PRELIMINARY STUDIES OF NANOREXIN ON AGE-RELATED MEMORY LOSS

By

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To my family

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Abstract of Thesis Presented to the Graduate School
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Orexin, an endogenous neuropeptide released by the hypothalamus within the brain, has been shown to improve memory performance in sleep-deprived primates. Orexin has been studied in the context of sleep and metabolism, but few studies have focused on its relationship with memory. Based on the hypothesis of improving age-related memory loss, orexin was formulated into a proprietary peptide delivery system for intranasal delivery in rats. Memory-impaired, 22 – 24 month old Fischer 344 rats were identified by selecting the lowest performing 25% of the Morris Water Navigation Task in Acquisition. Impaired rats were divided into two groups for a crossover study. Each group received drug or vehicle daily, alternating in week-long intervals for four weeks. Memory effects were assessed daily by the Y-maze test paradigm. At the end of the four weeks, all rats were re-tested on the Morris Water Maze. There were no significant differences found in alternating percent of the Y-maze or trial times in the Morris Water Maze. The study, however, had a small sample, and treated groups trended to better outcomes. Future directions should lead into further elucidation of the orexin mechanism and its relation to memory.

CHAPTER 1
INTRODUCTION

Introduction to Age-Related Memory Loss

Age-related cognitive impairment reduces quality of life and represents the most feared aspect of aging [1]. Age-related cognitive decline and Alzheimer's Disease appear to share vulnerability in the same areas of the brain [3, 4]. In the United States, over 5.3 million people are estimated to have Alzheimer's Disease [5]. The disease is a leading socioeconomic problem in developed countries [6].

Overview of Orexin

Orexin, also known as orexin-A or hypocretin-1, is a neuropeptide released by populations of neurons in the hypothalamus. The peptide is coded by the gene *preproorexin*, which also codes for the related peptide, orexin-B. Orexinergic neurons also release orexin-B, or hypocretion-2. The projections of these neurons reach throughout the brain. Orexin-A binds to orexin-1 receptors and orexin-2 receptors with equal affinity [7].

Two independent groups reported the discovery of orexin in 1998 [7, 8]. Since then, research has found that the orexin system regulates or interacts with numerous activities throughout the body. The most profound effect of orexin on the body appears to be the enhancement of arousal and reduction of REM stage sleep. Orexin neurons are responsive to the sleep/wake cycle [9]. Orexin proteins are also highly expressed in the brain region controlling food intake. Lateral cerebroventricular injection of orexin-A has a potent effect on feeding behavior on rats, especially during the light phase [10]. Central administration of orexin affects the sympathetic nervous system. Arterial blood pressure and renal sympathetic nerve activity are increased after intracerebroventricular

injection in rats. The HPA axis, the stress system of an organism, is also activated [11, 12].

In a widely-publicized 2007 paper, *Deadwyler et al.* found that intranasally-administered orexin improved cognitive performance of sleep-deprived primates [13]. Two groups of monkeys were sleep-deprived for a night and were tested by the delayed match-to-sample task which examines short-term memory. In this study, the authors also found improved effect from intranasal administration over intravenous administration. Imaging via PET scan revealed higher cerebral glucose metabolism in affected areas from the liquid orexin formulation. The intranasal administration of orexin was also pioneered by authors Dhuria, Frey, and Hanson [14]. In their study, they intranasally dosed anesthetized rats, and they found that the majority of drug entered the central nervous system directly through the nasal passage. This route of administration is an effective strategy for getting across the blood brain barrier. Using a nasal administration would also reduce the necessary amount of drug normally required for blood circulation.

In light of this research, few studies have focused on the relation of orexin and memory. There have been no studies on orexin and age-related memory loss. This study aimed to explore any benefits from the dosing of orexin to aged animals. It has been found that orexin receptors are highly expressed in the hippocampus, a brain structure involved in spatial learning and memory. Orexin antagonists have been found to impair memory and learning performance of rats [15].

The capability of orexin to improve memory performance from loss of sleep may affect age-related memory loss. Sleep disorders are common in elderly populations.

Disturbances of circadian rhythms increase with age. Such disturbances may be contributing to age-related memory loss [16, 17]. The question arises of whether orexin can affect age-related memory loss. Cognitive decline in aging has a high correlation with sleep disorders. Given that orexin improved cognitive performance after sleep deprivation, the neuropeptide could possibly improve memory function in aged subjects.

This study employed the use of a novel orexin formulation referred to as Nanorexin. This drug is a proprietary formulation produced by the local pharmaceutical specialty company, Nanotherapeutics (www.nanotherapeutics.com). The formulation acts as a drug delivery system by coating peptide with a degradable hydrocarbon. The resulting formulation allows for sustained release. Also, the encapsulated orexin has an increased stability for a longer half-life in the body, potentially lasting for several hours. Such a formulation would be optimal for practical use if translated. The formulation is in liquid form, which can be used for intranasal administration.

Introduction to Experimental Design

The aim of the study was to test the effects of the orexin formulation, Nanorexin, on the memory performance of aged and impaired rats. The test subjects of the experiment were a population of animals selected for memory impairment as a result of Morris Water testing done by the lab of Dr. Tom Foster. To evaluate Nanorexin, subjects were tested in the Y-maze and re-tested in the Morris Water Maze. Both behavioral tasks demand spatial learning and memory. The Y-maze Spatial Alternation Task tests the working memory of rodents. The Morris Water Navigation Task, or the Morris Water Maze, tests the several aspects of learning and memory of rodents.

The Morris Water Maze requires a rodent to swim and to find a hidden platform in a small pool. The amount of time it takes for the animal to find the platform is recorded

for each trial. The small pool is filled with water made opaque with non-toxic paint, and the platform is a few centimeters below surface level. Visual cues placed on the sides of the wall facilitate spatial orientation. Normally, the escape times of rats decrease after repeated trials. The decrease in time is the indicator in learning [18].

The Morris Water Maze is further separated into the Acquisition Phase, the Retention Phase, and the Reversal Phase. All phases are sensitive to hippocampal dysfunction. The Acquisition Phase tests short-term memory; the test subject learns the task during this phase. The Retention Phase tests the long-term memory of the subject. In this phase, the animals are tested 24 hours after their last exposure to the platform. The Reversal Phase tests the ability to learn when rules have changed. During this phase, the test subject must find the submerged platform in a new location. Animals are gauged by the amount of time spent in the correct quadrant of the tub.

The sensitivity of the Morris Water Maze decreases on subjects with repeated testing. The task tests the capacity of learning, and animals are no longer naïve after repeated testing. In this study, the Morris Water Maze was utilized twice for each subject. The maze was used initially to screen for memory impairment, and the second utilization of the Morris Water Maze comprised the Nanorexin drug trials for spatial learning. Out of a group of 32 rats, two experimental groups that each consisted of four rats were selected after an initial testing with the Morris Water Maze in Acquisition trials (data not shown). The lowest-performing 25% of rats are considered to be memory-impaired. This paradigm has been accepted in the field after the observation of a recurring trend in the performance of aged rats in the Morris Water Maze. The results

of a typical sample of aged rats tend to fall into a bimodal distribution, with the lower peak resembling an impaired population [19].

Orexin is also implicated in energy homeostasis and physical activity. Previous literature publications have indicated that orexin administration increases locomotive activity [20]. Running wheel activity is an indicator of physical activity, and it may serve as a possible behavioral assay of orexin activity independent of memory effects. This *in vivo* assay could serve as a possible control, confirming that the orexin formulation was reaching intended targets. To observe activity, rats were housed in cages fitted with running wheels. Running wheel revolutions were recorded during testing and 12 days prior to any drug administration.

For the Y-maze, a crossover study was performed on both groups of rats. Animals were tested in the Y-maze for five days a week with both groups receiving either Nanorexin or vehicle before testing. Each group received Nanorexin or control for one week (5 days of administration) before switching treatment. To assess any differences between the times of day, testing and dosing took place during the dark/active phase for the first two weeks. Testing and dosing took place during the light/inactive phase for the last two weeks. The crossover Y-maze study lasted for four weeks.

Subsequently, all eight experimental rats were tested in the Morris Water Navigation Task for a second time. The Morris Water Maze testing lasted two days and consisted of all three phases of learning and memory. Each group became permanently Nanorexin or control for the remainder of the study. After dosing with drug or control, the animals were given six blocks of three trials in the first day for the Acquisition Phase. The next day, the rats received three blocks of three reversal trials

for the Reversal Phase. In either day, a block consists of three trials. The distances traveled and trial times from the Morris Water Maze trials were recorded and analyzed statistically. In the beginning of the second testing day, each capable rat received one probe trial in the Retention Phase.

CHAPTER 2
MATERIALS AND METHODS

Animals

All animals used were male Fischer 344 rats aged 22-24 months, purchased from Jackson Labs. During all studies, rats were kept in 12:12 h light:dark cycles under constant room temperature. All rats were single-housed in cages that contained running wheels and revolution counters. Running wheel revolutions were recorded for the duration of the study. All rats used were memory-impaired. Memory impairment is defined by the lowest-performing quarter of subjects in the Morris Water Maze task.

Nanorexin was administered as a liquid solution of 1.0 to 1.5 μg per dose. The drug was administered by pipette through the nasal passage of an anesthetized rat. Rats were anesthetized via gas chamber using a mixture of isoflurane (4%) and medical-grade oxygen gas. An increased dose of 2.0 to 3.0 μg per dose was administered for the Morris Water Navigation Task.

Y-maze Spatial Alternation Task

Rats were tested by the Y-maze Spatial Alternation Task by placing them into a y-shaped maze and exploiting their natural tendency to alternate paths in exploration. The Y-maze apparatus is a maze with three identical arms made out of clear Plexiglas. Each arm is approximately one meter long and 10 centimeters wide. Each rat was tested individually in the apparatus; the apparatus was wiped clean with 70% ethanol between animals. All testing and training were done in the animal storage room.

Before testing, rats are first trained for the task. Training Phase 1 is for the acclimation of the rats to the maze. For six days, animals were given 10 trials. In each trial, rats were placed at the end of one arm while having one of the three arms blocked.

The rats can only move down the other open arm. Food pellets are placed at the end of the open arm to encourage movement. The trial concludes at either the end of two minutes or if the rat reaches the end of the open arm. After the trial ends, the rat was placed at the end of the same starting arm for the next trial, repeating this process until the 10th trial. After the first phase, Training Phase 2 began for the reinforcement of the selection of alternate arms. Each animal was given 10 trials daily for six days. In Training Phase 2, each trial consisted of a rat being placed at the end of an arm with both arms open. Initially, both open arms contain food pellets at each end. In the following trials, food pellets were placed only in "correct" arms. The trial ended either at the end of two minutes or at the arrival of the rat at an open arm. After the trial ends, the rat was placed at the end of the same arm for the next trial until the 10th trial.

Testing began at the end of the Training Phase 2. Rats participated in 10 trials per day. Trials during testing were conducted as described in Training Phase 2. All animals were dosed daily with either drug or control before any trials began.

Rats were scored at the end of each trial based on selection. Selections recorded were Left Arm, Right Arm, Return, or No Selection. Left Arm and Right Arm scores required the rat to enter an arm with all four paws. If a rat entered another arm with 1-3 paws and returned to the starting arm, Return was recorded for the trial. Otherwise, No Selection was recorded for the trial at the end of two minutes.

Morris Water Navigation Task

The Morris Water Navigation Task took places in a gray circular pool filled with water made opaque by the addition of non-toxic dark paint. A small Plexiglas platform, of dimensions 4x4 inches, was hidden approximately 2 cm below the surface of the water. Several fixed spatial cues were placed around the pool. Performance was

recorded by an overhead video camera that was linked to a computerized tracking system. Rats were acclimated to both the pool and testing room before trials begin [18].

Trials begin with the Plexiglas platform placed into one quadrant of the pool. One rat was released in the water with its head pointed at the side of the pool at one of three predetermined points around the pool. The time it takes for the animal to find the submerged platform was recorded. The trial concludes either when the animal reaches the platform or at the end of 60 seconds. If the animal had not found the platform by the end of 60 seconds, the animal was guided to the platform; and the trial time of 60 seconds was recorded. Subsequently, animals were allowed to remain on the platform for 10-15 seconds and were removed from the water. The rat was given a rest period of at least 5 minutes while testing resumed for other rats. Each block consisted of 3 trials per rat at different release points around the pool. Release points are the locations where rats were placed in the pool; the release points around the pool were separated by 120 degrees. The blocks differed from each other by time; subsequent blocks were started after a 5 minute wait period in between blocks. The first day of Morris Water Maze testing consisted of trials in the Acquisition Phase. The second day of testing consisted of trials in the Reversal Phase. During the Reversal Phase, the location of the platform was placed in the opposite quadrant. All animals received 6 blocks of testing during the Acquisition Phase and 3 blocks of testing during the Reversal Phase.

In the Retention Phase, rats received probe trials at the beginning of the second day of testing. All subjects in the Retention Phase had gone 24 hours before being last exposed to the platform of the tub. In a probe trial, the platform is removed, and the rat is placed into the tub at the first release point for 60 seconds of time. The movement of

the rat is recorded by an overheard video camera. The time spent in each quadrant of the tub is determined by computer software. This amount of time is the indicator of long-term memory.

Statistical Analysis

Statistics were analyzed primarily by multivariate ANOVA with the General Linear Model procedure (SAS). Main factors were drug and phase of day. Factors were considered significant at the .05 level. The experimental testing by the Morris Water Navigation Task was also analyzed by the analysis of variances for repeated measures and the linear mixed effects model.

CHAPTER 3
RESULTS

Results for the Y-maze Spatial Alternation Task

Collapsed over the light and dark cycle administrations, there was no significant effect of Nanorexin on overall alternation percentage (Figure 3-1). There was a trend for reduced alternation in rats that received Nanorexin and were tested in the light phase (Figure 3-2). Most of this was due to reduced alternation on 4 of the 5 days for the first drug cohort in the light phase (Figure 3-3).

Results for the Morris Water Navigation Task

In the trials for the Morris Water Navigation Task, the trial time and distance traveled were recorded for each trial. In the first six blocks of testing, there were no significant differences between the Nanorexin and Control groups in the averages or sums of the outcomes measured (Figure 3-4). In the reversal trials, there were also no significant differences found. The Nanorexin group, however, displayed a trend of having a smaller trial times and distances traveled (Figure 3-5).

Further analysis of the data sorted by blocks was attempted using more sophisticated statistical models. The data, however, did not meet the necessary assumptions for the analysis of variance for repeated measures, likely due to the small sample size. The linear mixed effects model was also used. The model estimated a smaller travel time for the Nanorexin group over the Control group for four out of the six groups, however, with insignificant p-values (values not shown). A larger sample size may have given significant p-values. Additionally, the Retention Phase showed no meaningful pattern from the small sample size available (values not shown).

Results of Running-Wheel Monitoring

During the crossover study, running wheel revolutions were recorded daily. Nanorexin groups and control groups had no significant difference in overall revolutions completed (Figure 3-6). Individual rats did not show effect to drug; there were no apparent responders or non-responders (Figure 3-7). Additionally, individual rats showed no effect between drug administration during the light phase and the dark phase. Over multiple doses, there were no additive effects of the drug. No order effect was observed in the assay; the rats did not show signs of tolerance or buildup to the drug.

There was a one deviation observed across all animals. At the beginning of the study, running wheel activity declined for both groups (Figure 3-8). Running wheel activity appeared to return to normal levels after 2 weeks of testing.

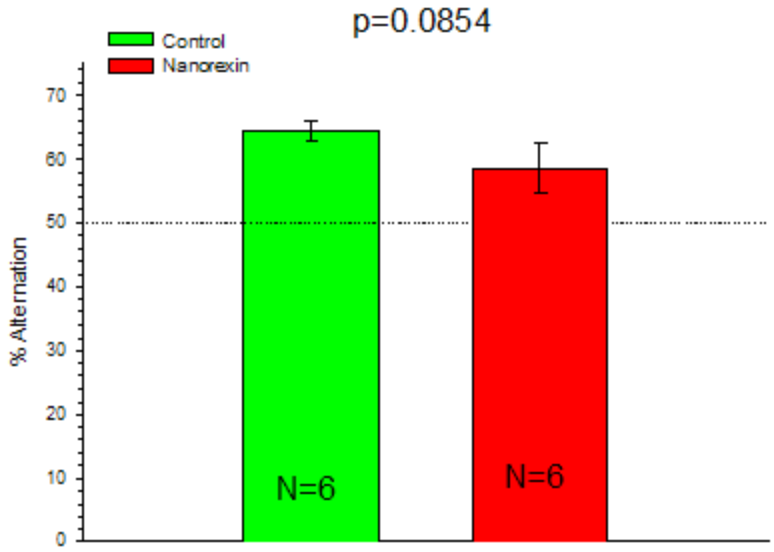

Figure 3-1. Overall Alternation Percentage. There was no significant difference in overall alternation percentage between Nanorexin and Control groups. The "% Alternation" represents the percentage of corresponding choices of Y-maze arms that alternated with the previous choice of the rat. A higher percentage correlates with an increase of alternate choices in the Y-maze.

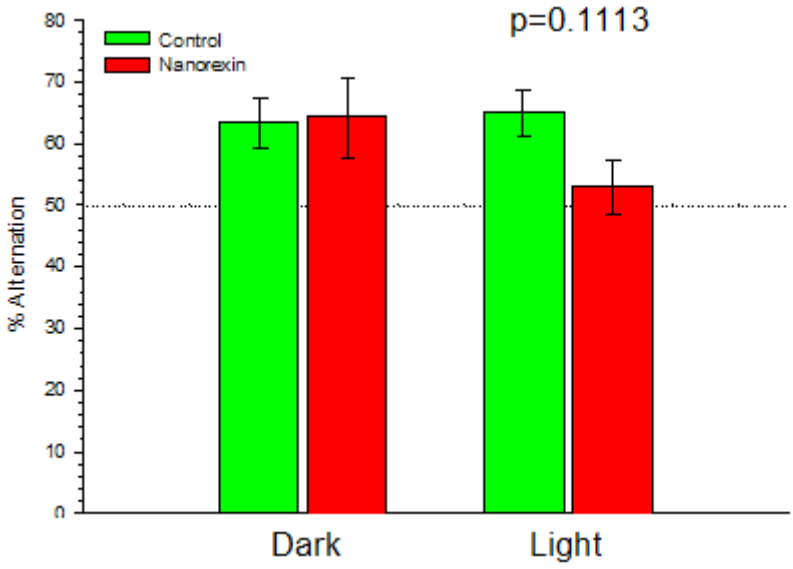

Figure 3-2. Alternation Percentage by Phase. There was no significant difference in overall alternation percentage in either the dark of light phases. The percentage number represents the percentage of corresponding choices of Y-maze arms that alternated with the previous choice of the rat. A higher percentage correlates with an increase of alternate choices in the Y-maze.

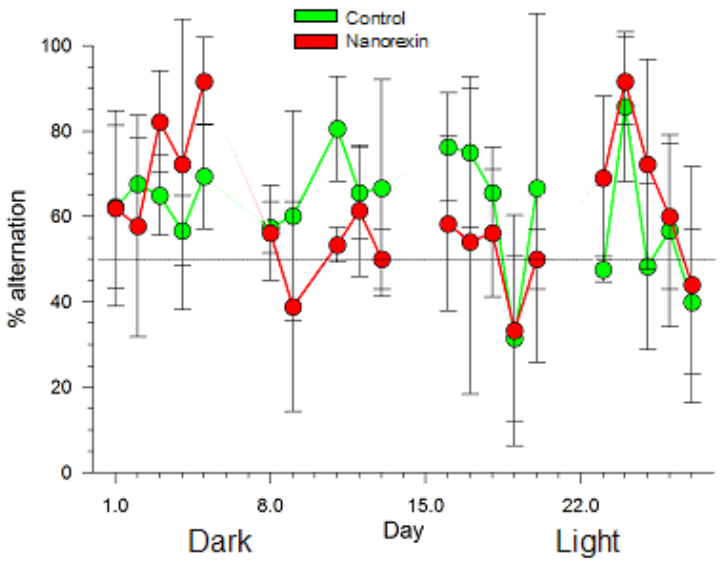

Figure 3-3. Alternation Percentage by Individual Day. No consistent group effects found in the individual day scores. . Each point represents the mean alternation percentage of the day for an individual rat. A higher percentage correlates with an increase of alternate choices in the Y-maze.

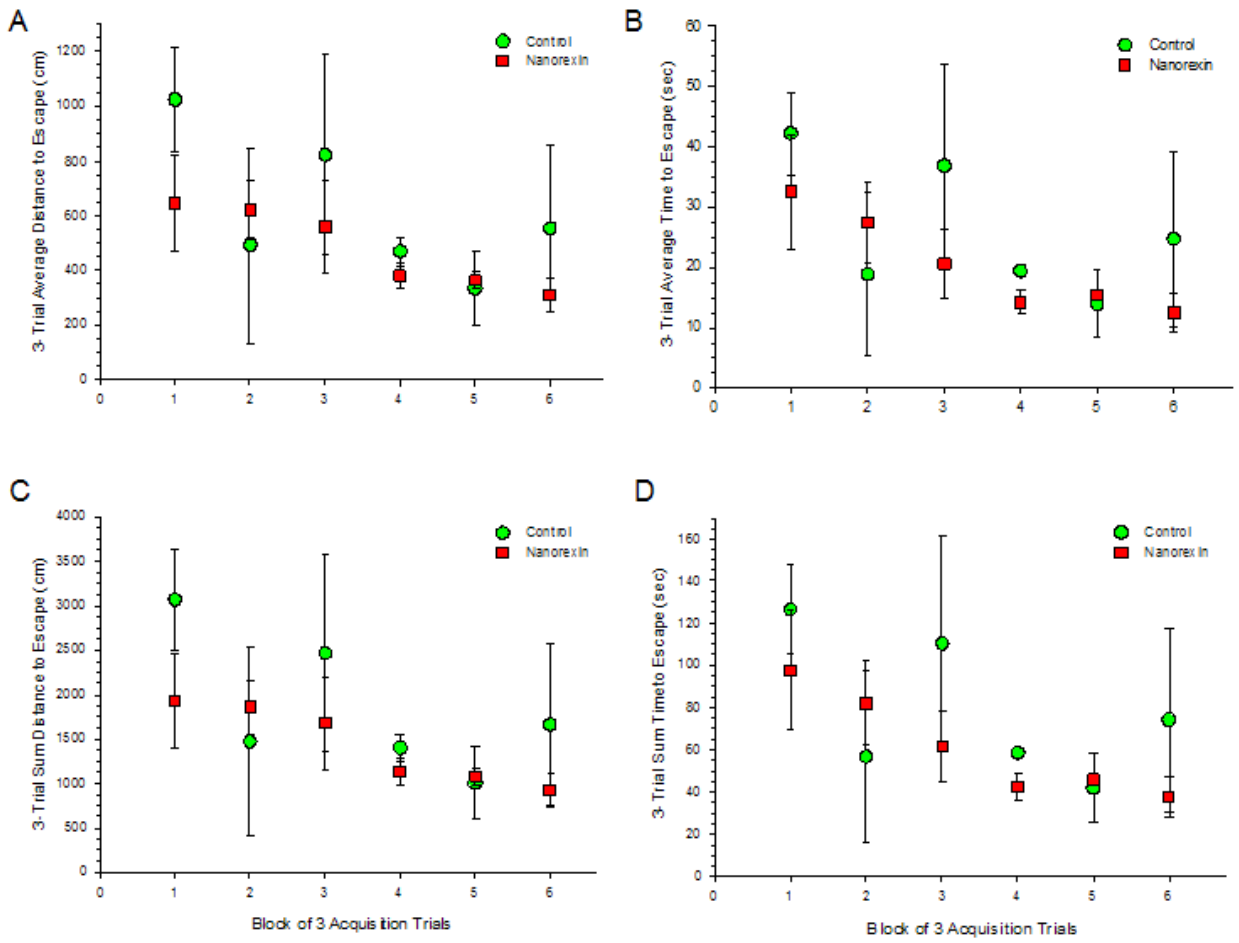

Figure 3-4. The First 6 Blocks of the Morris Water Navigation Task. A) The 3-trial average distance to finding the escape platform in cm. Points represent treatment group means (± s.e.m.) of within-animal averages of 3 Acquisition trials per block. B) The 3-trial average time to escape in sec. C) The 3-trial sum distance to escape in cm. D) The 3-trial sum time to escape in sec. (Nanorexin group n = 3, Control group n = 2)

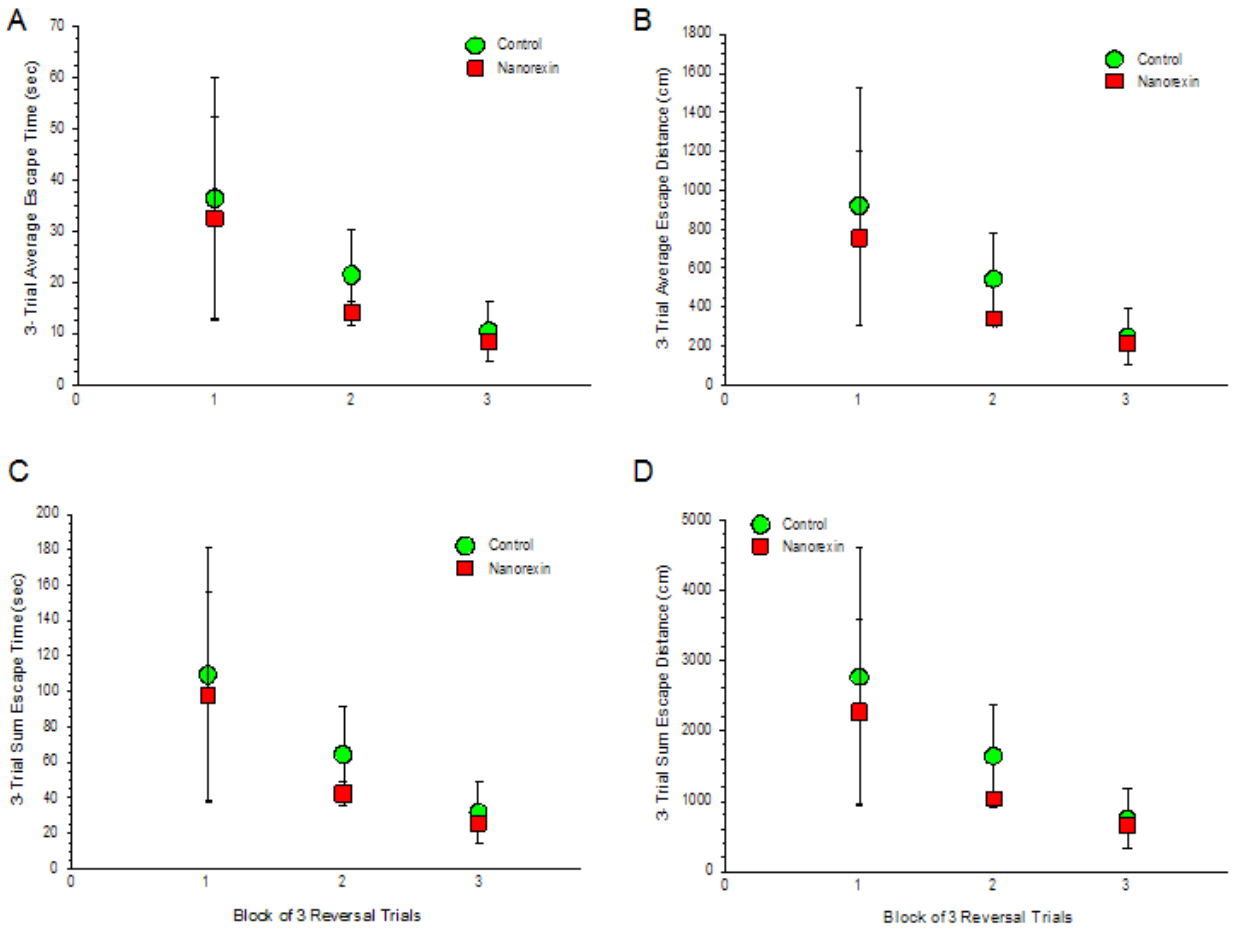

Figure 3-5. The 3 Blocks of Reversal Trials of the Morris Water Navigation Task. A) The 3-trial average escape time to finding the escape platform in sec. Points represent treatment group means (± s.e.m.) of within-animal averages of 3 Reversal trials per block. B) The 3-trial average escape distance to escape in cm. C) The 3-trial sum time to escape in sec. D) The 3-trial sum distance to escape in cm. (Nanorexin group n = 3, Control group n = 2)

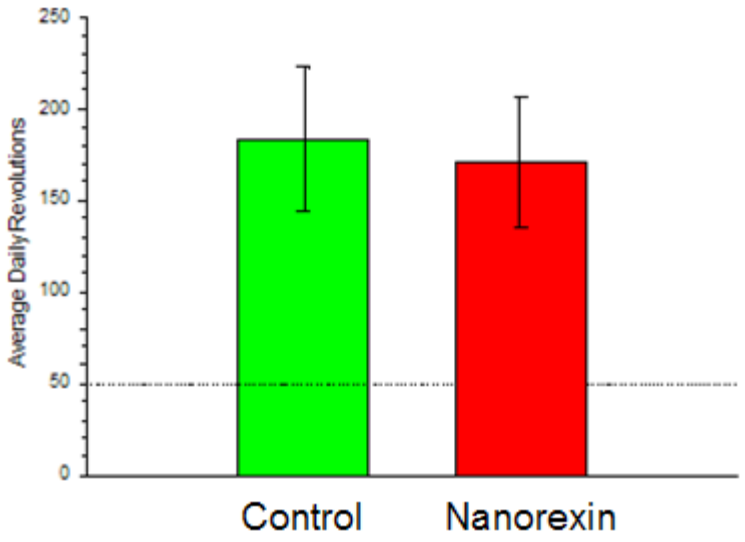

Figure 3-6. Average Daily Running Wheel Revolutions. There was no significant difference found between Nanorexin and Control groups. Each revolution is a complete turn of the running wheel within the cage.

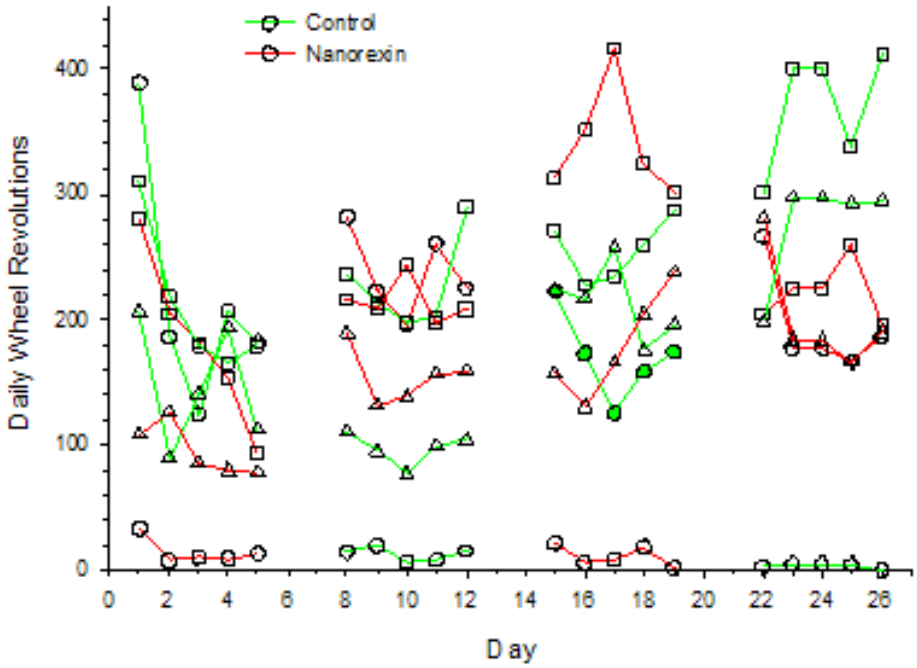

Figure 3-7. Running Wheel Revolutions by Individual. No major deviations were observed in the running wheel data by individuals. . Each point represents the number of revolutions completed since the previous day. The first 14 days resemble drug administration and testing during the dark phase.

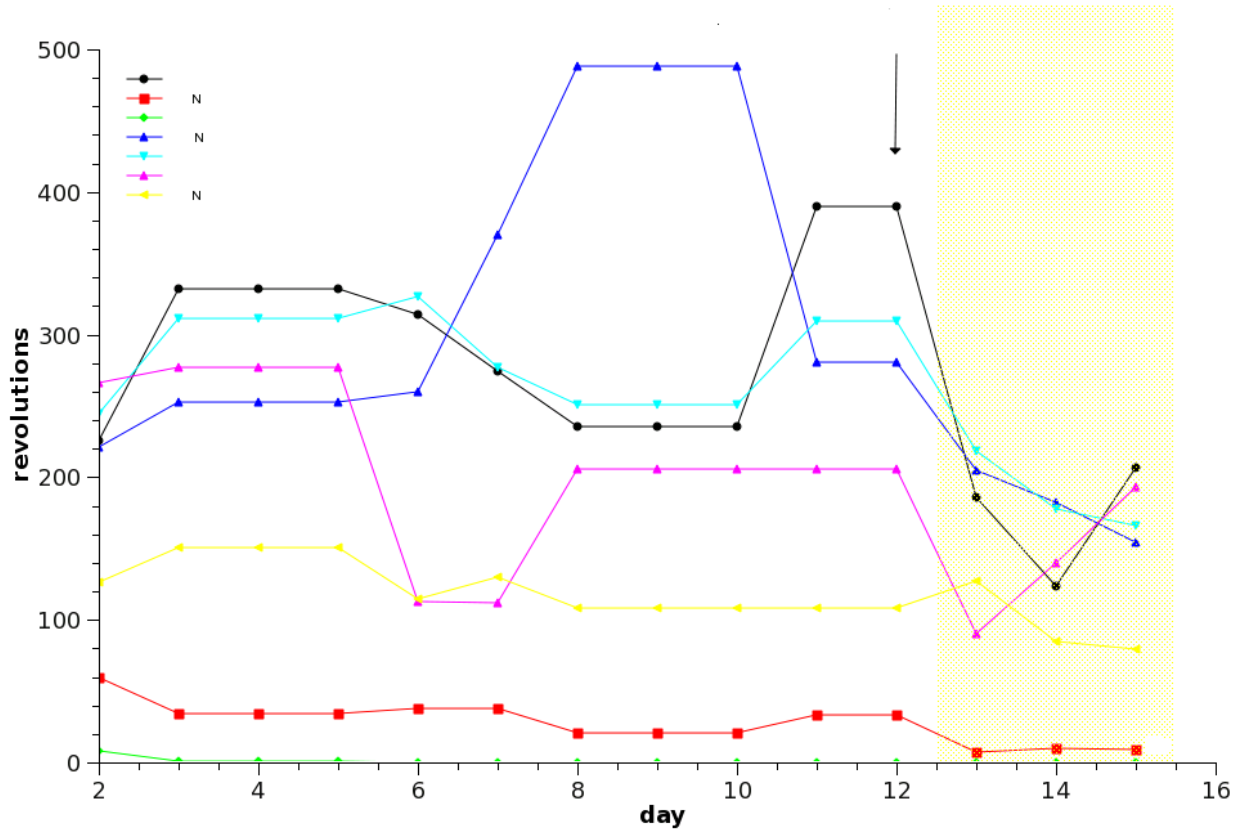

Figure 3-8. Running Wheel Revolutions of Individuals per Day. The red, blue, and yellow datalines (marked by N in the top-left Legend) represent individual rats beginning drug treatment at Day 12. The Arrow at Day 12 points out the decline in running wheel activity. The highlighted area displays the recordings during drug/control administration and anesthesia.

CHAPTER 4
DISCUSSION

In both behavioral tasks, the results were inconclusive in finding an effect of orexin-A on age-related memory loss. Control groups performed as well as the drug groups statistically. In addition to the lack of significant differences being found, there was no positive confirmation of the biological activity of Nanorexin.

Since there were no results for any positive control, this study cannot conclusively determine that orexin A had a lack of effect on age-related memory loss. No differences were found in running wheel activity between groups. Thus, the results from this behavioral assay did not confirm that orexin was reaching the intended targets. More conclusive results would have been achieved with additional positive controls. Furthermore, a dose range correlating to physical activity may have to be characterized in order to use running wheel activity as a successful positive control. To confirm that Nanorexin is activating the intended receptors, a bioassay could be used. In the literature, administration of orexin-A into the lateral hypothalamus increased cFos in the surrounding brain regions of rats. Rats were sacrificed after injection, and tissue sections were sliced and analyzed for cFos immunoreactivity. The experiment could be repeated with Nanorexin injected into the lateral hypothalamus. The bioactivity of Nanorexin would be confirmed if the immunoreactivity imaging mirrored the results from the pure orexin-A paper [21].

The uncharacterized dose of Nanorexin represents a limitation of the study. The dose was estimated from amounts used in the Deadwyler paper [13]. Additionally, the uncharacterized nasal administration of the Nanorexin formulation represents another unknown that should be addressed with an assay. Also, a known limitation of the

crossover treatment design is the possibility of washover effects between the drug and control groups. In crossover studies, control groups may still have residual drug within their systems. Experimental groups experiencing repeated regimens are also no longer naïve to initial testing conditions.

At first glance, the decrease in running wheel activity for all animals at the onset of the crossover study may seem to represent a limitation of the experimental design. The drug and control administration method may have altered physical activity and possibly any memory effect, particularly with the anesthesia. Recent studies, however, have shown that isoflurane does affect the long-term cognitive outcome of aged rats [22]. Running activity at least partially returned to pre-experimental levels after two weeks into the experiment; this was when the light phase dosing began. Instead, the decrease in running activity may reflect the disruption of normal wheel running in the dark phase by the experimental testing and dosing. Nevertheless, a fully intranasal spray formulation with corresponding distribution equipment may have been optimal.

The results for the Morris Water Navigation Task also had no significant differences. The sample sizes, however, were extremely small (n=3, n=2) due to financial constraints. The Nanorexin groups showed a trend of having small trial times and distances traveled, particularly in the reversal trials. This may indicate that the Nanorexin-treated animals had improved learning, since the reversal trials were done the second day of testing. Furthermore, the smaller estimations of trial times by the linear mixed effects model also indicated the trend of Nanorexin animals. Although the p-values were insignificant, a larger sample size may have given significant p-values.

Lastly, the possibility remains that the drug was active and delivered to the brain. Nanorexin may simply lack an effect to the memory of impaired, aged rats. Assuming this was the case, the results could still be interpreted in light of the original Deadwyler paper. The results would indicate that orexin-A improved memory performance in sleep-deprived animals through mechanisms different by those affected by age-related memory loss. The mechanisms improved by orexin-A in sleep-deprived animals are likely under circadian regulation.

CHAPTER 5
FUTURE RESEARCH DIRECTIONS

Due to financial constraints, this study never examined the pharmacokinetics of Nanorexin. Future studies involving this unique orexin formulation will involve finding dose-concentration curves for confirming appropriate dosages. An essay of finding the drug concentration within the cerebrospinal fluid of a rat such as an ELISA may be used.

Additionally, orexin is implicated in several activities of the brain. Future research may involve branching out to the fields of obesity and addiction, where the mechanisms of orexin have been further explored. In terms of memory, this research warrants the elucidation of the orexin mechanism and its relation to memory and cognitive performance.

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BIOGRAPHICAL SKETCH

David Demosthenes received a Bachelor of Science degree from the University of Florida in 2007. He majored in finance. During his undergraduate education, David helped conduct clinical research in the area of neuroscience at the University of Florida in Gainesville and the Albert Einstein College of Medicine in New York City. After his bachelor's degree, David received a Master of Science degree in Entrepreneurship from the University of Florida in 2008. In December of 2010, David Demosthenes received his second Master of Science degree, in medical science, from the University of Florida.