.10 B**). Cu has been demonstrated to inhibit mitochondrial MAO activity isolated from rat brains (Khatri and Juvekar, 2016). MAO is the primary enzyme necessary for the oxidative turnover of DA to DOPAC and is one of the enzymes essential for the oxidative turnover of DA to HVA. MAO inhibition is of considerable interest in drug discovery, where variants of MAO inhibitors can be used as a potent therapy for neurodegenerative disorders like PD (Elkamhawy et al., 2021). Although flies do not have the orthologue coding for MAO and COMT enzymes, it is apparent that the fly brain possesses analogous enzymatic pathways for DA catabolism (Yamamoto and Seto, 2014). Therefore, it is possible that Cu intervention not only restores the DA pool in the brain but also inhibits MAO analogous activity, thereby preventing oxidative turnover of DA to its downstream metabolites (Das, 2022). Consequently, the preservation of DA also serves to prevent the generation of ROS and promotes neuroprotection during the HP and TP.

The observed depletion in DA and DOPAC levels, alongside unchanged HVA levels, no DAergic neurodegeneration and no motor defects (particularly during HP) in case of single hit exposure (PQ-NM-Su), suggests a compensatory mechanism within the DAergic system. This compensatory response likely involves increased activity at the residual DAergic terminals to maintain neurotransmitter function (Colle et al., 2020; McCormack et al., 2002).

In adulthood, mice exposed to pesticides during the early postnatal period exhibited a recovery of mitochondrial complex I and II activities (PQ + MB//saline group),

suggesting that mitochondrial function was restored following postnatal exposure and absence of significant motor defects (Colle et al., 2020). Mitochondria are organelles that are capable of responding to stress conditions or stimuli. Recent literature has described changes in mitochondrial dynamics due to exposure to environmental contaminants. After such exposures, modifications in mitochondrial morphology and the expression of proteins that modulate mitochondrial fission and fusion have been observed. Furthermore, this may function as an adaptive mechanism to prevent cell mortality at low concentrations (Meyer et al., 2017). Agim and Cannon, (2018) found that a single dosage of the genotoxin PhIP affects dopamine metabolites and turnover at 8 hours but not 24 hours, indicating some recovery between 8 and 24 hours. Additionally, DAergic neurons exhibited an increase in nitrotyrosine levels 8 hours following PhIP treatment; however, these levels normalized by 24 hours. These results indicate that PhIP induces acute, but not persistent, toxicity, which may be attributed to the activation of antioxidant response mechanisms, transient inhibition of dopamine metabolism enzymes, or the rapid elimination of PhIP. Given the vulnerability of DAergic neurons in PD, this transient recovery suggests that similar environmental toxins may contribute to early, reversible changes in neurochemistry, which, over time, could potentially lead to the progressive neurodegeneration observed in PD.

DA uptake into vesicles and its metabolism to DOPAC via ALDH regulate DOPAL levels for a given rate of cytoplasmic DA synthesis. DOPAL levels are significantly elevated by the inhibition of ALDH and the blockade of vesicular uptake. Post-mortem studies have demonstrated that putamen tissue from PD patients contains a "double hit" of decreased ALDH activity and vesicular storage defects, indicative of a potential mechanism contributing to the disease (Goldstein, 2021).

Goldstein (2021) and Goldstein and Kopin (2018) suggest that the compensatory increase in activity at the remaining functional nerve terminals initially aids in the stabilization of neurotransmitter levels. By increasing the workload of the surviving terminals, this increased activity compensates for neuronal loss. However, this also imposes additional stress on the surviving terminals, which may result in the further depletion of DA and its metabolite, DOPAC. Although no immediate DAergic neurodegeneration is observed, this situation may establish favorable conditions for developing lethal positive feedback loops. In such cases, the compensatory mechanisms may ultimately result in neuronal dysfunction or degeneration if the underlying stressors become more severe or persistent, which is observed in case of re-challenged neurotoxicant exposure.

In the early life stages of *Drosophila* exposure to PQ, Koza et al. (2023) observed no motor defects or observable DAergic neurodegeneration. This observation suggests the possibility of cell type-specific variations in DA and its metabolites. Consequently, the early onset of sexual dysfunction in these flies can function as a sensitive early marker for PD, rendering courtship behavior a valuable indicator for early detection and intervention. In a recent study, it was found that *Drosophila* larvae possess a compensation system wherein certain neurons may identify and counterbalance the loss of neighboring neurons by expanding synapses and enhancing neurotransmitter release (Wang et al., 2021).

In a study of 353 PD patients and 60 healthy controls, both approximately 60 years old, researchers found that compensatory activity in the parieto-premotor cortex is crucial for improving symptoms, particularly in those with a mild-motor predominant subtype. This is despite motor defects being associated with basal ganglia dysfunction in PD. Future treatments should prioritize enhancing these compensatory mechanisms to improve overall neurological function and delay disease progression, as this cortical activity is

linked to reduced bradykinesia severity and improved cognitive performance (Johansson et al., 2024).

Additionally, research on PD emphasizes lifestyle interventions, including exercise, to identify modifiable risk factors and cell transplants to restore dopamine loss through stem cell transplantation, as evidenced by ongoing studies like TRANSEURO (Barker, 2019). Among the strategies implemented to slow the progression of the disease are the targeting of α-synuclein and the repurposing of treatments (Brundin and Wyse, 2019). Additional research is necessary to generate dependable disease-modifying therapies, as certain treatments have shown potential but are not as effective. Additionally, developing reliable biomarkers for early diagnosis and improving access to care and education in marginalized populations are critical areas of development (Bloem et al., 2021).

Relying solely on a single treatment approach can be myopic, as it overlooks the benefits of alternating between various strategies. Compensation mechanisms, when excessively automated and internalized, become increasingly dependent on the defective basal ganglia. Therefore, a more comprehensive and less myopic approach to managing PD involves alternating strategies to preserve the effectiveness of compensatory mechanisms.

3.4. Conclusion

The current study elucidates the neuroprotective efficacy of curcumin during the adult health and transition phases of *Drosophila*, emphasizing its role in sustaining tyrosine hydroxylase and dopamine synthesis during critical brain developmental stages. Results indicate that neuroprotection is substantially influenced by altered feeding regimens with curcumin, as they maintain dopamine levels and improve motor function. Moreover, this study demonstrates that developmental exposure to neurotoxicants, such as paraquat, might result in "silent toxicity," which appears only under additional stresses later in life. Curcumin shows promise as a nutraceutical compound that augments one's diet rather than a medication regimen. Small, consistent doses of Curcumin administered throughout one's lifespan may have more beneficial (and potentially less toxic) effects than current PD drugs. While many studies have focused on younger models, where neuroprotection and compensatory mechanisms are more robust, this study provides novel insights by using flies in their transition phase, representing an older model. This approach is more reflective of PD, a late-onset neurodegenerative disorder. By employing older models, we gain a deeper understanding of how neurodegeneration progresses with age, offering a more accurate assessment of curcumin's prophylactic nutraceutical potential to mitigate pesticide-induced neurotoxicity in aging systems.

Further investigation into the underlying molecular networks in an adult life stage-specific manner will significantly contribute to understanding the pathophysiology of PD and advancing therapeutic strategies for neurodegenerative disorders.

(Contributions: 1. Animal treatments and climbing assay: Nukshimenla Jamir; 2. Immunofluorescence experiment and analysis: Rahul Chaurasia 3. HPLC experiment and analysis: Dr. Abhik Das and Dr. Mohamad Ayajuddin)

CHAPTER 4

Prenatal Developmental Feeding of Curcumin Rescues Paraquat Induced Mobility Defects in Adult Transition Stage of a *Drosophila* Model

4.1. Introduction

Toxicity arises when a substance exhibits contradictory effects due to its inherent properties or excessive usage. Evidence from studies consistently supports that excessive and imprudent exposure to various substances, such as pesticides, herbicides, heavy metals, fungicides, metal-based nanoparticles, food additives, air pollutants, and dietary components etc, can increase the risk of diseases, and the prevalence of those diseases at any stage of life (Srivastava et al., 2023; Arab and Mostafalou, 2022; Nabi and Tabassum, 2022; Jankowska-Kieltyka et al., 2021; Lerner and Benzvi, 2021).

Exposure to environmental chemicals can adversely impact the nervous system across all life stages, with particular concern during developmental periods. Development of the brain is an extraordinary process that commences during pregnancy and persists until adolescence (Lubrano et al., 2024). The intricate nature of brain development might correspond to a common progenitor cell responsible for the development of nearly every variety of brain cells, such as neurons, astrocytes, and glial cells. Prenatal and postnatal brain development encompasses developing and positioning neurons, neuronal receptors, synapses, and synaptic connections, establishing the foundation for encoding information throughout life (Jauhari et al., 2022).

Exposure to low levels of certain compounds can impair the developing nervous system's structure and function due to its sensitivity (Bennett et al., 2016; Grandjean and Landrigan, 2014; Rice and Barone, 2000). The developing nervous system may be impacted by adverse chemical-dependent effects that result from the exposure of the developing progeny (including in utero and postnatal) to the time of sexual maturation. This "developmental neurotoxicity" (DNT) can have a long-term impact, extending beyond the exposure period, and can differ across the lifespan. Such developmental impacts are of significant regulatory concern due to their potential long-term

consequences (Tal et al., 2024; Spalding et al., 2013; Costa et al., 2004; Eriksson et al., 1998).

The multiple hit hypothesis that combines toxic stress from dopamine oxidation or mitochondrial dysfunction and, or loss of function can particularly be suited to explain the etiology of complex neuropsychiatric and neurologic diseases that share a highly sporadic nature characterized by genetic and environmental risk factors, which illustrates the long latency between exposure and disease, as subclinical deficits occurred during specific windows of developmental plasticity that do not manifest without a second hit occurring later in life (Jamir and Yenisetti, 2021; Heindel et al., 2015; Lahiri et al., 2009) (**Figure 4.1**).

Neurotoxicity refers to the occurrence of anatomical, morphological, physiological, biochemical, and behavioral abnormalities throughout the embryo's development to the fetus, which can result in malformations in the adult. The term "neurotoxicant" refers to the agent that causes neurotoxicity (Mishra et al., 2023; Coyle et al., 1976).

In determining the impact on human brain development, the timing of chemical exposure—whether prenatal or postnatal—and the type of toxicant are critical factors (Grandjean et al., 2019; Heyer and Meredith, 2017; Bennett et al., 2016). Neural progenitor cells are more sensitive to toxicants than glial cells or mature neurons, causing the prenatal period, particularly the first and second trimesters, to be more vulnerable to environmental toxins (Fritsche et al., 2018; Heyer and Meredith, 2017; Druwe et al., 2015). Chemical-induced disruptions during these vulnerable stages of neurogenesis, neuronal development, and migration have been shown to increase the susceptibility to PD and other neurodegenerative disorders in adulthood, according to experimental evidence (Kisby and Spencer, 2021; Schaefers and Teuchert-Noodt, 2013).

During development, a network of genes interacts to develop the nervous system, which controls an animal's physiology and behavior. Prolonged exposure to stressors can cause chronic stress, resulting in allostatic load and molecular alterations in the brain, the primary regulator of the nervous system (Min and Condron, 2005). Neurotoxicants have the potential to impair the stability of the CNS, resulting in behavioral changes in later developmental phases by altering neuronal symmetry, chemistry, or connections (Alves-Pimenta et al., 2018). Toxin exposure during development can also have an impact on the neurological and endocrine systems, resulting in behavioral abnormalities in adulthood (Hirsch et al., 2012). Neurotoxicity is assessed using a variety of methods, such as morphological assessments, biochemical assays, and behavioral tests.

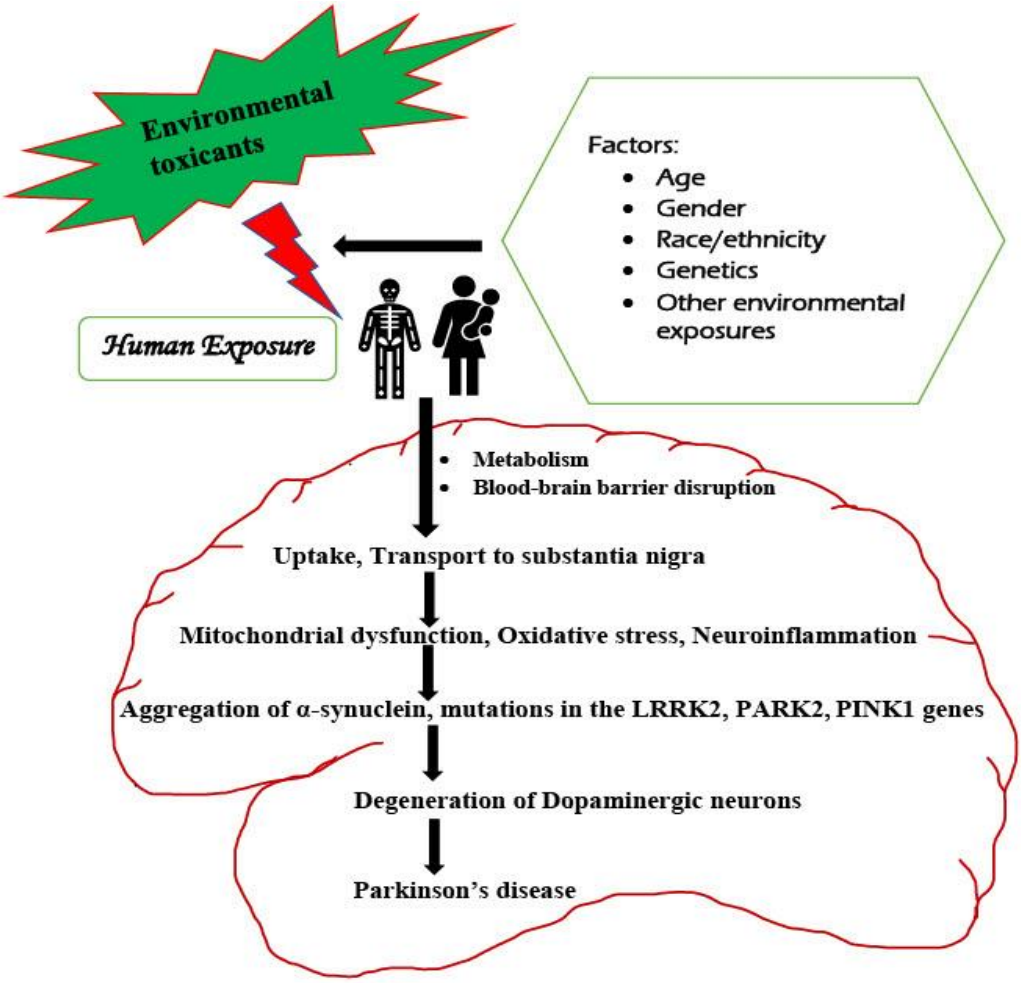


Figure 4.1. Mechanisms of gene-environment interaction in the early development of sporadic/idiopathic neurodegenerative diseases.

According to the DOHaD (Developmental Origins of Health and Disease) hypothesis, early-life environmental exposures can increase disease risk in adulthood (Fukunaga, 2021; Gillman, 2005). PD is a neurological degenerative disease that primarily impacts older adults, with the highest occurrence observed between the ages of 70 and 79 (Jiménez-Salvador et al., 2023; Hirsch et al., 2012). A significant pathogenic aspect of PD is nigrostriatal DAergic neuron degradation, which can occur long before motor symptoms are established (Giguère et al., 2018).

The current guidelines (OECD TG 426 and US EPA 712-C-98-239; OECD, 2007; US EPA, 1998) heavily rely on *in vivo* experiments, which are costly, time-consuming, and unsuitable for testing large numbers of chemicals, despite the critical need for data in neurotoxicity and DNT research (Tal et al., 2024; Bal-Price, 2012). To resolve this disparity, there is an increasing emphasis on developing alternative models incorporating novel testing strategies for more mechanistic approaches, such as mammalian cells in culture or non-mammalian systems (Xie et al., 2020). These methods are designed to detect biomarkers specific to a particular pathway and can be used to predict neurotoxic outcomes (Bal-Price and Meek, 2017).

The prevalence of PD rises after the age of 60 (Elbaz et al., 2016), but pre-clinical investigations frequently employ younger animals, which may not effectively represent age-related neurodegeneration (Sun et al., 2020). Laboratory mice are often investigated before 12 weeks of age and are considered sexually immature, while studies using rats seldom last more than 12 months, which equates to around 30 years in humans (Wang et al., 2020; Dutta and Sengupta, 2016; Flurkey et al., 2007). The disease is more effectively replicated by utilizing older animal models, as aging is a fundamental characteristic of PD (Sun et al., 2020).

Environmental neurotoxins, particularly pesticides, have been related to PD and aging. Several ideas have been offered, including one that proposes that early-life exposure to neurotoxicants such as PQ may create an initial, subclinical insult to the nigrostriatal DA pathway, with aging exacerbating the damage (Kanthasamy et al., 2019; Calne and Langston, 1983). Another concept holds that early exposure causes compensatory DA production, which eventually strains the system and leads to more neuronal death (Blesa et al., 2017; Thiruchelvam et al., 2003; Barbeau, 1984). Furthermore, there is evidence that elderly people may be more vulnerable to neurotoxins, while younger people are more resistant (Colle et al., 2020; Date et al., 1990).

PQ, which is chemically similar to MPP^+, has been linked to PD, with recent studies demonstrating its ability to disrupt the nigrostriatal pathway and preferentially trigger neuronal death (Vaccari et al., 2019; McCormack et al., 2002). Furthermore, PQ has been demonstrated to oxidize the ras-dependent oncogene product DJ-1, boosting its expression, which is associated with early-onset PD (Skou et al., 2024; Bonifati et al., 2003). Exposure to PQ and the fungicide maneb (MB) in combination has been demonstrated to cause a potentiated loss of nigrostriatal DAergic neurons and reduced striatal DA in mice, even at dosages that generate minor effects individually (Colle et al., 2020; Thiruchelvam et al., 2002). Furthermore, aside from the well-established harmful consequences of PQ exposure, there are also indications of PQ exposure during gestation due to its ability to easily pass through the placenta (Ait Lhaj et al., 2023; Berry et al., 2010). This may lead to developmental or post-developmental impacts in rodent offspring, such as behavioral abnormalities, cognitive impairment, and altered dopamine metabolism (Zuo et al., 2023; Mittra et al., 2020; Ait-Bali et al., 2016). PQ's adverse effects on mitochondrial function, DAergic neurons, striatal dopamine metabolism, and

trace element dysregulation in neurological disorders underscore the necessity of therapeutic interventions.

So far, only a few studies have explored neurotoxicity's delayed expression, where symptoms may remain hidden for months or years post-exposure (Kraft et al., 2016; Reuhl, 1991). Hormesis, a biphasic phenomenon, shows beneficial responses at low doses and harmful effects at high doses of environmental agents (Berry and López-Martínez, 2020; Mattson, 2008). Silent neurotoxicity, in contrast, involves long-term low-dose exposures, especially during critical developmental stages, leading to cellular stress and eventual neurodegeneration. The "event threshold" concept also suggests that neurodegenerative signs, such as those in PD, may only appear after significant neuronal loss (Costa, 2017).

Evelyn Witkin's groundbreaking discovery of the bacterial SOS system (Witkin, 1976) illustrates the classical notion that protecting responses can be elicited by low concentrations of hazardous substances. Mild environmental stress and stress-response mutations can increase the lifetime of animals. ROS can trigger cell-protective mechanisms in animals. Modest amounts of the toxin PQ or inhibition of mitochondrial superoxide dismutase can enhance mitochondrial superoxide dismutase levels to extend the lifespan of *Caenorhabditis elegans* (Wei and Kenyon, 2016; Lee et al., 2010). Studies demonstrated that low doses of PQ enhanced the antioxidant defense and diminished overall ROS production (Krůček et al., 2015; Hosamani and Muralidhara, 2013). In *Drosophila* species, hormesis-like benefits have been reported in relation to radiation (Moskalev et al., 2011), temperature (Gómez et al., 2009; Sørensen et al., 2008), and larval crowding (Lushchak et al., 2019; Henry et al., 2018), demonstrating that hormesis-like responses are widespread.

Nutrition, a significant environmental factor, plays a critical role in shaping animal physiology, metabolism, and traits such as survival and fitness (Klepsatel et al., 2020; Monaghan, 2008). Understanding how specific nutrients contribute to the animal phenotype is a central challenge in biology, given the complexity of food composition (Martelli et al., 2024). Animal models, particularly the fruit fly *Drosophila melanogaster*, offer valuable insights into genotype-environment interactions and their molecular underpinnings. These models are widely used in medical and biological sciences due to shared physiological and metabolic processes between flies and humans (Shin et al., 2018; Ugur et al., 2016).

Drosophila has gained particular attention in neurotoxicological research, owing to its neurological and developmental pathways, which closely mirror those of vertebrates (Rand, 2010). Its utility as a model organism for studying neurodevelopment and behavior makes it an excellent tool for assessing the impacts of environmental toxicants. With its short life cycle, high fecundity, and well-characterized genetics, *Drosophila* allows researchers to quickly study disease mechanisms and therapeutic interventions. The development of a fertilized egg to adulthood takes about 9–10 days, with the egg chamber undergoing 14 distinct stages, culminating in a fully developed oocyte (Schüpbach, 2019). These attributes not only facilitate rapid experimentation but also make *Drosophila* ideal for developmental and reproductive toxicity studies (Cabrita et al., 2022; Rand et al., 2019).

In recent years, *Drosophila* has also become increasingly recognized for its capacity to evaluate various compounds and their modes of action in relation to neurotoxicity and neurological disorders (Peterson et al., 2008). Its conserved molecular pathways and behavioral traits make it a powerful model for toxicological assays. While numerous studies have focused on general toxicity endpoints such as mortality, motor assays, and

enzyme activity (Cabrita et al., 2022; Cox et al., 2016; Rand et al., 2014), *Drosophila's* developmental and behavioral assays provide a more nuanced understanding of how specific compounds affect neurological functions.

Exposure to toxic chemicals can disrupt normal physiological functions in *Drosophila*, making it a reliable model for testing the neuroprotective potential of various compounds. Among these, plant-derived substances have garnered significant interest, particularly for their potential nutraceutical effects. Curcumin (Cu), a well-studied phytochemical, has attracted attention for its neuroprotective properties and has been the subject of extensive research (Garodia et al., 2023; Kunnumakkara et al., 2023). Its evaluation in *Drosophila* provides a promising avenue for understanding how natural compounds might mitigate the harmful effects of environmental toxicants.

Curcumin, a compound derived from turmeric, has demonstrated the potential to address cellular senescence and alleviate age-related symptoms, thereby extending lifespan. Its efficacy as a therapeutic agent for neurodegenerative diseases is suggested by its ability to cross the blood-brain barrier and its neuroprotective effects (Kunnumakkara et al., 2023; Kundu et al., 2016; Soh et al., 2013). The lifespan of the fly was extended by the addition of curcumin to sucrose-yeast diets, which resulted in the upregulation of superoxide dismutase (Shen et al., 2013) and protection against oxidative stress (Soh et al., 2013; Lee et al., 2010). The lifespan of flies raised on a diet supplemented with curcumin was significantly increased. The viability of the offspring of flies that were raised on curcumin was increased (Chandrashekara et al., 2014).

This study is the first to investigate whether prenatally fed Cu can act as a prophylactic nutraceutical molecule across different life stages beyond the health span in a PD model and sustain the DAergic neuroprotective efficacy of Cu during the adult transition phase in the *Drosophila* model of PD. As previously in our lab studies, Cu intervention was

found to rescue motor deficits during the health phase, but it failed during the transition phase (Phom et al., 2014). This observation raises the question of whether the intervention of Cu during early life sustains the genetic targets responsible for protecting DA during the health span, extending through the transition stage.

In the present study, I aim to examine the effects of PQ and Cu on the developmental viability, lifespan, and locomotor activity of flies. These metrics will be assessed from the prenatal stage until eclosion, which serves as a critical indicator for evaluating symptoms associated with PD phenotypes.

Additionally, this study will investigate whether prenatal curcumin intervention can sustain neuroprotection during the flies health and transition phases, even when re-challenged with PQ in later adult life stages. By assessing changes in motor symptoms and lifespan, this research aims to determine if prenatal Cu feeding can rescue mobility defects during the health and transition phases in the fly model of PD.

4.2. Results

4.2.1. *Assessing the PQ toxicity on pre-natal developmental stages of Drosophila*

Paraquat (PQ), a well-known neurotoxin commonly used as a standard in neurotoxicity screenings, was applied to study its effects on developing *Drosophila melanogaster* by transferring fertilized eggs into food containing defined concentrations of the compound. Curcumin, widely recognized for its therapeutic potential, was also utilized in the study, following the feeding regimen illustrated in **Figure 2.5.**

Analyses of various PQ doses were employed to investigate the effect of PQ toxicity. The screening was performed at concentrations 1 mM PQ to 1.5 mM PQ, 2.5 mM PQ, 5 mM PQ, and 7.5 mM PQ. PQ exposure did not cause any influence in their transition from egg-pupae, pupae-adult viability (**Figure 4.2 A**). However, when the concentration of PQ increased, the eclosion rate decreased, revealing a sharp decline in the adult fly eclosion rate at doses of 5 mM PQ and 7.5 mM PQ. The number of adults emerging successfully from the pupal case was reduced by 25% at 5 mM (**$p<0.01$) and 33% at 7.5 mM (***$p<0.001$), compared to the control, whereas in the 1 mM PQ, 1.5 mM PQ, 2.5 mM PQ no significant differences were observed in their eclosion (**Figure 4.2 B**) compared to the control group. For further Co-feeding experiments, PQ concentration 1 mM PQ was picked up, as it did not affect the survival of developing *Drosophila*, with a similar number of flies reaching adulthood and pupal stages compared to control.

When modeling PD pathogenesis in animal systems, including *Drosophila*, it is critical to avoid lethal concentrations that kill the animal before the DAergic neurons degrade. In such cases, the mechanistic insights generated by researchers utilizing animal models may be irrelevant to the clinical condition. Therefore, to study the developmental and behavioral markers associated with PD flies, exposure to 1 mM PQ was selected, as no

mortality was observed at any developmental stages of *Drosophila* with this feeding procedure.

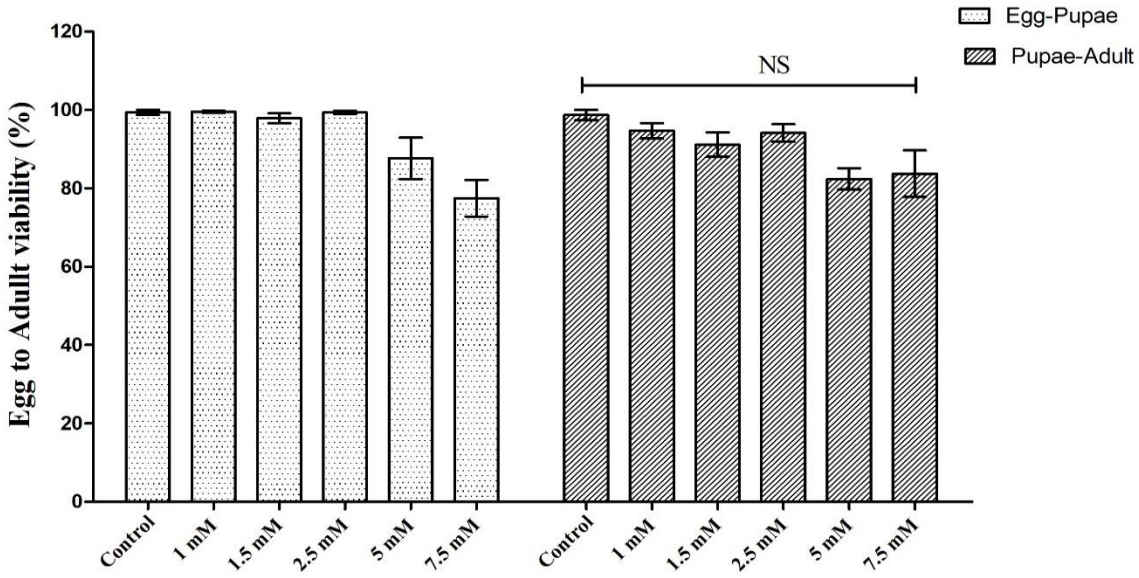


Figure 4.2 A: Assessing the PQ toxicity on prenatal developmental stages of *Drosophila*. The survival of developing *Drosophila* was evaluated by scoring eggs following the Delcour method (Delcour, 1969). Viability was recorded from egg-to-pupae and pupae-to-adult stages, reared on medium containing varying concentrations of paraquat viz., 1 mM PQ,1.5 mM PQ, 2.5 mM PQ, 5 mM PQ and 7.5 mM PQ. No significant differences were observed between egg-pupae and pupae-adult. The results were evaluated using two-way ANOVA Bonferroni post-test and represented as means ± SEM. [NS: Not significant].

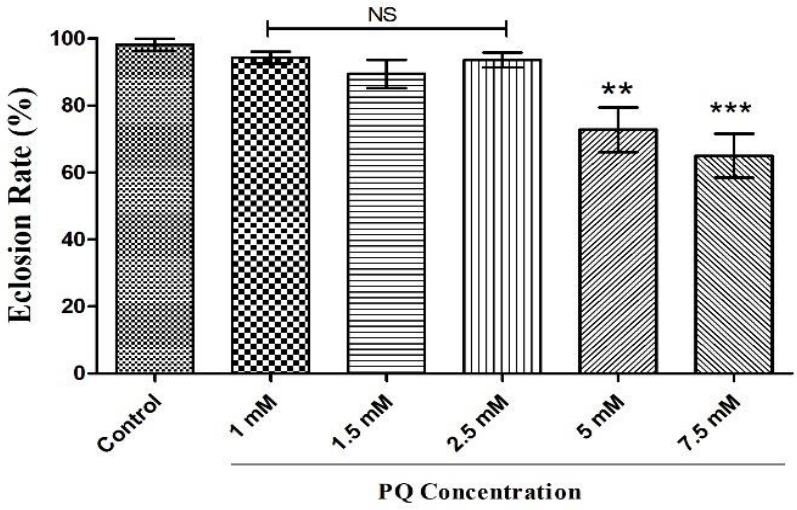


Figure 4.2 B: Effects on the eclosion rate of developing *Drosophila* when fed on diets containing increasing concentrations of PQ. *Drosophila* eggs were scored following the Delcour method (Delcour, 1969), reared on medium containing different concentrations of PQ viz., 1 mM PQ, 1.5 mM PQ, 2.5 mM PQ, 5 mM PQ and 7.5 mM PQ. The number of adults emerging successfully from the pupal case was reduced at 5 mM and 7.5 mM, compared to the control, whereas in the 1 mM PQ,1.5 mM PQ, 2.5 mM PQ there were no significant differences observed in their eclosion when compared to the control. Results were presented as means ± SEM and differences were statistically determine using one-way ANOVA and Tukey Post hoc test. [**p<0.01; ***p<0.001; NS: Not significant].

4.2.2. *Assessing the Cu toxicity on pre-natal developmental stages of Drosophila*

Analyses of various Cu concentrations were done to investigate the effect of Cu toxicity. The screening was performed at concentrations 5 µM, 10 µM, 25 µM, 50 µM,100 µM, 500 µM,1 mM, 2.5 mM, and 5 mM Cu. The Cu concentration versus the flies egg-to-adult eclosion rate was plotted as shown in **figure 4.3 A, B**. 100 µM, 50 µM, 10 µM, 25 µM, 5 µM Cu did not show any significant difference with egg-pupae when compared to pupae-adult viability, however on increasing concentrations, significant deleterious toxicity with pupal death and adult eclosion were observed in concentrations 500 µM, 1 mM, 2.5 mM and 5 mM Cu (**Figure 4.3 A**).

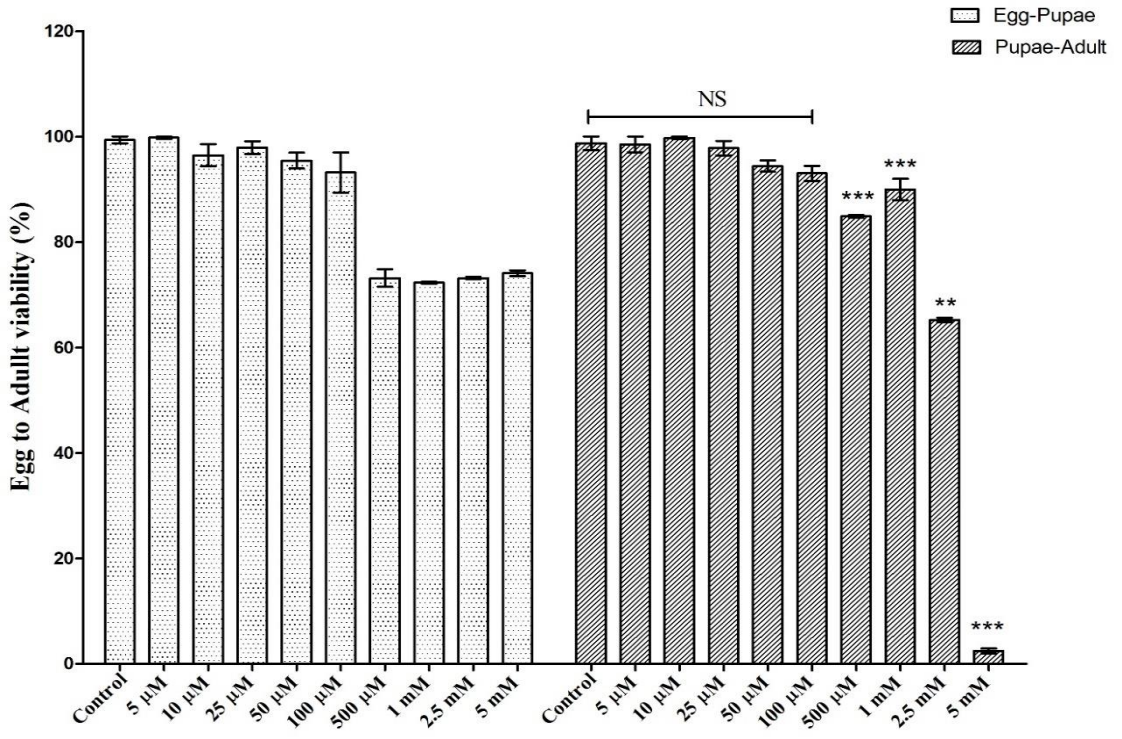


Figure 4.3 A: Assessing the Cu toxicity on pre-natal developmental stages of *Drosophila*. The survival of developing *Drosophila* was evaluated by scoring eggs following the Delcour method (Delcour, 1969). Viability was recorded from egg-to-pupae and pupae-to-adult stages, reared on medium containing varying concentrations of Cu concentrations viz., 5 µM, 10 µM, 25 µM,50 µM,100 µM, 500 µM, 1 mM, 2.5 mM, 5 mM. Cu concentrations such as 5 µM, 10 µM, 25 µM,50 µM,100 µM did not show any significant difference with egg-pupae when compared to pupae-adult, however on increasing concentrations, significant toxicity with pupal death and adult eclosion were observed in Cu concentrations 500 µM, 1 mM, 2.5 mM and 5 mM. The results were evaluated using two-way ANOVA Bonferroni post-test and reported as means ± SEM. [**p<0.01; ***p<0.001; NS: Not significant].

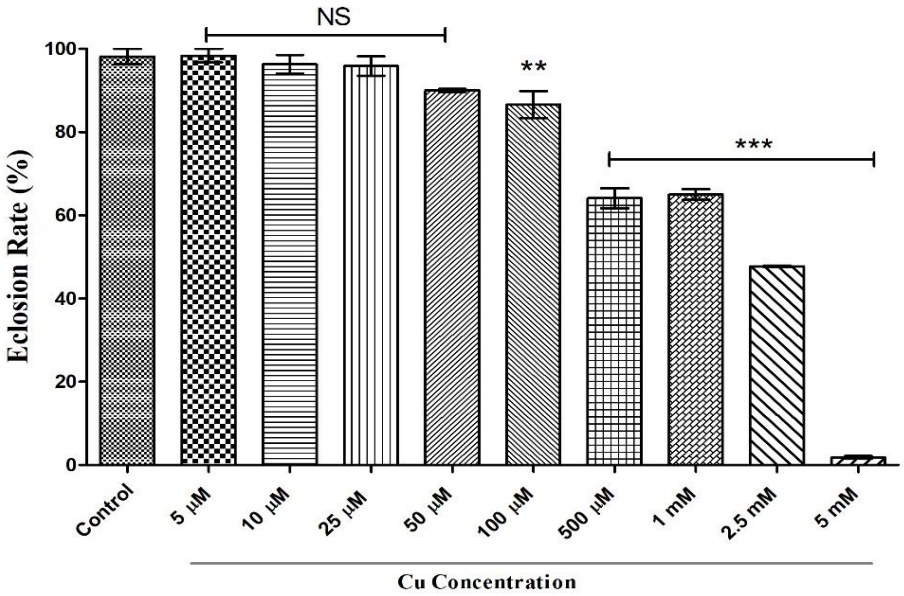
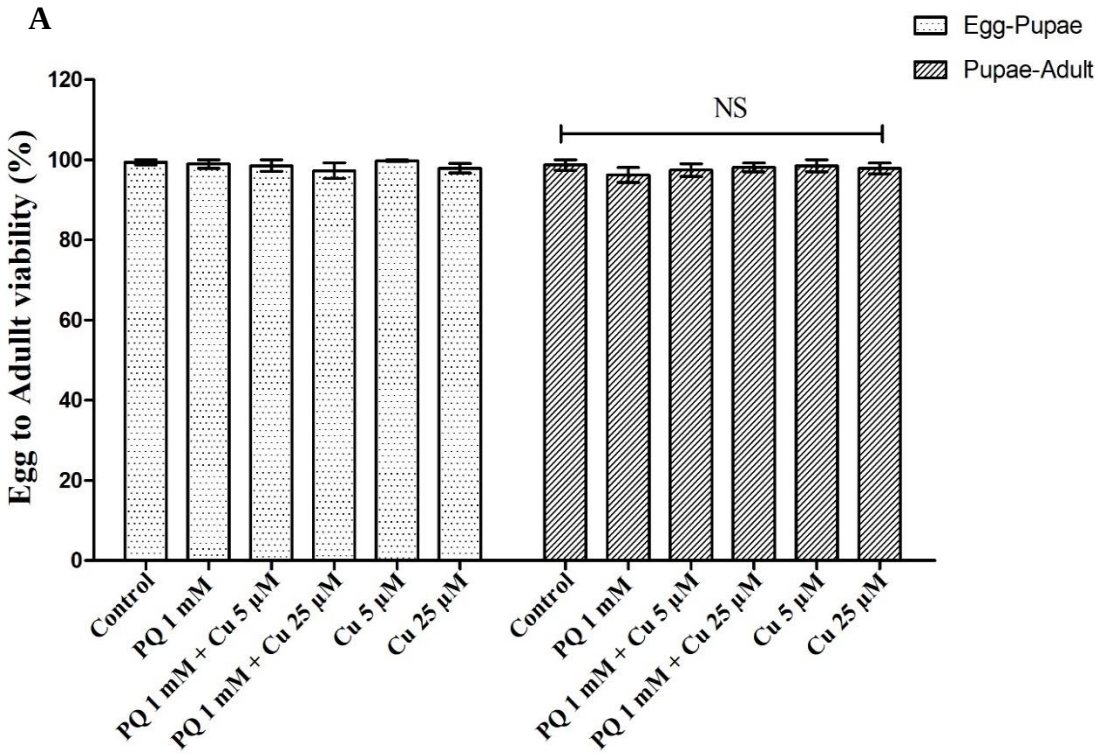


Figure 4.3 B: Effects on the eclosion rate of developing *Drosophila* when exposed to increasing food concentrations of Cu. *Drosophila* eggs were scored following the Delcour method (Delcour, 1969) and reared on a medium containing different concentrations of Cu concentrations viz., 5 µM, 10 µM, 25 µM, 50 µM, 100 µM, 500 µM,1 mM, 2.5 mM, 5 mM. There was a sharp decline and toxicity in the adult fly eclosion rate at doses of 100 µm, 500 µM, 1 mM, 2.5 mM and 5 mM Cu, compared to the control, whereas at the 5 µM, 10 µM, 25 µM, 50 µM Cu there were no significant differences observed in their eclosion compared to the control. The results were represented as means ± SEM, and significant statistical differences were assessed by one-way ANOVA. Tukey post hoc test. [**p<0.01; ***p<0.001; NS: Not significant].

The number of adults emerged from the pupal case was reduced by 12% at 100 µM (**p<0.01), 34% at 500 µM (***p<0.001), 33 % at 1 mM (***p<0.001), 50% at 2.5 mM (***p<0.001), and with the highest toxicity of 96% at 5 mM (***p<0.001) Cu compared to the control, whereas in 5 µM Cu, 10 µM Cu, 25 µM Cu , and 50 µM Cu there were no significant differences observed in their eclosion (**Figure 4.3 B**). For further Co-feeding experiments, Cu concentrations 5 µM and 25 µM were picked up, as it did not affect the survival of developing *Drosophila*, with a similar number of flies reaching pupal stages and adulthood as control.

4.2.3. *Prenatal curcumin and paraquat co-feeding does not affect Drosophila development*

Results show that egg-to-adult viability exhibited no significant differences between the egg-pupae and pupae-adult stages, and adult eclosion rates also displayed no significant differences. The co-feeding concentrations and the fly egg-to-adult eclosion rate were plotted as shown in **Figure 4.4 A, B**. It did not affect the survival of developing *Drosophila*, with a similar number of flies reaching pupal stages and adulthood compared to the control.



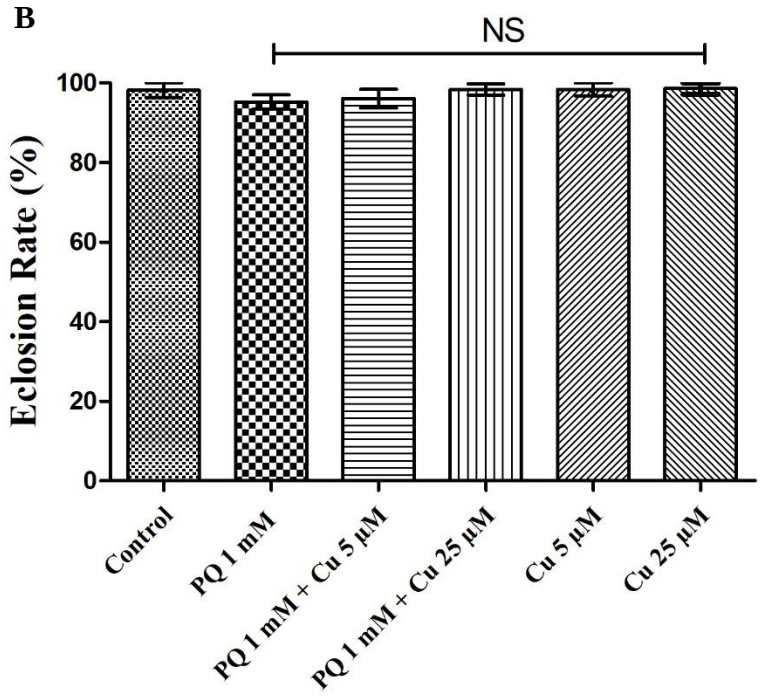


Figure 4.4: Effect on the survival of developing *Drosophila* when exposed to different prenatal co-feeding conditions. *Drosophila* eggs were scored following the Delcour method (Delcour, 1969), reared on a medium containing PQ 1 mM, Cu 5 µm, Cu 25 µM, and co-feeding with both the Cu and PQ concentrations. Egg-to-adult viability was observed with no significant difference in egg-pupae vs pupae-adult viability (A), and adult eclosion rates were observed with no significant differences (B). The results were presented as means ± SEM and evaluated using two-way ANOVA with Bonferroni post-test. (A). Statistical significant differences were determined by one-way ANOVA and Tukey post hoc test (B). [NS: Not significant]

4.2.4. *Developmental co-feeding of curcumin rescues paraquat-induced mobility defects in the adult transition phase*

Following the feeding regimen illustrated in **Figure 2.5**, a negative geotaxis assay, often known as the climbing assay, was performed to determine whether Cu can rescue PQ-induced mobility deficits. This test assesses motor deficits induced by PQ, which are associated with DAergic neurodegeneration and movement issues and are comparable to symptoms reported in PD patients.

PQ-fed flies, had significant motor difficulties within 24 hours, including resting tremors and bradykinesia. These flies struggled to ascend the walls of the climbing tube, frequently falling off to the bottom, with some displaying restlessness and constant wing flicking. In both the HP (**Figure 4.5 A**) and the TP (**Figure 4.5 B**), PQ-fed flies showed significant motor impairment to ascend within 12 seconds compared to controls (***$p<0.001$).

However, for flies that were Co-fed prenatally with Cu and PQ, their motor abilities improved significantly during the HP (**Figure 4.5 A**), and the flies climbed considerably more than those fed with 1 mM PQ alone (*$p<0.05$). During the transition phase, co-feeding PQ with 25 µM Cu significantly improved motor performance (**$p<0.01$), while 5 µM Cu co-feeding had no significant impact when compared with 1 mM PQ. Hence, developmental sub-lethal feeding of concentration of PQ induces mobility defects during adult life phases, while Cu feeding rescues the motor defects.

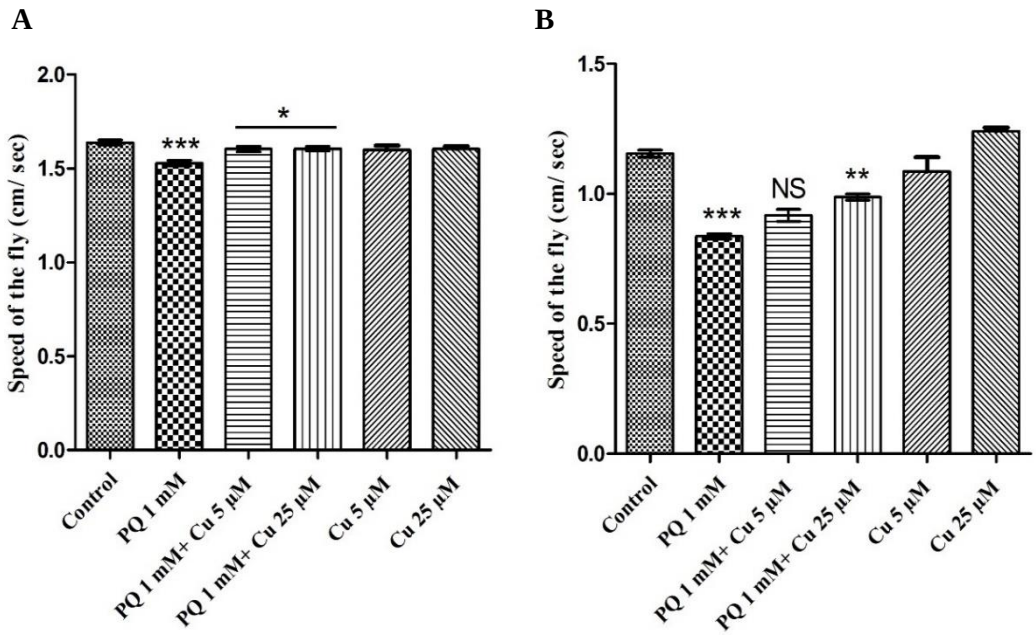


Figure 4.5: Assessment of negative geotaxis assay in flies during the health (A) and transition phases (B) under different prenatal feeding conditions. Climbing ability was evaluated on the 10 day for health-phase flies (A) and on the 50th day for transition-phase (B) flies. Prenatal feeding of the fly with PQ led to a significant reduction in climbing ability; this decline was significantly ameliorated when 5 µM and 25 µM curcumin was introduced as part of their co-feeding prenatal diet during both the HP (A) and the TP (B) of the fly, while 5 µM Cu co-feeding had no significant impact during the TP. However, feeding the flies with Cu alone did not produce any discernible impact on their mobility. Statistical significant differences were determined by one-way ANOVA followed by Tukey post hoc test. [***p<0.001; **p<0.01; *p<0.05; NS: Not significant].

Cu has been reported to alleviate survival rates and climbing defects when exposed to acute PQ concentrations (Park et al., 2012), hence the longevity of *Drosophila* prenatally fed with Cu at doses of 5 µM, 25 µM, and 1 mM PQ was examined to investigate the protective effects of Cu co-feeding (PQ+Cu) against PQ toxicity. After eclosion, lifespan was measured on a standard sucrose-agar medium (**Figure 4.6**). Compared to the Cu-fed group, we observed a substantial decrease in the viability of PQ-fed flies. The median lifespan of the 25 µM Cu, 5 µM Cu, and 1 mM PQ groups was 78 days, 70 days, and 61 days, respectively. However, when co-fed with Cu, PQ-treated flies median lifespan increased to 68 days, implying prenatal developmental Cu co-feeding rescues PQ-induced reduced longevity phenotype. Notably, the fly longevity of these groups was between 110 and 118 days, which was our criteria for selecting concentrations that caused minimal to

no mortality compared to the control groups. Survivorship was observed and reported every 24 hours until all flies died, with 100 flies used at each concentration.

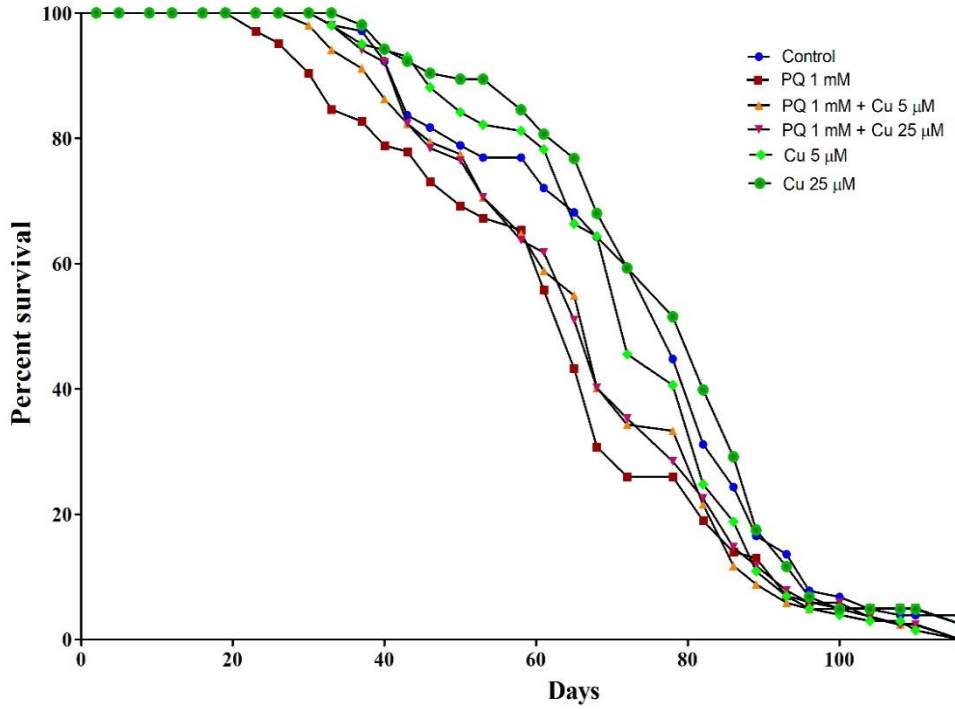


Figure 4.6. Time dependent mortality rate was studied under different prenatal feeding conditions to assess the effect of curcumin (Cu) on survival. Improved median lifespan were observed in Cu co-fed (PQ+Cu) flies and Cu per se compared to PQ fed alone. Live fly counts were recorded every 24 hours until all mortalities were observed, and survival percentages were plotted accordingly. Survival curves revealed a substantial variation in response across tested concentrations (log-rank [Mantel-Cox] test, ($P < 0.0001$).

4.2.5. *Prenatal curcumin feeding rescues paraquat-induced mobility defects during health (10 Day) and transition phase (50 Day) of the Drosophila model of PD*

The negative geotaxis assay of the PQ-induced PD model fly of adult health and transition phase fly were assessed to decipher the mobility alterations under Cu feeding conditions. The climbing ability of flies was assessed 24 hours after exposure to 10 mM PQ on Whatman filter paper. The assay was performed on both 10-day-old and 50-day-old flies that were prenatally fed with 25 µM Cu, 5 µM Cu, or 1 mM PQ. Another control group, which was raised on standard sucrose-agar medium throughout their lifespan, was also exposed to 10 mM PQ. The distance climbed in 12 seconds was measured for each group

to evaluate motor function and the effects of PQ exposure. After 24 hours of exposure to 10 mM PQ, flies solely exposed to PQ during their health and transition phase exhibited slowness in movement (***p<0.001) compared to control, as indicated by a significant decrease in the speed (bradykinesia), which is the characteristic clinical feature of PD in humans.

Prenatal feeding with Cu and exposed to 10 mM PQ during the HP i.e.,Cu 5 µM (DCu 5 µM-PQ) (*p<0.05) and 25 µM (DCu 25 µM-PQ) (***p<0.001) showed significant increase in their motor performance when compared to those exposed to 10 mM PQ alone (**Figure 4.7 A**), where the phenotype is rescued by the Cu feeding, as evidenced by the increased speed of the flies, indicating the protective efficacy of Cu.

Interestingly, flies prenatally fed with 5 µM Cu and 25 µM Cu *per se* and exposed to 10 mM PQ, i.e., DCu 5 µM-PQ and DCu 25 µM-PQ during the TP showed an increase in motor ability by 37% (***p<0.001) and 57% (***p<0.001) when compared to those exposed to 10 mM PQ alone (**Figure 4.7 B**).

Prenatal feeding with 1 mM PQ, followed by exposure to 10 mM PQ (DPQ 1 mM-PQ) during the HP, did not affect the fly's mobility performance compared to those fed PQ alone. This implies a hormetic effect or a compensatory defense mechanism in their motor pathways. Despite two-time insults, pre- and post-natally induced by the same neurotoxin, there was no significant difference in mobility. Further investigation into the molecular mechanisms underlying this outcome could be a promising area of research.

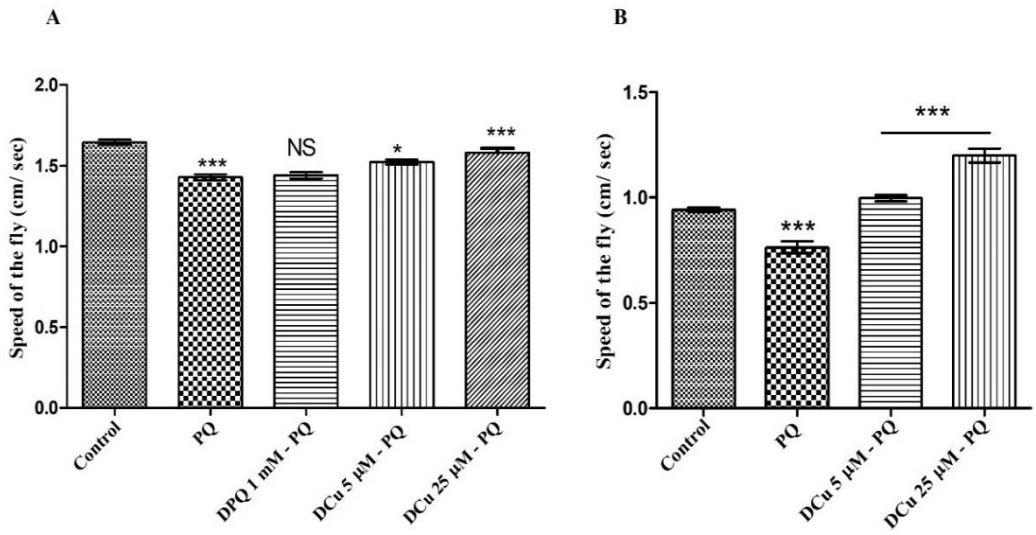


Figure 4.7: Assessment of negative geotaxis assay in flies during the health and transition phases under different prenatal feeding conditions. Climbing ability was evaluated on the 10 day for health-phase flies and the 50 day for transition-phase flies. Exposure to 10 mM PQ for 24 hours during both the health phase (A) and transition phase (B), prenatally fed Cu flies could restore their mobility when the PQ exposure was given to the fly. [PQ- treated with 10 Mm PQ; D - Developmentally fed 1 mM PQ, 5 µM Cu, and 25 µM Cu, exposed to 10 mM PQ (PQ) for 24 hours, i.e., DPQ 1 mM-PQ, DCu 5 µM-PQ & DCu 25 µM-PQ]. Statistical significant differences were determined by one-way ANOVA followed by the Tukey post hoc test. [*p<0.05; ***p<0.001; NS: Not significant]

Further, prenatally fed flies upon eclosion, the lifespan was recorded in a standard sucrose-agar medium (**Figure 4.8**). Exposure to 10 mM PQ for 24 hours during the HP on day 10 and the TP on day 50 substantially reduces the lifespan of PQ-treated flies alone, fed in a sucrose-agar medium from the developmental stages throughout their lifespan. However, flies fed solely on prenatal Cu show substantial resistance to exposure (DCu 5 µM-PQ & DCu 25 µM-PQ), improving median lifespan by 63% (**Figure 4.8 A**) compared to the PQ-treated flies during the HP, and also improving the maximum lifespan by 22%.

The longevity of PQ-induced flies prenatally fed with Cu (DCu 5 µM-PQ & DCu 25 µM-PQ) showed a notable improvement in lifespan, demonstrating Cu's ability to mitigate neurotoxicant effects during TP (**Figure 4.8 B**), with a maximum lifespan of 110 days compared to the PQ-treated flies alone with a lifespan of 90 days, indicating a 22%

increase in Cu prenatally fed survivability, implying Cu's neuroprotective impact. Hence, prenatal developmental Cu feeding rescues PQ-induced reduced longevity phenotype. Interestingly, in the case of flies prenatally fed with 1 mM PQ and re-challenge to 10 mM PQ (DPQ 1 mM -PQ) during the HP—indicating a two-hit model—there appears to be a hormetic effect or a compensatory defense mechanism in their lifespan. Instead of experiencing a more severe impact than those treated with PQ only once, this group of flies exhibited improved lifespan, increasing median lifespan by 38% (**Figure 4.8 A**). In most experimental studies, the optimum hormetic lifespan benefits are found in the 15-25% range when responses are optimized. While most human-based benefits are likely to be below this upper range. It is crucial to address the issue of hormetic effects; however, the current data suggests that these advantages are also restricted by the constraints of biological plasticity (Calabrese et al., 2024; Calabrese, 2008).

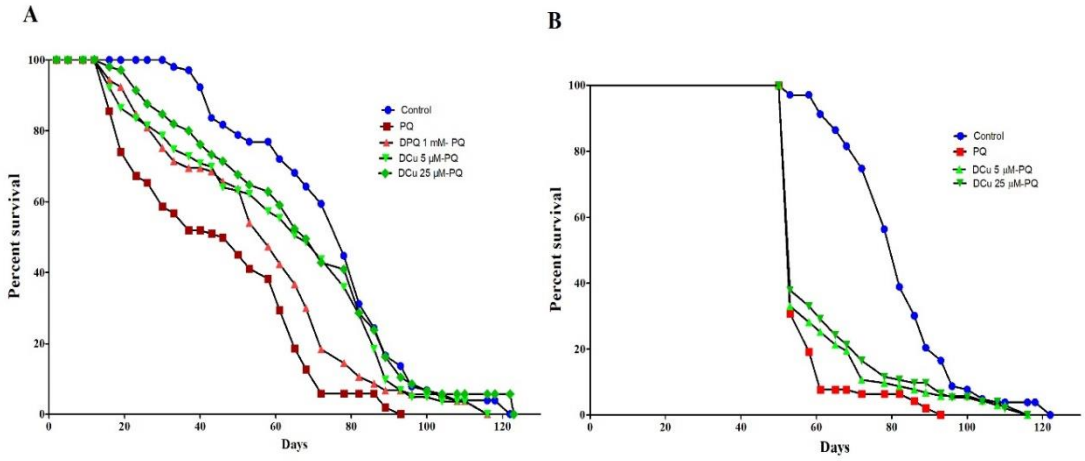


Figure 4.8. Prenatal developmental Cu feeding rescues PQ-induced reduced longevity phenotype. Exposure to 10 mM PQ for 24 hours during the HP on day 10 (A) and the TP on day 50 (B) results in a notable reduction in the lifespan of PQ-treated flies. Flies fed solely on prenatal Cu show substantial resistance to exposure (DCu 5 µM-PQ & DCu 25 µM-PQ), increasing median lifespan compared to the PQ-treated flies during the HP(A), also improving the maximum lifespan. The longevity of PQ-induced flies prenatally fed with Cu (D.Cu 5 µM-PQ & DCu 25 µM-PQ) showed a notable improvement in lifespan, demonstrating curcumin's ability to mitigate neurotoxicant effects during the TP with a maximum lifespan of 110 days compared to the PQ-treated flies alone with a lifespan of 90 days, indicating a 22% increase in Cu prenatally fed survivability, implying Cu's neuroprotective impact. Live fly counts were recorded every 24 hours until all mortalities were observed, and survival percentages were plotted accordingly [PQ- treated with 10 Mm PQ; D - Developmentally fed 1 mM PQ, 5 µM Cu, and 25 µM Cu, exposed to 10 mM PQ for 24 hours, i.e., DPQ 1 mM -PQ, DCu 5 µM-PQ & DCu 25 µM-PQ]. A study of survival curves revealed a substantial variation in response across tested concentrations (log-rank [Mantel-Cox] test, ***$p<0.0001$).

4.2.6. *Prenatal feeding to sub-lethal PQ and Cu rescues from PQ-induced mobility defects during the adult transition stage of Drosophila model of PD*

The negative geotaxis assay was assessed to decipher the mobility alterations from the flies exposed to 10 mM PQ during their HP and then evaluate the assay in their TP under feeding conditions. This assay aimed to ascertain whether Cu's neuroprotective effects are sustained even during the transition phase. The distance the fly climbed in 12 sec was assayed in their 50th day fly to prenatally fed flies of 25 µM Cu, 5 µM Cu, and 1 mM PQ and another group that was solely on standard sucrose-agar medium throughout their lifespan, was exposed to 10 mM PQ during their HP. Flies solely exposed to PQ exhibited slowness in movement by 41% (***$p<0.001$) compared to control group, as indicated by a significant decrease in the speed (bradykinesia), which is the characteristic clinical feature of PD in humans.

Prenatally fed Cu 5 µM and 25 µM and exposed to 10 Mm PQ (DCu 5 µM-PQ & DCu 25 µM-PQ) significantly increased the motor performance compared to those exposed to 10 mM PQ alone by 21% (**$p<0.01$) and 33% (***$p<0.001$) (**Figure 4.9**) during their TP, where the phenotype is rescued by the prenatal Cu feeding, as evidenced by the increased speed of the flies, indicating the protective efficacy of Cu.

Prenatal feeding with 1 mM PQ, followed by exposure to 10 mM PQ (DPQ 1 mM -PQ) during the HP, resulted in a 25% increase in mobility (***$p<0.001$) during the TP when assessed by the climbing assay, compared to flies exposed to 10 mM PQ alone. This suggests that sub-lethal PQ exposure during development does not cause mobility defects but instead confers resistance to PQ-induced mobility defects in the adult transition stage, likely due to the phenomenon of hormesis. Also, the criteria for choosing the time and doses for exposure play a vital role in observing this effect.

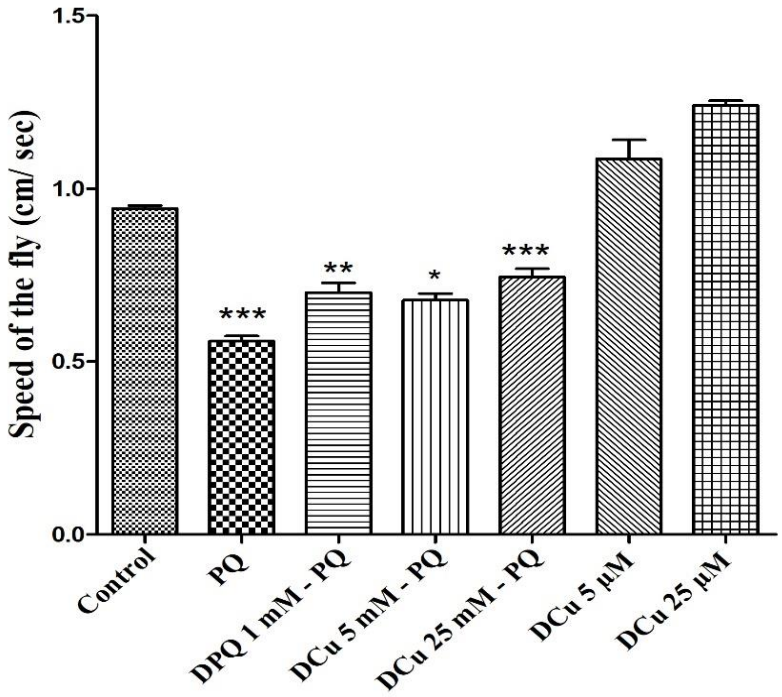


Figure 4.9: Prenatal feeding to sub-lethal PQ and Cu rescues from PQ-induced mobility defects during the adult transition stage of the *Drosophila* model of PD. PQ-exposed flies significantly reduced mobility; however, the prenatally fed Cu significantly alleviated this decline. Prenatal feeding with 1 mM PQ, followed by exposure to 10 mM PQ (DPQ 1 mM-PQ) during the HP, and performing climbing assay during their TP demonstrated increased mobility. It exhibited resistance to PQ and conferred to the phenomena of hormesis compared to those exposed to 10 mM PQ alone. Prenatal Cu feeding rescues the motor ability from early exposure to PQ by negative geotaxis assay in flies during the TP. Significant statistical differences were determined by one-way ANOVA, followed by a Tukey post hoc test. [***$p<0.001$; **$p<0.01$; *$p<0.05$, NS: Not significant]

4.3. Discussion

The developmental period is critical, during which the individual is susceptible to various environmental factors implicated in the pathogenesis of numerous diseases. In this regard, countless prevalent diseases that have experienced a significant increase over the past 40 years appear to be partially correlated with developmental factors associated with nutritional imbalance or exposure to environmental chemicals (Dieckmann and Czamara, 2024; Barouki et al., 2012). It is essential to mention that the central nervous system, in particular, is impacted by these environmental factors (Pamphlett and Bishop, 2022; Antonelli et al., 2017), which have been associated with the pathogenesis of PD and other NDDs (Lefèvre-Arbogast et al., 2024; Brouwer et al., 2017; Narayan et al., 2017). It has been reported that sporadic PD may be associated with events that occur during the early developmental period (in utero and/or early infancy) in addition to environmental factors of adult life (Barlow et al., 2007; Cory-Slechta et al., 2005; Thiruchelvam et al., 2002). Developmental consequences of pesticide exposures have been a critical field of study in recent decades, with studies demonstrating that exposure during embryonic life or early developmental stages might disrupt CNS maturation, which may cause cognitive and psychomotor deficits, as well as delays in the establishment of neural circuits, including motor pathways (Zuo et al., 2023; Ait-Bali et al., 2016). There is compelling evidence to suggest that neurodevelopmental toxicity is induced by PQ exposures (Hamdaoui et al., 2022; Colle et al., 2020; Thiruchelvam et al., 2002). A recent study found that exposure to low concentrations of PQ and MB in primary cultures of rat embryonic neural stem cells reduces cell proliferation by altering essential cell cycle regulator genes unrelated to cell death (Colle et al., 2018). Additionally, Colle et al. (2020) and Thiruchelvam et al. (2002) have demonstrated that the developmental exposure of rodents to the pesticides PQ and MB results in permanent neurotoxicity in the nigrostriatal DAergic system and

increases the system's susceptibility to a subsequent re-challenge later in life. Nevertheless, the mechanisms responsible for this elevated level of vulnerability are not yet fully understood.

Drosophila melanogaster has been characterized as one of the simplest and most efficient toxicity investigation models (Ayajuddin et al., 2022; Phom et al., 2014). Therefore, I examined the harmful impacts of PQ on different factors such as locomotor and developmental survivability using the *Drosophila melanogaster* as a PD model and evaluated the effectiveness of Cu and the combination of Cu and PQ as a potential remedy for induced toxicity. Numerous investigations have confirmed that Cu is a known anti-inflammatory molecule. In addition, Cu is a proven phytochemical that exhibits regressive effects on PQ-induced toxicity.

The primary objective of these experiments was to investigate whether prenatal developmental feeding of Cu could rescue PQ-induced mobility defects in adult *Drosophila* during both the HP and TP. Building on the neuroprotective effects observed during the transition phase in earlier studies, I sought to determine whether Cu's efficacy extends to the prenatal stage, potentially offering protection against PQ-induced neurotoxicity across the adult lifespan of the flies. This experiment aimed to understand if early developmental feeding to Cu could provide long-term benefits in mitigating mobility impairments caused by PQ. The second paradigm of this study focused on assessing whether co-feeding of Cu and PQ (Cu+PQ) during prenatal development could enhance survival and developmental viability, while minimizing the adverse effects on locomotor function in adulthood. By combining Cu with the neurotoxicant PQ, this approach explored whether the nutraceutical properties of Cu could mitigate or delay the onset of neurotoxic symptoms triggered by diets containing PQ. Ultimately, this study

aimed to identify an effective strategy for protecting against developmental neurotoxicity and improving motor function during the adult life phases of the flies.

Locomotion, a crucial nervous system output, necessitates both effective anatomical features and accurate neuronal processes for sensory information processing (Aggarwal et al., 2019; Levine, 2007). Extensive research on various animal models has demonstrated that precise regulation of neural activity is crucial for synchronized movement, and any disturbances can result in notable motor disabilities. Negative geotaxis assay in *Drosophila* has been a fundamental component of locomotor behavior extensively investigated for over a century (Phom et al., 2021; Botella et al., 2004; Carpenter, 1905). Climbing assays have been utilized to analyze the molecules and markers implicated in motor control, PD, aging, and degenerative motor function disorders such as spinal muscular atrophy (Akitake et al., 2015; Phom et al., 2014; Feany and Bender, 2000). Carpenter, (1905) examined several combinations of phototaxis and geotaxis in flies in his seminal research. He hypothesized that light causes movement, whereas gravity causes a sense of direction in flies that can move freely. The phenomenon of positive phototaxis concerning negative geotaxis has been extensively investigated in the countercurrent test by Benzer and other research groups. These assays involve mechanical agitation as an essential component (Aggarwal et al., 2019; Inagaki et al., 2010). Hence, precise neuronal regulation is emphasized in research on NDDs, as severe locomotor impairment is a common symptom, such as PD, where the fruit fly *Drosophila* has been extensively utilized as a model organism.

The basal ganglia regulate gait by altering rhythmic step patterns from spinal central pattern generators in response to environmental events (Pozzi et al., 2019; Takakusaki, 2013). This circuit, which enables adaptive gait, is disrupted in PD as a result of the impaired basal ganglia component of the locomotor network, resulting in gait disorders;

a combination of bradykinesia, reduced postural control (Farashi, 2021; Wahid et al., 2015), and impaired gait variability (Pozzi et al., 2019). Given the similarities in locomotor control mechanisms between humans and *Drosophila*, investigating such impairments in the fruit fly model provides valuable insights into PD pathology. *Drosophila* models of PD have allowed researchers to study the molecular and genetic factors involved in locomotor dysfunction, offering a platform to explore potential neuroprotective strategies, such as curcumin, which may mitigate the debilitating motor symptoms associated with neurodegenerative disorders.

In the present study, it was observed that prenatal feeding of Cu acts as a prophylactic nutraceutical across various life stages, extending beyond the health span of the *Drosophila* PD model and sustaining its motor protective effects during the adult TP.

As previously reported in our lab studies, Cu feeding was found to rescue motor deficits during the HP, but it failed during the TP (Ayajuddin et al., 2022; Phom et al., 2014). Negative geotaxis assay accurately and quantitatively characterizes the condition of the motor system. (i) Assessed the survival of developing *Drosophila* when fed on diets containing nine concentrations of Cu and five concentrations of PQ. Egg-to-adult viability and eclosion rate were evaluated by supplementing the prenatal diet with these compounds.

Present study also showed that larvae exposed to Cu 2.5 mM and Cu 5 mM, particularly, experienced developmental delays and pupation, with significantly less eclosion of the flies, indicating Cu toxicity. In a dose-response study, Von Hellfeld et al. (2024) observed that turmeric concentrations beyond 1% had detrimental effects on larvae growth without any observable positive impacts. Oviposition of adult females and the viability of eggs to adults in *Drosophila melanogaster* larvae were reduced as the concentration of turmeric in the diet increased. High turmeric levels (>5%) resulted in a complete developmental

arrest. This could be one of the possible reasons for the developmental delayed and reduced eclosion recorded in the present study.

Although the molecular mechanisms of turmeric-induced toxicity in insects are unclear, Rahman et al. (2022) showed that higher turmeric concentrations (>1%) lowered β-tubulin levels in the brain and altered many physiological features. Nevertheless, an optimal dosage of 0.5% turmeric enhanced numerous health metrics, such as viability, locomotor activity, fertility, tolerance to oxidative stress, and ocular health, potentially by increasing β-tubulin expression. These data indicate that turmeric has a non-linear, hormesis-like impact, in which low quantities may be advantageous up to a point, after which toxicity leads to reduced survival.

The eclosion rates of the flies were diminished by copper treatment (1 mM); however, the emergence rate of flies was comparable to that of the control group when flies were co-treated with copper and Curcumin (0.2 and 0.5 mg/kg) (Abolaji et al., 2020). Chandrashekara et al. (2014) reported that flies fed with Cu-supplemented diet (10 μM) exhibited an increase in egg-to-adult viability and fertility.

Hence, the concentration that did not affect the survival of developing *Drosophila* and resulted in a similar number of flies reaching pupal stages and adulthood compared to the control was chosen for further DNT investigations. When modeling PD pathogenesis in animal systems, including *Drosophila*, it is critical to avoid lethal concentrations that kill the animal before the DAergic neurons degrade. In such cases, the mechanistic insights generated by researchers utilizing animal models may be irrelevant to the clinical condition (Phom et al., 2014).

The model organism *Drosophila melanogaster* has numerous advantages for studying longevity extension, a critical fitness trait influenced by genetic and environmental factors (Lee et al., 2023; Partridge et al., 2005). Modulating adult diets is the primary way to

extend the lifespan (Jin et al., 2020; Chippindale et al., 1993). Present study aimed to test the age-old phrase "prevention is better than cure" by modifying the prenatal diet and observing the effects on adults. This was done by leveraging the fact that *Drosophila melanogaster* has distinct prenatal and adult phases known to affect adult traits by selective pressures.

The lifespan of an organism, which is the duration between its birth and death, is a critical evolutionary adaptation (Sharma et al., 2020). In the past, numerous studies have effectively extended model organisms' lifespan through dietary supplements that contain reactive oxygen species (ROS)-scavenging substances (Shields et al., 2021). Nevertheless, the correlated response to increased longevity has been reduced fecundity in most diet perturbation studies (Tu and Tatar, 2003; Chippindale et al., 1993). Any manipulation that lowers fecundity will impact the fitness of the organism(s). Additionally, lifestyle changes necessitate identifying and assessing nutrients that can extend reproductive health.

(ii) Examined the dose-response relationships under the prenatal co-feeding regime of Cu and PQ exposure during their health and transition phase where there were no differences in their viability (**Figure 4.4**), and it was found that Cu improved the motor defects and could sustain till the transition stage (**Figure 4.5 A, B**), which is the first report to highlight on age-time related benefits and also have a significant impact in their lifespan, implying prenatal developmental Cu co-feeding rescues PQ-induced reduced longevity phenotype (**Figure 4.6**).

(iii) Previously, in our laboratory, it was demonstrated that Cu did not improve motor performance throughout the transition period (Ayajuddin et al., 2022; Phom et al., 2014). However, in our present study, through feeding regime during the developmental prenatal stage, Cu feeding rescues PQ-induced mobility defects during the health and transition

phase of the *Drosophila* model of PD, also flies solely exposed to PQ exhibited slowness in movement (***p<0.001), as indicated by a significant decrease in the speed (bradykinesia), as an indication of neural impairment, which is the characteristic clinical feature of PD in humans.

Interestingly, sub-lethal PQ exposure during development does not cause mobility defects but instead confers resistance to PQ-induced mobility defects in the adult health stage (**Figure 4.7 A**), likely due to the phenomenon of hormesis, despite two-time insults by the same neurotoxin there was no significant difference in mobility, which was more evident by the increasing median lifespan by 38% (**Figure 4.8 A**).

Long-term PQ treatment, administered through both feeding diets and Whatman filter paper (at concentrations of 10^{-3} and 10^{-4} mM PQ) enhanced the transcriptional activity of HTT and non-telomeric elements in *Drosophila*, leading to increased telomere length (Korandová et al., 2018). Similarly, exposure to low levels of hydrogen peroxide (H_2O_2) resulted in elevated telomeric transcript levels where the pro-oxidants like PQ and H_2O_2 exhibit a hormetic effect on telomere dynamics, with low doses promoting telomere elongation, while higher doses cause telomere erosion (Korandová et al., 2018). When exposed to stressful environments, transposable elements were identified as modifiers of the genetic response in previous studies on various model organisms (Mourier et al., 2014). The activity of transposable elements results in insertional mutagenesis, which, when triggered by environmental stress, generates highly effective adaptive mechanisms to adapt to shifting conditions and specific stressors.

These findings underscore the importance of understanding stress responses and adaptive mechanisms in model organisms. Integrating these insights with our results highlights the complex interplay between dietary interventions, genetic responses, and environmental stressors. The evidence suggests that prenatal developmental feeding regimes and

hormetic responses can significantly impact motor function and longevity, offering potential strategies for mitigating neurotoxicity in PD models.

In the TP (**Figure 4.8 B**), PQ (10 mM) treated flies had a severe decrease in their lifespan upon exposure compared to the control group. However, prenatal developmental Cu feeding rescues PQ-induced reduced longevity phenotype (DCu + PQ) improving a maximum lifespan of 22%, compared to the PQ-treated flies, implying Cu's neuroprotective impact.

In addition to alleviating the cardinal PD motor symptoms (Choi et al., 2020) and enhancing quality of life (Chen et al., 2020), physical exercise can improve mobility, as demonstrated by numerous meta-analyses on various exercise types. However, the efficacy of this approach is contingent upon the patients' dedication to long-term training (Mak et al., 2017). PD patients encounter a variety of challenges that hinder their ability to adhere to exercise regimens, which include motor symptoms, pain, fatigue, and depression (Schootemeijer et al., 2020). Consequently, it is essential to identify appealing sports to optimize the therapeutic effects of exercise (Zaman et al., 2021). Sport climbing enhances gait speed in regular and fast walking and functional mobility in individuals diagnosed with PD (Langer et al., 2024).

(iv) Additionally, I investigated whether early-life intervention with Cu sustains the genetic targets responsible for protecting dopamine throughout the health span and into the transition stage. A negative geotaxis assay was assessed, which serves as a key indicator to evaluate the symptoms of PD phenotype to decipher the mobility alterations from the flies exposed to PQ (10 mM) during their HP and then evaluate the assay in their TP under feeding conditions. The results demonstrated that Cu prenatal feeding rescued the motor function significantly in the TP after HP exposure to PQ (10 mM). Cu feeding showed its strongest effect when administered during the developmental stage, suggesting

that larval or adult feeding of Cu of *Drosophila* normal-lived flies may act—possibly via the TOR pathway—to significantly extend the health span and delay the onset of senescence, and there exists a curcumin-sensitive component during development that exerts long-term delayed effects on the adult organism (Soh et al., 2013).

Additionally, the results were more apparent to the 1 mM PQ prenatal feeding group, followed by a re-challenge to 10 mM PQ (DPQ 1 mM -PQ) during the HP, and performing climbing assay during their TP demonstrated increased mobility (***$p<0.001$) compared to those exposed to PQ (10 mM) alone. This indicates a hormetic effect or a compensatory defense mechanism involved in their motor pathways, and also, the criteria for choosing the time and doses for exposure play a vital role in observing this kind of effect.

Hormesis is a process in which protective responses that increase longevity are triggered by moderate stressors, such as nonlethal heat shock, likely through an ADS-dependent increase in the expression of the heat-shock protein (*Hsp*) genes (Arking et al., 2002). The extended lifespan of centenarians, who frequently experience compression of morbidity (Perls, 1997), and a decrease in late-life mortality among the U.S. white female population between 1960 and 1980 (Myers and Manton, 1984) are examples of this effect in humans. It has been demonstrated that heterozygous individuals for the *Klotho FV* gene, which also plays a role in longevity hormone in PD, exhibit improved late-life survival (Luthra et al., 2023; Arking et al., 2002). These findings emphasize the potential of stress responses and genetic predispositions to promote healthful aging and an improved lifespan.

The importance of early-life interventions in influencing neuroprotection and longevity is emphasized by this study, and supported by other research as well. Research suggests that TOR is downregulated in both larvae and young adults, underscoring its critical role in the longevity-promoting effects of Cu. The results indicate that Cu diet has a significant

impact on the expression patterns of *4e-BP* (Thor) and *S6k*, which are components of the TOR signaling pathway. Consequently, the extension of lifespan is further influenced. During the larval and young adult stages, *Sir2* is inhibited, which implies that it is involved in the effects of Cu on longevity. *Foxo*'s nuanced function in lifespan regulation is reflected in its complex expression pattern, which includes transient decreases in young adults. The extended lifespan phenotype is associated with the robust overexpression of the chaperone gene *hsp22* in adults following larval Cu exposure. Furthermore, the expression levels of *Cat* and *Sod* in larvae exhibit a range of variations, suggesting that they are involved in the management of oxidative stress in a manner that is related to longevity (Rahman et al., 2022; Chattopadhyay et al., 2017; Soh et al., 2013). Additionally, the critical function of programmed cell death pathways, which include caspase-independent mechanisms such as parthanatos, in early development is essential, as they affect tissue homeostasis and the susceptibility to NDDs (Tarayrah-Ibraheim et al., 2021).

Emphasizing these molecular mechanisms could provide significant insights into how prenatal feeding with sub-lethal doses of PQ and Cu might rescue mobility defects and sustain genetic targets responsible for protecting DAergic neurons throughout the adult life stages. Integrating these findings into future research can help explore how modifying these pathways and programmed cell death pathways mechanisms during early development can prevent or delay the onset of diseases like PD. This approach underscores the importance of prenatal care and early-life nutrition in shaping long-term health outcomes and advancing our understanding of longevity and neuroprotection. A deeper understanding of these mechanisms may significantly enhance Cu’s potential to sustain neuroprotection and support dopamine function throughout the lifespan, offering therapeutic benefits for Parkinson’s disease.

4.4. Conclusion

The present study underscores the importance of the developmental period in determining susceptibility to environmental toxins such as PQ, while also exploring the neuroprotective potential of Cu in a *Drosophila* model of PD. The developing brain is a complex matrix that is particularly vulnerable to neurotoxic exposure, with such exposures adversely affecting neurotransmitter function across the brain and potentially triggering the onset of neurological diseases like PD. Epidemiological studies and hypotheses proposed by various investigators support this notion by providing insights into the etiology of PD, suggesting that neurotoxic exposure during early developmental stages, particularly targeting the DAergic system, may lead to the late onset of the disease. This study aligns with these hypotheses by demonstrating that prenatal exposure to PQ can induce neurotoxic effects in *Drosophila*, resulting in motor impairments later in life. However, prenatal co-feeding with Cu revealed significant protective effects, as it successfully rescued flies from PQ-induced motor impairments and improved their lifespan especially during the TP—findings that were not observed in previous lab research.

Notably, while higher concentrations of Cu (above 500 µM) led to developmental delays and reduced eclosion rates, lower doses exhibited protective effects consistent with the principle of hormesis. Prenatal Cu feeding not only rescued motor defects but also sustained protective effects into the adult transition phase. Flies co-fed with Cu and PQ during development showed a significant improvement in lifespan compared to those exposed to PQ alone, highlighting the Cu's neurotoxicant ameliorative property. Additionally, the hormetic response observed in flies exposed to sub-lethal PQ during development suggests that early exposure may confer resistance to later neurotoxic insults, a finding with potential clinical relevance. Overall, this study provides a

foundational understanding of PQ's prenatal effects and demonstrates Cu's efficacy in alleviating PQ-induced toxicity. Given the improvements in both behavior and longevity, the results suggest that prenatal developmental feeding of Cu effectively rescues PQ-induced mobility defects in the adult transition stage of a *Drosophila* model of PD, highlighting its potential neuroprotective role. Furthermore, Cu demonstrates potential as a nutraceutical compound that can augment one's diet (lifestyle) rather than as a therapeutic agent. The findings also support the notion that low-dose prenatal administration of PQ has a beneficial effect in countering hormesis, as it improves the lifespan and alleviates motor symptoms.

Furthermore, incorporating novel strategies, such as focusing on organic food, could complement traditional methods for reducing neurotoxic risks, while considering that "let not thy food be confused with thy medicine." A deeper understanding of the molecular mechanisms involved could significantly enhance Cu's nutraceutical potential in addressing neurodegenerative diseases like PD.

SUMMARY

"Developmental neurotoxicity" (DNT) can have a long-term impact, extending beyond the exposure period, and can influence across the lifespan. Such developmental impacts are of significant regulatory concern due to their potential long-term consequences. Several ideas have been offered, including one that proposes that early-life exposure to neurotoxicants such as paraquat (PQ) may create an initial, subclinical insult to the nigrostriatal DA pathway, with aging exacerbating the damage leading to onset of Parkinson's disease (PD).

The study explores the neuroprotective potential of curcumin (Cu) in the context of PQ-induced neurotoxicity in *Drosophila melanogaster*, particularly focusing on the dopaminergic (DAergic) system during different stages of life. The aim was to assess Cu's capacity to mitigate PQ-induced neurotoxicity, especially during the transition phase (TP) of the flies' lifespan, a stage associated with late-onset neurodegeneration in humans.

The study specifically explores two feeding regimens:

1. Adult 10-Day Curcumin Feeding Regimen (Chapter 3): In this approach, adult flies were fed with Cu for 10 days to evaluate its ability to rescue PD-like pathology during both the health phase (HP) and, importantly, the transition phase, which mimics the onset of late-stage neurodegeneration in humans. Previous lab studies demonstrated that Cu could rescue DA, mobility defects (Ayajuddin et al., 2022; Phom et al., 2014) and DAergic neurons (Ayajuddin et al., 2022; Das, 2022) only during the HP and failed to provide sustained protection during the TP. These observations indicated a need to reconsider feeding strategies and involving young animal models in the study of late-onset neurodegenerative disorders like PD.

(i) In the present study, a 10-day adult life-course feeding regimen with Cu during the HP and TP significantly rescued motor impairments caused by PQ exposure. Additionally, a re-challenge model was introduced, where flies were initially

exposed to PQ, then fed with Cu and exposed to PQ again. The results revealed that Cu not only restored motor function but also sustained DAergic neurodegeneration during the TP, which had not been observed in prior studies. The re-challenge with PQ induced more severe motor impairments and DAergic neurotoxicity, however Cu feeding ameliorated these effects, improving locomotor function, reducing oxidative stress, and preventing DAergic degradation.

(ii) 10 day Cu feeding conferred sustained neuroprotection during both the HP and TP, with improvements in dopamine (DA) levels and reduced DA turnover. Cu feeding specifically reduced the degradation of DA into its metabolites, such as DOPAC and HVA—key markers of DA deficiency in PD. This preservation of DA levels and reduced metabolite turnover highlights Cu's neuroprotective efficacy in counteracting PQ-induced toxicity during both the HP and TP stages.

(iii) Upon single PQ exposure (PQ-NM-Su), DA and DOPAC depletion were observed; however, no changes in HVA levels, motor impairment, or DAergic neurodegeneration were detected, particularly during the HP. This indicates a compensatory mechanism in which surviving DAergic terminals increase activity to maintain neurotransmitter function, as described by Goldstein (2021) and Goldstein & Kopin (2018). While this initially stabilizes DA levels, it places additional stress on the system, risking further DA depletion and potential dysfunction during subsequent neurotoxic exposures.

The findings suggest that developmental neurotoxic insults like PQ exposure may have cumulative effects when followed by later-life insults, reinforcing the significance of early neuroprotective interventions. However, further research is needed to develop consistent therapeutic strategies and reliable biomarkers for early diagnosis of PD.

Integrating multiple treatment approaches, including dietary feeding interventions and pharmacological strategies, may provide a more comprehensive approach to managing neurodegenerative diseases such as PD.

The study underscores the importance of not relying solely on a single treatment approach for managing PD, as compensatory mechanisms can become overly dependent on defective basal ganglia if not periodically re-evaluated. A more comprehensive strategy that incorporates varying treatment modalities may help preserve the effectiveness of these compensatory mechanisms and improve overall disease management.

2. Prenatal Life-Course Curcumin Feeding Approach (Chapter 4): Building on the neuroprotective efficacy of Cu observed during the TP in the adult feeding regimen, this study extended Cu feeding to the prenatal stage to explore its protective effects against developmental neurotoxicity induced by PQ. This chapter highlights the significance of early-life neurotoxic exposures, aligning with previous research that sporadic PD may originate from developmental events, including in utero or early infancy, and exacerbated by environmental factors encountered later in life (Cole et al., 2020; Barlow et al., 2007; Cory-Slechta et al., 2005; Thiruchelvam et al., 2002).

(i) *Curcumin's prenatal co-feeding with PQ (Cu+PQ) rescue motor defects during HP and TP:* In this study, prenatal feeding diets containing PQ led to long-lasting neurotoxic effects, which manifested as motor impairments in both the health and transition stage. However, prenatal Cu co-feeding was able to prevent these impairments, rescuing the flies from PQ-induced neurotoxicity. These findings contrast with previous lab results, where Cu failed to rescue motor impairments during TP when administered only in adulthood, underscoring the importance of early intervention for long-term protection.

(ii) *Curcumin's prenatal feeding rescues PQ-induced motor defects during HP and TP*: The findings demonstrated that prenatal Cu feeding successfully rescued PQ-induced mobility defects, when exposed to 10 mM PQ during the HP and TP. Additionally, another experiment was carried out where flies exposed to PQ during the HP were tested for motor function during the TP, revealing that Cu's neuroprotection persisted into later life stages. This suggests that early Cu intervention provides sustained protection by preserving motor function.

(iii) *Hormesis effect of prenatal sub-lethal PQ exposure*: In addition to the Cu feeding, the study explored the effects of prenatal feeding diets containing PQ and rechallenged with the same neurotoxicant later in life. Flies exposed to sub-lethal doses of PQ during development exhibited a hormetic response, where early neurotoxic stress primed the flies to better withstand neurotoxic challenges later in life. This hormesis effect was marked by improved motor function and improved longevity in the flies, suggesting that low-level early-life exposure to PQ conferred resilience to subsequent neurodegenerative insults. This phenomenon provides valuable insights into how developmental stressors may modulate neuroprotective pathways, with potential implications for human neurodegenerative diseases.

(iv) Additionally, while higher concentrations of Cu (above 500 μM) resulted in developmental delays and reduced eclosion rates, lower concentrations demonstrated protective effects consistent with the principle of hormesis. Flies co-fed with Cu and PQ during development showed a significantly improved lifespan compared to those exposed to PQ alone, highlighting Cu's ability to mitigate neurotoxicant effects. The hormetic response observed in flies exposed to sub-lethal doses of PQ during development also resulted in an improved

lifespan, suggesting that early neurotoxic exposure may confer resilience to later insults—a finding with potential clinical implications for human neurodegenerative diseases.

This research provides a foundational understanding of PQ's prenatal neurotoxic effects and highlights Cu's efficacy in alleviating these neurotoxic effects. The observed improvements in both behavior and lifespan suggest that prenatal developmental feeding of Cu effectively rescues PQ-induced motor impairments in the adult transition stage of a *Drosophila* model of PD, underscoring its neuroprotective potential.

The present study sheds light into the potential of nutraceutical-mediated pre-and postnatal developmental intervention strategies for PD, a condition with few effective therapeutic options that primarily manage symptoms rather than halt or slow down disease progression. In addition, exploring novel strategies, such as incorporating organic food into the diet, could complement traditional approaches to reducing neurotoxic risks, further enhancing the prophylactic potential of nutraceuticals like curcumin in the fight against neurodegenerative disorders such as PD.

SUPPLEMENTARY INFORMATION

ANNEXURE I

Chapter 3: 10-day Cu feeding preceded and followed by PQ treatment rescues DAergic neurodegeneration during both the HP and TP of the *Drosophila* model of PD

I. Figure: 3.5B, 3.9B (Cluster wise DA neuronal number in the whole fly brain of different experimental groups)

HP																								
Neuronal Cluster	Su-NM-Su				PQ-NM-Su				PQ-Cu-Su				PQ-NM-PQ				PQ-Cu-PQ				Su-Cu-Su			
PAL	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
PPL1	26	24	27	26	26	26	26	26	26	27	26	25	25	26	26	25	26	26	26	26	24	26	25	24
PPL2	14	14	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	13	12	13	12
PPM 1/2	16	17	16	18	18	16	18	16	16	16	18	17	18	18	16	16	18	16	18	16	18	17	17	17
PPM3	12	12	12	12	12	12	12	11	12	11	12	12	14	12	11	12	12	12	12	11	12	11	12	12
VUM	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3

TP																								
Neuronal Cluster	Su-NM-Su				PQ-NM-Su				PQ-Cu-Su				PQ-NM-PQ				PQ-Cu-PQ				Su-Cu-Su			
PAL	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
PPL1	26	26	26	26	26	26	24	27	26	26	25	26	28	26	26	26	26	26	24	27	26	26	26	28
PPL2	15	14	12	13	12	14	13	13	12	12	13	13	12	12	14	12	12	14	13	13	14	12	12	12
PPM 1/2	14	14	16	17	16	16	15	15	16	16	16	16	16	14	16	16	16	16	15	15	16	17	16	16
PPM3	12	14	11	13	12	11	12	10	12	12	14	12	12	11	10	12	12	11	12	10	12	12	12	11
VUM	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3

II. Figure: 3.5 C, 3.9 C (Total DA neuronal number in the whole fly brain of different experimental groups)

HP						
Su-NM-Su	PQ-NM-Su	PQ-Cu-Su	PQ-NM-PQ	PQ-Cu-PQ	Su-Cu-Su
81	81	79	82	81	80
79	79	81	79	79	78
80	81	79	77	81	80
81	78	82	77	78	78

TP					
Su-NM-Su	PQ-NM-Su	PQ-Cu-Su	PQ-NM-PQ	PQ-Cu-PQ	Su-Cu-Su
78	79	79	81	79	81
82	80	79	76	80	78
76	77	81	79	77	76
81	78	80	79	78	80

III. Figure 3.5 D, 3.9 D (Cluster-wise FI of DA neurons in the whole fly of different experimental groups)

HP																		
Neuronal Cluster	Su-NM-Su			PQ-NM-Su			PQ-Cu-Su			PQ-NM-PQ			PQ-Cu-PQ			Su-Cu-Su		
PAL	100	100	100	105.4049	125.8423	131.3993	115.4837	135.5441	143.0067	62.17061	68.06348	80.78738	109.5502	121.9849	149.6889	115.0551	155.4628	151.1695
PPL1	100	100	100	144.7959	124.549	115.4058	123.808	133.6114	130.3221	81.33935	83.30485	71.47755	133.8143	133.5657	114.8505	119.7769	134.9214	124.0674
PPL2	100	100	100	99.22785	90.35821	72.55143	90.45094	85.06462	77.81224	77.14178	76.43874	71.13489	93.88083	85.11688	75.8451	78.20592	76.65032	83.44476
PPM 1/2	100	100	100	111.8224	99.58014	106.045	102.869	83.98967	110.8774	75.35872	70.54087	71.75455	104.3308	83.08092	98.76684	107.9735	107.0922	106.4459
PPM3	100	100	100	108.1526	91.99727	97.7718	150.5369	129.8626	125.7764	75.67104	73.1937	78.7074	132.3049	105.6791	101.2286	143.7628	148.8157	105.4647

TP																		
Neuronal Cluster	Su-NM-Su			PQ-NM-Su			PQ-Cu-Su			PQ-NM-PQ			PQ-Cu-PQ			Su-Cu-Su		
PAL	100	100	100	80.45353	77.16321	94.37719	75.78595	104.0817	105.0106	70.79854	71.42178	51.36415	88.87438	98.56292	104.2367	105.3587	89.65165	109.0551
PPL1	100	100	100	118.8024	100.9028	99.38665	100.4721	106.0634	128.947	80.19619	77.66714	66.5705	111.5259	123.9805	95.332	95.53042	121.3234	93.448
PPL2	100	100	100	61.62222	80.24058	110.7318	92.31426	109.0782	150.5016	77.80858	51.65698	61.82264	173.5457	118.4903	100.7968	102.4002	149.2703	128.909
PPM 1/2	100	100	100	109.166	69.24038	133.1455	96.46083	77.00163	130.1776	69.75466	62.25171	67.37776	91.71361	87.54089	103.6144	126.928	95.33228	134.1719
PPM3	100	100	100	105.223	98.02803	55.35588	83.44859	107.8985	68.33875	59.27901	67.74807	40.2603	85.64922	127.2033	100.7686	65.03854	114.8364	66.04562

IV. Figure 3.5 E, 3.9 E (Total FI of DA neurons in the whole fly brain of different experimental groups)

HP						
Su-NM-Su	PQ-NM-Su	PQ-Cu-Su	PQ-NM-PQ	PQ-Cu-PQ	Su-Cu-Su
100	119.0034	115.655	75.80309	116.4635	113.1748
100	106.6289	110.2628	75.00904	104.7445	121.8991
100	104.8756	117.1121	73.56185	105.858	112.8334

TP					
Su-NM-Su	PQ-NM-Su	PQ-Cu-Su	PQ-NM-PQ	PQ-Cu-PQ	Su-Cu-Su
100	101.8959	92.05492	72.39389	106.1156	100.3783
100	84.87198	97.30264	67.86647	108.6219	110.5753
100	97.25986	113.7666	58.11826	100.2133	101.786

ANNEXURE II

Chapter 3: 10-day Cu feeding preceded and followed by PQ treatment rescues DA and its metabolites during both the HP and TP the *Drosophila* model of PD

I. Figure 3.7A, 3.10 A (DA, DOPAC, and HVA amount in the fly brain of different experimental groups)

HP															
	Su-NM-Su			PQ-NM-Su			PQ-NM-PQ			PQ-100 µM Cu-PQ					
DA	0.41	0.41	0.46	0.20	0.18	0.19	0.19	0.17	0.21	0.28	0.28	0.3			
DOPAC	1.62	1.70	1.67	1.12	0.99	1.08	0.97	0.90	0.99	0.52	0.54	0.49			
HVA	0.26	0.10	0.18	0.16	0.05	0.15	0.13	0.05	0.10	0.14	0.05	0.14			
TP															
	Su-NM-Su			PQ-NM-Su			PQ-NM-PQ			PQ-100 µM Cu-PQ			PQ-10 µM Cu-PQ		
DA	0.23	0.30	0.25	0.12	0.18	0.15	0.12	0.15	0.13	0.23	0.37	0.26	0.22	0.20	0.21
DOPAC	1.63	1.51	1.62	1.55	1.38	1.49	0.96	1.13	1.10	1.74	1.64	1.64	1.86	1.44	1.49
HVA	0.07	0.16	0.12	0.11	0.09	0.11	0.12	0.07	0.11	0.08	0.04	0.08	0.12	0.09	0.12

II. Figure 3.7B, 3.10 B (DA turnover ratio in the fly brain of different experimental groups)

HP					
(DOPAC+HVA)/DA	Su-NM-Su	PQ-NM-Su	PQ-NM-PQ	PQ-100 µM Cu-PQ	
	4.652853993	6.434144026	5.74187411	2.352205827	
	4.436216325	5.798413504	5.518039038	2.152767691	
	4.02173913	6.473684211	5.19047619	2.1	
TP					
(DOPAC+HVA)/DA	Su-NM-Su	PQ-NM-Su	PQ-NM-PQ	PQ-100 µM Cu-PQ	PQ-10 µM Cu-PQ
	6.552052986	13.35789381	9.121932759	6.295960004	9.126137794
	5.544151223	8.327939212	7.776648369	4.587738121	7.597823566
	6.96	10.66666667	9.307692308	6.615384615	7.666666667

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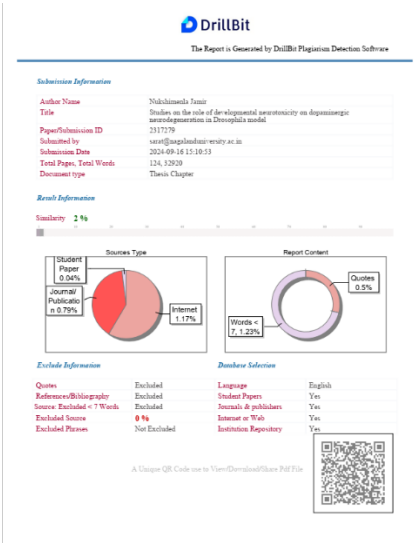

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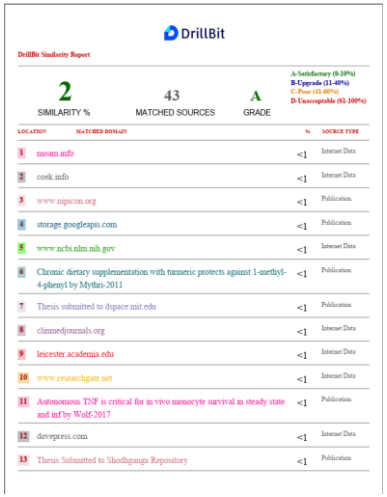

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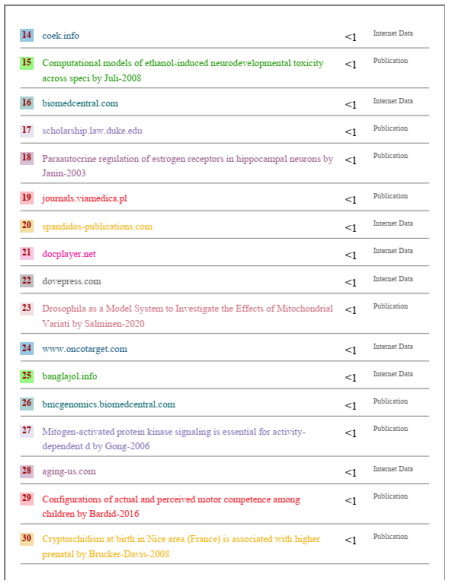

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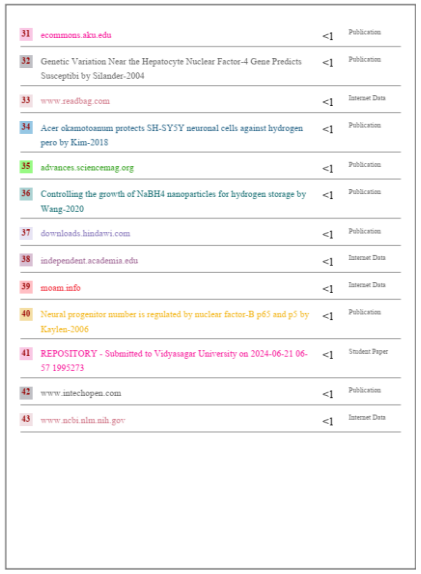
22	dovepress.com	<1	Internet Data
23	Drosophila as a Model System to Investigate the Effects of Mitochondrial Variati by Salminen-2020	<1	Publication
24	www.oncotarget.com	<1	Internet Data
25	banglajol.info	<1	Internet Data
26	bmcgenomics.biomedcentral.com	<1	Publication
27	Mitogen-activated protein kinase signaling is essential for activity-dependent d by Gong-2006	<1	Publication
28	aging-us.com	<1	Internet Data
29	Configurations of actual and perceived motor competence among children by Bardid-2016	<1	Publication
30	Cryptorchidism at birth in Nice area (France) is associated with higher prenatal by Brucker-Davis-2008	<1	Publication



31	ecommons.aku.edu	<1	Publication
32	Genetic Variation Near the Hepatocyte Nuclear Factor-4 Gene Predicts Susceptibi by Silander-2004	<1	Publication
33	www.readbag.com	<1	Internet Data
34	Acer okamotoanum protects SH-SY5Y neuronal cells against hydrogen pero by Kim-2018	<1	Publication
35	advances.sciencemag.org	<1	Publication
36	Controlling the growth of NaBH4 nanoparticles for hydrogen storage by Wang-2020	<1	Publication
37	downloads.hindawi.com	<1	Publication
38	independent.academia.edu	<1	Internet Data
39	moam.info	<1	Internet Data
40	Neural progenitor number is regulated by nuclear factor-B p65 and p5 by Kaylen-2006	<1	Publication
41	REPOSITORY - Submitted to Vidyasagar University on 2024-06-21 06-57 1995273	<1	Student Paper
42	www.intechopen.com	<1	Publication
43	www.ncbi.nlm.nih.gov	<1	Internet Data

CONFERENCES/SEMINARS/WORKSHOPS

International Conferences

- **Poster Presentation**

  4th International Conference on Nutraceuticals and Chronic Diseases

  IIT Guwahati, Assam, India

  Date: September 23-25, 2019

  Topic: Sustaining the dopaminergic neuroprotective efficacy of curcumin during the adult transition phase: Insights from *Drosophila* model of Parkinson's disease.

- **Oral Presentation**

  3-Day Virtual International Conference on Impacts & Consequences of Environmental Degradation on Animal Health and Human Wellbeing

  Abhayapuri College, in association with Department of Zoology, Gauhati University and Aaranyak, Assam, India

  Date: September 2-4, 2021

  Topic: Developmental Neurotoxicity and Adult Onset of Parkinson's disease: Understandings from Animal models with special reference to *Drosophila* and implications for human health.

National Conferences

- *National Conference on Contemporary Excitement in New Biology (CENB-2018)*

  Department of Zoology, Nagaland University, Lumami, Nagaland, India (Sponsored by UGC)

  Date: October 30-31, 2018

  Topic: Sustaining the genetic targets of curcumin during adult life phases: Insights from *Drosophila* model of Parkinson's disease.

- *National Seminar on Climate Change and Sustainable Development with Special Focus on North East India (NSCCSD)*

  Nagaland University Teachers' Association, Nagaland University, Hqs. Lumami, Nagaland, India

  Date: May 17-18, 2017

  Topic: Hunting genomic regions responsible for dopaminergic neurodegeneration in the *Drosophila* model.

Workshop Attended

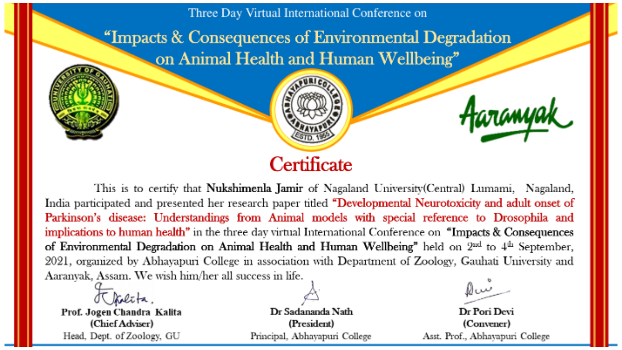
- *One-week MHRD-GIAN Course*
  Anti-Inflammatory Lifestyle for Prevention and Treatment of Cancer and Neurodegeneration: Facts and Fiction
  Department of Zoology, Nagaland University, Lumami, India
  Date: October 1-5, 2019
  Supported by: MHRD, India-GIAN (Global Initiative of Academic Networks).

- *Online Workshop CRISPR: A Game–Changing Genetic Engineering Technique*
  CytoGene Research & Development, Lucknow
  Date: September 3-5, 2021.

Fellowship Availed

• Awarded and availed **DST-SERB** Junior research fellowship and Senior research fellowship
Department of Science and Technology Government of India, New Delhi
Date:19/04/2017 – 31/03/2020

• Awarded and availed **UGC Non-NET fellowship**
University Grant Commission (UGC) through Nagaland University
Date: 1/09/2020 – 31/08/2024


Three Day Virtual International Conference on
"Impacts & Consequences of Environmental Degradation on Animal Health and Human Wellbeing"
Aaranyak
Certificate
This is to certify that Nukshimenla Jamir of Nagaland University(Central) Lumami, Nagaland, India participated and presented her research paper titled "Developmental Neurotoxicity and adult onset of Parkinson's disease: Understandings from Animal models with special reference to Drosophila and implications 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


CERTIFICATE
OF PARTICIPATION
Reg. No.: 2109014
CRISPR: A GAME–CHANGING GENETIC ENGINEERING TECHNIQUE
THIS IS TO CERTIFY THAT PROF./DR./MRS./MISS./MR.
Nukshimenla Jamir
Research Scholar, Department of Zoology, Nagaland University, Lumami (Nagaland)
has actively participated in the Online Workshop on "CRISPR: A Game–Changing Genetic Engineering Technique" from 03 to 05 of September, 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
CytoGene

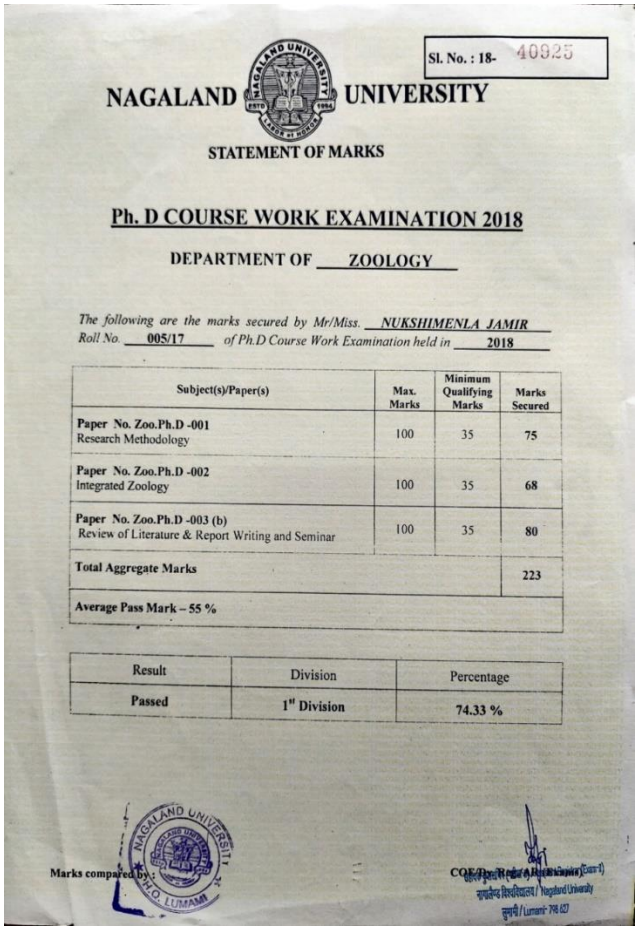

Subject Code: 174053H01
Nagaland University
Gian
Government of India Ministry of Human Resource Development
Global Initiative of Academic Network (GIAN)
This is to Certify that Prof./Dr./Mr./Ms. Nukshimenla Jamir from Nagaland University, Lumami
participated in the course Anti-Inflammatory Life Style for Prevention and Treatment of Cancer and Neurodegeneration: Facts and Fiction
conducted during 1st – 5th October 2019
Date: 05/10/2019
Place: Department of Zoology, Nagaland University, Lumami, Nagaland, India
Dean Prof.(Dr.) T Sangyu Yaden Nagaland University
Local & Course Coordinator Prof.(Dr.) Sarat C. Yenisetti Dept. of Zoology, Nagaland University


Celebrating 25 Glorious Years of IIT GUWAHATI
INTERNATIONAL CONFERENCE ON "NUTRACEUTICALS AND CHRONIC DISEASES" INCD 2019
Certificate
OF APPRECIATION
This is to certify that Nukshimenla Jamir
has presented a poster entitled Sustaining the dopaminergic neuroprotective efficacy of Curcumin during adult transition phase: insight from drosophila model of Parkinson's disease 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. NUKSHIMENLA JAMIR of NAGALAND UNIVERSITY participated/presented a paper (Key Note Talk/Invited Talk/Presentation Talk/Platform Talk) titled: SUSTAINING THE GENETIC TARGETS OF CURCUMIN DURING ADULT LIFE PHASES: INSIGHTS FROM DROSOPHILA MODEL OF PARKINSON DISEASE 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. Nukshimenla Jamir
has presented a paper
entitled "Hunting Genomic Regions Responsible for Dopaminergic Neurodegeneration in Drosophila Model"
in the two day National Seminar on Climate Change and Sustainable Development with Special focus on North East India organized by NUTA, Nagaland University, held on 17-18 May, 2017.
Organising Secretary NSCCSD 2017
Convener NSCCSD 2017

COURSE WORK COMPLETION CERTIFICATE



Sl. No. : 18- 40825

NAGALAND UNIVERSITY

STATEMENT OF MARKS

Ph. D COURSE WORK EXAMINATION 2018

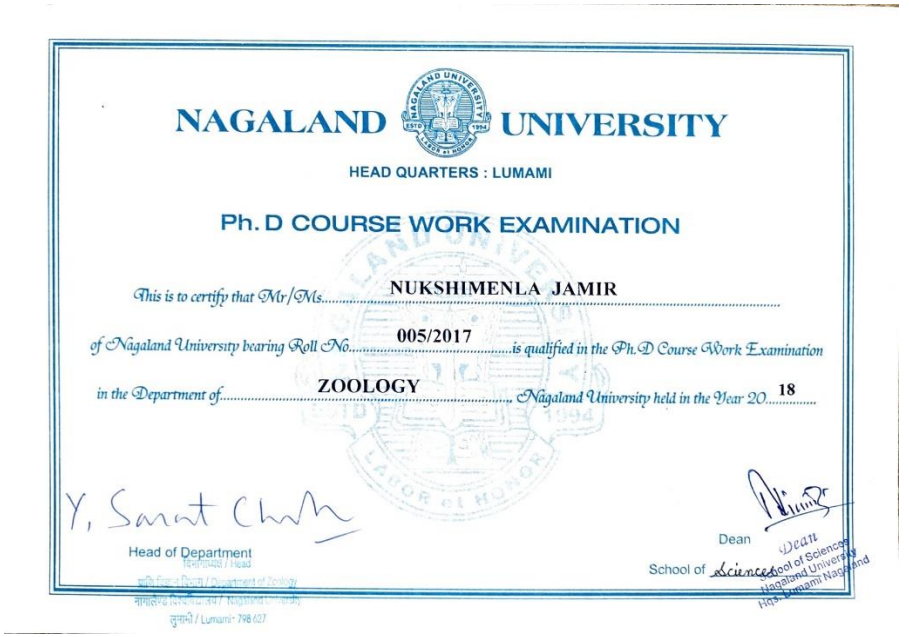
DEPARTMENT OF ZOOLOGY

The following are the marks secured by Mr/Miss. NUKSHIMENLA JAMIR
Roll No. 005/17 *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	75
Paper No. Zoo.Ph.D -002 Integrated Zoology	100	35	68
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

HEAD QUARTERS : LUMAMI

Ph. D COURSE WORK EXAMINATION

This is to certify that Mr/Ms. NUKSHIMENLA JAMIR
of Nagaland University bearing Roll No. 005/2017 *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 Sciences

PUBLICATIONS

Research/Review 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. *Front. Mol. Neurosci* (Impact Factor: 6.261). 15:896183. DOI:10.3389/fnmol.2022.896183.
- Pukhrambam RS, Thepa A, **Jamir N**, Phom L, and Yenisetti SC (2017). Parkinson's Disease and Therapeutic Strategies. International Journal of Neurology and Neurosurgery. 9:172-186.

Research Methodologies

- Neikha K, **Jamir N**, Thepa A, Walling B and Yenisetti S.C (2021) Utility of paper microscope (Foldscope) in class room teaching of genetics. In: Experiments with *Drosophila* for Biology Courses (eds: S.C. Lakhotia & H.A. Ranganath). Indian Academy of Sciences, Bangalore, India. pp. 97-102. ISBN 978-81-950664-2-1.
- Modi P, Thepa A, **Jamir N** and Yenisetti S.C (2021) Extraction and processing of fly brain protein for western blotting. In: Experiments with *Drosophila* for Biology Courses (eds: S.C. Lakhotia & H.A. Ranganath). Indian Academy of Sciences, Bangalore, India. pp. 381-388. ISBN 978-81-950664-2-1.
- Phom L, Ayajuddin M, Koza Z, Modi P, **Jamir N** and Yenisetti S.C (2021) A primary screening assay to characterize mobility defects in *Drosophila* model, In: Experiments with *Drosophila* for Biology Courses (eds: S.C. Lakhotia & H.A. Ranganath). Indian Academy of Sciences, Bangalore, India. pp. 477-480. ISBN 978-81-950664-2-1.
- 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. Ed: Joshi PC, Joshi N, ReshmanYasmin, Mansotra DK (Today and Tomorrow publishers, New Delhi, India). ISBN 10:81-7019-661-5.

- Walling B, Neikha K, Thepa A, **Jamir N**, and Yenisetti S.C (2020) Utility of paper microscope (Foldscope) in classroom teaching of genetics 397-403. Ed: Joshi PC, Joshi N, Reshman Yasmin, Mansotra DK (Today and Tomorrow publishers, New Delhi, India). ISBN 10:81-7019-661-5.

Book Chapters

- **Jamir N** and Yenisetti SC (2021) Developmental neurotoxicants and Parkinson's disease. In: Biological Spectrum of Northeast India (ed: Hemen Chandra Majumdar) Annual Bioscience Communication (Vol-1), EBH Publishers, Guwahati, India. pp. 294-305. ISBN 978-93-90434-19-0.
- 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 (Impact Factor: 0.02), London, UK] pp 157- 192. ISBN: 978-953-51-3854-9. DOI: 10.5772/66545. ISBN 978-953-51-5484-6.