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Activation of microglia in the rotenone Lymnaea stagnalis model of Parkinson's disease

Xiomara Galvan
The University of Texas Rio Grande Valley

ACTIVATION OF MICROGLIA IN THE ROTENONE LYMNAEA STAGNALIS MODEL OF PARKINSON'S DISEASE

A Thesis

by

XIOMARA GALVAN

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COMMITTEE MEMBERS

Dr. Megan Keniry
Chair of Committee

Dr. Robert Edwards
Committee Member

Dr. Angela Chapman
Committee Member

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ABSTRACT

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Parkinson's disease (PD) is the most common irreversible neurodegenerative movement disorder affecting approximately ten million people worldwide. In the central nervous system (CNS), PD is known by the selective loss of Dopamine (DA) cells of the substantia nigra pars compacta. Although the disease has been very widely studied, the underlying mechanisms are not yet understood. Several hypotheses have arisen as result of injury of the mitochondria, some experiments include microglia activation, microtubule damage, and reactive oxidative species, which all result in the death of DA cells. The theory of microglia activation upon inflammation was explored in this study. Using the snail *Lymnaea stagnalis*, Lipopolysaccharide (LPS) was administered to induce inflammation in the brain, and the neurotoxin rotenone was used to artificially induce inflammation on the brain of the snail by allowing it to be absorbed for several days and Isolectin B4 was used to stain the microglia cells. LPS and rotenone treated snails resulted in morphological changes of the microglia cells in the snail. Based on current results, rotenone in *Lymnaea stagnalis* could be used as model to determine if microglia phagocytizes dopamine cells during Parkinson's disease.

DEDICATION

The completion of my thesis would have not been possible without the support of my parents, my husband, and family. My mother, Guadalupe Galvan, my father, Omar Galvan, and my husband Rogelio A. Ramos. Thank you for your love and patience.

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CHAPTER I

INTRODUCTION

Parkinson's disease (PD) is the most common neurodegenerative disorder (Surmeier et al., 2010). Selective degeneration of Dopamine (DA) neurons in the substantia nigra (SN) causes the major PD symptoms, but there is often widespread neurodegeneration and pathology in other regions of the brain, including the protein inclusions called Lewy bodies (LBs). (Terzioglu and Galter, 2008). It is characterized clinically by motor dysfunction that exhibits gait, bradykinesia, rigidity and tremor of the extremities and head, and postural instability. Other non-motor symptoms occur as well, such as sleep disturbance, depression, sensory loss and subsequent dementia are common (Trinh and Farrer, 2013). By the time clinical manifestations appear, at least 50% of the dopamine neurons in the SN are already lost. Disease occurrence rises exponentially above the age of 65. Because life expectancy is increasing as a result of improvements in health care, the number of PD patients is expected to grow dramatically in the coming years, reaching over 2 million in the United States by 2030. (Surmeier et al., 2010)

Although the cause for PD still remains unknown, it is considered to be a result of a combination of genes and environmental factors. Several hypotheses have arisen as result of injury to mitochondria; some experiments include microglia activation (Barcia et al., 2012) microtubule damage (Bauereis et al., 2011), and reactive oxidative species (ROS) (Bosse et al., 2013) which all result in the death of dopamine cells.

Numerous neurotoxins have been identified such as 1,2,3,6-tetrahydropyridine (MPTP) (Przedborski et al., 2001), 6-hydrocypodamine (6-OHDA) (Cohen, 1984), and Rotenone (Cannon and Greenamyre, 2010). There are important physiological effects of these neurotoxins such as impaired movement and loss of DA neurons, therefore are known and used to mimic many PD symptoms.

Rotenone is an organic compound frequently used as a pesticide. Rotenone also acts as a complex I inhibitor for the mitochondria electron transport chain. Chronic exposure to rotenone produces Parkinsonian symptoms in rats, including nigrostriatal degeneration and formation of Lewy body-like intracellular inclusions (Ahmadi et al., 2008). Rotenone also causes selective toxicity to DA cells and behavioral impairment in several animal models (Cannon and Greenamyre, 2010). Rotenone models provide an advantage since its effects are similar to human PD.

Dopaminergic neurons decline with chronic rotenone exposure, specifically in the nigrostriatal pathway of the midbrain. The process of neuronal degeneration is not well-defined, though it has previously been attributed to microglia mediated cell death. Microglia are specialized macrophages of the central nervous system (CNS). Stimulation by proinflammatory factors can activate microglia causing the cell itself to change shape once activated and become highly phagocytic (Gao et al., 2002). Microglia can phagocytize dying or dead neurons to prevent the release of damaging proinflammatory components in the brain. In recent studies, however, it has been noted that activated microglia can phagocytize viable neurons specifically eliciting DA neuron death (Emmrich et al., 2013)

Recent studies have shown the relevance of rotenone animal models demonstrating that these models induce PD like symptoms in rodents (Betarbet et al., 2000). Of note rotenone

animal models induce the formation of alpha-synuclein aggregates in the CNS similar to those observed in PD. One of the most common invertebrate models used for PD is that of *Drosophila melanogaster.* After exposure to rotenone, the fly has decreased locomotion as well as loss of DA cells, although some studies report no death of DA neurons at all (Meulener et al., 2005).

A more widely used invertebrate model for neurobiology is the fresh water snail *Lymnaea stagnalis*. This snail has a CNS of about 20,000 neurons thus making it a useful and accessible model to study. Moreover, the neuronal pathways including physiological process such as locomotion and feeding have been elucidated in detail in the fresh water snail (Bavan et al., 2012). The advantages of *Lymnaea* as compared to the *Drosophila* model is the neurons in the CNS are easy to find and larger in size, and all the DA neurons in the snail have been identified (Elekes et al., 1991). Rotenone has been found toxic to the snail, causing progressive and irreversible behavioral impairment. It is still unknown as to how rotenone mechanisms are responsible for the effects in *Lymnaea stagnalis*.

Microglia cells in *Lymnaea stagnalis* have not been well studied. It is hypothesized that they get activated once inflammation of the brain is triggered, thus phagocytizing Dopamine cells. My work focused on the overarching concept that Microglial activation leads to a decrease in DA cells. Since rotenone can induce Parkinsonian symptoms in the snail *Lymnaea,* it is my hypothesis that once *Lymnaea stagnalis* is exposed to the toxin, microglia become activated, which is commonly associated with morphological changes. My work studied the effects rotenone had on the activation of microglia in *Lymnaea stagnalis*.

CHAPTER II

REVIEW OF LITERATURE

Parkinson's Disease Pathology

Parkinson's disease (PD) is the most common, irreversible neurodegenerative disorder affecting ten million people worldwide (Vehovszky et al., 2007). The classical signs of the disease are characterized clinically by tremor at rest; rigidity, bradykinesia and postural instability; as well as in the late stages symptoms which involve gait dysfunction, swallowing and speech difficulties (Alberio et al., 2012). In the central nervous system (CNS), PD is known by the selective loss of Dopamine (DA) cells of the substantia nigra pars compacta (SNc) and constant decrease of DA levels in the striatum (Shimizu et al., 2003). PD is also know to have formation of fibrillary cytoplasmic inclusions known as Lewy bodies primarily composed of alpha synuclein (Spillantini et al., 1997). Characterization of the symptoms and neuropathology has been established in PD patients, but the underlying cause of the disease is still a mystery.

The cause of PD is not clear, involving a complex interaction between environmental and genetic factors (Johnson and Bobrovskaya, 2015). PD can be inherited or sporadic. Both forms of PD share the same pathology, and many exhibit early onset. These cases are correlated with a single gene mutation in any one of the Park 1 through 10 genes. These genes include alpha synuclein, parkin, DJ1, PINK1, AND LRRK2 (Dias et al., 2013).

These genes play different roles in cell death, but are all involved in mitochondrial function (Dias et al., 2013).

Dopamine Cell Death in Parkinson's Disease

In the CNS, PD is characterized by the selective loss of dopaminergic (DA) cells in the substantia nigra pars compacta (SNc) and subsequent decrease of DA levels in the striatum (Shimizu et al., 2003). The SNc of the brain is considered a harsh region, it is dopamine rich and contains neuromelanin and high tissue iron content. DA neurons, throughout their life spam, are constantly exposed to factors that puts them in high risk, therefore evolving them to have a high defense mechanism to survive. Furthermore, DA neurons can absorb toxins through transporter channels, thus increasing their potential for cellular damage and death. PD symptoms appear only after the loss of more than half of nigral dopaminergic neurons. It is unknown but possible, that interactions between extracellular and intracellular factors in the SNc, are involved in the pathological mechanism that is responsible for dopaminergic neurodegeneration in PD (Barzilai et al., 2003).

Injury of Mitochondria

Several hypotheses have arisen as result of mitochondrial injury studies, some experiments include microglia activation (Barcia et al., 2012), microtubule damage (Bauereis et al., 2011), and reactive oxidative species (ROS), (Bosse et al., 2013) which all result in the death of dopamine cell. Dysfunction of the mitochondrial electron transport chain generates ROS, leading to oxidative stress. Oxidative stress and imbalance of ROS causes to oxidative damage to macromolecules (DNA, proteins and lipids). Furthermore, there is evidence that suggest

thatoxidative stress and mitochondrial dysfunction lead to selective degeneration of DA cells in PD (Schapira and Gegg, 2011).

Mitochondrial dysfunction can also lead to a decrease in ATP supply to microtubule motor proteins, which results in mitochondrial axon transport disruption. Importantly, alteration in microtubule dynamics can leads to defects in alpha-synuclein transport. Alpha-synuclein is normally transported by fast axon transport; disruption of intracellular trafficking may lead it to mis-localization and accumulate which is commonly found in PD patients (Esteves et al., 2014).

As a result of mitochondrial dysfunction, unknown cells release free radicals that activate nearby microglia cells. Microglial cells are the macrophages of the CNS. It is hypothesized that the glial cells emit inflammatory cytokines that bind to and damage nearby dopaminergic neurons or simply phagocytizes them. This feedback continues over years and slowly contributed to the loss of dopaminergic neurons that leads to Parkinson symptoms (Barcia et al., 2013).

Microglia Activation

Microglia are the macrophages of the CNS. They react to injury and diseases of the brain thus causing them to become morphologically activated. Microglia are scavengers of dead cells and act as immune-effector cells in the brain (Nakajima K, 2001). According to several researchers microglia size is about 10μm in diameter. When damage in the brain is detected, microglia cells launch a specific program that results in changes in their resting state to full-blown microglial activation taking an amoeboid form, becoming phagocytic (Barcia et al., 2013). The activated microglia then can migrate through the brain tissue until reaching the site of injury. The initial signal that triggers microglia activation is unknown. It is thought that abnormal

molecules, inflammation, or abnormal concentrations of present molecules can activate these cells (Nakajima K et al., 2001).

Inflammatory response in PD has been focused for many years on the study of microglia. Although there is an increase in publications over this topic in the last two decades, microglia's role in PD is still not fully understood. Microglia cells have been known to remain activated throughout the years in patients with PD. Most importantly, patients with PD show correlation between microglia activation and the terminal loss of dopaminergic cells (Barcia et al., 2013). In studies with humans, and non-human primates, dopaminergic degeneration is a result of activation of microglia in the first stages of PD, shortly after the onset of rapid degeneration, microglia activation is maintained for years after a threshold of activation has been reached (Peruzzi et al., 2004)

Lipopolysaccharide Induce Activation of Microglia

Gram negative bacteria membrane derived lipopolysaccharide (LPS) can induce inflammation to affect dopaminergic neurons in non-PD related mechanisms. The microglial response to LPS has been previously characterized. Administration of LPS to the midbrain in rodents induces dopaminergic degeneration together with release of cytokines. It also induces proinflammatory responses that are toxic to dopaminergic neurons. Furthermore, administration of LPS causes neurodegeneration and ATP to massively be released from damaged neurons thus signaling to initiate microglial activation (Barcia et al., 2013).

Labeling Lectin-IB4 in Microglia

Ret (rearranged during transfection) receptor tyrosine kinase is part of the signaling component receptor complex for the family of the glial cell line derived neurotropic factor

(GDNF). Ret regulates microglial neuronal survival and maturation. The plant lectin *Griffonia (Bandeiraea) simplicifolia* lectin I, Isolectin B4 (IB4), a microglia marker in the brain, bind to the extracellular domain of Ret on the surface of living fibroblast cells. Lectins are heterogeneous plant proteins that bind to the carbohydrates on glycoproteins and glycolipids. Furthermore, IB4 staining in rodents has identified resting and activated Ret-expressing microglia cells (Boscia et al., 2013).

Toxin-induced Models of Parkinson's Disease

Treatment with environmental factors such as the neurotoxins 1,2,3,6-tetrahydropyridine (MPTP) (Przedborski et al., 2001), 6-hydrocypodamine (6-OHDA), paraquat (PQ) (Cohen, 1984), and Rotenone (Cannon and Greenamyre, 2010) mimic PD. MPTP was recognized as a neurotoxin in the early 1980s, when several young drug addicts mysteriously developed PD-like symptoms after intravenous use of the street preparation of the drug meperidine that, unknown to anyone, was contaminated with MPTP (Przedborski et al., 2001). MPTP was found to be an inhibitor of the mitochondrial electron transport chain complex 1 and has been used since as a model of PD in different mammalian species (Bove et al., 2005). The neurotoxin causes several PD like symptoms such as impaired movement and depletion of DA cells (Przedborski et al., 2001).

A prototypic toxin, paraquat, is a potent herbicide known to produce PD like symptoms through oxidative stress. Paraquat toxicity comes from the interaction of redox cycling with cellular diaphorase such as nitric oxide synthase, therefore producing excessive reactive oxidative species (ROS) in the cell (Bove et al., 2005). It used to be theorized that paraquat only affected the lung, liver, and kidney, and many experimental studies only focused on these effects. It was not until later that significant damage to the brain was noted in individuals who died from

paraquat exposure; thus studies suggested that paraquat exposure increased the risk for PD, raising the possibility that paraquat could be an environmental parkinsonian toxin. However, paraquat treatment has been reported to lead to severe to minimal phenotypes ranging from loss of DA neurons, to impaired motor activity to no symptoms at all (Bove at al., 2005).

6-OHDA is a neurotoxin used to model PD in rats and has been employed in both *in vitro* and *in vivo* studies. This toxin, once in the peripheral nervous system, destroys sympathetic neuron nerve terminals. It damages catecholaminergic neurons since it has high affinity for the catecholamine transporters due to its similarity with catecholamines like norepinephrine and dopamine. (Bove and Perier, 2012). Normally DA cells are impaired in PD; several techniques need to be implemented in order to avoid norepinephrine cells. By a combination of ROS and quinones, it has been theorized that 6-OHDA induces DA cell death (Cohen et al., 1984).

Rotenone is the most newly discovered neurotoxin used as a model for PD research. It is a very potent member of the rotenoids extracted from various parts of the Leguminosae plants. Rotenone is a mitochondrial poison used around the world as an insecticide and piscicide to kill fish because of its short half-life (Jankovic et al., 2015). Since the year 2000, rotenone has been widely used as a PD model with animals in the lab. Rotenone is a selective inhibitor of mitochondrial complex I, thus resulting in reduced ATP, reduced glutathione levels and increased oxidative stress. (Mostafalou and Abdollahi, 2013). The rotenone model of PD reproduces many features of PD, it induces motor dysfunction, formation and aggregations of Lewy body-like inclusions, and iron accumulation in substantia nigra. Furthermore, it has also been associated with the selective death of DA cells in the SNc in mice (Cannon et al., 2009). Although it has been a good model for PD in rats, it has become very difficult to replicate.

Rotenone Models

Rotenone treatment in rats can reproduce PD symptoms found in patients including oxidative damage, microglia activation, L-DOPA-responsive motor deficits, and alpha-synuclein accumulation and aggregation with formation of Lewy body-like inclusions (Cannon et al., 2009). Rotenone exposure to rats has been known to result in the loss of Tyrosine hydroxylase (TH) expressing cells in DA cells, and the impaired motor function (Betarbet et al., 2000). This model has its disadvantages, most of the studies report a high mortality rate in rats shortly after exposure to rotenone, and therefore adjustments usually tend to be made for the rats to last enough time for the experiment. Rodent models also lack the reproducibility in the DA lesions, the location of the lesions, the magnitude and the mortality rate (Cannon et al., 2009).

Caenorhabditis elegans is a nematode that has a well-defined nervous system that is also genetically tractable, thus making this organism an effective model to explore human neurobiological diseases. These organisms are a good model because of amenable transgenic expression of Parkinson human genes and the study of the impact on dopaminergic neurons in several environmental conditions (Calahorro and Ruiz-Rubio, 2011). Once treated with rotenone, the organism shows a significant loss of DA neurons and decreases in movement resulting in abnormalities (Mocko et al., 2010). *Caenorhabditis elegans* as a model has a downside since it lacks the human Parkinson related genes PARK1 and LRRK2 (Calahorro and Ruiz-Rubio, 2011).

Drosophila melanogaster is one of the first models used to study PD; particularly because of the completion of the fly genome-sequencing project. Some advantages of *Drosophila* used as a model is a short life cycle, and the pathology of human PD can be replicated using this model, including age-dependent loss of DA neurons and formation of Lewy-

bodies (Pienaar et al., 2010). Once the flies are treated with rotenone, usually by ingestion, there is a significant depletion of DA cells in the CNS, and impaired movement (Coulom and Birman, 2004). *Drosophila* as model has not shown consistent results; some studies report DA cell death, while others found no loss at all. Furthermore, there have been some studies that report decreased in serotonin levels as a result of rotenone ingestion, which is not consistent with PD pathology (Navarro et al., 2014).

Lymnaea stagnalis is a fresh water snail commonly known for all DA neurons being identified and mapped. It is a widely used model in invertebrate neurobiology since its brain only contains nine ganglia and has a well-described dopamine system (Chase et al., 2002). The snail acts as a good model since is shows simple behaviors that can be quantified and explored; these include locomotion, respiration and feeding. When rotenone is introduced in *Lymnaea*, it occurs through skin absorption and by digestion. Rotenone has been found to be toxic in the snail at a concentration of 0.1 to 5.0μM, creating irreversible and progressive behavioral impairment (Vehovszky et al., 2004). *Lymnaea stagnalis* has been shown to be a possible model for PD, however the mechanisms responsible for the effects of rotenone have not been well characterized.

CHAPTER III

MATERIALS AND METHODS

Animals

Several *Lymnaea stagnalis* breeding stocks were maintained in "snail water". The snail water was made by dissolving 1 gram of "instant Ocean" aquarium salts per 1 gallon of deionized water. The snails were fed with Romaine lettuce and maintained under water with air filters. Animals used in the study were considered adults with a shell of 2-3 cm in length.

Dissection and Cell Culture

Animals were anesthetized by immersion for 45 minutes in 16nM 1-phenoxy-2propanol solution. Ganglia were dissected and placed in a 5% solution of protease type XIV (Sigma Aldrich) for 15 minutes. After protease treatment, brains were washed twice with 2 mL of saline water. To snail saline water was made with concentrations of 40.0 mM NaCl, 1.7 mM KCL, 4.1 mM $Cacl_2$, 1.5 mM $MgCl_2$, and 10.0 mM HEPES. Ganglia were then mechanically dissociated using glass pipets in a 50 ml tube; later the dissociated cells were placed into a poly-D-Lysine coated glass-bottom dish with IB4 solution.

DAPI Staining

14.4mM DAPI (Thermo Fisher) solution was used to stain the cell nuclei. After dissection, and before dissociation, brains were suspended in a micro tube with 1µl of DAPI and

0.999 ml of deionized water for 25 min in a shaker. Washing the brain twice with 10 ml of saline solution for 2 minutes.

Isolectin B4

GSL I-B4 Isolectin (Vector Laboratories) was used to fluorescently label (green) potential microglia cells. Dissociated brain cells were plated in poly-D-lysine coated glass-bottom dishes in a 1 mg/mL IB4 in PBS buffer for a total solution of 500µl for 4 hours for control snail brains, and 1 hour for treated snail brains.

Confocal Fluorescence Microscopy

For confocal imaging brains were dissected and placed in a 5% solution of Protease type XIV (Sigma) for 15 min to be later stained with DAPI, dissociated with IB4 and placed in a poly-D-lysine coated glass-bottom dish. The dish was then visualized on a Fluoview (FV10i) Olympus Confocal Microscope using DAPI and FITC excitation and emission channels. Composite digital images were then converted to JPG format, imported into Adobe Illustrator Artwork 16.0 (Adobe Illustrator C16; Adobe Systems INC., San Jose CA), and color balance was adjusted for presentation.

Lipopolysaccharide Treatment

2 mg Lipopolysaccharides from *Escherichia coli* (Sigma Aldrich) was placed in a container with 100 ml of deionized water. The snail was placed inside for 24 hours, with food, and covered with Parafilm to prevent evaporation, with small incisions in the covered area.

LPS Confocal Fluorescence Microscopy

For confocal imaging, after LPS treatment, brains were dissected and placed in a 5% solution of Protease type XIV (Sigma) for 15 min to be later stained with DAPI, dissociated with IB4 and placed in a poly-D-lysine coated glass-bottom dish for 1 hour. The dish was then visualized on a Fluoview (FV10i) Olympus Confocal Microscope using DAPI and FITC excitation and emission channels. Composite digital images were then converted to JPG format, imported into Adobe Illustrator Artwork 16.0 (Adobe Illustrator C16; Adobe Systems INC., San Jose CA), and color balance was adjusted for presentation.

Behavioral Analysis

Lymnaea stagnalis locomotion was analyzed by video pre and post rotenone treatment. Each snail was placed in a container with 100 ml of snail water, and its crawling behavior was video recorded for 15 minutes. The video then was processed using Image J analysis software (Wayne Rasband, National Institutes of Health, version 1.36b), and the individual traces made by a single snail were measured and analyzed using additional plug in MtrackJ (Biomedical Imaging Group Rotterdam). Statistical tests were performed on the mean distance crawled using MS Excel, with Student's two sample t-test for significant level.

Rotenone Treatment

Stock solutions of 10mM of rotenone were made in dimethyl sulfoxide (DMSO) and diluted in snail water to reach a final concentration of 0.5µM rotenone. Snails were placed in separate containers with a volume of 650 ml of snail water for 48 hours. After rotenone treatment, the snail's brain was dissected and dissociated with 500µl-centrifuged IB4, then placed in a poly-D-lysine coated glass-bottom dish for 30 min.

Confocal Fluorescence Microscopy

For confocal imaging, after rotenone treatment, brains were dissected and placed in a 5% solution of Protease type XIV (Sigma) for 15 min to be later stained with DAPI, dissociated with IB4 and placed in a poly-D-lysine coated glass-bottom dish for 1 hour. The dish was then visualized on a Fluoview (FV10i) Olympus Confocal Microscope using DAPI and FITC excitation and emission channels. Composite digital images were then converted to JPG format, imported into Adobe Illustrator Artwork 16.0 (Adobe Illustrator C16; Adobe Systems INC., San Jose CA), and color balance was adjusted for presentation.

CHAPTER IV

FINDINGS

IB4 Staining Microglia Cells

Microglia cells are known to be characterized by using the marker IB4, which only binds to the Ret receptor of the microglia cell (Boscia et al., 2013). In order to show that the snail *Lymnaea stagnalis* does in fact contain microglia cells, the snail brain was dissected and dissociated. The cells were stained with IB4 and DAPI and confocal microcopy images were taken using DAPI and FITC excitation and emission channels. **Fig 1. A** shows confocal images of the *Lymnaea* brain cells stained by IB4, as compared **Fig 1.B** with just DAPI staining.

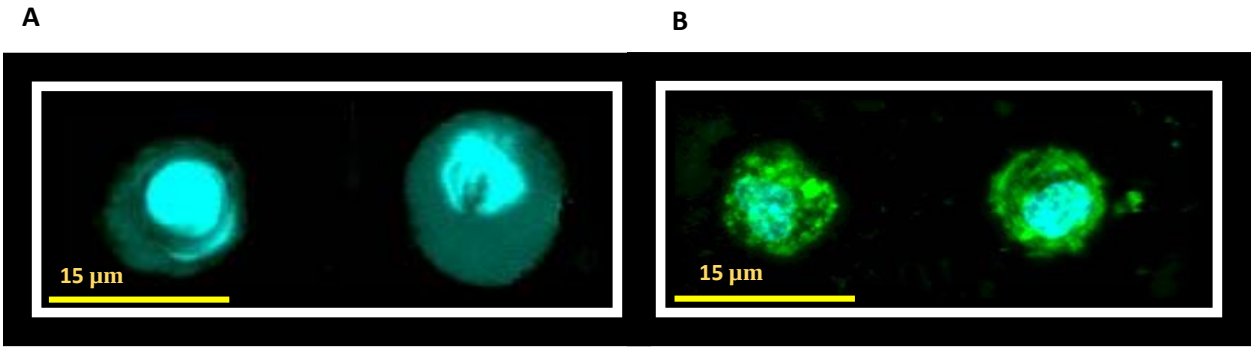
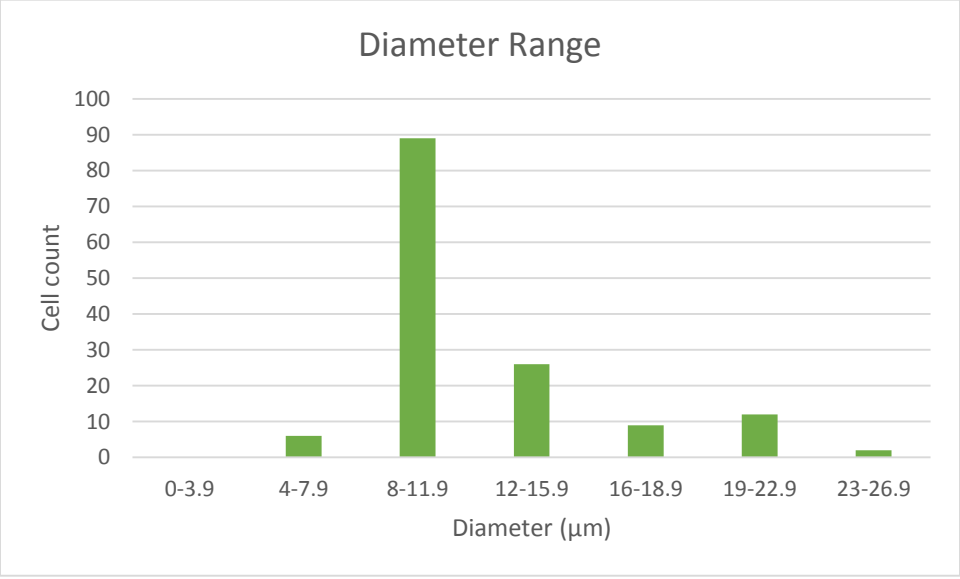


Figure 1. Confocal microscopy of potential microglia cells. Confocal images of dissociated snail brain cells in a poly-D-lysine coated glass-bottom dish and stained with fluorescence green IB4 for outer membrane and DAPI (blue) for presence of nuclei. Data presented are representative of 8 independent experiments. Scale bar, 15 µm. (**A)** Cells only stained with DAPI. **(B)**Stained cells with IB4 and DAPI.

Size Distribution of IB4 Labeled Cells

Several studies suggest that microglia can range in the size of 10 ±3 µm, depending on the organism in study, although it has not been well studied in the snail (Boscia et al., 2013). One way to prove that the IB4 stained cells in the *Lymanea* snail are microglia cells is by comparing their size. The dissociated brain cells were stained with IB4 and DAPI and confocal microcopy images were taken using DAPI and FITC excitation and emission channels. As shown on in **Fig. 2 A**, about 78% of the total IB4 stained cells in the control experiment were of the size of 10±3 µm in diameter. **Fig. 2 B** depicts the mean length and width of the total counted cells of the experimented snail brains, with no significant difference from each other.

A



B

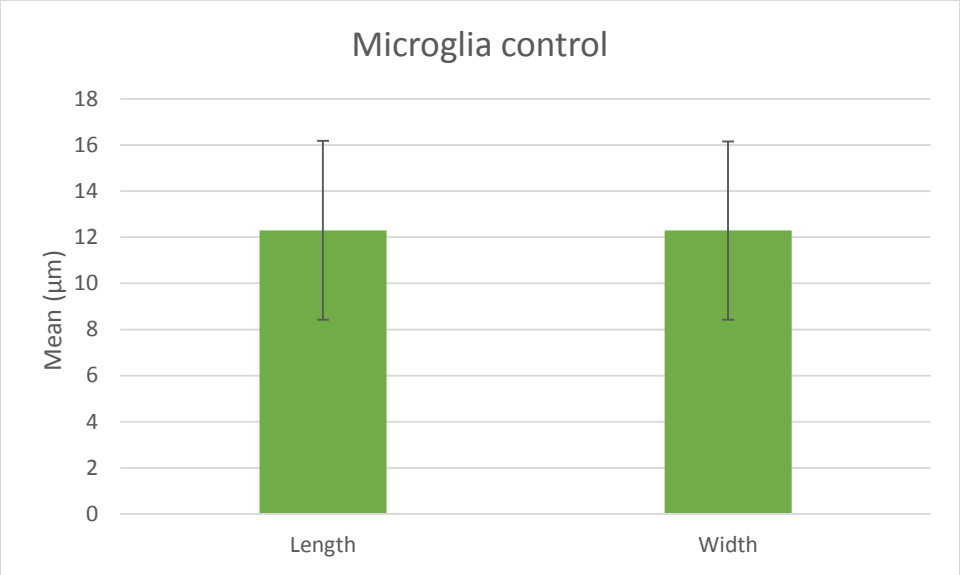


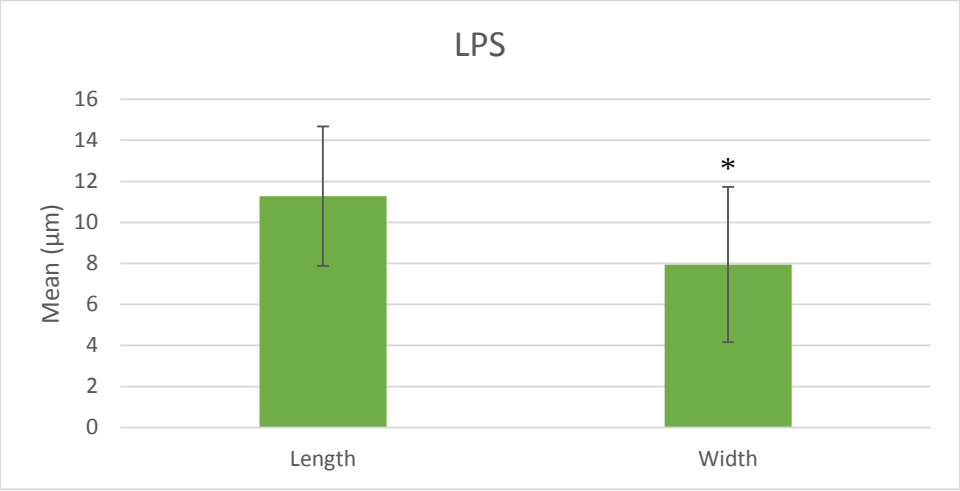
Fig 2. Size distribution of IB4 Labeled Cells. Analysis of the potential microglia cells is done through fluorescence green IB4 for outer membrane and DAPI (blue) for presence of nuclei using DAPI and FITC excitation and emission channels. Data presented are representative of 7 independent experiments with a total of 150 IB4 stained cells counted. **(A)** The majority of the counted cells have a diameter of 10 ±2 µm. **(B)** The mean average diameter of the cells are around 12 µm with a standard deviation of 3.87 with a p value of 0.164.

LPS Induces Inflammation and Activates Microglia

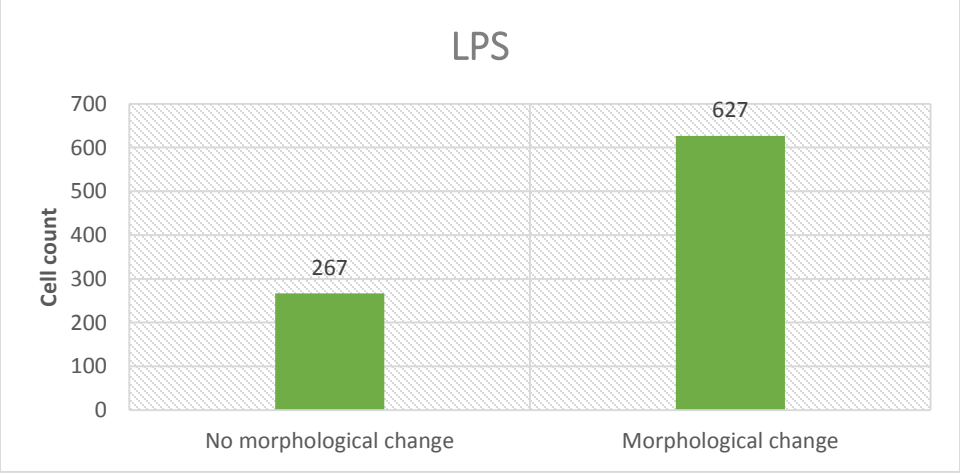
The stained cells need to go through activation to confirm *Lymnaea* contain microglia cells. Lipopolysaccharide can induce inflammation in the brain of the snail and cause morphological changes in microglia cells. The snails were treated with LPS for 24 hours, and the dissociated brain cells were stained with IB4 and DAPI and confocal microcopy images were taken using DAPI and FITC excitation and emission channels. **Fig. 3 A** represents the length and

width of the stained cells after LPS treatment, there is a decrease in the width of the cells because of the morphological change in the cell. **Fig. 3 B** depicts the total number of cells that showed morphological change or no change at all. About 70% of the stained cells showed some morphological change. **Fig. 3 C** Shows the difference between length and width of the cell, any number higher than 1 represents a significant change in shape. Statistical analysis preformed shows a significant change in width with $p<0.05$. **Fig 4.** Represents images taken on the confocal microscope of LPS treated cells showing visually their size and morphological change.

A



B



C

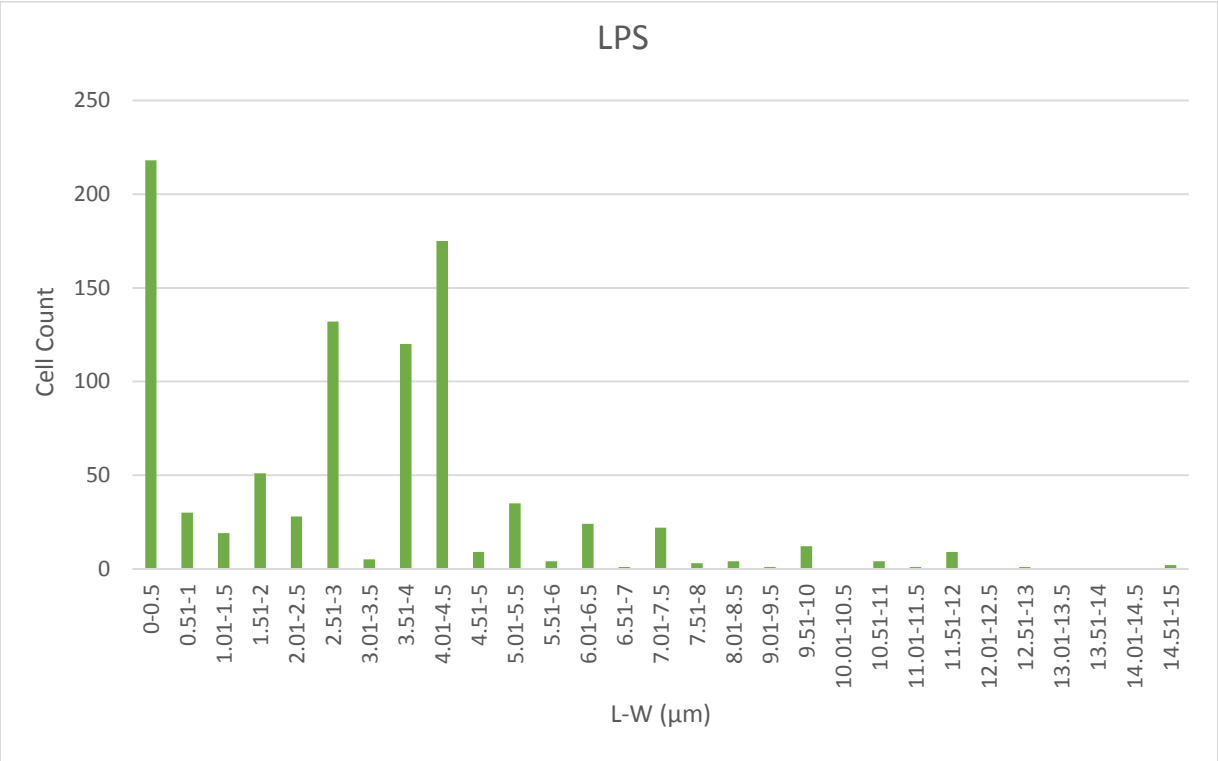


Fig. 3. Morphological change after LPS treatment (A) Mean average of length and width of treated lipopolysaccharide brain cells. **(B)** Total number of cells that show both morphological and no change at all after snail treatment of LPS. **(C)** Length minus width difference after LPS treatment in brain cells. Data presented are representative of 12 independent experiments with 894 IB4 and DAPI stained cells counted and * denotes $p<0.05$.

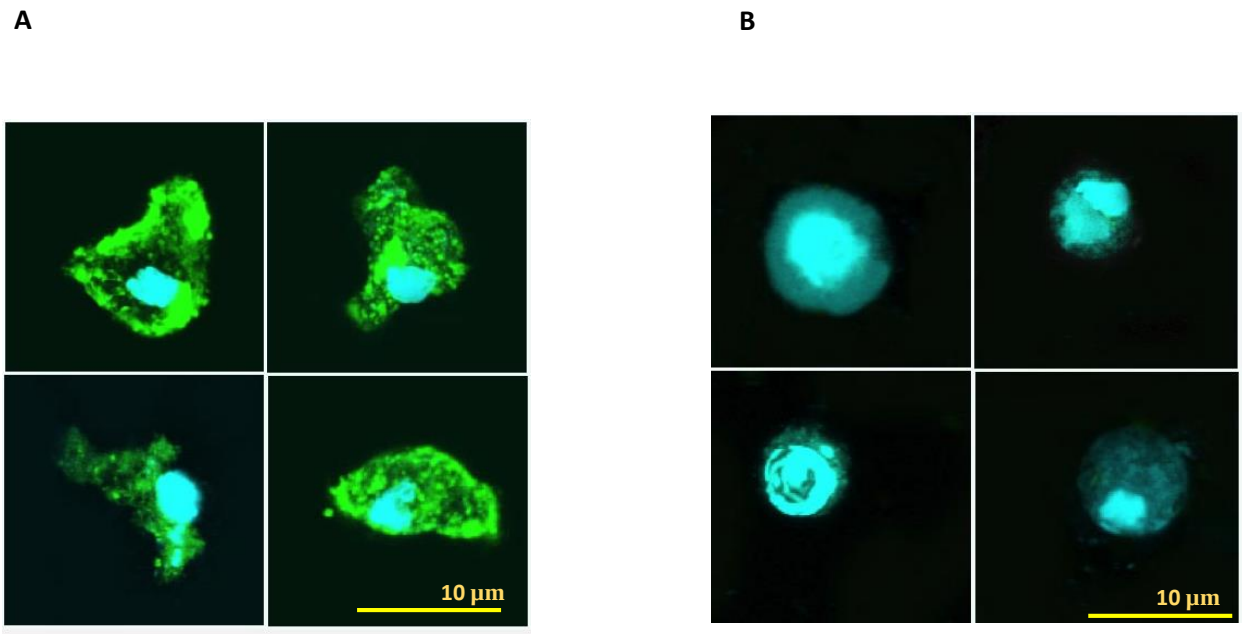


Fig. 4 Confocal microscopy of LPS treated cells. **(A)** There are morphological changes in the IB4 and DAPI LPS treated brain cells compared to **(B)** DAPI stained LPS treated cells. All figures represent different snail brains treated with LPS.

Snail Movement Analysis

In order to test rotenone toxicity in the snail, it was placed in a container for 48 hours with 0.5 µM rotenone and 600 ml of snail water. The locomotion of both control and the 0.5 µM rotenone treated were analyzed and measured using Image J and analyzed using Anova. Previous research in rodents with rotenone had shown a deleterious effect in the locomotion of the animal (Vehovszky et al., 2007). As shown in **Fig. 5** there is a decrease of almost 50% in locomotion in the rotenone treated snails after 48 hours of treatment compared to non-treated snails.

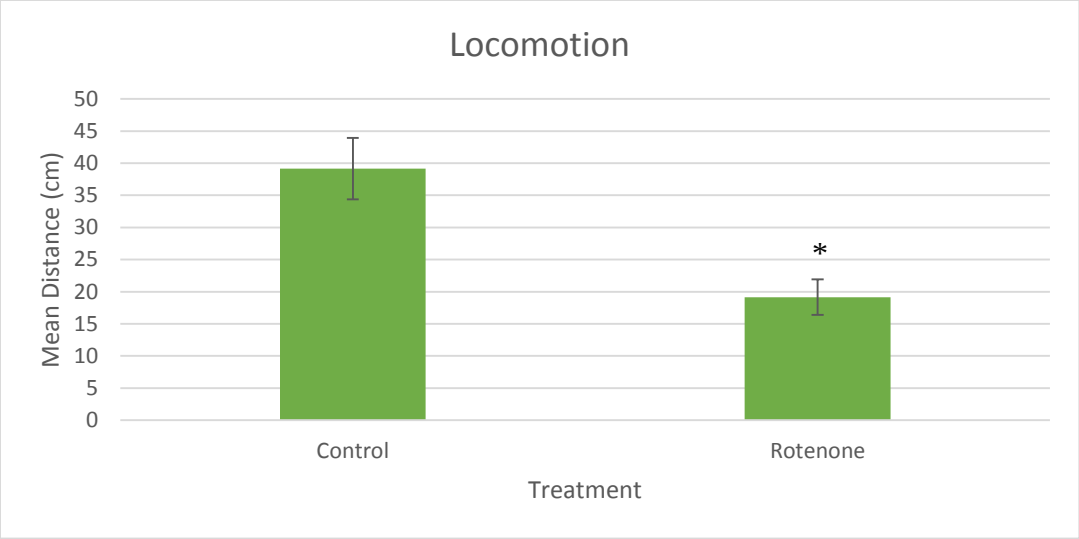
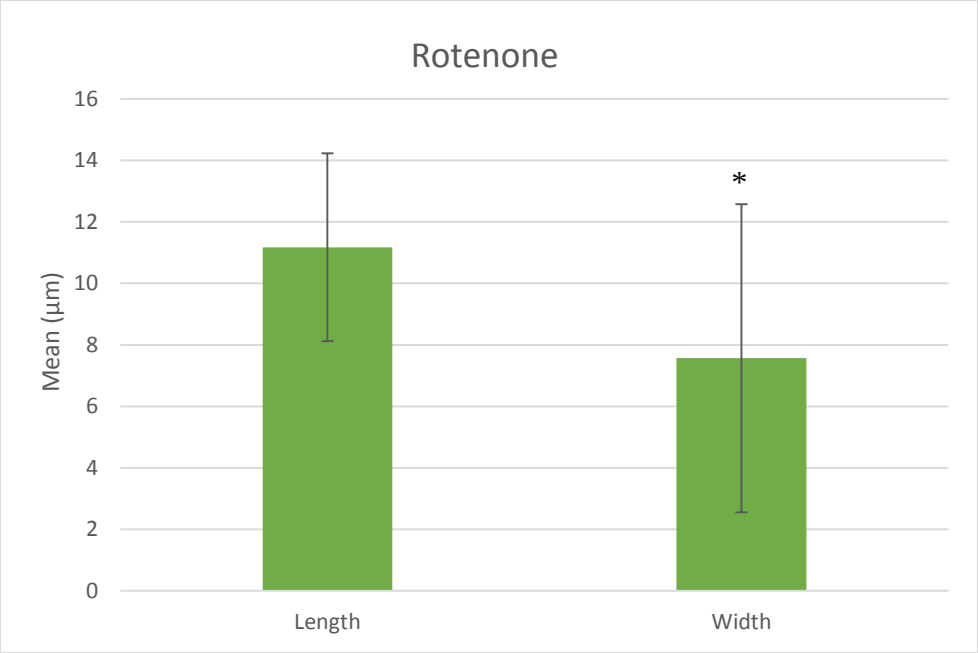


Fig. 5. Locomotion decrease after rotenone treatment. Rotenone treated snails show a decline in the amount of crawling. Bars show mean distance traveled by snails in both control and treated groups. Control groups exhibit a mean of 39 ±5cm; rotenone treated groups show a mean of 19 ±3cm. * Indicates statistically significant at $p<0.001$, n=12.

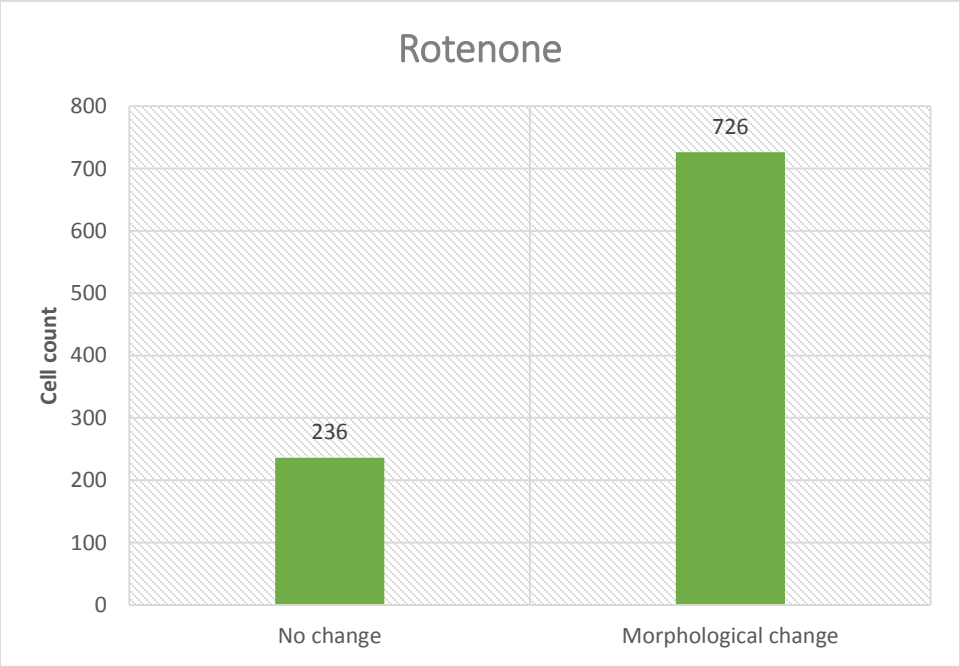
Rotenone Activates Microglia Creating Morphological Change

The neurotoxin rotenone has been known to create similar parkinsonian symptoms in human and animal models, although the mechanism is unknown. *Lymnaea stagnalis* was treated with rotenone for 48 hours, the dissociated brain was stained with IB4 and DAPI and confocal microcopy images were taken using DAPI and FITC excitation and emission channels. **Fig. 6 (A)** represents the length and width of the stained cells after rotenone treatment, compared to the control; there is a decrease in the width of the cells because of the morphological change in the cell. **Fig. 6 (B)** depicts the total number cells that showed morphological changes. About 84% of the stained cells showed some morphological change. **Fig. 6 (C)** shows the difference between length and width of the cell after treatment as compared to the control, no longer a circular cell.

A


Rotenone
16
14
12
10
8
6
4
2
0
Mean (µm)
*
Length
Width

B


Rotenone
800
700
600
500
400
300
200
100
0
Cell count
236
726
No change
Morphological change

C

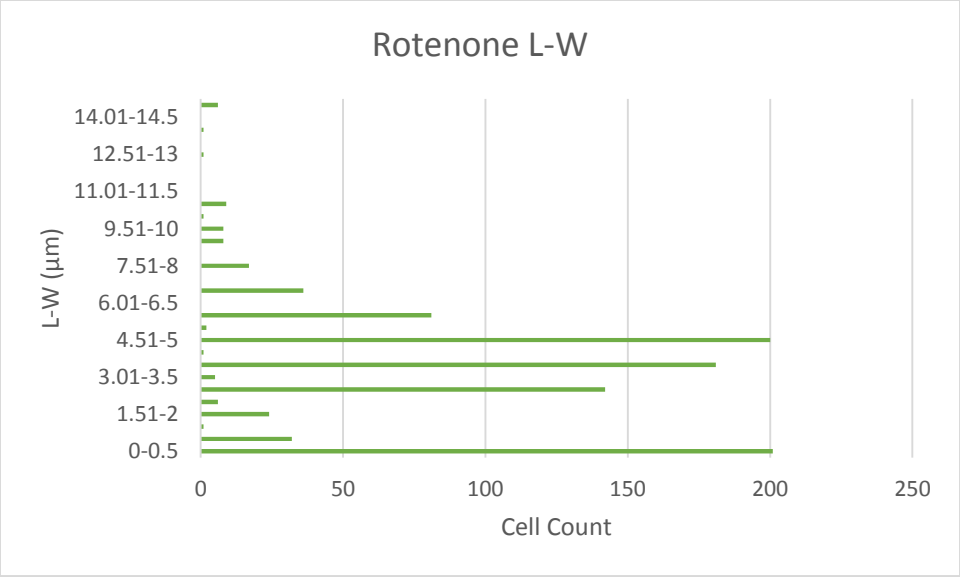


Fig. 6. Morphological change after rotenone treatmnent. (A) Mean average of length and width of treated rotenone brain cells. **(B)** Total number of cells that show both morphological and no change at all after snail treatment of rotenone. **(C)** Length minus width difference after rotenone treatment in brain cells. Data presented are representative of 12 independent experiments with 962 cells counted with IB4 and DAPI stains after 48 hours of 0.5 µM rotenone treatment and * denotes $p<0.05$.

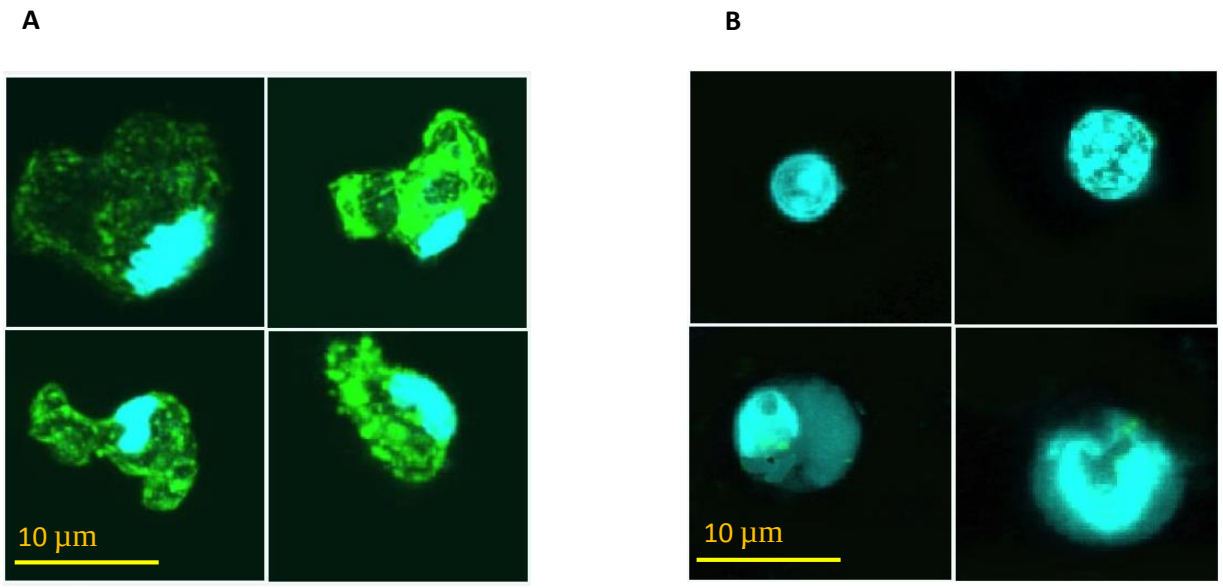


Fig. 7 Confocal microscopy of rotenone treated cells (A) shows there are morphological changes in the snail brain cells stained with IB4 and DAPI after 48 hours of 0.5 µM rotenone treatment. **(B)** DAPI stained cells after rotenone treatment. All images represent different snail brain cells treated with Rotenone.

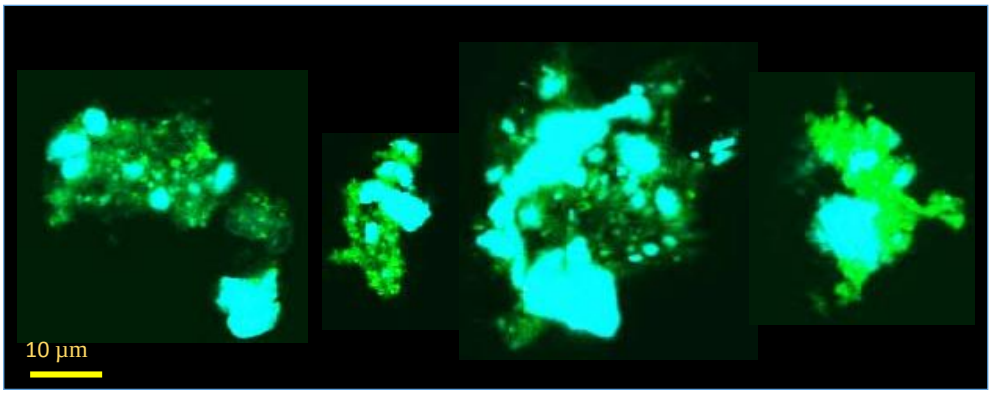


Fig 8. Confocal microscopy of clustered cells after rotenone treatment. IB4 and DAPI stained cells after rotenone treatment appear as clusters. Images represent different snail brain cells after 48 hours of 0.5 µM rotenone treatment.

CHAPTER V

RESULTS

The experiment show that *Lymnaea stagnalis* can serve as a model for Parkinson's disease using rotenone as an inducer of Parkinson-like symptoms in the snail. Previous researchers have been able to use IB4 as a marker specifically for microglia cells in rodents (Boscia et al., 2013). Additionally, these data confirmed the role of IB4 as a marker in microglia of the snail *Lymnaea stagnalis.* Evidence for this role in my experiments is provided by the small subpopulation of fluorescent green in the brain cells as shown in **(Fig. 1).** Furthermore, microglia cells are known to be at about 10±3 μm in diameter, depending on the organism being studied (Barcia et al., 2013). This small subpopulation of IB4 stained cells also fit into the appropriate size of a microglia resulting in the majority of the cells to average a diameter of 8-11.0 μm **(Fig 2 A).**

Administration of the gram negative bacteria membrane derived lipopolysaccharide (LPS), causes inflammation and neurodegeneration and ATP to massively be released from damaged neurons thus signaling to initiate microglia activation in rodents (Nakajima K et al., 2001). Microglia activation can be identified by changing their state from resting to active state and causing a morphological change. Consistently with the theory of LPS inducing inflammation, the data shows that as a cause of inflammation by LPS, the IB4 stained cells were morphologically changed thus suggesting microglia activation **(Fig. 4).** Their dimensions in length and width size

changed, as compared to the control to an average of ±3 µm **(Fig. 3A).** Cell length and width were statistically significant after treatment with LPS, resulting in a 70% of the cells to change morphologically **(Fig. 3B).** Further statistical analysis show a significant difference of the cells Length minus Width greater than 1.01 µm in the same 70th percentile, with the majority of the cells having a difference of 3.0-4.5µm (**Fig. 3C).**

Previous rotenone models have been used to see the pathological factors of Parkinson's disease in different animals. Using the snail *Lymnaea* as a model, it has been recently known to have caused behavior impairment after treatment with rotenone. My experiments have shown that the pathology of rotenone-treated pond snail *Lymnaea stagnalis* is consistent with the effects. In other models and humans, one major characteristic in primary changes in motor function is changes in locomotion, which is consistent in the snail (**Fig. 5**). 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.*

Besides locomotion being affected, the brain also shows evidence of microglia activation after rotenone. The IB4 labeled cells treated with rotenone can be seen with different shapes other than a circular cell, the IB4 stained cells were morphologically changed thus suggesting microglia activation **(Fig. 7).** Their dimensions in length and width size changed, as compared to the control to an average of a decrease of 3 µm **(Fig. 6A).** Cell length and width were statistically significant after treatment with rotenone, resulting in 84% of the cells to change morphologically **(Fig. 3B).** Further statistical analysis shows a significant difference of the cells Length minus

Width greater than 1.01 µm in the same 84th percentile, with the majority of the cells having a difference of 3.5-5.5µm (**Fig. 3C).**

An unexpected finding in my experiment is clustering of the IB4 and DAPI stained cells seen after rotenone treatment, this evidence can suggest that there is microglia being recruited to the injured site or phagocytizing another cell **(Fig 8).**

Further research is needed to confirm the reason of this IB4 stained cells clustering phenomenon in the snail *Lymnaea stagnalis*. Additionally, IB4 stained other cells of diameters higher than 14 µm, although a very small percentage and did not change morphologically, further research needs to be done to identify and characterized the types of cells in the snail *Lymnaea* these are.

Although the mechanism by rotenone to activate microglia in the snail is yet unknown, this can be the starting point of using the snail *Lymnaea stagnalis* as a model for Parkinson's disease. The mechanism of rotenone can be studied using microglia cells in the future to gain understanding in how microglia can phagocytize dopamine cells for the hopes of preventing the disease.

LITERATURE CITED

Ahmadi, F.A., Grammatopoulos, T.N., Poczobutt, A.M., Jones, S.M., Snell, L.D., Das, M., and Zawada, W.M. (2008). Dopamine selectively sensitizes dopaminergic neurons to rotenone-induced apoptosis. Neurochemical research *33*, 886-901.

Alberio, T., Lopiano, L., and Fasano, M. (2012). Cellular models to investigate biochemical pathways in Parkinson's disease. The FEBS journal *279*, 1146-1155.

Barcia, C. (2013). Glial-mediated inflammation underlying parkinsonism. Scientifica *2013*, 357805.

Barcia, C., Ros, C.M., Annese, V., Carrillo-de Sauvage, M.A., Ros-Bernal, F., Gomez, A., Yuste, J.E., Campuzano, C.M., de Pablos, V., Fernandez-Villalba, E., *et al.* (2012). ROCK/Cdc42-mediated microglial motility and gliapse formation lead to phagocytosis of degenerating dopaminergic neurons in vivo. Scientific reports *2*, 809.

Barzilai, A.a.M.E. (2003). Molecular mechanisms of selective dopaminergic neuronal death in Parkinson's disease. Trends in Molecular Medicine *9*, 126-132.

Bauereis, B., Haskins, W.E., Lebaron, R.G., and Renthal, R. (2011). Proteomic insights into the protective mechanisms of an in vitro oxidative stress model of early stage Parkinson's disease. Neuroscience letters *488*, 11-16.

Bavan, S., Straub, V.A., Webb, T.E., and Ennion, S.J. (2012). Cloning and characterization of a P2X receptor expressed in the central nervous system of Lymnaea stagnalis. PloS one *7*, e50487.

Betarbet, R.S., T. B.; MacKenzie, G.; Garcia-Osuna, M.; Panoc, A. V.; and Greenamyre, J. T. (2000). Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nature Neuroscience *3*, 1301:1306.

Boscia, F., Esposito, C.L., Casamassa, A., de Franciscis, V., Annunziato, L., and Cerchia, L. (2013). The isolectin IB4 binds RET receptor tyrosine kinase in microglia. Journal of neurochemistry *126*, 428-436.

Bosse, T., ter Haar, N.T., Seeber, L.M., v Diest, P.J., Hes, F.J., Vasen, H.F., Nout, R.A., Creutzberg, C.L., Morreau, H., and Smit, V.T. (2013). Loss of ARID1A expression and its relationship with PI3K-Akt pathway alterations, TP53 and microsatellite instability in endometrial cancer. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc *26*, 1525-1535.

Bove, J., and Perier, C. (2012). Neurotoxin-based models of Parkinson's disease. Neuroscience *211*, 51-76.

Bove, J.P., D.; Perier, C. and Przedborski (2005). Toxin-Induced Models of Parkinson's Disease. NeuroRx: The Journal of the American Society for Experiemental NeuroTherapeutics *2*, 484-494.

Calahorro, F., and Ruiz-Rubio, M. (2011). Caenorhabditis elegans as an experimental tool for the study of complex neurological diseases: Parkinson's disease, Alzheimer's disease and autism spectrum disorder. Invertebrate neuroscience : IN *11*, 73-83.

Cannon, J.R., and Greenamyre, J.T. (2010). Neurotoxic in vivo models of Parkinson's disease recent advances. Progress in brain research *184*, 17-33.

Cannon, J.R., Tapias, V., Na, H.M., Honick, A.S., Drolet, R.E., and Greenamyre, J.T. (2009). A highly reproducible rotenone model of Parkinson's disease. Neurobiology of Disease *34*, 279-290.

Chase, R. (2002). Behavior and Its Neural Control in Gastropod Molluscs (Oxford University Press).

Cohen, G. (1984). Oxy-radical toxicity in catecholamine neurons. Neurotoxicology *5*, 77-82.

Coulom, H., and Birman, S. (2004). Chronic exposure to rotenone models sporadic Parkinson's disease in Drosophila melanogaster. The Journal of neuroscience : the official journal of the Society for Neuroscience *24*, 10993-10998.

Dias, V., Junn, E., and Mouradian, M.M. (2013). The role of oxidative stress in Parkinson's disease. Journal of Parkinson's disease *3*, 461-491.

Elekes, K.H., G. ; Hiripi, L..; Geffard, M.; and Benjamin, P.R. (1991). Dopamine-Immunoreactive Neurones in the Central Nervous System of the Pond Snail Lymnaea stagnalis. The Journal of comparative neurology *307*, 214-224.

Emmrich, J.V., Hornik, T.C., Neher, J.J., and Brown, G.C. (2013). Rotenone induces neuronal death by microglial phagocytosis of neurons. The FEBS journal *280*, 5030-5038.

Esteves, A.R.a.C.S.M. (2014). The rescue of microtubule-dependent traffic recovers mitochondrial funtion in Parkinson's disease. . Molecular Basis of Disease *1842*, 7-21.
Gao, H.M., Hong, J.S., Zhang, W., and Liu, B. (2002). Distinct role for microglia in rotenone-induced degeneration of dopaminergic neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience *22*, 782-790.

Jankovic, J.a.T., E. (2015). Parkinson's Disease & Movement Disorders, 6th edn (Philadelphia, PA: Wolters Kluwer).

Johnson, M.E., and Bobrovskaya, L. (2015). An update on the rotenone models of Parkinson's disease: their ability to reproduce the features of clinical disease and model gene-environment interactions. Neurotoxicology *46*, 101-116.

Meulener, M., Whitworth, A.J., Armstrong-Gold, C.E., Rizzu, P., Heutink, P., Wes, P.D., Pallanck, L.J., and Bonini, N.M. (2005). Drosophila DJ-1 mutants are selectively sensitive to environmental toxins associated with Parkinson's disease. Current biology : CB *15*, 1572-1577.

Mocko, J.B., Kern, A., Moosmann, B., Behl, C., and Hajieva, P. (2010). Phenothiazines interfere with dopaminergic neurodegeneration in Caenorhabditis elegans models of Parkinson's disease. Neurobiol Dis *40*, 120-129.

Mostafalou, S., and Abdollahi, M. (2013). Pesticides and human chronic diseases: evidences, mechanisms, and perspectives. Toxicology and applied pharmacology *268*, 157-177.

Nakajima K, K.S. (2001). Microglia: activation and their significance in the central nervous system. . J Biochem *130*, 169-175.

Navarro, J.A., Hessner, S., Yenisetti, S.C., Bayersdorfer, F., Zhang, L., Voigt, A., Schneuwly, S., and Botella, J.A. (2014). Analysis of dopaminergic neuronal dysfunction in genetic and toxin-induced models of Parkinson's disease in Drosophila. Journal of neurochemistry *131*, 369-382.

Peruzzi, E., Fontana, G., and Sonetti, D. (2004). Presence and role of nitric oxide in the central nervous system of the freshwater snail Planorbarius corneus: possible implication in neuron-microglia communication. Brain research *1005*, 9-20.

Pienaar, I.S., Gotz, J., and Feany, M.B. (2010). Parkinson's disease: insights from non-traditional model organisms. Progress in neurobiology *92*, 558-571.

Przedborski, S., Jackson-Lewis, V., Naini, A.B., Jakowec, M., Petzinger, G., Miller, R., and Akram, M. (2001). The parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): a technical review of its utility and safety. Journal of neurochemistry *76*, 1265-1274.

Schapira, A.H., and Gegg, M. (2011). Mitochondrial contribution to Parkinson's disease pathogenesis. Parkinson's disease *2011*, 159160. Shimizu, K., Matsubara, K., Ohtaki, K., and Shiono, H. (2003). Paraquat leads to dopaminergic neural vulnerability in organotypic midbrain culture. Neuroscience research *46*, 523-532.

Shimizu, K.M.K.O.K.a.S., H. (2003). Paraquat leads to dopaminergic neural vulnerability in organotypic midbrain culture. Neuroscience research *46*, 523-532.

Spillantini, M.G., Schmidt, M.L., Lee, V.M., Trojanowski, J.Q., Jakes, R., and Goedert, M. (1997). Alpha-synuclein in Lewy bodies. Nature *388*, 839-840.

Surmeier, D.J., Guzman, J.N., Sanchez-Padilla, J., and Goldberg, J.A. (2010). What causes the death of dopaminergic neurons in Parkinson's disease? *183*, 59-77.

Terzioglu, M., and Galter, D. (2008). Parkinson's disease: genetic versus toxin-induced rodent models. The FEBS journal *275*, 1384-1391.

Trinh, J., and Farrer, M. (2013). Advances in the genetics of Parkinson disease. Nature reviews Neurology *9*, 445-454.

Vehovszky, A., Szabo, H., and Elliott, C.J. (2004). Octopamine-containing (OC) interneurons enhance central pattern generator activity in sucrose-induced feeding in the snail Lymnaea. Journal of comparative physiology A, Neuroethology, sensory, neural, and behavioral physiology *190*, 837-846.

Vehovszky, A., Szabo, H., Hiripi, L., Elliott, C.J., and Hernadi, L. (2007). Behavioural and neural deficits induced by rotenone in the pond snail Lymnaea stagnalis. A possible model for Parkinson's disease in an invertebrate. The European journal of neuroscience *25*, 2123-2130.

BIOGRAPHICAL SKETCH

The author, Xiomara Galvan, was born on September 14, 1990 in Monterrey, Nuevo Leon, Mexico; and is now currently living in 8426 N. 18th LN, McAllen, TX 78504 as the daughter of Guadalupe and Omar Galvan.

She took her primary education at Zavala elementary, secondary education at Morris Middle School and high school education at McAllen High School in McAllen, TX. She was a consistent honor student and received several distinguished achievement program awards such as National Honor Society and was awarded the McAllen PART scholarship for high achievement, ambitiousness and leadership. In 2008, she was admitted to the University of Texas Pan American under the Bachelor of Science in Biology, with minors in Chemistry and Psychology. In 2014, she was admitted to the graduate program at the University of Texas Pan American under the Masters of Science in Biology, where she earned a Master's degree in Biology in 2015.

Xiomara became a member of the Golden Key Honor Society and Alpha Lambda delta Honor Society. Before working in research in biology, Xiomara also gained experienced in psychology research and became one of the presenters at a local psychology conference in 2011. She became a laboratory Teaching Assistant in 2011 and taught general biology I and II until 2015. As a graduate student she help with several Pre-AP and AP high school teachers at an Advance Placement Summer Institute for Biology lab. She also engages in Community Service by volunteering at the Exxon Mobile summer camp at her University to teach dissections to students from middle school. Xiomara hopes to continue to exceed with the help of her family.