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The Use of 3-Nitropropionic Acid to Create a Model of Aging in Mice

by

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A thesis

Presented to the faculty of

Towson University

in partial fulfillment

of the requirements for the degree

Masters of Arts

Department of Psychology

Towson University
Towson, Maryland 21252

May, 2016

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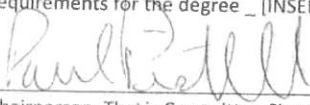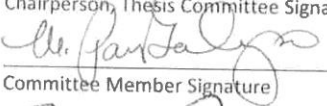
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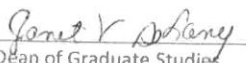The Use of 3-Nitropropionic Acid

to Create a Model of Aging in Mice

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Acknowledgements

The author would like to give special thanks to her mentor and committee chairperson, Dr. Paul Pistell. His support and feedback have allowed this thesis to be successful. The author also wishes to thank her committee members, Dr. M. Paz Galupo and Dr. Bryan Devan, for their guidance during the thesis process. Lastly, the author would like to thank her research assistant, Kari Haines, for all of her help during the duration of this project.

Abstract

The Use of 3-Nitropropionic Acid to Create a Model of Aging in Mice

Cherish Ardinger

This experiment was designed to create an aging model in mice that is more cost-effective, time-efficient, and easier for the animals to recover from than traditional models. This model was designed using intraperitoneal (IP) injections of 3-nitropropionic acid (3NP), a mitochondrial toxin that is known to damage the striatum. The striatum has been shown in previous literature to play a large role in the aging process. This experiment compared two cumulative doses of 3NP: 540 mg/kg, 720 mg/kg, and a saline control. The 540 mg/kg dose was given at either a steady dose every other day or an increasing dose given twice a day. These dosing schedules were both compared to one another and control mice to assess which was the most effective at creating cognitive and motor deficits when mice receiving these respective treatments were tested on the Stone T-Maze (STM) and rotarod. It was found that an escalating dose of 3NP impaired retention in the STM and motor function on the rotarod task.

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Chapter One:

Introduction

Aging Models: Past and Present

Traditional models of aging in the laboratory have focused on the use of rats in spatial memory tasks, such as the Morris Water Maze and radial arm maze. These tasks are allocentric in their design, requiring the animals to become familiar with spatial cues to successfully complete the task. This type of effort engages the hippocampus and has been used to study aging, particularly of this brain region, for many years (Gage, Dunnett, & Bjorklund, 1984; Rapp, Deroche, Mao, & Burwell, 2002). Recent models have continued this trend (Wilson et al., 2004; Zhang, Kadar, Sirimanne, MacGibbon, & Guan, 2012).

While the hippocampus does have many implications in the aging process, it is important to remember that aging does not solely affect one region of the brain. Aging in humans and animals is associated with deficits in both motor and cognitive functioning. Past research has traced many of these age-related deficits to a brain structure known as the striatum. The striatum is composed of the caudate nucleus and the putamen and is a central part of the basal ganglia system. This brain structure receives input from the cortex, substantia nigra, and thalamus. The striatum plays a large role in habit formation, as well as body-centered or egocentric movement (Pinel, 2010).

Umegaki, Roth, and Ingram (2008) found that the striatum requires a particular balance of dopamine and acetylcholine to help humans and animals maintain effective motor control. The balance of these neurotransmitters is often lost during the aging

process. These researchers also found that aging affects the physical structure of the striatum.

Previous studies have examined the effects of aging in the striatum and hippocampus by using both egocentric and allocentric tasks. For example, Devan et al. (2014) assessed the cognitive functioning of both young and aged rats that were treated with phosphodiesterase inhibitors. The goal of this experiment was to test whether sildenafil and vardenafil were effective in the preservation of memory in 24-month old rats on both the egocentric Stone T-Maze (STM) task and the allocentric Morris water maze place task. For the STM task, this older cohort of subjects was compared to middle-aged (6-7-month old) and young (3-month old) rats. For STM training, rats were tested using a version of the maze that delivered mild foot shocks to subjects if they did not clear a section of the maze within a certain timeframe. Other than this key design difference, the procedure used for testing is very similar to the one utilized in the current study where subjects completed straight run training, 15 acquisition trials, and then retention trials one week later. Prior to acquisition testing, rats received intraperitoneal (IP) injections of either saline or four different doses of sildenafil. Results of this experiment show that 24-month old rats commit significantly more errors than the middle-aged and young rats during acquisition testing. When tested one week later, rats given 3 mg/kg of sildenafil showed significantly less errors in the maze. This effect shows that the drug may help preserve the long-term retention of information.

Devan et al. (2014) showed this same effect of cognitive preservation using vardenafil-treated 24-month old and 11-month old rats. These rats were tested in the Morris water maze where they were trained to swim to a hidden platform during

acquisition testing. One week later, memory for the task was assessed using a probe test in which there was no platform in the pool. Video tracking software recorded where the subjects spent their time searching for the platform. Results of this experiment showed no effect of the drug during acquisition, but did show that aged rats given injections of vardenafil prior to and during acquisition improved both their heading angle and spent more time in the quadrant where the platform was located during the probe test. These results, like the STM results, suggest that phosphodiesterase inhibitors may improve retention in aged animals.

Aging Models: Future Directions

Devan et al. (2014) were able to study their drug of interest in relation to age-related memory decline because they had a sample of young, middle-aged, and old animals. For some laboratories obtaining a sample of this nature may be problematic as older animals tend to be costlier than younger animals. Alternatively, waiting for young animals to age naturally may take an average of two years.

A new and effective mouse model of aging in the laboratory setting could prove to be very beneficial. An increased interest in models of aging in mice was pointed out by Ingram and Jucker (1999). In this review paper, the authors discuss how rats were the traditional rodent of choice for most aging researchers; however, mice are becoming more appealing due to the increasing use of transgenic mice in this field of research. As speculated by these researchers, there are working models of aging in transgenic mice (Park et al., 2013). While these models have many benefits, transgenic mice are expensive and can be hard to acquire.

While past aging models have focused on the rat hippocampus, a few models of aging in the laboratory have chosen to focus solely on striatal function. These models include using direct excitotoxic striatal lesions to assess the function of this brain region as it relates to aging. This was demonstrated in Pistell et al.'s (2009) experiment. In this experiment, rats with lesions in both the medial and lateral striatum had worse performance than control rats exhibited by both errors and latency in the STM. The results of this work show that direct impairment of the striatum does impact learning and memory when assessed using the STM.

Along similar lines, a recent study from our laboratory (Gilman & Pistell, 2013, Unpublished manuscript) assessed the cognitive functioning of mice with excitotoxic lesions in either their striatum or hippocampus. These mice were compared with control mice who underwent the same surgical procedure, but no lesion was produced in the brain. Using the same STM protocol as the current experiment, it was found that these impaired animals had significantly more errors than control animals in the water-motivated version of the mouse STM. This increase in errors was relatively equal for both lesion groups.

While direct lesions to the striatum in rats and mice do mimic an age-related decline in performance in the STM, the surgeries to create these lesions are often hard for the animals to recover from. This may be especially true for mice, where previous studies show a mortality rate of about 50% in lesioned subjects (Gilman & Pistell, 2013, Unpublished manuscript).

Aging, 3NP, and the Current Experiment

Past research on 3-nitropropionic acid (3NP) has shown how this toxin creates both motor (Fernagut et al., 2002; Jadiswami et al., 2014) and cognitive (Lohmann & Riepe, 2007) deficits in mice by damaging the striatum. It is known that age-related cognitive and motor decline in both animals and humans is also associated with striatal damage (Umegaki, Roth, & Ingram, 2008). Based upon the results of the experiments that will be shared in this introduction, it is reasonable to believe that 3NP could be used to create a model of aging in mice. The current experiment strives to create such a model. Once this model is established, pharmacological and/or dietary interventions could be put in place, as it has been shown that various interventions for 3NP damage can be effective (Kumar, Kalonia, & Kumar, 2011; Tasset, Pontes, Hinojosa, de la Torre, & Tunez, 2011).

3NP intoxication in a laboratory mouse via IP injection is not costly and does not require surgery. This novel approach should theoretically produce similar deficits on cognitive and motor tasks as previous aging models, based upon the literature reviewed regarding this neurotoxin. An aging model created with the use of this drug will also focus on striatal function, which has received less attention than the hippocampus in the realm of aging research.

3NP and Motor Function

To explore the relationship between 3NP intoxication and motor functioning, Fernagut et al. (2002) tested male C57/Bl6 mice, divided into three groups. One group received steadily increasing doses of 3NP via IP injection, starting at 10 mg/kg 3NP and ending at 50 mg/kg 3NP, over a total of 7 days for a total cumulative dose of 340 mg/kg 3NP. The second group also received 3NP treatment on a steadily increasing regimen, for

a total of 560 mg/kg over 7 days. The third group served as a control group and received a saline injection using the same schedule as group two (Fernagut et al., 2002). After treatment, mice were tested on several motor tasks, including the rotarod, the pole test, a measure of stride length, and a test of open field spontaneous activity. For all of these tasks, performance was measured at baseline then again at week one and four post-3NP or control treatment.

Performance on the rotarod task was assessed considering total time spent on the rod during a 10-trial session. Mice were trained to stay on the rod as it rotated at 5 r.p.m., with a maximum time of 180 seconds per trial. The pole test consisted of mice being placed on a wooden pole 50 cm high. The time to turn their head completely downward and the time each mouse took to descend from the pole was recorded as a measure of motor ability. A measure of stride length was completed by painting the hindlimb paws and then forelimb paws of each mouse and having them run down a strip of paper. Measurements of paw prints were taken over three trials to assess stride length. Lastly, open field spontaneous activity was measured by placing mice in an open field apparatus equipped with computer technology that measured a variety of variables, such as traveling distance, mean velocity and time spent in a central compartment during a five minute session (Fernagut et al., 2002).

Results of the experiment completed by Fernagut et al. (2002) show that, when compared to both their own baseline scores and control subjects, mice receiving a cumulative dose of 340 mg/kg 3NP had a statistically significant increase in their forelimb/hind limb stride length difference as well as a significant reduction in number of rearings during the test of open field spontaneous activity. This was a much more mild

impairment than the mice receiving a cumulative dose of 560 mg/kg 3NP. Mice receiving

the higher dose of this toxin showed a significant difference in performance, as compared

to their baseline scores and control mice, on all tests of motor function on at least one

variable measured. It is also noteworthy that three mice receiving the higher dose of the

3NP died, whereas this attrition was not seen in the control or lower dose group. Lastly,

histology revealed that mice receiving 340 mg/kg 3NP had a significant reduction in their

striatal volume when compared to control mice. Mice receiving 560 mg/kg 3NP had both

a significant reduction in striatal volume and a lateral lesion in the antero-posterior

aspects of the striatum. This experiment shows that 3NP's damaging effects are dose-

dependent, and 3NP treatment can significantly impair a mouse's motor functioning as

demonstrated by performance on several motor tasks.

Another experiment that assesses the effects of 3NP on motor functioning in mice

is Jadiswami et al. (2014). In this experiment, 40 adult male Swiss albino mice were

divided into 5 groups and treated with either 3NP, piroxicam, or a combination of the two

at varying doses. Piroxicam is a Cyclooxygenase (COX) inhibitor that is generally used

to treat osteoarthritis. In some groups of mice, this drug was used to see if it had a

neuroprotective effect when administered before 3NP injections (Jadiswami et al., 2014).

In the experiment done by Jadiswami et al. (2014), mice were randomly divided

into five groups with each group getting 2 treatments per day for 14 days. Treatments

were given 30 minutes apart. Group one served as the control group and received saline

for both treatments. Group two served as a way to look at piroxicam alone and received

20 mg/kg piroxicam then saline. Group three tested 3NP damage without intervention;

mice in this group received saline then 15 mg/kg 3NP. Group four received 10 mg/kg

piroxicam then 15 mg/kg 3NP. Lastly, group five received 20 mg/kg piroxicam then 15 mg/kg 3NP.

On the last day of treatment, Jadiswami et al. (2014) assessed the motor ability of all mice using several tests. These tests included a movement analysis, assessment of locomotor activity, a beam walking test, and a hanging wire test. Several biochemical measures were also taken from all mice. Movement analysis involved assigning a neurological score to each mouse based upon Ludolph et al.'s (1991) procedure. Scores on the movement analysis ranged from 0 (normal behavior) to 4 (incapacity to move resulting from fore limb and hind limb impairment). Locomotor activity was tested using an actophotometer, which recorded activity of the mouse for five minutes. The beam walking task was used to test motor coordination. This task involved mice walking on a narrow beam suspended 50 cm above a cushion. Mice were given two minutes to walk across the beam. Latency to cross the beam was recorded. The hanging wire test required mice to hang onto a steel wire also 50 cm above a cushion. Latency to fall from the wire was measured.

Jadiswami et al. (2014) found that for all of these motor tasks, mice receiving only 3NP treatment performed significantly worse than control mice. Also on all tasks, mice pre-treated with piroxicam performed significantly better than the 3NP-only treated mice; their performance was comparable to that of the control group. These findings support the idea that 3NP causes significant motor impairment in mice as well as suggesting that piroxicam offers neuroprotection from 3NP damage.

Biochemical measures taken from mice in this experiment show a significant increase in lipid peroxidation (LPO), reduced glutathione (GSH), and decreased brain

catalase in 3NP-only mice when compared to control mice. Mice receiving piroxicam and 3NP showed improvement on these measures. These results show that 3NP creates brain oxidative stress when administered to mice via IP injection and this stress can be combated with neuroprotection, such as piroxicam, that offers antioxidant properties (Jadiswami et al., 2014).

3NP and Huntington's Disease

As discussed, 3NP causes distinct motor impairments in rodents, which is related to the striatal damage that is caused by this toxin. Many researchers have applied this knowledge to create a model of Huntington's disease using 3NP. While this is not directly related to creating a model of aging, it is noteworthy to recognize that this model is widely accepted, and further provides evidence of striatal damage in rodents caused by 3NP. Reviews discussing the use of 3NP intoxication to replicate Huntington's disease in lab animals began appearing in the late '90s. During this time, Brouillet et al. (1999) discussed the ability of a chronic, low dose of 3NP in rats via subcutaneous osmotic minipumps to mimic features of Huntington's disease.

More recent work by Brouillet (2014) includes a protocol for creating a model of Huntington's disease using 3NP. This protocol suggests two main methods of 3NP treatment to replicate Huntington's disease in rats. One method is to use IP injections of 3NP over a period of time. Another suggested method is placing osmotic pumps in the animals to deliver the 3NP treatments subcutaneously (Brouillet, 2014).

Transgenic mouse models of this disease have also utilized 3NP to create striatal damage. Hickey and Morton (2000) treated adult mice (R6/2) transgenic for the Huntington's disease gene with a chronic dose of 3NP and compared them to wild type

mice. They found that R6/2 mice had smaller striatal lesions than the control, wild type mice post-treatment. The authors theorize that the transgene may provide a neuroprotective mechanism around 7 - 10 weeks of age in these mice, which may be related to the late onset of neurological symptoms seen in humans with Huntington's disease.

3NP Damage and Pharmacological and Dietary Intervention

While many studies have been focused on creating models of striatal damage with 3NP, some experiments are focused on practical interventions to alleviate this damage. An example of this is research done by Kumar, Kalonia, and Kumar (2011). In this experiment, Kumar et al. (2011) assessed the neuroprotective properties of antidepressants against 3NP striatal damage in adult male Wistar rats. The experiment's schedule lasted 14 days and involved rats receiving an antidepressant followed by an injection of 3NP or a nitric oxide modulator, then antidepressant, followed by 3NP, exceptions to this were the control groups which received saline or 3NP treatments alone. Antidepressants tested include sertraline, venlafaxine, trazodone, and imipramine. All antidepressants were administered via oral route and at varying doses. L-arginine and L-NAME were used as nitric oxide modulators and were administered via IP injection. 3NP was also administered via IP injection at 10 mg/kg (Kumar et al., 2011).

On days 1, 5, 10 and 15 of the experiment Kumar et al. (2011) tested all rats on the rotarod as a measure of motor performance. Results show that rats treated only with 3NP had a significant decrease in motor performance over the 14-day experiment. Rats treated with sertraline and venlafaxine performed better on the rotarod task than those treated with imipramine and trazodone or controls. This result is explained by the fact

that more recently developed antidepressants (i.e., sertraline, venlafaxine) have different mechanisms than traditional ones (i.e., imipramine, trazodone), such as serotonin and norephineprine reuptake. These drugs seem to have an antioxidant-like effect, which may explain their ability to act as a neuroprotective mechanism against 3NP damage (Kumar et al., 2011). Similar studies have also shown extra-virgin olive oil to be neuroprotectant against 3NP treatment (Tasset et al., 2011).

3NP and Maze Tasks

One study has assessed the effects of 3NP on mouse cognitive abilities using complex maze tasks. Lohmann and Riepe (2007) treated 4.5-month-old and 9-month-old CD-1 female mice with 20 mg/kg 3NP via IP injection every other day for 18 days. Same age mice were treated with saline as controls. One day following treatment, all mice were tested in a complex maze task. This maze used food pellets as motivation. All mice were tested in this complex maze five times a day for eight days. Latency to finish the maze task was recorded (Lohmann & Riepe, 2007).

After completing the complex maze task, Lohmann and Riepe (2007) allowed the saline-treated 9-month-old and 3NP-treated 4.5-month-old mice to age to 22 months. These mice were then tested for any residual effects of their injections using a mirrored version of the complex maze. Much like the original version, mice were tested five times a day for eight days as escape latency was recorded. These 22-month-old mice were also tested on their cognitive abilities using the radial arm maze. This maze also used food pellets as a motivation. For this task mice were tested 3 times a day until they had consumed the food in each arm of the maze, or for a maximum of 300 seconds per trial. Errors were characterized as a mouse entering an arm they had previously visited.

Latency to complete the radial arm maze (visit all arms) was also recorded (Lohmann & Riepe, 2007).

Through this research, Lohmann and Riepe (2007) found that 4.5-month-old mice had significantly better escape latencies than 9-month-old mice on the complex maze. Mice of both age groups treated with saline performed significantly better in this maze than mice treated with 3NP. Mice 22 months of age tested in the mirror version of the maze task showed similar results. Trials in the radial arm maze showed that mice treated with 3NP had both a higher number of errors and used more time to visit all arms of the maze than mice receiving saline injections. These results suggest that 3NP may have an age effect on mice, where it affects older mice more strongly than young or middle-aged mice (Lohmann & Riepe, 2007).

The Present Experiment

The present experiment was designed to test a novel model of aging in mice and as such strived to explore different doses of 3NP and different treatment schedules to determine which regimen most effectively creates age-related cognitive and motor decline in young mice.

Pre-treatment, mice were tested for baseline scores on the rotarod. Mice were then randomly assigned to treatment schedules and dosing groups. Once treatments were completed and mice had recovered, all subjects were tested on a striatal-dependent task, the Stone T-maze (STM). Performance on this task was measured by assessing errors and latency in the maze. Motor function was then evaluated using the rotarod. It was hypothesized that mice would display a 3NP dose-dependent higher number of errors and greater latency in the STM as compared to control subjects, as well as a dose-dependent

lower latency on the rotarod task, where animals receiving a higher dose of 3NP would

display the lowest cognitive and motor functioning as measured by these tasks.

Chapter Two:

Method

Subjects

Fifty female C57/Bl6 6-7-month-old mice were used in this experiment. Mice used in this experiment were bred at Towson University. The breeder mice were acquired from Charles River Laboratories. Mice were randomly assigned to either one of three 3NP treatment groups or one of two saline groups. These groups operated on either an escalating or steady dosing schedule. All mice were housed 3-4/cage in large, plastic cages in the Towson University vivarium. The room the mice were housed in was kept at 70-72°F with a 12 hour light/dark cycle. Water and food was provided ad libitum. All procedures in this study were approved by the Towson University Animal Care and Use Committee.

Treatment Schedules

Injection schedule one was inspired by Fernagut et al's (2002) protocol. Mice in this group were going to receive 3NP treatments once every 12 hours for 7 consecutive days. These treatments were to be dosed at 20 mg/kg (x 4), 40 mg/kg (x 4), and 60 mg/kg (x 5). This was to result in a cumulative dose of 540 mg/kg 3NP over the treatment period. However, due to a high mortality rate in this group, mice randomly assigned to this group did not receive that full cumulative dose and treatments were stopped after the 3rd 60 mg/kg injection. This left the remaining mice (n=6) having had received a cumulative dose of 420 mg/kg 3NP. Table 1 offers a comparison of survival rates between the groups. There was an accompanying saline control group for this schedule, which will be referred to as the escalating dose schedule.

Injection schedule two was inspired by Lohmann and Riepe's (2007) protocol. Mice in this group received 60 mg/kg 3NP every-other-day for 18 days. This resulted in a cumulative dose of 540 mg/kg 3NP over the treatment period. Another group of mice followed the same schedule but received 80 mg/kg 3NP. This resulted in a cumulative dose of 720 mg/kg 3NP over the treatment period. There was one accompanying saline control group that followed this schedule, which will be referred to as the steady dose schedule.

Injections

Mice were randomly assigned to one of three 3NP treatment groups or two saline groups that followed either a steady or escalating dose schedule. 3NP solutions were mixed fresh each day that injections were given. Before injections, all mice were weighed. All treatments, including saline control treatments, were administered via IP injection. The side of the mouse in which the injection was administered was alternated between left and right during each treatment for the mouse's comfort. All mice were given a five-day recovery period before beginning straight run training prior to STM trials.

Water-Motivated Stone T-Maze (STM)

A mouse water-motivated version of the STM was used to assess cognitive deficits by measuring errors and escape latency of the mice during maze trials. The water-motivated version of this maze is a somewhat new apparatus used to test learning and memory deficiencies in mice, but a rat version has been around for many years (Pistell & Ingram, 2010). This study is one of the first to assess the effects of 3NP in the mouse water-motivated STM.

As per Pistell and Ingram's design (2010), the STM has three main components. These include the start box, the maze, and the goal-box. The start box features a plunger and a sliding door. The mice were placed into the start box with the sliding door closed. Once the mice were securely in the box, the sliding door was opened into the maze. The plunger was then lightly pressed to assist the mice in entering the maze. Once the mice entered the maze, the sliding door was closed so they could not re-enter the start box (Pistell & Ingram, 2010).

Once the mice entered the maze, a video recording of their trial using Any-Maze (Stoelting; Wood Dale, IL) began. The mice then had to find the correct path of 13 left and right turns to get to the dry, dark goal box. The maze was filled with water that was 21°F and filled to a level that required the mice to wade, but not swim through the water. A clear, Plexiglas ceiling prevented the mice from rearing. Pistell and Ingram (2010) found that water is an effective motivation for mice in the STM, as water is aversive to mice. The STM also features five guillotine and five false guillotine doors. The guillotine doors serve the purpose of closing off a section of the maze once the mouse had completed it, so the mouse could not backtrack. The false guillotine doors were implemented so the mice cannot use the guillotine doors as visual clues to get to the goal box. This task is striatal-dependent in nature due to the lack of visual cues in the testing environment. This engages the striatum as the subject uses habit formation and egocentric movement to navigate the turns needed to complete the maze (Pistell & Ingram, 2010).

While the mice were navigating the maze, the number of errors and latency they took to complete a trial were tracked by both the researchers and the video software. An error was characterized by both ears of the mouse crossing the line of an incorrect path.

The mice completed the maze when they entered the opaque black acrylic goal box. At the door of the goal box, a ramp allowed the mice to exit the water. The ceiling of the goal box is removable, which allowed the researchers to remove the mice once they finished their trial. A picture of the STM is shown in Figure 1 below.
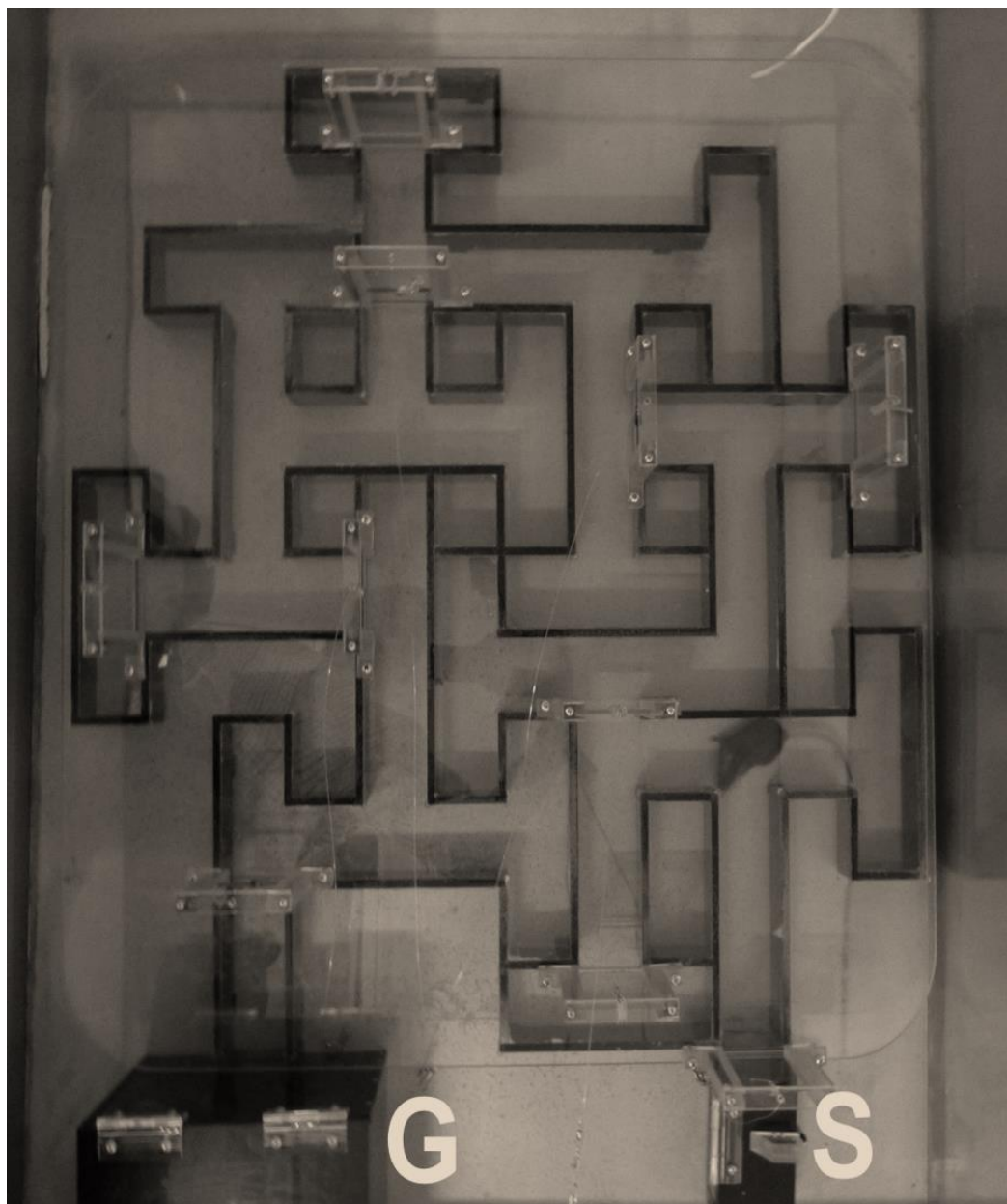

Figure 1. The water-motivated Stone T-maze, where "S" represents the start box and "G" represents the goal box.

STM Training Schedule

Before the mice were tested in the STM, they were pre-trained in a straight run. The straight-run is made out of black acrylic and placed with an identical start and goal box to the STM. Straight run training allows the mice to learn the contingency that moving forward in the water and then into the goal box allows them to escape from the water. The mice were required to reach the goal box in 15 seconds or less on 7/9 trials, with a maximum of 30 trials allowed. Any mouse unable to complete this task was excluded from further testing. For both the control and experimental group, STM training occurred the following day.

During STM trials, the mice from the 3NP and saline groups were assigned numbers and then randomly selected for the order in which they ran their trials. Which group the mouse was in was unknown to the researcher, controlling for any bias in error counting. Each mouse then performed 15 acquisition trials in the STM in a single day, with their escape latencies and number of errors being recorded both by the researcher and the computer video software Any-Maze (Stoelting; Wood Dale, IL). Trials were terminated after 5 minutes. If a mouse was still in the maze after 5 minutes, the mouse was removed from the trial, and the trial was considered a failed trial and was considered in statistical analyses. If a mouse failed 3 trials, the mouse was taken out of initial acquisition testing. Mice were tested for their retention of the maze task one week later using three additional STM trials. These trials were completed using the same procedure as acquisition testing.

Rotarod

One week before treatment, all mice were assessed for their baseline latency scores on three trials of the rotarod (Med-Associates, St. Albans, VT). Mice were then

evaluated again on the rotarod task nine days after STM testing. This test is a rotating rod that the mice tried to stay on as it rotated beginning at 4 r.p.m. and gradually increased to 40 r.p.m. Each mouse was tested for 3 trials and average latency to fall from the rotarod across the three trials was examined as a measure of motor function. All trials were terminated at five minutes, to focus on the assessment of motor function and coordination rather than endurance.

Histology

After all the mice finished STM and rotarod testing, they were perfused and histological procedures will take place. This manipulation check assures that increased errors and/or latency in the maze made by the experimental 3NP treated groups were in fact a result of striatal damage from the 3NP and not any potential confound. These biological results will provide additional context for the behavioral data.

Procedure

One week before treatment, mice were tested for baseline latency on the rotarod. Mice were then administered their appropriate treatments determined by schedule and dose assignment. After a 5-day recovery period, the first group of eight mice (group 1) were trained using the straight run procedure. The following day, these eight subjects were tested using 15 trials of acquisition in the STM where errors and latency were recorded. Another group of eight (group 2) were trained in the straight run the same day that group 1 received STM acquisition training. This procedure went on until all mice had been tested in both the straight run and STM. Testing groups were randomly assigned and contained mice from various schedule and dose groups. One week later, mice were tested for three additional trials in the STM using the same procedure as acquisition. Nine days

after acquisition testing, mice were tested for three trials using the rotarod. Mice were

then perfused several days after rotarod testing.

Chapter Three:

Results

STM Acquisition

     STM Latency. Results for STM trial acquisition latency were analyzed using a 2 (schedule) x 3 (dose) x 5 (trial block) mixed methods ANOVA. The 15 trials were collapsed into 5 trial blocks for ease of interpretation. A total of 40 mice were used for this analysis, where mice were missing from either death during treatment or failure to complete acquisition trials (Table 1). Results of this analysis show a main effect of trial block, $F (4, 140) = 7.63$, $p < .05$, partial $\eta^2 = .18$, observed power = .99, where mice were completing the trials more quickly as they progressed through the trial blocks. There were no other significant effects found, including interaction effects between trial block and treatment, trial block and schedule, and main effects for dose and schedule. Descriptive statistics for acquisition latency in the STM can be found in Table 2. A visual display of these results can be found in *Figure 2* below.

Figure 2. Mice in all treatment groups show lower latencies as they progress through the trials. Error bars represent the standard error of the mean.

STM Errors. Results for acquisition errors committed in the STM were also analyzed using a 2 (schedule) x 3 (dose) x 5 (trial block) mixed methods ANOVA. Results of this analysis show a main effect of trial block, F (4, 140) = 31.32, $p < .05$, partial η^2 = .47, observed power = 1.00, where mice were committing less errors in the STM as they progressed through the trial blocks. There were no other significant effects found, including interaction effects between trial block and treatment, trial block and schedule, and main effects for dose and schedule. Descriptive statistics for errors committed in the STM can be found in Table 3. A visual display of these results can be found in Figure 3 below.

Errors Committed by Mice During STM Acquisition

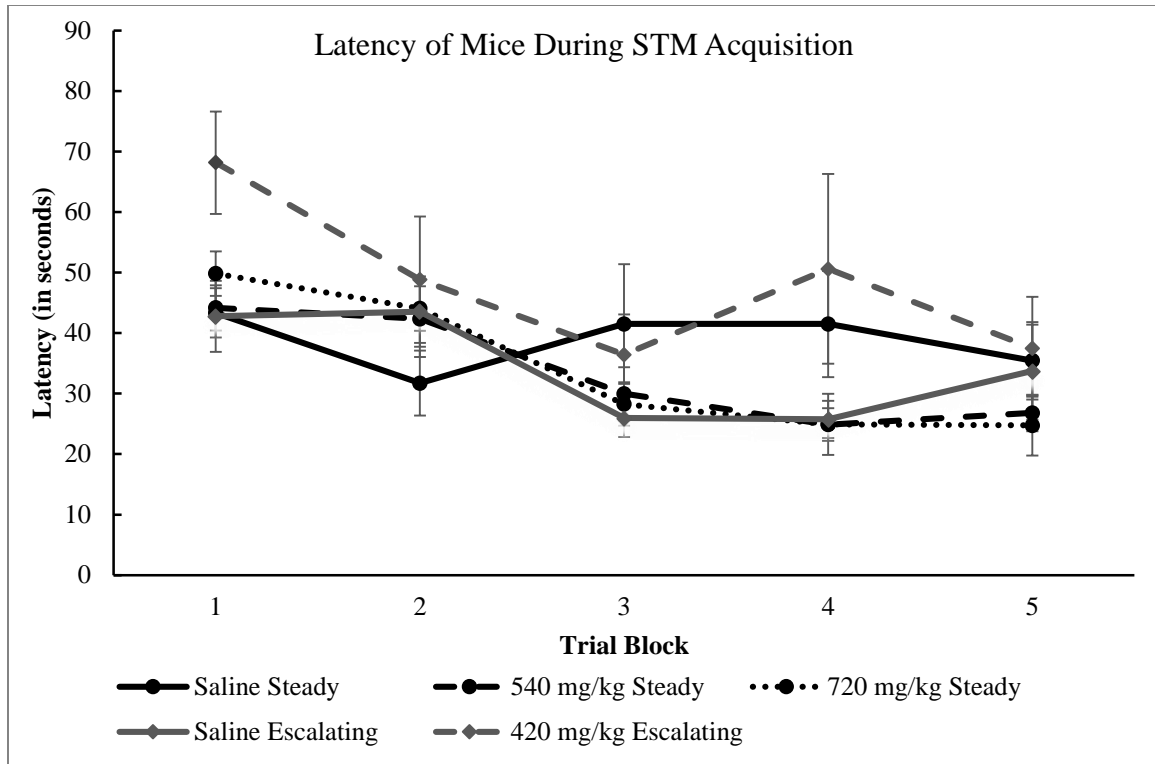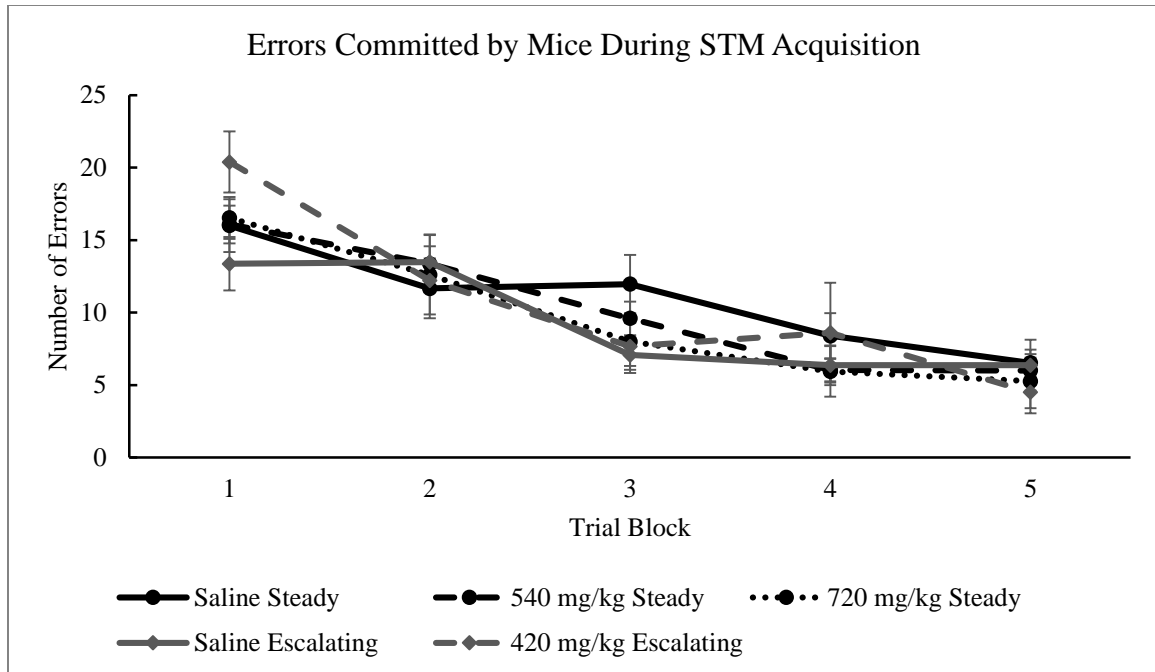*Figure 3.* Mice in all treatment groups show less errors as they progress through the trials. Error bars represent the standard error of the mean.

STM Retention

Retention Latency. Results for retention trials were analyzed using a 2 (schedule) x 3 (dose) x 2 (trial) mixed methods ANOVA. The two trials compared were the last acquisition trial (trial 15) and the first retention trial, which was completed one week later. These trials were chosen for analysis because of the nature of the STM task. Theoretically, mice should know the STM task the best on the last acquisition trial. Similarly, given the week-long break between acquisition and retention testing, the first trial of retention testing may be the best indicator of how well a mouse remembers the task as it is reminded of the task after the first trial. Therefore, a comparison of these two trials offers an analysis of the mouse's recall of this task.

Results for latency using this analysis do not show a significant main effect of trial, dose, or schedule. There were also no significant interaction effects between trial block and treatment, trial block and schedule (Table 4, Figure 4).
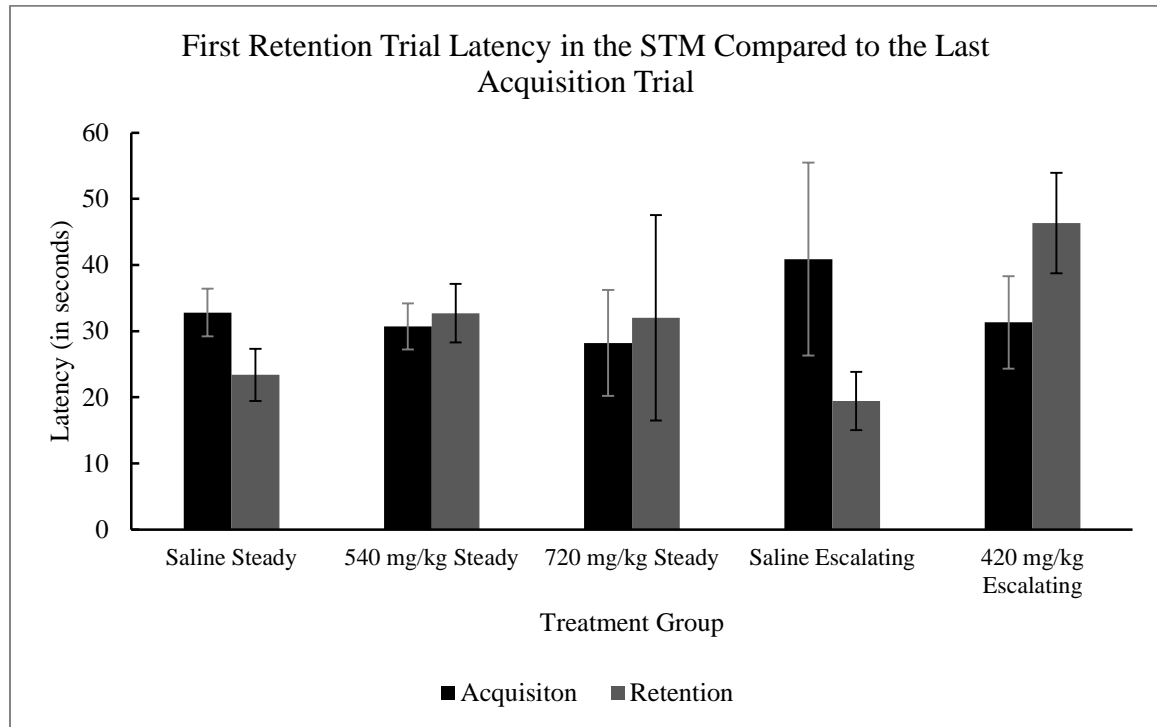
Figure 4. A comparison of latencies from acquisition to retention in the STM. Error bars represent the standard error of the mean.

Retention Errors. Results for errors using this analysis show a main effect of trial, $F (1, 35) = 10.21$, $p < .05$, partial $\eta^2 = .27$, observed power $= .87$, where mice commit more errors when tested in the STM one week later. There was no significant interaction effect between trial and schedule and no significant main effects for dose and schedule. For errors, there was a significant interaction effect of dose by trial, $F (3, 35) = 4.73$, $p < .05$, partial $\eta^2 = .21$, observed power $= .76$. To further examine these results, a follow-up paired samples T-test was completed and indicated a significant difference between the two trials considered for the 420 mg/kg escalating dose group, $t (5) = -3.63$,

p < .05, *d* = 4.79 [95% CI: 3.88, 5.70]. An independent samples T-test allowed for a

comparison of the 420 mg/kg escalating dose group to the schedule-matched saline

control group. Results of this analysis indicate that on the last trial of acquisition, there

was no significant difference between these groups, *t* (12.5) = -1.17, *p* > .05, *d* = -0.56

[95% CI: -3.71, 2.54]. However, during the first trial of retention, there was a significant

difference between the groups, *t* (9.43) = 3.63, *p* < .05, *d* = 2.14 [95% CI: -0.94, 5.23].

Similar results were found for the 540 mg/kg steady dose group. In this group, there was

also a significant difference between the two trials considered, *t* (9) = -2.33, *p* < .05, *d* =

1.00 [95% CI: .30, 1.71]. However, this group did not differ significantly from the steady

saline group during either the last trial of acquisition or the first trial of retention (Table

5, Figure 5).

First Retention Trial Errors in the STM Compared to the Last Acquisition Trial

Figure 5. The 540 mg/kg steady dose group and 420 mg/kg escalating dose group commit significantly more errors during retention trials when compared to acquisition trials. Error bars represent the standard error of the mean.
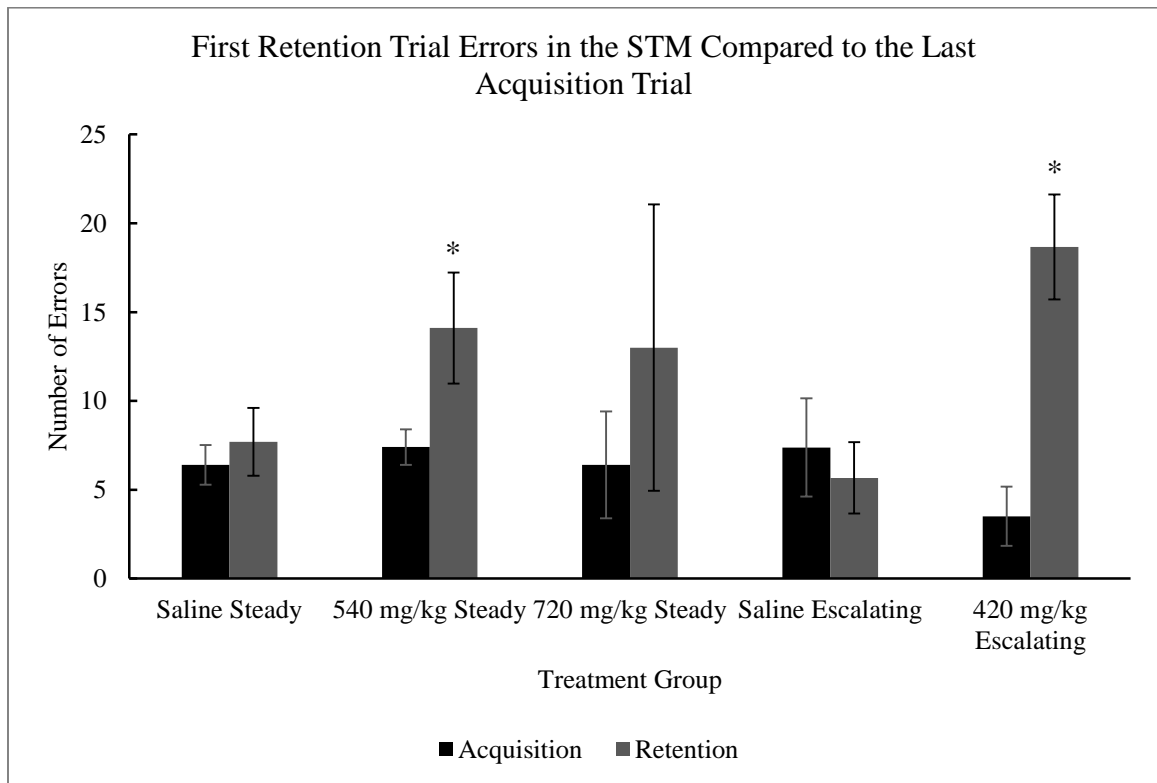
Rotarod Latency

Rotarod Latencies Post-Treatment. Baseline rotarod latency scores were first analyzed using a 2 (schedule) x 3 (dose) x 3 (trial) mixed methods ANOVA and showed that there were no significant differences between the groups pre-treatment. This analysis considered 44 mice, where missing mice had died during treatment (Table 1). The same analysis was also used to test the 3 trials conducted post-treatment and showed a main effect of trial, F (1.72, 67.03) = 5.57, p < .05, partial η^2 = .13, observed power = .80, where mice were able to stay on the rotarod longer as they progressed through the trials. There was also a main effect of dose, F (2, 39) = 4.94, p < .05, partial η^2 = .20, observed power = .78, trial, F (1, 39) = 5.43, p < .05, partial η^2 = .12, observed power = .62. There was also a significant interaction effect between dose and schedule, F (1, 39) = 7.62, p < .05, partial η^2 = .16, observed power = .77. A post-hoc analysis shows that the 420 mg/kg 3NP escalating dose group had significantly lower latencies than the schedule-matched saline group at every trial (Table 6, Figure 6).
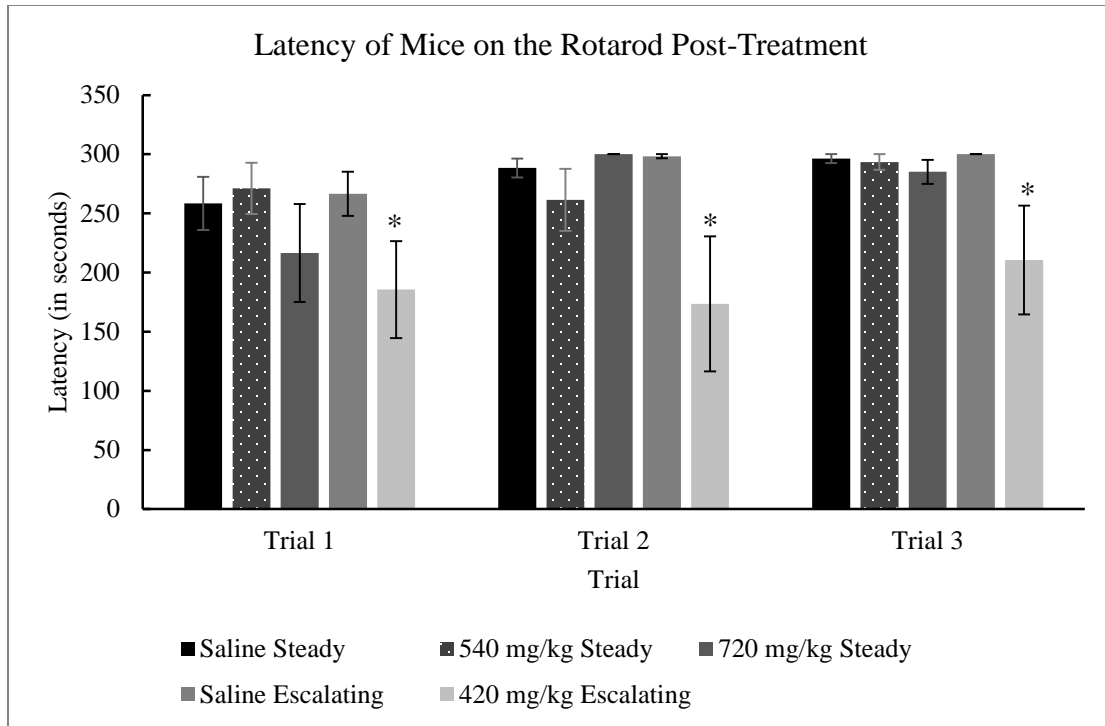
Figure 6. The 420 mg/kg escalating dose group shows a significantly lower latency on the rotarod than all other treatment groups during all three trials. Error bars represent the standard error of the mean.

Rotarod Latencies Baseline Versus Post-Treatment. To compare post-treatment rotarod results to pre-treatment baseline scores, a 2 (schedule) x 3 (dose) x 2 (average) ANOVA was conducted, where average indicates an average of baseline scores and an average of post-treatment scores. Results of this analysis show a main effect for trial, $F (1, 38) = 7.20$, $p < .05$, partial $\eta^2 = .16$, observed power $= .74$, where most mice show higher latencies on the rotarod task post-treatment. There was also an interaction effect of trial and dose, $F (2, 38) = 4.52$, $p < .05$, partial $\eta^2 = .19$, observed power $= .74$, where mice receiving 420 mg/kg 3NP were the only group to have a higher latency during baseline than post-treatment (Table 6, Figure 7).
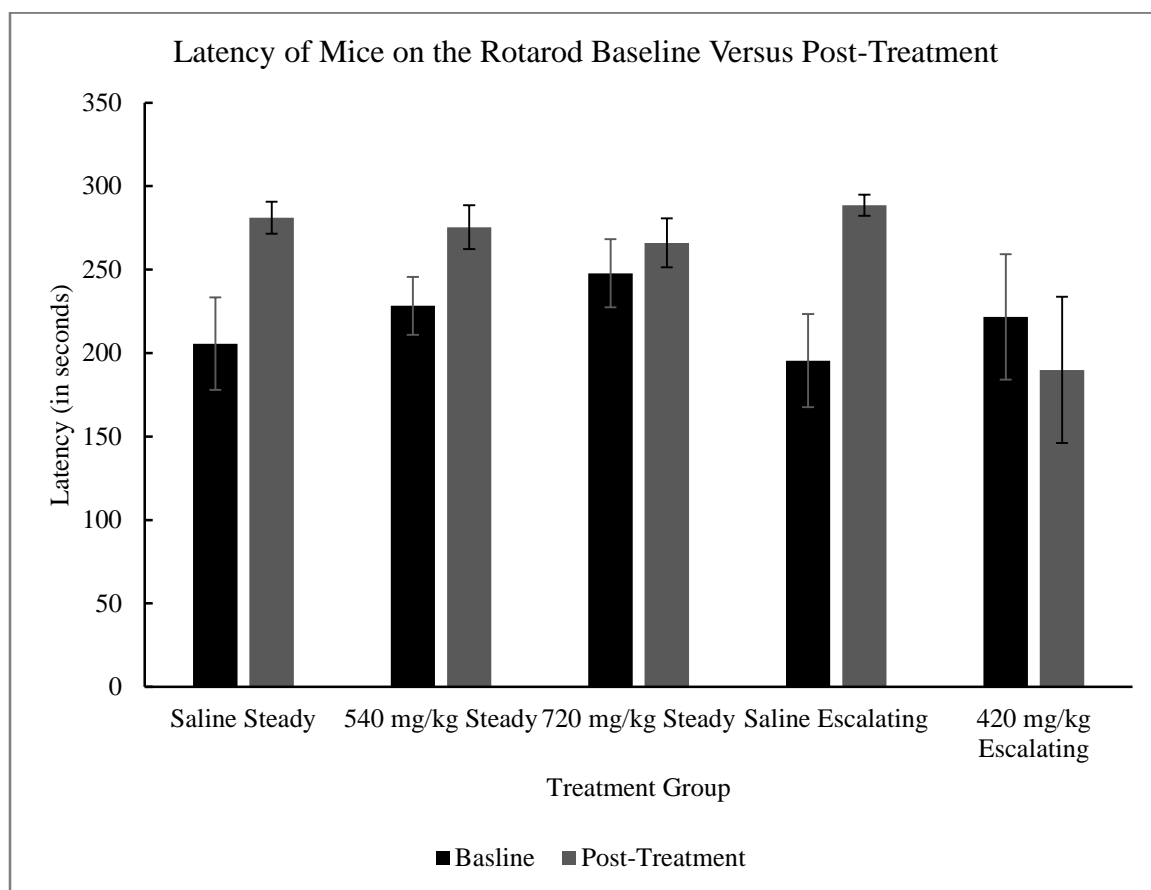
Figure 7. The 420 mg/kg escalating dose group is the only group whose average rotarod latency does not improve from baseline to post-treatment. The other 3NP groups who do improve may be showing a practice effect (see discussion). Error bars represent the standard error of the mean.

Chapter Four:

Discussion

Review of Findings

For STM acquisition testing, this experiment produced no significant interaction effects between the various schedules, doses, and trial blocks when considering either latency or errors. Interestingly, a significant interaction effect of dose by trial for retention errors shows that both the 420 mg/kg escalating dose group and 540 mg/kg steady dose group performed significantly worse on their first trial of retention when compared to their last trial of acquisition. The 420 mg/kg escalating dose group also had errors which were significantly higher than their respective schedule-matched saline control group during retention. This effect was not seen when these groups were compared during the last trial of acquisition. This finding shows that 3NP treatment creates problems with recall. These problems with recall may be more prominent when 3NP is administered at an escalating dose, as this group of mice had the worst performance of all groups during retention testing (Figure 5).

Rotarod testing shows that mice in the 420 mg/kg escalating dose group have significantly lower latencies than the schedule-matched saline control group at every trial of post-treatment testing (Figure 6). This effect was not found for any of the steady schedule dose groups. When post-treatment scores were compared to baseline scores, all mice except for the 420 mg/kg escalating dose group show a practice effect. This is where mice became familiar with the rotarod apparatus during baseline testing and then all performed well when tested post-treatment (Table 6, Figure 7). Given that the only group who did not experience this effect was the 420 mg/kg escalating dose group, it is

fair to say that this is a robust finding of motor impairment and may also speak to deficiencies in recall due to striatal damaged caused by 3NP that is specific to the escalating dose schedule. These results are consistent with the hypothesis that mice would display a dose-dependent lower latency on the rotarod task, where animals receiving a higher dose of 3NP would perform worse on this motor task than control mice.

Limitations

Attrition. A limitation was a small sample size. This experiment started with an adequate sample size for each treatment group (Table 1). However, there was a notable loss of 40% of the 420 mg/kg escalating dose group due to death during treatment. There were also two deaths in the 720 mg/kg steady dose group, also during treatment. Three mice in this group also failed STM acquisition trials, which left gaps in this data. Future research should consider boosting initial sample sizes, particularly of escalating dose treatment groups, in case of unexpected attrition.

Cryostat. A limitation of this experiment is the current absence of a working cryostat. Mice were perfused and their brains are currently being stored in refrigeration in a 10% formalin solution. When the equipment is present for these brains to be analyzed, this biological insight will provide context to the behavioral data that has already been gathered and will allow us to see if a lesion was produced in the striatum or not.

Future Research

Future research on this topic should focus efforts on escalating doses of 3NP. Deficits in both learning and memory recall and motor functioning were found when 3NP was administered on an escalating, but not steady, regimen. Future research should also

consider testing both male and female mice. It is known that estrogen, more commonly found in female mice, has neuroprotective properties (for a review, see Simpkins, Singh, Brock, & Etgen, 2012). Therefore, it is possible that a protocol to establish an aging model using 3NP may utilize different doses between males and females. Knowing the effective doses for different sexes may become beneficial if pharmacological and dietary interventions for this aging model are tested in the future. Future projects may also be interested in the way that different strains of mice respond to 3NP. Like sex, this variable should also be considered when creating an aging model using this drug. Lohmann and Riepe (2008) saw a loss of zero female CD-1 mice during treatment, where the current experiment lost two mice that were given the same steady dose of 3NP. Where possible, future research may also consider testing animals of varying ages.

Summary

This experiment utilized two dosing schedules of 3NP inspired by the work of Lohmann and Riepe (2007) and Fernagut et al. (2002). These dosing schedules were both compared to one another and control mice to assess which was the most effective at creating cognitive and motor deficits when mice receiving these respective treatments are tested on the STM and rotarod. It was found that an escalating dose of 3NP impaired mouse recall of the STM and motor function on the rotarod task.

This experiment was novel in both its comparison of dosing schedules and assessment of 3NP-treated mice in the STM. With this novel design came unexpected results. The ultimate goal was to create an aging model where mice receiving 3NP treatments show cognitive and motor deficits when compared to mice receiving saline treatments. While there were no significant differences in STM acquisition testing, we are

still able to study impaired recall in the mice receiving the 420 mg/kg escalating dose of 3NP. Unlike their peers, these mice showed poor performance when tested for retention of the STM one week after acquisition testing. This group was also the only group to do worse on the rotarod task post-treatment when compared to baseline scores. This is an indication that all other groups may have benefitted from practicing the task before receiving their appropriate treatments.

Further research with a goal of establishing an aging model using 3NP should assess varying escalating doses to determine which doses create this deficit of recall and if any dose could create the full desired impairments. If this aging model is established, pharmacological and dietary interventions could be tested.

Table 1

Mouse survival and maze failure rates.

		Steady Schedule			Escalating Schedule	
	Saline	540 mg/kg	720 mg/kg	420 mg/kg	Saline
Pre-treatment					
n	10	10	10	10	10
Post-treatment					
n	10	10	8	6	10
STM failure					
n	0	0	3	0	1

Table 2

Latency of mice in the STM during acquisition testing.

	Steady Schedule			Escalating Schedule	
	Saline	540 mg/kg	720 mg/kg	420 mg/kg	Saline
Trial Block 1					
M	43.33	44.17	49.83	68.17	42.78
SD	12.90	11.75	8.22	20.73	17.61
Trial Block 2					
M	31.73	42.37	44.06	48.83	43.56
SD	16.99	19.96	8.20	25.59	17.40
Trial Block 3					
M	41.50	29.98	28.27	36.44	25.96
SD	31.29	13.91	8.04	16.26	9.41
Trial Block 4					
M	41.50	24.87	24.93	50.61	25.74
SD	27.76	8.52	11.29	38.41	9.24
Trial Block 5					
M	35.45	26.80	24.73	37.50	33.74
SD	18.84	9.58	11.11	20.79	24.21

Table 3

Errors committed by mice in the STM during acquisition testing.

		Steady Schedule		Escalating Schedule	
	Saline	540 mg/kg	720 mg/kg	420 mg/kg	Saline
Trial Block 1					
M	16.00	16.08	16.53	20.39	13.37
SD	5.73	4.11	3.24	5.18	5.51
Trial Block 2					
M	11.67	13.37	12.60	12.22	13.48
SD	6.52	6.26	2.17	5.76	5.70
Trial Block 3					
M	11.97	9.60	8.00	7.67	7.07
SD	6.36	3.66	3.74	3.95	3.71
Trial Block 4					
M	8.40	6.03	5.93	8.61	6.37
SD	4.94	2.46	3.90	8.44	4.11
Trial Block 5					
M	6.53	6.00	5.27	4.50	6.37
SD	2.91	2.82	4.16	3.57	5.26

Table 4

Latency in the STM during acquisition and retention.

		Steady Schedule			Escalating Schedule	
	Saline	540 mg/kg	720 mg/kg	420 mg/kg	Saline
Acq. Trial 15					
M	32.80	30.70	28.20	31.33	40.89
SD	11.42	11.03	17.91	17.12	43.77
Retention Trial 1					
M	23.40	32.70	32.00	46.33	19.44
SD	12.47	14.00	34.72	18.62	13.18

Table 5

Errors in the STM during acquisition and retention.

		Steady Schedule			Escalating Schedule	
	Saline	540 mg/kg	720 mg/kg	420 mg/kg	Saline
Acq. Trial 15					
M	6.40	7.40	6.40	3.50	7.11
SD	3.53	3.17	6.73	4.09	7.80
Retention Trial 1					
M	7.70	14.10	13.00	18.67	5.67
SD	6.04	9.86	18.01	7.26	6.04

Table 6

Latency of mice on the rotarod post-treatment.

		Steady Schedule			Escalating Schedule	
		Saline	540 mg/kg	720 mg/kg	420 mg/kg	Saline
Trial 1						
	M	258.50	271.30	216.50	185.67	266.60
	SD	71.05	68.36	117.18	100.41	59.16
Trial 2						
	M	288.40	261.40	300.00	173.50	298.30
	SD	25.46	83.22	0.00	139.70	5.37
Trial 3						
	M	296.30	293.50	285.13	210.67	300.00
	SD	11.70	20.56	28.61	112.69	0.00

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