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Naturally Derived Compounds as Preventatives of Proteinopathies

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NATURALLY DERIVED COMPOUNDS AS PREVENTATIVES OF
PROTEINOPATHIES

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Master's Program in Chemistry

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Dedication

Quiero dedicar mi tesis a mis padres, Luis Fernando y Rosa Maria Mendez, y a mis hermanas, Elvia Rosa, Abril y Salma. Les estoy eternamente agradecida por el apoyo incondicional que me han brindado no solo durante mi maestría, sino durante el transcurso de mi vida como estudiante. Gracias por ser mis porristas y ser además mi centro, ese que me ayuda a recordar que es necesario tener balance en la vida cuando se me olvida. Los amo muchísimo, gracias.

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PROTEINOPATHIES

by

LOIS MENDEZ, B.S.

THESIS

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Abstract

Neurodegenerative diseases, such as Parkinson's disease (PD), afflict millions worldwide. Of greater concern is the fact that a main risk factor this disease is aging and the baby boomer population is now reaching a geriatric age. Despite the active research in this area, little progress has been made in the development of therapeutic strategies and furthering of our understanding in pinpointing the causal events that onset these disease processes. Our lab has focused on studying the protein homeostasis of key biomarkers in neurodegenerative diseases and examining whether natural compounds can act as neuro-protectants and, thus, attenuate the pathology that ensues in these conditions. First, we sought to determine whether Na- β -hydroxybutyrate (Na β HB) could serve as a prophylactic for PD. We did so by using an organismal, vertebrate model (rat) to induce a Parkinsonian condition via chronic injection of rotenone, a known mitochondrial complex I inhibitor. Animals underwent behavioral testing to ascertain the presence of motor deficits. After the behavioral testing, tissue was collected to perform immunohistochemical analysis. The potential of Na β HB to rescue neurons and protect from damage was determined by counting the number of TH-positive neurons in the Substantia Nigra pars compacta (SNpc). The exact causal agents of Parkinson's disease and Alzheimer's disease (AD) are unknown; however, there are biochemical and cellular changes that are common to both, such as: oxidative stress, mitochondrial dysfunction and protein aggregation. Given the commonalities of these two disease processes, we aimed to examine whether we could intercede in the pathogenesis caused by the prion-like protein, β -amyloid (AD associated), when introduced to a non-native environment and allowed to interact with another prion-like protein, α -synuclein. The molecule of choice was Tanshinone IIA, a polyphenol found in red sage, which has the potential to scavenge free radicals and has shown promise in other areas of biomedical research.

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Chapter 1: Background and Introduction

1.1 Parkinson's Disease

1.1.1 Disease Overview

Parkinson's disease (PD) is the second most common neurodegenerative disorder; it afflicts approximately half a million people in the United States, and about 50,000 people are newly diagnosed every year.¹ Males are about two times as likely as females to develop the disease. The disease is characterized by both motor and non-motor symptoms, but most classically known for the motor aspect. The most common motor symptoms include: tremors, rigidity/stiffness, slowness of movement, and unsteady gait due to a loss of balance and coordination.² However, some people exhibit non-motor symptoms even prior to exhibiting motor symptoms, such as: apathy, excessive daytime sleepiness, anhedonia, loss of smell and taste, constipation, insomnia, depression and anxiety.³ Furthermore, many of these patients might go on to develop cognitive deficits as well, this might be in part due to the overlap in pathologies, such as is seen in Lewy body dementia. Hanagasi et al. found that approximately 33% of patients have mild cognitive impairments at the moment of diagnosis and that about 50% of those that do not will go on to present cognitive deficits two to six years from PD diagnosis.⁴ Thus, this disease is debilitating to the patient in the sense that they are limited in their day to day activities and eventually might develop a co-morbidity (i.e. PD with dementia).⁵ Not only is this a burden to the patient but to the family and caretakers as well. Furthermore, there are few treatments/therapies for Parkinson's disease despite the ongoing research efforts, such as that of the Michael J. Fox Foundation. This highlights the importance of furthering our knowledge of this disease process and in doing so, developing new and better treatment options, if not a cure, and also, finding ways to prevent the disease from happening in the first place.

1.1.2 Molecular Outcomes

1.1.2.1 Oxidative Stress

Oxidative stress seems to be a ubiquitous outcome or causative agent in many diseases, ranging from: cancer to heart disease, and allergies to neurodegenerative diseases.⁶⁻¹³ The human body -living organisms in general- produces free radicals, such as reactive oxygen species (ROS), as part of normal biological processes, namely cellular respiration.³ Nevertheless, our bodies are built with self-defense mechanisms such as glutathione, catalase and superoxide dismutase(SOD) enzyme which can 'neutralize' these radicals. However, the problem arises when this balance is perturbed, whether it be because of a genetic mutation in one of these enzymes (as is the case in some patients with Amyotrophic Lateral Sclerosis with mutation in SOD gene) or because environmental exposures are increasing these radical species in our organism. It is then that these highly reactive species are able to attack lipids and cause lipid peroxidation or attack DNA or proteins, causing subsequent damage. Oxidative stress is particularly of interest in neurodegenerative diseases given that the brain is a high energy organ (undergoing high levels of cellular respiration) and that there are many compounds (e.g. dopamine) that can be readily oxidized to toxic species.⁷ Furthermore, the Substantia Nigra pars compacta (SNpc) -the brain region mainly affected in PD- is rich in iron, thus rendering it more susceptible to oxidative damage as iron can undergo Haber-Weiss and Fenton reactions to produce radicals.¹³ The study of oxidative stress as a mechanism in PD was potentiated by the finding of the effects of the metabolite of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). This compound was ingested unknowingly by consumers of an illicit drug (MPPP) and they shortly after showed strikingly similar symptoms to PD; it was later found that the metabolite of MPTP, 1-methyl-4-phenylpyridinium (MPP⁺), is an inhibitor of complex I of the ETC (electron transport chain).¹⁴

1.1.2.2 Mitochondrial dysregulation

Given the pivotal role that the mitochondria has in cellular respiration, it is no surprise that mitochondrial dysfunction has been found to be a hallmark of neurodegenerative disorders, namely PD.^{9, 22-23} The importance of the mitochondria in neurodegenerative diseases has been highlighted by the many models used for PD, such as rotenone and MPTP whose toxic effects derive from their ability to inhibit complex I of the electron transport chain. Mitochondrial dysfunction is

dangerous for many reasons: 1) a ‘faulty’ mitochondria decreases the amount of energy being produced, 2) calcium (an important secondary messenger) homeostasis is disrupted, and 3) an apoptotic cascade can be initiated.⁹ Familial versions of the disease have led to the discovery of important proteins involved in the disruption of mitochondrial homeostasis.²³ Thus, dysfunctional mitochondria are a hallmark of PD and therapeutically targeting this or preventing it is of clinical interest.

1.1.2.3 Protein aggregation and α -synuclein

Parkinson’s disease is characteristically defined histologically by the presence of Lewy body inclusions predominantly in the Substantia Nigra pars compacta.¹⁵⁻²⁰ These intracellular inclusions are made up of misfolded, aggregated proteinaceous material, including: ubiquitinated debris, synphilin-1, tubulin, SOD2 (superoxide dismutase 2), and many other components, but the major component is α -synuclein.²¹ Given its large presence in Lewy bodies and some genetic variations of the disease are due to SNCA(α -synuclein gene) mutations, there has been extensive research on α -synuclein. Its function is still unclear but it appears to play a role in synaptic trafficking and possibly other neuronal functions. Recently, given its presence in other diseases as well (e.g. Lewy body dementia, Multiple System Atrophy), it has been proposed that perhaps α -synuclein has the potential to act as a prion-like protein and, upon being misfolded, may recruit other proteins to misfold as well. This theory has been supported by the fact that many researchers have found that Lewy bodies or α -synuclein oligomers, after transplanting these into the brain, can cause a PD like pathology in cellular models (i.e. SH-SY5Y neuroblastoma cell line) and multiple organismal models (e.g. non-human primates, mice –transgenic and non-transgenic- and rats).^{16-17, 19-20}

1.1.3 Etiology/PD models

Given that the underlying etiology of idiopathic or sporadic PD is unknown, different models have been developed to study the disease (in addition to genetic models to study the genetic aspects of the disease); however, they are all imperfect and thus the model is chosen based on the mechanisms one wishes to study. The most common cellular and organismal models, specifically

for the study of toxin based or impact of environmental factors in PD, are: 6-hydroxydopamine (6-OHDA), rotenone, MPTP, paraquat, and alpha-synuclein/Lewy body infusion.²⁴⁻²⁹ Some of these models are best to study behavioral outcomes, such as the unilateral 6-OHDA model which allows you to compare the effects of an unaffected versus an affected hemisphere. However, this model does not reproduce the classical Lewy body inclusions.²⁷ The MPTP model is very effective at producing nigrostriatal lesions, however its efficacy as a behavioral model is not as robust in rat models as it is in nonhuman primates.^{26,28} Furthermore, it does not show alpha-synuclein aggregation. The rotenone model has been reported to show motor impairments as well as alpha-synuclein aggregation; however, there is a high mortality rate.²⁵ The paraquat model shows decreased tyrosine hydroxylase (TH) levels in the striatum but does not cause motor deficits nor does it produce alpha-synuclein aggregation.²⁹ These models have been used largely in part due to the fact that they allow researchers to study the environmental toxin theory for sporadic PD. People could be exposed to MPTP, or a similar analog, via recreational illicit drug use (very unlikely), and rotenone and paraquat have been used as a pesticide and herbicide, respectively. However, there is limited information in the literature about people who were exposed to these toxins environmentally and later on developed the disease. Nevertheless, given the molecular and/or behavioral outcomes associated with these toxins, they are useful models for the development of prophylactics and therapeutics for sporadic PD.

1.2 Antioxidants –Nature as Nurture

Hippocrates said “Let your food be your medicine and your medicine be your food”. Presently, many current pharmacological therapies have their origins in naturally derived compounds. Ranging from digitoxin - a chemical originally found in the foxglove plant- which is used to treat heart problems, to penicillin – produced by a fungus- to treat infections, time and time again in history, we have found answers to medicinal ailments in nature.^{6,30} Many naturally derived compounds have been acclaimed for their antioxidant capabilities and many areas of nature await to be tapped into to unearth their potential.

Chapter 2: Tanshinone IIA – Antioxidant or Fibril mitigator

2.1 Introduction

Tanshinone IIA, 1,6,6-trimethyl-8,9-dihydro-7H-naphthol[1,2-g]benzofuran-10,11-dione, belongs to the group of chemicals Tanshinones found in Chinese sage (*Salvia miltiorrhiza*), of those it is the most abundant.³¹⁻³⁵ This diterpene quinone, commonly referred to as Dan Shen ketone, has been used in traditional Chinese medicine to treat cardiovascular diseases. However, given that it has been reported to have antioxidant properties, it has gained popularity in other areas of research where oxidative stress is a key player in disease onset and/or progression. Furthermore, studies by Wang, et al. (2013) showed that Tanshinone IIA could directly interact with full length

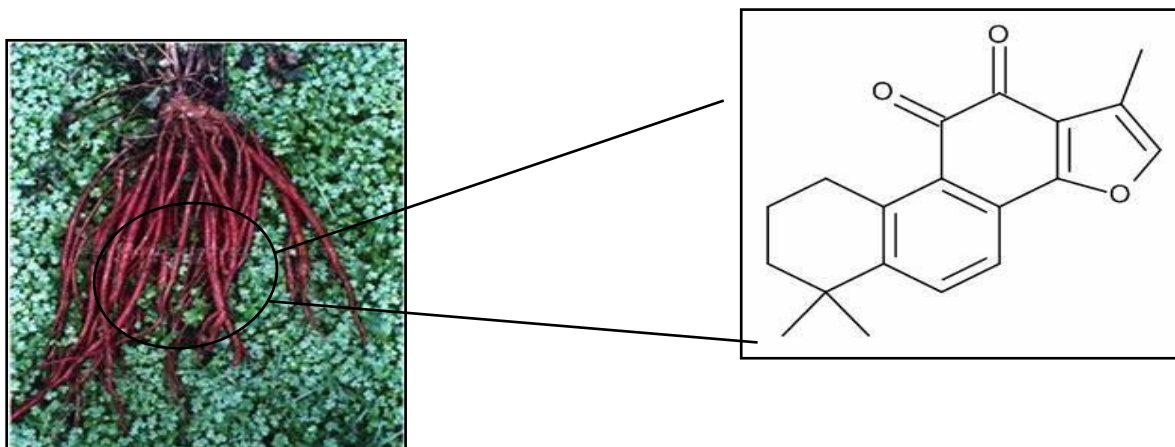


Image 1: To the left is an image of the Chinese sage roots from which the lipophilic Tanshinone IIA (structure shown on the right) is extracted.

peptide sequence of beta-amyloid ($A\beta_{1-42}$) and prevent the formation of fibrils and, of greater scientific interest, disassemble pre-formed fibrils. Thus, Tanshinone IIA is a perfect candidate to use as a molecule capable of intersecting the prion-like trajectory that can be initiated by the beta-amyloid peptide.³⁶

2.2 Specific Aims

Specific Aim 1: To evaluate Tanshinone IIA as a potential neuroprotectant by first determining the concentration at which it is not toxic to the neuroblastoma cell line, SH-SY5Y

Specific Aim 2: Evaluate if Tanshinone IIA can intervene in a heterotypic Parkinsonian model and rescue SH-SY5Y from A β ₂₅₋₃₅ insult

2.3 Methodology

2.3.1 Chemical reagents and Biological agents

SH-SY5Y cell line was purchased from ATCC [ATCC CRL-2266]. SH-SY5Y cells were transfected with pCMV6-SNCA plasmid made by a previous member of the lab.¹ Beta-amyloid (25-35) [H-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-OH] peptide was purchased from Anaspec. Tanshinone IIA (TIIA) was purchased from Selleckchem [S2365], and sodium- β -hydroxybutyrate (Na β H) and rotenone (RT) were purchased from Sigma-Aldrich [T4952 and 54965]. Hoechst 33342, Propidium iodide (PI), and H2DCFDA were purchased from Life Technologies [H1399, P1304MP, and D399, respectively].

Cell Culture: SH-SY5Y cells were cultured in Dulbecco's Modified Eagle's Medium/ F-12 formulation (DMEM/F-12) supplemented with 10% fetal bovine serum and 100 units penicillin/100 μ g of streptomycin antibiotic as a prophylactic. Cells were grown in an incubator set at 37°C and 5% carbon dioxide. Cell line utilized was stably transfected to express α -synuclein by previous lab members.³⁶ Cells were detached with Gibco Trypsin-EDTA (0.25%) [25200072]

Amyloid-Beta preparation: The following preparation was followed for all experiments involving the use of the amyloid-beta (25-35) peptide. The A β ₂₅₋₃₅ peptide was purchased as a lyophilized powder from Anaspec (AS-24228). The powder was stored at -20°C in original container until preparation. Prior to use, powder was allowed to equilibrate to room temperature, the entire contents of the glass vial were then used to prepare a 5 mM stock solution in ultra-pure (milliQ) water. The solution was then either used immediately for experiments or stored at -20°C for future use.

2.3.2 Dose determination (Cytotoxicity Assay)

In order to use Tanshinone IIA as a mitigator of beta-amyloid induced damage, we needed to determine an optimal concentration at which Tanshinone IIA would not be toxic to the SH-

SY5Y cells. A 96-well plate was seeded with 20,000 cells/well. A stock concentration of 5 mM Tanshinone IIA was prepared in fresh DMSO once the cells were confluent. Serial dilutions were made so as to add a range of concentrations (500 nM, 1 μ M, 2.5 μ M, 5 μ M, 7.5 μ M, 10 μ M, 12.5 μ M, 25 μ M, 50 μ M) to the cells with DMSO at no greater than 1% (v/v) in the culture medium. A negative control of cells with media only was used and a solution of 100 μ M hydrogen peroxide was used as a positive control for cell death. This experiment was conducted both for a 24 h period and a 48 h period. An hour before the conclusion of the 24 h and 48 h incubation period, Hoechst 33342 and Propidium Iodide were added to the cells at a final concentration of 1 μ g/ml. After an hour incubation of these dyes, the plate was read in an InCell 2000 Analyzer Bioimaging system (GE Healthcare). Eight replicates were performed for each test, means are shown and +/- S.D. bars are shown.

2.3.3 *Oxidative Stress*

A 96 well plate with 20,000 cells/well was seeded. Once plate reached confluency, SH-SY5Y cell were then pretreated with Tanshinone IIA (2.5 μ M and 7.5 μ M) for 1 hour prior to the addition of 20 μ M Amyloid-beta peptide (prepared as described previously). A stock solution of dichlorofluorescein diacetate- H_2 was prepared in DMSO immediately prior to use. After 24 hour incubation of treatments, the dye was added at a final concentration of 20 μ M in phenol red free media. Loading of the cells with the dye was allowed for 30 min under dark conditions at 37°C. After this period, the relative intensities (A.U.) were then read in a microplate reader (Thermo Fisher Fluoroskan Ascent) with filters set for an excitation of 485 nm and emission of 527 nm. Images of fluorescence were captured with LSM 700 Zeiss confocal microscope using 5x objective. Quadruplicate replicates for each treatment were performed, graphs show means with +/- S.D. error bars.

2.3.4 *Mitochondrial Dysregulation*

Rhodamine 123 is a probe used to measure mitochondrial membrane potential. SH-SY5Y cells were seeded at a confluency of 20,000 cells/well in a 96 well plate. Once cells were at 80-90% confluency, they were treated with 2.5 μ M and 7.5 μ M for 1 hour prior to exposing them to

20 μM $\text{A}\beta_{25-35}$ for a 24 hour period. After the 24 h incubation, rhodamine 123 dissolved in DMSO was added to the cells to a final concentration of 10 μM (1% v/v [DMSO/media]). The dye was allowed to permeate the cells for 30 min in the dark at 37°C in phenol-free media. After the 30 min incubation, relative fluorescence intensities were read in a Fluoroskan Ascent microplate reader with filters set at excitation of 485 nm and emission of 527 nm. Quadruplicate replicates for each treatment were performed, graphs show means with +/- S.D. error bars.

2.3.5 *Apoptosis Detection via Phosphatidylserine externalization*

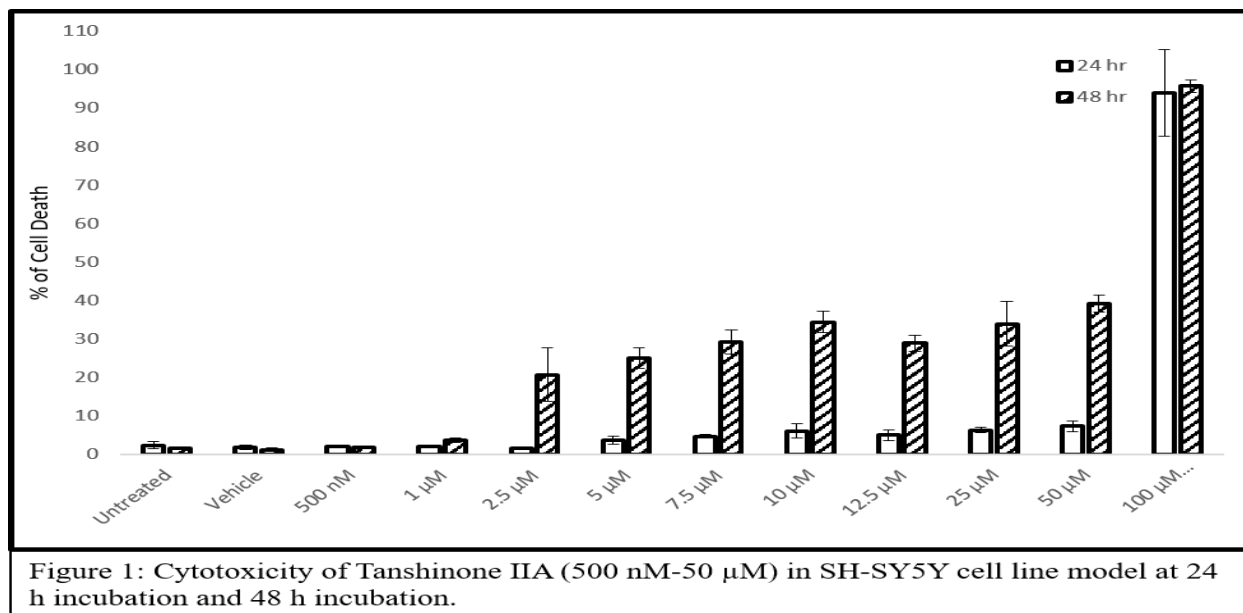
SH-SY5Y cells were seeded at 100,000 cells/ well in a 24-well tissue culture plate. Once confluent, treatment of 2.5 μM and 7.5 μM TIIA was applied 1 hour prior to exposing the cells to 20 μM $\text{A}\beta_{25-35}$. After 24 hour incubation, the culture media was transferred to flow tubes set on ice, the remaining cells were detached using 0.25% EDTA-Trypsin and added to their respective flow tube. The culture media cell mixture was then centrifuged at 1200 RPM for 5 min at 4°C on a table top centrifuge. The supernatant was then discarded and cells were re-suspended in 100 μL of 1 X binding buffer (BD Pharmingen) with Propidium Iodide (1 $\mu\text{g}/\text{mL}$) and Annexin V-FITC (BD Pharmingen). The cells were then incubated in this mixture in the dark, on ice for 15 min. After this incubation, an additional 400 μL of 1 X binding buffer was added to each flow tube. Samples were then read on Gallios Flow Cytometer (Beckman Coulter). Parameters were set so that the instrument would detect 10,000 events per sample, with a time limit of 3 minutes. Triplicates of each treatment were performed, values shown are representative means with +/- S.D. bars shown.

2.4 *Results and Discussion*

2.4.1 *Optimal Dose Determination*

The toxicity of Tanshinone IIA (TIIA) at varying concentrations (500 nM-50 μM) was determined using a differential nuclear stain method. Cells were incubated under standard conditions with the compound for 24 and 48 hours; proper negative (media only) and positive (hydrogen peroxide) controls for cell death were used. In order to determine the live/dead cell ratio, Hoechst 33342 – a dye that readily permeates both live and dead cells- and Propidium Iodide –a dye that can only permeate cells with an un-intact membrane- were used at a concentration of

1 $\mu\text{g/mL}$. Thus, Hoechst gave the count of total number of cells, whereas Propidium iodide positive cells reflected the dead cells. As can be observed in *Figure 1*, after 24 hours, there was a dose dependency to cell toxicity, but even at a concentration of 50 μM there were low percentages of

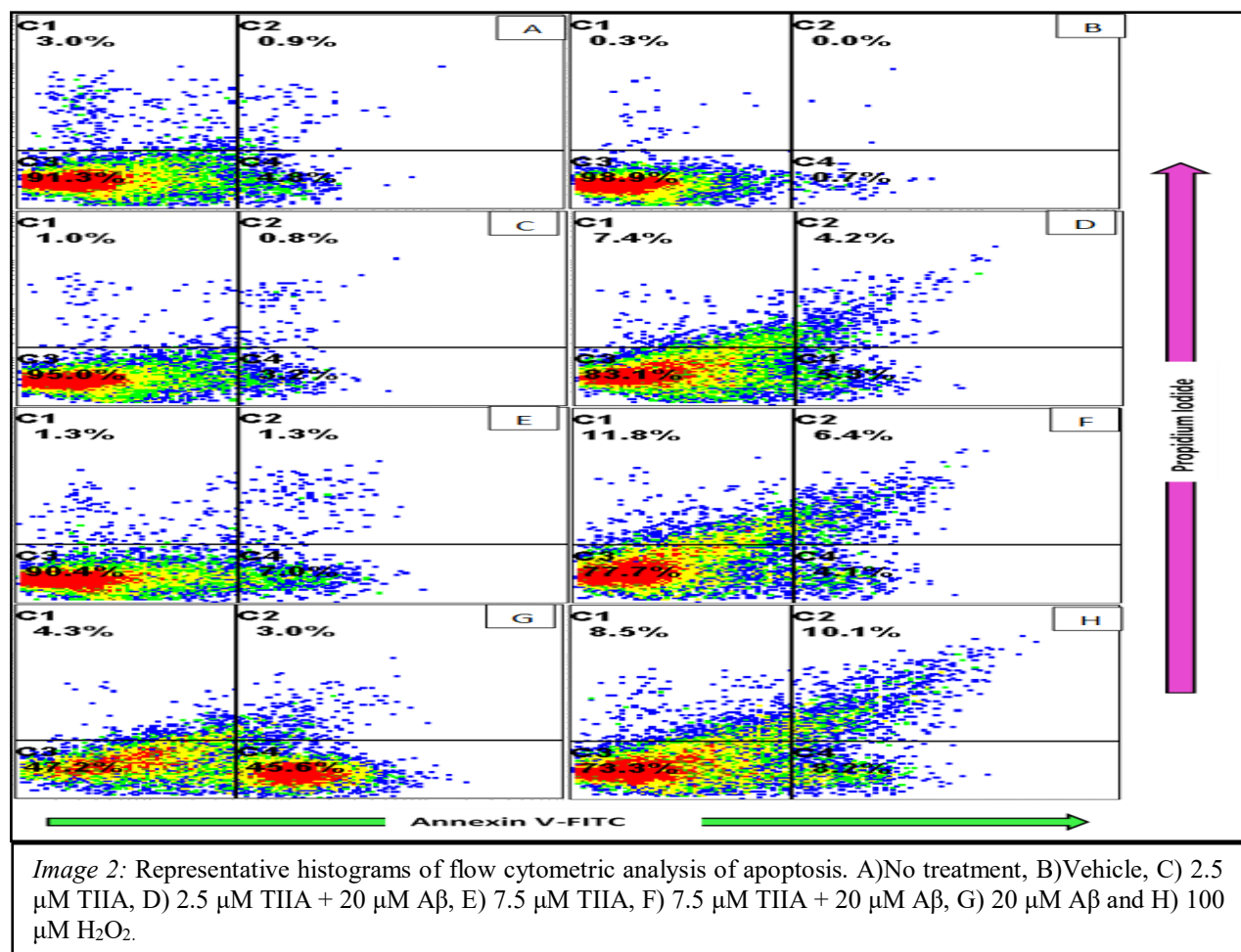


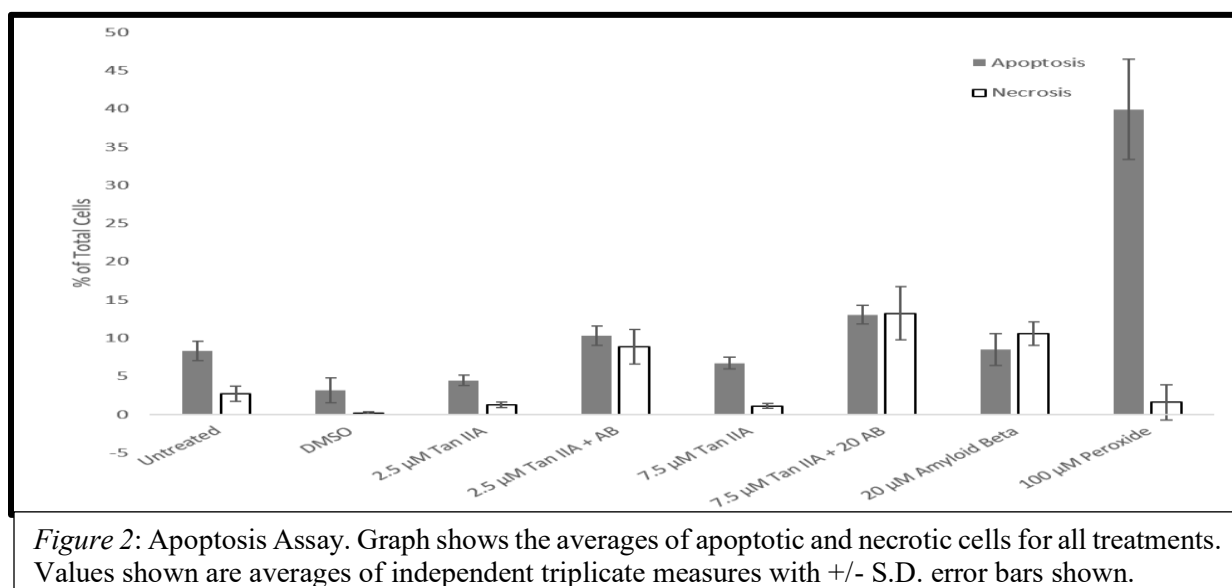
cell death (~8%) as compared to 100 μM hydrogen peroxide (~94%). However, the % of cell death for 2.5 μM -50 μM TIIA was greater after a 48 hour incubation period. Given that all subsequent experiments were carried out in 24 hour periods only, the 24 h results were used to determine the optimal working concentrations. The concentration of 2.5 μM was the highest concentration that still had approximately the same percentage of death as the vehicle alone (~1.6% for 2.5 μM , ~1.8% for DMSO alone) indicating that the compound itself is not toxic at this concentration; thus, this concentration was used for consecutive experiments. Additionally, although 7.5 μM showed a higher % of cell death (~4.6%), this concentration was used to establish proof of concept given that this concentration has been reported to be therapeutic/bioactive by the literature.³³⁻³⁴

2.4.2 Programmed Cell Death

Apoptosis (Type I programmed cell death) has been well established as a mechanism that takes place in the neurons affected in Parkinson's disease. Consequently, many of the PD models have been known to induce apoptosis –both in cell lines (e.g. PC12, SH-SY5Y) and in organismal models (e.g. mice and rats). Protein aggregation, mitochondrial dysfunction and oxidative stress

(all events that occur in PD) can ultimately lead to apoptotic death. Thus, we set forth to determine if Tanshinone IIA could protect the neuroblastoma cell line from amyloid-beta induced cell death. However, as can be seen in *Figure 2*, there was no statistical significance in the difference of apoptotic death in the A β treated cells as compared to the cells that were in standard culture conditions with no treatment ($p>0.05$). Thus, rescuing potential of Tanshinone IIA could not be measured, given that the insult itself did not cause apoptotic death. Nonetheless, it is noteworthy to mention that despite the fact that the beta-amyloid peptide did not cause a statistically significant change in cell death (apoptotic or necrotic) as can be seen in *Image 2*, there is a signature pattern in the side scatter of cells that were treated with the amyloid beta peptide (note panel D, F and H as compared to the others). Thus, although amyloid beta is not inducing phosphatidylserine





externalization to signal apoptosis, the cellular morphology is undergoing changes in response to the presence of the peptide.³⁷⁻³⁸

2.4.3 Intracellular Reactive Oxygen Species

Increase in reactive oxygen species (ROS) and in turn, oxidative stress, is a hallmark of PD. This intracellular cascade can be replicated in many disease models of PD, such as RT and MPTP. Previous lab members of our group sought out to determine whether A β ₂₅₋₃₅ insult, would induce an increase in reactive oxygen species.³⁶ As *Figure 3* shows, we were able to reproduce their work and found that despite the toxic effect of A β ₂₅₋₃₅, it does not directly cause an increase in the production of ROS, unlike hydrogen peroxide treatment which even at low concentrations (100 μ M) produces statistically significant ($p < 0.05$ as per a student's t-test) higher amounts of ROS than those found in cells with no insult.³⁹⁻⁴¹

2.4.4 Mitochondrial Membrane Potential

Given that the mitochondria is the hearth of energy production in the cell, mitochondrial integrity and homeostasis is vital to cell function and its disruption is a hallmark of many disease processes, including PD. Oxidative stress and nitrosative stress are known to cause a cascade of cellular events that ultimately result in decreased mitochondrial membrane potential (MMP).⁴² As

can be seen in *Figure 4*, the Beta-amyloid (25-35) peptide at a concentration of 20 μM caused a decrease in mitochondrial membrane potential, just as the positive control hydrogen peroxide, as compared to the cells that received no treatment. Furthermore, Tanshinone IIA was able to restore the MMP when the cells were pre-treated with TIIA prior to amyloid-beta insult both at 2.5 μM ($p < 0.01$ [$**$]) and 7.5 μM ($p < 0.05$ [$*$]). These results indicate that TIIA is capable of interfering with the A β and attenuating its toxic effects to the cell.

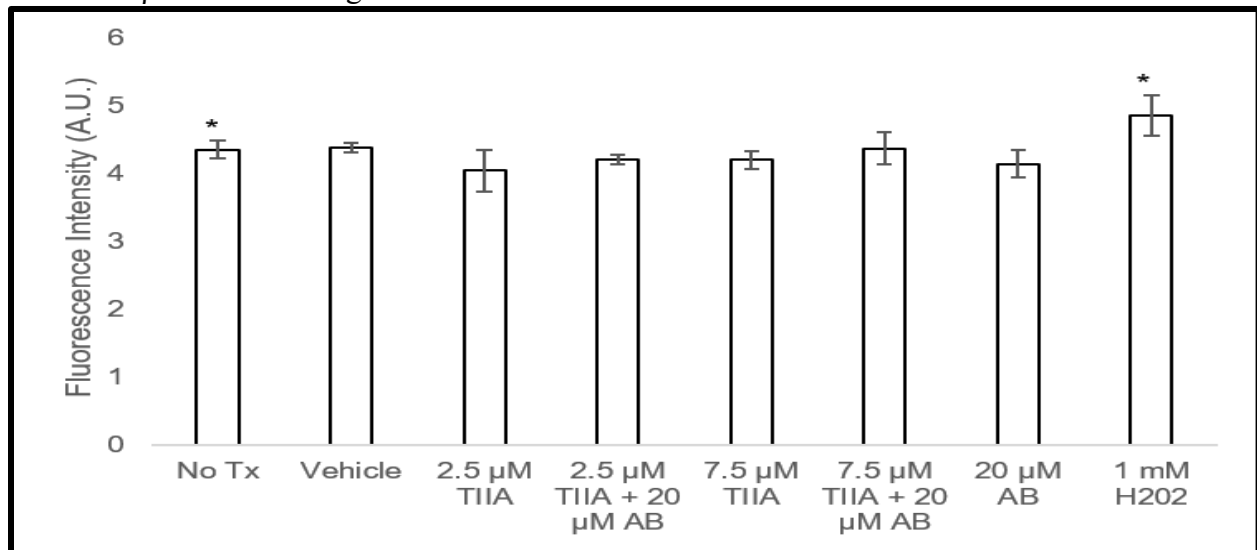


Figure 3: ROS Assay: Bar graphs show averages for quadruplicate measures for each treatment. S.D. +/- shown.

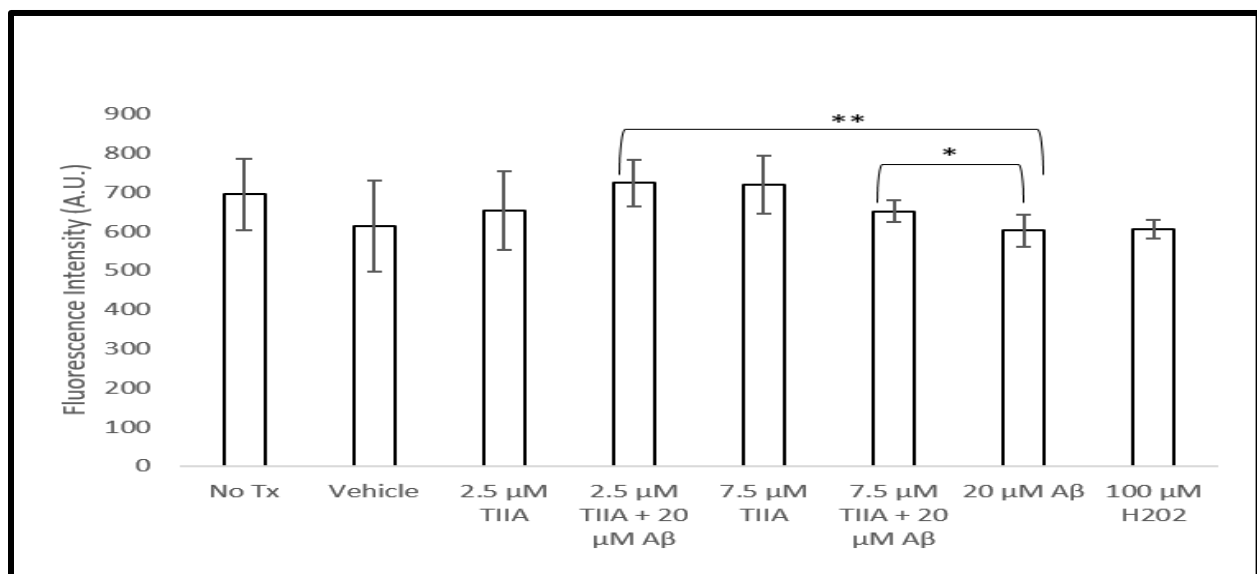


Figure 4: MMP Assay: Averages for quadruplicate measures for each treatment. S.D. +/- shown.

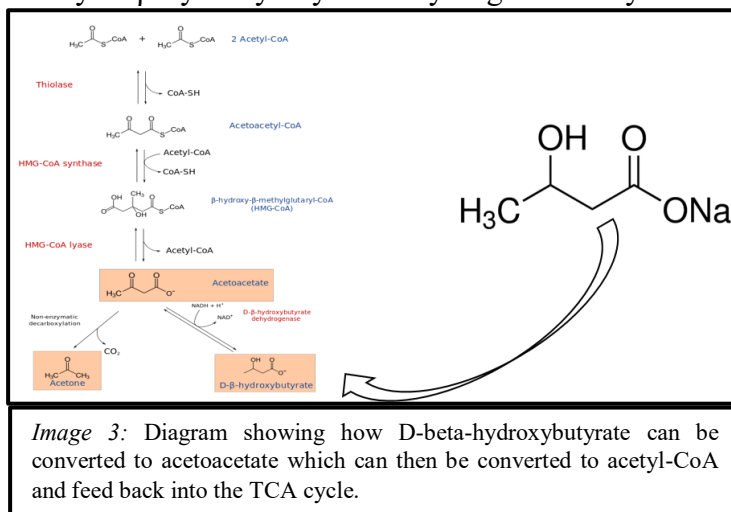
2.5 Conclusion

Collectively, we have used well-established and standard metrics to evaluate the ability of a natural compound, Tanshinone IIA -which has been lauded for its antioxidant, anti-inflammatory and lipase inhibitory activity- to rescue a neuroblastoma cell line in a heterotypic model where a prion-like protein, A β , was used as a toxicant to reproduce hallmark cellular aberrations of a PD model. Overall, Tanshinone IIA was non-toxic to the SH-SY5Y neuroblastoma cell line and capable of protecting the cells from A β -induced mitochondrial dysregulation when the cells were prophylactically treated with the compound. However, further assessments of Tanshinone IIA's therapeutic potential could not be made given that the Beta-amyloid peptide at a concentration of 20 μ M did not induce a marked increase in apoptosis or ROS production; which is contrary to the case for most PD models. This suggests that the mechanism of toxicity of this prion-like protein is distinct to that of current commonly used toxins used to model PD in a cell model (i.e. rotenone, 6-OHDA, MPTP). Thus, different experimental procedures to those used for such models should be used to examine the effects of amyloid beta on a dopaminergic-like cell line. Previous literature has shown that Tanshinone IIA might directly interact with specific residues in the full length peptide sequence of A β ₁₋₄₂,³⁴ this supports the idea that TIIA is not serving a role as a ROS scavenger in this scenario but perhaps directly interacting with the peptide to prevent fibrillization instead. Overall, none of the classical models of sporadic/idiopathic PD - toxin models such as paraquat, rotenone, MPTP, or 6-OHDA- fully reproduce all of the cellular mechanisms associated with the disease. This is corroborated by the fact that we have failed to develop new drugs or therapies which can halt the disease progression, or cure it. Thus, new approaches need to be taken, such as looking at the possible interaction of two distinct proteins, namely A β and α -synuclein, namely) and how the two can together cause a mass exodus of protein misfolding. Then, with a deeper understanding, we can test compounds which have demonstrated therapeutic potential and to determine how they fare in a heterotypic landscape.

Chapter 3: A ketone as a prophylactic for PD in a vertebrate model

3.1 Introduction

D-β-hydroxybutyrate is a ketone, mainly the byproduct of fatty acid oxidation in the liver but it can also be made from ketogenic amino acids via first producing acetoacetate that is then converted to D-β-hydroxybutyrate by the enzyme β-hydroxybutyrate dehydrogenase enzyme. This ketone can be used by the brain as a fuel source when there are low levels of glucose. This small compound is of interest given that it is produced endogenously during states of fasting and thus would intuit that it would have little to no side effects if used as a prophylactic agent. Furthermore,



Kabiraj, et al. (2016) showed in SH-SY5Y cell studies that NaβHB was able to rescue from rotenone induced damage, and Tieu, et al. (2003) demonstrated that this specific stereoisomer, D-β-hydroxybutyric acid, readily crosses the blood brain barrier (BBB) and rescued mice from MPTP induced nigrostriatal damage when administered prophylactically via osmotic pumps at 1.6 mmol/kg/day in normal saline.⁴³ Thus, D-β-hydroxybutyrate, shows promise as a prophylactic agent that can protect from the pathology that ensues in toxin induced-PD models.

3.2 Specific Aims

Specific Aim 1: We proposed to evaluate whether the salt of D-β-hydroxybutyrate was capable of preventing rotenone-induced motor deficits in a Lewis rat model via a battery of well-established behavioral tests.

Specific Aim 2: We proposed to examine the extent, if any, to which Sodium-β-hydroxybutyrate was capable of protecting the dopaminergic neurons of the Substantia Nigra pars compacta from rotenone-induced death.

3.3 Methodology

3.3.1 *Animals*

Initially a pilot study was conducted with 21 male Lewis (LEW/Hla®CVF®) rats purchased from Hilltop Lab Animals Inc. (Scottsdale, Pennsylvania). For the expanded study, forty male Lewis rats of 6 months of age, weighing approximately 400-600 g were purchased from Envigo®. The holding room for the animals was on a reverse light cycle (lights off at 8:00 A.M., lights on at 8:00 P.M). The rats were pair-housed in IVC cages in a temperature controlled setting (22+/-2°) and provided water and standard laboratory chow *ad lib*. All experiments with animals were performed in accordance with the Institutional Animal Care and Use Committee of the University of Texas at El Paso.

3.3.2 *Materials:*

Rotenone (RT), (+/-)-Sodium 3-hydroxybutyrate (NaβHB), and Sunflower seed oil from *Helianthus annus* were all purchased from Sigma Aldrich (R8875-1G, 59465-10G-F and 88921-1L-F, respectively). Thionin acetate (229840250) was purchased from Acros Organics as a high purity reagent. Ethanol (histological grade), xylene and dimethylsulfoxide (DMSO) were purchased from Fisher Scientific (A405-20, X3S-4, and BP231-1, respectively).

3.3.3 *Experimental Outline*

In order to test the prophylactic/neuroprotective capability of sodium-β-hydroxybutyrate in a rodent PD model, the following injection regimen was followed (as can be seen in *Image 4*).

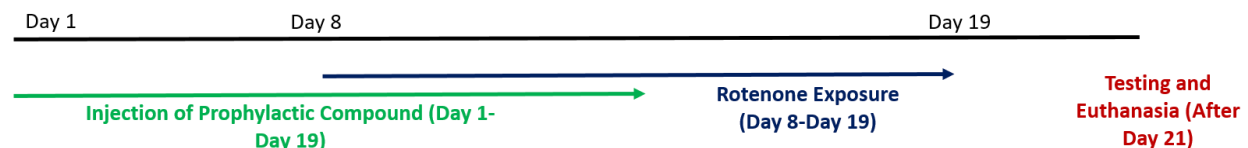


Image 4: Scheme showing injection regimen.

Overall there were 5 groups: 1) no injection/control group, 2) vehicle (V), 3) sodium-β-hydroxybutyrate, 4) sodium-β-hydroxybutyrate + rotenone, and 5) rotenone only. The no injection group received no injection, these animals underwent the same handling practices, behavioral testing and husbandry conditions as their rat mates but they were never injected or administered

any treatment. The vehicle group received an injection corresponding to the same volume that the rotenone rats would receive to serve as a control for the emulsion in which the rotenone was prepared, which was made of 2%(v/v) DMSO in Mygliol medium chain triglycerides (gifted from OLEO Chemicals) for the pilot study and 2% (v/v) DMSO in Sunflower seed oil for the expanded study. The Na β HB administered to those in the Na β HB only group and those in the Na β HB and rotenone group was prepared at a concentration of 50 mg/mL in normal saline (0.9% [w/v] sodium chloride in ultra-pure, sterile water) and was administered at a dose of 200 mg/kg. The rotenone powder was prepared as a 30 mg/mL stock solution in DMSO and then diluted Sunflower Seed Oil to a 3 mg/mL solution; this solution was prepared fresh every 3-4 days, stored in the dark, and re-suspended thoroughly prior to ensure a homogenous solution.²⁵

3.3.4 *Behavioral Testing*

A total of four behavioral tests were performed to gauge the degree of motor deficits, if any. All the behavioral tests mentioned below have been used by other neurodegeneration research laboratories as a measure for PD model to determine presence/absence of motor deficits and severity. The tests performed were as follows: 1) Adhesive removal test, 2) Forelimb Placement test, 3) Cylinder test and 4) Akinesia Test.⁴⁴

3.3.4.1 *Adhesive Removal Test*

This behavioral test, also known as the “sticky dot test” is meant to measure the somato-motor capabilities of the rat, to include: 1) somato-sensation and 2) motor. Avery® circular adhesive sticky dots (12 mm diameter, red glow color) were placed bilaterally on the glabrous pad of the forelimb paws. After the sticky dots have been secured on both the right and left forepaws, the animal was then set inside of a clean cage and the timer was started, experimenters then recorded time to contact (the amount of time it took for the animal to note that it had a foreign stimulus and touch it to its mouth) and the time to removal (the total amount of time from when the rat was placed down until when it removed the sticky dot from its paw. If the animal had not yet touched or removed the sticky dot after three minutes, a time of 180 seconds was marked as the final score for that measure. The time to contact is a measure of the animal’s sensory capability,

whereas the time to removal is a reflection of its motor abilities. This test was conducted three times and averaged. All data shown are averages and error bars reflect +/- S.D.

3.3.4.2 *Forelimb Placement Test*

A variety of the forelimb placement test was used that is meant to measure the rat's reflexes. For this experiment, the rat was placed on the edge of a tabletop so that all its limbs were on a solid surface, then, one of its forelimbs was slid off of the tabletop so that it did not have a solid support. The amount of time it took for the rat to place its forelimb back on the tabletop was measured. This was done a total of three times for each forelimb. Values shown in Figure ... are averages and error bars represent +/- S.D.

3.3.4.3 *Cylinder Test*

This test is meant to measure the animal's rearing behavior by exploiting its natural exploratory tendencies. The rat will be placed in a Plexiglas cylinder of dimensions 30 cm (height) x 20 cm (diameter). The test was conducted during the animal's awake cycle in the dark and then video recorded for 2 min. The number of rears (defined as when the animal is supporting itself only on its two hind limbs and using its forelimbs to touch the walls of the cylinder) was then calculated by two experimenters blinded to the treatment whom watched the videos and recorded all paw touches within a 120 second time span. The test was just conducted once per animal given that the very nature of this experiment relies on its novelty to the animal. The values of both experimenters had to be at a minimum of a 95% concordancy. The values shown in *Figure 11* reflect the averages and +/- S.D. error bars are shown.

3.3.4.4 *Akinesia Test*

For this experiment, a rat was placed in a Plexiglas chamber shaped as a triangle with dimensions as follows: 15 cm (length) x 11 cm (width) x 13 cm (height), where the base side of the triangle was open ended. In this experiment, the rat was placed so that its nose was touching the inside of the apex. The experimenter then recorded the amount of time it took the animal to exit the enclosure, start time was recorded when the rat was touching apex of the triangle and after it was released, and end time was recorded when the nose of the animal passed the boundary line

of the triangle (i.e. open ended side at the base of the triangle). The animal was allowed two minutes to exit the enclosure, if they had not exited after the time allotted, a time of 120 seconds was recorded and animal was removed from enclosure. This test was performed a total of three times for each animal and values shown are averages with error bars shown being +/- S.D.

3.3.5 *Neurochemistry*

3.3.5.1 *Monoamine assay*

For pilot study, after the conclusion of behavioral testing animals were sacrificed for tissue collection. The striatum (Caudate nucleus and Nucleus accumbens) were excised from the tissue while the brain was on ice and then rapidly weighed. Immediately afterwards, tissue was placed in a microcentrifuge tube in the internal standard solution [8 μ M EDTA (ethylenediamine tetraacetic acid), 400 μ M sodium metabisulfite ($\text{Na}_2\text{S}_2\text{O}_5$) in 0.05 N Perchloric acid with DHBA (dihydroxybenzylamine) at 0.1 ng/ μ L] followed by sonication. The tissue was then centrifuged for 30 minutes at 10,000 x g at 4°C. Tissue was then filtered and placed in HPLC tubes and analytes were processed within two weeks of isolation. Samples were ran on an ESA 542 autosampler HPLC using an isocratic system with a C_{18} column coupled with an electrochemical detector. The mobile phase was as follows: 69 mM sodium monobasic phosphate, 33 mM citric acid, 0.127 mM EDTA, and 1.72 mM sodium octyl sulfate in 6% (v/v) methanol in water, pH 2.5. Analytes detected were: dopamine, DHBA, 5-hydroxyindoleacetic acid, 5-hydroxytyramine, homovanillic acid, 3, 4-dihydroxyphenylacetic acid (DOPAC), and norepinephrine (chromatogram not shown). Figure 7 shows results for dopamine only as ng of dopamine present per mg of tissue analyzed.

3.3.5.2 *Nissl Staining*

In order to identify the region of interest, a representative section (one tissue section from each of the five per well) was taken for Nissl staining. Nissl staining is a process which is based on the use of a basic dye, such as Thionin acetate or Cresyl violet, to stain the negatively charged nucleic acids (i.e. DNA and RNA). This staining method derives its name from the fact that Nissl was a term used to refer to the endoplasmic reticulum (ER), and given the high abundance of ribosomal RNA in the rough ER this region is heavily stained. Thus, with this method, nuclei and

rough ER create characteristic patterns (as has been documented in atlases such as the Swanson Atlas) in histological preparations which allow for the identification of different structures.⁴⁵ (Swanson) Brain tissue sections were removed from the cryo-protectant and washed in 1 X Phosphate Buffered Saline then mounted onto previously subbed slides. The slides were then left to dry overnight. The following day the sections underwent the following process for staining: dehydration -using solutions of increasing ratio of ethanol/water (100% water to 100% ethanol)-, then de-fattening – left in Xylene for 30 min-, then rehydrated, then placed in 0.25% Thionin acetate in a solution of pH 4.5 for 30 seconds, and finally, sections were dehydrated as before and after the xylene, tissue sections were allowed to dry and then cover slipped and sealed with CytoSeal60 (Thermo Scientific; Catalog# 8310-16). Images were taken using BZ-X700 Keyence All-In-One Fluorescence microscope with 2x objective, mosaic of images were made using the instrument's software.

3.3.5.3 *Immunohistochemical detection of TH-positive neurons*

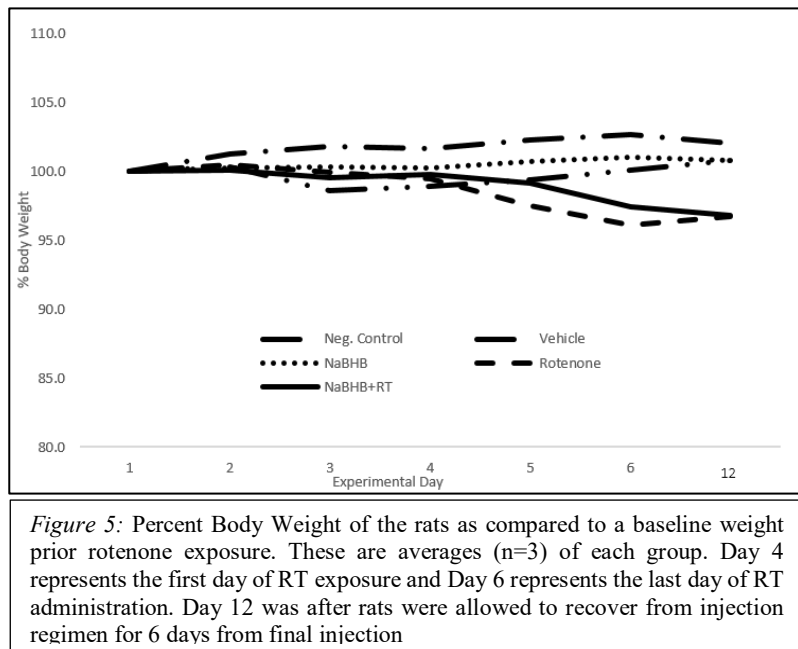
For the longitudinal study, in order to determine the extent of the nigrostriatal lesion, if any, the brain tissue of the rats was analyzed by immunohistochemistry. The rats were perfused with approximately 500 mL of normal saline (0.9% sodium chloride in water) followed by approximately 250-350 mL of 4% (w/v) paraformaldehyde (PFA) in 1X phosphate buffered saline (PBS). The rat brain was then extracted and stored in 4% PFA in 1 X PBS overnight at 4° C. The following day, brains were transferred to a 30% sucrose (w/v) in 1 X PBS solution for 48-72 hours. The brains were then placed on a pedestal and covered in OTC (optimal temperature cutting solution) and placed in a -20°C Leica CM1850 Cryostat for 30 – 120 min to allow complete freezing of the brain prior to slicing. Once frozen, the brain was cut in coronal sections in 30 µm thick sections. Brain sections were collected in a serial fashion, where 5 consecutive sections were placed into the same well so that that singular well could account for the same atlas level. The sections were stored in a cryoprotectant solution [30% (v/v) ethylene glycol/20% (v/v) glycerol in 1 X PBS] and stored at -20°C for later use. Sections were then immunolabeled for detection of TH (tyrosine hydroxylase) positive neurons. The tissues were first washed in 1 X PBS, each section

was washed three times for 8 minutes/wash. After washing off the cryoprotectant solution, tissues were then blocked in blocking solution [2% normal goat serum (NGS) in 1 X PBS + 0.1% (v/v) Triton X-100], to prevent non-specific interactions and for tissue permeabilization, for 2 h on a shaker at room temperature. Afterwards, tissues were washed three times in 1 X PBS, for 8 min/wash. The sections were then placed in the primary antibody solution [Anti-tyrosine hydroxylase (Mouse) (1:100) in 1 X PBS; Santa Cruz Biotechnology, Catalog#: sc-25269] overnight at 4°C for 24 hr. After the conclusion of the incubation in primary antibody, tissues were washed in 1 X PBS for 8 min/wash, three times. Tissues were then placed in secondary antibody solution [Anti-Mouse- Alexa 488 conjugated antibody (1:2000 in blocking solution); Thermo Scientific, Catalog# A11001] for a 2 h incubation at room temperature in the dark. The tissue sections were then washed three times in 1 X PBS for 8 min/wash. Finally, tissues were incubated for 10 minutes in Hoechst 33342 (10 µg/mL) in 1 X PBS at room temperature on a shaker for a nuclear counterstain. Washes were then performed as previously mentioned. After staining, tissue sections were mounted onto microscope slides; once tissue sections were dried, tissues were coverslipped using 50% (v/v) glycerol in 0.1 M sodium bicarbonate solution and sealed with clear nail polish. Slides were allowed to dry overnight and then stored in 4 °C until imaging. Slides were imaged using LSM 700 Zeiss Confocal Microscope. Images were taken using 10 x objective and the same parameters were used for all images: 1024x1024 pixels, laser power of 2.0, speed of 7, averaging of 8 and at 1.0 airy unit. Number of TH-positive neurons were quantified using FIJI (Fiji Is Just Image J) software with cell counter plugin. The tyrosine hydroxylase (TH) positive neurons of the Substantia Nigra pars compacta were quantified, a representative section corresponding to page 37 of Swanson atlas (as corroborated via Nissl staining-mentioned previously) was used for all animals. Experimenter was blinded to treatment.

3.4 Results and Discussion

3.4.1 Pilot Study

A pilot study was conducted to determine the effectiveness of a 2.5 mg/kg/day dose of rotenone over a span of three days. The same groups as mentioned above (No injection, vehicle, Na β HB, Na β HB +RT, and RT only) were utilized; given that this was a pilot study only three animals were used per group.



The prophylactic compound was administered for 7 days prior to the exposure to rotenone and then the prophylactic was administered in conjunction with rotenone for 3 more days for a total of 10 days of Na β HB I.P. (intraperitoneal) injections and 3 days of rotenone I.P. injections. As can be seen in *Figure 5*, rats experienced a drop (>5%) in body weight but nowhere near experimental endpoints. There were no unexpected mortalities in this study. Overall, the forelimb placement test showed no differences (data not shown, average ~0.18 seconds for all groups) amongst groups (statistical analysis not shown) as this test measures the reflex ability of the rat, which is not necessarily affected by nigrostriatal lesion. For the adhesive removal test, two measures were recorded, the time to contact (defined as the amount of time it took for the rat to notice a foreign stimuli on its paw) and the time to removal (as data is shown here, the amount of time from when rat noticed foreign stimuli-contact- to the time it removed the foreign stimuli). *Figure 8* shows both measures, with time to contact shown on the left panel and time for removal on the right panel. As can be observed, there were no differences amongst the group for time to contact. Given that this is a measurement of somatosensory affects, it shows that the animal's ability to sense (feel) stimuli was not affected. However, although there were no statistically significant differences, for the time to removal there is a pattern indicating that the group of rats treated with

only rotenone took longer to perform the task; indicating that there might have had a slight motor deficit. The cylinder test data, as can be seen in *Figure 6*, However, no conclusions can be drawn from this behavioral data given that there was not sufficient power in this study determine effect and to render any of the data statistically significant. In addition to the behavioral tests, a neurochemical analysis was performed of the brain tissue to measure the levels of catecholamines and their metabolites. Overall, serotonin, 5-HIAA, DOPAC, HVA, norepinephrine and dopamine were measured. *Figure 7* shows the levels of dopamine (ng/mg) in striatal tissue, each bar graph is the average per group (n=3). The data obtained from the pilot study was used to design a more robust experimental design for the rotenone administration in the subsequent study.

3.4.1.1 Behavioral Data

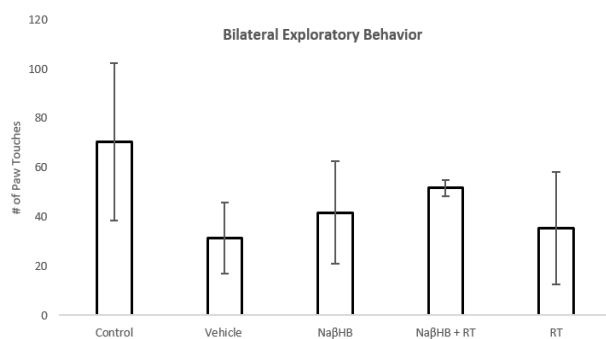


Figure 6: Cylinder Test Data is shown here as averages (n=3/group) with +/- S.D. error bars. Total # of rears (paw touches) with both forelimbs were summed.

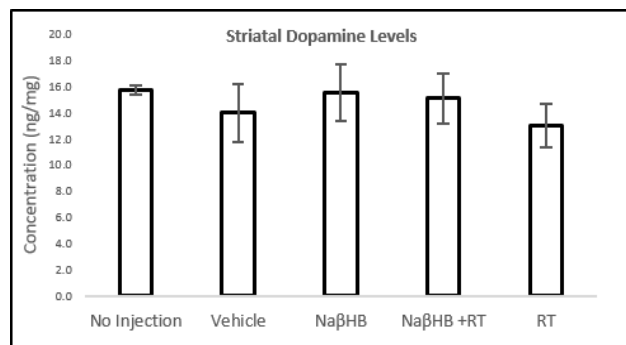


Figure 7: Monoamine assay. Levels of dopamine (ng of dopamine/mg tissue) in the striatum were assayed via HPLC-ECD analysis.

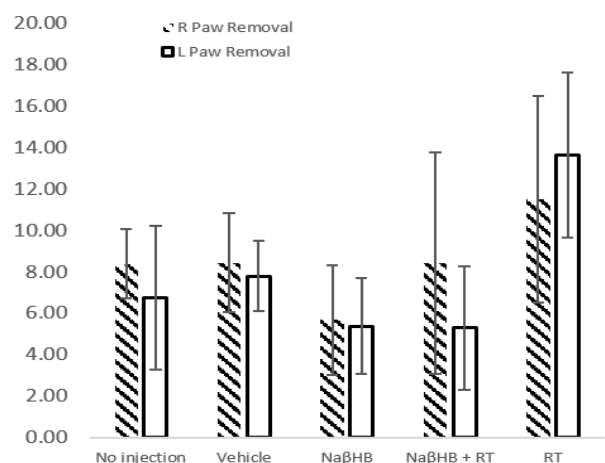
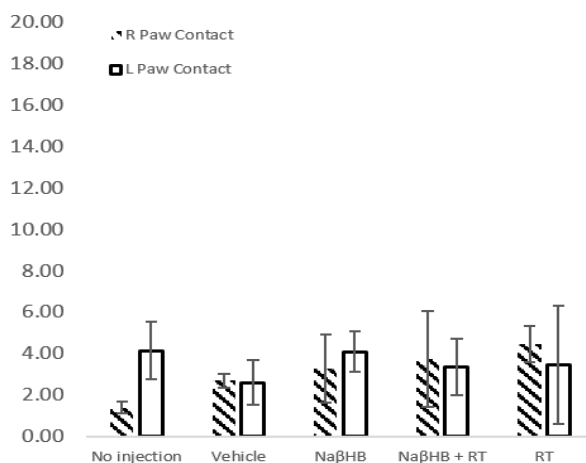
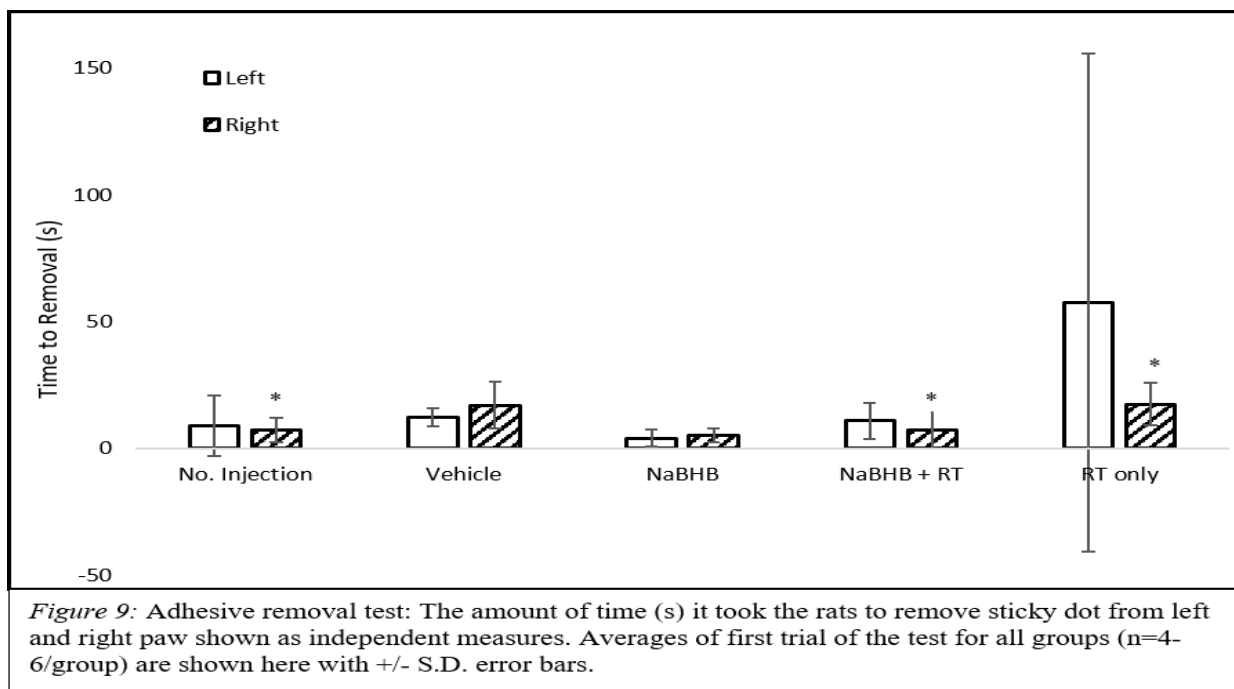


Figure 8: Adhesive Removal Test: For the Adhesive removal test two measures were recorded: time to contact and time for removal. Both graphs show the averages for the left and right paw independently. The graph on the left shows the time to contact, the Graph on the right shows the amount of time it took to remove the sticky dot. Values shown here are averages (n=3/group) with +/- S.D. error bars.

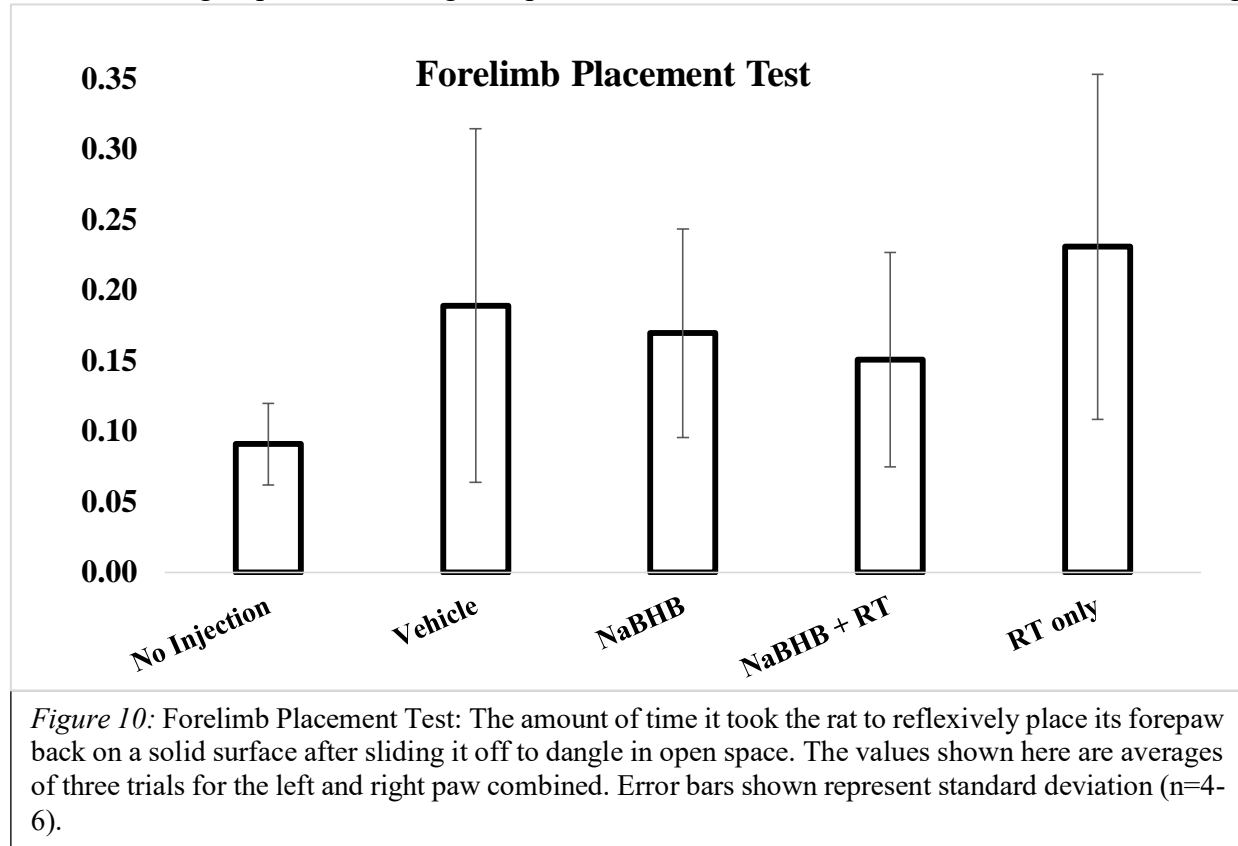
3.4.1.1.1 Adhesive Removal Test

The adhesive removal test was performed in the same manner as it was for the pilot study. Just as was the case in the pilot study, there were no statistically significant differences in the amount of time it took the rats to notice that they had a foreign stimuli on their forepaws, indicating that their somato-sensation remained unaffected by rotenone treatment. As for the amount of time it took for the rats to remove the foreign stimuli (sticky dot) there was great variation within the groups, as can be seen in *Figure 9*. However, an unpaired student's t- test showed that there was a statistically significant difference between the average time it took the rotenone rats to remove the sticky dot as compared to the control group ($p < 0.05$) indicating that the rotenone did have an effect on the motor ability of the rat to remove the foreign stimuli. Furthermore, the rat's pre-treated with NaBHB seemed to have had a protectant effect from the rotenone as the amount of time they took on average was less than that of the rotenone group ($p < 0.05$). However, interestingly so, this was only seen for the Right paw. As for the left paw, there were no statistically significant differences between the aforementioned groups. This may be explained by the fact that the rotenone group did not perform uniformly (heterogeneous population), there was great variation within the group.



3.4.1.1.2 Forelimb Placement Test

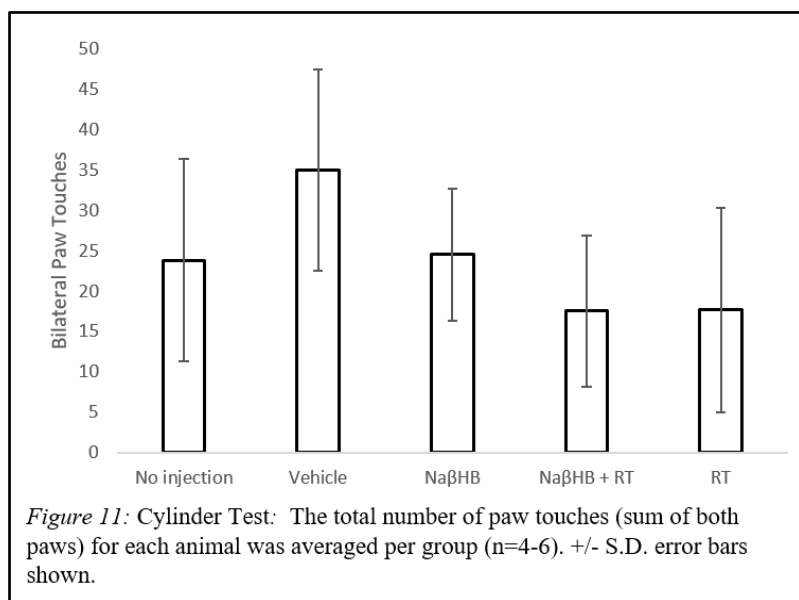
Most rats performed uniformly for this task, except for the rotenone group. There was one rat within this group that took longer to perform the task and thus the vast difference in the range



for this task. Overall, when the groups were compared using statistical analyses (student's t-test), there were no statistically significant differences ($p\text{-value} > 0.05$) between the groups, as can be seen in *Figure 9*. These results were expected given that only animals with (>80%) large bilateral lesions would be expected to show a delayed response given that this behavioral test has more of a reflex component to it and does not greatly involve the basal ganglia system and thus, would be unperturbed by a PD model.

3.4.1.1.3 Cylinder Test

The cylinder test is a behavioral test commonly used to assess motor behavior in rat models. The advantages of this test are that it requires no training given that this test exploits the rat's

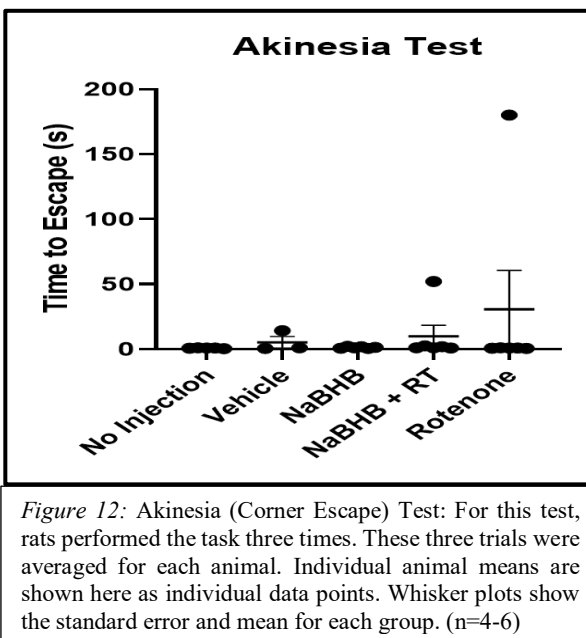


innate desire to explore novel environments. This test requires the animal to be able to stand on its hind paws and touch the walls of the cylindrical enclosure using an open paw with all digits touching the wall (behavior known as rearing). Given that rotenone was administered systemically (via I.P. injection)

motor deficits, if any, were expected in both hemispheres; however, the effects might be more pronounced on one side versus the other. Thus, for this test, the animals were recorded over a period of two minutes, the videos were then watched take-by-take every one-hundredth of a second on Windows Photos program. Experimenter recording total paw touches (Left and Right) was blinded to the treatment group. Although, there seemed to be a trend of less activity (less paw touches) in the rotenone treated group, as well as the NaβHB + RT group, there were no statistically significant differences as per a student's t-test ($p\text{-value} > 0.05$). As was the case for most behavioral tests, there was a lot of variability in task performance within the group.

3.4.1.1.4 Akinesia Test

The akinesia test was meant to measure the latency period for an animal to exit an enclosed space, in this case a triangular enclosure. The animals don't want to feel trapped so when placed in this enclosure, given that they have their full motor capabilities, they should readily



exit the apparatus. However, if the animal has motor difficulties, they would take a longer period of time to escape.

3.4.1.1.5 Immunohistochemical Data

In order to determine the extent of the nigrostriatal lesion, if any, tissue sections were stained for tyrosine hydroxylase (the rate limiting enzyme in the synthesis of dopamine). Images of the fluorescently labeled neurons were taken on a Zeiss 700 LSM confocal microscope. Number of TH-positive neurons were quantified for a singular representative section using Image J software. Representative Images can be seen in *Image 3*. The averages of TH-positive neurons per

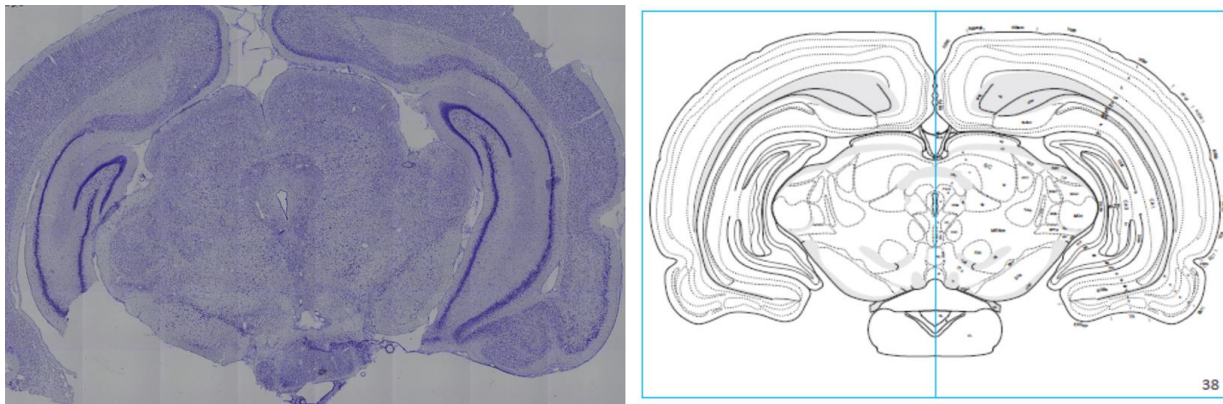


Image 5: Nissl Staining: Nissl staining was carried out on all sections (1 of every 5) in order to compare the staining pattern (as can be seen on the left) to the Swanson atlas (seen on the right) and determine the level and brain regions present. All sections immuno-stained for TH (tyrosine hydroxylase) were sections found to be comparable to level 38 of the Swanson atlas as per the representative Nissl section.

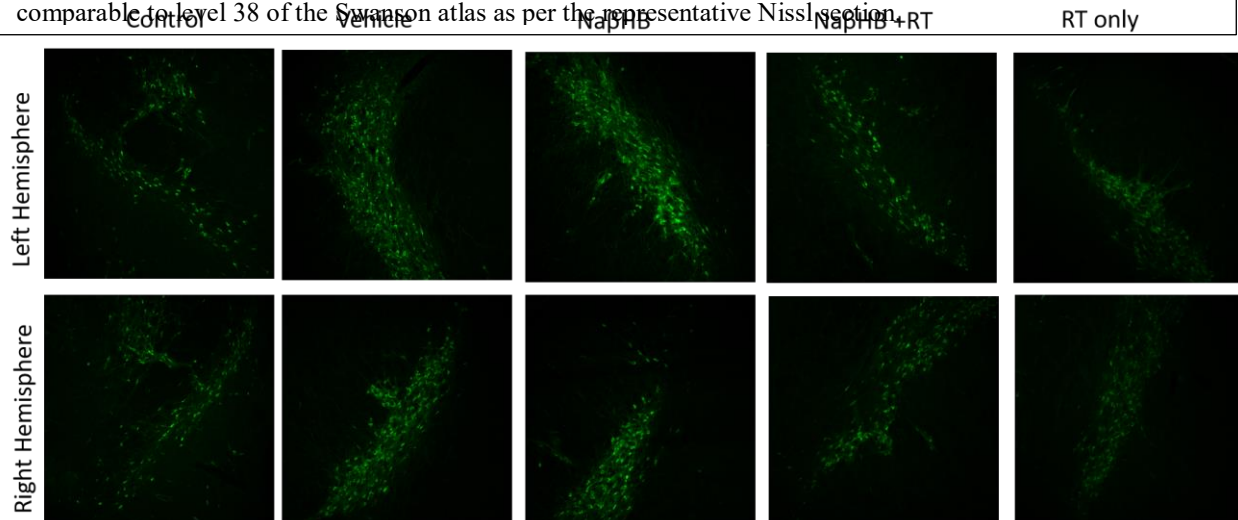


Figure 13: TH immunostaining: In order to quantify the amount of dopaminergic neurons in the SNpc, representative sections (as determined via Nissl staining) were incubated with anti-tyrosine hydroxylase (Mouse) and then with anti-mouse conjugate with Alexa488. Sections were imaged with LSM 700 Zeiss confocal microscope.

group are shown on *Figure 13*. As was the case for the behavioral tests, there was large variability within groups in number of TH-positive neurons and overall, the averages for each group were comparable to each other, showing no statistically significant differences as per a student's t-test ($p\text{-value} > 0.05$).

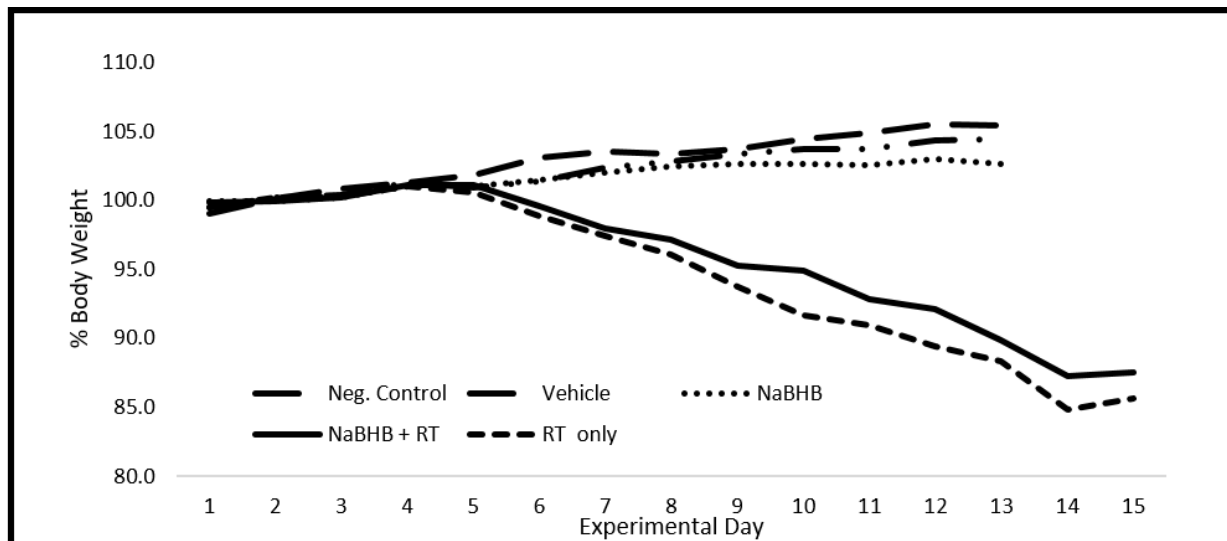


Figure 14: The percent body weights of all animals were calculated. Day 0 represents the baseline weight of the animals three days prior to receiving any rotenone injections. Lines represent averages for all animals ($n=4-6$) in the group. The animals receiving rotenone, both with and without NaBHB as a prophylactic had a substantial decrease in % body weight.

3.4.2 Conclusion

Rotenone has been repeatedly used as a PD model, including Cannon, *et al.* publishing in 2009 declaring it “A highly reproducible rotenone model”. However, the literature is riddled with many other publications citing different doses, different routes of exposure, and different lengths of exposure in order to recreate a PD model. It has been our experience that this model is shortcoming in the terms of being reproducible. We did not see any signs of a nigrostriatal lesion and none of the four behavioral assays showed any motor deficits across the group. Furthermore, if we do a singular per animal analysis, the effect varied greatly from rat to rat. There was one rat within the rotenone group that showed cataleptic signs (thus the large standard deviation in the rotenone group); however, immunostaining did not show statistically significant diminishment in dopaminergic neurons in the SNpc but odd histological markings were present throughout the

tissue. Additionally, many of the rats had white fibers/plaques in or atop of the liver. Bacterial infections were ruled out by autopsy, but otherwise, the causative agent for these physiological abnormalities could not be determined. In addition, there were four mortalities throughout the study, two died immediately after injection and the other two from rotenone overdose. Although one pitfall of our study was small number of animals per group (n=4-6), we would be reluctant to perform the study again to evaluate the potential of prophylactic compounds given the vast variability in effect of the toxin. Although the rotenone model seems to be the ideal one when testing for attenuation of Lewy body formation or protective capability of compounds as antioxidants, the lack of consistency in this model makes it affects the ability of the researcher to properly evaluate the efficacy of this compounds and it would appear unwise to refer to it solely as a PD model given the many other organ systems that are being affected.

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Vita

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