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The role of oxidative stress in the Rotenone model Lymnaea stagnalis

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THE ROLE OF OXIDATIVE STRESS IN THE ROTENONE MODEL LYMNAEA STAGNALIS

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ABSTRACT

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Parkinson's disease (PD) is the most common neurodegenerative movement disorder, characterized by progressive depletion of dopamine (DA). Rotenone, a common pesticide, can induce PD like symptoms in animal models. The goal of this thesis is to quantify the levels and effects of reactive oxygen species (ROS) in brain tissue of the common neurobiological gastropod model, *Lymnaea stagnalis,* after treatment with Rotenone. The results show that rotenone causes a behavioral deficit and increases ROS production, especially superoxide. Furthermore, glutathione was depleted and protein carbonyl formation increased. These results are consistent with findings in human PD and the high levels of ROS we find in the *Lymnaea* model suggest that it is a useful avenue to explore the possible causative role of oxidative stress in dopaminergic neurodegeneration.

DEDICATION

The completion of my thesis would not be possible without the support of my parents and family.

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I will always be grateful to Dr. Plas chair of my thesis committee, for all the advice, support and mentoring. My thanks go to my committee members: Dr. Terry, Dr. Gilkerson, and Dr. Ahmad. Their advice, input, and comments on my career helped ensure the quality of my work.

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CHAPTER I

INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disorder, it severely compromises the quality of life of more than 1.5 million Americans, and costs billions of dollars each year to treat. It is clinically characterized by symptoms including, bradykinesia, tremors, rigidity, and the loss of dopaminergic (DA) neurons within the substantia nigra pars compacta (SNpc), a region of the midbrain (Shimizu et al., 2003). The affected neurons commonly exhibit protein inclusions called Lewy bodies (Spillantini et al., 1997). The main protein found in these bodies is α- synuclein, a synaptic protein whose functions still remain obscure (Dias, V. et al., 2013). Although many pathologies of the disease have been proposed, the cause of the disease still unclear.

While a cause for PD still remains unknown, it is not clear that a single entity can be pinpointed. Instead, it may be caused by several different, but related, factors (both genetic and environmental (Johnson ME & Bobrovskaya L, 20014). A small percentage of PD cases are caused by inherited mutations, which have been identified in humans and observed in model organisms: e.g. fruit fly and mice (Dauer & Przedborski, 2003). These genetic models are beginning to provide some understanding of the genetic causes of PD, but the environmental factors still remain to be understood

Some neurotoxins have been identified as playing a role in the pathology of some PD cases, including 6-hydroxydopamine (6-OHDA), 1,2,3,6,-tetrahydropyridine (MPTP), paraquat

(PQ), and rotenone. The effects of these neurotoxins are known to mimic many of the PD symptoms including impaired movement and loss of DA neurons (Przedborski et al.,2001; Bove et al,. 2005; Cohen G, 1984; Cannon J.R. & Greenamayer J. T. , 2010)

Rotenone is an organic compound produced in several plant such as jicama, and was commonly used as a pesticide. Rotenone is a hydrophobic compound which readily crosses the blood-brain barrier and acts as a potent complex I inhibitor of the mitochondrial electron transport chain. The neurotoxin has been reported to cause a selective toxicity to DA cells and behavioral impairment in animal models (Cannon, J. R., & Greenamyre, J. T., 2010). In addition, the effect of rotenone in animal models share many other similarities with human PD. Idiopathic PD has been characterized by an activity reduction of complex I, and the formation of alpha-synuclein protein aggregates which can also be mimicked by rotenone toxicity (Cannon, J. R., & Greenamyre, J. T., 2010)

Evidence supports that rotenone toxicity to DA neurons is due to the inhibition of the complex I of the mitochondrial respiratory chain (Nianyu Li et al., 2002). The effect of inhibiting the complex I is the release of reactive oxygen species (ROS), which in high amounts can lead to oxidative stress (Lagston JW et al., 1983). Oxidative stress is the unbalance between ROS and antioxidants in the cell, which can potentially damage macromolecules. Many experiments suggest that oxidative damage leads to the selective death of DA neurons (Dias, V. et al., 2013). This evidence is strongly supported by human postmortem experiments showing increased levels of macromolecules damaged by ROS (Floor E, & Wetzel M, 1998). Rotenone increases the level of ROS in animal models (Dias, V. et al., 2013).

There are severed rotenone animal models that are currently being studied in order to elucidate the etiology of PD. Rotenone can induce PD like symptoms in rodents (R. et al., 2000), and is known to show selective DA cell death in the SNpc (Cheng et al, 2010). The greater advantage of this model is the ability to induce the formation of alpha-synuclein aggregates in the CNS (Betarbet R. et al., 2000). The major disadvantage of this model is the lack of reproducibility in the animals exhibiting DA lesions, which vary in magnitude and location (Cannon, J. R., & Greenamyre, J. T., 2010). *Drosophila melanogaster* is the most common invertebrate model used for PD. After rotenone treatment, the fruit fly exhibits a reduction in locomotion and selective loss of DA cells (Meulener et al., 2005). However, the loss of DA neurons in the fruit fly can vary significantly, with some labs reporting no loss at all (Navarro et al., 2014).

Lymnaea stagnalis, a fresh water pond snail, is a widely used model in invertebrate neurobiology. The snail shows relatively simple behaviors that can be quantified including, locomotion, respiration, and feeding (Willows AD, 2003) The advantage of using this model compared to *Drosophila* is that the neurons in the central nervous system (CNS) are larger and identifiable. For example RPeD1 , is a giant pedal DA neuron of the snail which can be up to 250 μm in diameter. In addition all the DA neurons of the snail have been identified (Elekes et al.,1991).Vehovszky, and colleagues, found that a concentration from 0.1 to 5 μM of rotenone was toxic for the snail. The neurotoxin caused irreversible and progressive behavioral impairment in the snail. In addition, immunostaining showed that tyrosine hydroxylase, an enzyme responsible for DA production, was decreased below detectable levels. These experiments lay the ground work for the usage of *Lymnaea stagnalis* as possible model organism

for PD. However, the mechanisms which are responsible for the effects of rotenone in *Lymnaea stagnalis* have not been explored.

The purpose of this study is to elucidate the mechanisms of roteneone's role in the CNS, specifically addressing the extent and effect of oxidative stress in *Lymnaea stagnalis.* The snails were treated with a 0.5 μM rotenone as indicated by Vehovsky et al. in 2007. The first series of experiments I performed were designed to measure the production of reactive oxygen species (ROS) in control and rotenone-treated animals. For this, I used the fluorescent probes, 2' 7' DCF and Mitosox, visualized by confocal microscopy. The quantification of these results showed that the levels of ROS in the CNS of rotenone treated snails increased by several fold. In an effort to further correlate the production of ROS with what is found in human PD, I characterized the role of glutathione, a peptide that serves as a defense mechanism for ROS in cells. The results led me to analyze the effects that ROS have on macromolecules. Because PD is known to result in protein misfolding and accumulation, I proceeded to examine the damage ROS causes to proteins, specifically in carbonyl group formation. Carbonyl groups are detrimental to proteins, causing misfolding and loss of function (Levine R.L. et al., 1994). I used a spectrophotometric assay based on the reaction of protein carbonyl groups with DNPH as suggested by Levine and his lab, and the results revealed that carbonyl formation in neurons after rotenone treatment was significantly increased. Though not unexpected, these results were an important step in characterizing the *Lymnaea stagnalis* rotenone model and established that similar mechanisms are at work, compared to rodent models. Once I established the high levels of protein damage, I began to explore the cellular response. There are several mechanisms by which a cell can dispose of damaged proteins including proteolysis via autophagy and the ubiquitin-proteasome system. By immunoblotting I was able to show substantial increases in the overall level of the protein

ubiquitination and by using lysotracker, a commercially available dye for autophagosomes, I was able to quantify the levels of autophagy and proteolysis. Finally, in order to show that rotenone is related to neuronal death, I was able to demonstrate via immunoblotting that levels of cleaved-caspase 3, a common apoptosis marker, significantly increased. My data suggests that rotenone induces oxidative stress, protein damage, high level of ubiquitinated proteins, and apoptosis, similar to human PD. Furthermore, this experiments contribute to the potential use of the pond snail as a model organism for PD.

CHAPTER II

REVIEW OF LITERATURE

Parkinson's' Disease Pathology

Parkinson's disease (PD) is the second most common neurodegenerative disorder. It is clinically characterized by symptoms that include, bradykinesia, tremors, rigidity, postural instability, flexed posture, and freezing gait (Fah S, 2006). In the CNS, PD is characterized by the selective loss of dopaminergic (DA) cells in the substantia nigra pars compacta and subsequent decrease of DA levels in the striatum (Shimizu et al., 2003). The affected neurons commonly exhibit fibrillary cytoplasmic inclusions called Lewy bodies, containing ubiquitin and alpha synuclein. (Spillantini et al., 1997) Although, the neuropathology and the symptoms have been characterized, the etiology underlying the disease still remains to be elucidated.

While a cause for PD still remains unknown, it is not clear that a single entity can be pinpointed. Instead, the disease may be caused by the interplay of several different, but related, factors, both environmental and genetic (Johnson ME & Bobrovskaya L, 2014). PD commonly arises as a sporadic condition, but, in a few cases the disease can be inherited (Dauer & Przedborski, 2003). These cases are correlated with single gene mutations in any one of the genes Park 1 through 10, which include alpha-synuclein, parkin, PINK1, LRRK2,and DJ1 (Dias, V. et al., 2013). Sporadic and genetic forms of PD are almost indistinguishable, meaning that

they share the same pathology, though, many of the inherited forms exhibit early onset. The discovery of these genes has yielded some insight into the molecular mechanisms of PD. These genes have very different roles in the cell, but there is one key element that unifies them: they are involved in mitochondrial function (Dias, V. et al., 2013).

Mitochondrial Dysfunction in Parkinson's disease

Mitochondria are dynamic organelles with the ability to perform many functions, including ATP synthesis, calcium homeostasis, and apoptosis. Damage to the mitochondria is detrimental for the cell and is associated with PD (Zhu J. & Chu CT, 2010) and many other diseases. Mitochondria possesses a double membrane structure, and on the inner membrane it contains the machinery to generate ATP, called the electron transport chain (ETC). The ETC is the main source of reactive oxygen species (ROS) in the cell (Turrens JF, 2003), and dysfunction can lead to oxidative stress. The general mechanism of ROS production involves the 'leakage' of electrons from the ETC (Turrens JF, 2003). Mitochondrial dysfunction was initially linked to PD when the neurotoxin MPTP was recognized to induce Parkinson-like symptoms (Lagston JW et al., 1983). MPTP is able to cross the blood brain barrier where is taken up by astrocytes and transformed into MPP+. MPP+ is a substrate that can be taken up by the dopamine transporter in the DA cells, where it shown to inhibit the complex I of the mitochondria. Complex I (NADH: ubiquinone oxidoreductase) is a multi-subunit protein that catalyzes the first step in the mitochondrial electron transport chain. It receives a high-energy electron from NADH and transfers it to ubiquinone, yielding a ubiqunol. Ubiquinol transfers electrons to Complex III. Complex I dysfunction has been observed in PD and it may lead to neuronal degeneration (Lagston JW et al.,1983).

Oxidative Stress and Parkinson's disease

Dysfunction of the mitochondrial electron transport chain generates ROS (Turrens JF, 2003), which in high amounts leads to oxidative stress. Oxidative stress is the inbalance between the levels of ROS and the defense mechanisms of the cell, leads to the oxidative damage to macromolecules. ROS is the activation of molecular oxygen, including hydrogen peroxide (H_2O_2), superoxide anion radical (O_2^-) and hydroxyl radical ($^*OH$). Superoxide anion is primarily produced by dysfunction of complexes I and III, and it has the capability to cross the mitochondrial membrane where it is highly reactive with macromolecules (Muller FL et al., 2004).

The human brain during normal function produces a high amount of ROS. The brain uses about 20% of the entire oxygen supply of the body, and a relatively large amount of that is converted to ROS (Johnson WM et al., 2012). The electron transport chain is known to be the main contributor of ROS, but there are other sources that are still being investigated (Yan MH et al., 2011). Evidence suggests that oxidative stress and mitochondrial dysfunction leads to the selective degeneration of DA cells in PD (Schapira AH. & Jenner P, 2011). For example in human postmortem brains. There are high levels of 4-hydroxyl-2-nonetal (HNE), a by-product of lipid peroxidation (Jenner P, 2003), proteins with carbonyl modifications (Floor E. & Wetzel M, 1998), and DNA oxidative damage in form of 8-hydroxygeoxyguanosine (Muller FL et al., 2004) are found.

Carbonyl (CO) groups, aldhydes and ketones, are produced when proteins are exposed to ROS. Proline, arginine, lysine and threonine are the amino acids, that when oxidized, form carbonyl groups. These CO modifications can also be introduced to proteins by HNE, a by-

product of lipid peroxidation and generated as a reaction of reducing sugars. The concentration of CO groups in proteins is the most common indicator of protein oxidation, and it is used in order to detect oxidative stress in many diseases (Berlett BS et al., 1997).

It has been shown that there is a decreased amount of glutathione (GSH) in postmortem brain tissue of PD patients. (Dias, V. et al., 2013). GSH is a peptide consisting of three amino acids, glutamate, cysteine and glycine. In presence of ROS, cysteine's thiol group forms a sulfide bond with and adjacent GSH peptide and generating glutathione disulfide molecule. The tripeptide is a well known antioxidant molecule (Dias, V. et al., 2013). GSH is synthesized in the cytoplasm, and then is transported to the mitochondria. The levels of GSH have been recognized as a maker for mitochondrial homeostasis. GSH has been shown to decrease upon impairment of the complex I of the mitochondria. Furthermore, it has been suggested that down regulation of GSH in rat brain is correlated with neurodegeneration of DA cells in the SNpc. (Dias, V. et al., 2013)

Ubiquitin Proteasome System (UPS)

The ubiquitin-proteasome system (UPS) is the principal pathway for cells to degrade unwanted proteins (Olanow CW & McNaught KS, 2006). During oxidative stress, carbonyl groups are the main modification when proteins are exposed to ROS (Levine R. L. et al., 1994). These covalent modifications result in the misfolding of proteins leading to a loss of function (Levine R. L. et al., 1994). The clearance of these proteins by the proteasome is considered to be a defense mechanisms, since it will prevent detrimental aggregates. The UPS system also plays an important role in the degradation of dysfunctional mitochondria, preventing generation of ROS (Bennett MC et al., 1999). Mutation in the genes Parkin and UCH-L1, which are linked to

PD and are components of the UPS, indicate the potential role of UPS in PD (Olanow CW & McNaught KS, 2006).

During oxidative stress, the UPS systems has been shown to be dysfunctional due to ROS damage. Inhibition of Complex I in the mitochondria is suggested to impair proteoseomal activity due to oxidative modifications of the proteosomal complex (Shamoto-Nagai M et al., 2003). This evidence is supported by McNaugh and his lab, where they show that in the SNpc of PD patients there impaired UPS with structural proteosomal alterations which include the loss of the alpha-subunit (McNaught St. P et al., 2003). Therefore, this suggest that oxidative stress can cause UPS dysfunction, which can exacerbate the vulnerability of DA cells in PD.

Autophagy in Parkinson's disease

Autophagy is a cellular defense pathway that engulfs dysfunctional organelles or misfolded proteins in a double membrane system called the autophagosome. Autophagosomes are suggested to play a role in the pathogenesis of neurodegenerative diseases including PD (Chinnery P.F., & Schon, E. A., 2003). The first experiments that link the important role of autophagy and PD came from the demonstration that alpha-synuclein is degraded by macroautophagy (Karbowski, M., 2010). Besides degradation of alpha-synuclein, autophagy is also responsible to degrade dysfunctional mitochondria. Damaged mitochondria increase the production of ROS, therefore it is important that cells have a mechanism to degrade them. The selective degradation of mitochondria by autophagy is called mitophagy. It has been reported that mitochondrial DNA in the DA neurons of PD patients exhibit a higher rate of oxidative mutations (Bender, A et al., 2006). Additionally, mutations in PINK1 and PARKIN, genes involved in mitophagy, are known to cause autosomal recessive PD (Banerjee R. eta al., 2009). PARKIN, an E3 ubiquitin ligase, is responsible for tagging dysfunctional mitochondria for

degradation. Mutant PARKIN is associated with associated with high levels of protein carbonyls, and DNA and lipid oxidative damage (Hyun DH et al., 2002). Suggesting that failure to eliminate damaged mitochondria plays an important role in the degeneration of DA cells in PD.

Cell death in Parkinson's disease

Neurodegeneration is generally characterized by a progressive course of neuronal death. There are two main forms of cell death that are known to happen: necrosis and apoptosis. Necrosis is the result of cellular injury inducing premature cell death. Necrosis can be caused by factors in the cellular environment such as toxins, infection, or trauma. Apoptosis is known as programmed cell death. Apoptosis is a key process in the physiology of all living organisms because it serves to balance tissue development by degrading unwanted cells, selectively and without damaging neighbor cells. This process is described by several phenotypical features including nuclear condensation, cell shrinkage, and DNA degradation. These events are characterized by a cascade of events in which the family of cysteine proteases known as caspases cleave several cellular substrates. In an apoptotic cell death there is a change of expression of genes, mainly oncogenes, which will allow for the apoptotic process to be efficient (Often et al, 2000). Evidence of apoptotic cell death has been identified through all stages of PD, in patients and animal models of PD. Oxidative stress has been the leading hypothesis as the main mechanism in which DA cells enter apoptosis. This was supported by several cell culture studies that showed that treatment with antioxidants will significantly decrease neurodegeneration via apoptosis (Lev et al., 2003)

Toxin Induced Models of Parkinson's disease

In order to elucidate the role of environmental factors in PD, some neurotoxins have been used, including 6-hydroxydopamine (6-OHDA), 1,2,3,6,-tetrahydropyridine (MPTP), paraquat (PQ), and rotenone. The correlation between PD and oxidative stress is further supported by the observed effects of these toxins in animal models.

MPTP was first discover in the early 1980s, where drug users of California developed PD like symptoms following intravenous injections of the street drug, meperidine. After identification of this drug, MPTP was found to be a side product of the illicit manufacture of the narcotic. MPTP is characterized as a selective Complex inhibitor I, ands has been used in order to mimic PD pathology in a variety of animal models, ranging from mammalian to invertebrates (Przedborski S et al., 2001). The neurotoxin causes PD like symptoms and pathology, including impaired movements and the depletion of DA cells (Przedborski S et al., 2001). It is notable, though, that the formation of Lewy bodies (LB), a hallmark of PD, has not been observed using MPTP (25).

Paraquat is a potent herbicide that is known to produce PD like symptoms through oxidative stress. Paraquat toxicity comes from the interaction with cellular diaphorase such as nitric oxide synthase, increasing the amount of ROS (Day BJ et al., 1999). Epidemiological studies have linked paraquat exposure with PD in humans (Bove et al,. 2005).When this toxin is administered to mice, there have been some confusing results, ranging from impaired motor activity and loss of DA neurons to no symptoms at all (Bove et al,. 2005).

6-OHDA is an extensively used neurotoxin used for both *in vitro* and *in vivo* studies of PD. This toxin shares a structural similarity with catecholamines like norepinephrine and

dopamine. The similarity allow for 6-OHDA to be transported selectively into DA and norepinephrine neurons through cell transporters (Bove et al,. 2005). Since in PD DA cells are mainly affected, in order to avoid norepinephrine cells, extensive techniques should be implemented. It is well accepted that 6-OHDA induces DA cell death through the combination of ROS and quinones (Cohen G, 1984).

Rotenone is the most recent neurotoxin used for PD research. Rotenone is a potent member of the rotenoid family, natural toxins extracted from Leguminosa plants. The neurotoxin is commonly used as a pesticide around the world, since its half-life is very short. Epidemiological data has linked human exposure to rotenone to a higher risk of having PD (Butterfield PG et al., 1993).Rotenone is a hydrophobic compound, which allows it to freely cross cellular membranes and gain access to the brain independent of transporters. The neurotoxin is a high affinity Complex I inhibitor, leading to the reduction of ATP levels and electron leak leading to production or ROS in the form of superoxide, subsequently leading to reduce of glutathione levels and oxidative stress(Degli Espositi M. et at., 1998). Similar to PD, rotenone induces the motor dysfunction, formation of cytoplasmic inclusions, Lewy bodies, containing alpha synuclein and ubiquitin (Cannon, J. R., & Greenamyre, J. T., 2010). Furthermore, rotenone is also associated with the selective death of DA cells in the SNpc in mice (Cannon, J. R., & Greenamyre, J. T., 2010). However, the administration of the neurotoxin to rats yields high mortality rates, and is difficult to replicate.

Rotenone Models

The rodent rotenone model recently gained attention after it was shown that the neurotoxin applied systematically to rats con reproduce the hallmarks of PD (Cannon, J. R., & Greenamyre, J. T., 2010). The major advantage of using the rat as a model is the formation of

cytoplasmic inclusions, Lewy bodies, containing alpha-synuclein and ubiquitin, leading to impaired behavior, therefore replicating the pathology seen in human PD (Cannon, J. R., & Greenamyre, J. T., 2010). Additionally, there is a strong correlation between the loss ofTyrosine hydroxylase (TH) expressing cells (in DA cells) and the impaired motor function (Cannon, J. R., & Greenamyre, J. T., 2010). Rotenone exposure to rats has been shown to result in high levels of protein carbonyls and oxidative stress damage of other macromolecules (Betarbet R. et al., 2000). A key disadvantage of the rodent rotenone model, is the lack of reproducibility in the DA lesions, the magnitude of the lesion, the location, and the mortality rate (Cannon, J. R., & Greenamyre, J. T., 2010).

C. elegans are transparent nematodes, usually of 1mm in length commonly found in temperate soil environments. The nematode was the first organism that had its whole genome sequenced and is the only organisms that has its CNS connectome completed. *C. elegans* has a simple nervous system making it ideal for neurodegeneration studies. The organisms is grown in pre-coated plates treated with rotenone, and show a significant loss of DA neurons and movement abnormalities (Mocko JB et al., 2010). The DA cells of the nematode show no alpha-synuclein aggregates since the protein is a vertebrate exclusive protein. No further studies have been done in the nematode in order to investigate the pathology of the *C. elegans* rotenone model.

Drosophila melanogaster was the first model used in order to study PD. The fly has elucidated the molecular function and the pathways involved in many genes associated with familial PD. Some of the main advantages of this model is the short life cycle, advance genetic tools, and the reduced area needed in order to contain them. In order to elucidate the effects of rotenone in *Drosophila,* the flies are fed with food containing rotenone. Rotenone causes

significant depletion of DA cells in the CNS of the fly (Coulom H et al., 2004. The concentration of rotenone used is also correlated to the impaired movement (Coulom H et al., 2004). Conversely, the DA cell death reported in the fly is not consistent and it varies depending on the laboratory. Some laboratories show considerable loss and some show no loss at all (Navarro JA et al., 2014). Furthermore, some studies suggest that administration of rotenone to *Drosophila* causes a decreased in serotonin levels, which is not congruent with PD pathology (Coulom H et al., 2004).

Lymnaea stagnalis, a freshwater pond snail, is a widely used model in invertebrate neurobiology. The snail shows relatively simple behaviors that can be quantified and explored, including locomotion, respiration and feeding (Willows A.D., 2003). An advantage of using this model is that the rotenone can be dissolved in water, hence the exposure occurs though skin absorption and by swallowing during feeding. Furthermore, *L.stagnalis* contains neurons in the CNS that can measure up to 250µm in diameter, such as RPeD1 which is a giant pedal DA neuron. In addition all the DA neurons of the snail have been identified and mapped (Elekes et al.,1991). Vehovszky, and his lab, found that a concentration from 0.1 to 5 µM of rotenone was toxic for the snail. The neurotoxin caused irreversible and progressive behavioral impairment in the snail. In addition, immunostaining showed that tyrosine hydroxylase, enzyme responsible for DA production, was decreased below detectable levels. These experiments lay the ground work for the usage of *Lymnaea stagnalis* as possible model organism for PD. However, the mechanisms that are responsible for the effects of rotenone in *Lymnaea stagnalis* have not been elucidated.

CHAPTER III

METHODOLOGY AND FINDINGS

Materials and Methods

Animals A breeding stock of *Lymnaea stagnalis* was maintained in 'snail water' made by dissolving 1gram/gallon of 'Instant Ocean' aquarium salts in deionized water. The snails were fed with Romaine lettuce ad libitum. Animals used in the study had a shell length of 2-3cm.

Rotenone Treatment Stock solutions (10 mM) of rotenone were made in dimethyl sulfoxide (DMSO) and diluted in snail water to reach a final concentration of 0.5 μM rotenone. Experimental animals were placed in a separate tank with a volume of 650ml for 48hrs.

Behavioral Analysis Snail locomotion was analyzed pre- and post-rotenone treatment. Each animal was placed in a small container and its crawling behavior was video recorded for 15 minutes. The video file was processed using ImageJ analysis software (Wayne Rasband, National Institutes of Health, version 1.36b), and the individual traces made by a single animal were measured and analyzed using the additional plugin MtrackJ (Biomedical Imaging Group Rotterdam). Data are reported as mean ± SEM. Statistical tests were performed on the mean distance crawled using MS Excel, with Student's t-test for significance level.

Dissection and Cell culture. Animals were anesthetized by immersion for 45 minutes in 16mM 1-phenoxy-2-propanol solution. Ganglia were dissected and placed in a 5% solution of Protease

type XIV (Sigma) for 15 min. Ganglia were mechanically dissociated using glass pipets, and placed into poly-D-lysine coated glass-bottom dishes.

DCF, Mitotracker, MitoSox and Lysotracker staining Neuronal cells were stained with MitoSOX red (100 nM, Molecular Probes or DCF (1mM)) and imaged with an Olympus FV1000 Scanning Laser Confocal Microscope. For these experiments, cells in each sample were randomly chosen for imaging and all confocal laser and detector settings during image acquisition were the same. Fluorescence level was quantified by calculating the corrected total cell fluorescence (CTCF) using Image J. This procedure subtracts background and calculates the average fluorescence per unit area

Western Blot Pedal and Cerebral ganglia were separated from the rest of the brain, and where homogenized in a glass homogenizer using Tris HCL buffer (20mM Tris-HCL, pH 7.4, and a cocktail of protease inhibitors containing AEBSF, Aprotinin, Bestatin, E64, EDTA, Leupeptin, and Pepstain A). Lysate was then homogenized at 13,000RPM at 4ºC for 20mins. Lysates of whole brain were separated by SDS-PAGE and transferred to a nitrocellulose membrane using a transfer apparatus according to the manufacturer's protocols (Bio-Rad). After incubation with 5% nonfat milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20) for 60 min, the membrane was washed three times for 10 minutes with TBST and incubated with antibodies against ubiquitin (Santa Cruz, 1:2000), cleaved caspase-3 (Cell Signaling, 1:1000), tyrosine hydroxylase (Immunostar, 1:2000), and actin (Sigma, 1:10,000) at 4 °C for 12 h. Membranes were washed three times for 10 min and incubated with a 1:5000 dilution of horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies for 2 h. Blots were washed with TBST three times and developed with the ECL system (Amersham Biosciences) according to the manufacturer's protocols.

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Carbonyl Assesment Carbonyl assessment was performed as described by Reznick and Packer 1994. Briefly, whole brain was homogenized in a glass homogenizer with.2 ml of homogenizer buffer (0.1% digitonin, 50mM potassium buffer, pH 7.4, with a protease inhibitor cocktail and 1mM EDTA). The lysate was incubated for 15 minutes at room temperature, and then it was centrifuged at 6000g for 10 mins. The supernatant was analyzed for nucleic acid, which can interfere with the carbonyl assay. The supernatant was separated in two .05ml aliquots. Dinitrophenyl hydrazine (10mM in 2.5M HCL) was added to one aliquot, and 2.5M HCL to the other. Proteins were incubated for 1hr in the dark at room temperature. Protein pellets were then

dissolved in .1ml of 6M guanidine hydrochloride. Carbonyl concentrations were calculated as nmol/mg of protein as described by Reznick and Packer, 1994.

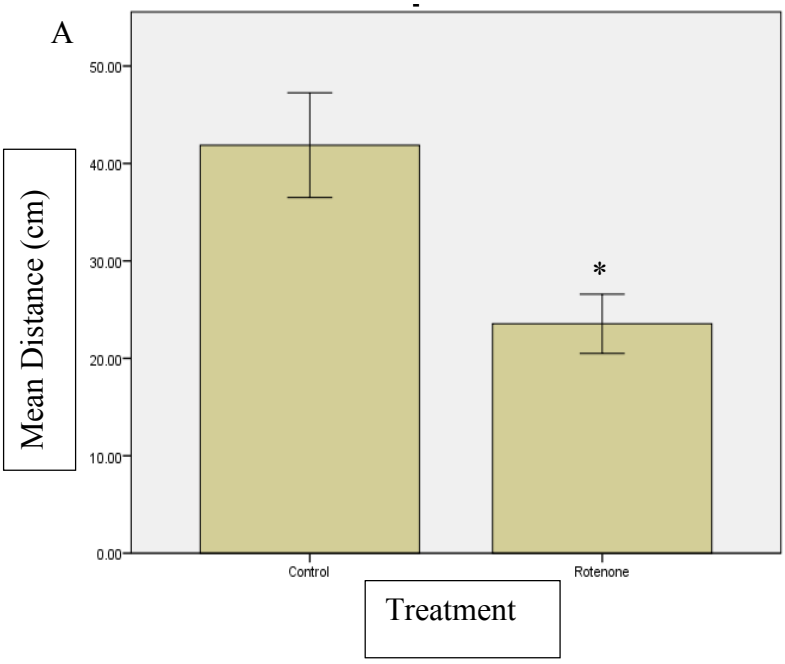
Glutathione Determination. Whole brain was homogenized in glass homogenizer using 1X Phosphate buffer (1mg per 3.3µL of buffer). The lysate was centrifuged ant 13,000RPM for 15mins at 4º C. Supernatant was then mixed with precipitating solution containing m-phosphric acid, sodium chloride, EDTA dissolved in water. Mixture was centrifuge for 5 minutes at 10,000RPM. Supernatant was separated in .1ml aliquots. .1ml of DTNB reagent was added along with .9ml of .3M odium phosphate dibasic. Glutathione concentrations were calculated in µM/ml according to Hassan Ahmad

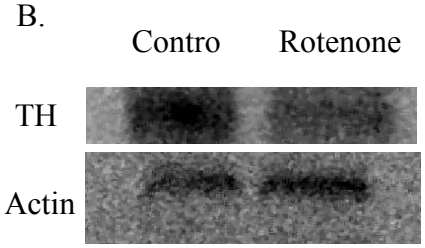
Results

Movement and Tyrosine Hydroxylase Analysis

In order to test rotenone toxicity, the spontaneous locomotion of both control and the .5μM rotenone treated snails were measured using Image J and analyzed using SPSS. As shown by Vehovsky et al. in snails and Greenamyre et al. in rats, rotenone has a deleterious effect in the locomotion of animals. As shown in figure 1A, the rotenone treated snails show almost a 50% reduction of locomotion after 48 hours of treatment compared to nontreated snails (42 ± 5 vs 24 ± 3 cm, $P<0.01$ n=140).These results are consistent with the finding of Vehovsky et al. Furthermore, in order to test rotenone impact on dopamine cells, western blots for Tyrosine Hydroxylase (TH) were performed in snail whole brain lysate (Fig 1B). Results show that Rotenone causes a significant decrease of TH expression shown in the western blot analysis (Fig 1C)

Fig.1 Rotenone Reduces Locomotion and Tyrosine Hydroxylase Expression.





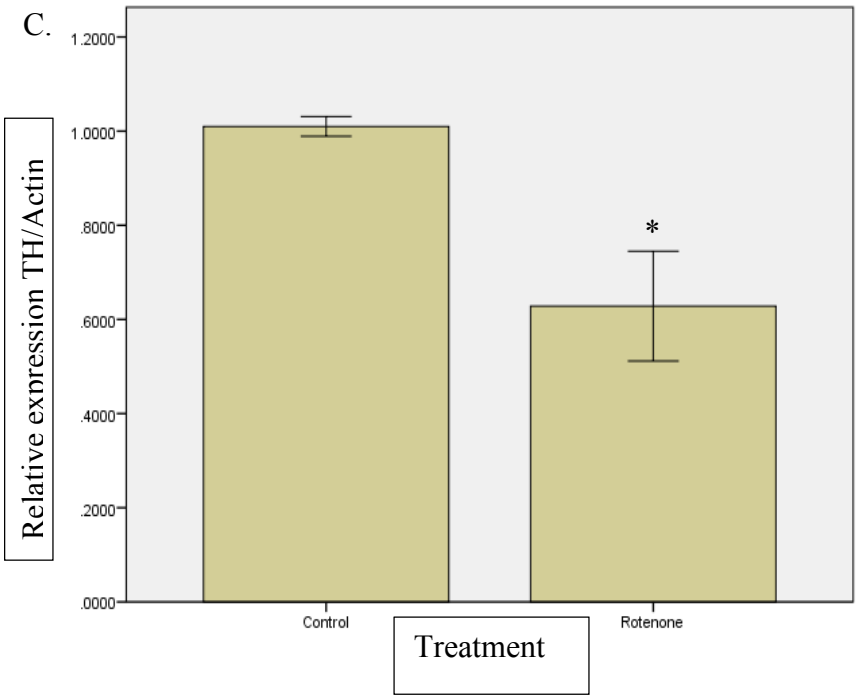


Fig. 1. (A) Rotenone treated snails show a decrease in locomotion. Bars show mean distance traveled by snails in control and rotenone treated groups. Control group exhibits a mean of 42±5cm; rotenone-treated group exhibits a mean of 24±3cm. *Indicates statistically significant at $p<0.05$, n=140. (B) Representative images of western blot experiments. (C) Relative levels of Tyrosine Hydroxylase in control and rotenone treated cells. Control group exhibits a mean of 1.01 ±.02 and rotenone treated group exhibits a mean of 0.62 ± .11 * Indicates statistically significant for $p<0.05$, n=3, using Students t-test

Rotenone Increases Cytoplasmic Levels of ROS

In order to examine the effects of rotenone on levels of ROS in the CNS, neurons were treated with common markers used for the identification of reactive oxygen species. MitoSOX (Life Technologies), was used in the snail CNS as a specific superoxide marker. Confocal microscopic imaging showed a significant increase in superoxide fluorescence of MitoSOX in *Lymnaea* neurons treated with .5 μM Rotenone for 48hrs (Fig. 2A). All images were gathered using random sampling at 600X total magnification. Laser sensitivity and intensity settings were held constant for all images. Histogram analysis shows an increase in the corrected total cell fluorescence (Fig. 2B).Quantitative measurements of the corrected total cell fluorescence demonstrated a two fold increase (1346 ± 232 and 2797 ± 313, $P<.001$ n=58) in the exhibited MitoSOX intensity of rotenone treated neurons.

Figure 2. Rotenone increases cytoplasmic superoxide concentration.

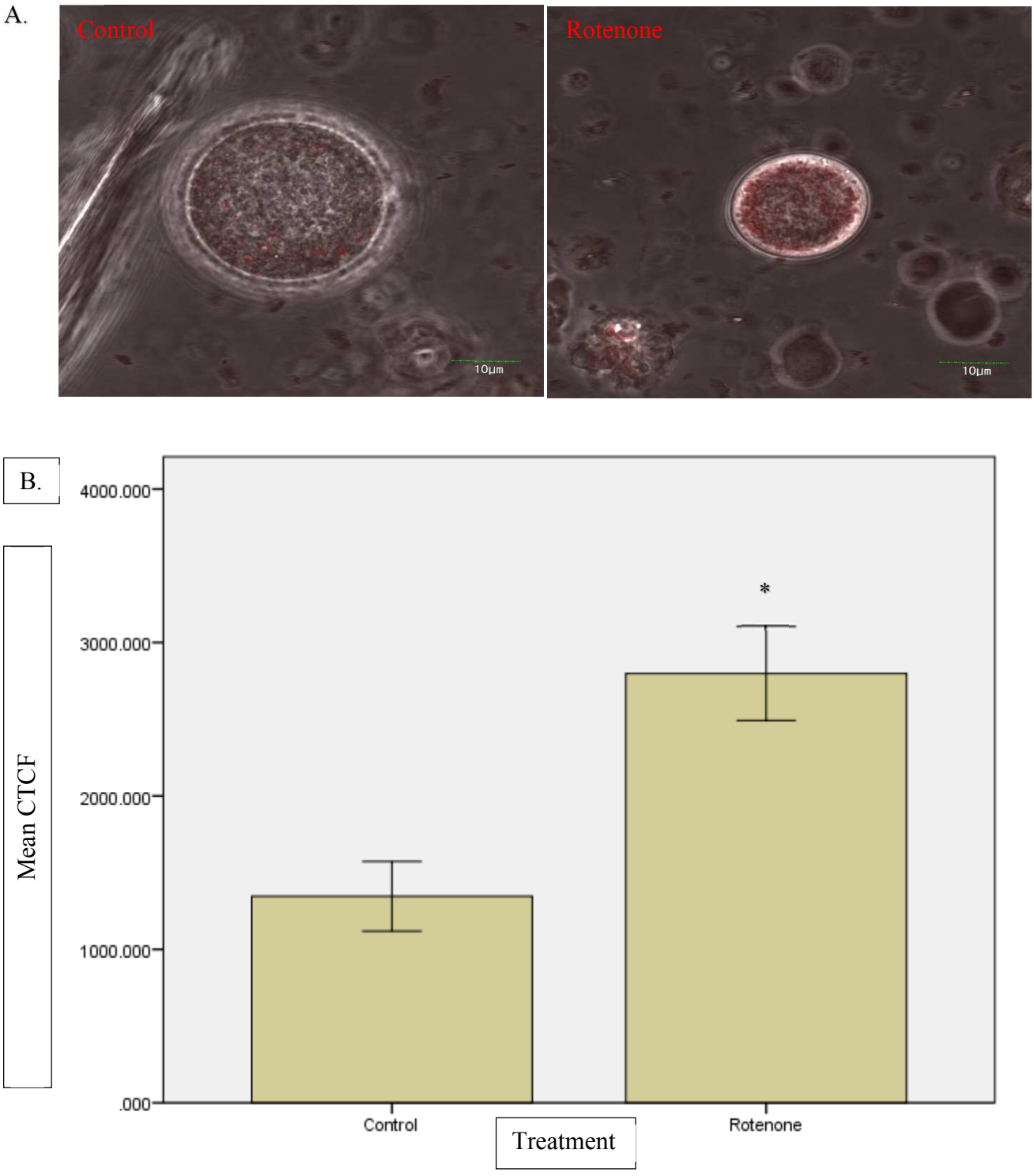


Figure 2. Rotenone-treated snails show an increase in concentration of intracellular superoxide species. (A) MitoSOX fluorescence labeling of superoxide species in neurons. (B) Corrected total cell fluorescence shows a two-fold increase in superoxide-specific fluorescence in rotenone-treated snails. Control group exhibits a mean of 1346 ± 232 and rotenone-treated group exhibits a mean of 2797 ± 313. * Indicates statistical significance at $p < 0.001$, n=5

To further supplement the data taken from MitoSOX experiments, treatment with the fluorescent dye, 2'7' dichlorofluroescein (DCF), which is indicative of all ROS, was used to assess the change in generalized ROS concentrations in the cell. DCF is a common probe that gets trapped in the cytoplasm of the cell and is easily oxidized by any ROS, transforming it into fluorescent dichlorofluorescein. Confocal microscopic imaging showed a significant increase in fluorescence of DCF in *Lymnaea* neurons treated with .5 μM Rotenone for 48hrs (Fig. 3A). All images were gathered using random sampling at 600X total magnification. Laser sensitivity and intensity settings were held constant for all images. Histogram analysis showed an increase in the corrected total cell fluorescence (Fig. 3B).Quantitative measurements of the corrected total cell fluorescence demonstrated a threefold increase (2726 ± 307 and 7028 ± 617), in the exhibited DCF intensity of rotenone treated neurons.

Glutathione (GSH) has to be transported to the mitochondria, where it functions as an antioxidant molecule. Since the GSH levels have become recognized as an important marker in the event or oxidative stress. The reaction between DTNB and GSH was measured by using a standard spectrophotometer assay, as described by Hassan Akhmad. Rotenone treated snails show decrease in mean concentration of GSH (Fig 4). Rotenone treated snails exhibit significant increase in mean concentration of glutathione concentration (Fig. 4A). Control snails exhibit a mean of 15.48± 3.8 μM and rotenone treated snails 6.09 ± 5.05 μM

Figure 3. Rotenone increases overall levels of intracellular ROS

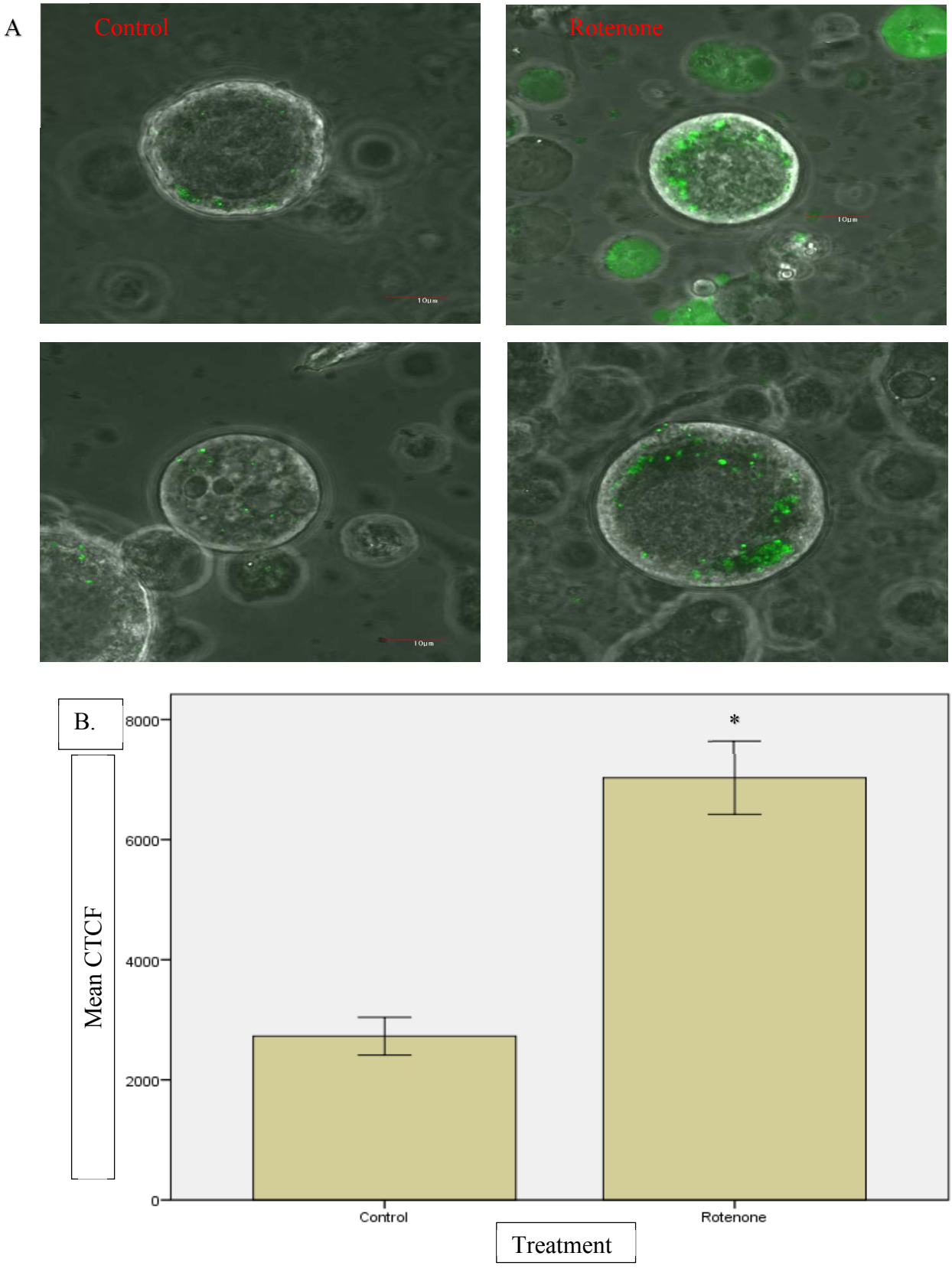


Fig. 3. (A) DCF fluorescence labeling of reactive oxygen species in neurons. (B) Corrected total cell fluorescence analysis shows a three-fold increase in DCF reactivity in rotenone treated cells. Control group exhibits a mean of 2726 ± 307 and rotenone treated group exhibits a mean of 7028 ± 617. * Indicates statistically significant for $p<0.05$, n=45.

Figure 4. Rotenone decreases intracellular Glutathione concentration

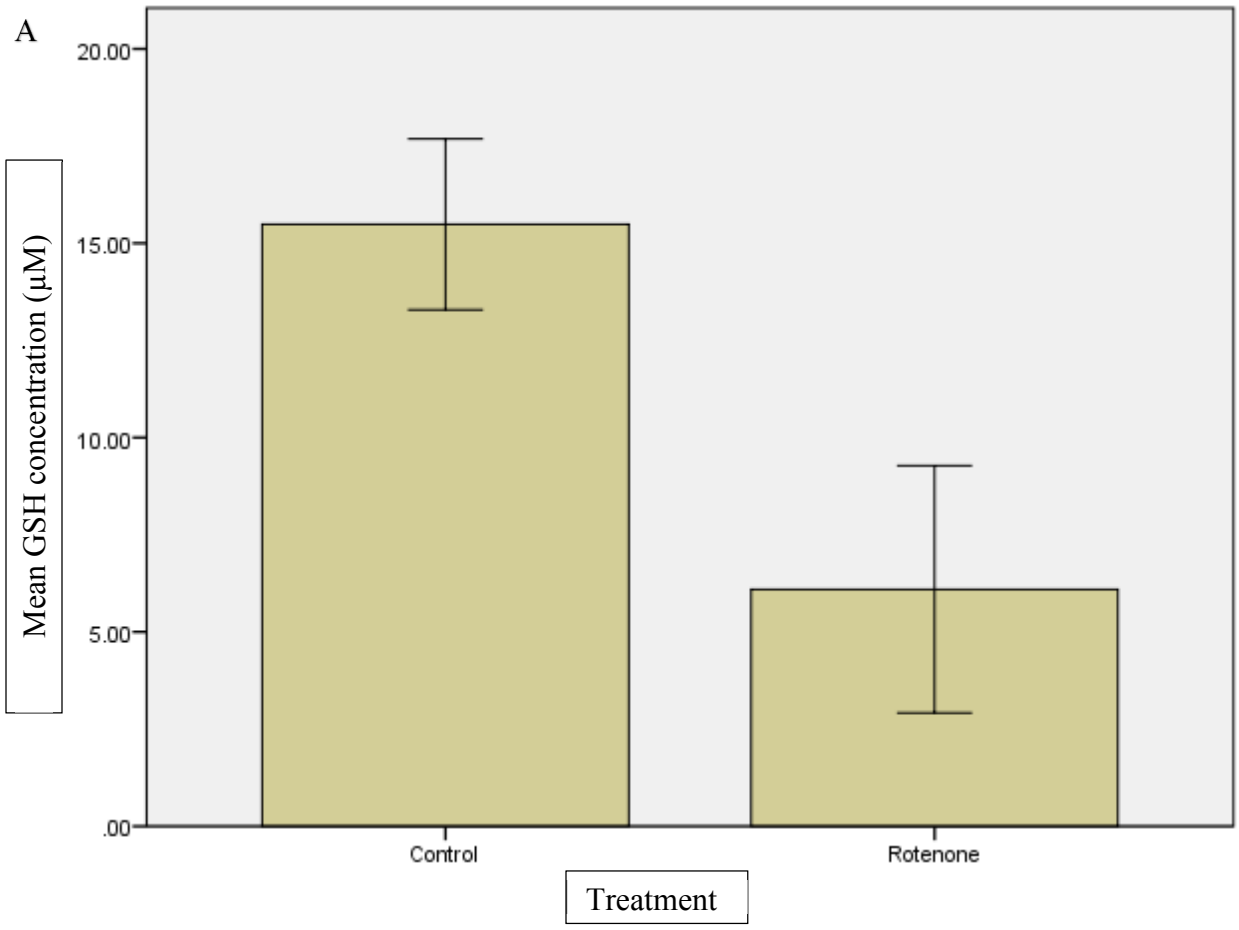


Fig. 4. Rotenone decreases intracellular Glutathione concentration. A) Bars shows SPSS

analysis of mean concentration of GSH in μM. Control snails exhibit a mean of 15.48± 3.8 μM and rotenone treated snails 6.09 ± 5.05 μM $p<0$

Protein Damage

To test the hypothesis that an increase in ROS concentration will cause protein damage within the cell, overall protein carbonyl modifications were analyzed. The reaction by 2,4 Dinitrophenyl hydrazine (DNPH) was measured by using a standard spectrophotometer, as described by Reznick and Packer 1994. Rotenone treated snails exhibit significant increase in mean concentration of protein carbonyl modifications (Fig. 5A). Control snails exhibit a mean of 0.59±.01nmol/ml and rotenone treated snails 5.86 ± .91nmol/ml.

In order to show that protein carbonyl modifications were detrimental for the function of cytoplasmic proteins, ubiquitin expression of rotenone-treated neurons was analyzed. Western Blot analysis was utilized to compare intracellular expression of ubiquitin. Rotenone-treated snails exhibit an overall increase in protein ubiquitination (Fig 6A). Intensity of ubiquitin expression from western blot analysis relative to expressed actin controls was analyzed and compared. Rotenone-treated snails exhibit a significant increase in mean expression of protein ubiquitination (Fig. 6B). Control snails' relative expression of ubiquitin/actin is 3.69 ±.31 and rotenone treated snails 7.45 ± 1.1.

Figure 5. Rotenone increases intracellular protein carbonyl concentration.

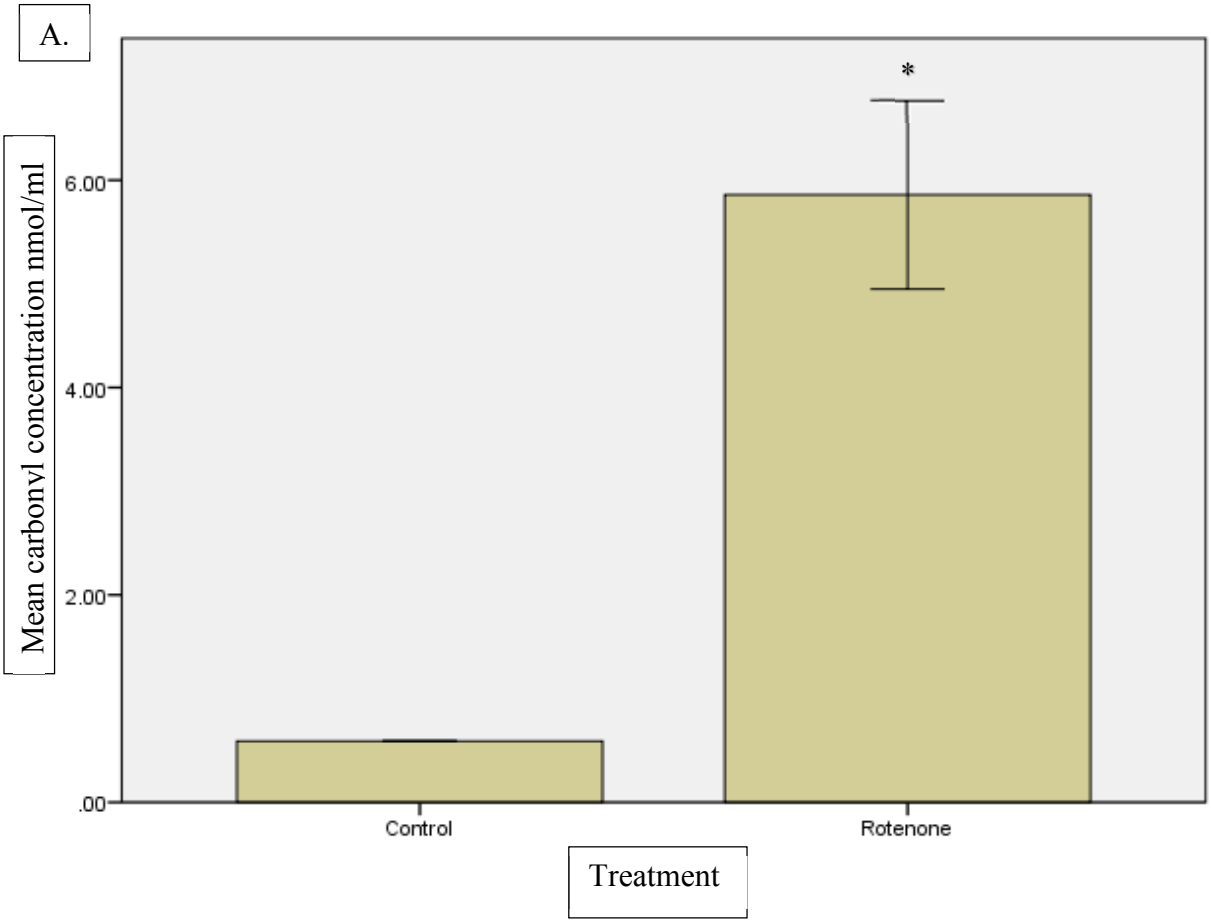


Fig. 5. Intracellular protein carbonyl concentration. A) Bars shows SPSS analysis of mean concentration of carbonyl in mmol/ml. Control snails exhibit a mean of 0.59±.01nmol/ml and rotenone treated snails 5.86 ± .91nmol/ml. * Indicates statistically significant for $p<0.05$, n=3.

Figure 6. Rotenone increases overall protein ubiquinylation

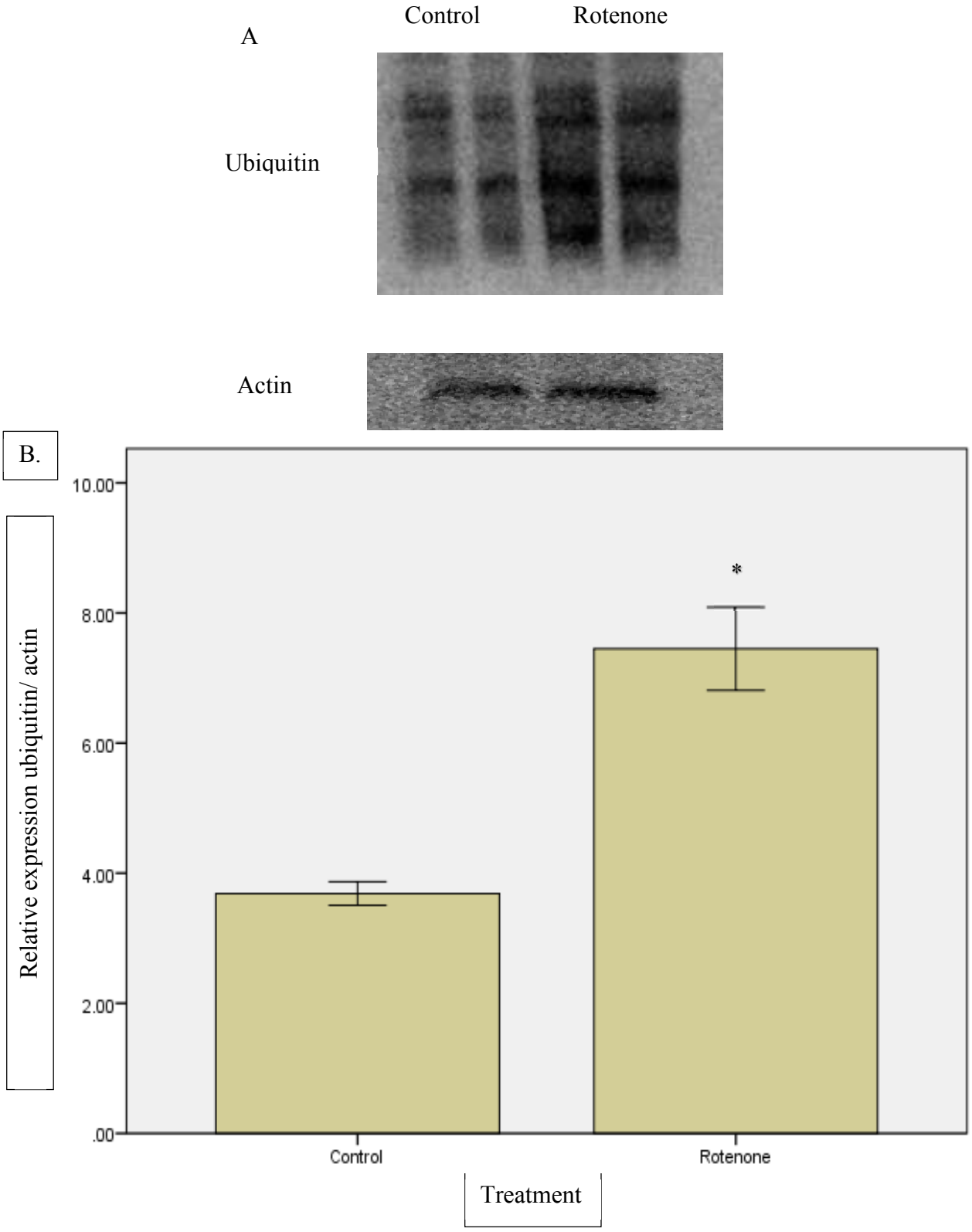


Fig. 6. Western blot analysis of ubiquitin. A) Representative images of western blot experiments B) Bars shows SPSS analysis of relative levels of ubiquitin in control and rotenone treated cells. Control group exhibits a mean of 69 ±.31and rotenone treated group exhibits a mean of 7.45± 1. * Indicates statistically significant for $p<0.05$, $n=3$.

Autophagy levels are a common indicator that the cell is undergoing oxidative stress. In order to quantify the levels of autophagy in the cell, Lysotracker, a specific autophagosome fluorescent probe, was used in the snails' CNS. Confocal microscopic imaging showed significant increase of Lysotracker's fluorescence in Lymnaea neurons treated with .5mM rotenone for 48hrs (Fig. 7A). All images were gathered using random sampling at 600X total magnification. Laser sensitivity and intensity setting were held constant for control and experimental images. T-test analysis shows an increase in the corrected total cell fluorescence (CTCF) (Fig. 7B). Quantitative measurement of CTCF demonstrated a significant increase in the mean fluorescence of rotenone treated snails 5.20 ±.81 as compared to control 0.92 ± .1.

Figure 7: Rotenone Upregulates Autophagy

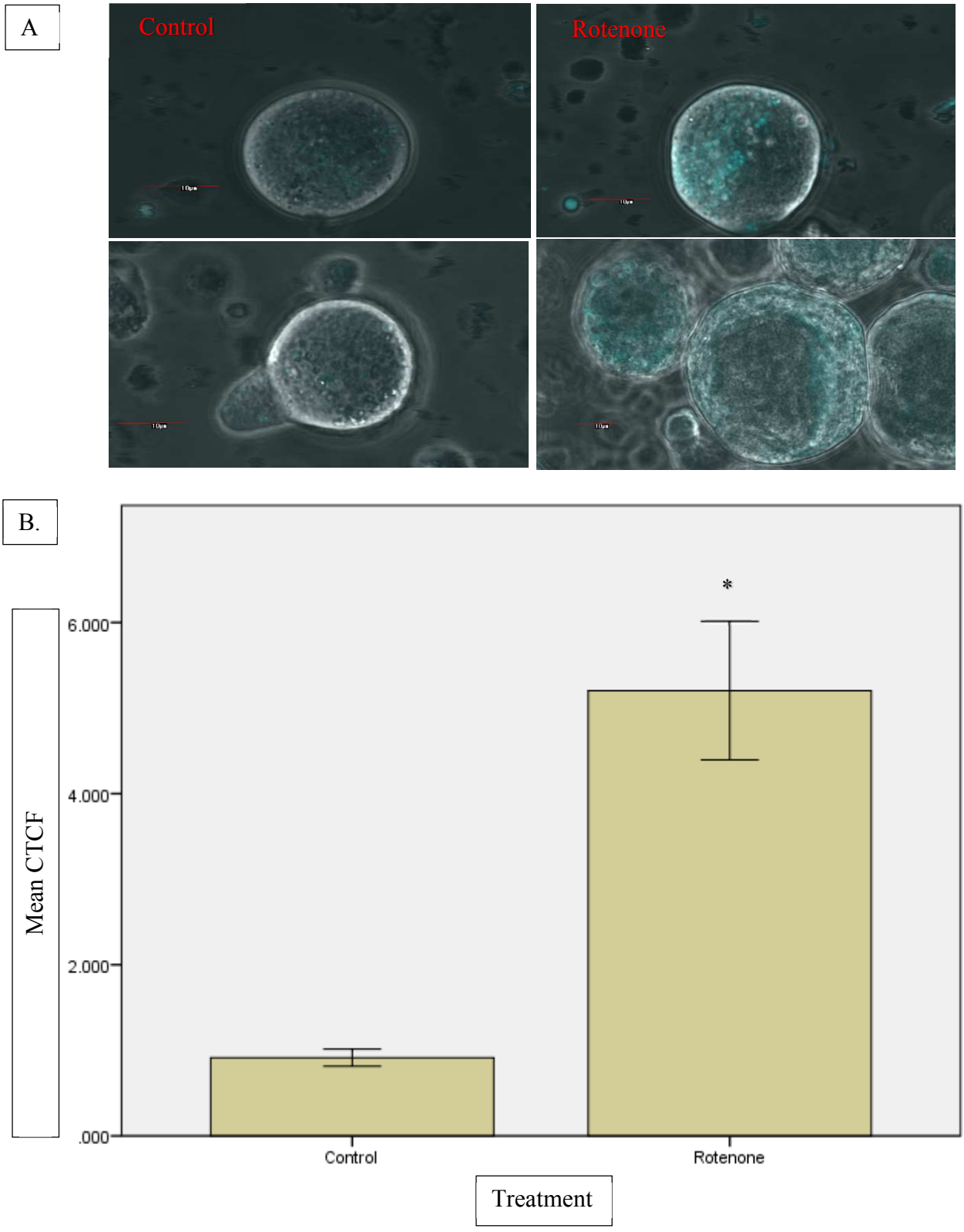


Fig. 7. (A) Representative images of Lysotracker fluorescence labeling of autophagy s in neurons. (B) Corrected total cell fluorescence analysis shows an increase in Lysotracker reactivity in rotenone treated cells. Control group exhibits a mean of 0.92 ± .1 and rotenone treated group exhibits a mean of 5.20 ±.81. * Indicates statistically significant for $p<0.05$, n=65.

Rotenone Increases Apoptotic Cell Death

Oxidative stress is the leading hypothesis for the upregulation of apoptotic neurons in PD. Detection of caspase 3 concentration is a reliable marker for the detection of apoptosis. Western blot analysis was utilized to compare intracellular expression of brain lysate cleaved caspase 3. Intensity of cleaved caspase 3 expression from western blot analysis relative to expressed actin controls was analyzed and compared (Fig 8A). Rotenone-treated snails exhibit a significant increase in mean expression of cleaved caspase 3 (Fig 8B). Control snails' relative expression of cleave caspase 3/actin is 0.64 ± .14 and rotenone treated 1.16

Figure 8: Rotenone Increases Overall Cleaved Caspase 3 Expression

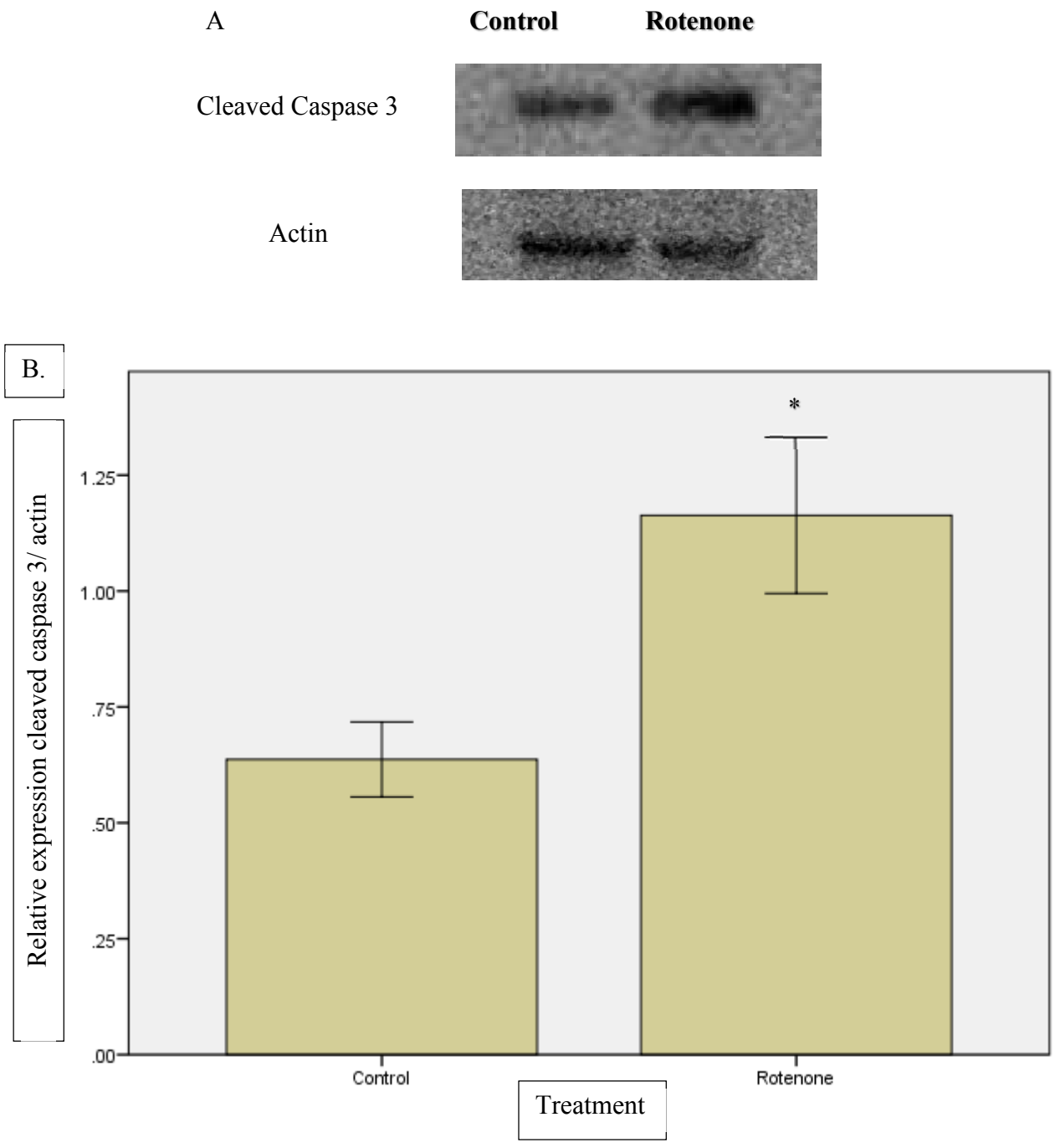


Fig. 8. : Rotenone Increases Overall Cleaved Caspase 3 Expression A) Representative images of western blot experiments B) Bars shows SPSS analysis of relative levels of cleaved caspase 3 in control and rotenone treated cells. Control group exhibits a mean of 0.64 ± .14 and rotenone treated group exhibits a mean of 1.16 ± .29. * Indicates statistically significant for $p<0.05$, n=3.

CHAPTER IV

SUMMARY AND CONCLUSION

The results of these experiments show that *Lymnaea stagnalis* can serve as an effective animal model for Parkinson's disease through the use of rotenone as an inducer of Parkinson's like symptoms in the snail. Symptomatically, rotenone is shown to induce changes in snail locomotion consistent with the characteristic primary changes in motor function in other animal models as well as in the human form of the disease in addition to decreasing intracellular concentrations of the dopamine precursor tyrosine hydroxylase (Fig. 1). This suggests that the symptoms of rotenone toxicity in the snail model are analogous to the loss of motor function and decrease in dopamine production characteristic of Parkinson's disease in humans. This is consistent with the findings of Vehovsky, et al. in 2007 which showed these symptoms in *Lymnaea*.

Additionally, these data confirm the role of oxidative stress within the cell as a common primary mechanism of the pathology of the disease. Evidence for this role is provided by the unique increase in intracellular reactive oxygen species exhibited by rotenone-treated *Lymnaea* neurons well beyond normal levels in control quantity (Fig 3). Furthermore, MitoSOX experiments provide evidence that superoxides, reactive oxygen species specific to the mitochondria, are also increased, indicating that oxidative stress within the mitochondria plays a primary role in the progression of the disease (Fig 2)

Consistent with the theory of reactive oxygen species as the primary mechanism of rotenone toxicity, the data show that oxidative modifications of proteins is prevalent in rotenone-treated animals. Analysis of intracellular carbonyl-modified proteins showed a significant increase in this type of protein damage in rotenone-treated snails over their healthy counterparts (Fig 6). It is hypothesized that the accumulation of this type of protein damage is at least partially responsible for the selective death of dopamine neurons in Parkinson's disease (Cannon, J. R., & Greenamyre, J. T., 2010). In addition to ROS-specific protein damage, rotenone is shown here to induce generalized protein damage as evidenced by the upregulation of protein ubiquitylation in cells exposed to the drug (Fig 6). Consistent with human PD, the levels of autophagy in neurons significantly increase, suggesting that cells are undergoing oxidative stress (Fig. 7)

PD is characterized by the selective degeneration of DA neurons, complex I dysfunction in the mitochondria, oxidative stress, protein damage, and proteasomal impairment. It has been shown by Greenamyre and his lab that rotenone is able to reproduce these effects in rats. My experiments have shown that the pathology of rotenone-treated pond snail *Lymnaea stagnalis* is consistent with all of the mentioned pathology of PD, contributing the body of knowledge which was initiated by Vehovsky, et al. The snail model shows compelling evidence that mitochondrial impairment can cause selective neurodegeneration of DA cells, and exposure can cause Parkinson like symptoms. Moreover, these experiments have laid the foundation for further research to be done using this manipulative model of PD.

Though the disease is sometimes caused by single gene mutations, most cases of PD are likely caused by an interaction between genetic susceptibility and accumulation of toxin exposure over the course of a lifetime. The *Lymnaea* model has shown that it shares some common mechanisms with PD. Although the extent of this research does not address the link between oxidative damage and the selective neurodegeneration of DA cells, we can speculate that inhibition of complex I, by rotenone, can lead to this phenomena. It is known that rotenone can interact with the mitochondria in all cells and that particular groups of cells, including DA cells, will have higher susceptibility, implying that failure to deal with these toxic effects lead to neurodegeneration across cell types. If so, we can be hopeful that neuroprotective treatments intended to treat one form of the disease may be more broadly useful for other aspects of the disease.

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BIOGRAPHICAL SKETCH

Homero Cantu graduated from The University of Texas-Pan American (UTPA) in 2013 with a B.S. in Biology and minors in Chemistry and Medical Spanish. He received a M.S. in biology from UTPA in May 2015. Since 2012 he has worked in Dr. Plas' lab where research is focused on neurodegenerative diseases. The lab is engaged in developing a new animal model that will contribute to the understanding of the cellular processes that lead to dopaminergic degeneration in Parkinson's disease. Using a rotenone snail model, Homero has explored the role of reactive oxygen species in dopamine cell death. Homero has co-authored six poster presentations at different conferences. In 2014, he presented a first-author poster explaining findings on early neuronal consequences of rotenone at the Society for Neuroscience annual meeting. Currently, he is writing up two manuscripts that summarize his work over the past year and a half in the lab. He will be the first author on a paper characterizing oxidative stress in the rotenone model, and has worked closely with another student and will be a co-author on a second paper describing the effects of rotenone on neuronal mitochondria. During his undergraduate career, Homero was an active member of the "Student Association for Medical Spanish". The association's main goal was to educate low-income communities about preventable diseases. In addition to his research and pursuit of master's degree, Homero has taught undergraduate anatomy and physiology laboratories for four semesters. In fall of 2015, Homero will begin a Ph.D. program in the department of neurobiology at Georgetown University. His current address is Relaxation St., Edinburg, Texas 78539.