Toxicant-Induced Mitochondrial Dysfunction and Dopaminergic Neurodegeneration in

*Caenorhabditis elegans*

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in Environment in the Graduate School
of Duke University

2017
ABSTRACT

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Abstract

Mitochondrial dysfunction appears to be a hallmark of many neurodegenerative diseases. The link between mitochondrial and neuronal health is particularly strong in Parkinson’s disease (PD). Genes that have been shown to cause PD when mutated are known to interact with mitochondria to varying degrees; particularly, two of the genes associated with mitochondria-specific autophagy (mitophagy), PINK1 and PARK2, have been shown to cause autosomal recessive PD. Deletions and mutations in mitochondrial DNA (mtDNA) have been detected in the substantia nigra of PD patients. Furthermore, toxicants used to model PD in the laboratory are known to exert their toxicity by disrupting mitochondrial function. Despite all the advances in PD research, the precise mechanisms through which this disease arises are still not well understood.

The central goal of this dissertation was to investigate the role that mtDNA damage and mitochondrial dysfunction play in causing dopaminergic neurodegeneration in the context of PD. This work was performed with a particular focus on mitochondrial toxicants: their mechanism of action and effect on neuronal health, and how these toxic exposures might exacerbate genetic susceptibilities. Another goal was to identify windows of susceptibility to mtDNA damage, with early life and old age being of particular interest. To explore these ideas, the nematode Caenorhabditis elegans was utilized as a model organism.
To characterize age-related sensitivity to mtDNA damage, worms at different life stages were exposed to ultraviolet C (UVC) to accumulate mtDNA damage and the effect of this damage on mitochondrial genome copy number and dopaminergic neuron health was assessed. To evaluate the role of autophagy in maintaining mitochondrial and neuronal health after toxicant exposure, mitophagy gene knockouts (KOs) and wild type worms were exposed to 6-hydroxydopamine (6-OHDA) and UVC; to study non-selective macroautophagy, starvation was used to induce it, and nematodes were subsequently subjected to a toxic exposure. Dopaminergic neuron morphology, targeted gene expression, and mitochondrial morphology were evaluated. Lastly, the mechanism of toxicity of rotenone in *C. elegans* was investigated; specifically, mitochondrial respiration (with the Seahorse Bioanalyzer), targeted metabolomics, ATP levels, and targeted gene expression were evaluated.

Due to technical complications with our UVC exposure model and PCR-based evaluation of mtDNA damage in *C. elegans* eggs, the windows of susceptibility to mtDNA damage hypothesis could not be properly tested. However, our current results do not support this idea. Our studies on the role of autophagy in the context of toxicant-induced dopaminergic neurodegeneration suggest that both non-selective macroautophagy and mitophagy have a protective effect. We saw protection from 6-OHDA-induced neurodegeneration after starvation treatment, suggesting that macroautophagy could be playing a protective role. In the context of mitophagy
mutations, we saw different responses to UVC and 6-OHDA. Both pink-1 and pdr-1 mutants were more sensitive to UVC-induced dopaminergic neurodegeneration than the control strain; however, only the pdr-1 strain was more sensitive than the control strain to 6-OHDA. The pink-1 mutant was actually protected from neurodegeneration caused by 6-OHDA. We proceeded to evaluate antioxidant responses in these strains (since 6-OHDA exerts its toxicity mainly via oxidative stress) but saw no differences across strains. We are currently testing the hypothesis that changes in the mitochondrial network in the pink-1 mutant after 6-OHDA exposure allow it to better withstand its toxic effect. Our studies into the mechanism of action of rotenone in C. elegans gave us surprising results: we saw no major alterations in oxygen consumption or ATP levels. However, various metabolites were altered, signaling a metabolic restructuring similar to that observed in C. elegans complex I mutants. Also similar to complex I mutants, it appears that the glyoxylate pathway is upregulated after rotenone exposure; this alternate pathway parallel to the citric acid cycle might be allowing the nematodes to acclimate to rotenone exposure, explaining our respiration and ATP results.

Overall, this dissertation work supports the link between mitochondrial dysfunction and dopaminergic neurodegeneration, and provides new hypotheses for research in this field.
Dedication

This work is dedicated to my wonderful familia, who has always believed in me; especially my husband Brian, whose love, patience, and support carried me when I could not continue on my own.
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List of Abbreviations

6-OHDA, 6-hydroxydopamine  
KO, knockout

AD, Alzheimer’s disease  
MPTP, 1-methyl-4-phenyl-1,2,3,6-

tetrahydropyridine

ADE, anterior deirid  

ALS, amyotrophic lateral sclerosis  
mtDNA, mitochondrial DNA

ANOVA, analysis of variance  
nDNA, nuclear DNA

CEP, cephalic neurons  
NER, nucleotide excision repair

DCCD, N,N’-Dicyclohexylcarbodiimide  
NRTI, nucleoside reverse transcriptase

DOHaD, developmental origins of  
inhibitors

health and disease  
OCR, oxygen consumption rate

DSB, double strand breaks  
OXPHOS, oxidative phosphorylation

FCCP, carbonyl cyanide-4-

(trifluoromethoxy)phenylhydrazone  
PAH, polycyclic aromatic hydrocarbon

PD, Parkinson’s disease  
PDE, posterior deirid

FET, Fisher’s Exact test  
ROS, reactive oxygen species

FuDR, 5’-fluoro-2-deoxyuridine  
UVC, ultraviolet C

HD, Huntington’s disease

HIV, human immunodeficiency virus
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1. Introduction

Mitochondrial dysfunction has been increasingly associated with neurodegenerative disorders (Beal 1998; Johri and Beal 2012). Mutations in genes related to mitochondrial dynamics or involved in mitochondrial function have been associated with familial cases of neurodegenerative diseases such as Parkinson’s disease (PD) and amyotrophic lateral sclerosis (ALS) (Martin 2012). Furthermore, mitochondrial dysfunction and bioenergetic deficits are hallmarks of the pathophysiology of other common neurodegenerative diseases such as Alzheimer’s disease (AD) and Huntington’s disease (HD) (Browne and Beal 2004; Swerdlow et al. 2010). It has also been documented that mitochondria interact with a variety of proteins involved in neurodegenerative disease etiology (Lin and Beal 2006). The relationship between mitochondrial function and neuronal health is also highlighted by the fact that many mitochondrial diseases have neurological symptoms (McFarland et al. 2010). At a more basic level, neurons appear to be particularly susceptible to mitochondrial dysfunction when compared to other cell types, as the brain has one of the biggest energetic demands in the body (Albers and Siegel 1999).

There is particularly strong evidence for the link between mitochondrial and neuronal health in PD. As mentioned above, several genes associated with familial PD code for proteins that are directly or indirectly involved in mitochondrial function.
$PINK1$ and $PARK2$ cause early-onset PD via autosomal recessive inheritance, and are involved in mitochondrial-specific autophagy (i.e. mitophagy) (DP Narendra et al. 2010); $DJ-1$ also causes autosomal recessive early-onset PD, and is believed to be involved in the mitochondrial response to oxidative stress (Bonifati et al. 2003). $LRRK2$, the main cause of autosomal dominant PD, codes for a kinase mostly present in the cytoplasm (also present in the mitochondrial outer membrane) that has been shown to participate in mitochondrial dynamics by interacting with the fission protein DRP1 and inducing mitochondrial fragmentation (Wang et al. 2012).

In addition to the compelling evidence presented by genetic inheritance studies, studies into the mitochondrial genome and mitochondrial toxicants further highlight the involvement of mitochondrial dysfunction in PD. High levels of mutations and deletions in mitochondrial DNA (mtDNA) have been detected in the substantia nigra (primary region for neuron degeneration in PD) of both PD patients and aged-matched controls (Bender et al. 2006; Kraytsberg et al. 2006). Furthermore, toxicants used to model Parkinson’s disease (PD) in laboratory organisms, such as rotenone, MPP+ and paraquat, inhibit complexes of the mitochondrial electron transport chain (Dawson and Dawson 2003).

The last two decades of PD research have unveiled many of the relevant factors playing a role in the development of this disease; a major takeaway has been that
genetics alone cannot explain PD etiology (Rappaport 2016; Wirdefeldt et al. 2011). This dissertation aims to further elucidate the role environmental toxicants and mitochondrial biology play in dopaminergic neurodegeneration (in the context of PD), by looking at novel endpoints of toxicity using the nematode *Caenorhabditis elegans*.

### 1.1 Mitochondria

Mitochondria are dynamic double-membrane bound organelles, best known for being the site of oxidative phosphorylation (OXPHOS). However, mitochondria also participate in calcium regulation, iron-sulfur cluster synthesis, fatty acid oxidation, heme synthesis, and amino acid metabolism (Alberts 2008; Wallace 2005). Although traditionally depicted as isolated “kidney bean”-shaped organelles, mitochondria exist as dynamic networks that regularly fuse and divide based on cellular cues (Liesa and Shirihai 2013). Mitochondria have their own, generally circular, genome. It is about 16,000 bp in length in metazoans, and it encodes the 16S & 18S ribosomal RNAs, 22 transfer RNAs, and 13 OXPHOS polypeptides (Wallace 2005). The number of mitochondrial genome copies varies; in humans, it ranges between 1,000 to 10,000 mtDNA copies per cell and 1-10 copies per mitochondrion, where it is organized in DNA-protein complexes known as nucleoids (Clayton 1982; Satoh and Kuroiwa 1991).

MtDNA integrity is required for good cellular function. Due to its close proximity to the electron transport chain (nucleoids are anchored to the inner
mitochondrial membrane), mtDNA is particularly at risk for oxidative damage. Moreover, some types of mtDNA damage cannot be repaired, as mitochondria lack nucleotide excision repair (NER), and the handling of double-strand breaks (DSBs) is unclear (Meyer et al. 2013).

1.1.1 Oxidative phosphorylation

One of the main functions of mitochondria is producing ATP via electron transfer from the reducing agents NADH and FADH₂ ultimately to molecular oxygen. This process allows for proton pumping from the matrix to the intermembrane space, creating the proton motive force necessary to power ATP production (Berg et al. 2015). This is modulated by the electron transport chain, as follows: complex I (NADH dehydrogenase) receives electrons from NADH, and complex II from FADH₂; these electrons are transferred to ubiquinone (coenzyme Q), which via complex III (ubiquinol-cytochrome c reductase) passes electrons to cytochrome c. Complex IV transfers electrons to oxygen from cytochrome c, producing water. Complexes I, III, and IV are responsible for proton pumping; complex V (ATP synthase) then uses the electrochemical gradient created by proton accumulation in the intermembrane space to produce ATP. Complex V allows for reentry of protons into the matrix, and harnesses the energy by phosphorylating ADP into ATP. Because oxygen is the ultimate receptor of electrons, this process is called OXPHOS (Chandel 2015).
1.2 Macroautophagy and mitophagy

Autophagy is a process through which cellular components are recycled via degradation of macromolecules in the lysosome or vacuole (Klionsky et al. 2011). Macroautophagy is a type of autophagy characterized by the formation of a double-membrane structure called the autophagosome (Yang and Klionsky 2010). One type of selective macroautophagy, mitophagy, specifically removes mitochondria that are damaged or no longer necessary (Kim et al. 2007). In mammals, mitophagy is thought to involve PINK1, an outer mitochondrial membrane kinase, and parkin, a cytosolic E3 ubiquitin ligase. If mitochondria are damaged and lose membrane potential, PINK1 accumulates in the outer mitochondrial membrane and phosphorylates ubiquitin on proteins in the outer mitochondrial membrane (Kane et al. 2014; Kazlauskaite et al. 2014; Koyano et al. 2014). This recruits parkin to the mitochondria, where it further ubiquitinates proteins, signaling autophagy receptors to the mitochondria. (Geisler et al. 2010; Lazarou et al. 2015; D Narendra et al. 2010; Wong and Holzbaur 2014). This marks the organelle for engulfment by isolation membranes which then fuse with the lysosome where the mitochondria are degraded (Youle and Narendra 2011).

Several studies implicate PINK1 and parkin in mitochondrial dysfunction and PD onset. Mutations in PINK1 and parkin can cause hereditary early onset PD, highlighting the importance of removing damaged mitochondria in order to preserve
neuronal health (Kitada et al. 1998; Valente et al. 2004). Past studies in Drosophila show that PINK1 and parkin defects can cause locomotor problems, and even dopaminergic neurodegeneration (Clark et al. 2006; Greene et al. 2003; Park et al. 2006).

1.3 Mitochondrial dynamics

Besides macroautophagy and mitophagy, other processes are involved in maintenance of the mitochondrial network. As briefly mentioned earlier, fusion and fission of the mitochondrial network can occur based on cellular cues, and this is crucial in maintaining mitochondrial and cellular health. Fusion allows for healthy mitochondria to transfer RNA and protein to faulty mitochondria by fusing with them. This process is known as functional complementation, and is effective to a degree; past a threshold of 80-90% mutation load, mitochondrial dysfunction is apparent (Schon and Gilkerson 2010). Fusion has also been related to bioenergetics, with fused mitochondrial networks being frequently observed in cells with high energy demands, suggesting that fusion might promote better mitochondrial function (Westermann 2012). Mitochondrial fission is also necessary for mitochondrial health; fragmentation of the mitochondrial network allows for the isolation of defective mitochondria, which can then be degraded via autophagy (van der Bliek et al. 2013; Youle and van der Bliek 2012), and may also facilitate allocation of mitochondria to daughter cells during cell division (Katajisto et al. 2015; Mishra and Chan 2014). Also highlighting the importance of these pathways is the
fact that some human diseases directly result from mutations in fusion and fission genes (e.g. Charcot Marie tooth type 2A and autosomal dominant optic atrophy). Furthermore, many other diseases, such as AD, PD, HD, diabetes and even cancer, have been associated with disruptions in normal mitochondrial dynamics (Babbar and Sheikh 2013). In the case of PD, defects in fusion (loss of Mfn2 specifically) have been shown to directly result in loss of dopaminergic neurons in conditional knockout mice (Lee et al. 2012; Pham et al. 2012).

1.4 Mitochondria as targets for environmental toxicants

Mitochondria are a target for environmental toxicants: their high lipid content attracts lipophilic compounds; the negatively charged matrix attracts and accumulates metal cations; and the mitochondrial calcium transporter can be hijacked by cationic metals to enter the organelle. Additionally, the mitochondrial cytochrome P450s can activate compounds that have accumulated in the organelle. As mentioned in section 1.1, mtDNA is at a high risk for oxidative damage due to being in close proximity to the electron transport chain, and does not have as robust DNA repair mechanisms as nuclear DNA (nDNA). Despite this, relatively few toxicants have been tested for mitochondrial genotoxicity (Meyer et al. 2013) (Figure 1).
Some precedent for mitochondria susceptibility to chemical exposures has been documented in the context of drugs with off-target effects. A well-known example is the effect nucleoside reverse transcriptase inhibitors (NRTIs) used against the human immunodeficiency virus (HIV) have on mitochondrial polymerase $\gamma$; the inhibition of this polymerase caused by NRTIs can cause mtDNA depletion, mutations, and as a result mitochondrial toxicity (Kohler and Lewis 2007; Poirier et al. 2015).
Further compounding the adverse effects environmental toxicants might have on mitochondria, is the fact that the number of mitochondrial genome copies is variable. MtDNA copy number varies by tissue type and at different developmental stages, creating windows of mtDNA vulnerability to toxic insult (Meyer et al. 2013; Miller et al. 2003; Shoubridge and Wai 2007).

1.5 The nematode Caenorhabditis elegans

*C. elegans* is a free-living nematode commonly found in decaying matter such as leaf litter (Kiontke and Sudhaus). Originally introduced as a laboratory organism in 1965, this nematode remains one of the most utilized model organisms in biological research to this day. Its microscopic size, short life cycle, fully mapped genome, and high homology with mammalian genes make it very amenable to less expensive, *in vivo*, high-throughput research (Riddle et al. 1997). The worm’s lifecycle takes approximately three days, and each hermaphrodite worm lays ~300 eggs (Lewis and Fleming 1995). At least twelve of the thirteen proteins encoded by the mammalian mitochondrial genome are also encoded by *C. elegans* mtDNA, making it an excellent model to study mitochondrial biology (Maglioni and Ventura 2016; Tsang and Lemire 2003). These same strengths have made it an increasing popular model for the study of toxicology (Boyd et al. 2012; Leung et al. 2008).
The *C. elegans*’ dopaminergic system consists of only 8 neurons in the hermaphrodite: four cephalic neurons (CEP), two anterior deirids (ADE) and two posterior deirids (PDE) (Chase and Koelle) (Figure 2). The simplicity of the *C. elegans* dopaminergic system (and its nervous system in general) has both advantages and disadvantages. Because it is a comparatively simple system, it allows for mechanistic studies to take place in a much more approachable context than the mammalian brain. The nematode being transparent also makes it possible to take full advantage of fluorescent imaging for evaluation of neuronal morphology and health. However, the dopaminergic system is simple to the point that it risks being too reductionist. There are some marked differences in *C. elegans* glial function compared to higher organisms; glial trophic support is not required for neuronal survival, and there is no axonal myelination (Shaham 2015). Also, microglia do not seem to be present in *C. elegans* (Stout et al. 2014). These differences are particularly relevant for PD research, as neuroinflammation and activation of glial cells appear to be common features of this disease (Hirsch et al. 2012). Another important difference is the lack of endogenous α-synuclein in *C. elegans*, a protein that is known to aggregate and form fibrils that can be found in Lewy bodies in PD (Dexter et al. 2012).
1.6 Dissertation objectives and experimental approach

The central hypothesis of my dissertation is that mtDNA damage and dysfunction, caused by environmental toxic exposures, play a central role in triggering dopaminergic neurodegeneration. I also hypothesized that environmental exposures at vulnerable ages/life stages or in susceptible genetic backgrounds would have a more potent effect on mitochondria, and in turn, in dopaminergic neurons. In order to explore this, a few well-studied toxicants were selected to induce mitochondrial dysfunction: rotenone and 6-hydroxydopamine (6-OHDA), both widely used to model PD in the laboratory, and ultraviolet C (UVC), used as a proxy for other environmental chemicals such as polycyclic aromatic hydrocarbons (PAHs) that cause bulky, helix-distorting lesions to DNA (Bess et al. 2012). Endpoints used to evaluate the effects of these compounds include: a visual dopaminergic neurodegeneration assessment; PCR-based
analyses of DNA integrity, genome copy number, and gene expression; mitochondrial morphology assessment using confocal microscopy; mitochondrial oxygen consumption (using the Seahorse Bioanalyzer); microplate reader-based ATP measurements; and targeted metabolomics. These experiments were performed on nematodes of different genetic backgrounds (depending on endpoint of interest). All nematode strains used (described in detail in each chapter) are listed in Table 1.

Table 1: Summary of *C. elegans* strains used in this dissertation.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Gene</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY200</td>
<td>$\text{P}_{\text{dat-1}}$::GFP</td>
<td>GFP in dopaminergic neurons</td>
</tr>
<tr>
<td>UA226 (pink-1::BY200)</td>
<td>$\text{pink-1} ; ; \text{P}_{\text{dat-1}}$::GFP</td>
<td>$\text{pink-1}$ KO (homolog of human <em>PINK1</em>); GFP in dopaminergic neurons</td>
</tr>
<tr>
<td>UA227 (pdr-1::BY200)</td>
<td>$\text{pdr-1} ; ; \text{P}_{\text{dat-1}}$::GFP</td>
<td>$\text{pdr-1}$ KO (homolog of human <em>parkin</em>); GFP in dopaminergic neurons</td>
</tr>
<tr>
<td>BY250</td>
<td>$\text{P}_{\text{dat-1}}$::GFP</td>
<td>GFP in dopaminergic neurons</td>
</tr>
<tr>
<td>N2 (Bristol)</td>
<td>NA</td>
<td>Wild type</td>
</tr>
<tr>
<td>JK1107</td>
<td>$\text{glp-1}$</td>
<td>Temperature-sensitive sterility</td>
</tr>
<tr>
<td>PE255</td>
<td>$\text{glp-4} ; ; \text{sur-5}::\text{luc+::gfp}$</td>
<td>Temperature-sensitive sterility; Luciferase- and GFP-expressing</td>
</tr>
<tr>
<td>RB766</td>
<td>$\text{icl-1}$</td>
<td>Isocitrate lyase KO</td>
</tr>
</tbody>
</table>
This dissertation is organized in three specific research aims briefly described below:

**Specific Aim 1 (Chapter 2): Characterize age-related sensitivity to mitochondrial DNA damage in *Caenorhabditis elegans***

Worms at different life stages were exposed to UVC to accumulate mtDNA damage, and the effect of this damage on dopaminergic neuron health was assessed.

**Specific Aim 2 (Chapter 3): Non-selective macroautophagy and mitophagy aid in maintaining mitochondrial and neuronal health in *C. elegans* after toxicant exposure***

Mitophagy gene knockouts (KOs) and wild type nematodes were exposed to 6-OHDA and UVC; starvation was used to induce macroautophagy. The role of autophagy in dealing with mitochondrial damage was investigated.

**Specific Aim 3 (Chapter 4): Evaluate rotenone’s mechanism of toxicity in *C. elegans* utilizing new techniques to measure mitochondrial function***

Young adult *C. elegans* were exposed to rotenone for 48 hours; mitochondrial oxygen consumption, ATP and metabolite levels were measured post exposure.

The weaknesses, strengths, and broader implications of this dissertation work are discussed in Chapter 5.
2. Characterize age-related sensitivity to mitochondrial DNA damage in *Caenorhabditis elegans*

2.1 Introduction

Mitochondria are believed to play an important role in many neurological disorders (DiMauro and Schon 2008; Gomez et al. 2007). This is in part due to the fact that neurons require high amounts of energy, and as such they rely on mitochondria for their energy supply; if that supply is somehow disrupted, neurological dysfunction would be expected (Weissman et al. 2007). Furthermore, this high metabolic activity results in higher intrinsic levels of reactive oxygen species (ROS) in neurons than in other cells; this, along with an elevated inflammatory response and an increased sensitivity to ROS-mediated signaling, makes the brain particularly susceptible to any further increases in ROS (Pinto et al. 2012; Wang and Michaelis 2010). Another line of evidence for the link between mitochondria and neurological disease is provided by studies into mtDNA integrity and neurodegeneration, which found associations between mutations and deletions in mtDNA and PD (Bender et al. 2006; Kraytsberg et al. 2006; Pinto and Moraes 2013).

The majority of PD cases are of unknown etiology, and it is very probable that some of these cases result from environmental exposures, or a combination of environmental exposures and genetic predisposition (Bronstein et al. 2009; Goldman 2014). Several epidemiological studies have identified an association between
neurodegeneration and exposure to environmental chemicals (Bronstein et al. 2008; Cannon and Greenamyre 2011; Dinis-Oliveira et al. 2006; Guilarte 2010; Tanner et al. 2011; van der Mark et al. 2012). Laboratory studies also support this theory (Benedetto et al. 2009; Betarbet et al. 2000; Brooks et al. 1999; Tieu 2011). There is also evidence that the timing of toxicant exposure matters; research shows that there are critical windows of exposure throughout life, during which the effects of toxicants on neuronal health are potentiated. One such window could be old age, when protective mechanisms in the brain are in decline (Hindle 2010; Weiss 2000). Another vulnerable time appears to be early development; research studies have provided abundant evidence supporting the developmental origins of health and disease (DOHaD) hypothesis, which states that damage that occurs early on in development can have deleterious consequences later in life (Barker 1990). In the context of PD and environmental exposures, this hypothesis focuses on the effect neurotoxic compounds have in utero or during early life, and how this damage can remain latent until later in life when disease ensues (Landrigan et al. 2005). Patients with PD do not show symptoms until 60% of neurons in the substantia nigra have degenerated (Koller 1992); it is believed that the remaining cells can compensate for the loss of neurons to a degree, until neuronal death crosses that 60% threshold and the clinical signs associated with the disease appear (Fearnley and Lees 1991; Zigmond et al. 1990). Toxic exposures early in life could decrease the number of
neurons at an early age or cause an accelerated rate of neuronal degeneration over time, until the threshold is reached later in life when compensatory systems fail and clinical symptoms of disease become apparent (Landrigan et al. 2005; Reuhl 1991).

As mentioned earlier, mitochondrial dysfunction is observed in most neurodegenerative diseases, and appears to play an important role in the etiology of PD (Lin and Beal 2006). Of particular interest to us is the contribution of mtDNA damage to the development of this disease, a topic that has not been a major focus of PD research (Sanders and Greenamyre 2013). Also of interest to us is the effect of environmental toxicants on mtDNA, which has been understudied as well (Meyer et al. 2013).

Returning to the idea of critical windows of exposure and DOHaD, it is important to mention that there is a “mitochondrial bottleneck” during development; primordial germ cells, which will eventually give rise to mature oocytes, have as little as 10 mitochondria per cell (Shoubridge and Wai 2007). If this mitochondrial bottleneck also correlates with a reduced number of mitochondrial genomes, this would make primordial germ cells very vulnerable to mitochondrial genotoxicant exposure. All mitochondrial genome copies in the organism arise from replicating the few mtDNA copies present in primordial germ cells; as a result, early-life damage to mtDNA could have deleterious later-life effects in neuronal cells, particularly once an organism becomes more vulnerable to toxicants and disease with old age. Further increasing old
age mitotoxicant vulnerability is the fact that mutated mtDNA copies undergo preferential replication in a cell as an organism ages. This phenomenon is known as clonal expansion (Larsson 2010). Wild-type copies can maintain a normal phenotype until they become too low in number, an occurrence called the phenotypic threshold effect (Rossignol et al. 2003). If experimental testing corroborates these ideas, mtDNA damage during the mitochondrial bottleneck resulting in increased neuronal decline would be another great example of the DOHaD hypothesis.

For this research aim, I hypothesize that age or life stage is an important factor in determining neuronal vulnerability to toxicants that cause mtDNA damage. Developing embryos and old nematodes would be the most vulnerable to mtDNA damage, with embryos being more sensitive due to the mitochondrial bottleneck, and old nematodes due to failing compensatory mechanisms. I postulate that damage in embryo mtDNA (caused as primordial germ cells) will become apparent as the worms ages (later-life effects), by making them more sensitive to a second toxicant exposure later in life (“two-hit” exposure model). In this “two-hit” model of neuronal sensitivity to mtDNA damage, damaged mitochondrial genomes in the primordial germ cells (due to genotoxicant exposure) are replicated, generating and passing on mutations that can undergo clonal expansion as the worm ages. Early damage could also result in alterations in mitochondrial function that predispose mitochondria to dysfunction.
(Leung et al. 2013). This would result in a higher number of mutated mitochondrial genomes and therefore faulty mitochondria in old nematodes, making sensitive tissues like neurons more vulnerable to a “second hit” mitotoxicant exposure and inducing neurodegeneration.

2.2 Materials and Methods

2.2.1 C. elegans culture

Unless stated otherwise, populations of *C. elegans* were maintained on K agar plates (Williams and Dusenbery 1988) seeded with OP50 *E. coli* at 20 °C for up to 32 hours; at that point, they were transferred to K-agar plates with 400 µM 5’-fluoro-2-deoxyuridine (FUdR) and seeded with 20X concentrated UVC-inactivated *E. coli* (UvrA-deficient strain, which is an enzyme required for NER in *E. coli* (Croteau et al. 2008)). UVC-inactivated *E. coli* was generated via exposure to 1000 J/m² UVC (UVLMS-38 EL Series Lamp, UVP, Upland, California; primarily emitting at 254 nm), as previously described in Meyer et al. (2010).

Synchronized populations of nematodes were obtained by hypochlorite treatment of gravid adults in order to isolate the eggs (Lewis and Fleming 1995). L1 growth-arrested (starved) larvae were obtained by allowing eggs to hatch for 12 hours in K+ medium, previously referred to as “complete K-medium” (Boyd et al. 2009). Transfers
were made by washing nematodes off agar plates and rinsing (after centrifugation at 2200 g for 2 min) with K medium.

The transgenic strain BY200 [vtls1; P_dat-1::GFP, rol-6(su1006)] was generously provided by Guy and Kim Caldwell (University of Alabama).

2.2.2 Exposure to UVC to induce mtDNA damage

UVC was used as a proxy for environmental compounds that preferentially cause helix-distorting mtDNA damage that cannot be repaired in mitochondria, such as PAHs and the mycotoxin aflatoxin β1 (Backer and Weinstein 1982; Niranjan et al. 1982). A proxy was chosen in the hopes of studying the contribution of mtDNA damage to neurodegeneration, isolated from other deleterious and potentially confounding forms of macromolecular damage caused by environmental compounds. Old worms and primordial germ cells are the focus of this work, representing potential periods of vulnerability to mtDNA damage. In order to study old age while avoiding the physiological changes in dying nematodes, ten-day-old worms were selected for “old age” studies. Exposing primordial germ cells was accomplished by exposing L1 nematodes; at this larval stage the progeny of the primordial germ cell P4, the Z2 and Z3 cells, are present and will not develop further unless food is present (Fukuyama et al. 2006).
Ten-day-old and L1 nematodes were exposed to UVC (0, 25, 50, and 75 J/m² each day for ten-day-old worms, and 0 and 4 J/m² each day for L1s) for three days in a row in K-agar plates with no peptone; this allows for mtDNA to accumulate damage while nDNA is repaired (Bess et al. 2012). L1 nematodes were kept on the no-peptone plates, without food, during those three days to prevent growth and cell division of the Z2 and Z3 cells that could dilute mtDNA damage. Ten-day-old worms were exposed to UVC in no-peptone plates, but were otherwise kept on agar plates with food, as at that age developmental cell division has ceased and dilution of mtDNA damage via mtDNA replication is not a concern (Rooney et al. 2014; Sulston and Horvitz 1977). L1 nematodes (F0 generation) were allowed to grow on K-agar OP50 plates until gravid, at which point they were transferred to new plates and allowed to lay eggs for 2 hours. F0 worms were then washed off the plate, and F1 eggs were collected for further experiments. Eggs (F1 generation) were hatched in liquid K⁺ medium and were then plated and allowed to grow as described in section 2.2.1 until they reached young adulthood. F1 worms were then dosed with a second hit of UVC for three days (0, 25, and 50 J/m² each day) in no-peptone plates, and kept on plates with food otherwise.

2.2.3 mtDNA damage and copy number assay

Six worms were picked and pooled immediately after the last UVC exposure (i.e. 0 hours post exposure) in a single tube per biological replicate, and three biological
replicates were taken per treatment in two experiments separated in time (three experiments for copy number assay). MtDNA damage and copy number were evaluated using PCR-based methods as previously described (Gonzalez-Hunt et al. 2016). In the DNA damage assay, control samples are set as undamaged, and decreases in amplification efficiency in the treated samples relative to the controls are used to quantify lesion frequency (Meyer 2010). Two mitochondrial genome targets (10.9 kb for long-amplicon PCR, and 75 bp for real-time PCR) were amplified. The long amplicon is used to measure lesion frequency in the DNA damage assay, while the short amplicon is used to exactly measure the mitochondrial genome copy number as well as to normalize the long-amplicon PCR amplification to DNA concentration (Gonzalez-Hunt et al. 2016; Hunter et al. 2010).

Utilizing FUdR for these experiments allowed us to study DNA damage in adults composed mostly of non-dividing cells while causing minimal disruptions to mitochondrial biology (Rooney et al. 2014). Adult and young adult C. elegans have a rapidly proliferating germ line, and DNA damage caused by toxic exposure could be “diluted” by the new DNA produced in dividing germ cells (Meyer et al. 2007). The effect of DNA replication in the germline can be minimized by FUdR sterilization; however, this is not a perfect solution, as even 400 µM FUdR does not completely prevent germ line proliferation. Despite this, no eggs laid were viable, having stopped
cell division and growth before gastrulation occurs (González-Hunt, unpublished observations). This level of egg production does not seem to confound the PCR results obtained for these experiments.

2.2.4 Dopaminergic neurodegeneration assay

Synchronized BY200 were exposed to UVC as described in section 2.2.1. The worms were then transferred to seeded agar plates, and sampled at 0 and 48 hours after exposure (“two-hit” worms were also sampled at 10 days old).

Treated C. elegans were picked onto a 2% agar pad and immobilized with 15µl of 156 mM tetramisole hydrochloride (Sigma-Aldrich). Nematodes were examined using a Zeiss Axioskop microscope and neuronal morphology was assessed by individual observation of each cephalic (CEP) neuron. Neurons were assigned a score from 0 to 2 based on the amount of morphological abnormalities present (Figure 3). The score categories were as follows: 0 = no observable damage; 0.5 = blebs on equal or less than half of dendrite, no breaks; 1 = blebs on more than half of dendrite, no breaks; 1.5 = Breaks, equal or more than half of dendrite still present; 2 = Breaks, less than half of dendrite still present (includes completely absent dendrite).

Each experiment was repeated three times with 10 worms analyzed per treatment at each time point. All scoring was double-blinded.
Figure 3: Dopaminergic neurodegeneration observed in *C. elegans* after toxic insult. Image from Gonzalez-Hunt et al. (2014).

### 2.2.5 Statistical analyses

DNA damage and mtDNA copy number data were analyzed with JMP Pro for Mac (Version 13.0.0, SAS Institute Inc., Cary, NC). One- or two-factor analysis of variance (ANOVA) was used to evaluate the effect of UVC dose. For evaluation of UVC on neurodegeneration (effect of dose), the Kruskal-Wallis test was used and each time point was analyzed independently.
The Fisher’s exact test (FET) was used to evaluate the effect of the UVC “first-hit” on neurodegeneration after the “second-hit,” with each dose and time point assessed independently. A p-value of less than 0.05 was considered statistically significant.

2.3 Results

2.3.1 UVC caused expected amounts of DNA damage, and had no effect on mitochondrial genome copy number

Exposure to UVC caused a dose-dependent increase in lesions to the mitochondrial genome of old worms as expected based on previous experiments (one-way ANOVA p=0.0007*; Tukey-Kramer HSD compared to control, 50 J/m² p=0.0241*, 75 J/m² p=0.0004*) (Figure 4). No change was seen in the number of mitochondrial genome copies after exposure (one-way ANOVA p=0.8069).

![Figure 4. Dose-dependent increase in mtDNA damage but no change in copy number after exposure to UVC in old worms.](image)
In the “two-hit” adult worms, an increase in mtDNA lesions with increasing UVC exposure was observed as well; however, the first UVC hit on the germline had no effect on the amount of lesions observed in the adults after a second hit (two-way ANOVA; p=0.3602 for pre-dose x dose interaction, p<.0001* for dose effect) (Figure 5).

The number of mitochondrial genome copies was not affected by the first or second UVC hit (two-way ANOVA; p=0.6715 for pre-dose x dose interaction, p=0.0542 for dose effect, Figure 5).

Figure 5: UVC caused dose-dependent mtDNA damage but no change in copy number for young adult "two-hit" worms.
2.3.2 *UVC exposure did not increase background levels of neurodegeneration*

Old worms exhibited some blebbing due to age, but it did not worsen after exposure to UVC (no statistically significant comparisons with either Kruskal-Wallis test or FET of dosed groups compared to control) (Figure 6).

![Figure 6: Neurodegeneration levels observed in old worms 0 hours (left panel) and 48 hours (right panel) after exposure to UVC.](image)

Adult worms exposed to a first (as germ cells) and second (as young adults) hit of UVC also did not exhibit increased dopaminergic neurodegeneration at 0 or 48 hours post exposure, or at ten days old (Figure 7). These results for old and adult “two-hit” worms are at odds with previous data from the lab showing increased dopaminergic neurodegeneration in young adult worms after UVC exposure (data not shown).
2.3.3 Higher levels of neurodegeneration and altered mtDNA copy number, but same levels of DNA damage in adult worms were observed with different UVC source

Prior to obtaining the results reported in 2.3.1 and 2.3.2, we had attempted to perform these experiments with a different, older UVC light bulb (the lamp itself was the same). For these experiments, we utilized a pre-dose of 7.5 J/m² and secondary exposures of 0, 25, 50, or 75 J/m² UVC.

Figure 7: UVC did not cause an increase in neurodegeneration in "two-hit" worms at 0 or 48 hours after exposure, or at 10 days old.
It is important to mention that within the 7.5 J/m² pre-dosed group, worms were not being uniformly affected by UVC; some were growth delayed, and this was obvious from a quick visual inspection. To account for this, worms were separated before evaluation; as a result, there are two groups of worms pre-dosed with 7.5 J/m²: the normal-sized group (labelled as “7.5”) and the growth-delayed group (labeled as “7.5b”). This size disparity was not observed upon repetition of these experiments with a new light bulb and a pre-exposure of 4 J/m² UVC as presented in sections 2.3.1 and 2.3.2.

We observed similar levels of DNA damage after exposure to 7.5 J/m² UVC (first “hit”) and a second exposure to 0, 25, 50, or 75 J/m² UVC (second “hit”) to those observed with 4 + 0, 25, 50, 75 J/m² in section 2.3.1 (only one experimental rep, three biological reps picked from that experiment, Figure 8). However, we saw different results for copy number, with pre-dose (UV hit #1) and dose (UV hit #2) but not their interaction having statistical significant effects (two-way ANOVA; pre-dose x dose interaction p=0.5985, effect of pre-dose p=0.0055*, effect of dose p=0.0095*, three experimental reps with three biological reps each) (Figure 9). Overall, the UVC exposure decreased mtDNA copy number, and the pre-dose increased it.

We also performed the neurodegeneration assay after the “two-hit” exposure with the old light bulb. The results are different from what we observed from the “two-
hit” exposure with the new bulb (described in section 2.3.2). The Kruskal-Wallis test was statistically significant for several exposure groups; there was an effect of the secondary UVC exposure (“second-hit”) at 0 hours for the 7.5b group (p=0.0004*), at 48 hours for the 7.5 (p=0.0076*) and 7.5b (p<.0001*) groups, and at 9 days old for the pre-dose control (p<.0001*) and 7.5 (p<.0001*) groups (Figure 10).

The pairwise comparisons performed with the FET also were statistically significant in some instances. However, in a few comparisons the pre-dosed groups exhibited less neurodegeneration than the pre-dose controls; also, in some comparisons it was unclear whether the controls or the dosed groups showed more neurodegeneration. The statistically significant p-values are presented in Table 2. Groups that showed more degeneration are highlighted in bold; if it was unclear which group exhibited more degeneration no group was highlighted. Scores for these comparisons are depicted in Figure 10.
Figure 8: Levels of DNA damage caused by pre-exposure to 7.5 J/m² and a secondary exposure to 25, 50, and 75 J/m² UVC with a different light bulb.

Worms labeled “C” were not pre-exposed, worms labeled “7.5” were pre-exposed and had normal growth, worms labeled “7.5b” were pre-exposed and showed growth delay.

Figure 9: MtDNA copy number after pre-exposure to 7.5 J/m² and a secondary exposure to 25, 50, and 75 J/m² UVC with a different light bulb.

Worms labeled “C” were not pre-exposed, worms labeled “7.5” were pre-exposed and had normal growth, worms labeled “7.5b” were pre-exposed and showed growth delay.
Table 2: FET comparisons and corresponding p-values. Group highlighted in bold exhibited the most neurodegeneration.

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>FET comparisons 1st Dose – 2nd Dose (J/m²)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr</td>
<td>C-C vs 7.5b-C</td>
<td>0.0002*</td>
</tr>
<tr>
<td>48 hr</td>
<td>C-C vs 7.5b-C</td>
<td>0.0091*</td>
</tr>
<tr>
<td></td>
<td><strong>C-25 vs 7.5b-25</strong></td>
<td>0.0074*</td>
</tr>
<tr>
<td></td>
<td>C-75 vs 7.5b-75</td>
<td>0.0077*</td>
</tr>
<tr>
<td>9-day-old</td>
<td>C-25 vs 7.5-25</td>
<td>0.0138*</td>
</tr>
<tr>
<td></td>
<td><strong>C-50 vs 7.5b-50</strong></td>
<td>&lt;.001*</td>
</tr>
<tr>
<td></td>
<td>C-75 vs 7.5-75</td>
<td>0.0006*</td>
</tr>
<tr>
<td></td>
<td><strong>C-75 vs 7.5b-75</strong></td>
<td>0.0141*</td>
</tr>
</tbody>
</table>
Figure 10: Neurodegeneration observed after pre-exposure to 7.5 J/m² and a secondary exposure to 25, 50, and 75 J/m² UVC with a different light bulb.

Worms labeled “C” were not pre-exposed, worms labeled “7.5” were pre-exposed and had normal growth, worms labeled “7.5b” were pre-exposed and showed growth delay

2.4 Discussion

Overall, our experiments do not provide sufficient evidence to suggest that mtDNA damage alone is responsible for dopaminergic neurodegeneration in *C. elegans*, at least under these circumstances. Furthermore, our results are not supportive of the existence of windows of susceptibility to mtDNA damage in the nematodes. However,
drawing any conclusions is complicated due to the confounding effect our UVC source might be having. This is particularly concerning because approximately the same number of DNA lesions caused different responses in our copy number and neurodegeneration assays. With the old light bulb, we observed an effect of the UVC pre-dose (first-hit) and an effect of the secondary exposure (second-hit) in mitochondrial copy number, yet the effect of the interaction was not significant; this suggests that although the presence of mtDNA lesions is altering the number of mitochondrial genome copies, there is no synergistic effect of exposing the germline to UVC and then exposing the adult worms that originate from these cells once again to UVC. However, when we performed this same experiment with the new light bulb (albeit with a slightly smaller pre-dose of 4 J/m²) all effects on mitochondrial genome copies disappeared. A similar observation can be made for our neurodegeneration assay. UVC exposure with the old light bulb appeared to cause an increase in neurodegeneration, although this was not consistent; whether or not the first dose of UVC sensitized the neurons to a second dose cannot be answered, as the results were contradictory at times (worms without a first-hit had more neurodegeneration than worms with two-hits in some instances). Yet, when the experiment was repeated with the new light bulb, no degeneration due to UVC was observed. It is difficult to explain what might be causing these disparities; although unlikely, it is not impossible that the old UV light bulb might be emitting light
at more wavelengths than just 254 nm, potentially increasing its negative effect on the nematodes. This could also explain why we started seeing nematode growth delays at 7.5 J/m², a dose that had never caused such growth delays in our hands before.

Despite the issues with our UVC source, our results consistently suggest that the “two-hit” exposure model does not increase the amount of neurodegeneration due to mtDNA observed in *C. elegans*. There is an important caveat to this assertion, however: our whole-worm DNA damage assay does not allow us to ensure that our first hit on germ cells was being effective. There is evidence in the scientific literature that *C. elegans* cull their germ cells during oogenesis or due to environmental damage, just as mammals do (Gartner et al.; Krakauer and Mira 1999). Given our results, we suspect that germ cells that received damaged mitochondrial genome copies during germline proliferation were selected against during oogenesis, making it unlikely that mtDNA damage passed on to the F1 generation. If this were the case, our current two-hit exposure model would be invalid. In an attempt to shed light on this possibility, we tried to measure mtDNA damage in the F1 generation. We ran our long amplicon DNA damage assay in eggs laid by the F0 generation after the first hit of UVC; if germ cells are being culled, then no mtDNA damage would be present in these eggs. As of this writing, this work has not been completed due to issues adapting our whole-worm method to eggs.
Lastly, it is worth mentioning that this work provides further evidence of mild dopaminergic neurodegeneration happening due to normal aging in *C. elegans*, an observation that has not been well documented in the scientific literature.
3. Non-selective macroautophagy and mitophagy aid in maintaining mitochondrial and neuronal health in C. elegans after toxicant exposure

3.1 Introduction

Autophagy is a cellular mechanism to deliver intracellular components to the lysosome for degradation (Mizushima 2007). Macroautophagy, a type of autophagy, is characterized by the engulfment of cellular components to be degraded by a double-membrane structure called the autophagosome (Yang and Klionsky 2010). Non-selective macroautophagy refers to the bulk catabolism of cytoplasmic components by the cell under conditions of nutrient deprivation or stress (Feng et al. 2014). Mitophagy, a type of selective macroautophagy, is the targeted removal of damaged or spare mitochondria (Kim et al. 2007). In mammals, the main enzymes involved in mitophagy are PINK1, a mitochondrially-targeted kinase, and parkin, a cytosolic E3 ubiquitin ligase (Narendra et al. 2008; DP Narendra et al. 2010). PINK1 constantly undergoes proteolysis in healthy mitochondria, but if mitochondrial dysfunction and loss of membrane potential occurs this degradation is disrupted and accumulation of PINK1 in the outer mitochondrial membrane occurs (DP Narendra et al. 2010). There, PINK1 phosphorylates ubiquitin on outer mitochondrial membrane proteins and this signals parkin to the mitochondria (Kane et al. 2014; Kazlauskaitė et al. 2014; Koyano et al. 2014). Parkin proceeds to further ubiquitinate outer mitochondrial membrane proteins, creating ubiquitin chains that
recruit autophagy receptors such as p62, optineurin, NDP52, and NBR1 to the mitochondria (Geisler et al. 2010; Lazarou et al. 2015; D Narendra et al. 2010; Wong and Holzbaur 2014). This begins the process of engulfment of the mitochondria by the autophagosome, which then delivers the faulty mitochondria to the lysosome for degradation (Shaid et al. 2013).

Studies with PINK1 and parkin implicate these proteins and mitochondrial dysfunction in the development of PD. Parkin mutations cause autosomal recessive early-onset PD (Kitada et al. 1998). Mutations in PINK1 are also associated with autosomal recessive early-onset PD (Valente et al. 2004). Studies in Drosophila parkin null mutants show decreased lifespan, locomotor defects and male sterility (Greene et al. 2003). PINK1 loss-of-function Drosophila mutants and KOs exhibit male sterility, abnormal mitochondrial morphology, and muscle and dopaminergic neuron loss (Clark et al. 2006; Park et al. 2006). Interestingly, rodent studies of PINK1 and parkin in the context of neurodegeneration frequently show altered dopamine release and other disruptions to the nigrostriatal system, yet loss of dopaminergic neurons has not been consistently reported (Gautier et al. 2008; Gispert et al. 2009; Goldberg et al. 2003; Itier et al. 2003; Kitada et al. 2007). A similar pattern has also emerged for teleost fish studies (Anichtchik et al. 2008; Flinn et al. 2009; Matsui et al. 2013; Xi et al. 2010).
Additionally, studies of PINK1 and parkin deficiencies in the context of environmental exposures show emergence or enhancement of mitochondrial and neurological defects with chemical exposure; however, sensitization to toxin-induced neurodegeneration has not been consistently observed (Bornhorst et al. 2014; Haque et al. 2008; Haque et al. 2012; Perez et al. 2005; Sallinen et al. 2010).

The goal of this work was to further shed light on the role PINK1 and parkin play in the neurodegenerative response to toxic exposures. We hypothesized that impaired removal of damaged mitochondria due to KO of mitochondrial autophagy genes would sensitize the nematodes to the deleterious effects of mito- and neurotoxicants. Compromised removal of damaged mitochondria would result in or worsen dopaminergic neurodegeneration after toxicant exposure. Conversely, inducing macroautophagy (and the resulting removal of damaged mitochondria) would decrease levels of dopaminergic neurodegeneration caused by toxicants that cause mitochondrial dysfunction.

In order to investigate this, we chose UVC light and 6-OHDA as our model mito- and neurotoxicants. UVC was used as a proxy for environmental compounds that preferentially cause helix-distorting mtDNA damage that cannot be repaired in mitochondria, such as PAHs and the mycotoxin aflatoxin β1 (Backer and Weinstein 1982; Niranjan et al. 1982). The main advantage of working with UVC is the ability to
approximate studying the toxicity caused by mtDNA damage on its own, isolated from other negative effects caused by environmental compounds. The neurotoxicant 6-OHDA was selected for being a potent dopaminergic neurodegenerative agent. It is believed to exert its toxicity by creating ROS leading to cell death (Choi et al. 1999; Lotharius et al. 1999). Another advantage of using these compounds was that this allowed us to study the ability of autophagy to protect neurons from toxicants that exert their effects through mostly different mechanisms of action.

Using *C. elegans* as our model organism, we looked at dopaminergic neurodegeneration, mRNA levels of antioxidant genes, and mitochondrial morphology in muscle wall cells after exposure to UVC and 6-OHDA. We observed sensitization to UVC-induced neurodegeneration in the *pink-1* and *pdr-1* (*C. elegans* homolog for PARK2) KO strains as we expected. However, only the *pdr-1* mutant was more sensitive to 6-OHDA-induced neurodegeneration; the *pink-1* mutant exhibited less neurodegeneration than the control and the *pdr-1* strains. We then proceeded to investigate if this was due to an elevated antioxidant response that could confer protection, as 6-OHDA acts predominantly by causing ROS (as discussed above); no statistically significant inductions in the mRNA levels of *ctl-2*, *trx-1*, or *gst-10* were observed. We are now looking at changes in mitochondrial morphology, as increases in fusion or fission of the mitochondrial network can be beneficial to the cell when dealing with mitochondrial
dysfunction. Mitochondrial fusion—which allows mitochondria to exchange contents and compensate for defects—has been shown to protect the cell from insults that can lead to cell death (Chen et al.; Cheung et al. 2007; Cribbs and Strack 2007; Nakada et al. 2001; Yoneda et al. 1994); mitochondrial fission could help withstand mitochondrial stress by aiding in the isolation and removal of faulty mitochondria (Twig et al. 2008; Youle and van der Bliek 2012). Preliminary results suggest that inter-strain differences in mitochondrial morphology might be in part responsible for the differences we see in neurodegeneration levels caused by 6-OHDA in the strains tested.

3.2 Materials and Methods

3.2.1 C. elegans culture

Populations of C. elegans were maintained on K agar plates (Williams and Dusenbery 1988) seeded with OP50 E. coli at 20 °C until young adulthood, when they were dosed and evaluated.

Synchronized populations of nematodes were obtained by hypochlorite treatment of gravid adults in order to isolate the eggs (Lewis and Fleming 1995). Worms were allowed to hatch for no more than 14 hours in K⁺ medium, previously referred to as “complete K-medium” (Boyd et al. 2009). Worms were transferred by washing nematodes off agar plates or treatment wells and rinsing (after centrifugation at 2200 g for 2 min) with K medium.
C. elegans strains used were BY250 (vtIs7; Pdat-1::GFP), BY200 [vtIs1; Pdat-1::GFP, rol-6 (su1006)], UA226 [pink-1 (tm1779); vtIs1; Pdat-1::GFP, rol-6 (su1006)] and UA227 [pdr-1 (tm598); vtIs1; Pdat-1::GFP, rol-6 (su1006)]. UA226 and UA227 are pink-1 and pdr-1 KOs crossed with BY200, a nematode strain with a dopamine transporter/GFP fusion protein that allows for easy observation of all eight dopaminergic neurons present in C. elegans. BY200, UA226, and UA227 were generous gifts from Guy and Kim Caldwell (University of Alabama). BY250 was generously provided by Michael Aschner (Vanderbilt University).

3.2.2 Toxic Exposures

For UVC exposures, young adult nematodes were exposed to UVC (0, 25, 50, or 75 J/m² each day) for three days in a row in unseeded K-agar plates with no peptone; this allows for mtDNA to accumulate damage while nDNA is repaired (Bess et al. 2012). In between doses, worms were kept on seeded agar plates, as at that age developmental cell division has ceased and dilution of mtDNA damage is not a concern (Sulston and Horvitz 1977).

For pink-1 and pdr-1 6-OHDA exposures, young adult nematodes were washed off food plates and allowed to clear their guts for 15 minutes. They were then exposed one time to a 0, 33, or 100 mM 6-OHDA solution in ascorbic acid (to delay oxidation; ascorbic acid dose was 20% of the 6-OHDA dose used). Exposure was done in 1.7 ml
tubes (1000 worms per tube) and lasted 1 hour. Afterwards, worms were washed 4 times in K medium and either transferred to corresponding media with food or evaluated.

Worms subjected to starvation conditions were used to investigate the effects of non-selective macroautophagy on mitochondrial dysfunction and neurodegeneration. Starvation is the most commonly used and well-studied trigger of macroautophagy, and it has been used in a variety of species (Li et al. 2013; Mizushima et al. 2004; Mizushima 2007; Mortimore and Poso 1987). After a 48-hour starvation treatment (L1 nematodes kept in K+ medium), the nematodes were exposed to 0 mM, 15 mM, and 50 mM 6-OHDA and immediately transferred to seeded plates to be evaluated at a later timepoint.

3.2.3 Dopaminergic neurodegeneration assay

After toxic exposure, worms were sampled at 24, 48, and/or 72 hours for evaluation of dopaminergic neuron morphology. Treated C. elegans were picked onto a 2% agar pad and immobilized with 15µl of 156 mM tetramisole hydrochloride (Sigma-Aldrich). Nematodes were examined using a Zeiss Axioskop microscope and neuronal morphology was assessed by individual observation of each cephalic (CEP) neuron. Neurons were assigned a score from 0 to 2 based on the amount of morphological abnormalities present (Figure 3). The score categories were as follows: 0 = no observable damage; 0.5 = blebs on equal or less than half of dendrite, no breaks; 1 = blebs on more
than half of dendrite, no breaks; 1.5 = Breaks, equal or more than half of dendrite still present; 2 = Breaks, less than half of dendrite still present (includes completely absent dendrite).

Each experiment was repeated three times with 10 worms analyzed per treatment at each time point. All scoring was double-blinded.

3.2.4 Targeted gene expression measurements

The mRNA of approximately 1000 worms was extracted using the RNeasy mini kit (QIAGEN) 24 hours after 6-OHDA exposure. Worms were washed off plates and allowed to clear their guts for 15 minutes. They were then transferred to 1 ml RLT buffer plus 10 µl β-mercaptoethanol and flash-frozen in liquid nitrogen. After bringing them to room temperature, zirconia/silica beads were added to the tubes and samples were homogenized using a bullet blender (Next Advance). The homogenate was transferred to a new 1.7 ml tube and kept at room temperature for 5 minutes. These tubes were then centrifuged, and the supernatant was transferred to a new microcentrifuge tube. We then followed steps 5-12 of the RNeasy Mini handbook. Once the mRNA was extracted and quantified, it was converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression was measured by real-time PCR as described in Leung et al. (2013). Primers used were as follows: cdc-42 forward - 5’- GAG AAA AAT GGG TGC CTG AA -3’, reverse - 5’-CTC GAG CAT TCC TGG ATC
AT-3’ (111 bp, published in Leung et al. (2013)); ctl-2 forward –
ACCCAGAGCGTAATCCACA reverse – CCTTGAGTTGGCTTGAAATGGA (224 bp);
trx-1 forward – AGCGGAAGATCTTTGTTCCA reverse –
AATTGCCTCTCATTCTTG (76 bp); gst-10 forward –
TGGGAAGAGTCATGGCTTG reverse – AACTTCATAGACCTCCGG (172 bp).

Annealing temperature was 60 °C for all primers. All samples were run in triplicate, and the experiment was repeated three times.

3.2.5 Mitochondrial morphology evaluation

This protocol was adapted from Luz et al. (2015a). After exposure to 33 mM 6-OHDA, worms were kept in OP50 plates for 20 hours at 20 °C. They were then washed with K medium and allowed to clear their guts for 15 minutes. Worms were then transferred to 12-well plates at a density of 1000 worms/well (one well per treatment and strain). Each well had MitoTracker Red CMXRos (Molecular Probes) at 3.7 µM, 100 µl of 2X UVC-inactivated E. coli, and K medium up to 1 ml. UVC-inactivated E. coli was generated via exposure to 1000 J/m² UVC with the UVLMS-38 EL Series Lamp primarily emitting at 254 nm (UVP, LLC), as previously described in Meyer et al. (2010). Worms were incubated in the dye for 4 hours at 20 °C, then washed off with K medium and allowed to clear their guts for 15 minutes. They were then transferred to a fresh 12-well
plate with 100 µl of 2X UVC-inactivated *E. coli* and K medium up to 1 ml (per well) for imaging.

Confocal imaging was performed immediately after the washes (rep 1 with a Zeiss 510 upright confocal; reps 2 and 3 with a Zeiss 780 upright confocal). Treated *C. elegans* were picked onto a 10% agar pad and immobilized with 15 µl of 156 mM tetramisole hydrochloride (Sigma-Aldrich). A z-stack of the area adjacent to the worm pharynx (between pharyngeal bulbs) was taken at 63X, 2.9 zoom, and at 0.5 µm intervals. Three worms per treatment group were imaged (six per strain total). The experiment was repeated three times.

3.2.6 Statistical analyses

Data were analyzed with JMP Pro for Mac (Version 13.0.0, SAS Institute Inc.). One- or two-factor analysis of variance (ANOVA) was used to evaluate the effect of the toxic exposures on mRNA levels and mitochondrial morphology. For evaluation of UVC or 6-OHDA on neurodegeneration, the Fisher’s exact test (FET) was used to compare the effect of the exposures on each mutant and/or dosed strain with the effect on the control strain. Each dose and time point assessed independently. A p-value of less than 0.05 was considered statistically significant.
3.3 Results

3.3.1 Inducing macroautophagy via starvation protects the worms from 6-OHDA-induced dopaminergic neurodegeneration

Previous work published in Gonzalez-Hunt et al. (2014) led us to the realization that a 48-hour starvation period as L1s protected the nematodes from subsequent exposure to 15 mM and 50 mM 6-OHDA and the ensuing dopaminergic neurodegeneration (neuron morphology evaluated 48 hours post exposure) (Figure 11); others have made the same observation (Nass and Hamza 2007). In Gonzalez-Hunt et al. (2014), we hypothesized that this was likely due to an induction of macroautophagy, which by removing dysfunctional mitochondria and damaged mitochondrial genomes was protecting the neurons from degeneration. To look into this potential mechanism for the protection we observed, we compared the DNA damage levels caused by the mitochondrial toxicant paraquat with or without a starvation period. We measured slightly (~25%) less mtDNA damage in starved worms dosed with 6 mM and 20 mM paraquat (Figure 12), but this was not statistically significant (three-way ANOVA, p=0.1411 for the interaction between dose, genome, and starvation status) (Gonzalez-Hunt et al. 2014). These results suggest that the protective effect of a fasting period and induction of macroautophagy is not due to removal of damaged mitochondrial genomes. Whether or not removing dysfunctional mitochondria by itself was enough to observe a protective effect was not tested.
Figure 11: Fasting protects against 6-OHDA–induced dopaminergic neurodegeneration.

Neuronal damage was scored from 0 (lowest) to 2.5 (highest) and assessed statistically using the Fisher’s Exact Test. From Gonzalez-Hunt et al. (2014).
Figure 12: Starvation-induced macroautophagy did not remove damaged mitochondrial genomes in young adult *C. elegans* 48 hours after paraquat exposure. From Gonzalez-Hunt et al. (2014)

3.3.2 UVC caused more neurodegeneration in pink-1 and pdr-1 mutant strains than in the control strain

MtDNA damage due to UVC exposure caused neurodegeneration in all three strains (BY200, UA226, and UA227). Increased neurodegeneration was observed mostly at the 50 and 75 J/m² exposures, and also appeared to worsen as time went on. At the 24 hour time point, the *pdr-1* mutant exhibited more neurodegeneration than the BY200 controls at all doses of UVC; the *pink-1* mutant exhibited more neurodegeneration at 50 and 75 J/m². At 72 hours, once again the *pdr-1* mutant exhibited more neurodegeneration than the BY200 controls at all doses of UVC, but the *pink-1* mutant only had significantly
more degeneration at 75 J/m\(^2\). No statistically significant differences were observed at 48 hours post exposure (Figure 13). P-values are listed in Table 3.

Figure 13: Exposure to UVC caused more dopaminergic neurodegeneration in \textit{pdr-1} and \textit{pink-1} than in the control BY200 worms.
Table 3: P-values (Fisher’s exact test) for strain comparisons after UVC exposure in young adult C. elegans. Each timepoint and dose analyzed separately.

<table>
<thead>
<tr>
<th>vs</th>
<th>Timepoint</th>
<th>Dose (J/m²)</th>
<th>pdr-1::BY200</th>
<th>pink-1::BY200</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY200</td>
<td>24 hr</td>
<td>0</td>
<td>0.8599</td>
<td>0.7267</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>0.0438*</td>
<td>0.2343</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>0.0119*</td>
<td>0.0005*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>75</td>
<td>&lt;.0001*</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td></td>
<td>48 hr</td>
<td>0</td>
<td>0.0517</td>
<td>0.1928</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>0.7275</td>
<td>0.4445</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>0.1419</td>
<td>0.5351</td>
</tr>
<tr>
<td></td>
<td></td>
<td>75</td>
<td>0.3884</td>
<td>0.3332</td>
</tr>
<tr>
<td></td>
<td>72 hr</td>
<td>0</td>
<td>0.5833</td>
<td>0.754</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>0.0124*</td>
<td>0.9593</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>0.0002*</td>
<td>0.9467</td>
</tr>
<tr>
<td></td>
<td></td>
<td>75</td>
<td>0.0001*</td>
<td>&lt;.0001*</td>
</tr>
</tbody>
</table>

Exposure to 6-OHDA caused neurodegeneration in all three strains as well, with the highest dose causing the most neurodegeneration. The 100 mM dose caused more degeneration in the pdr-1 mutant than in the BY200 control at all time points; the 33 mM dose caused more degeneration in the pdr-1 mutant than in the BY200 control at 24 and 48 hours. Unexpectedly, the pink-1 mutant showed less degeneration than the BY200 control at all time points after exposure to 100 mM 6-OHDA; it also showed less degeneration than the BY200 control after exposure to 33 mM at the 72 hour timepoint (Figure 14). P-values are listed in Table 4.
Figure 14: Exposure to 6-OHDA caused more neurodegeneration in the *pdr-1* mutant but less in the *pink-1* mutant than in BY200 control.

3.3.3 Antioxidant gene expression was not elevated basally or in response to 6-OHDA in the mutant or control strains

Our neurodegeneration results indicated that the *pink-1* mutant is less sensitive to 6-OHDA than the wild-type and *pdr-1* mutant strains, yet as sensitive as *pdr-1* and more sensitive than the BY200 control to UVC. These findings led us to test if antioxidants responses could be elevated in the *pink-1* mutant, which could explain
Table 4: P-values (Fisher’s exact test) for strain comparisons after 6-OHDA exposure in young adult C. elegans. Each timepoint and dose analyzed separately.

<table>
<thead>
<tr>
<th>vs</th>
<th>Timepoint</th>
<th>Dose (mM)</th>
<th>pdr-1::BY200</th>
<th>pink-1::BY200</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY200</td>
<td>24 hr</td>
<td>0</td>
<td>&lt;.0001*</td>
<td>0.6336</td>
</tr>
<tr>
<td></td>
<td></td>
<td>33</td>
<td>&lt;.0001*</td>
<td>0.3738</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>&lt;.0001*</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td></td>
<td>48 hr</td>
<td>0</td>
<td>0.0030*</td>
<td>0.1894</td>
</tr>
<tr>
<td></td>
<td></td>
<td>33</td>
<td>&lt;.0001*</td>
<td>0.0108*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>&lt;.0001*</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td></td>
<td>72 hr</td>
<td>0</td>
<td>0.4511</td>
<td>0.205</td>
</tr>
<tr>
<td></td>
<td></td>
<td>33</td>
<td>0.068</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>&lt;.0001*</td>
<td>&lt;.0001*</td>
</tr>
</tbody>
</table>

why it was protected from the oxidative stress caused by 6-OHDA yet not from the DNA damage caused by UVC.

We initially measured the mRNA levels of the antioxidant genes coding for catalases 1, 2 and 3 (ctl-1, ctl-2, ctl-3), thioredoxin (trx-1), glutathione s-transferase (gst-10), 2-Cys peroxiredoxin (prdx-2), and the copper/zinc, and iron/manganese superoxide dismutases (sod-1, sod-2, sod-3, sod-4) in two separate experiments. We evaluated whether or not gene expression was altered in the mutants compared to the control strain, with and without 6-OHDA exposure; most genes seemed to have similar expression across strains, and this did not change after 6-OHDA exposure (data not shown). Based on this preliminary screen we selected three genes that appeared to have
a response for more thorough testing: ctl-2, gst-10, and trx-1. We looked at fold-change increases in mRNA levels again compared to the control BY200 (0 mM 6-OHDA) and compared to each strain’s own 0 mM 6-OHDA control. When comparing to the BY200 control, there was no statistically significant effect of 6-OHDA dose, strain, or their interaction for ctl-2 or trx-1. For gst-10, there was no statistical significance for the interaction between strain and 6-OHDA dose or for the effect of strain alone; however, there was an effect of dose (two-way ANOVA p=0.0231*) (Figure 15).

![Figure 15](image.png)

**Figure 15:** mRNA levels for antioxidant genes were not elevated basally or in response to 6-OHDA in the BY200 control, ping-1, or pdr-1 mutant strains when compared to BY200 0 mM control.
When comparing to each strain’s own 0 mM 6-OHDA control, there was no variance among strains in the fold change in expression for any of the genes (one-way ANOVAs; \(ctl-2\) \(p=0.1046\), \(gst-10\) \(p=0.1264\), and \(trx-1\) \(p=0.1264\)) (Figure 16).

\[
\text{Figure 16: mRNA levels were not elevated in the BY200 control, pink-1, or pdr-1 mutant strains after exposure to 6-OHDA when comparing to each strain's own control.}
\]

3.3.4 Mitochondrial morphology might be altered in response to 6-OHDA

Due to the lack of an elevated antioxidant response (measured as mRNA levels), we set out to explore differences in mitochondrial morphology among the strains basally or in response to 6-OHDA. Visual evaluation of the acquired images led us to hypothesize that the mitochondrial network is fusing after 6-OHDA exposure,
particularly in the pink-1 mutant (Figure 17). A future direction for this work is performing a proper, quantitative evaluation of this hypothesis. We have selected representative slices from the confocal z-stack and are classifying mitochondrial morphology using the naïve Bayes and $k$-nearest neighbors classifier algorithms. Mitochondria will be classified as enlarged, fragmented or networked; total mitochondrial area will also be measured.

Figure 17: Raw images of mitochondrial morphology in *C. elegans* body wall muscle after exposure to 6-OHDA.

We hypothesize that the mitochondrial network (particularly in the pink-1 mutant) is fusing in response to 6-OHDA exposure.
3.4 Discussion

Our results support the hypothesis that autophagy plays an important role in the physiological response to toxicant-induced mtDNA damage and oxidative stress. We present evidence that starvation treatment (and the accompanying induction of macroautophagy) provides dramatic protection from 6-OHDA-induced neurodegeneration; whether or not this is specifically due to the removal of damaged mitochondria via macroautophagy was not tested. We do not see a decrease in the number of mitochondrial lesions caused by another toxicant (paraquat) after starvation treatment, indicating that at least protection due to starvation is likely not due to clearing of damaged genomes. Nonetheless, our results indicate that the role of starvation and macroautophagy in the response to a mitochondrial insult in the context of dopaminergic neurodegeneration warrants further study.

Our experiments with the mitophagy mutants pink-1 and pdr-1 also suggest that autophagy (and more specifically, mitophagy) plays an important role in the response to toxicant-induced mtDNA damage and oxidative stress, and the dopaminergic neurodegeneration these stressors might cause. We hypothesized that knocking out the main genes involved in mitophagy (pink-1 and pdr-1) would sensitize the nematodes to mitochondrial toxicity and neurodegeneration. This hypothesis proved correct for the neurodegeneration caused by mtDNA damage due to UVC, with both mutant strains
being more sensitive to it than the control strain. Interestingly, this was not the case for the neurodegeneration caused by 6-OHDA; while the *pdr-1* mutant was more sensitive to 6-OHDA than the control strain as we expected, the *pink-1* mutant was unexpectedly less sensitive to 6-OHDA than the control strain. These results led us to hypothesize that the reason for this difference might be the mechanism of action of the toxicant: with 6-OHDA being known for causing oxidative stress, we postulated that the *pink-1* mutant had an elevated antioxidant response, either basally or due to 6-OHDA exposure. To test this, we measured mRNA levels for the antioxidant genes *ctl-2*, *gst-10*, and *trx-1* in all three strains after 6-OHDA exposure. No statistical significance was found for most comparisons, however; only the effect of dose for *gst-10* was statistically significant when all strains and doses were compared to the BY200 0 mM control. We were unable to analyze the data further to see which strain (or strains) was responding to 6-OHDA because there was no effect of the interaction for the two-way ANOVA. To further evaluate the role of antioxidant responses in modulating the differences we see between strains, we are planning on performing antioxidant rescue experiments and challenges due to oxidative stress-causing agents (other than 6-OHDA).

We are also considering differences in mitochondrial morphology, as changes in mitochondrial morphology could help compensate for damaged mitochondria (if mitochondrial fusion is increased) or aid in the removal of damaged mitochondria (if the
network is more fragmented). Using machine learning algorithms, we will classify the mitochondrial area into networked, enlarged, or fragmented, and also estimate average mitochondrial size for each strain with and without exposure to 6-OHDA.

Overall, our work provides more evidence for the involvement of macroautophagy and mitophagy in the neuronal response to mitotoxicants, and highlights the need for future experiments detailing these protective mechanisms.
4. Evaluate rotenone’s mechanism of toxicity in *C. elegans* utilizing new techniques to measure mitochondrial function

4.1 Introduction

The pesticide rotenone was discovered to act via complex I inhibition in the early 1960s (Lindahl and Öberg 1961). This mechanism of action became of particular interest to the PD research community after it was discovered that there was significant complex I inhibition in parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and that postmortem substantia nigra samples from PD patients exhibited decreased complex I function (Langston et al. 1983; Schapira et al. 1990). Rotenone has since become a widely used tool in the study of PD, particularly after a study by Betarbet et al. (2000) showed that chronic, systemic infusion of this chemical can recapitulate hallmarks of parkinsonism (including substantia nigra neurodegeneration). Research into rotenone’s mode of action, particularly how systemic complex I inhibition can lead to such region-specific neurodegeneration, has helped unveil its effects on mitochondrial and neuronal health beyond inhibiting complex I. It is now known that rotenone exposure causes increases in oxidative stress, ATP depletion, and cell death (Li et al. 2003; Sherer et al. 2003). This increase in oxidative stress results in damage to cellular macromolecules (Sanders and Greenamyre 2013). Other discoveries more directly provide tools to explain rotenone’s heightened effect on dopaminergic neurons:
it has been documented that rotenone causes increases in calcium influx via the NMDA
receptor (which is constantly active in dopaminergic neurons), leading to excitotoxicity;
also, a dopamine metabolite causes structural modification and oligomerization of \( \alpha \)-
synuclein (protein that aggregates to form Lewy bodies in PD) following rotenone
exposure (Costa et al. 2008; De Miranda et al. 2016; Follmer et al. 2015). Both of these
studies help shed light on rotenone’s selectivity for dopaminergic neurons.

Another research focus for rotenone toxicity has been its effects on bioenergetics
and metabolism, which is particularly interesting given the link between PD and
bioenergetic alteration (Requejo-Aguilar and Bolaños 2016). Studies have revealed
alterations in mitochondrial bioenergetic parameters, reporting decreases in oxygen
consumption, increases in glycolysis, and alteration in the levels of organic and amino
acids after rotenone exposure (Dranka et al. 2012; Giordano et al. 2012; Karlsson et al.
2016; Lei et al. 2014; Xu et al. 2011). Increases in lipid \( \beta \)-oxidation have also been
reported (Worth et al. 2014). However, most of these experiments were performed in _vitro_,
so more studies on this topic using _in vivo_ models are important given the typically
abnormal metabolism of most cells in culture (Young 2013). One such model is the
nematode _C. elegans_, a medium- to high-throughput _in vivo_ model that allows for faster
and less expensive studies compared to vertebrate models. Few studies into the
bioenergetic disturbances caused by rotenone have been performed with _C. elegans_.

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Those that have been published largely focus on inhibition of electron transport chain enzymatic activities and respiration rates (Grad and Lemire 2004; Schouest et al. 2009; Ved et al. 2005). In contrast, many studies have been performed on the bioenergetic alterations caused by mutations in complex I subunits, and these alterations are potentially similar to and informative regarding those caused by rotenone-induced inhibition of complex I. The complex I subunit mutant gas-1 exhibits a decrease in complex I-dependent respiration, and an increase in complex II activity (Kayser et al. 2001; Pujol et al. 2013). Gas-1 also exhibits signs of disrupted pyruvate metabolism (with alterations in the levels of lactate, pyruvate, and alanine) and widespread changes in amino acid levels (Falk et al. 2008; Morgan et al. 2015; Schrier Vergano et al. 2014). Knockdown or mutations in the gene coding for another complex I subunit, nuo-1, also causes changes in lactate and pyruvate levels, impaired respiration, and increases in mRNA levels for genes involved in fatty acid β-oxidation, gluconeogenesis, and glycolysis (Grad and Lemire 2004; Zuryn et al. 2010). Interestingly, several of these studies also presented evidence of upregulation of the glyoxylate cycle in complex I mutants (Morgan et al. 2015; Pujol et al. 2013; Zuryn et al. 2010). This pathway, which does not appear to be present in higher metazoans, can use acetyl-CoA to make malate and succinate. Isocitrate lyase, the main enzyme in this pathway, can take isocitrate produced by the citric acid cycle and make glyoxylate and succinate; glyoxylate can then
be transformed into malate, bypassing two NADH-producing steps in the citric acid cycle (Braeckman et al.; Kondrashov et al. 2006). This might be important in the context of complex I inhibition as NADH might accumulate without a properly functioning complex I, and increasing levels of NADH are associated with increases in superoxide production. Furthermore, disrupted electron transfer from NADH to ubiquinone can cause increases in ROS (De Miranda et al. 2016). Inducing the glyoxylate pathway can then theoretically reduce oxidative stress by reducing the need for complex I to catalyze electron transfer from NADH to ubiquinone, and as a result prevent complex I-mediated dysfunction (Morgan et al. 2015). An increase in succinate levels due to the glyoxylate cycle could also account for the complex II upregulation observed in complex I mutants, and would permit at least partial maintenance of energy production despite the decrease in complex I activity.

The goal of this study was to further investigate the effect rotenone has in C. elegans, with the goal of furthering our ability to utilize this model organism in understanding mitochondrial dysfunction and dopaminergic neurodegeneration. Previous work in our lab evaluated DNA damage levels and mitochondrial genome copy number in C. elegans following rotenone exposure; we were unable to detect significant alterations (Gonzalez-Hunt et al. 2014). We also have been unable to detect significant levels of dopaminergic neurodegeneration after rotenone exposure.
(Gonzalez-Hunt, unpublished observations); neurodegeneration was evaluated using the scoring system previously published in Gonzalez-Hunt et al. (2014). These results were surprising since rotenone has caused mtDNA damage and neurodegeneration, as well as mitochondrial dysfunction, in other systems. Therefore, in this study we examined specific functional mitochondrial endpoints. We evaluated oxygen consumption utilizing the Seahorse XF24 Bioanalyzer, performed targeted metabolomics analysis, and measured ATP levels following rotenone exposure. We did not see major alterations in oxygen consumption or ATP levels, but saw alterations in amino acids, organic acids, and acylcarnitines similar to those previously reported for complex I mutants or after rotenone exposure in other model systems. Our data also suggest that there is an upregulation of complex II activity and an induction of the glyoxylate pathway. Overall, our results suggest that rotenone’s inhibition of complex I is similar in C. elegans to other organisms, but that C. elegans’ response to that inhibition is different. They also highlight the importance of considering C. elegans biology and how it might differ from other metazoans when performing experiments, particularly when the goal is to extrapolate results to higher organisms.
4.2 Materials and methods

4.2.1 C. elegans culture

Synchronized populations of nematodes were obtained by harvesting eggs from gravid adults via hypochlorite treatment (Lewis and Fleming 1995). Worms were hatched for no more than 14 hours in K medium, referred to as “complete K-medium” in Boyd et al. (2009). Worm transfers were performed by washing nematodes off agar plates or treatment wells and rinsing (after centrifugation at 2200 g for 2 min) with K medium.

C. elegans strains used were N2 (Bristol), JK1107 glp-1(q224) III, PE255 glp-4(bn2); sur-5::luc::gfp, and RB766 icl-1(ok531). The N2, JK1107, and RB766 strains were obtained from the Caenorhabditis Genetics Center (University of Minnesota). The PE255 strain was generously provided by Cristina Lagido (University of Aberdeen, Aberdeen, UK).

For experiments, populations of C. elegans were grown on OP50-seeded K agar plates (Williams and Dusenbery 1988) at 25 °C until dosing. For maintenance purposes, N2 and RB766 were kept at 20 °C on OP50-seeded K agar plates; PE255 and JK1107 were maintained at 15 °C until ready for experiments due to their temperature-sensitive sterility.
4.2.2 Rotenone exposures

After liquid hatch in K⁺ medium, worms were grown at 25 °C for 48 hours and then transferred to K medium and allowed to clear their guts for 15 minutes. They were then exposed for 48 hours to rotenone (dissolved in K⁺ medium) and fed 2X concentrated UVC-inactivated UvrA-deficient *E. coli* (bacteria were exposed to 1000 J/m² UVC with the UVLMS-38 EL Series Lamp emitting at 254 nm [UVP, LLC], as previously described in Meyer et al. (2010)). Following the exposure, worms were washed, allowed to clear their guts for 15 minutes, and measured as described in the next sections. The rotenone concentrations were chosen based on a high dose that would cause no lethality but minimal growth inhibition for each strain.

For oxygen consumption analysis with the Seahorse Bioanalyzer and targeted metabolomics, 4500 JK1107 (*glp-1*) nematodes were exposed to 0, 0.25, or 5 µM rotenone in a 25 cm² vented cell culture flask. For gene expression measurements, 500 JK1107 (*glp-1*) worms/well were exposed to 0 or 5 µM rotenone in a 12-well plate. For the ATP measurements using the PE255 nematodes, 4500 worms were exposed to 0, 0.25, or 1 µM rotenone in a 25 cm² vented cell culture flask (PE255 nematodes are more sensitive to rotenone than JK1107 worms so we reduced the highest dose to avoid lethality). For ATP measurements using the Promega kit, 1000 N2, JK1107 (*glp-1*), or RB766 (*icl-1*) nematodes/well were exposed to 0 or 5 µM in a 6-well plate.
4.2.3 Oxygen consumption analysis

We measured oxygen consumption rate (OCR) in age-matched young adult *glp-1* mutant nematodes using an XFe24 Bioanalyzer (Seahorse Bioscience). We then calculated the following parameters of mitochondrial performance: basal and maximal respiration, ATP-linked respiration, spare respiratory capacity, and proton leak as previously described (Luz et al. 2015a; Luz et al. 2015b).

Following exposure, worms were diluted to 1.0±0.2 nematode/µl in unbuffered EPA water and pipetted into a 24-well utility plate as follows: 50 worms/well for carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) plate, and 60 worms/well for sodium azide + N,N’-Dicyclohexylcarbodiimide (DCCD) plate. An aliquot of worms was stored at -80 °C for protein determination using the BCA protein assay kit (Thermo Fisher Scientific) as described in (Luz et al. 2015b). Seven or eight wells were run per treatment (with another two wells used as blanks) and final well volume was 525 µl. We performed 8 basal OCR measurements, followed by 8 OCR measurements after exposure to 25 μM FCCP, or 14 OCR measurements after exposure to 20 μM DCCD and then 4 measurements after exposure to 10 mM sodium azide (DCCD and azide exposures were done sequentially).

We calculated spare respiratory capacity by subtracting average basal OCR from average maximal respiration (induced by FCCP) for each well. To calculate ATP-linked
respiration we subtracted the DCCD response from the basal OCR for each well. For proton leak, we subtracted the response to sodium azide from the response to DCCD for each well. All OCR measurements were normalized to total protein.

4.2.4 Targeted Metabolomics

This protocol was performed as described in Luz et al. (2016a). Following 24 and 48 hours of rotenone exposure performed upon aged-matched young adults, nematodes were prepared for metabolomics analysis. After a rinse with cold PBS, worms were resuspended in 300 µl of an aqueous solution of 0.6% formic acid and flash-frozen in liquid nitrogen. Samples were kept at -80 °C until ready to continue the extraction.

During metabolite extraction, samples were thawed and kept on ice. After thawing, samples were sonicated with five 30-second pulses at 20% power (Biologics, Inc.). A third of each sample was then stored for protein analysis using the BCA protein assay kit (Thermo Fisher Scientific). The remaining sample was mixed 1:1 with acetonitrile and then aliquoted into two: one aliquot for quantifying amino acids and acylcarnitines, and another for organic acids. Metabolites were measured in collaboration with Matthew Hirschey’s laboratory. Amino acids and acylcarnitines were measured as described in An et al. (2004) and Wu et al. (2004). Organic acids were measured as described in Jensen et al. (2006). Metabolite concentrations were normalized to total protein.
4.2.5 *Metabolic inhibition assay*

This protocol was performed as described in Luz et al. (2016b). Rotenone-exposed age-matched young adult nematodes were incubated with various metabolic inhibitors and ATP was measured following the incubation as a way to assess performance. The principle of this assay is as follows: following inhibitor treatment, larger reductions in ATP in a rotenone-exposed group than in a control group would indicate increased function of the electron transport chain complex or metabolic pathway being inhibited in the rotenone-exposed group; the converse, lesser or no reductions in ATP following inhibitor treatment in a rotenone-exposed group than the reductions observed in a control group, indicate that the electron transport chain complex or metabolic pathway being inhibited is not playing a major role in energy production in the rotenone-exposed group.

Fifty aged-matched young adult nematodes/well were added to a 96-well white plate (diluted with unbuffered EPA water to 1.0±0.2 nematode/µl). For each experiment, 4 wells were run for each rotenone dose and inhibitor combination (including unbuffered EPA water or 1% DMSO as vehicle controls for the inhibitors). The experiment was repeated at least three times for each inhibitor. Final inhibitor concentration, target and incubation time are listed in Table 5.
Table 5: Inhibitor targets, final concentrations and incubation times as used in the mitochondrial inhibitor assay.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Target</th>
<th>Final Concentration</th>
<th>Incubation Time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rotenone</td>
<td>Complex I</td>
<td>20 µM</td>
<td>1</td>
</tr>
<tr>
<td>Malonate</td>
<td>Complex II</td>
<td>10 mM</td>
<td>1</td>
</tr>
<tr>
<td>Antimycin A</td>
<td>Complex III</td>
<td>150 µM</td>
<td>1</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>Complex IV</td>
<td>250 µM</td>
<td>1</td>
</tr>
<tr>
<td>DCCD</td>
<td>ATP synthase</td>
<td>20 µM</td>
<td>1</td>
</tr>
<tr>
<td>FCCP</td>
<td>Mitochondrial coupling</td>
<td>25 µM</td>
<td>1</td>
</tr>
<tr>
<td>Perhexiline</td>
<td>Fatty acid oxidation</td>
<td>100 µM</td>
<td>1</td>
</tr>
<tr>
<td>2-DG</td>
<td>Glycolysis</td>
<td>50 mM</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Following incubation, ATP levels were measured as previously described (Lagido et al. 2008; Lagido et al. 2015). First, we measured GFP fluorescence for each well using a FLUOstar Optima plate reader (BMG Labtech). We then injected luminescence buffer (140 mM Na₂PO₄, 30 mM citric acid [pH 6.5], 1% DMSO, 0.05% Triton X-100, 100 µM D-luciferin) into each well, incubated for 3 minutes, and then measured luminescence on the plate reader. All values were normalized to GFP.

4.2.6 ATP measurements

ATP levels after rotenone exposure were measured for N2, and the glp-1 and icl-1 mutants using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). This protocol was adapted from previously published methods (Bailey et al. 2016; Todt et al. 2016).
Following rotenone exposure, 50 age-matched young adult worms per well (at a concentration of 4 nematodes/µl in K medium) were added to a 96-well white plate. We then added 50 µl of CellTiter-Glo reagent and measured luminescence immediately after. Ten measurements were taken (30 minutes total measuring time, with a measurement occurring every 3 minutes). The last three measurements were averaged and used for ATP calculations. Three wells were run per dose and strain combination for each experiment; the experiment was repeated five times. ATP is reported as percent of the 0 µM rotenone control luminescence for each strain.

4.2.7 Targeted gene expression measurements

The mRNA of approximately 500 age-matched young adult worms was extracted using the RNeasy mini kit (QIAGEN) following rotenone exposure. After rinses, worms were allowed to clear their guts for 15 minutes. We then transferred the worms to 1 ml RLT buffer plus 10 µl β-mercaptoethanol and flash-froze them in liquid nitrogen. After bringing them to room temperature, zirconia/silica beads were added to the samples and they were homogenized using a bullet blender (Next Advance). The homogenate was transferred to a new 1.7 ml tube and kept at room temperature for 5 minutes; tubes were then centrifuged, and the supernatant was transferred to a new 1.7 ml tube. We then followed steps 5-12 of the RNeasy Mini handbook. Following extraction and quantification, mRNA was converted to cDNA using the High-Capacity cDNA Reverse
Transcription Kit (Applied Biosystems). Gene expression was measured by real-time PCR as described in Leung et al. (2013). Primers used were as follows: cdc-42 forward - 5’- GAG AAA AAT GGG TGC CTG AA-3’, reverse - 5’-CTC GAG CAT TCC TGG ATC AT-3’ (111 bp, published in Leung et al. (2013)); pmp-3 forward – AAG ATG ATT GGC CGG ATG AT, reverse – GCA ACG AGA GCA ACT GAA CT (102 bp); tba-1 forward – TCA TCT CGC AGG TTG TGT CT, reverse – GGT AAG CCT TGT CAG CAG AG (159 bp); mev-1 forward – GTTGGACAGATCTACAAATCGGG reverse – TCTTGTTGCTCTTGTCTGGC (100 bp); sdhA-1 forward – TCGCAGCTCAAGGAGGAATC reverse – ATGGCATCCTGATCTCCGAG (115 bp); and icl-1 forward – TGCTCATCCAGGATTGGTGC reverse – CTGAGCCAAGAGTGAGGTATCCA (198 bp). Annealing temperature was 60 °C for all primer sets except the primers for icl-1 (59 °C). All samples were run in triplicate, and the experiment was repeated three times. Replicates were averaged, and normalized to the expression levels of each housekeeping gene (cdc-42, pmp-3, tba-1). These values were then averaged and presented as fold change.

4.2.8 Statistical analyses

Data were analyzed with JMP Pro for Mac (Version 13.0.0, SAS Institute Inc.). One- or two-factor analysis of variance (ANOVA) was used to evaluate the effect of the
rotenone exposures on all endpoints. A p-value of less than 0.05 was considered statistically significant.

**4.3 Results**

4.3.1 Rotenone did not cause major alterations in oxygen consumption

We first measured oxygen consumption rate (basal and following chemical challenges) using the Seahorse XF-24 Bioanalyzer after a 48-hour rotenone exposure of age-matched young adult *glp-1* nematodes. Our chemical challenges were: DCCD (inhibitor of the FoF1 ATP synthase and ATP-linked oxygen consumption), FCCP (protonophore and uncoupling agent, induces maximal respiration), and sodium azide (inhibitor of the electron transport chain complex IV and all mitochondria-related oxygen consumption) (Benz and McLaughlin 1983; Fillingame 1975; Wharton and Tzagoloff 1967). We observed slightly higher OCR in the rotenone-treated groups compared to control after DCCD injection (one-way ANOVA p=0.0205*, Tukey-Kramer HSD vs control for 0.25 µM p=0.0399*, for 5 µM p=0.0447*), and also a small decrease in OCR in the rotenone-treated groups compared to control after the azide injection (one-way ANOVA p=0.0060*, Tukey-Kramer HSD vs control for 5 µM p=0.0079*) (Figure 18).

We then used these values to calculate parameters of mitochondrial performance. We do not see any alterations due to rotenone exposure in ATP-linked respiration (basal OCR minus OCR after DCCD injection) or spare respiratory capacity
(FCCP-induced OCR minus basal OCR); only proton leak (OCR after DCCD injection minus OCR after azide injection) was altered by rotenone exposure (Figure 19; one-way ANOVA p<.0001*, Tukey-Kramer HSD vs control: p=0.0107* for 0.25 µM, p<.0001* for 5 µM).

Figure 18: Rotenone did not cause major alterations in the response to the mitochondrial stress test (measured as oxygen consumption after chemical challenge) in *C. elegans*. 
Figure 19: Rotenone caused a statistically significant increase in proton leak, but not ATP-linked respiration or spare capacity.

4.3.2 Rotenone caused changes to levels of amino acids, organic acids, and acylcarnitines

We also evaluated the effect of rotenone (24- and 48-hour exposures) on young adult *glp-1* C. elegans metabolism by measuring metabolite levels using mass spectrometry. Changes were observed for all metabolite categories analyzed.

For amino acids, we saw an increase (normalized to control) in alanine levels after the 48-hour 5 µM rotenone exposure (one-way ANOVA p=0.0001*, Tukey-Kramer HSD vs control: p=0.0001* for 5 µM). We saw decreases in ornithine after the 24-hour exposure for both rotenone doses (one-way ANOVA p=0.0028*, Tukey-Kramer HSD vs
control: p=0.0088* for 0.25 µM, p=0.0042* for 5 µM). All measured amino acid levels (as Log2 fold change from control) are presented in Figure 20.

We also saw changes for organic acids, with lactate, malate, and pyruvate showing increases after the 5 µM exposure. Lactate and malate were both increased only after the 48-hour exposure (for malate: one-way ANOVA p=0.0165*, Tukey-Kramer HSD vs control: p=0.0140*; for lactate: one-way ANOVA p=0.0008*, Tukey-Kramer HSD vs control: p=0.0011*). Pyruvate was increased after both 24- and 48-hr exposures to 5 µM rotenone (for 24-hr exposure: one-way ANOVA p=0.0129*, Tukey-Kramer HSD vs control: p=0.0344*; for 48-hr exposure: one-way ANOVA p<0.0001*, Tukey-Kramer HSD vs control: p<.0001*). All measured organic acid levels (as Log2 fold change from control) are presented in Figure 21.

We saw widespread decreases in short, medium and long chain acylcarnitines. Altered acylcarnitines, exposure time, concentration, and p-values are presented in Table 6; all acylcarnitines presented in this table were decreased, with the exception of C4-OH which was increased after both exposure times. All measured acylcarnitines levels (as Log2 fold change from control) are presented in Figure 22, Figure 23, and Figure 24.
Figure 20: Metabolomics analysis of amino acid levels in *C. elegans* after rotenone exposure.
Figure 21: Metabolomics analysis of organic acid levels in *C. elegans* after rotenone exposure.
Figure 22: Metabolomics analysis of short chain acylcarnitine levels *in C. elegans* after rotenone exposure.
Figure 23: Metabolomics analysis of medium chain acylcarnitine levels in *C. elegans* after rotenone exposure.
Figure 24: Metabolomics analysis of long chain acylcarnitine levels in *C. elegans* after rotenone exposure.
Table 6: Levels of acylcarnitines were altered by rotenone. One-way ANOVA and posthoc Tukey-Kramer HSD p-values.

<table>
<thead>
<tr>
<th>Acylcarnitine</th>
<th>Exposure Time (hrs)</th>
<th>Rotenone (µM)</th>
<th>One-way ANOVA p-value</th>
<th>Tukey-Kramer HSD vs control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Short chain</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td>48</td>
<td>5</td>
<td>0.0271*</td>
<td>0.0361*</td>
</tr>
<tr>
<td>C4/Ci4</td>
<td>24</td>
<td>5</td>
<td>0.0154*</td>
<td>0.0121*</td>
</tr>
<tr>
<td>C4-OH</td>
<td>48</td>
<td>5</td>
<td>0.0023*</td>
<td>0.0045*</td>
</tr>
<tr>
<td>C4-DC/Ci4-DC</td>
<td>24</td>
<td>5</td>
<td>0.0026*</td>
<td>0.0090*</td>
</tr>
<tr>
<td>C6</td>
<td>48</td>
<td>5</td>
<td>0.0400*</td>
<td>0.0423*</td>
</tr>
<tr>
<td><strong>Medium chain</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C10:1</td>
<td>24</td>
<td>0.25</td>
<td>0.0141*</td>
<td>0.0168*</td>
</tr>
<tr>
<td>C14:1</td>
<td>24</td>
<td>5</td>
<td>0.0141*</td>
<td>0.0430*</td>
</tr>
<tr>
<td>C16:2-OH/C14:2</td>
<td>48</td>
<td>5</td>
<td>0.0097*</td>
<td>0.0132*</td>
</tr>
<tr>
<td><strong>Long chain</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18</td>
<td>48</td>
<td>5</td>
<td>0.0053*</td>
<td>0.0205*</td>
</tr>
<tr>
<td>C18:1</td>
<td>48</td>
<td>5</td>
<td>0.0016*</td>
<td>0.0053*</td>
</tr>
<tr>
<td>C18:2-OH/C16:2</td>
<td>48</td>
<td>5</td>
<td>0.0012*</td>
<td>0.0024*</td>
</tr>
<tr>
<td>C20</td>
<td>48</td>
<td>5</td>
<td>0.0001*</td>
<td>0.0002*</td>
</tr>
<tr>
<td>C20:1</td>
<td>48</td>
<td>5</td>
<td>0.0102*</td>
<td>0.0208*</td>
</tr>
<tr>
<td>C20:2</td>
<td>48</td>
<td>5</td>
<td>0.0003*</td>
<td>0.0006*</td>
</tr>
</tbody>
</table>

4.3.3 Metabolic inhibitor assay suggests increased complex II activity.

The results from the metabolomics analysis are suggestive of widespread metabolic disruption by rotenone. To further test this and investigate the mechanistic changes responsible, we assayed the contribution of different mitochondrial energy production pathways. We measured ATP levels following chemical treatment to inhibit
electron transport chain complexes I, II, III, IV, and V (ATP synthase), as well as coupled mitochondrial respiration, fatty acid oxidation, and glycolysis.

We first examined the effect on ATP of a 48-hour rotenone exposure followed up by an incubation with inhibitor vehicle controls (EPA water and 1% DMSO). We did not observe any decreases in ATP due to rotenone exposure (Figure 25).

![Figure 25: Rotenone exposure did not cause decreases in ATP in adult PE255 C. elegans.](image)

We then proceeded to examine the effect of metabolic inhibition after rotenone exposure. After complex II inhibition with malonate, we observed larger ATP decreases in the 1 µM rotenone-exposed group than in the control group, suggesting increased complex II function after 1 µM rotenone exposure (One-way ANOVA p=0.0373*, Tukey-Kramer HSD vs control 0.0314*). We also saw a larger decrease in ATP in the 0.25 µM rotenone-exposed group than in the control group after inhibition of the ATP synthase with DCCD, suggesting increased ATP synthase function after 0.25 µM rotenone exposure (One-way ANOVA p=0.0215*, Tukey-Kramer HSD vs control 0.0227*).
was also a trend towards bigger ATP decreases after mitochondrial uncoupling with FCCP in the rotenone-exposed groups, but it was not statistically significant (One-way ANOVA p=0.0510). ATP levels after metabolic inhibition are presented in Figure 26.

4.3.4 Rotenone caused similar changes to ATP in N2, glp-1, and icl-1 (isocitrate lyase) mutant strains

The results from the metabolomics analysis and the metabolic inhibition assay (particularly the increases in malate and complex II function, yet absence of effects on ATP levels after rotenone exposure) led us to hypothesize that the nematodes were able to compensate for complex I inhibition by inducing the glyoxylate pathway in order to maintain functionality of the electron transport chain and thus ATP levels. If this were the case, then a glyoxylate pathway mutant strain (isocitrate lyase KO) should exhibit reductions of ATP levels after rotenone exposure. To test this, we exposed N2, glp-1, and icl-1 mutants to 5 µM rotenone (we used the higher dose since we needed to see a depletion in wild-type in order to leave room for the possibility of less depletion in icl-1). Unexpectedly, we saw ATP reductions of 50-70% for all three strains (compared to each strain’s 0 µM control), and there was no statistically significant difference among them (One-way ANOVA p=0.3901; Figure 27).
Figure 26: ATP levels after metabolic inhibition in rotenone-exposed PE255 C. elegans.
Figure 27: Rotenone caused similar ATP decreases in N2, glp-1, and icl-1 mutant strains.

4.3.5 mRNA levels for complex II subunits and isocitrate lyase were elevated after rotenone exposure

To continue testing whether or not the glyoxylate pathway might be induced in C. elegans following rotenone exposure, we measured expression of the genes coding for isocitrate lyase (icl-1) and two complex II subunits, succinate dehydrogenase subunit A and cytochrome b560 (sdha-1 and mev-1, respectively). We found that mRNA levels for all three genes were elevated after rotenone exposure (Figure 28; two-tailed t-test p-value: icl-1 p=0.0041*, mev-1 0.0201*, sdha-1 0.0097*).
Figure 28: Rotenone exposure induced isocitrate lyase (*icl-1*) and both complex two subunits (*mev-1* and *sdha-1*).

4.4 Discussion

Based on our previous inability to detect rotenone-induced mtDNA damage or dopaminergic neurodegeneration in *C. elegans*, we set out to characterize the effect rotenone has on *C. elegans* bioenergetics downstream from complex I inhibition. We did not see major differences in OCR between rotenone-treated and control worms; this was very surprising, as it is widely accepted that rotenone decreases mitochondrial respiration (De Miranda et al. 2016). This is unlikely to be a trivial methodological discrepancy, because the Seahorse Bioanalyzer has been used to show that rotenone causes major alterations in oxygen consumption in cell culture (Dranka et al. 2012; Giordano et al. 2012). We did see an increase in proton leak, suggesting that rotenone might affect ATP levels by increasing the amount of uncoupled oxygen consumption;
however, this was not a huge increase, so we would not expect major ATP changes because of it. Furthermore, we did not see decreases in ATP levels due to rotenone exposure, supporting the idea that exposed worms are able to use compensatory mechanisms to maintain ATP levels. Overall, these data were consistent with our previous results, suggesting that rotenone-exposed nematodes are able to use compensatory mechanisms to deal with chemical complex I inhibition.

Consistent with this, we saw alterations to metabolites in all categories analyzed. Although there is not a precisely defined, published metabolic signature for the effect of rotenone in mammalian models, there are some patterns that can be gleaned from the literature; overall, our results align with these patterns. Particularly, increases in lactate have been consistently reported (Karlsson et al. 2016; Lei et al. 2014; Xu et al. 2011). Xu et al. (2011) also saw an increase in alanine, while Lei et al. (2014) reported a decrease. An increase in pyruvate following rotenone exposure was also observed by Karlsson et al. (2016). It is worth noting that very similar results have been observed in complex I mutants as briefly described in the Introduction (Falk et al. 2008; Grad and Lemire 2004; Morgan et al. 2015; Schrier Vergano et al. 2014; Zury et al. 2010), supporting the assumption that the primary, direct mode of action of rotenone is indeed complex I inhibition.

These results indicate a disruption in pyruvate metabolism. Pyruvate can be converted into alanine and lactate; very high levels of pyruvate might explain the
increases we see in both of them (Chandel 2015). Based on our data, we cannot unequivocally say which pathways are responsible for these alterations. This result would be consistent with upregulation of glycolysis, an observation that has also been made in other metabolic studies on the effect of rotenone (Dranka et al. 2012; Giordano et al. 2012; Karlsson et al. 2016; Lei et al. 2014). However, we did not detect an alteration in reliance on glycolysis for ATP production in our 2-DG inhibition experiment.

We also analyzed levels of acylcarnitines after rotenone exposure, and saw decreases in short, medium, and long chain acylcarnitines. These widespread reductions are consistent with increased fatty acid β-oxidation, an observation that has been made previously in cell culture (Worth et al. 2014). Increases in fatty acid oxidation could help replenish acetyl-CoA levels, which might be reduced due to increased pyruvate conversion to alanine and lactate. However, we did not detect an alteration in reliance on fatty acid oxidation for ATP production in our perhexiline inhibition experiment.

We observed increases in malate after rotenone exposure, which have also been previously reported (Xu et al. 2011). Interestingly, increases in malate have also been reported in complex I mutants, and they were attributed to a possible increase in the glyoxylate cycle (Schrier Vergano et al. 2014). Indeed, evidence of glyoxylate cycle upregulation in complex I mutants was presented in Falk et al. (2008) and Morgan et al. (2015). These reports in complex I mutants made us consider that a similar glyoxylate pathway upregulation could be caused by complex I inhibition by rotenone. If that were
the case, then the glyoxylate pathway would provide a way to produce FADH$_2$ for oxidative phosphorylation and ATP production, while “skipping” two NADH-producing steps in the citric acid cycle; this would reduce the NADH pool and consequently some of the deleterious effects of complex I inhibition. The glyoxylate pathway would allow mitochondria to maintain a somewhat functional electron transport chain and ATP levels, with increased reliance on complex II function; this idea is supported by reports of increased complex II activity in complex I mutants (Kayser et al. 2001; Pujol et al. 2013). Furthermore, an upregulation in this pathway could explain the lack of evidence in our inhibition assays (in contrast to our metabolomic results) for reliance on fatty acid oxidation and glycolysis—if there are multiple compensatory pathways engaged, inhibition of any one would have a dampened effect on ATP levels.

Consistent with this idea, we found evidence that rotenone-exposed worms upregulated complex II (as well as V). To further test the hypothesis that the glyoxylate cycle was helping the nematodes maintain their ATP levels, we exposed two control strains (N2 and *glp-1* mutants) and a glyoxylate pathway mutant (*icl-1*) to rotenone and measured ATP levels using a different method from the one used in the metabolic inhibition assay. Surprisingly, we saw reduced ATP levels due to rotenone exposure in all three strains, but there was no difference in the response among them. It is worth mentioning that unlike ATP measurements with PE255 nematodes, ATP measurements in this assay are not normalized to the amount of worms measured. This could be a
confounder, as rotenone causes nematodes to stick to the exposure well; loss of worms during transfer to the measuring plate could artificially cause ATP levels in exposed worms to seem lower than those in control worms. Furthermore, our 5 µM dose causes a mild growth inhibition that could also skew the results. Our third test of potential glyoxylate-mediated acclimation to rotenone did support this hypothesis; we saw significant increases in transcripts for three glyoxylate pathway genes in exposed groups compared to control.

Taken together, our results show that rotenone causes various alterations in metabolite levels in *C. elegans* at doses that did not cause major changes in oxygen consumption or ATP levels. These changes are consistent with a multi-pathway metabolic restructuring that helps maintain oxidative phosphorylation and ATP production: we found evidence for concomitantly increased glycolysis and fatty acid oxidation—pathways that also function in compensatory fashion in mammals—as well as the glyoxylate pathway, which appears to be absent in mammals. Induction of the glyoxylate pathway could help the worms deal with complex I inhibition and maintain baseline levels of ATP and oxygen consumption, and could explain why we did not see increases in DNA damage levels or alterations to copy number in Gonzalez-Hunt et al. (2014), or why we have failed to see neurodegeneration following rotenone exposure (Gonzalez-Hunt, unpublished observations). On the other hand, other groups have seen mitochondrial dysfunction and toxicity after rotenone exposure in *C. elegans*, including
decreased respiration and neurodegeneration (Chikka et al. 2016; Grad and Lemire 2004; Ray et al. 2014; Schouest et al. 2009; Ved et al. 2005; Zhou et al. 2013); it is possible that these discrepancies are because of the exposure protocol used. Indeed, when we expose adult worms to higher doses of rotenone for just an hour (as seen in the metabolic inhibition assay described above), we see decreases in ATP levels. We propose that these are in fact entirely consistent results; the longer, 48-hour exposure at a lower concentration likely allows the nematodes to “acclimate” to complex I inhibition by inducing the glyoxylate pathway. Similarly, even with the availability of an additional compensatory pathway (the glyoxylate pathway), high enough doses will eventually result in the same downstream results of mitochondrial inhibition. It is also important to consider the medium for rotenone exposures and exposure duration. In aquatic environments, rotenone has been estimated to have a half-life of 14 hours at 24 °C (Gilderhus et al. 1986); although laboratory conditions are more controlled (and therefore have less biotic and abiotic degradation), this is an important consideration as it can be a confounding factor, especially when comparing the effects of acute versus chronic rotenone exposures. Thus, it is very likely that the exposure protocol used dictates the magnitude of the effects caused by rotenone and the potential for acclimation to complex I inhibition (i.e. whether or not the glyoxylate pathway becomes upregulated).
The main takeaway from this study is that nematode biology needs to be considered when using *C. elegans* to study complex I inhibitors, as the nematodes have an alternate pathway that increases their ability to compensate for such inhibition. This compensation could confound results from toxicological studies on the effect of complex I inhibitors, in particular in the context of environmentally-relevant long-term, low-dose experiments, and appropriate controls should be included.
5. Conclusions

5.1 Summary

The main goal of my dissertation work was to investigate the role mtDNA damage plays in causing dopaminergic neurodegeneration. Particularly, I wanted to explore the effect of large, helix-distorting lesions such as thymine dimers on neuronal and mitochondrial health. Such lesions can be caused by common environmental toxicants such as polycyclic aromatic hydrocarbons, and are irreparable in mitochondria. To this end, I performed experiments to evaluate the effect of mtDNA damage in Caenorhabditis elegans, a free-living nematode that presents an opportunity to do this mostly mechanistic work in vivo.

In chapter 2, I hypothesized that nematodes (their dopaminergic neurons in particular) were more sensitive to irreparable mtDNA damage inflicted at certain life stages. In other words, I postulated that there were windows of neuronal vulnerability to mtDNA damage. The results do not support this hypothesis; we did not observe increased vulnerability in older worms, and inflicting DNA damage on germ cells did not make the worms more sensitive as young adults. However, these results need to be corroborated due to potential technical issues with the UVC source (our source of irreparable mtDNA lesions), and the more biologically interesting possibility that germ cells are being culled prior to egg laying (so the mtDNA damage we caused is effectively removed from the viable population).
In chapter 3, we tested the hypothesis that autophagy, by removing dysfunctional mitochondria and damaged mitochondrial genomes, protects dopaminergic neurons from toxicant-induced neurodegeneration. We saw marked protection from 6-OHDA-induced neurodegeneration after a 48-hour starvation period (used to induce macroautophagy), although we did not test whether or not this protective effect was due to an induction in autophagy alone. We also evaluated the effect of genetic inhibition of mitophagy, and saw different responses to different toxic insults. Both mitophagy mutants were more sensitive to UVC-induced mtDNA damage as we expected; however, the pink-1 mutant was resistant (compared to the control strain) to 6-OHDA-induced neurodegeneration, while the pdr-1 mutant was more sensitive. This led us to hypothesize that antioxidant responses are elevated in the pink-1 mutant, protecting it from oxidative stress caused by 6-OHDA. However, our gene expression measurements of antioxidant genes do not provide support for this hypothesis. We are currently evaluating mitochondrial morphology, as increased fusion or fission of the mitochondrial network could help the pink-1 mutant better deal with mitotoxic insults and maintain mitochondrial function.

Lastly, in chapter 4 we set out to expand our knowledge of the mechanism of toxicity of rotenone, a mitochondrial complex I inhibitor widely used to study the effects of mitochondrial dysfunction on dopaminergic neurons in the context of Parkinson’s disease. At the tested doses, we did not see major alterations in oxygen consumption or
ATP levels, both endpoints widely reported in the literature as altered due to rotenone exposure in other laboratory models. Nonetheless, at the same doses, we saw alterations in metabolite levels indicating that metabolic restructuring due to rotenone exposure was taking place in *C. elegans*. Particularly, we found evidence that the glyoxylate pathway, an alternate pathway parallel to the citric acid cycle not present in higher metazoans, was being induced by rotenone exposure. Follow-up experiments provided more evidence for this, with our metabolic inhibition assay suggesting upregulation of complex II and V, and with gene expression measurements showing increases in mRNA levels for *sdha-1* and *mev-1* (complex II subunit genes) and *icl-1* (gene coding for isocitrate lyase, the main glyoxylate pathway enzyme).

### 5.2 Broader implications and limitations

The results presented in this dissertation further highlight the importance of the link that exists between mitochondrial dysfunction and neurodegeneration (Johri and Beal 2012). Specifically, we investigated the effect of genetic defects in mitophagy in the context of dopaminergic neurodegeneration. The dysregulation of mitophagy in the context of neurodegenerative disease has been studied widely, yet many questions remain as we translate *in vitro* work in non-neuronal cell lines to other experimental models (Ashrafi and Schwarz 2013; Palikaras and Tavernarakis 2012). Our work shows that removal of dysfunctional mitochondria (via selective or non-selective autophagy) appears to play an important role in maintaining neuronal health and preventing cell
death in vivo. We also see that different mitophagy-related gene mutations are not equal when it comes to dealing with toxic insults; in our work, the pink-1 and pdr-1 mutants responded differently to the same 6-OHDA treatment, yet both had a similar response to UVC-induced mtDNA damage. These results are particularly relevant as it is not clear if mitophagy defects sensitize dopaminergic neurons to toxic insult (Bornhorst et al. 2014; Haque et al. 2008; Haque et al. 2012; Perez et al. 2005; Sallinen et al. 2010). This work also highlights the importance of considering mechanism of action when working with mitochondrial toxicants. Simply determining that a toxicant is a mitochondrial poison is not enough; digging deeper into how it exerts its toxic effects is important. This is particularly important in our model organism, C. elegans; our work with rotenone showed that in response to inhibition of complex I, alterations in metabolic pathways may be providing the nematodes with a way to cope with mitochondrial dysfunction. This idea has been postulated previously in the context of a complex I mutant (Morgan et al. 2015). The pathway involved in this response, the glyoxylate pathway, is not present in mammals. This work is a reminder to always consider the unique biology of our laboratory model, particularly when the goal is to tease out the mechanisms of human disease.

In addition to the new ideas uncovered in this work, there are some limitations to these studies that need to be discussed. Unfortunately, we were unable to fully test the windows of vulnerability hypothesis in chapter 2 due to difficulties with our UVC
source and measuring mtDNA damage in C. elegans eggs. Drawing definitive conclusions regarding windows of vulnerability, therefore, will require additional experiments. In chapter 3, it is possible that antioxidant responses play an important role in the response to 6-OHDA and gene expression measurements do not capture the whole story. More experiments are needed to fully explore this idea. In chapter 4, although our data suggest that complex II and isocitrate lyase activity is upregulated, we did not specifically test enzymatic activities. We also did not test if inhibiting the glyoxylate pathway (e.g. via RNAi) sensitizes the worms to the effects of rotenone. These additional experiments would be necessary to fully show that the glyoxylate pathway is getting induced in C. elegans.

Although follow-up experiments are necessary, the research presented in this dissertation provided more evidence for the theory linking mitochondrial dysfunction and the etiology of neurological disease. It also produced new hypotheses to continue exploring this link, such as the idea that the mechanism of toxicity of different mitotoxicants plays a major role in determining the magnitude of neurodegeneration observed in dopaminergic neurons. This could be especially true in the context of genetic susceptibility, such as mitophagy defects, and might be relevant as we continue exploring the multiple hit (gene x environment) hypothesis of PD (Ross and Smith 2007).
Appendix A – Dopaminergic neuron damage can be regenerated in Caenorhabditis elegans


The regenerative potential of the CEP dendrites has not been determined. To assess whether these neurons are capable of regenerating after damage, we tested their ability to respond to pulsed laser surgery in collaboration with the Hammarlund laboratory. Many neurons in C. elegans can respond to laser surgery by initiating regeneration (Bejjani and Hammarlund 2012) but the dendrites of the dopamine neurons have not been tested.

We found that CEP dendrites are capable of regenerating after laser surgery, and that the dlk-1 MAP kinase pathway might be implicated. We then investigated if a similar response could occur after chemical exposure, so we evaluated the response to 6-OHDA-induced neurodegeneration in mutants of the dlk-1 MAP kinase pathway. The
mutant worms appeared to be more sensitive to 6-OHDA than the control strain, but no regeneration over time was observed.

**A.1 Materials and methods**

**A.1.1 C. elegans culture**

Populations of *C. elegans* were maintained on K agar plates (Williams and Dusenbery 1988) seeded with OP50 bacteria. Synchronized populations of nematodes were obtained by bleach-sodium hydroxide isolation of eggs. L1 larvae were obtained by hatching eggs in K+ medium, previously referred to as “complete K-medium” (Boyd et al. 2009). All transfers were made by washing nematodes off of agar plates and rinsing (after centrifugation at 2200 g for 2 min) in K medium.

The transgenic strain BY250 (*vtls7[Pdat-1::GFP]*, expressing GFP only in dopaminergic neurons) was generously provided by Michael Aschner (Vanderbilt University). Strain XE1311 (*vtls7[Pdat-1::GFP];mkk-4(ju91)*) was generated by crossing the *mkk-4(ju91)* mutation into the *vtls7* background.

**A.1.2 Chemical exposures**

6-OHDA was dissolved into a solution of ascorbic acid in K+ medium. *C. elegans* were treated in 1.7 ml tubes, and each tube contained 1 ml of the treatment solution and 1000 L1 larvae. Exposure duration was one hour. The worms were then washed with K medium twice, transferred to seeded K agar plates, and sampled 24h, 48h, 96 h, and 9 days after the exposure.
A.1.3 Dopaminergic neurodegeneration

Treated C. elegans were picked onto a 2% agar pad and immobilized with 15µl of 1% sodium azide (Sigma-Aldrich). Nematodes were examined using a Zeiss Axioskop microscope and neuronal morphology was assessed by individual observation of each cephalic (CEP) neuron. Neurons were assigned a score from 0 to 2 based on the amount of morphological abnormalities present. Ten to fifteen worms were analyzed per treatment for each time point, and the experiment was repeated twice. All scoring was double-blinded.

A.1.4 Laser surgery

Laser surgery was performed by collaborators in the Hammarlund laboratory. The dendrites of the CEP neurons were severed with a pulsed laser essentially as described (Byrne et al. 2011). L4 stage BY250 vtlIs7[Pdat-1::GFP] and XE1311 vtlIs7[Pdat-1::GFP];mkk-4(ju91) worms were used. 2 CEP neurons were cut in each worm, at the point just anterior to the curve made around the anterior pharyngeal bulb. Worms were recovered onto NGM plates at 20°C for 24 h before being remounted and scored for regeneration. Regeneration was defined as anterior growth beyond the cut site. Representative images were collected on a Zeiss LSM710 point scanner and analyzed with ImageJ (U. S. National Institutes of Health, Bethesda, Maryland, USA).
A.1.5 Statistical analysis.

Dopaminergic neurodegeneration data were analyzed using the statistical software JMP Pro for Windows (Version 11.0.0, SAS Institute Inc., Cary, NC). The nonparametric Kruskal-Wallis test was used to test for differences between dosage levels for each chemical at each time point. Due to sparseness in the cross-tabulations of dosage levels and scores, Fisher’s exact test (FET) was used when testing independence of chemical dosage levels and scores at each time point. FET was also used to analyze laser axotomy data. A $p$-value of less than 0.05 was considered statistically significant.

A.2 Results and discussion

A.2.1 Dopaminergic neuron damage can be regenerated in C. elegans

We found that the CEP dendrites are capable of injury-induced growth after laser surgery: 13 out of 16 severed dendrites exhibited anterior growth beyond the cut site (Figure 29). Next, we asked whether injury-induced growth of the CEP dendrites requires the critical $dlk-1$ MAP kinase pathway, which is important for regeneration in a variety of neuron types in C. elegans (Hammarlund et al. 2009; Yan et al. 2009). We found that animals defective for $dlk-1$ pathway signaling ($mkk-4$ mutants) have reduced CEP regeneration (Figure 29), suggesting that regeneration of the CEP dendrite has some molecular similarities to axon regeneration.

We then explored the effect of the $dlk-1$ MAP kinase pathway in the response to chemical-induced neurodegeneration, as chemically induced neuronal damage could be
different from that caused by laser surgery and therefore trigger a different regenerative response. We tested the effect of the mkk-4 mutation on the response to 6-OHDA, as this chemical causes the widest range of neuronal damage scores. The XE1311 worms (mkk-4 KO) were more sensitive to 6-OHDA than wild type BY250 at almost every time point and dose as determined by the Fisher’s exact test (Figure 30). However, we did not observe regeneration in the BY250 worms; damage worsened over time for both strains of C. elegans. These results suggest that the MAP kinase pathway response to neuronal damage depends on the type of damage induced, as the responses we saw to chemical injury and laser surgery were different. Furthermore, exposure to different chemicals may or may not trigger regenerative pathways in response to neuronal damage, with damage caused by some toxins (paraquat and AFB1) but not others (6-OHDA) exhibiting apparent regeneration over time.

Together, these data suggest that recovery of CEP dendrite morphology after a toxic exposure could be mediated by injury response pathways, possibly involving the conserved dlk-1 MAP kinase pathway.
Figure 29: Damage to dopaminergic neurons caused by laser ablation is repaired.

A, after laser ablation, 81% of BY250 worms showed neuronal anterior growth, compared to 17% of the XE1311 worms. B, representative images of dopaminergic neurons after laser ablation. Left to right: uncut, no response, response.
**Figure 30:** A mutation in the mkk-4 gene required for neuronal regeneration worsens dopaminergic neurodegeneration in 6-OHDA-exposed nematodes.
Appendix B – Paraquat toxicity in Caenorhabditis elegans

For the purposes of exploring mitochondrial dysfunction and neurodegeneration in C. elegans we also set out to evaluate the effect of paraquat exposures on dopaminergic neuron health, mitochondrial copy number, and oxygen consumption.

B.1 Materials and methods

B.1.1 C. elegans culture and exposure

C. elegans were maintained on K agar plates (Williams and Dusenbery 1988) seeded with OP50 bacteria. Synchronized populations of nematodes were obtained by bleach-sodium hydroxide isolation of eggs, and larvae were obtained by hatching eggs in K+ medium (referred to as “complete K-medium” in Boyd et al. (2009)). All transfers were made by washing nematodes off plates with K medium.

C. elegans strains used were BY200 [vtIs1; P/dat-1::GFP, rol-6 (su1006)], UA226 [pink-1 (tm1779); vtIs1; P/dat-1::GFP, rol-6 (su1006)], UA227 [pdr-1 (tm598); vtIs1; P/dat-1::GFP, rol-6 (su1006)], VC1024 pdr-1 (gk448; outcrossed 3x), pink-1 (tm1779), N2 (Bristol), and JK1107 glp-1(q224) III. The N2, pdr-1 and glp-1 strains were obtained from the Caenorhabditis Genetics Center (University of Minnesota). UA226 and UA227 are pink-1 and pdr-1 KOs crossed with BY200. BY200, UA226, UA227, and pink-1 were generous gifts from Guy and Kim Caldwell (University of Alabama).
For copy number measurements, N2, *pink-1*, and *pdr-1* L1 nematodes were exposed for 48 hours in 0, 200, or 400 µM paraquat K agar plates seeded with 20X concentrated UV-inactivated *E. coli* (UvrA-deficient); doses were based on a high dose that would cause minimal growth reductions (worms evaluated 0 hours after exposure). For neurodegeneration evaluation, BY200, UA226, and UA227 L1 nematodes were exposed for 48 hours in a 12-well plate to 18 and 54 µM paraquat in K medium with 2.5X concentrated UV-inactivated *E. coli*; doses were based on a high dose that would not cause noticeable growth reductions throughout the duration of the experiment (out to 9 days old). For oxygen consumption measurements, 4500 JK1107 (*glp-1*) nematodes were exposed to 0, 50, or 500 µM paraquat in a 25 cm² vented cell culture flask with 2X concentrated UV-inactivated *E. coli*; doses were based on a high dose that would cause minimal growth inhibition for each strain.

**B.1.2 Genome copy number assay**

Six worms were picked and pooled immediately after the paraquat exposure (i.e. 0 hours post exposure) in a single tube per biological replicate, and three biological replicates were taken per treatment in two experiments separated in time. Genome copy number were evaluated using RT-PCR-based methods as previously described (Gonzalez-Hunt et al. 2016). Mitochondrial genome target was 75 bp, nuclear genome target was 164 bp.
B.1.3 Dopaminergic neurodegeneration assay

Worms were sampled at 48hrs, 96hrs, and 7 days after exposure for evaluation of dopaminergic neuron morphology. Treated *C. elegans* were picked onto a 2% agar pad and immobilized with 15µl of 1% sodium azide (Sigma-Aldrich). Nematodes were examined using a Zeiss Axioskop microscope and neuronal morphology was assessed by observation of each cephalic (CEP) neuron. Neurons were assigned a score based on the amount of morphological abnormalities present (Figure 3). The scoring categories were: 0 = no observable damage; 0.5 = blebs on equal or less than half of dendrite, no breaks; 1 = blebs on more than half of dendrite, no breaks; 1.5 = Breaks, equal or more than half of dendrite still present; 2 = Breaks, less than half of dendrite still present (includes completely absent dendrite).

The experiment was repeated three times for the 48-hour timepoint, and twice for the 96-hour and 7-day timepoints. Ten worms were analyzed per treatment at each time point. All scoring was double-blinded.

B.1.4 Oxygen consumption analysis

We measured OCR in *glp-1* mutant nematodes using an XFe24 Bioanalyzer (Seahorse Bioscience). We then calculated parameters of mitochondrial performance: basal and maximal respiration, ATP-linked respiration, spare respiratory capacity, and proton leak as previously described (Luz et al. 2015a; Luz et al. 2015b).
Following exposure, worms were diluted to 1.0±0.2 nematode/µl in unbuffered EPA water and pipetted into a 24-well utility plate as follows: 50 worms/well for FCCP plate, and 65 worms/well for sodium azide + DCCD plate. An aliquot of worms was stored at -80 °C for protein determination using the BCA protein assay kit (Thermo Fisher Scientific) as described in (Luz et al. 2015b). Seven or eight wells were run per treatment (plus two wells used as blanks) and final well volume was 525 µl. We performed 8 basal OCR measurements, then 8 OCR measurements after exposure to 25 µM FCCP, or 14 OCR measurements after exposure to 20 µM DCCD and then 4 measurements after exposure to 10 mM sodium azide (DCCD and azide exposures were done sequentially, separate from FCCP).

We calculated spare respiratory capacity by subtracting average basal OCR from average maximal respiration (induced by FCCP) for each well. We calculated ATP-linked respiration by subtracting the DCCD response from the basal OCR for each well. For proton leak, we subtracted the response to sodium azide from the response to DCCD for each well. Measurements were normalized to total protein.

B.1.5 Statistical analyses

Data were analyzed with JMP Pro for Mac (Version 13.0.0, SAS Institute Inc.). One- or two-factor analysis of variance (ANOVA) was used to evaluate the effect of paraquat on copy number and oxygen consumption. For evaluation of neurodegeneration, the Fisher’s exact test (FET) was used to compare the effect of the
exposures on each mutant with the effect on the control strain. Each dose and time point assessed independently.

**B.2 Results and discussion**

**B.2.1 Paraquat exposure decreases copy number equally for N2, pink-1, and pdr-1 strains**

Nematodes were evaluated right after the 48-hour paraquat exposure to avoid the confounding effects of egg production. In all strains tested (N2, pink-1, and pdr-1), both doses of paraquat caused approximately a 40% decrease in mitochondrial genome copies (mitochondrial genome copies to nuclear genome copies ratio, normalized to N2 control) (Figure 31). For the two-way ANOVA, there was no interaction or effect of strain, but there was an effect of dose (p<.0001*).

![Figure 31: Paraquat similarly decreases copy number in all strains tested (N2, pink-1, pdr-1).](image)
B.2.2 The pink-1 mutant appeared to be more resistant to paraquat-induced neurodegeneration

We evaluated dopaminergic neuron morphology at 48 hours, 96 hours, and 7 days after paraquat exposure. When we compared the pdr-1 mutant to the control strain, most comparisons were not statistically significant; the exceptions were the 54 μM exposure after 48 hours (p<.0001*) and the 18 μM exposure after 7 days (p=0.0068*). There does not seem to be a difference in paraquat sensitivity between these strains (Figure 32). However, in both of those instances it is not obvious which strain was more affected (our statistical test only can determine if the distributions were different).

Interestingly, when we compared the pink-1 mutant to the control strain, the pink-1 mutant appeared to be less sensitive to paraquat than the control strain in several comparisons (Figure 32). P-values for the pink-1 vs BY200 comparisons are in Table 7. Shaded boxes represent comparisons in which the pink-1 mutant appeared to be less degenerated than control.

<table>
<thead>
<tr>
<th>BY200 vs pink-1:BY200</th>
<th>48 hrs</th>
<th>96 hrs</th>
<th>7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 μM</td>
<td>0.0462*</td>
<td>n.s.</td>
<td>0.0148*</td>
</tr>
<tr>
<td>18 μM</td>
<td>n.s.</td>
<td>0.0182*</td>
<td>0.0008*</td>
</tr>
<tr>
<td>54 μM</td>
<td>&lt;.0001*</td>
<td>n.s.</td>
<td>0.0072*</td>
</tr>
</tbody>
</table>

This is particularly interesting given the fact that we have also seen the pink-1 mutant be less sensitive than control when we exposed them to 6-OHDA (see chapter 3).
However, we must take into consideration that the neurodegeneration assay was only performed twice for the 96-hour and 7-day timepoints. In those instances, only 20 worms were evaluated, so caution must be exercised when drawing conclusions from this data.

Figure 32: Neurodegeneration score distributions for BY200, pink-1:BY200, and pdr-1:BY200 at 48 hrs, 96 hrs, and 7 days after exposure to paraquat.

**B.2.3 Paraquat did not alter the rate of oxygen consumption in glp-1 mutants**

Using a Seahorse XF-24 Bioanalyzer, we measured the oxygen consumption rate (OCR) in *glp-1* mutants following paraquat exposure. We did not observe changes in
basal or maximal respiration, or in coupled respiration, but we saw a decrease in oxygen consumption in the 50 µM group after the sodium azide injection (one-way ANOVA p=0.0385*, Tukey HSD p=0.0446*; Figure 33). This could signal a difference in the amount of proton leak (or differences in non-mitochondrial respiration). We then calculated mitochondrial parameters (ATP-linked respiration, spare respiratory capacity, and proton leak) in each treatment group; no statistically significant differences were found (Figure 34).

Overall, our OCR measurements indicate that paraquat exposure does not disrupt mitochondrial respiration in *C. elegans*.

Figure 33: Oxygen consumption rates after chemical challenges in paraquat-exposed *glp-1* nematodes.
Figure 34: Mitochondrial parameters following paraquat exposure in *glp-1* nematodes.
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Biography

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