CHAPTER 3

To investigate the effect of excess ROS generation on mitochondrial homeostasis and susceptibility to environmental stress in aging

This chapter results are part of the published work: *Sur et al.* "Sarm1 induction and accompanying inflammatory response mediates age-dependent susceptibility to rotenone-induced neurotoxicity." Cell death discovery 4 (2018).

3.1. Introduction

3.1.1. Aging and neurodegeneration

Physiological brain aging occurs via loss of synaptic contacts and neuronal apoptosis that mediates age-dependant reduction of sensory processing, cognitive function and motor performance (Rossini et al., 2007). Age is a risk factor for several neurodegenerative diseases like Alzheimer's and Parkinson's disease that are often incurable (Brookmeyer et al., 1998). Neurodegenerative diseases like Parkinson's disease (PD), Huntington's disease (DH) and Alzheimer's disease (AD) affect the central nervous system (CNS), leading to progressive loss of structure and function of neuron (Cannon and Greenamyre, 2011a; Kim et al., 2013). These diseases are characterized by age-dependent deterioration in memory functions, learning and movement coordination (Ali et al., 2011). Elevated innate immune and pro-inflammatory responses are found to be associated with many age-related disorders in humans, including Alzheimer's disease and atherosclerosis (Zerofsky et al., 2005). Reduction of autophagy has been also associated with accelerated aging, whereas induction of autophagy might have potent anti-aging effects (Rubinsztein et al., 2011). Mitochondrial dysfunction and associated oxidative damage have been considered to be the important factors of aging. However, data on age-related alteration in activities of mitochondrial electron transport chain (ETC) complexes become controversial and molecular mechanisms responsible for the dysfunction of ETC are still largely unknown. Effect of aging has been studied to determine the activities of ETC complexes in cardiac mitochondria from old and senescent rats. With age, ETC complexes I-IV displayed various extent of inhibition. The most significant decline has been shown to occur in complex IV activity, whereas no change in activity was found in complex II in old rats and was only reduced slightly in senescent rats (Tatarková et al., 2011). However, effect of aging on ETC complexes activities has not been well established yet in neurons. The interaction between gene and environment has been implicated in the increased occurrence of the neurological disorders and environmental toxins like pesticide exposure may increase age-related neurodegenerations.

Exposure to the pesticide rotenone in rats mimics numbers of PD like symptoms. Several experimental models have been developed over the years to study neurodegenerative diseases like PD. Since deregulation of mitochondrial complex I

activity has often been observed in sporadic PD, rotenone treatment is a commonly used model. In Drosophila model, it has been shown that chronic exposure of rotenone in flies resulted in dopaminergic (DA) neuronal loss associated with increased ROS generation and motor deficits (Coulom, 2004; Varga et al., 2014). However, these studies fail to explain the fact that dopamine replenishment relieve initial symptoms of PD but cannot provide a long-lasting cure to the locomotor deficits, which at later stage of the disease, may be associated with a rapid loss of neurons. Further, these studies only examined the effect of chronic exposure of rotenone in younger flies (7-day old) but age-dependent vulnerability to low-level rotenone exposure is not well established yet. It has been found that rotenone induces serious learning and memory impairment in *Drosophila melanogaster* (Yy et al., 2011). However, these studies fail to correlate the rotenone mediated memory loss with increased age. Previous studies have also reported that rotenone disrupts basal synaptic transmission and induces neurodegeneration in primary neuronal culture (Kimura et al., 2012). Studies have now confirmed that rotenone mediates dysregulation of autophagy that leads to the accumulation of damaged organelles, as is found in PD. Particularly in PD brains, autophagy is deregulated. Many studies have shown that rapamycin acts as a neuroprotective agent against PD through the enhancement of autophagy (Zhou et al., 2016). However, it has not been well established yet how rotenone deregulates autophagy machinery in aged organisms.

Loss of axon is an early sign of aging where axons start to gradually degenerate from the synaptic region to the cell body in a 'dying back' phenomenon (Kanaan et al., 2013; Salvadores et al., 2017), although the molecular mechanisms behind that axonal retraction during aging is not established. Recently, in *Drosophila melanogaster* a protein named dSarm/SARM1 was found to play an essential role in programmed axonal degeneration (Osterloh et al., 2012) and NAD^+ depletion and subsequent energy deficit in the axons played a pivotal role in this process (Essuman et al., 2017; Summers et al., 2016). However, role of dSarm/SARM1 in age-associated neurodegeneration remains undetermined so far. Previously, rotenone has also been shown to initiate an inflammatory response through the activation of microglia (Gao et al., 2003; Liang et al., 2015) . However, these studies failed to correlate this increased inflammatory response with the loss of dopaminergic neurons or the increased locomotor deficits, in an aging experimental model of PD.

3.1.2. Environmental toxin induced neurodegeneration

Environmental toxins like pesticides, fungicides and herbicides have received the maximum attention as risk factors for PD. The identification of rural living, exposure to agricultural chemicals and well water consumption have highlighted the risks of exposure to such compounds (Gatto et al., 2009; Gorell et al., 1998; Smargiassi et al., n.d.). Rotenone and paraquat exposure were identified as major risk factors for PD (Fig. 52) (Tanner et al., 2011). Paraquat is a broad-spectrum herbicide widely used in developing countries. Although its use in the United States is highly restricted, it is used as a most common herbicide worldwide (Wesseling et al., 2001). Paraquat was postulated to be a critical neurotoxicant due to its structural similarity to the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), MPP+. The role of this pesticide in the etiology of PD has received a great deal of attention. Paraquat is demonstrated to enter the brain via a neutral amino acid carrier (McCormack and Di Monte, 2003). However, it has been reported that paraquat does not directly inhibit complex I (Richardson et al., 2005). Paraquat has been known to produce reactive oxygen species (ROS) by redox cycling and it seems a possible reason to directly play a role in dopaminergic neurons (Fisher et al., 1973; Ilett et al., 1974). Systemic paraquat injection in mice results in dose-dependent (5–10 mg/kg) decrease in dopamine cell counts in the substantia nigra and body movement (Brooks et al., n.d.). Repeated administration of paraquat produces loss of dopaminergic neurons (McCormack et al., n.d.). In Drosophila, paraquat is generally administered by feeding for 1–2 days (Rzezniczak et al., 2011). Paraquat administration in Drosophila resulted in oxidative damage of brain and other parts of the CNS (Cassar et al., n.d.).

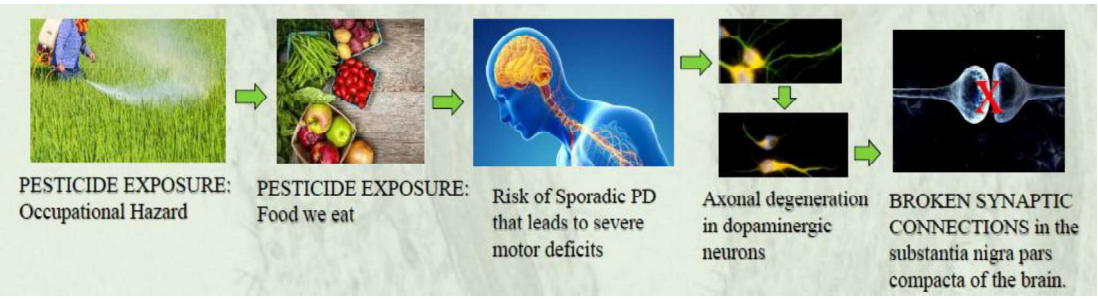

Fig. 52. Pesticide exposure in agricultural fields may induce risk of sporadic PD that is associated with axonal degeneration and broken synaptic connections in the dopaminergic neurons

Extensive use of rotenone has been reported as an insecticide and as a piscicide to kill fish. Rotenone is a naturally occurring compound which is found in the leaves and roots of various plant species. Rotenone is a very well-known, high-affinity and selective inhibitor of mitochondrial complex I which has been used for decades in biological experiments (Ravanel et al., 1984). This has been directly associated with PD (Jr et al., n.d.; Schapira et al., 1990). The fact that specific dopaminergic neurons (those lost in PD), are mostly sensitive to complex I inhibition lead to its testing in animals. Rotenone is a highly lipophilic compound which is able to cross the blood brain barrier (BBB) rapidly (Talpade et al., 2008). Early *in vivo* experiments that administered rotenone systemically at 10–18 mg/kg/day in mice, was not capable to yield selective pathology consistent with PD (Ferrante et al., n.d.; Heikkila et al., n.d.; Rojas et al., 2009). However, chronic administration of rotenone at much lower doses that achieved complex I inhibition, produced highly selective nigrostriatal degeneration in rats (Betarbet et al., 2000). Critical pathological markers like cytoplasmic alpha-synuclein-positive inclusions same as Lewy bodies, were found in surviving dopamine neurons. Hence, the rotenone model provided the very first proof that systemic mitochondrial impairment could generate selective loss of dopaminergic neurons. These findings also support the hypothesis that dopaminergic neurons show a unique sensitivity to complex I inhibition. Hence, rotenone and other environmental toxins having similar mechanisms may be significant risk factors for PD (Cannon and Greenamyre, 2011b).

Exposure of high quantities of lead, manganese and arsenic have negative effects on nervous system. It was shown that arsenic exposure can induce neurodegeneration. Inorganic form of arsenics linked with memory impairment and its long-term exposure affects brain performance. Arsenic-mediated toxicity can be explained considering the induced apoptotic effect in the hippocampus. Experiments proved that arsenic exposure induced disorganized thinking, hallucinations, and obsessive-compulsive symptoms (Avram et al., 2019). Various other classes of environmental agents have been linked to PD, including carbamates, organophosphates and organochlorines. However, currently, animal-based and laboratory-based research on environmental toxin induced neurodegeneration is mostly lacking (Cannon and Greenamyre, 2011b).

3.1.3. Drosophila: an ideal genetic tool for studying age-associated neurodegeneration

There is a lack of a proper model system to study the nature and molecular mechanism of age-associated neurodegeneration. Though, several mouse models have been developed for the research purposes to study PD, but the dissimilarities between inbred strains and the huge difference in their responses to toxic substances brings serious limitations in their use for aging studies.

Two model systems were studied extensively for performing genome wide genetic screens in relation to neuronal function: the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*. Interestingly, in *C.elegans* Wallerian degeneration is a rare phenomenon. In fact, there was a fusion between the intact severed axon and regenerating axons for the restoration of axonal integrity and function (Neumann et al., 2011). However, in Drosophila, severed axons underwent Wallerian degeneration which was molecularly and morphologically very similar to vertebrate axons (MacDonald et al., 2006). Therefore, fruit fly appeared ideal to study the axon death program. First forward genetic screen was performed to identify the mutants that could probably block Wallerian degeneration after injury. SARM1 was identified as the first gene required for lesioned axons to undergo Wallerian degeneration. The role of SARM1 in injury induced axonal degeneration is highly conserved, since axons from SARM1 KO mice were also protected from Wallerian degeneration. In fact, protection from Wallerian degeneration in SARM1 mutant organisms showed similar pattern of protection, found in Wld^{s} expressing axons (Osterloh et al., 2012).

3.1.4. Mitochondrial complex I inhibitor rotenone and ROS production

Complex I and complex III of the electron-transport chains are the two major sites of ROS generation (Sugioka et al., n.d.; Turrens and Boveris, 1980). Rotenone inhibits complex I and increases ROS production in submitochondrial particles (Turrens et al., n.d.; Turrens and Boveris, 1980). Previously it was shown that in the presence of NADH, isolated complex I generates $O_2^{\bullet -}$ and rotenone enhances this generation which binds to the CoQ-binding site (Cadenas et al., n.d.). The mechanism of $O_2^{\bullet -}$ generation by isolated complex I is well understood now (Hirst et al., 2008; Kussmaul and Hirst, 2006). The isolated complex yields $O_2^{\bullet -}$ from the reaction of the fully reduced FMN with O_2, and the proportion of the FMN which is fully reduced

(P_R) is the result of NADH/NAD^+ ratio (Hirst et al., 2008; Kussmaul and Hirst, 2006). This model explains why complex I inhibition with rotenone induces $O_2^{\bullet-}$ generation, as it will result in a backup of electrons onto FMN that will generate $O_2^{\bullet-}$(Takeshige and Minakami, 1979; Votyakova and Reynolds, 2001).

3.1.5. Mitochondrial dysfunction and Parkinson's disease

Widely accepted pathological mechanisms in Parkinson's disease (PD) patients are aberrant mitochondrial form and function. Recently, in the PD genetics nomenclature, 18 specific genes including *PARK* (Klein and Westenberger, n.d.) *SNCA* (Polymeropoulos et al., n.d.), *UCHL1*, *LRRK2* (Funayama et al., 2002), *HTRA2* (Martins et al., 2004), *Parkin* (Mizuno et al., 2008), *PINK1* (Hatano et al., 2004), *DJ-1*(Bonifati et al., 2003) and *ATP13A2* (Ramirez et al., 2006) were reported to be associated with PD. However, the pathological mechanisms in familial PD resulting in mitochondrial dysfunction require further investigation at the molecular level. The (PTEN)-induced putative kinase 1 (*PINK1*) gene encoding a serine/threonine kinase is localized in mitochondria and is sub-localized in different cellular regions including outer mitochondrial membrane, inner mitochondrial membrane and intermembrane space. *PINK1* recessive mutation was responsible for a familial form of early-onset PD (Valente et al., 2004). *PINK1* knockout mouse and human dopaminergic neurons have reduced membrane potential, increased ROS generation, abnormalities in mitochondrial morphology, and higher tendency to apoptosis. *PINK1* gene mutation leads to swollen or enlarged mitochondria. Moreover, null mice for *PINK1* or *Parkin* show synaptic dysfunction in neurons projecting towards the striatum that correlates with increased oxidative stress and progressive loss of mitochondrial function in the striatum with age (Johri and Beal, 2012). Also, *PINK1* or *Parkin* loss-of-function mutations are associated with mitochondrial dysfunction in cells from patients with familial forms of parkinsonism (Johri and Beal, 2012). PINK1 is found to show a strong cytoprotective role in the maintenance of mitochondrial homeostasis via different mechanisms. In SH-SY5Y neuroblastoma cells, overexpression of wild type *PINK1* stabilizes mitochondrial membrane potential and suppresses autophagy (Dagda et al., 2009). Several groups found that expression of PINK1 is essential for the recruitment of Parkin to dysfunctional mitochondria (Pilsl and Winklhofer, 2012). Moreover, PINK1 plays a role to phosphorylate the mitochondrial molecular chaperone heat shock protein 75

kDa (Pridgeon et al., 2007). Mutation of *PINK1* results in severe alterations in mitochondrial homeostasis, observed as aberrations in mitochondrial dynamics, mitochondrial cytoarchitecture, calcium homeostasis, increased mitochondrial ROS and biosynthetic pathways, ultimately mediating a robust increase in mitophagy (Exner et al., 2007). Mitophagy is the selective autophagy process of mitochondria and is a major quality control mechanism that is required for the elimination of excess or damaged mitochondria (Narendra et al., 2008). Defective mitophagy is one of the important pathological mechanisms that underly dysfunction of mitochondria in autosomal recessive forms of PD, including those caused by *PINK1* and *Parkin* mutations (Narendra et al., 2008). Interestingly, the neurodegenerative molecule SARM1 has been shown to interact with PINK1 and stabilize it on the mitochondrial surface (Murata et al., 2013). The implications of such an interaction have not been studied yet.

3.1.6. Neuroinflammatory responses in experimental models of PD

Neuroinflammation has been implicated as an integral component of the progressive neurodegenerative process and associated with the pathogenesis of PD. Microglial activation is a hallmark of neuroinflammation that is frequently found in PD patients and experimental animal models. In fact, rotenone toxicity is markedly enhanced via the activation of microglia and that rotenone-induced microglial activation preceded dopaminergic neurodegeneration (Gao et al., 2002). It has been revealed that rotenone induces microglia activation and generation of pro-inflammatory factors includinginterleukin-1β IL-1β, tumor necrosis factor-α (TNFα) etc in PD (Gao et al., 2003; Liang et al., 2015). Improved animal models of Parkinsonism and inflammatory responses are necessary to understand the pathophysiology of disease and for subsequent testing of potential therapeutics. Among the most widely used Parkinson's disease (PD) models, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) are very common. Depending on the different available protocols, MPTP generates large variations in nigral cell loss, behavioural deficits, inflammatory response and striatal dopamine loss. Although motor difficulties do not replicate fully those seen in PD, but MPTP mouse models mimic many aspects of the disorder and are therefore beneficial tools for understanding PD (Meredith and Rademacher, 2011). Other than this some of the prominent existing mouse models of PD and inflammation are as described below.

The M83 transgenic mice express the mutant human A53T alpha-synuclein under the promoter of mouse prion protein (Giasson et al., n.d.). Hualpha-Syn (A53T) transgenic mice model is also useful for studying PD as they display an age-dependent phenotype including neuronal loss, progressive motor deficits and intraneuronal inclusion bodies (Lee et al., n.d.). TH-Cre transgenic mice have the rat tyrosine hydroxylase (TH) promoter that directs the expression of Cre recombinase in catecholaminergic cells, and are useful for studying Parkinson's disease, inflammatory response, dopaminergic cell function and other neurodegenerative disorders (Savitt et al., 2005). LRRK2 G2019S knock-in mice have two nucleotide substitution mutations in exon 41 of the *Lrrk2* gene for substitution of the amino acid G2019S which makes an autosomal dominant and late-onset Parkinson's disease (Yue et al., n.d.).

3.1.7. Concluding remarks

In our previous objectives, we were able to determine the toxic effect of rotenone in neuroblastoma cells. However, in this objective, we tried to correlate rotenone induced neurotoxicity in an age-dependent manner to determine how age acts as a trigger for the increased susceptibility to neuronal loss.

3.2. Materials and Methods

3.2.1. Fly strains

w^{1118} flies were a generous gift from Dr. Rupak Datta, IISER, Kolkata. Both male and female flies were grown at 22° C and 12-h light/dark cycle except for the rotenone, resveratrol, sodium butyrate and NR treated flies (see below). The diet was composed of 10 ml of standard jaggery/yeast Drosophila medium (vide Appendix) and changed every 3-5 days. Only male flies were used in this study, since, female flies show altered physiological condition because of the reproductive development.

3.2.2. Fly exposures

A. Rotenone, sodium arsenite and NAC treatment

Rotenone was freshly prepared as 100 mM stock solution in DMSO (Structure shown in Fig. 8A). Sodium arsenite (Structure shown in Fig. 53) and N-Acetyl Cysteine (Structure shown in Fig. 34 G) were prepared freshly as 100 mM and 100 mg/ml stock respectively in water. Cohorts of twenty flies of both genders in each vial were placed on the rotenone containing diet at 1-day, 10-day, and 20-day post-

eclosion. Rotenone was given to the fly food for achieving final concentrations of 50, 100, and 200 µM. Flies were transferred to rotenone containing fresh medium every 2-3 days and were monitored for survival and locomotor activity as described below. For aging studies, flies were grown in regular medium following eclosion for 20-days. At day-20 they were transferred in rotenone containing diet and experiments were performed as described. For some of the experiments flies were exposed to either rotenone or sodium arsenite (360 µM) in the presence or absence of 1 mg/ml of N-acetyl-L-cysteine (NAC).

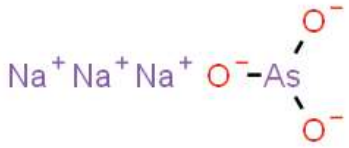

Fig. 53. Chemical structure of Sodium arsenite

B. Resveratrol treatment

Resveratrol was freshly prepared as 10 mM stock solution in DMSO (Structure shown in Fig. 34 D). Resveratrol was given to the fly food for achieving final concentration of 1 µM in the presence or absence of rotenone (200 µM). Flies were placed on resveratrol containing media on the day of eclosion and transferred every 2 days to fresh food and collected at 1 and 10-days post treatment.

C. NR treatment

NR was freshly prepared as 20 mM stock in water (Structure shown in Fig. 8 C). NR was added to the fly food to achieve final concentration of 1 mM in the presence or absence of rotenone (100 and 200 µM). Flies were placed on NR containing media on the day of eclosion and transferred every 2 days to fresh food and collected at 1- and 10-days post treatment.

3.2.3. Fly survival assay

Flies were exposed to control media and various concentrations of rotenone (50, 100 and 200 µM) mixed to the diet. Each concentration had three replicates. The number of dead and alive flies was counted daily for 20 days. For each condition, at least five vials containing twenty flies of both genders were raised on food with or without rotenone from day one post eclosion and monitored daily to check survival

for up to 40-days. Every other day, food was changed, and all deaths were recorded. The same has been performed for resveratrol, sodium butyrate and NR treatment.

3.2.4 Negative geotaxis assay

The negative geotaxis assay was performed in a similar manner to that published by Gargano et al (Gargano et al., 2005) with minor modifications. 15 treated and 15 control male flies were transferred once a day in the early afternoon without anaesthesia into a vertical glass climbing vial (length, 25cm; diameter, 3cm) to perform the assay. The climbing vials were placed in a rectangular frame in order to keep them upright. After a ten-minute acclimation period, the vials were tapped three times to initiate the negative geotaxis assay and climbing was captured with photographs. After 10 s, the percentage of flies crossing the 5 cm mark was noted for each trial. This procedure was repeated a total of ten times. All behavioural experiments were performed at room temperature under standard light conditions.

3.2.5. Whole brain mount immunofluorescence labelling

The heads of adult flies were isolated mechanically under a dissection microscope. The fly brains were dissected under stereo zoom microscope (Olympus, Japan). After dissection, brains were fixed immediately in 4% PFA for 30 min. Fixed brain tissues were incubated in anti-tyrosine hydroxylase solution overnight at 1:400 dilution in PBT (5% BSA and 0.3% Triton X-100 in PBS). Tissues were incubated with anti-rabbit Alexa 488 secondary antibody at 1:500 dilution in PBT for 3 h to detect primary antibody. After immunostaining tissues were mounted in fluoroshield mounting medium. In Apotome 2 microscope whole brain images were taken (Zeiss, Germany). In motorized stage, 9–12 tiles were taken and in each tile images of 5 slices of 0.32 µm were taken and by using ZEN software, images were reconstructed.

3.2.6. Real-Time PCR

A. RNA isolation from fly heads

The extraction of total RNA from fifteen adult male fly heads per sample was performed using Trizol according to the manufacturer's protocol (vide Section 1.2.2.8 A). At least three independent RNA extractions were prepared for each sample. An additional centrifugation step was used at 10,000 x g for 10 min to remove the cuticles prior to chloroform addition. The concentration of total RNA isolated from each sample was measured with a Synergy H1 microplate reader (BioTek).

B. cDNA synthesis

1µg of total RNA were digested with DNAse I and followed by reverse transcription using the iScript Reverse Transcription Supermix with oligo (dT) primers. cDNA synthesis reaction was as follows: Priming for 5min at 25ºC, Reverse transcription for 20 min at 46ºC, RT inactivation for 1min at 95ºC.

C. Real-Time PCR analysis

Quantified real-time PCR was performed on CFX96 Real-Time System (BIO-RAD) using the SYBR Green qPCR Master Mix (2X) from SsoFast EvaGreen Supermix following the manufacturer's instructions. PCR cycling conditions were: 95°C for 30 s for initial denaturation, and 40 cycles of 95°C for 5 s and 60°C for 10 s. Relative expression levels of mRNA were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The quantification was performed using the comparative Ct method. Real-time PCR was repeated three times to verify results, and the mean mRNA expression level was used for subsequent analysis. Primer sequences used in this study are listed in Table 9.

Table 9. Sequences of primers used in this study

Target genes	Sequences (size)
GAPDH (R)	5'-TTGATGTTGGCCGGGTCGC-3' (19 mer)
GAPDH (L)	5'-GTGGCCGTCAACGATCCCTTC-3' (21 mer)
nAChRbeta (R)	5'-CGTTTGGTGTCCGGATTTGCCTC-3' (23 mer)
nAChRbeta (L)	5'-GTGGCATTGGAGTTTGTTGTGTGTTTTC-3' (28 mer)
Nrx-1 (R)	5'-CGATTTCGCTGCTGATCCATGTCC-3' (24 mer)
Nrx-1 (L)	5'-GGCGCCACATAGTGCAACATATCAG-3' (25 mer)
EAAT (R)	5'-GATGATGAAGCCGATGAGTCCACC-3' (24 mer)
EAAT (L)	5'-CCCAAACAGGATGGCGGC-3' (18 mer)
p120 (R)	5'-GTTTGCAGGCTCGTGCGTCAAT-3' (22 mer)
p120 (L)	5'-CGCGATCTCTCAAACACAGCCT-3' (22 mer)
Pink1 (R)	5'-GCTTCTTGTCTTCAATTGATTCTGGTCC-3' (28 mer)
Pink1 (L)	5'-CTGTGAGACTGCTGACCGTGC-3' (21 mer)
Hsp70 (R)	5'-TGAAAGCCACGTAGGACGGC-3' (20 mer)
Hsp70 (L)	5'-CCTGCTATTGGAATCGATCTGGGC-3' (24 mer)
dSarm (R)	5'-ACTTTGCACCGTAATACTGGAGGCC-3' (25 mer)
dSarm (L)	5'-AAGGAAGCCCTCTCACTCCGC-3' (21 mer)
Eiger (R)	5'-TGGTCGCTTTGGCCGGAAAA-3' (20 mer)
Eiger (L)	5'-GACTGCCGAGACCCTCAAGC-3'(20 mer)
Atg5 (R)	5'-ATGCCCACGATCTCGTCCC-3'(19 mer)
Atg5 (L)	5'-TGCGAATGATCTGGGAGGGC-3' (20 mer)
Atg3 (R)	5'-ACACCTGTCTCACGGAATTTCG-3'(22 mer)
Atg3 (L)	5'-GTCAAGGGCACAGCGCTC-3' (18 mer)

Target genes	Sequences (size)
Ndufv1 (R)	5'-TCGTCTGTGGCGGAGGAGT-3' (19 mer)
Ndufv1 (L)	5'-AGATTGTTGCGACTTTGCCGG-3' (21 mer)
Ndufs2 (R)	5'-CCTGAGAAGTTAACGCGGCCG-3' (21 mer)
Ndufs2 (L)	5'-ATGGCCAATATAATGAGAAGAACGTTGA-3' (28 mer)
Cox5B (R)	5'-AGGATCGTTCATCATTTTGCAGAAGC-3' (26 mer)
Cox5B (L)	5'-ATGGCATCGATCTGTGGACGC-3' (21 mer)
Sir 2 (R)	5'-TTCGTAATTTTCGAGCGTAGTTTCTGGAA-3' (29 mer)
Sir 2 (L)	5'-ACATACACACACAGGCGCAGCTA-3' (23 mer)
Tep1 (R)	5'-CAGAGTAGTCAGGCCCACTT-3' (20 mer)
Tep1 (L)	5'-TCTGTAAAGCGGGGTGAAGT-3' (20 mer)
Tep2 (R)	5'-AGCAATTACCTCACCTCGCT-3' (20 mer)
Tep2 (L)	5'-CCTCATGGGTGGTTACTGGT-3'(20 mer)
Tot M (R)	5'-GCTGTCGATGTTCCGGTATT-3' (20 mer)
Tot M (L)	5'-GCCAAGCCTGCACTATGAAT-3' (20 mer)
Upd1 (R)	5'-AACTGCTATCACTGTTCCTCCCTGA-3' (25 mer)
Upd1 (L)	5'-GCATCGAAGCACCCGACC-3' (18 mer)
Upd2 (R)	5'-ATCACTAGCAGCACCTGCCG-3' (20 mer)
Upd2 (L)	5'-GCCAGCCAACAGAGCCAAA-3'(19 mer)
Upd3 (R)	5'-CAGGCGAATCAGGCGACTTTG-3' (21 mer)
Upd3 (L)	5'-TTCGTCCAGCCGCGATATAAAGATAC-3'(26 mer)
Wnt 1 (R)	5'-AGGAGAGTGGTGCCTGGAAC-3' (20 mer)
Wnt 1 (L)	5'-TATTACGAGCACTCTGGCAGCAG-3'(23 mer)
Ampk (R)	5'-GATGTCAAAGTGGTTGTGGATGTGTC-3' (26 mer)
Ampk (L)	5'-GCCGCGCTCGAATAGAAATCCA-3' (22 mer)

3.2.7. Measurement of ROS generation

To assess the ROS levels, 1-day old flies were grown in rotenone containing diet in the presence of absence of NAC. At least 15 heads from 10-day old male flies treated as described were homogenized with 150 µl of T-PER extraction buffer. 10 µl of head homogenate was added to 140 µl of PBS containing 50 µM DCFDA. The reaction mixture was incubated at room temperature for 60 min and the fluorescence was measured at 485 nm excitation/530 nm emission on a cell plate reader (Synergy, BioTek). The assay was repeated three times. Treated sample data were compared with control samples to determine ROS levels.

3.2.8. Western blot analysis

Twelve adult fly heads were homogenized per sample using a motorized pestle in lysis buffer (1M NaCl, 1M Tris, 0.5% Triton-X100 with protease inhibitor cocktail) at 4°C. Homogenized samples were sonicated for 1 min, with 30 s pulse and 30 s pause to inhibit sample heating. After sonication, the lysates were cleared by centrifugation at 14,000 rpm for 15 minutes. The protein contents were subjected to

Bradford protein assays to ensure equal protein loading (~15µg). The protein samples were then heated for 5 min at 95°C and loaded into the wells. The proteins were separated on 12% SDS-PAGE gels and transferred to Nitrocellulose membranes. The membranes were blocked in 1X TBS (25 mM Tris, 150 mM NaCl, 2 mM KCl, pH 7.4) and 5% fat free milk for 1 h and then incubated with the anti-Tyrosine Hydroxylase antibody overnight at 4°C followed by 2 h incubation with the HRP-conjugated anti-rabbit secondary antibody. The antigen-antibody complexes were then visualized by enhanced chemiluminescence (ECL). β-Actin was used at 1:5000 dilution as loading control for Western blotting experiments. Blots were quantified using ImageJ. Antibodies used in this study are listed in Table 10.

Table 10. List of primary and secondary antibodies used in this study

Protein to be detected	**Primary antibody used and its dilution**	**Time of incubation**	**Secondary antibody used**
β-actin	Anti- β-actin mouse monoclonal antibody (abcam) used at a dilution of 1:1000	*2h **overnight	Goat anti-mouse IgG H&L (HRP) preadsorbed (abcam) at 1/5000 dilution
Tyrosine hydroxylase	Anti- TH rabbit polyclonal antibody (Merck) used at a dilution of 1:1000	*2h **overnight	Goat anti-rabbit IgG H&L (HRP) preadsorbed (abcam) at 1/5000 dilution
*Blocking with 5% skimmed milk for 30 min at room temperature. **Incubation with antibody for 2 h at room temperature or overnight at 4-degree C.			

3.2.9. Mathematical modelling

Our model is based on a set of coupled ordinary differential equations that describes the time evolution of the following quantities: induced SARM1 level (S), number of active dopaminergic neurons in the flies (N) and the level of the neurotransmitter dopamine (D) following rotenone treatment. An associated derived quantity is the locomotor ability of the flies (L). The dynamics of the SARM1 level was modelled by Eqn. (1) based on the assumption that SARM1 is induced by the intake of Rotenone through food and the food intake itself is affected by the flies' motor deficits only when the locomotion falls appreciably below its normal value L_0 ,

$$\frac{dS}{dt} = \frac{a}{1 + (\frac{L_0 - L}{L_0})^{\alpha}} + \frac{S}{N}\frac{dN}{dt}. \quad (1)$$

a denoting the maximum rate of SARM1 intake corresponding to normal neuronal conditions and α denoting the dependence of the flies' consuming ability with their motor deficit. The second term in the right-hand side of Eqn. (1) comes from the consideration that the reduction in SARM1 levels correlates with the loss of dopaminergic neurons in these flies. In Eqn. (1), the variable corresponding to locomotor abilities (L) is assumed to have the following dependence on dopamine level (D) and number of neurons (N):

$$L = \frac{L_0}{1 + (\frac{D_0 - D}{D_0})^{\beta}} \frac{N}{N_0} \tag{2}$$

where the movement of the flies is significantly reduced only when the dopamine level D falls appreciably below its normal value D_0 and this dependence is parameterized by including a positive parameter β. The locomotion ability is also controlled irrespective of dopamine levels by the fraction of healthy and undamaged dopaminergic neurons among the total number of such neurons ($N=N_0$) for flies not exposed to rotenone We also assume by simple first-order kinetics that the degradation of dopaminergic neurons and subsequent decrease of the dopamine level both are dependent on SARM1 level S:

$$\frac{dN}{dt} = -bSN \tag{3}$$

$$\frac{dD}{dt} = -cSD \tag{4}$$

with two positive rate constants b and c. The equations are now set in a simpler form using the dimensionless variables s, l, d, n and τ and the parameter γ defined in the following equation

$$l = \frac{L}{L_0}, n = \frac{N}{N_0}, d = \frac{D}{D_0}, \gamma = \frac{b}{c}, s = \sqrt{\frac{c}{a}}\, S, \tau = t\sqrt{ca}\ . \tag{5}$$

With these, Eqn. (1)-(4) now become

$$\frac{ds}{d\tau} = \frac{1}{1 + (1 - l)^{\alpha}} + \frac{s}{n}\frac{dn}{d\tau} \tag{6}$$

$$l = \frac{n}{1 + (1 - d)^{\beta}} \tag{7}$$

$$\frac{d(d)}{d\tau} = -sd \tag{8}$$

$$\frac{dn}{d\tau} = -\gamma sn \tag{9}$$

The above equations were solved in MATHEMATICA® with the initial conditions $s = 0, l = d = n = 1$ at $\tau = 0$ corresponding to a healthy and active fly and the numerical solutions were plotted accordingly. To further understand the susceptibility of neurons to damage induced by SARM1 with increasing age, we assumed a linear time-dependence of the neuronal degeneration rate in presence of induced SARM1 so that a possible modification of Eqn. (9) becomes

$$\frac{dn}{d\tau} = -\gamma' sn\tau \qquad (10)$$

with a new rate constant γ and the subsequent solutions were plotted.

3.2.10 Statistical analysis

Kaplan-Meyer survival analysis was performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA, USA). The results are presented as mean ± SEM (standard error of mean) of three independent experiments performed in triplicate. Statistical significance was measured by one-way analysis of variance (ANOVA). Asterisks indicate levels of significance (*p <0.05, **p <0.01 and ***p < 0.001).

3.3. Results

3.3.1. Rotenone mediates decreased lifespan and progressive locomotor deficits in an age-dependent manner in w^{1118} strain of Drosophila

Environmental factors including pesticides like rotenone and paraquat have been shown to cause oxidative damage induced loss of DA neurons accompanied by motor deficits in animal models of Parkinson's disease. However, the age-related susceptibility to chronic exposure to such environmental toxins is poorly understood. To study the effect of rotenone on Drosophila survival, w^{1118} flies were exposed to increasing doses of rotenone 1-day after eclosion and survival of the flies were followed up to 40 days as represented in Fig. 54 A. As shown in Fig. 54 B flies were susceptible to increasing rotenone concentration (50, 100, and 200 µM) that was accompanied by severe motor deficits as shown in a negative geotaxis assay at 1-day (Fig. 54 C), 10-days (Fig. 54 D) and 20-days (Fig. 54 E) post rotenone treatment by their decreased climbing ability. One-way ANOVA suggests that climbing ability of adult flies in 200 µM rotenone exposure for 1-day, 10-days and 20-days was significantly ($P < 0.001$) less than those untreated control flies.

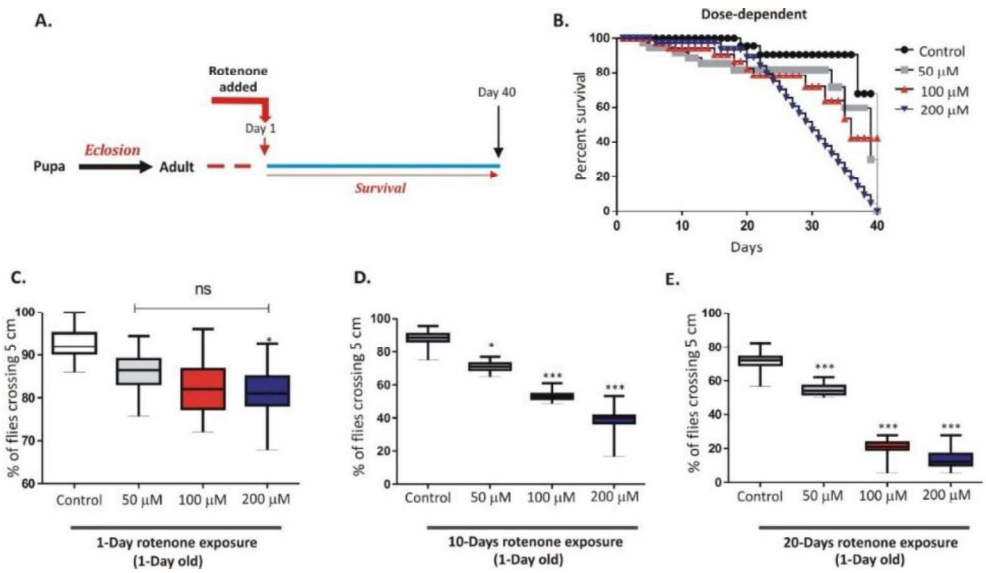

Fig. 54. (A) Schematic representation of rotenone treatment in the 1-day old flies for survival curve assay. **(B)** Survival curve of 1-day old flies exposed to varying concentration of rotenone (50, 100, and 200 µM). Fly viability was scored over a period of 40-days using a minimum of 100 flies per treatment. The statistical significance was calculated as log-rank using Mantel–Cox test. **(C–E)** Negative geotaxis assay of 1-day old flies exposed to rotenone at concentrations indicated above for 1-day **(C)**, 10-days **(D)** and 20-days **(E)**. *p < 0.05, **p < 0.01, and ***p < 0.001 compared to control flies

Age is a risk factor for several neurodegenerative diseases including Parkinson's disease. In order to determine the age-related susceptibility to environmental toxins like rotenone, 1-day, 10-day and 20-day old flies were exposed to 200 µM of rotenone (Fig. 55 A). Interestingly, the 20-day old flies exposed to rotenone showed a significantly increased ($P < 0.001$) death rate compared to the younger flies (Fig. 55 B) which was also accompanied by increased motor deficits (Fig. 55 C), indicating that age played a determining role in increased susceptibility to rotenone.

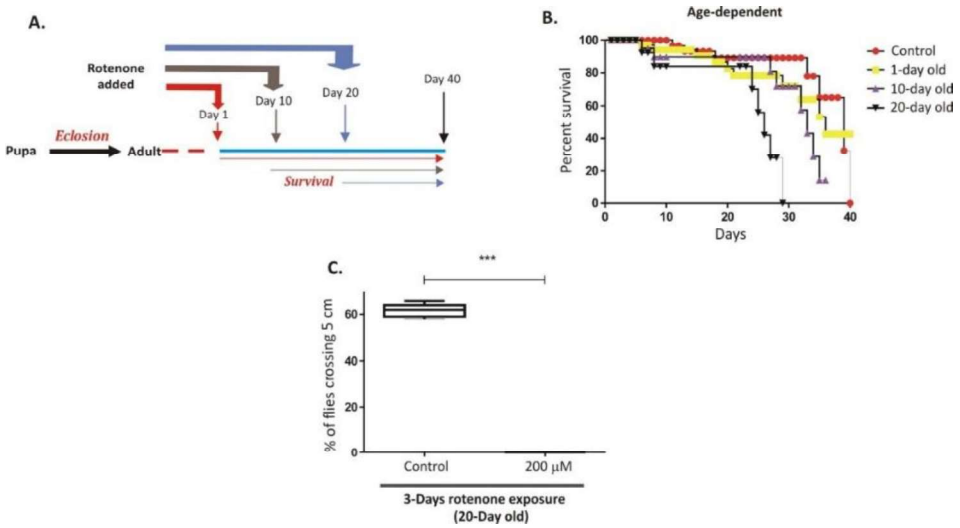

Fig. 55. (A) Schematic representation of rotenone treatment in the 1-day, 10-day, and 20-day old flies for survival curve assay. **(B)** Age-dependent survival of flies exposed to 100 µM of rotenone at 1-day, 10-day, and 20-day following eclosion. Fly viability was scored up to 40-days, post-eclosion using a minimum of 100 flies per treatment. **(C)** Negative geotaxis assay of 20-day old flies exposed to 200 µM rotenone for 3-days. *p < 0.05, **p < 0.01, and ***p < 0.001 compared to control flies

3.3.2. Rotenone induced locomotor deficit is accompanied by increased *dSarm* expression

Rotenone treatment induces loss of DA neurons in all of the brain clusters and loss of motor abilities in a Drosophila model of PD. It has also been shown that the neuronal loss as observed in several neurodegenerative diseases including PD is preceded by axonal and synaptic degeneration but the molecular mechanisms remain unclear. Here we determined the expression of one of the neurodegenerative molecule dSarm that has recently been implicated in injury induced axonal loss in the young (1-day old) and aged (20-days old) flies as schematic representation is provided in Fig. 56 A. Quite interestingly, we found an early induction of *Ect4* or *dsarm* (Drosophila homolog of SARM1) in the brains of flies that were exposed to rotenone 1-day after eclosion which peaked at 20 days following exposure to rotenone followed by a steep decrease at 30-days post exposure (Fig. 56 B). Our results indicate that rotenone exposure mediated heightened *dSarm* expression in w^{1118} flies. To understand further the age-dependent susceptibility to rotenone, 20-day old aged flies were exposed to rotenone (200 µM) and at 3-days and 5-days post rotenone exposure, brains were collected. These flies were very much susceptible to rotenone exposure and not even survived beyond 10-days post-exposure. In these aged flies there was a significant increase in *dSarm* expression (Fig. 56 C) as early as 3-days post rotenone exposure

followed by a rapid decrease at 5-days, as compared to the younger flies which correlated with the increased age-dependent susceptibility to rotenone. Our results indicate that rotenone exposure caused upregulation of *dSarm* expression in an age dependent manner in w^{1118} flies.

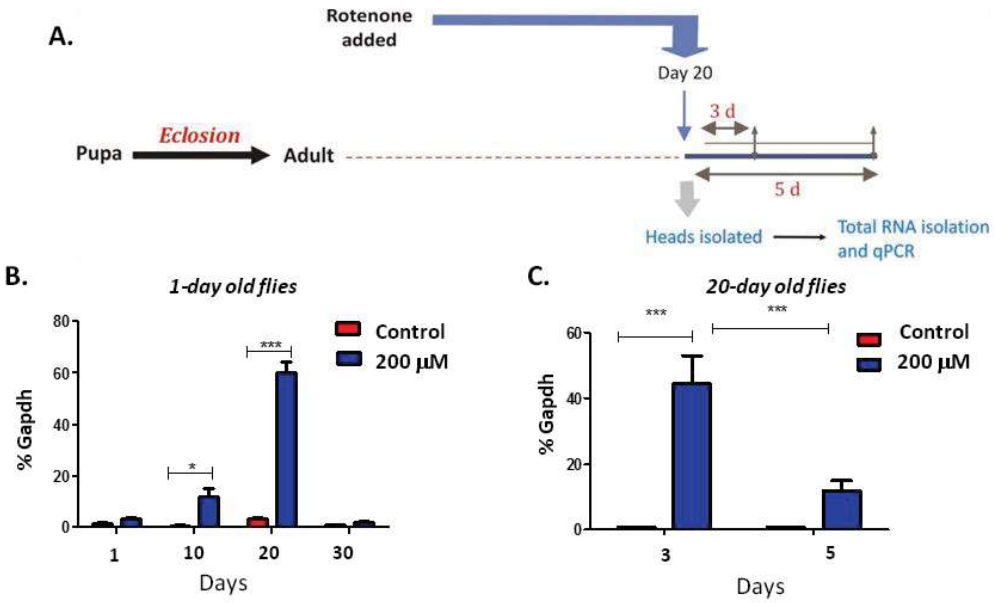

Fig. 56. (A) Schematic representation of experimental setup for rotenone treatment in the aged (20-days old) flies. **(B, C)** Expression of *dSarm* in young flies (1-day old) exposed to 200 µM of rotenone for 1-day, 10-days, 20-days, and 30-days **(B)** and aged flies (20-days old) to 200 µM of rotenone for 3-days and 5-days post-treatment (n = 5) **(C).** *p < 0.05, **p < 0.01, and ***p < 0.001 compared to control flies

Previously It has been shown that, rotenone treatment induces loss of dopaminergic neurons in younger flies (7-day old) (Hou et al., 2013). To determine whether rotenone treatment had any crucial effect on the dopaminergic neurons in aged flies, we stained fly brains with tyrosine hydroxylase (TH), a rate-limiting enzyme that converts tyrosine to DOPA. In agreement with our earlier data of age-dependent increased susceptibility to rotenone, we found a rapid and significant loss of dopaminergic neurons from the dopaminergic neuronal clusters after tyrosine hydroxylase (TH) staining of whole fly brains in the 20-day old flies exposed for 3-days to rotenone as compared to the age-matched control flies (Fig. 57 A-B). A significant reduction of TH protein levels was also observed in the 20-day old flies exposed for 3-days to 200 µM of rotenone as compared to age-matched controls (Fig. 57 C), indicating that rotenone treatment mediated loss of dopaminergic neurons in aged flies.

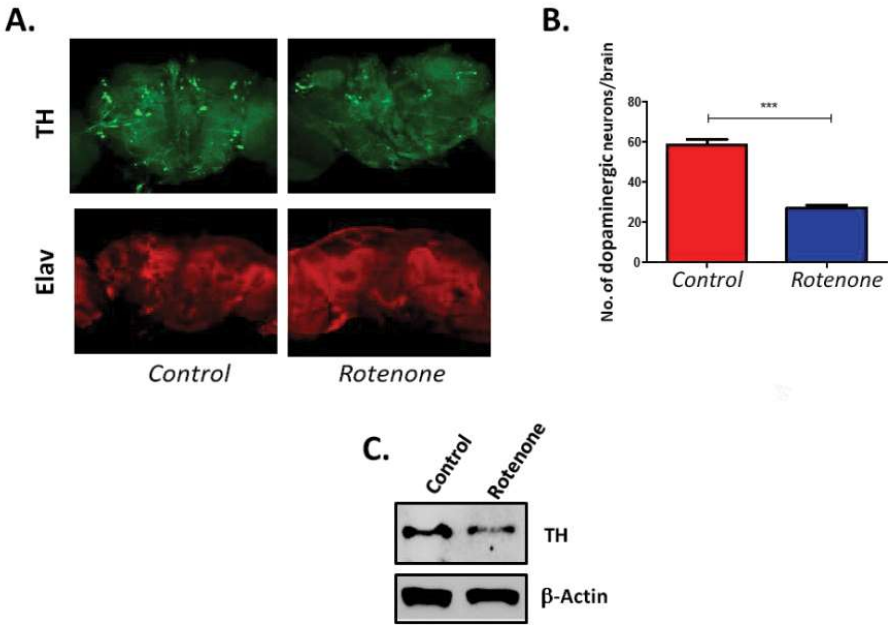

Fig. 57. (A) Multifocal confocal image of dopaminergic neurons following tyrosine hydroxylase (TH) immunostaining (green) and Elav immunostaining (red) in the brains of flies exposed to 200 µM rotenone at 20-days post-eclosion for 3-days (right-hand panel). Results were compared to age-matched rotenone untreated flies (left-hand panel). Scale bars = 200 µm. **(B)** Bar graph represents the number of dopaminergic neuron cell bodies in the control and rotenone (200 µM) treated fly brains (n = 3). **(C)** Representative western blot analysis of the dopaminergic neuronal marker tyrosine hydroxylase in 20-day old flies exposed to 200 µM rotenone and analysed at day-3 post-exposure. β-actin served as a loading control and data was compared with age-matched untreated control flies (n = 3). Results are representative of at least three independent experiments. *$p < 0.05$, **$p < 0.01$, and ***$p<0.001$ compared to control sample

3.3.3. An early induction of dSarm was necessary and sufficient for rotenone induced neurotoxicity

To understand whether the dSarm levels correlates with the loss of neurons and the initial induction of dSarm is sufficient to drive the locomotor deficits, an attempt was made to create a mathematical model in order to correlate rotenone intake, SARM1 induction and subsequent motor deficits. The profile of the plots (Fig. 58 A-C) qualitatively matches with our initial experimental findings that the locomotor ability of the flies incessantly decreases while the SARM1 level reaches a maximum and then drops monotonically. This indicates that an early induction of SARM1 acts like a switch to trigger the loss of dopaminergic neurons leading to the reduction in the levels of dopamine. However, the results also indicate that the SARM1 level tends to reach a constant value asymptotically in time contrary to the experimental findings where the level seems to decrease continuously. Inclusion of the age-dependent susceptibility of the neurons to rotenone induced stress in our

mathematical model showed that SARM1 levels continually decreased with time which correlated with the experimental findings.

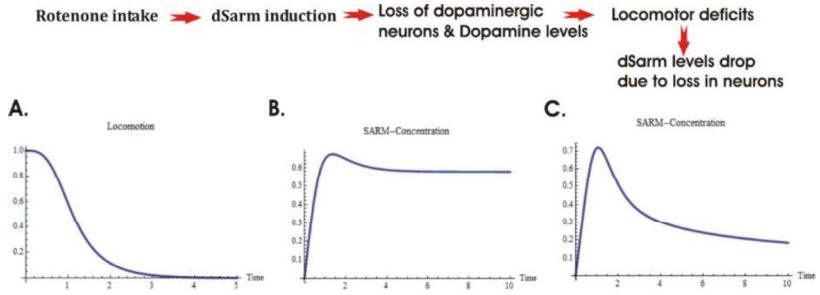

Fig. 58. (A-C) Mathematical model to correlate rotenone intake, SARM1 induction and subsequent motor deficits

To further verify and understand whether early induction of *dSarm* as found in Fig. 56 B was enough to initiate rotenone mediated neurotoxicity, 1-day old flies were exposed for 10-days to 200 µM of rotenone following which they were maintained in rotenone free media (Fig. 59 A). Early rotenone exposure in the younger flies (1-day old) for 10-days and subsequent removal of rotenone continued to induce death (Fig. 59 B) and locomotor deficits (Fig. 59 C). In these flies, significant ($P < 0.01$) upregulation of *dSarm* expression was found, following early exposure of rotenone (Fig. 59 D), that was indicated by a paired student t-test. Taken together, these data indicate that early induction of SARM1 may act as a switch to trigger the dopaminergic neuronal loss leading to rotenone-mediated motor deficits in flies.

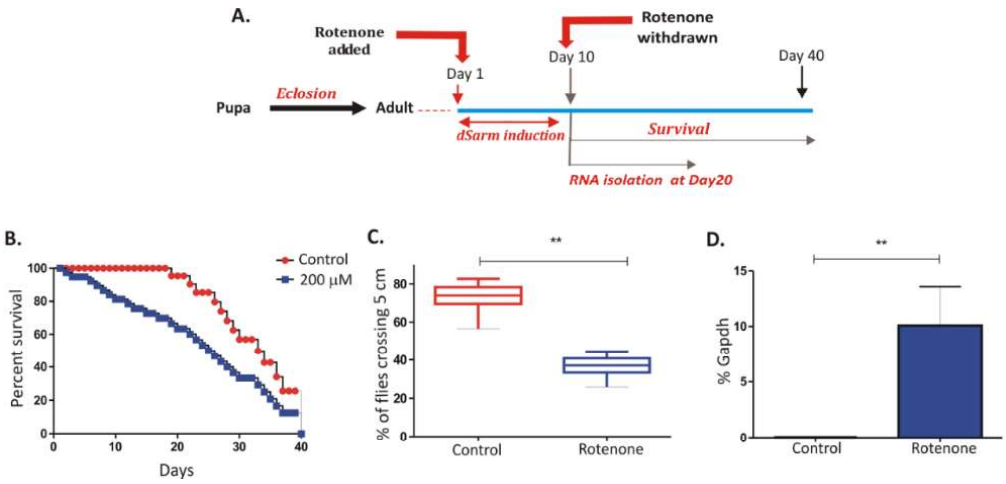

Fig. 59. (A) Schematic representation of rotenone treatment of 1-day old flies for 10-days followed by withdrawal and further experiments were performed at the time points indicated. **(B)** Survival curve of control adult flies and flies exposed for 10-days to 200 µM of rotenone and subsequent transfer of these flies to normal media. Fly viability was scored over a period of 40-days using a minimum of 100 flies per treatment. **(C)** Negative geotaxis assay of adult flies exposed to rotenone as indicated above for 10-days and experiment performed at day-20 post-exposure. **(D)** *dSarm* expression was analysed at day 20 of flies treated above and results

compared to age-matched untreated control flies. *p < 0.05, **p < 0.01 and ***p < 0.001 compared to control flies

3.3.4. Rotenone mediated neurotoxicity is independent of ROS generation

The free radical theory of aging presented by Harman et al. in 1950s proposes ROS accumulation contributes to aging (Harraan et al., 1955). However, this theory has been controversial and only in a few models, antioxidant therapy has been successful in reversing shortened lifespan (Fusco et al., 2007). Previous studies have shown that rotenone induces neuronal apoptosis via accumulation of ROS (Liu et al., 2016). Taking cue from these observations, we exposed w^{1118} flies to rotenone in the presence or absence of the antioxidant N-acetylcysteine (NAC). However, rotenone-induced toxicity and locomotor deficits were not reversed by NAC treatment (Fig. 60 A-B) in these flies. However, ROS levels were elevated moderately in these flies following exposure to 200 µM rotenone for 10-days which was reversed by the NAC addition (Fig. 60 C). To determine the specific role of ROS in environmental toxin mediated neurodegeneration, w^{1118} flies were also exposed to sodium arsenite (As) along with NAC. Interestingly, a significant reversal of arsenic mediated toxicity (Fig. 60 D) and locomotor deficits (Fig. 60 E) were observed in the presence of NAC. These results suggest that rotenone-induced ROS generation is not the sole player in the ongoing neurotoxicity and *dSarm* expression may act independently of ROS generation.

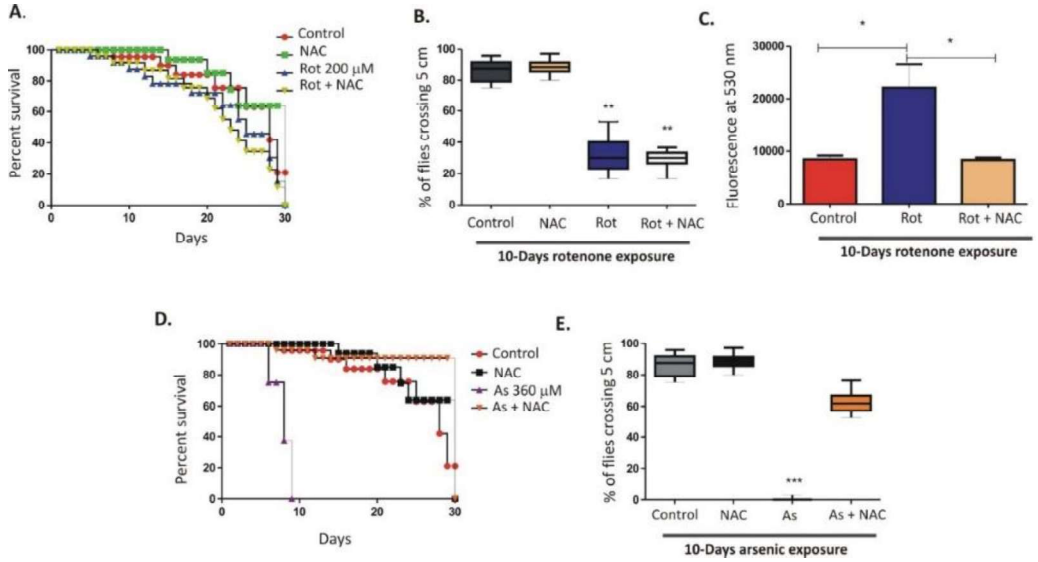

Fig. 60. (A) Survival curve of control adult flies and flies exposed to 200 µM of rotenone in the presence or absence of the ROS scavenger N-acetyl cysteine or NAC (1 mg/ml). Fly viability was scored over a period of 30-days, using a minimum of 100 flies per treatment. **(B)** Negative geotaxis assay of the flies at 10-days post-exposure. **(C)** ROS generation assay in 1-day old flies exposed to 200 µM rotenone for 10-days in the presence or absence of NAC. **(D)** Survival curve of control adult flies and flies exposed to 360 µM of Arsenic (As) along with

NAC (1 mg/ml). Fly viability was scored over a period of 30-days, using a minimum of 100 flies per treatment. **(E)** Negative geotaxis assay of the flies exposed to As in the presence or absence of NAC at 10-days post-exposure. *p < 0.05, **p < 0.01 and ***p < 0.001 compared to control flies

3.3.5. Rotenone induced locomotor deficits was accompanied by increased *PINK1* expression and reduction of autophagic flux

Recent works have suggested that PTEN-induced putative kinase 1 (PINK1) is stabilized during the loss of mitochondrial membrane potential and the accumulated PINK1 mediates translocation of Parkin (a PD-linked E3 ubiquitin ligase) from the cytoplasm to mitochondria. Parkin ubiquitinates mitochondrial outer membrane proteins such as VDAC1 and MFN1/2 and target the damaged mitochondria for the mitophagy. PINK1 has been shown to form complex with SARM1 and TRAF6, which helps in stabilization of PINK1 on depolarized mitochondria (Murata et al., 2013). To determine the status of *PINK1* in rotenone treated flies, 1-day old flies were exposed to 200µM rotenone for 1-, and 20-days and their brains were subjected to real time PCR analysis. A significant increase in *PINK1* expression was observed in the rotenone treated flies (Fig. 61 A).

It has been previously shown that rotenone causes autophagic vacuole accumulation. the mechanism of which has not been thoroughly investigated. This autophagic vacuole accumulation may result from a decrease in their effective lysosomal degradation (Mader et al., 2012). Defective autophagy has also been implicated in the neurons of the aging brain. To understand rotenone mediated autophagy gene regulation in the w^{1118} flies, 1-day old flies were exposed to 200µM rotenone for 1-, 10- and 20-days and their brains were subjected to real time PCR analysis. A significant decrease in *Atg3* and *Atg5* expression was observed following 1-day post treatment in the flies (Fig. 61 B-C). Taken together, our results indicate that rotenone exposure caused heightened *PINK1* expression and reduced expression of autophagy-related genes, suggesting that rotenone treatment might stimulate mitophagy but it is not completed leading to accumulation of damaged mitochondria and neuronal death.

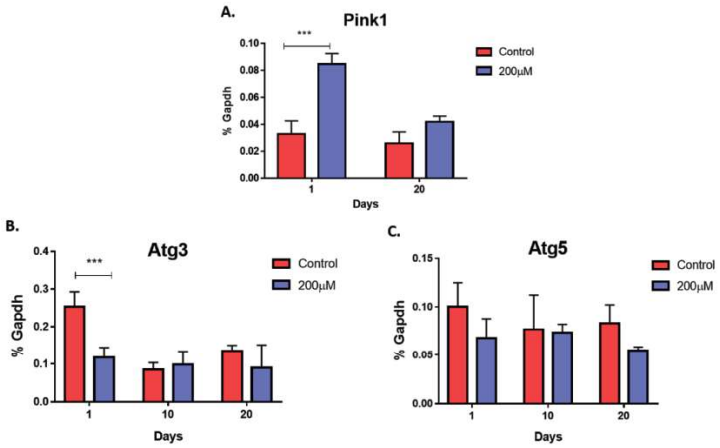

Fig. 61. Real-time PCR analysis of 1-, 10- and 20-day old flies, exposed to 200 µM of rotenone. **(A)** *Pink1*, **(B)** *Atg3* and **(C)** *Atg5* genes were analysed. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample

3.3.6. Rotenone exposure mediates decreased learning ability and synaptic deregulation in w^{1118} strain of Drosophila

It has been found that nAChR beta 3 is widely expressed in the CNS and able to regulate neurotransmitter release like acetyl choline, dopamine, norepinephrine, serotonin, glutamate and GABA. Similarly, another molecule EAAT also plays an essential role in the neural networks controlling memory, learning and motivity. They capture the neurotransmitter released in the synaptic cleft and prevent glutamate-induced neuronal death (or excitotoxicity) by maintaining a low extracellular concentration of this neurotransmitter (Besson et al., 1999). To determine the effect of rotenone on these molecules, 1-day old flies were exposed to 200µM rotenone for 1-, and 20-days and their brains were collected. A significant decrease in *nAChR beta 3* expression was observed without any effect on *EAAT* expression (Fig. 62 A-B) in the rotenone treated flies.

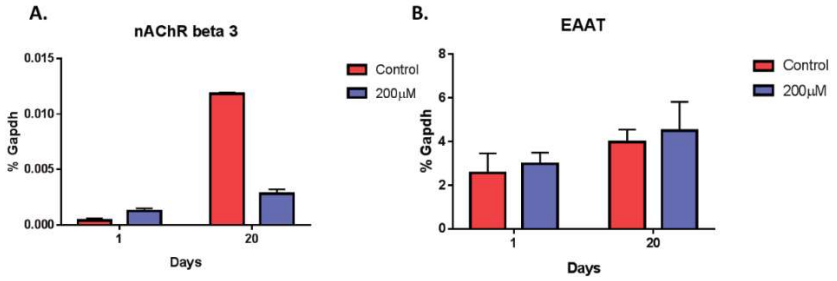

Fig. 62. Real-time PCR analysis of 1- and 20-day old flies, exposed to 200 µM of rotenone. **(A)** *nAChRbeta 3* and **(B)** *EAAT* genes were analysed. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample

To determine the *Nrx-1* expression in rotenone treated flies, we exposed w^{1118} flies to 200µM rotenone for 1-, 10- and 20-days and collected brains from them. *Nrx-1* expression was higher in rotenone exposed aged (20-days old) flies as compared to untreated control flies (Fig. 63 A). The pathophysiological changes associated with neurodegeneration include aggregation of misfolded proteins and synaptic and neuronal loss. In our model, rotenone (200 µM) induced neurotoxicity was also accompanied by deregulation of the synaptic proteins synapsin and syntaxin at 20-days post exposure (Fig. 63 B). The levels of synapsin showed a decrease while the late endosomal protein syntaxin expression was higher compare to control flies indicating synaptic dysfunction which may lead to subsequent neuronal loss.

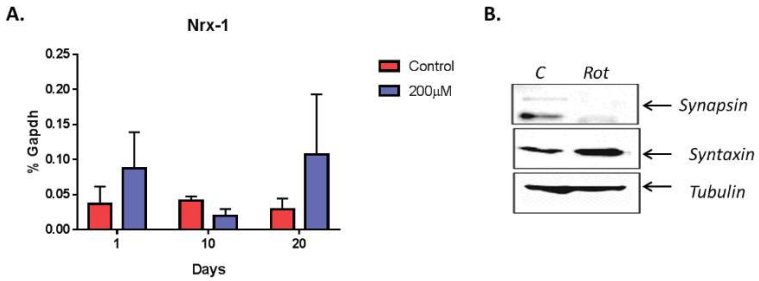

Fig. 63. (A) Real-time PCR analysis of 1-, 10- and 20-day old flies, exposed to 200 µM of rotenone. Nrx-1 gene was analysed. Results are representative of at least three independent experiments. **(B)** Representative western blot analysis of Synapsin and Syntaxin in 20-day old flies exposed to 200 µM rotenone. Tubulin served as a loading control and data was compared with age-matched untreated control flies (n = 3). *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample

3.3.7. Rotenone exposure in w^{1118} flies causes increased expression of stress related genes

It has been reported that in Drosophila, molecules like p120 and Hsp70 expression is required in stress response to increase animal life span (Stefanatos et al., 2013; Wheeler et al., 1995). Hsp70 performs chaperone function by stabilizing new proteins to ensure correct folding or by helping to refold proteins that were damaged in stress (Parsell and Lindquist, 1993). To determine the expression of these genes in rotenone exposed flies, we exposed w^{1118} flies to 200µM rotenone for 1-, and 20-days and collected brains from them. A significant increase in *Hsp70* and *p120* expression was found in rotenone exposed aged (20-days old) flies as compared to untreated control flies (Fig. 64 A-B).

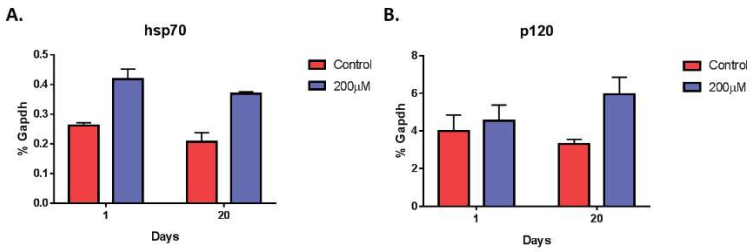

Fig. 64. Real-time PCR analysis of 1- and 20-day old flies, exposed to 200 μM of rotenone. **(A)** *hsp70* and **(B)** *p120* genes were analysed. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample

3.3.8. Age-mediated heightened susceptibility to rotenone was associated with increased inflammatory response

Inflammation in brain has been related to several neurodegenerative diseases and leads to the failure of general resolution mechanisms of the brain (Russell et al., 2016). In Parkinson's disease, non neuronal population like the microglia produce proinflammatory cytokines IL1□ and TNF-α that modulate neuronal loss (Pisanu et al., 2014; Russell et al., 2016). SARM1 is a molecule has been linked to modulate TNF-α production in restriction of viral infection (Szretter et al., 2009). Since rotenone-exposed Drosophila brain showed increased expression of *dSarm*, we also analysed *Eiger* (TNF-α homolog in Drosophila) expression in these flies. Significant increase (P < 0.01) in expression of *Eiger* was observed in the younger flies (1-day post-eclosion) brain exposed to 200 μM rotenone (Fig. 65 A). Interestingly, expression of *Eiger* followed a similar increase as found in *dSarm* as shown in Fig. 65 A followed by a reduction at 30-days post-exposure.

For the correlation of age-dependent susceptibility with heightened inflammatory response, 20-day old flies were exposed to rotenone for 3-days and 5-days and brains were collected at 3-days and 5-days post rotenone exposure. A significant increase in *Eiger* expression was observed compared to flies similarly exposed at 1-day post rotenone exposure. However, there was a significant reduction in inflammatory response in the 5-days rotenone treated flies compared to the 3-days post rotenone treatment that correlated with expression of *dSarm* in these flies (Fig. 65 B). However, we observed that the NF-κB protein *Relish* was upregulated in the younger flies following rotenone treatment for 10-days (Fig. 65 C) and therefore may act as an upstream activator of the Eiger pathway following rotenone treatment which needs to be further investigated.

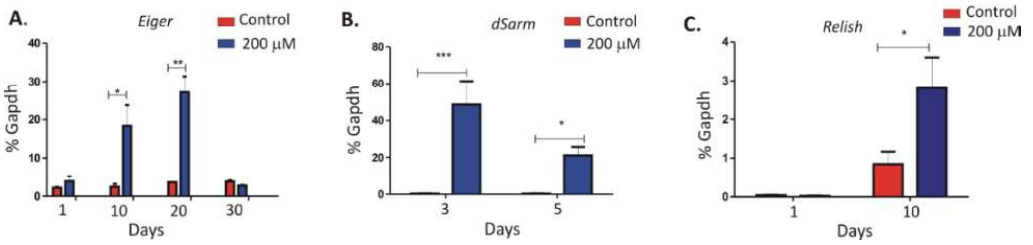

Fig. 65. *Eiger* expression in **(A)** younger (1-day old) flies exposed to 200 µM of rotenone for 1-day, 10-days, 20-days, and 30-days and **(B)** aged (20-days old) flies to 200 µM of rotenone for 3-days and 5-days post-treatment. **(C)** Expression of *Relish* in 1-day old flies exposed to 200 µM of rotenone for 1-day and 10-days. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample

Other immune response genes (Wnt, Upd1, Upd2 and Upd3) analysis showed no significant increase in the young as well as aged flies exposed to 200 µM of rotenone (Fig. 66 A-E).

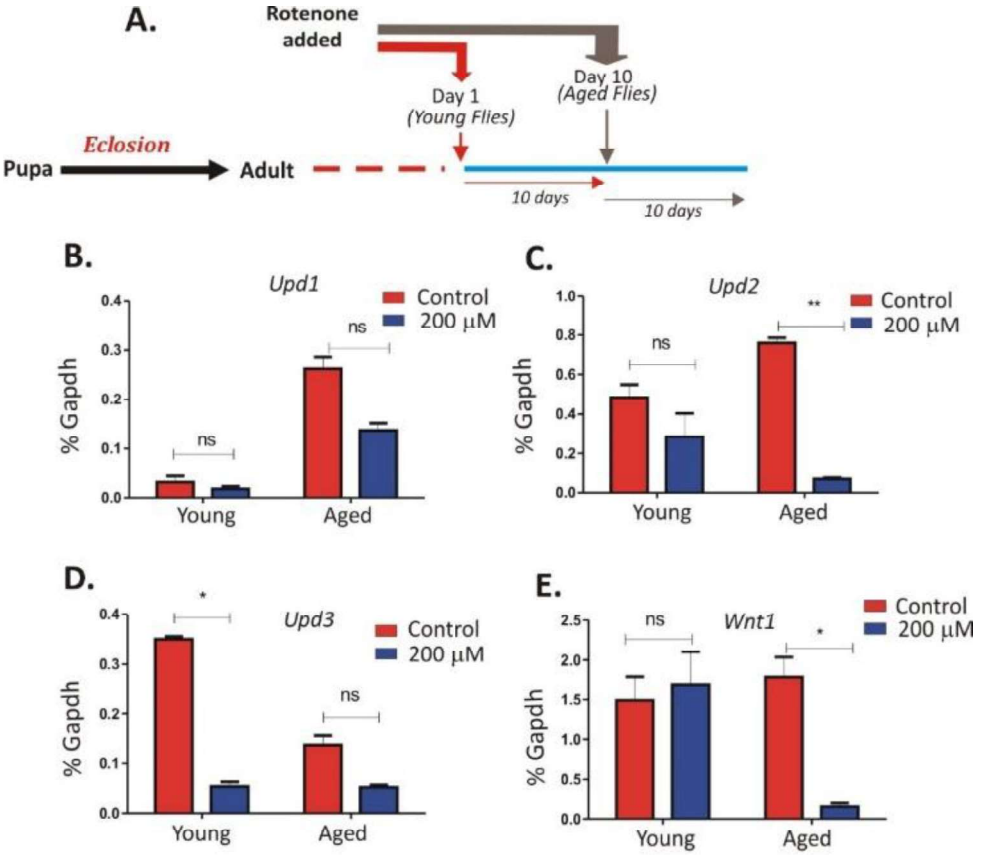

Fig. 66. (A) Schematic representation of rotenone treatment of 1-day and 10-day old flies followed by withdrawal in 10 days and further experiments were performed at the time points indicated. **(B-E)** Real-time PCR analysis of young and aged flies, exposed to 200 µM of rotenone. *Upd1* **(B)** ,*2* **(C)** ,*3* **(D)** and *Wnt1* **(E)** expression in younger (1-day old) flies exposed to 200 µM of rotenone and aged (10-days old) flies to 200 µM of rotenone. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample

3.3.9. The anti-inflammatory molecule resveratrol rescued rotenone-mediated locomotor defects and reduced *dSarm* Expression

Resveratrol (trans-3,4',5- trihydroxystilbene) is a polyphenolic compound and it is naturally found in nuts and grapes. Resveratrol is a promising neuroprotective agent acting via the downregulation of the inflammatory responses (Soleas et al., 1997). In order to determine whether rotenone-mediated inflammatory responses have a role in age-associated increased susceptibility, 1-day old w^{1118} flies were exposed to 200 µM rotenone in the presence or absence of resveratrol. Interestingly flies were even more susceptible to rotenone-mediated toxicity with increasing age in the presence of resveratrol (Fig. 67 A). This finding correlates with the concept that a basal level of inflammation has neuroprotective effects and complete inhibition of the inflammatory responses with continuous resveratrol exposure may have lethal effects in the CNS. As our previous data suggested that an early dSarm induction may play a major role in the subsequent age-related susceptibility to rotenone, we treated 1-day old flies with 200 µM rotenone with or without resveratrol (1 µM) and performed negative geotaxis assay at 10-days following exposure. In the adult flies at 10-days post-exposure, a significant reversal ($P < 0.001$) of locomotor deficits was found in the presence of resveratrol (Fig. 67 B). To further investigate whether resveratrol mediated suppression of the inflammatory responses had a major role in this mechanism, we analysed *Eiger* expression in these flies as early as 1-day post treatment. A significant reversal of the inflammatory responses as found by the reduction in *Eiger* upregulation was seen in these flies in the presence of resveratrol that was accompanied by a concomitant reduction in *dSarm* expression (Fig. 67 C-D) that strongly suggests that the inflammatory responses may work upstream of *dSarm* expression in these flies.

Recently, a study has suggested the important role of DAMPs (damage associated molecular pattern) in mediating a sterile inflammatory response through Jak-STAT pathway in flies (Srinivasan et al., 2016). To understand further whether such sterile inflammation functions in rotenone-mediated neurotoxicity, we analyzed a few JAK/STAT-dependent genes expression in flies exposed to rotenone in the presence or absence of resveratrol at 10-days post rotenone exposure. The genes including the turandot family member (*TotM*) belong to immune and stress response genes family and thioester containing protein (*Tep)* 1 and 2. A significant increase of

these genes expression was observed in 1-day old flies treated with 200 µM of rotenone (Fig. 67 E-G). Interestingly, significant reduction of the expression of these genes was found in rotenone treated flies in the presence of resveratrol suggesting that JAK/STAT-mediated sterile inflammation may play a major role in rotenone associated locomotor disabilities in addition to the Eiger pathway.

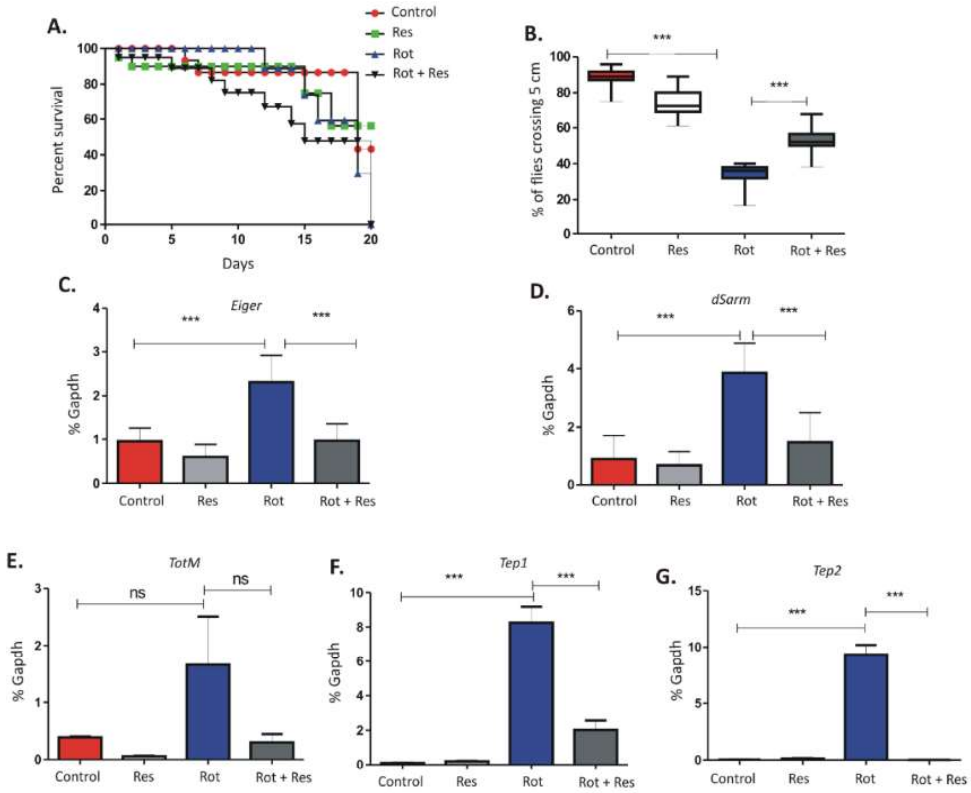

Fig. 67. (A) Survival curve of 1-day old flies exposed to 200 µM of rotenone in the presence or absence of 1 µM of resveratrol. Fly viability was scored over a period of 20-days, using a minimum of 100 flies per treatment. **(B)** Negative geotaxis assay of the same samples at 10-days post-exposure. **(C, D)** Real-time PCR analysis of fly heads as treated above. Both **(C)** *Ect4* and **(D)** *Eiger* genes were analysed in the 1-day old flies following 1-day post-exposure to rotenone (200 µM) in the presence or absence of resveratrol (1 µM) and results compared to age-matched untreated control flies. **(E-G)** Real-time PCR analysis of fly heads as treated above. **(E)** *TotM*, **(F)** *Tep1* and **(G)** *Tep2* genes were analysed in the 1-day old flies following 1-day post-exposure to rotenone (200 µM) in the presence or absence of resveratrol (1 µM) and results compared to age-matched untreated control flies. Results are representative of at least three independent experiments. *$p < 0.05$, **$p < 0.01$, and ***$p<0.001$ compared to control sample

Apart from the anti-inflammatory responses, resveratrol has been found to induce expression of *SIRT1*, a member of the NAD^+ dependent protein deacetylase family which plays an important role in aging and lifespan (Bonkowski and Sinclair, 2016; Imai and Guarente, 2014). Resveratrol has also been shown to regulate AMPK responses in both SIRT1- dependent and independent manner (Curry et al., 2018).

Our data suggests that resveratrol treatment in rotenone treated flies elevates the expression of *Sir2* gene (Drosophila homolog of mammalian *SIRT1*) (Fig. 68 A) without any effect on *AMPK* expression (Fig. 68 B), the mechanism of which needs to be further explored.

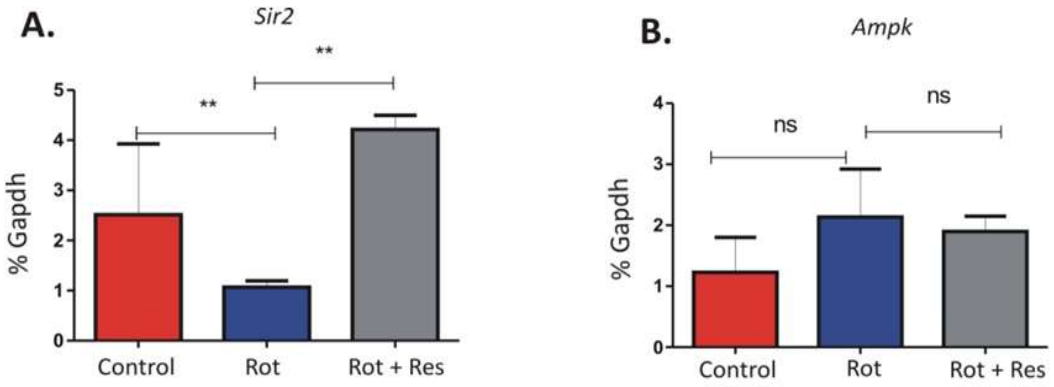

Fig. 68. (A, B) Real-time PCR analysis of fly heads. Both **(A)** *Sir2* and **(B)** *Ampk* genes were analysed in the flies following exposure to rotenone (200 µM) in the presence or absence of resveratrol (1 µM). Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample

3.3.10. The NAD^+ precursor Nicotinamide riboside (NR), rescued rotenone induced loss of survival and associated motor deficits

Nicotinamide riboside has been identified as a nutrient in milk. It is an anti-aging molecule and activates Sirtuins. It has been found that exogenous nicotinamide riboside extends the lifespan of certain animal models. Supplementation in mammalian cells and mouse tissues increases NAD^+ levels (Cantó et al., 2012). Nicotinamide Riboside is a precursor of nicotinamide adenine dinucleotide that is converted to nicotinamide mononucleotide by specific enzymes, like nicotinamide riboside kinases, Nrk1 and Nrk2 (Fletcher et al., 2017). It has been shown in recent years that following trauma or injury in neurons, SARM1 starts breaking down NAD^+ via its intrinsic NADase activity within the TIR domain. SARM1 induced axon degeneration could be counteracted by increased NAD^+ synthesis (Gerdts et al., 2015). However, the effect of exogenous administration of NR in Drosophila model following rotenone exposure is not understood well.

To study the effect of NR on rotenone mediated neurotoxicity, w^{1118} flies were exposed to 200 µM rotenone at 1-day and 10-day post-eclosion in the presence or absence of 1 mM NR and followed up to 30-days. Flies showed no reversal in rotenone induced toxicity at the higher dose (200 µM) (Fig. 69 B, C) but there was

significant reversal at 100 µM of rotenone in these flies in the presence of NR (Fig. 69 A). Interestingly, a significant reversal of rotenone induced locomotor deficits was also observed in these flies (Fig. 69 D).

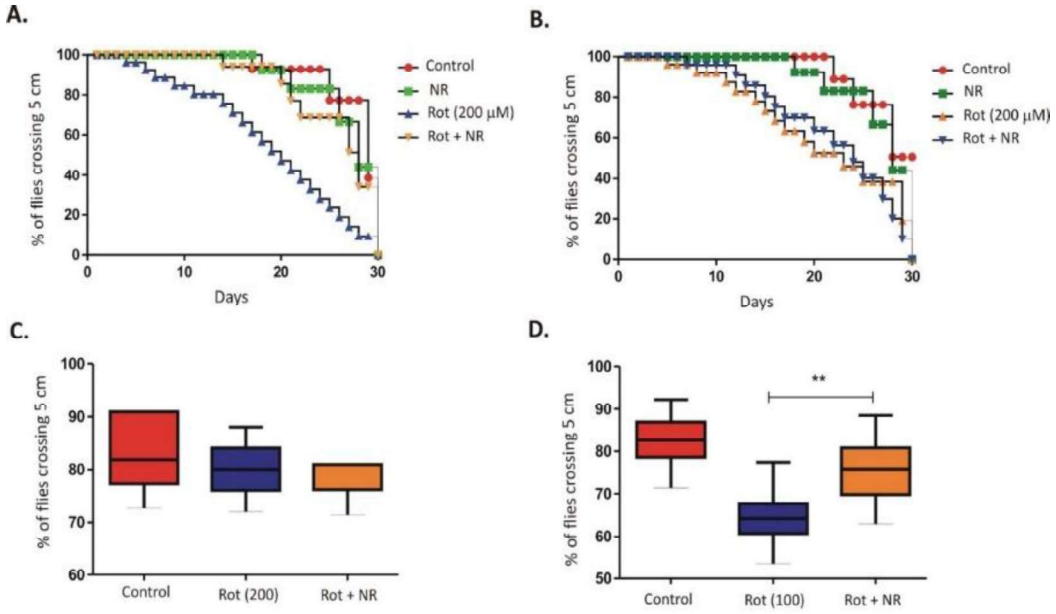

Fig. 69. (A, B) Survival curve of 1-day old flies exposed to 100 µM **(A)** and 200 µM **(B)** of rotenone in the presence or absence of 1 mM of NR. Fly viability was scored over a period of 30-days, using a minimum of 100 flies per treatment. **(C-D)** Negative geotaxis assay of the flies exposed to **(C)** 200 µM and **(D)** 100 µM of rotenone in the presence or absence of 1 mM of NR. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample

The expression of dSarm and the inflammatory genes Eiger and Relish was also reversed in the 1 and 10-day old flies grown in the presence of NR (Fig. 70 A-B).

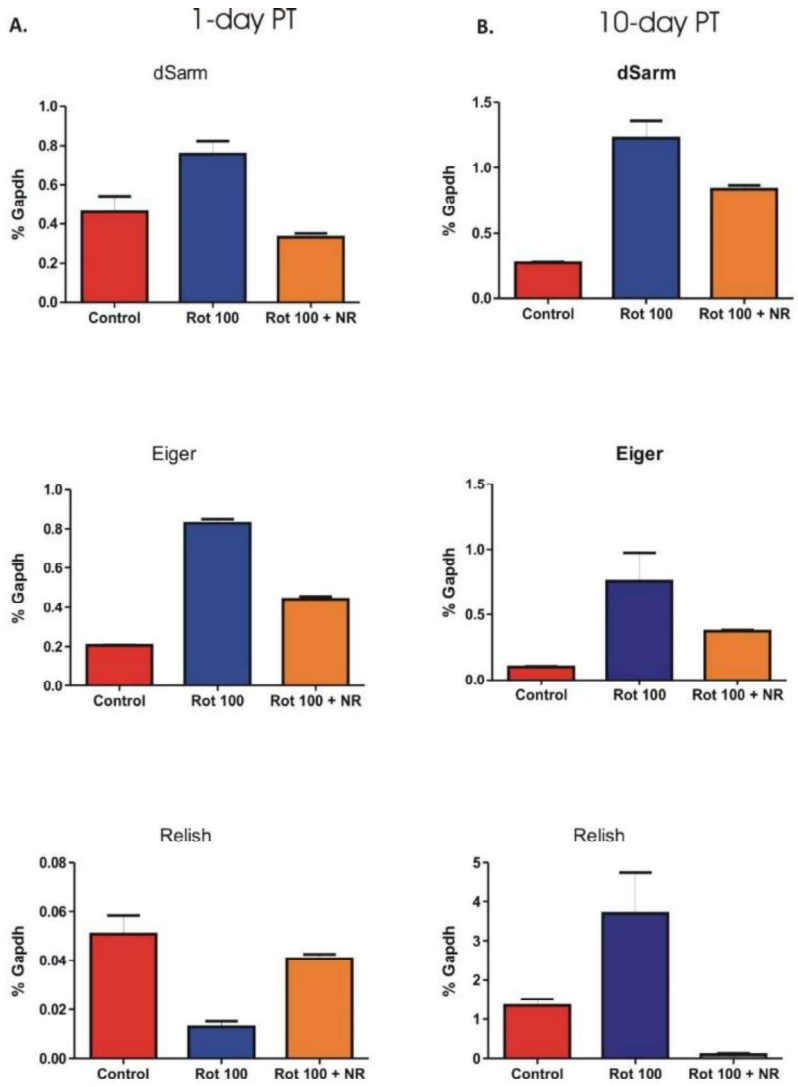

Fig. 70. Real-time PCR analysis of fly heads. *dSarm*, *Eiger* and *Relish* genes were analysed in the **(A)** 1-day and **(B)** 10-days old flies following exposure to rotenone (100 µM) in the presence or absence of NR (1 µM). Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample

To determine the age-related susceptibility to environmental toxins, 20-day old flies were exposed to 100 and 200 µM rotenone with or without NR. But NR had no effect on increased susceptibility and locomotory deficits of these flies to rotenone exposure (Fig. 71 A-B). Interestingly, NR pre-treated flies, exposed to even higher dose (200 µM) of rotenone at 10-day post-eclosion showed a significant reversal in rotenone induced toxicity (Fig. 71 C-D).

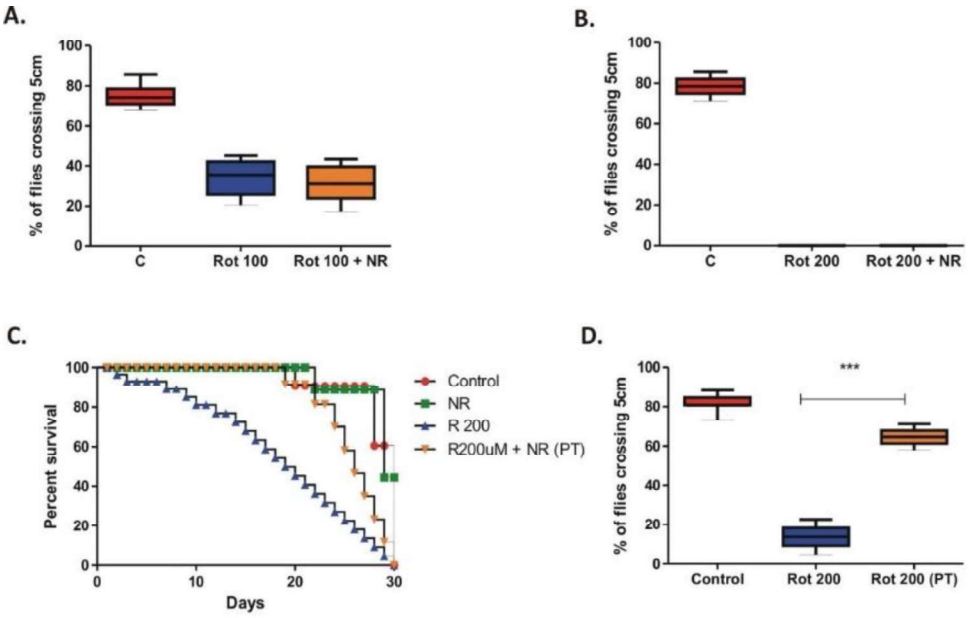

Fig. 71. Negative geotaxis assay of the 20-days old flies exposed to **(A)** 100 µM and **(B)** 200 µM of rotenone in the presence or absence of 1 mM of NR. **(C)** Survival curve of NR pre-treated 10-days old flies exposed to 200 µM of rotenone in the presence or absence of 1 mM of NR. Fly viability was scored over a period of 30-days, using a minimum of 100 flies per treatment. **(D)** Negative geotaxis assay of the same samples. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample

Reversal of rotenone induced toxicity in the presence of NR in the aged flies (20-day old) also resulted in reversal of increased induction of dSarm as well as Eiger (Fig. 72 A-B). Our results indicate that external supply of NR could rescue rotenone mediated loss of survival and associated motor deficits as well as increased *dSarm* and *Eiger* expression.

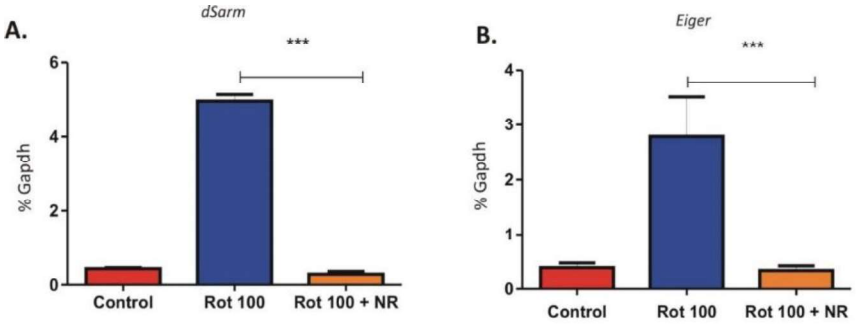

Fig. 72. Real-time PCR analysis of fly heads. **(A)** *dSarm* and **(B)** *Eiger* genes were analysed in the 20-days old flies following exposure to rotenone (100 µM) in the presence or absence of NR (1 µM). Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample

3.4. Discussion

The molecular switch that regulates the transition from 'physiological aging' to a degenerative state of the brain is still elusive. Several environmental risk factors like pesticide exposure and dietary lifestyle choices have been implicated in the increased incidence of age-associated neurodegenerative disorders like Parkinson's disease (PD) but the etiology remains unknown so far. There is also lack of a proper animal model system that provides a comprehensive understanding of age-associated neurodegeneration. Why there is specific loss of dopaminergic neuronal clusters in PD is a long standing question. Although mitochondrial dysfunction has emerged as key players in PD it is not yet known whether they are the disease drivers. Our results indicate that age plays a determining role in the increased mortality rate and locomotor impairment in w^{1118} flies. Aged flies (20-days old) were more susceptible to a sub lethal dose (100 µM) of rotenone as compared to the younger flies (1-day old) which correlated with the human form of PD where age plays a critical role in the increased incidence of these neurodegenerative diseases. Further, 20-day old flies could not survive beyond 10 days following 200 µM of rotenone exposure strongly suggesting that age poses a major risk factor in the increased susceptibility to neurodegeneration.

In several psychiatric disorders neuroinflammatory responses play an important role, but its role in age-associated neurodegeneration is emerging. In order to understand how inflammation could play a role in the heightened susceptibility to rotenone we analysed the expression of the inflammatory molecule *Eiger* in the 1-day old flies subjected to rotenone exposure. We demonstrated for the first time that rotenone mediated motor deficits and neurotoxicity is accompanied by an induction of the inflammatory molecule *Eiger* in both the young (1-day old) and aged (20-days old) flies. Our results also point towards an early induction of a JAK-STAT mediated sterile inflammatory response that has been previously been shown to be induced in damaged cells mediated by actin release.

Few studies have implicated that the neurodegenerative molecule SARM1 mediates a proinflammatory cytokine response in viral encephalitis (D. W. Summers et al., 2014). Interestingly, induction of *dSarm* followed a similar pattern as *Eiger* expression in the rotenone-treated flies and was induced early, reached its maxima at 20-days followed by its sharp decrease. We also observed several higher fold *dSarm* induction in the aged flies as early as 3-days post exposure with the levels decreasing

at 5-day post exposure which may correlate with the rapid dopaminergic neuronal loss in these flies. This corroborated our previous result of age related increased susceptibility to rotenone. We also demonstrated that rotenone exposure results in deregulation of synaptic proteins and increased expression of stress related proteins. These findings strongly indicate that SARM1 might be an important player in heightened susceptibility to age associated neuronal loss.

The reduction in the levels of *dSarm* after the initial peak following exposure to rotenone could be due to the neuronal loss, producing them per se in the rotenone-exposed flies. In agreement with this, we demonstrated that short term rotenone exposure results in a progressive decline in locomotor abilities with accompanying induction of *dSarm*. Following withdrawal of rotenone after 10-days exposure these flies manifested motor deficits with subsequent loss of survival indicating that an early induction of *dSarm* is necessary and sufficient for rotenone-mediated locomotor deficits in flies.

In order to understand whether ROS plays an important role in rotenone-mediated induction of *Sarm1* and accompanying inflammatory response, we analysed the ROS levels in the rotenone-exposed flies. Our results suggested that though there was an increase in the ROS levels following rotenone treatment in flies, there was no reversal in the survival or locomotor deficits in these flies when fed in media consisting of the ROS scavenger N-acetyl cysteine (NAC) in the presence of rotenone. In contrary to some of the earlier studies, where dopaminergic neuronal loss was associated with rotenone-induced ROS generation in mouse models of PD (Choi et al., 2015), our data indicates that ROS is not a sole player in rotenone induced loss of survival or locomotor deficits in these flies.

An important pathway that has been shown to be altered with aging is the brain's intrinsic waste disposal system or macroautophagy. Due to the accumulation of aggregated proteins and damaged organelles as often observed in several age-associated neurodegenerative diseases, an obvious question arises whether the cellular clearance mechanism is functional. In familial models of PD with mutations in *PINK1* and *Parkin*, one of the mechanisms for the clearance of damaged mitochondria termed mitophagy is defective. However, the precise mechanism of such a mitochondrial quality control process in the case of sporadic PD, that account for most of the PD cases worldwide, is still not known. Our results showed an increased expression of *PINK1* following rotenone exposure which could be due to mitophagic induction.

However, there was a reduction in the expression of the early autophagy genes *Atg3* and *Atg5* which indicates a defective autophagic clearance. Whether autophagy is blocked at the autophagosome formation stage or at the autophagic flux in the aging brain of the flies following rotenone exposure opens an exciting avenue to be explored in the future.

To further understand the implication of rotenone mediated inflammatory response, flies were treated with the naturally occurring polyphenolic compound resveratrol that has been shown to induce anti-inflammatory responses *in vitro*. Since early *dSarm* induction and the inflammatory responses were sufficient to mediate the loss of survival and locomotor deficits in these flies, we conducted our studies in the younger flies (1-day following eclosion). Interestingly, exposure of resveratrol not only reversed the activation of the inflammatory responses but also decreased *dSarm* expression significantly along with accompanying motor deficits thus suggesting that dSarm might act downstream of the inflammatory responses in these flies.

Nicotinamide adenine dinucleotide (NAD) is a key cellular component in the simplest living organism like bacteria to the complex multicellular organisms. Replenishing NAD^+ intermediates has shown to increase life span and reverse neuronal loss thus making it an attractive target to understand rotenone-induced dopaminergic neuronal loss. Our preliminary results in Drosophila model suggest that chronic low dose exposure to the pesticide rotenone results in upregulation of the neurodegenerative molecule SARM1 that regulates programmed axonal death via NAD^+ depletion. Prior exposure to Nicotinamide riboside (NR), a precursor of NAD^+ significantly reverses the locomotor deficits and loss of survival in flies exposed to rotenone in an age-dependent manner. Interestingly, prior exposure with NR reduced age-associated susceptibility to rotenone induced neurotoxicity.

Taken together, our results suggest a novel activation of SARM1 following rotenone treatment in flies is accompanied by a heightened inflammatory response that may subsequently mediate the loss of dopaminergic neurons as often observed in PD. Dopamine replacement therapy has limited restorative effects in PD mainly due to the fact that dopamine cannot reverse the ongoing loss of dopaminergic neurons. Thus, targeting SARM1 or the use of anti-inflammatory compounds/NAD^+ replenishing agents like NR could open up cost-effective exciting avenues in the treatment of PD and other age-associated neurodegenerative diseases.