5-2018

Effects of Norharmane and Nicotine on the Conditioned Place Preference of Mice

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EFFECTS OF NORHARMANE AND NICOTINE ON THE CONDITIONED PLACE PREFERENCE OF MICE

By

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THESIS

Submitted to
Northern Michigan University
In partial fulfillment of the requirements
For the degree of

MASTER OF SCIENCE

Office of Graduate Education and Research

04/2018

SIGNATURE APPROVAL FORM

Effects of norharmane and nicotine on the conditioned place preference of mice

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ABSTRACT

EFFECTS OF NORHARMANE AND NICOTINE ON THE CONDITIONED PLACE PREFERENCE OF MICE

By

Lindsey K. Galbo

Tobacco smoking in the United States is used by approximately 25% of adults. Many studies using animal models have suggested that nicotine has rewarding properties. Contrastingly, several studies have also found it to be a weakly reinforcing substance at low and high dose levels. Due to this, other tobacco constituents, such as the monoamine oxidase inhibitor norharmane which is found in tobacco leaf and smoke, may be responsible for tobacco addiction by potentiating the rewarding properties of nicotine. Several studies have attempted to observe this phenomenon, however, monoamine oxidase inhibitors that are not found in tobacco leaf or smoke have been used. Thus, the present study utilized a conditioned place preference paradigm to observe whether or not nicotine at dose levels of 0.15 and 0.30 mg/kg, and norharmane at dose levels of 5.0 and 10.0 mg/kg, could individually produce place preference, indicating drug seeking behavior of the rewarding properties of each compound. Finally, a combination of nicotine and norharmane were administered together, and the same parameters were observed. A significant place preference for either drug alone, nor in combination, produced significant results. In conclusion, norharmane was not found to potentiate the rewarding properties of nicotine, however the present study was able to provide data on the effects of norharmane in a conditioned place preference paradigm, which have never been observed before.

ACKNOWLEDGMENTS

The author wishes to thank her thesis director, Dr. Adam Prus, for his guidance, advice, and support throughout the entirety of the study; Dr. Joshua Carlson, for statistical expertise; Dr. Joseph Porter, for his expertise in behavioral analysis; Kailey MacDonald, Mike Zuidema, and Kevin Thomma for their assistance with various study procedures; My family, Mark Galbo, Deb Smith, and Andrea Galbo for their never ending support and encouragement. This project could not have been completed as efficiently without the help of these individuals.

This thesis follows the format prescribed by the *Publication manual of the American Psychological Association* and the Department of Psychological Science.

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LIST OF ABBREVIATIONS

MAO = Monoamine oxidase

MAOI = Monoamine oxidase inhibitor

CPP = Conditioned Place Preference

VEH = Vehicle

NIC = Nicotine

NOR = Norharmane

INTRODUCTION

Tobacco smoking is used by approximately 25% of adult in the United States, and tobacco cessation therapies are extremely unsuccessful. There are approximately 3,000 chemical constituents found in tobacco, therefore it is suspected that other constituents, such as monoamine oxidase inhibitors, must play a role in nicotine and tobacco addiction. Norharmane is naturally found in tobacco leaf and smoke. Other monoamine oxidase inhibitors used in nicotine self-administration studies have been found to potentiate the rewarding properties of nicotine, as well as locomotor activity. The aims of the present study were to utilize a conditioned place preference behavioral paradigm to study this phenomena. Using male and female mice, the present study attempted to establish place preference following nicotine administration, norharmane administration, and a combination of both drugs. Locomotor activity was also observed as an additional behavioral measurement.

Literature Review

Tobacco smoking in the United States is used by approximately 25% of adults. Among adults, 88% smoked their first cigarette before the age of 18 (U.S. Department of Health and Human Services, 2014). Life-long tobacco smoking leads to a 50% chance of premature death due to cancer, cardiovascular disease, pulmonary disease, and other various primary and secondary complications (Neal Benowitz, 2010). According to a report from the Centers of Disease Control and Prevention (2009), approximately 40% of tobacco smokers attempt to quit smoking for at least one day, however, only 3% of smokers quit successfully (Benowitz, 2010).

The rate of young adult smokers has remained relatively steady since the early 2000's according to a report from the Surgeon General (U.S. Department of Health and Human Services, 2014). However, this report does not include any analysis or projection of how the introduction of electronic nicotine delivery devices in recent years has and will affect nicotine addiction or tobacco use in the future (U.S. Department of Health and Human Services, 2014).

Nicotine

Nicotine acts physiologically by traveling through the lungs where it is absorbed into the blood stream and quickly passes through the blood-brain barrier via pulmonary venous circulation. Here, nicotine binds to nicotinic cholinergic receptors, which opens ion channels, allowing the entry of sodium and calcium. This further opens voltage-dependent calcium channels resulting in an influx of calcium that subsequently releases neurotransmitters, such as dopamine (Neal Benowitz, 2010). Dopamine is highly

involved in reinforcement and dependence. Nicotine also causes glutamate release which further aids in the release of dopamine, and ϒ-aminobutyric acid (GABA) which inhibits dopamine release in the mesolimbic area, primarily the ventral tegmental area and nucleus accumbens, corpus striatum, and the frontal cortex (Neal Benowitz, 2010). Over time nicotinic cholinergic receptors may become desensitized which decreases the inhibitory effect of GABA and increases the excitation of dopaminergic neurons, which ultimately potentiates the reaction to nicotine administration (Neal Benowitz, 2010).

The strength of nicotine as a reinforcing drug and its effects on behavior has been found to be dose dependent and many studies have suggested that nicotine has both aversive and rewarding properties (Manzardo, Stein, & Beluzzi, 2002; Villégier *et al*, 2006). For example, a study by Shoaib, Schindler, and Goldberg (1997) found nicotine to function as a reinforcer of intravenous self-administration. Three doses of nicotine (0.015, 0.03, and0.06 mg/kg/infusion) produced and maintained a nose-poke response to an active self-administration hole with very little to no responding in the inactive hole. In another study by Risinger and Oakes (1995), place preference in mice following conditioning to a range of subcutaneous nicotine doses (0.25, 0.5, 1.0, 2.0 mg/kg) was observed. Mice showed significant place preference at an intermediate dose of 0.5 mg/kg, suggesting that the rewarding properties of nicotine were reinforced during conditioning trials.

Contrastingly, several studies have found nicotine to be a weak reinforcer in comparison to other drugs of abuse, and may be considered aversive at lower and higher doses (Arnold, Loughlin, Belluzzi, & Leslie, 2014; Hall *et al*, 2014; Villégier *et al*, 2006). In addition to the place preference observed by Risinger and Oakes (1995),

significant place aversion was observed in animals conditioned using a nicotine dose of 2.0 mg/kg. In another study by Manzardo, Stein, & Belluzzi (2002) the degree of reinforcement of nicotine versus cocaine doses were observed. Animals self-administered lower doses of cocaine more frequently than nicotine, showing that nicotine is a less potent reinforcer.

Understanding that nicotine can be either rewarding or aversive based on dose, it has been suggested that some of the constituents found in tobacco and tobacco smoke may be responsible for potentiating the rewarding properties of nicotine in humans, thus strengthening tobacco addiction. These suggestions function as a possible explanation as to why nicotine replacement therapies and cessation products are not generally successful (Cao *et* al, 2007; Guillem *et al*, 2006; Hall *et al*, 2014). Etter and Stapleton (2006) conducted a meta-analysis of all randomized controlled trials of nicotine replacement therapies with participant follow ups more than a year after the start of treatment. Their analyses found significant but modest effects of nicotine replacement therapies successfully helping with tobacco cessation for more than one year, however, they also state that more than 90% of those treated for tobacco addiction do not successfully quit smoking for the long term (Etter & Stapleton, 2006).

Norharmane

Monoamine oxidase is an enzyme that exists in two subtypes, A and B, that both function to break down neurotransmitters. Both have been found to oxidize dopamine, and the MAO A subtype also functions to oxidize serotonin and norepinephrine. Therefore, when inhibition of these MAO enzymes occurs, smokers experience an excess of dopamine, serotonin, and norepinephrine in the brain (Fowler *et al*, 1996). Of

particular interest, research by Fowler *et al* (1996) using positron emission tomography (PET) scans has found that smokers have 30-40% lower levels of monoamine oxidase (MAO) A and MAO B activity in their brains versus non-smokers, due to smoking tobacco.

The same results have been reported in studies observing the effects of cigarette smoke on MAO inhibition in the mouse brain (Carr and Basham, 1991). Therefore, it is possible that the influx of dopamine potentiates the reinforcing effects of nicotine, suggesting why humans are easily addicted to tobacco when nicotine alone can be aversive in rodent models (Arnold *et al*, 2014). A human study by Pomerleau and Pomerleau (1991) observed the euphoric subjective effects of smokers for nicotine-induced euphoric sensations. Of their 22 subjects, 19 reported feelings of euphoria beginning approximately 2.5 minutes after the start of lighting a cigarette, and that euphoria was also more pronounced following overnight deprivation. Additionally, it has been suggested that individuals withdrawing from tobacco are possibly withdrawing from MAO inhibition caused by tobacco constituents rather than nicotine alone (Fowler *et al*, 1996). If so, we may be able to better understand why nicotine replacement therapies and cessation programs do not have higher success rates.

As a result of these opinions and conclusions, many studies have been conducted to determine the effects of different known MAO inhibitors (MAOI's) on the discriminative stimulus and self-administration of nicotine in rats and mice. The study of these effects is complicated due to the variability and selectivity of different MAO inhibitors. Many studies use compounds that have not been found in tobacco leaf or smoke, although they yield significant results, such as increases in nicotine self-

administration (Guillem *et* al, 2006; Hall *et al*, 2014; Smith *et al*, 2016; Villégier *et al*, 2006; Villégier *et al*, 2007; Wooters & Bardo, 2007). However, not using compounds found in tobacco leaf or smoke make their results difficult to generalize to the human population. For the purpose of the present study, norharmane was chosen to be used as an MAOI treatment because it is a reversible MAO A and B inhibitor (Arnold *et al*, 2014). Norharmane is a β-carboline alkaloid and can not only be found naturally in tobacco leaf and smoke, but may also be found in a number of other medicinal plants (Arnold *et al*, 2014; Farzin & Mansouri, 2006). Interestingly, however, the levels of norharmane found in tobacco smoke is approximately four times greater than the amount found in a gram of tobacco (approximately one tobacco cigarette), suggesting that there is some type of pyrolytic process that causes the increased levels found in tobacco smoke (Poindexter & Carpenter, 1962).

Conditioned Place Preference Paradigm

The conditioned place preference (CPP) paradigm was chosen to for the present study because it is frequently used to study the rewarding properties of drugs of abuse. It has been used frequently in the past and is still presently used to investigate the rewarding properties of nicotine. Also, despite all of the current information on the effects of MAOI's on nicotine reinforcement and addiction, there is a gap in the literature. A database search was conducted (May, 2017) using the key words 'norharmane', 'MAO', 'MAOI', 'nicotine', 'CPP', and 'place preference' did not result in any studies that have focused on the effects of norharmane on the conditioned place preference in rodents, nor on the effects on the conditioned place preference of animals who have received both norharmane and nicotine, in combination.

The conditioned place preference paradigm functions by conditioning animals in a two or three chambered apparatus where each chamber is paired with either a drug or a vehicle. Each chamber of the apparatus is composed of different visual, tactile, and olfactory stimuli so that the animal may associate stimuli with either drug or vehicle. During testing, the animal is placed in the apparatus and given a choice between chambers. Time spent in either chamber is acknowledged as a place preference (more time) or aversion (less time). Avoidance of the drug paired chamber is also considered place or drug aversion (Le Foll & Goldberg, 2005). Nicotine is particularly interesting to study using a conditioned place preference paradigm because of its controversial aversive properties, and whether or not place preference is obtainable is controversial as well (Le Foll & Goldberg, 2005). Le Foll and Goldberg (2005) reviewed 22 studies that investigated nicotine effects using conditioned place preference and suggested several variables that may influence whether or not place preference or aversion is found, including various ranges of nicotine doses, specific acclimation and conditioning procedures, habituation to the conditioned place preference apparatus, and strain of animal. Despite the variability in design and models of nicotine conditioned place preference, a large number of studies have still successfully used the conditioned place preference paradigm and observed nicotine place preference. Therefore conditioned place preference is still an ideal behavioral paradigm that may be used in order to understand how a MAOI may or may not affect the rewarding properties of nicotine in the present study.

Additionally, sex differences have been observed in nicotine conditioned place preference tests in several studies (Kotal *et al*, 2008; Yararbas, Keser, Kanit, and Pogun, 2010). Therefore, data analysis for the present study will consider sex as a variable.

Locomotor Activity

Locomotor activity is often measured in studies observing post dosing behavioral effects of nicotine and MAOI interactions (Guillem *et al*, 2005; Guillem *et al*, 2006; Villégier *et al*, 2006). Guillem *et al* (2005, 2006) and Villégier *et al* (2006) found some increases in locomotor activity after a pre-treatment of MAO inhibitors such as clorgyline, selegiline, norharmane, tranylcypromine, and phenelzine, when administered prior to nicotine administration. In contrast, past studies such as a study conducted by Kita, Nakashima, Shirase, Asahina, and Kurogrochi, (1988), have suggested that nicotine has hypoactive and ataxic effects on the locomotor activity of mice with a range of nicotine doses from 0.1 to 1.0 mg/kg. Thus, locomotor activity will be measured during testing procedures of the present study in order to observe the potential differences during a conditioned place preference test between experimental groups.

Rationale

As previously mentioned and to the best of my knowledge, there are currently no available studies investigating how MAOI's effect the place preference of rodents after administration of nicotine. In addition, no studies were found investigating norharmane using a conditioned place preference paradigm. Establishing place preference with these conditions may provide data that can be used to interpret the rewarding properties of these drugs. Therefore the aims of the present study were to establish nicotine place preference, establish norharmane place preference, establish place preference following

administration of both norharmane and nicotine, and to observe drug effects on locomotor activity. By achieving these goals, the data may support the theory that monoamine oxidase inhibitors play a role in tobacco addiction. If so, tobacco cessation therapies may be altered in order to better suit tobacco withdrawal by other means than simply nicotine replacement. The results of this study may also help scientists better understand how electronic nicotine delivery device smokers may be at a higher risk of becoming tobacco smokers in the future due to their pre-exposure to nicotine, but not norharmane.

The first aim of the present study was to establish place preference in animals exposed to nicotine. I hypothesized that using specific acclimation procedures and two doses of nicotine, place preference would be established in animals administered the high dose of nicotine (see Table 1). If place preference were established, the specific acclimation procedures may be used as a model for future studies taking into consideration the variability of nicotine conditioned place preference. I also hypothesized that animals administered the high dose of nicotine would have increased locomotor activity in comparison to the low dose group and vehicle group.

The second aim of the present study was to establish place preference in animals exposed to norharmane. There are currently no studies that have investigated norharmane using a conditioned place preference paradigm. I hypothesized that place preference would be observed in animals exposed to two doses of norharmane (see Table 2), which would add data to the literature suggesting that norharmane has rewarding properties. I also hypothesized that both doses of norharmane would show an increase in locomotor activity.

The final aim of the present study was to determine whether or not the pre-treatment of norharmane prior to nicotine administration would affect the conditioned place preference in animals. I hypothesized that a pre-treatment of norharmane and nicotine would potentiate place preference such that there would be an increase in the amount of time spent in the drug-paired chamber compared to animals who received just nicotine, just norharmane, or vehicle. I also hypothesized that the norharmane + nicotine group would have increased locomotor activity in comparison to all other conditions.

If the hypotheses were proven true, then the results of this experiment may suggest that MAO inhibitors such as norharmane that are found in tobacco strongly increase the rewarding properties of nicotine, thus strengthening an addiction to tobacco. This would further suggest that smokers of electronic nicotine delivery devices who are already addicted to nicotine may only need to smoke one or a few tobacco cigarettes, exposing themselves to the stronger reinforcing effects of norharmane, to quickly create a new and potentially stronger addiction to tobacco, rather than to just nicotine alone. Additionally, it may also suggest that the first time an e-cigarette smoker smokes a tobacco cigarette, the individual may not experience the commonly reported aversive effects that many first time tobacco users experience, because of the pre-exposure to nicotine. These results may cumulatively suggest that there will be an increase in tobacco smokers in the future due to e-cigarette smokers experimenting with tobacco products. Finally, investigating the interaction between MAOI's and nicotine may help us formulate alternative tobacco cessation programs in the future.

Methods

Animals

A total of 215 adult mice (108 male, 107 female; C57BL/6 strain) bred at Northern Michigan University were used. All animals were socially housed (unless separated due to cage mate aggression and housed individually) in standard plastic home cages in a temperature and humidity controlled vivarium with a 12 hour light/dark cycle. All study training and testing was conducted during the dark cycle because this is the active cycle for mice. Animals were provided at least one environmental enrichment device, food, and water *ad libitum* when in their home cage. All animals were adult mice, age 8 weeks or older, for the present study. All testing was conducted with approval from the Northern Michigan University IACUC Committee and in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).

Drugs

(-)-Nicotine was purchased from Sigma Aldrich (St. Louis, MO). Norharmane was purchased from BOC Sciences (Shirley, NY). (-)-Nicotine was dissolved in physiological saline (0.9% NaCl in water) vehicle to a concentration of 0.15 mg/kg and 0.3 mg/kg. Nicotine and vehicle were delivered to animals subcutaneously at a dose of 10 ml/kg 10 minutes prior to entering the CPP apparatus. These concentrations were chosen because a previous study by Grabus *et* al (2006) using C57BL/6J inbred strain mice found that there was variability in the ability of various nicotine dose levels to produce a conditioned place preference. Grabus *et al* (2006) found that an intermediate dose of 0.3 mg/kg showed a significant place preference, whereas there was no significant place

preference in lower doses (0.05 or 0.1 mg/kg), and at higher doses (0.5 or 0.7 mg/kg) the conditioned place preference disappeared. Other studies using various dose levels of nicotine found 0.5 mg/kg to have the most significant effect showing conditioned place preference, however, these studies did not use the same strain proposed for the present study (Berrendero, Kieffer, & Maldonado, 2002; Risinger & Oakes, 1995). Thus, the proposed range of doses were used in an attempt to show place preference using the C57 inbred strain of mice.

Norharmane hydrochloride was dissolved in saline vehicle to a concentration of 5.0 or 10.0 mg/kg. Norharmane was administered subcutaneously at a dose level of 10 ml/kg of body weight 20 minutes prior to entering the CPP apparatus. The concentration and dose volume were chosen based off of previous study by (2006) who found a dose dependent effect (2.5, 5.0, and 10.0 mg/kg) of norharmane on the immobility time of Swiss-Webster mice in a forced swim test.

Equipment

All place preference conditioning and testing was conducted in a customized three chamber (18.4 cm x 22.9 cm x 16.5 cm, per chamber) place preference apparatus with removable middle inserts to allow free movement between chambers. The left chamber had a barred floor (made from ¼" thick steel rods), black and white horizontally striped walls, and pine bedding (Kaytee, Chilton, WI). The right side chamber had a wire mesh floor, black and white circled walls, and corncob bedding (Kay Kob, Marquette, MI). All data collected while using the place preference apparatus was collected using Noldus Ethovision XT version 7.1.420 software program on a desktop computer running Windows XP operating system.

Place Conditioning Procedures

Several of the conditioned place preference handling, conditioning, and testing procedures were adapted from Grabus *et al* (2006).

Acclimation.

In an effort to eliminate the possibility of any confounds and to decrease the stress levels of the animals, specific procedures were followed to ensure acclimation to testing environments, handling, and dose administration. Some of the methods for acclimation have been adapted from Grabus *et al* (2006) due to their ability to show nicotine place preference in mice after performing specific acclimation procedures versus mice who were not acclimated or habituated prior to conditioning procedures.

Once animals reached the age requirement, experimental procedures began. Handling acclimation was conducted for three days, consisting of handling the animals for 1-2 minutes. As described by Grabus *et al* (2006), each mouse was grasped and stroked along the dorsal neck and thorax until they appeared to show less stressful behavior, such as minimal struggling. Afterwards, each animal was returned to its home cage.

For the next four consecutive days, all animals were placed in the testing room while remaining in their home cages for approximately two hours. The testing room remained free of heavy foot traffic, loud noises, and was dimly lit to simulate the dark cycle of the vivarium. Once the animals had the opportunity to acclimate to this environment, each animal was handled for approximately 1-2 minutes as before. Animals were then returned to their home cage and taken back to the vivarium.

After the four days of acclimation to the testing room and handling, animals were acclimated to the restraint hold used during dose administration for 3 consecutive days. Animals were taken to the testing room and given approximately two hours to acclimate. Animals were then placed in a subcutaneous injection restraint hold and pinched in the dorsal scapular area to mimic a subcutaneous injection, and then were returned to their home cage. On the following day, animals received a subcutaneous injection of 0.1 ml of saline to allow a pre-exposure to the injection process prior to beginning the conditioning sessions. After the injection, animals were returned to their home cage and then transported to the vivarium.

Pre-Testing.

A biased place conditioning design was used in the present study. This required that all animals be pre-tested in order to determine a side preference for each individual animal. The drug paired chamber was the least preferred side from the pre-testing trials, in order to attempt to diminish any bias prior to conditioning trials (Le Foll & Goldberg, 2005). There were two days of 15 minute pre-testing trials. All pre-testing procedures were conducted after the animals were placed in the testing room and given a minimum of two hours to acclimate. All middle inserts were removed from the CPP apparatus, and each animal was placed in the middle chamber and given free access to entire apparatus. For each day, seconds spent on either side was totaled and was used to determine the least preferred side for that particular animal, which became the side paired with drug treatment administration during the conditioning trials. In an analysis of several studies using the conditioned place preference paradigm to observe nicotinic effects, it was found that the majority used a biased procedure and successfully observed a conditioned place

preference to the side of the apparatus paired with nicotine administration. Fewer studies reported a conditioned place preference for nicotine when using an unbiased procedure (Le Foll & Goldberg, 2005).

Place conditioning.

After completing the pre-testing trials, all animals underwent six trials of place conditioning. Handling and dosing of animals was performed identically to the acclimation procedures described above.

Place conditioning occurred over the course of three days with two trials per day so that each animal received a total of three drug and three vehicle conditioning trials. The exception was the vehicle group, which received vehicle at every conditioning trial. Each trial lasted 30 minutes, with approximately three hours between trials. For the first trial, each animal was dosed with vehicle and then placed into the individual animal's preferred side of the apparatus where it was confined. Three hours after the initial trial, each animal was dosed with the drug treatment and placed into the individual animal's non-preferred side of the apparatus, where it remained confined (Grabus *et al*, 2006). On the subsequent days of conditioning, all procedures were conducted in the same manner as on the first day, except the order of dose administration was alternated from the previous day (such as: Vehicle-Treatment, Treatment-Vehicle, Vehicle-Treatment). This dosing schedule was meant to ensure that neither treatment condition was administered at the same time each day (Grabus *et al*, 2006).

Place preference testing.

On the testing day, handling and dosing of animals was performed identically to acclimation procedures listed above. After approximately two hours of acclimation, the

animal were placed on either side of the apparatus with the middle inserts removed. Each animal was placed in the neutral, middle chamber. The post-testing trial ran for 15minutes. Seconds spent on either side was totaled and locomotor activity was recorded.

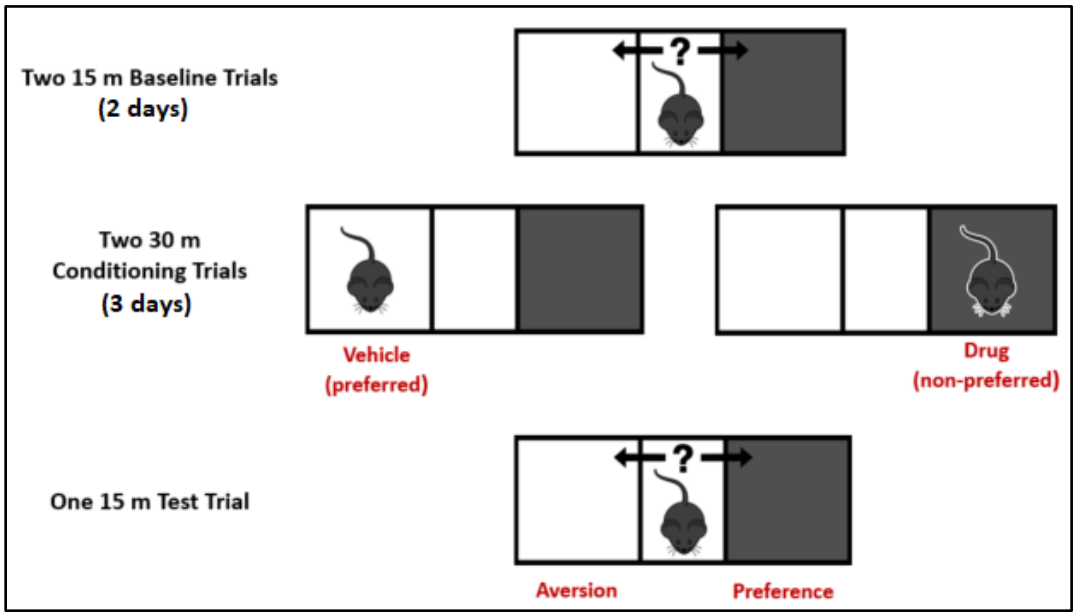

Figure 1. A graphical depiction of each of the three phases of the conditioned place preference design used for the present study.

Nicotine conditioned place preference.

All animals were randomly assigned to either treatment group as shown in Table 1. All groups were counterbalanced based on baseline pre-test chamber preference. All place conditioning procedures previously described were followed. On days of conditioning, animals received their assigned nicotine or vehicle dose 10 minutes prior to placement in the CPP apparatus.

Table 1: Vehicle and nicotine experimental groups. All animals were randomly assigned to one of three conditions (N=12; VEH=Vehicle, NIC-0.15=0.15 mg/kg nicotine, NIC-0.3=0.3 mg/kg nicotine).

Sex	VEH	NIC-0.15	NIC-0.3
Male	12	12	12
Female	12	12	12

Norharmane conditioned place preference.

All animals were randomly assigned to either group as shown in Table 2. All groups were counterbalanced based on baseline pre-test chamber preference. All place conditioning procedures previously described were followed. On days of conditioning, animals will receive their assigned norharmane or vehicle dose 20 minutes prior to placement in the CPP apparatus.

Table 2: Norharmane experimental groups. All animals were randomly assigned to one of two conditions (N=12; NOR-2.5=2.5 mg/kg norharmane, NOR-5.0=5.0 mg/kg norharmane). A vehicle group was not tested during the norharmane conditioned place preference because a vehicle group was tested during the nicotine conditioned place preference procedures.

Sex	NOR-2.5	NOR-5.0
Male	12	12
Female	12	12

Norharmane + nicotine conditioned place preference.

A Latin square was used to assign groups for the combination dose studies. All animals were randomly assigned to either group as shown in Table 3. All groups were counterbalanced based on baseline pre-test chamber preference. All place conditioning procedures previously described were followed. On days of conditioning, the first vehicle or drug dose of each group was administered 20 minutes prior to the second vehicle or drug dose. The second vehicle or drug dose was administered 10 minutes prior to placement into the CPP apparatus.

Table 3: Combination experimental groups. All animals were randomly assigned to one of four conditions (N=12; VEH-VEH=Vehicle, VEH-NIC=Vehicle + 0.3 mg/kg nicotine, NOR-VEH=5.0 mg/kg norharmane + vehicle [N=11], NOR-NIC=5.0 mg/kg norharmane + 0.3 mg/kg nicotine).

Sex	VEH-VEH	VEH-NIC-0.30	NOR-10.0-VEH	NOR-10.0-NIC-0.30
Male	12	12	12	12
Female	12	12	11	12

Data analysis.

The dependent variables for the present study consisted of time (seconds) spent in the non-preferred and preferred chamber, the percentage of time spent in the non-preferred chamber during the pre-test trials and during the place preference test trial, and for locomotor activity the mean path length (centimeters) traveled was measured in the non-preferred chamber and the preferred chamber. Data for all variables were reported as means (+/- standard error of the mean [SEM]). A paired samples t test was conducted for all variables in the vehicle, 0.15 mg/kg nicotine, 0.30 mg/kg nicotine, 5.0 mg/kg

norharmane, and 10.0 mg/kg norharmane groups to compare preference for the non-preferred versus the preferred chamber, the percent preference for the non-preferred chamber during pre-test baseline trials versus the place preference test trial, and locomotor activity in the non-preferred chamber versus the preferred chamber during conditioning trials. The same variables were analyzed for the vehicle-vehicle, vehicle-nicotine, norharmane-nicotine, and norharmane-nicotine groups, although their comparisons were made used a two factor between groups ANOVA (norharmane X nicotine) in order to examine main effects of norharmane and nicotine, and interaction effects between norharmane and nicotine. A Bonferroni post hoc test was used to examine group differences for statistically significant interaction effects. All analyses were conducted using GraphPad Prism 7, version 7.04 for Windows (GraphPad Prism, La Jolla, CA).

Results

During the baseline pre-test trials, the chamber where each animal spent the greatest duration of time was identified as the preferred chamber. In all groups the non-preferred chamber became the drug paired chamber, and the preferred chamber became the vehicle paired chamber. Because there was no drug used during the conditioning trials in the vehicle only group, the non-preferred and preferred chambers remained labeled as such. Also, some data points were not calculated in some groups due to technical difficulties. The true sample sizes used for statistical analyses are noted in the figure captions.

Vehicle Group

As noted in the methods, a conditioned place preference test was examined using vehicle paired for both chambers. The time spent (seconds) in the non-preferred chamber versus the preferred chamber during the place preference test trials is shown in Figures 2A (males) and Figure 2B (females). Neither the males (non-preferred, M = 187.2, +/- SEM = 33.86; preferred, M = 324.2, +/- SEM = 53.81; $t(5) = 1.77$, $p > 0.05$), nor females (non-preferred, M = 305.2, +/- SEM = 36.44; preferred, M = 258.5, +/- SEM = 47.27; $t(5) = 0.82$, $p > 0.05$), showed a significant preference for either side.

The percentage of time spent in the non-preferred chamber during the baseline pre-test trials versus the percentage of time spent in the non-preferred chamber during the place preference test trial (i.e., after the conditioning sessions) is shown in Figures 2C (males) and 2D (females). It was found that neither the males (baseline, M = 27.5, +/- SEM = 6.54; test trial, M = 37.48, +/- SEM = 6.16; $t(5) = 1.38$, $p > 0.05$) nor the females

(baseline, M = 42.01, +/- SEM = 4.88; test trial, M = 55.49, +/- SEM = 6.34; t (5) = 1.58, $p > 0.05$) showed a shift in preference for the non-preferred chamber between the baseline session and the test session.

Figure 2. Vehicle Group

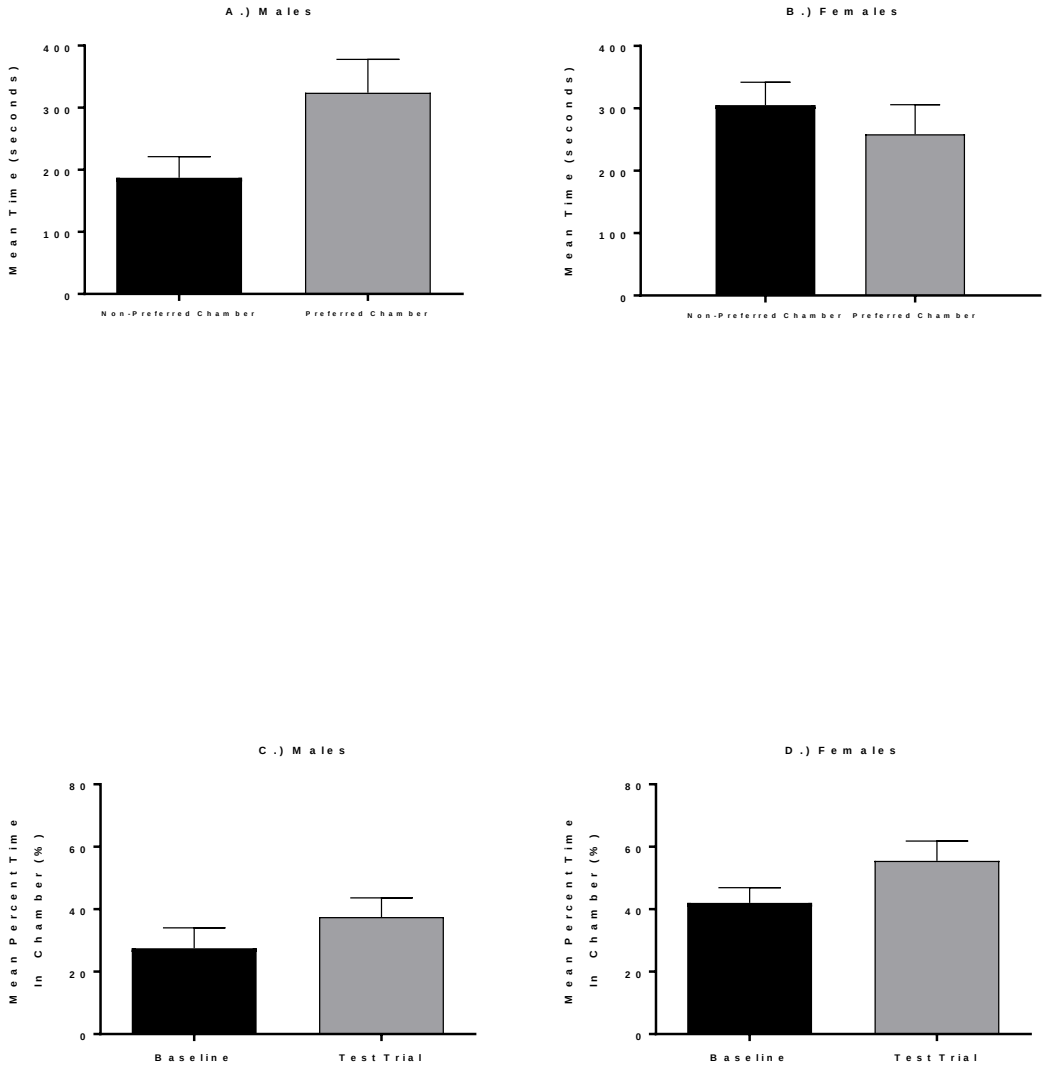

Figure 2. Vehicle group results. A., males mean time spent in the non-preferred and preferred chambers during the place preference test trial; B., females mean time

(seconds) spent in the non-preferred and preferred chambers during the place preference test trial; C., males percent of time spent in the non-preferred chamber during the baseline pre-test trials and the place preference test trial; D., females percent of time spent in the non-preferred chamber during the baseline pre-test trials and the place preference test trial. Bars represent means, +/- SEM, N=6.

Nicotine 0.15 mg/kg Group

The time spent (seconds) in the nicotine paired chamber versus the vehicle paired chamber during the place preference test trials is shown in Figure 3A (males) and Figure 3B (females). The males spent significantly less time in the nicotine paired chamber than the vehicle paired chamber (nicotine, M = 266.8, +/- SEM = 14.43; vehicle, M = 363.4, +/- SEM = 21.85; $t(11) = 2.99$, $p < 0.05$). The females, however, did not spend a significantly different amount of time in either chamber (nicotine, M = 307.7, +/- SEM = 25.43; vehicle, M = 236.8, +/- SEM = 23.89; $t(8) = 1.84$, $p > 0.05$).

The percentage of time spent in the non-preferred chamber during the baseline pre-test trials versus the percentage of time spent in the nicotine paired chamber during the place preference test trial is shown in Figures 3C (males) and 3D (females). The males showed a significant increase in the percent of time spent in the nicotine-paired chamber during the test trial compared to the baseline (baseline, M = 34.36, +/- SEM = 3.093; test trial, M = 46.22, +/- SEM = 1.81; $t(8) = 3.29$, $p < 0.05$). The females, however, did not show a significant change in the percent of time spent in the nicotine paired chamber during the test trial compared to baseline (baseline, M = 38.99, +/- SEM = 5.93; test trial, M = 43.64, +/- SEM = 3.7; $t(8) = 0.64$, $p > 0.05$).

Figure 3. Nicotine 0.15 mg/kg Group

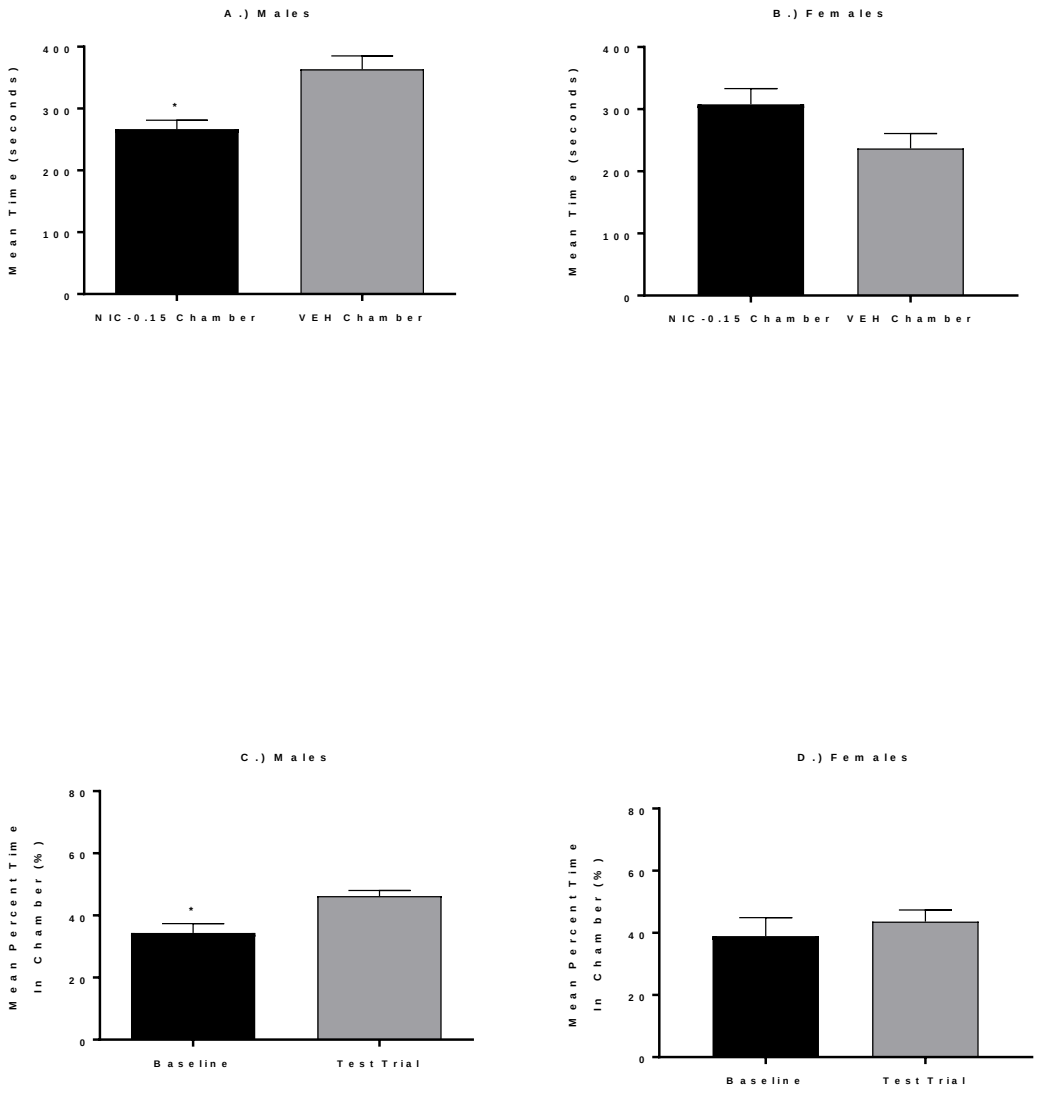

Figure 3. Nicotine 0.15 mg/kg group results. A., males mean time spent in the nicotine and vehicle paired chambers during the place preference test trial (n = 12); B., females mean time (seconds) spent in the nicotine and vehicle paired chambers during the place preference test trial (n = 12); C., males mean percent of time spent in the nicotine paired chamber during the baseline pre-test trials and the place preference test trial (n =

9); D., females mean percent of time spent in the nicotine paired chamber during the baseline pre-test trials and the place preference test trial (n = 9). Bars represent means, +/- SEM. (* denotes $p < 0.05$).

Nicotine 0.30 mg/kg Group

The time spent (seconds) in the nicotine paired chamber versus the vehicle paired chamber during the place preference test trials is shown in Figures 4A (males) and Figure 4B (females). Neither the males (nicotine, M = 246.8, +/- SEM = 24.78; vehicle, M = 315.9, +/- SEM = 34.51; $t(11) = 1.37$, $p > 0.05$) nor the females (nicotine, M = 276.5, +/- SEM = 24.21; vehicle, M = 292.5, +/- SEM = 20.52; $t(10) = 0.41$, $p > 0.05$) showed a significant preference for either chamber.

The percentage of time spent in the non-preferred chamber during the baseline pre-test trials versus the percentage of time spent in the nicotine paired chamber during the place preference test trial is shown in Figures 4C (males) and 4D (females). Neither the males (baseline, M = 40.57, +/- SEM = 3.77; test trial, M = 44.43, +/- SEM = 4.76; $t(9) = 0.51$, $p > 0.05$) nor the females (baseline, M = 37.25, +/- SEM = 5.06; test trial, M = 48.18, +/- SEM = 4.33; $t(8) = 2.16$, $p > 0.05$) showed a significant difference in percentage of time spent in the nicotine paired chamber during the place preference test trial versus the baseline.

Figure 4. Nicotine 0.30 mg/kg Group

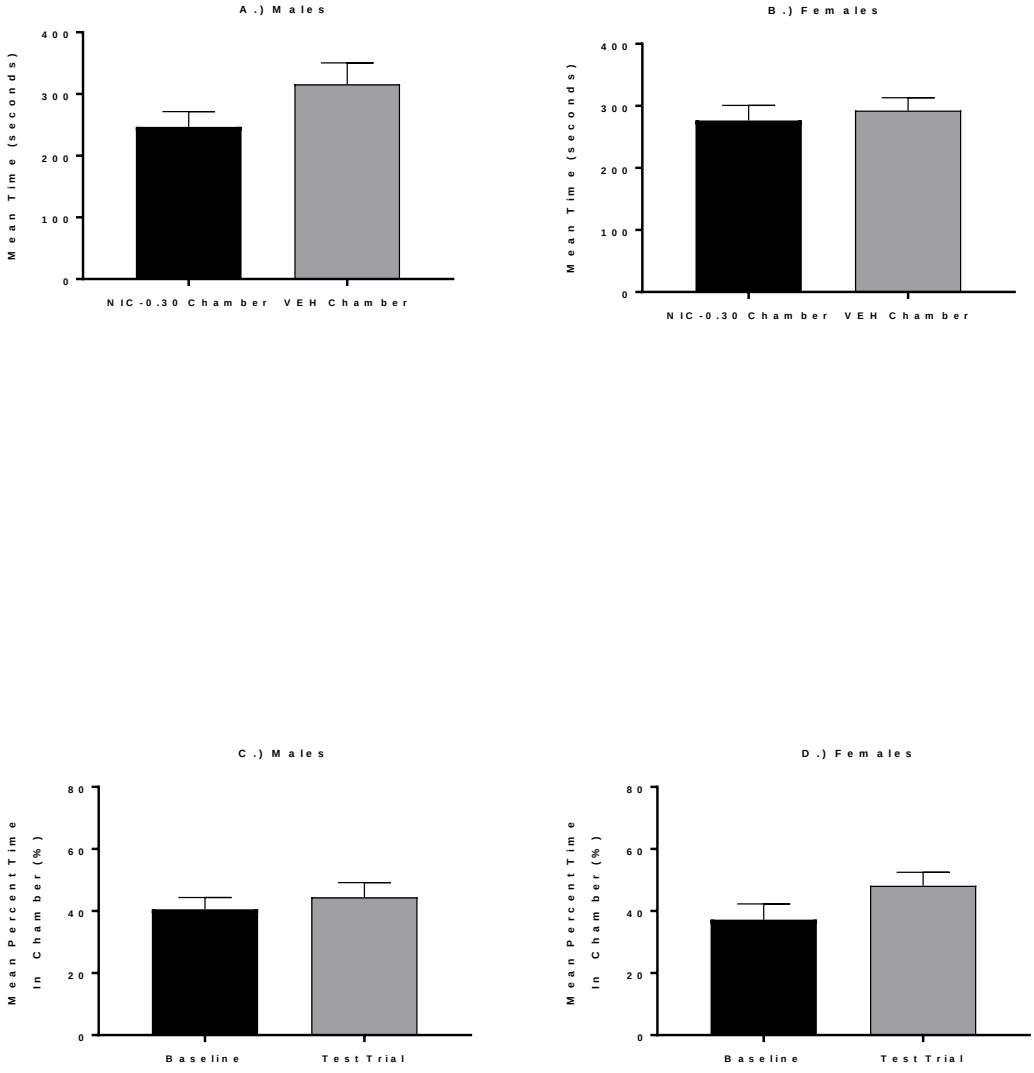

Figure 4. Nicotine 0.30 mg/kg results. A., males mean time spent in the nicotine and vehicle paired chambers during the place preference test trial (n = 12); B., females mean time (seconds) spent in the nicotine and vehicle paired chambers during the place preference test trial (n = 11); C., males mean percent of time spent in the nicotine paired chamber during the baseline pre-test trials and the place preference test trial (n = 10); D.,

females mean percent of time spent in the nicotine paired chamber during the baseline pre-test trials and the place preference test trial (n = 9). Bars represent means, +/- SEM.

Norharmane 5.0 mg/kg Group

The time spent (seconds) in the norharmane paired chamber versus the vehicle paired chamber during the place preference test trials is shown in Figures 5A (males) and Figure 5B (females). Neither the males (norharmane, M = 229.1, +/- SEM = 26.01; vehicle, M = 308.4, +/- SEM = 33.41; t (11) = 1.43, p > 0.05) nor the females (norharmane M = 244.5, +/- SEM = 30.32; vehicle, M = 292.9, +/- SEM = 27.36; t (11) = 0.92, p > 0.05) showed a significant preference for either chamber.

The percentage of time spent in the non-preferred chamber during the baseline pre-test trials versus the percentage of time spent in the norharmane paired chamber during the place preference test trial is shown in Figures 5C (males) and 5D (females). Neither the males (baseline, M = 42.03, +/- SEM = 4.11; test trial, M = 43.27, +/- SEM = 4.68; t (11) = 0.21, p > 0.05) nor the females (baseline, M = 37.41, +/- SEM = 3.34; test trial, M = 45.02, +/- SEM = 4.91; t (11) = 1.80, p > 0.05) showed a significant increase in percentage of time spent in the nicotine paired chamber during the place preference test trial versus the baseline.

Figure 5. Norharmane 5.0 mg/kg Group

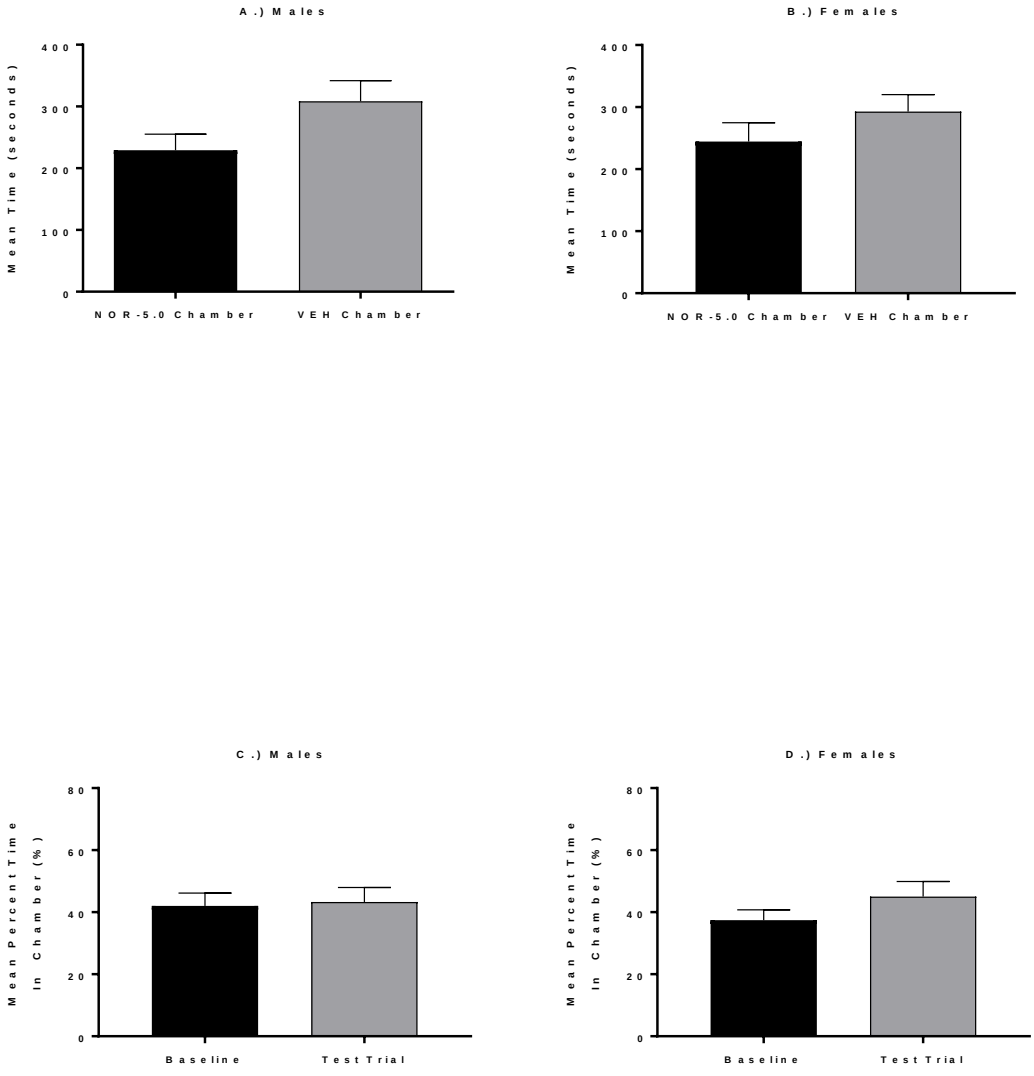

Figure 5. Norharmane 5.0 mg/kg group results. A., males mean time spent in the norharmane and vehicle paired chambers during the place preference test trial; B., females mean time (seconds) spent in the norharmane and vehicle paired chambers during the place preference test trial; C., males mean percent of time spent in the norharmane paired chamber during the baseline pre-test trials and the place preference

test trial; D., females mean percent of time spent in the norharmane paired chamber during the baseline pre-test trials and the place preference test trial. Bars represent means, +/- SEM; N=12 per bar.

Norharmane 10.0 mg/kg Group

The time spent (seconds) in the norharmane paired chamber versus the vehicle paired chamber during the place preference test trials is shown in Figures 6A (males) and Figure 6B (females). Neither the males (norharmane, M = 231.6, +/- SEM = 23.43; vehicle, M = 275.4, +/- SEM = 24.78; t (11) = 1.04, p > 0.05) nor the females (norharmane, M = 232.0, +/- SEM = 21.05; vehicle, M = 313.3, +/- SEM = 27.74; t (11) = 2.04, p > 0.05) showed a significant preference for either chamber.

The percentage of time spent in the non-preferred chamber during the baseline pre-test trials versus the percentage of time spent in the norharmane paired chamber during the place preference test trial is shown in Figures 6C (males) and 6D (females). Neither the males (baseline, M = 35.97, +/- SEM = 3.97; test trial, M = 45.6, +/- SEM = 4.07; t (11) = 1.92, p > 0.05) nor the females (baseline, M = 53.11, +/- SEM = 3.76; test trial, M = 42.95, +/- SEM = 3.41; t (11) = 1.98, p > 0.05) showed a significant difference in percentage of time spent in the nicotine paired chamber during the place preference test trial versus the baseline.

Figure 6. Norharmane 10.0 mg/kg Group

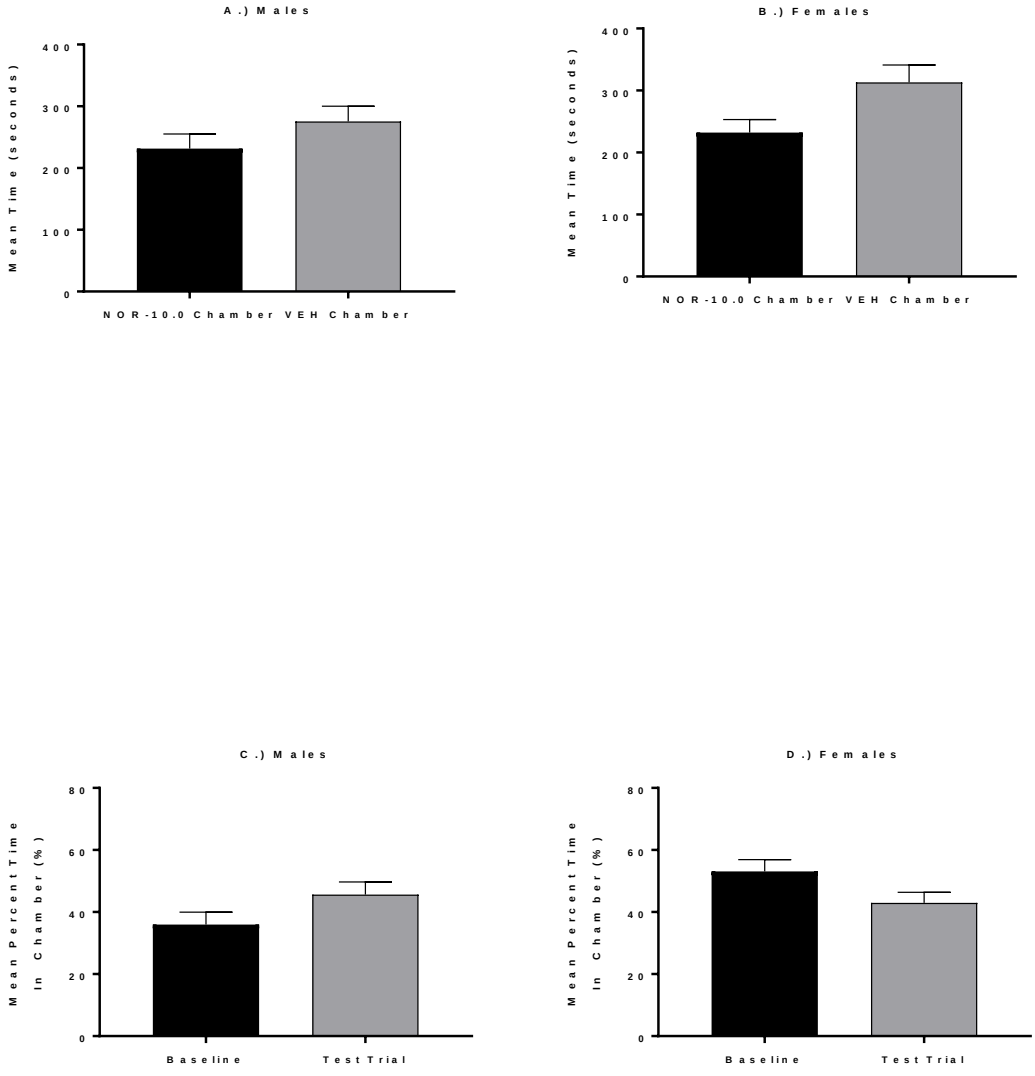

Figure 6. Norharmane 10.0 mg/kg group results. A., males mean time spent in the norharmane and vehicle paired chambers during the place preference test trial; B., females mean time (seconds) spent in the norharmane and vehicle paired chambers during the place preference test trial; C., males mean percent of time spent in the norharmane paired chamber during the baseline pre-test trials and the place preference test trial; D., females mean percent of time spent in the norharmane paired chamber

during the baseline pre-test trials and the place preference test trial. Bars represent means, +/- SEM; N=12 per bar.

Vehicle-Vehicle, Vehicle-Nicotine, Norharmane-Vehicle, & Norharmane-Nicotine Groups

A two-way between groups ANOVA (norharmane X nicotine) was used to examine statistically significant differences in the amount of time (seconds) spent in the drug paired chamber versus the vehicle paired chamber during the place preference test trial across the following groups: vehicle-vehicle, norharmane 10.0 mg/kg-vehicle, vehicle-nicotine 0.30 mg/kg, and norharmane 10.0 mg/kg-nicotine 0.3 mg/kg. Results are shown in Figures 7A (males) and 7B (females). Statistically significant differences were not found between the treatment conditions for the males (main effect nicotine, $F(1, 44) = 0.38$, $p > 0.05$; main effect norharmane, $F(1, 44) = 0.44$, $p > 0.05$; interaction effect, $F(1, 44) = 0.12$, $p > 0.05$), nor the females (main effect nicotine, $F(1, 43) = 1.71$, $p > 0.05$; main effect norharmane, $F(1, 43) = 0.03$, $p > 0.05$; interaction effect, $F(1, 43) = 3.23$, $p > 0.05$).

A two-way between groups ANOVA was also used to examine statistically significant differences between the percent of time spent in the drug paired chamber during the place preference test trial versus the baseline pre-test trials. Results are shown in Figures 7C (males) and 7D (females). Statistically significant differences were not found between the treatment conditions for the males (main effect of nicotine, $F(1, 44) = 0.35$, $p > 0.05$; main effect of norharmane, $F(1, 44) = 0.07$, $p > 0.05$; interaction effect, $F(1, 44) = 0.04$, $p > 0.05$). There were also no statistically significant main effects found across norharmane or nicotine for the females (main effect of nicotine, $F(1, 43) = 0.00$, p

> 0.05; main effect of norharmane, $F(1, 43) = 0.03$, $p > 0.05$), however, there was a statistically significant interaction effect between norharmane and nicotine (interaction effect, $F(1, 43) = 4.47$, $p < 0.05$). A Bonferroni post hoc analysis was run for pairwise statistical analysis but did not yield any significant findings.

Next, a two-way between groups ANOVA was also used to examine statistically significant differences in the mean path length traveled in the drug paired chamber during the three drug conditioning trials across the following groups: vehicle-vehicle, norharmane 10.0 mg/kg-vehicle, vehicle-nicotine 0.30 mg/kg, and norharmane 10.0 mg/kg-nicotine 0.3 mg/kg. Results are shown in Figures 7E (males) and 7F (females). Statistically significant decrease in mean path length was found in the norharmane-vehicle group in the males (main effect of norharmane, $F(1, 44) = 12.12$, $p < 0.05$) but not in the vehicle-nicotine group (main effect of nicotine, $F(1, 44) = 1.08$, $p > 0.05$). Additionally, there was a statistically significant interaction effect in the noharmane-nicotine group which showed a significant decrease in mean path length (interaction effect, $F(1, 44) = 4.68$, $p < 0.05$). A Bonferroni post hoc analysis was run for pairwise statistical analysis and it was found that the vehicle-vehicle group had a significantly greater mean path length versus the norharmane-nicotine groups. Additionally, the vehicle-nicotine group was found to have a significantly greater mean path length versus the norharmane-nicotine group. For the females, statistically significant differences were found across the norharmane group (main effect of norharmane, $F(1, 43) = 3.83$, $p < 0.05$) but not across the nicotine group (main effect of nicotine, $F(1, 43) = 1.64$, $p > 0.05$). Finally, there was not a statistically significant interaction effect for the females

between the nicotine and norharmane groups (interaction effect, $F(1, 43) = 0.33$, $p > 0.05$).

Anecdotally, although no additional tests or procedures were ran, it was noted that all male and female animals in the norharmane-nicotine group showed considerably decreased body movements unless startled or handled in their homecages following administration of norharmane and nicotine. The effect appeared to take place sometime following nicotine administration within the 10 minutes prior to placement into the norharmane-nicotine paired chamber, during each of the three drug conditioning trials. Following each 30 minute conditioning trial when each animal was removed from the chamber and returned to its home cage, the drug effect appeared to have diminished completely.

Figure 7. Norhamane + Nicotine Groups

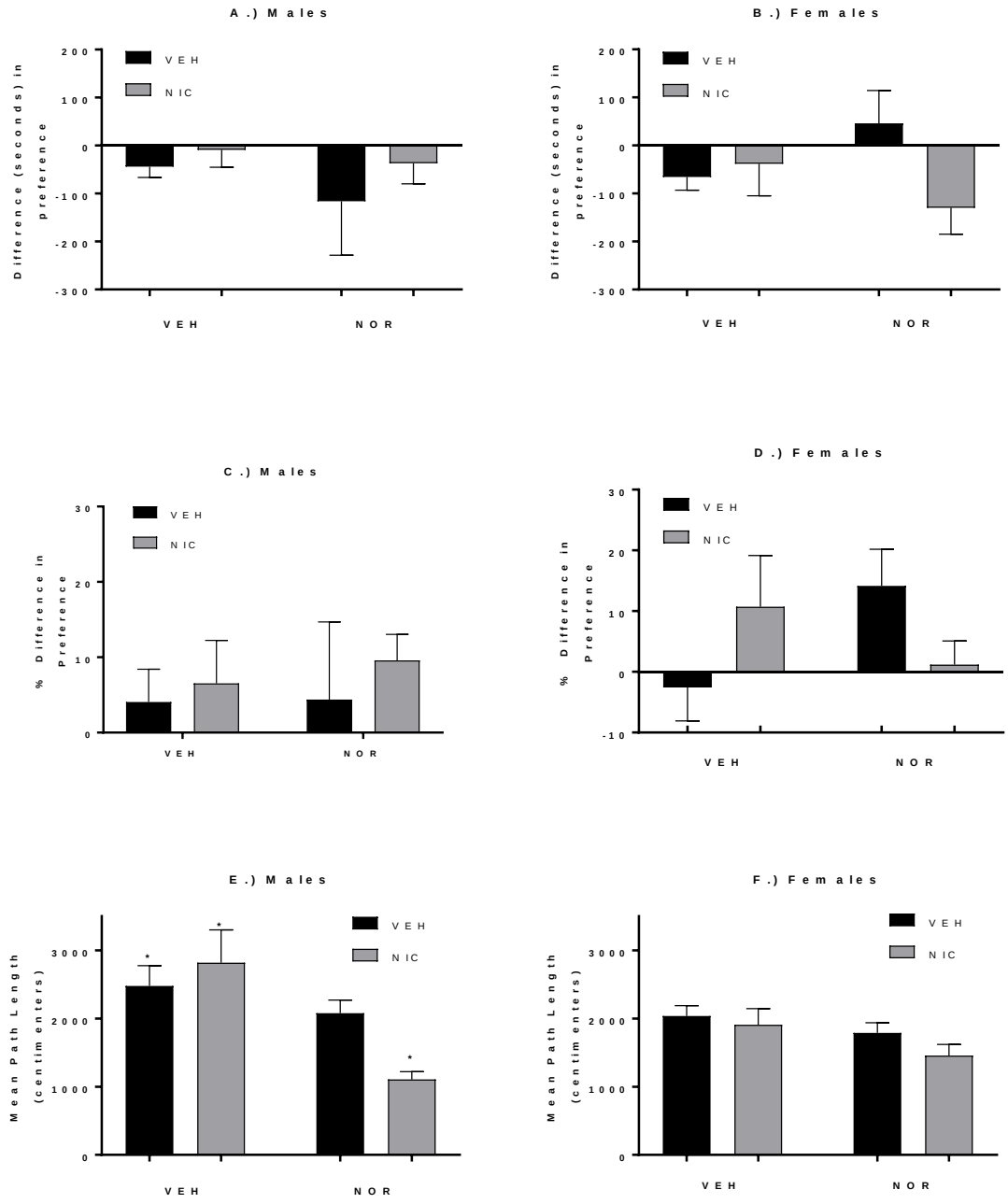

Figure 7. Combination groups results. All graphs show results for the following groups: vehicle-vehicle, vehicle-nicotine, norharmane-vehicle, and norharmane-nicotine.

A., males difference in time spent (seconds) in the drug paired chamber and the vehicle paired chamber (calculated as seconds of time spent in drug paired chamber – seconds of time spent in vehicle chamber); B., females difference in time spent in the drug paired chamber and the vehicle paired chamber; C., males difference in the percent of time spent in the drug paired chamber in the baseline pre-test trials and the place preference test trial (calculated as percent of time in drug paired chamber during test trial – percent of time in drug paired chamber during baseline trials). D., females difference in the percent of time spent in the drug paired chamber in the baseline pre-test trials and the place preference test trial; E., males mean path length traveled (centimeters, calculated from the three drug conditioning trials); F., females mean path length traveled. Bars represent means, +/- SEM; N=12 per bar except females in the norharmane-nicotine group, N=11. (* denotes $p < 0.05$).

Locomotor Activity

The mean path lengths (centimeters) traveled in the non-preferred, or drug paired, chamber versus the preferred, or vehicle paired, chamber during conditioning trials for males is shown in the graphs in Figures 8A (vehicle), 8B (nicotine 0.15 mg/kg), 8C (nicotine 0.30 mg/kg), 8D (norharmane 5.0 mg/kg) and 8E (norharmane 10.0 mg/kg).

The vehicle group did not show a significant difference in mean path length traveled between chambers (non-preferred, M = 2821, +/- SEM = 459.5; preferred, M = 1796, +/- SEM =331.0; $t(5) = 1.71$, $p > 0.05$). The males in the nicotine 0.15 mg/kg group showed a significant decrease in the mean path length traveled in the nicotine paired chamber versus the vehicle paired chamber (nicotine, M = 1534, +/- SEM = 210.5; vehicle, M = 2572, +/- SEM = 305.5; $t(8) = 4.15$, $p < 0.05$). The males in the nicotine

0.30 mg/kg group did not show a significant difference in the mean path lengths traveled in either chamber (nicotine, M = 1700, +/- SEM = 303.5; vehicle, M = 2607, +/- SEM = 359.6; $t(8) = 1.93$, $p > 0.05$). The males in the norharmane 5.0 mg/kg group also did not have a significant difference in the average path lengths traveled in either chamber (norharmane, M = 2513, +/- SEM = 252.8; vehicle, M = 2953, +/- SEM = 123.0; $t(11) = 1.8$, $p > 0.05$). Finally, the males in the norharmane 10.0 mg/kg group showed a significant decrease in the mean path length traveled in the norharmane paired chamber versus the vehicle paired chamber (norharmane, M = 2561, +/- SEM = 233.3; vehicle, M = 3984, +/- SEM = 297.9; $t(11) = 2.95$, $p < 0.05$).

Figure 8. Males Locomotor Activity

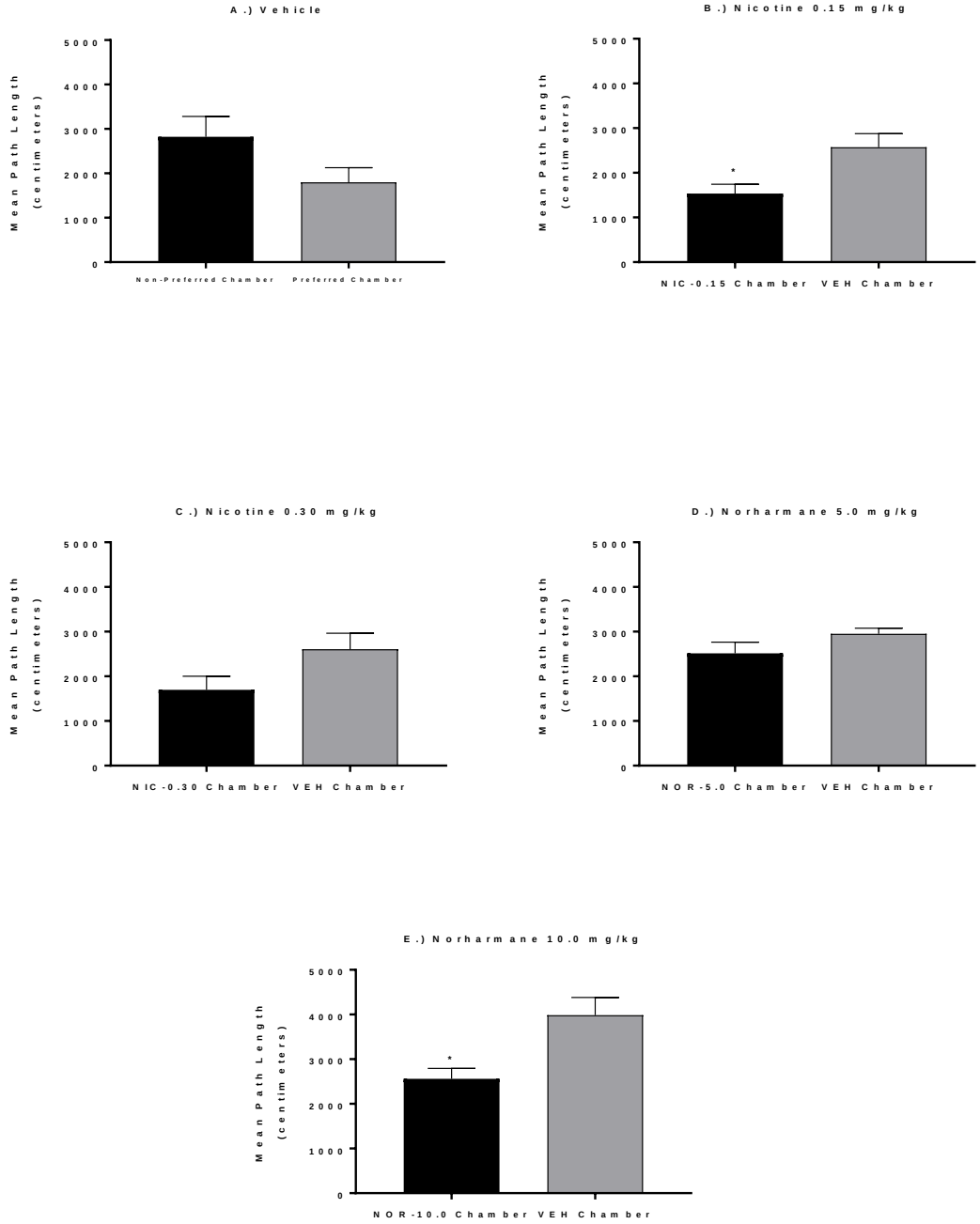

Figure 8. Males locomotor activity. Males mean path length traveled (centimeters) was calculated from three conditioning trials in the non-preferred, or drug paired, chamber and three conditioning trials in the preferred, or vehicle paired, chamber for the

A., vehicle group (N=6); B., nicotine 0.15 mg/kg group (N=9); C., nicotine 0.30 mg/kg group (N=9); D., norharmane 5.0 mg/kg group (N=12); E., norharmane 10.0 mg/kg group (N=12). Bars represent means, +/- SEM. (* denotes $p < 0.05$).

The mean path lengths (centimeters) traveled in the non-preferred, or drug paired, chamber versus the preferred, or vehicle paired, chamber during conditioning trials for females is shown in the graphs in Figures 9A (vehicle), 9B (nicotine 0.15 mg/kg), 9C (nicotine 0.30 mg/kg), 9D (norharmane 5.0 mg/kg) and 9E (norharmane 10.0 mg/kg).

The vehicle group showed a significant decrease in path length in the non-preferred chamber versus the preferred chamber (non-preferred, M = 2348 cm, +/- SEM = 294.0; preferred, M = 1456, +/- SEM = 154.4; $t(5) = 3.20$, $p < 0.05$). The females in the nicotine 0.15 mg/kg group showed a significant decrease in the mean path length traveled in the nicotine paired chamber versus the vehicle paired chamber (nicotine, M = 1712, +/- SEM = 160.3; vehicle, M = 2642, +/- SEM = 250.8; $t(8) = 3.21$, $p < 0.05$). The females in the nicotine 0.30 mg/kg group showed a significant decrease in the mean path length traveled in the nicotine paired chamber versus the vehicle paired chamber (nicotine, M = 1742, +/- SEM = 157.5; vehicle, M = 2317, +/- SEM = 183.8; $t(9) = 2.87$, $p < 0.05$). The females in the norharmane 5.0 mg/kg group showed a significant decrease in the mean path lengths traveled in the norharmane chamber versus the vehicle paired chamber (norharmane, M = 2456, +/- SEM = 165.1; vehicle, M = 3331, +/- SEM = 272.0; $t(11) = 3.35$, $p < 0.05$). Finally, the females in the norharmane 10.0 mg/kg group showed a significant decrease in the mean path lengths traveled in the norharmane paired chamber versus the vehicle paired chamber (norharmane, M = 3146, +/- SEM = 233.3; vehicle trial, M = 2051, +/- SEM = 140.7; $t(11) = 3.79$, $p < 0.05$).

Figure 9. Females Locomotor Activity

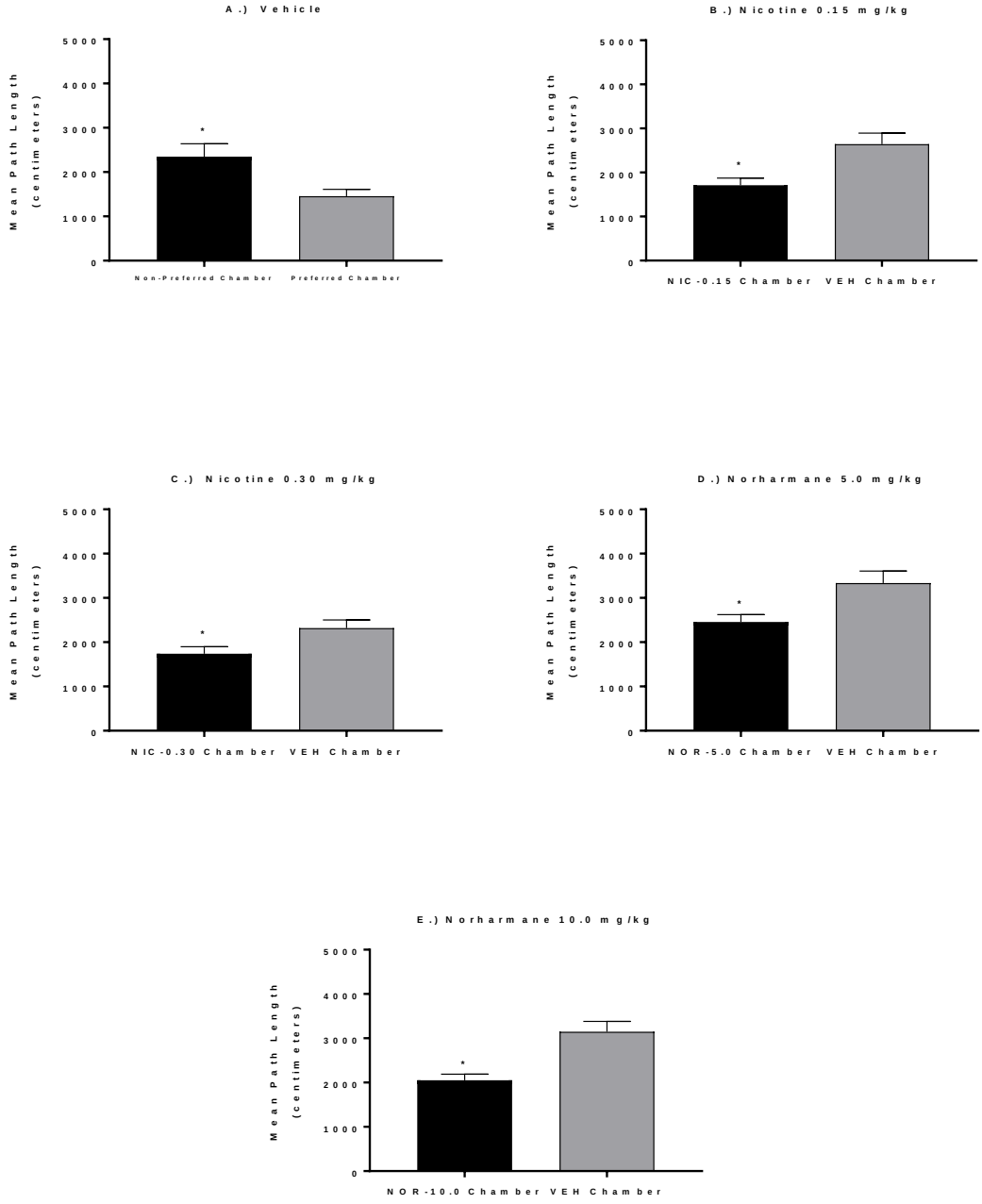

Figure 9. Females locomotor activity. Females mean path length traveled (centimeters) was calculated from three conditioning trials in the non-preferred, or drug paired, chamber and three conditioning trials in the preferred, or vehicle paired, chamber

for the A., vehicle group (N=6); B., nicotine 0.15 mg/kg group (N=9); C., nicotine 0.30 mg/kg group (N=10); D., norharmane 5.0 mg/kg (N=12); E., norharmane 10.0 mg/kg group (N=12). Bars represent means, +/- SEM; N=6 per bar.

Discussion

The aim of the present study was to determine if place preference may be established following a combination of nicotine with a monoamine oxidase inhibitor naturally found in tobacco, norharmane. Place preference may be defined as the pairing of formerly neutral stimuli within a particular environment that, over time, becomes associated with a particular drug or the absence of drug.(Thomas Tzschentke, 1998). Throughout this discussion, place preference will refer to this definition and was measured as an increase in time spent in a particular chamber of the conditioned place preference apparatus. In contrast, place aversion will refer to a decrease in time spent in a particular chamber.

A significant place preference for the drug paired chamber was not found in any experimental groups. Conditioned place aversion for the drug paired chamber was found in the 0.15 mg/kg nicotine group, and the females in the vehicle-vehicle group and norharmane-nicotine group. A significant increase in time spent in the drug paired chamber in the place preference test trial versus the baseline pre-test trials was only observed in the male 0.15 mg/kg nicotine group and norharmane-nicotine group, and only in the females norharmane-vehicle group. Finally, a statistically significant decrease in the mean path length traveled in the drug paired chamber during the three drug conditioning trials was found in every female group, and also in the males 0.15 mg/kg nicotine and norharmane-nicotine groups. The males in the vehicle only group showed an increase in the mean path length traveled in the non-preferred chamber.

Nicotine

While neither a place preference nor aversion was shown for a 0.30 mg/kg dose of nicotine, a conditioned place aversion was found in male, but not female, mice treated with 0.15 mg/kg nicotine. Thus, neither experimental condition demonstrated a conditioned place preference. Locomotor activity was decreased following 0.15 mg/kg nicotine administration in both males and females, whereas the 0.3 mg/kg dose reduced locomotor activity in only the male mice.

As noted earlier, studies report inconsistent findings on the ability to establish a conditioned place preference for nicotine, and some studies report a conditioned place aversion with nicotine. Grabus *et al* (2006) and Le Foll and Goldberg (2005) have acknowledged that many different variables may have an effect on the ability for the mouse to establish place preference following conditioning to nicotine, including but not limited to species, strain, sex, dose level, the level of habituation to handling, route of administration, utilizing a biased versus unbiased procedure, the number of and length of conditioning trials, and the type of apparatus. Unfortunately, the results from nicotine conditioned place preference studies vary widely and there are many discrepancies, therefore a standard procedure has not been established (Tzschentke, 1998).

In an effort to increase the likelihood of establishing a place preference for nicotine, the present studied closely followed the procedures described by Grabus *et al* (2006). These authors suggested that more thorough acclimation procedures may decrease the physiologic stress response in animals and therefore decrease any potential stress-mediated influence on a nicotine conditioned place preference. Yet, differences between the present study and the study described by Grabus *et al* (2006) did occur to

some extent. Although the present study used three days of handling acclimation performed in the vivarium, four days of handling acclimation performed in the test room, four days of dose restraint acclimation performed in the test room, and one day of dose administration acclimation via a single mock saline injection performed in the test room, none of the experimental treatment groups showed a place preference for the drug paired chamber. Grabus *et al* (2006) only exposed their animals to handling, restraint, or dose administration procedures for three days the week prior to baseline testing, with two days between the last day of acclimation and the first day of testing. It is possible that the extensive acclimation procedures used in the present study actually had a stress-inducing effect, rather than a stress-reducing effect, that diminished the ability for animals to establish place preference following nicotine administration. Assuming that this may be true, it may also explain the inability for any of the other experimental groups to establish place preference as well.

There are several characteristics of the animal model used in the present study that may have also contributed to the inability for animals to establish place preference following nicotine administration. C57 strain mice were chosen partially due to their use and success in the Grabus *et al* (2006) study. The C57BL/6 strain of mice (breeders purchased from Charles River Laboratories) were bred in house for the present study. However, the strains are still slightly different as the genetic lines were produced and patented in different breeding facilities. Thus, the animals cannot be considered genetically identical. Additionally, the Grabus *et al* (2006) study did not begin any experimental procedures until animals were 10 weeks of age. The animals in the present study ranged from 8 to 12 weeks of age at the beginning of experimental procedures,

including the acclimation procedures. Although mice of 8 weeks of age or older are considered sexually mature adult animals, it is possible that the 8 week old mice versus the 12 week old mice may have had an effect on the ability for animals to establish place preference in any of the experimental groups. In contrast, nicotine has been found to establish place preference in both adolescent and adult rodents (Ahsan *et al*, 2014). Unfortunately there are no data on the effects of age on the ability to establish place preference following norharmane administration. An additional database search was conducted using the keywords: 'norharmane', 'conditioned place preference', 'age', 'rodents'.

As previously mentioned, sex was a variable that was considered when collecting and analyzing data for the present study. A study by Yararbas *et al* (2010) showed that place preference following nicotine administration was unable to be established in female Sprague-Dawley rats, however, a significant effect was observed in male rats. In contrast, Kota *et al* (2008) observed nicotine place preference in both male and female ICR mice, however, males showed conditioning at lower nicotine dose levels versus females. Demaj (2001) stated that there is much complexity in male versus female response to nicotine which may be attributed to several factors including dose, pharmacokinetics, age, species, and strain – some variables which have been discussed in throughout this discussion. For the present study, minor differences between males and females were observed except in locomotor activity. Females in the majority of the experimental groups showed a decrease in locomotor activity in the drug paired chamber. Because no major differences between sexes were observed for the other dependent variables, additional statistical analyses were conducted combining the male and female data for mean time spent in the

drug paired versus vehicle paired chamber and for the mean percent of time spent in the drug paired chamber during the baseline pre-test trials versus the test trial, and may be found in Appendix A.

Several other differences may provide insight to the inability of the present study to observe nicotine place preference, unlike in the Grabus *et al* (2006) study. For example, the Grabus *et al* study used an unbiased procedure, whereas the present study used a biased procedure. A biased procedure was used in an attempt to eliminate the possibility that any observed place preference may have been due to an underlying preference to a particular chamber prior to nicotine administration rather than due to the animal seeking the rewarding properties of the drug. Although Grabus *et al* (2006) justified their use of an unbiased procedure with preliminary data that showed no bias in any of the strains tested, Le Foll and Goldberg's (2005) article showed that two thirds of the reviewed nicotine conditioned place preference studies were successful using a biased procedure. Therefore a biased procedure was utilized for the present study as it seemed most suitable without preliminary data to suggest there was not a prior bias of most animals. One shortcoming to using a biased procedure in the present study, however, is that the degree of bias for one chamber over another was not analyzed when assigning drug and vehicle paired chambers for each individual animal. It is possible that assigning animals whom were 'highly biased' versus animals who were 'mildly biased' or 'unbiased' differently could have created different results within each experimental group.

Second, Grabus *et al* (2006) allowed their animals five minutes to acclimate in the middle chamber prior to the 15 minute baseline pre-test trials. Although not reported in

the present study, the majority of the animals in all groups spent a substantial amount of time in the neutral middle chamber. It may be possible that animals would have spent less time in the middle chamber if given the time to acclimate. This potential effect may have also been eliminated by the use of a two chambered apparatus.

Additionally, the apparatus used by Grabus *et al* (2006) was described as having three chambers: a middle grey walled chamber, a black walled chamber, and a third white walled chamber. This is typical for many commercially available conditioned place preference apparatuses. In another study observing the effects of different conditioned place preference apparatuses on cocaine and morphine place preference, the reported data suggests that rats (female Sprague-Dawley) prefer dark colored chambers over light colored chambers (Roma & Riley, 2005). If the same is true for mice, it is possible that the present study's use of striped and circled black and white walls, although substantially different to the human eye, was not substantially different in the view of a mouse, therefore interjecting with their ability to establish place preference following drug administration.

Beyond these procedure differences, conditioned place preference studies using a wide range of nicotine doses have produced varying results. The dose levels of nicotine used in the present study were at 0.15 mg/kg and 0.30 mg/kg. These dose levels were justified as effective in establishing place preference in several studies (Berrendero, Kieffer, & Maldonado, 2002; Grabus *et al*, 2006; Risinger & Oakes, 1995). Le Foll and Goldberg (2005) also reviewed 22 rat studies (using various strains) and reviewed several variables across each, including dose level. In rats, the most effective range of nicotine that resulted in establishment of place preference was between 0.1 mg/kg and 1.0 mg/kg.

An almost equal number of animals, however, did not show any effect, place preference nor aversion, to nicotine. Thus, it is unlikely that the dose levels for the present study were ineffective and that other various aspects of the study were ultimately responsible for the lack of observed place preference.

Additionally, nicotine was administered to animals in the present study with a pre-treatment time of 10 minutes prior to being placed in the conditioned place preference apparatus for all appropriate conditioning trials. According to Matta *et al* (2007), peak brain levels of nicotine in the rat brain following subcutaneous administration occurs approximately 15 minutes following dose administration. Thus, the animals in the present study who received nicotine would have reached peak brain levels of nicotine within five minutes of being placed into the apparatus, and nicotine levels would then begin to decrease over the remaining 25 minutes of the conditioning trial. It is possible that nicotine place preference could not be established because drug effects were diminished after the first five minutes of the conditioning trial. Matta *et al* (2007) also stated that nicotine is metabolized more rapidly in females versus males. It is possible that place preference may have been established if animals were placed directly into the apparatus following nicotine administration during the conditioning trials.

Norharmane

Neither place preference nor place aversion was found in either the male or female 5.0 mg/kg or the 10.0 mg/kg norharmane groups. Locomotor activity in the drug paired chamber was decreased in females in both the 5.0 mg/kg and the 10.0 mg/kg norharmane groups. The male mice did not have a significant difference in locomotor activity in either groups.

There are apparently no published studies that have reported place preference findings using norharmane or any other monoamine oxidase inhibitors monoamine oxidase inhibitors. Because there is no available information on monoamine oxidase inhibitors, including norharmane, and conditioned place preference paradigms, there is obviously no information on how variables such as species, strain, sex, and dose level, among others, may affect the ability for animals to establish place preference following drug administration. As previously stated, the species and strain of animal was chosen based on previous nicotine research. The dose levels of norharmane used in the present study were justified from their use in a study conducted by Farzin and Mansouri (2006), however, they did use a different strain of mouse and also a different behavioral paradigm (forced swim test) when studying the behavioral effects of norharmane.

Norharmane + Nicotine

Among the combination groups, the female mice in the vehicle-vehicle group and the norharmane-nicotine group showed a statistically significant place aversion to the drug paired chamber. None of the groups of male mice showed any significant effects. Locomotor activity in the drug paired chamber was significantly decreased in all groups of females, but was only decreased in the male norharmane-nicotine group.

Due to previous studies observing monoamine oxidase inhibitor effects potentiating the motivation to self-administer nicotine, it was hypothesized that the present study would observe a significant increase in place preference, and also an increase in locomotion (Arnold *et al*, 2014; Guillem *et al*, 2005; Guillem *et al*, 2006; Villégier *et al*, 2006). Because neither were observed in the norharmane-nicotine group, nor in any of the nicotine or norharmane alone groups, it is possible that some of the

suggested variables previously described may have contributed, such as species, strain, sex, dose levels, and conditioned place preference procedures.

Locomotor Activity

As previously stated, locomotor activity was measured as an additional observed behavioral effect during each drug and vehicle conditioning trial following substance administration. Previous studies have found MAOI's, including norharmane, to have an interactive effect with nicotine increasing locomotor activity in rodents in studies utilizing self-administration of nicotine (Arnold *et al*, 2014; Guillem *et al*, 2005; Guillem *et al*, 2006; Villégier *et al*, 2006). These studies, however, did not observe the effects of norharmane alone on locomotor activity. A study conducted by Goodwin *et al* (2015) observed the locomotor effects of norharmane alone administered chronically in adolescent rats. It was found that norharmane, compared to harmane alone or nicotine alone, actually had the least significant effect on locomotion. Unfortunately, it is difficult to infer whether these effects would occur after only three doses and in adult mice, and there is no other data reported.

As previously stated, it has been reported that nicotine alone can cause a decreased locomotor response in a range of doses from 0.1 mg/kg to 1.0 mg/kg (Kita *et al*, 1988). This was observed in the present study in females in each experimental nicotine group, and in males in the 0.15 mg/kg group. It is possible that if the present study exposed animals to more than three nicotine conditioning trials, that animals may have shown an increase in locomotion, rather than decrease. A study by Prus *et al* (2008) found that Lewis rats, who are believed to be more sensitive to nicotinic effects, that were given a single nicotine injection had a decrease in locomotion, and an increase after one

or two weeks of twice daily nicotine treatment. They attributed this effect to acute behavioral tolerance to nicotine as it is continually administered despite the desensitized state of nACh receptors, resulting in increased locomotion. It may be possible that norharmane has a similar effect causing behavioral tolerance, although this is unknown. Chronic administration of monoamine oxidase inhibitors, including norharmane, have previously increased nicotine reward and locomotor activity, therefore it may be plausible (Arnold *et al*, 2014; Guillem *et al*, 2005; Guillem *et al*, 2006; Villégier *et al*, 2006). Further studies indexing behavioral tolerance to chronic monoamine oxidase inhibitor administration are necessary. Overall, behavioral tolerance may also have an effect on establishing place preference with either drug which, again, needs to be indexed using a conditioned place preference paradigm.

Limitations

As noted above, there were several limitations to the present study. Several variables with the animal model used in the present study, such as strain and age, may have affected the ability to observe statistically significant results. The particular conditioned place preference apparatus used, in addition to the procedures, such as acclimation to handling and restraint, the number of conditioning trials, the biased procedure, and the lack of middle chamber acclimation may have also limited the ability of observe nicotine place preference. Also, because the ability to observe nicotine place preference with different dose levels for different strains of mice is complicated in preclinical research, it makes it difficult to translate findings to the human population as individuals are extremely genetically different as well. Finally, the lack of reported data on establishing place preference to norharmane also caused a large limitation. Certain

procedures may have been carried out differently in the norharmane groups if using the compound in conditioned place preference were better understood.

Conclusion

Taken together, all of these finding reveal a greater need for standardized methodologies for observing nicotine effects using a conditioned place preference paradigm. Additionally, next to no information exists on the rewarding properties of monoamine oxidase inhibitors, including norharmane, and no studies have been reported describing whether or not place preference has been used to study monoamine oxidase inhibitors. Additionally, there is very little reported information on how the large number of variables in a conditioned place preference study may affect the ability to establish preference with monoamine oxidase inhibitors. Thus, these are expansive areas for future studies to engage in.

With this information, future studies may reveal a better understanding of the interactive neuromechanisms between monoamine oxidase inhibitors and nicotine. The overall purpose of the present study was to determine if another psychoactive component found in tobacco would enhance the pharmacological effects of nicotine in a way that would promote further tobacco use. With this information, nicotine cessation programs may be altered in a manner that allows for greater success for those who wish to quit smoking tobacco by focusing on the withdrawal from other psychoactive constituents, as well as nicotine. Finally, further studies may give insight to how individuals who smoke electronic nicotine delivery devices and are never exposed to the potentially addictive, psychoactive constituents of a tobacco cigarette may be at a higher risk of addiction after smoking a single tobacco cigarette, as these constituents may potentiate their current nicotine addiction.

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APPENDIX A

Further Statistical Analyses

Further statistical analyses were conducted in order to determine if combining the male and female data would produce any significant sex differences. First, an unpaired t test was conducted to compare the males and females difference in time (seconds) spent in either chamber (calculated as time spent in drug paired chamber – time spent in vehicle paired chamber).

This statistical analysis did not indicate significant differences in the difference in time spent in either chamber between males and females in the vehicle group (males, M = -50.07, +/- SEM = 57.66; females, M = 27.72, +/- SEM = 47.04; t (22) = 1.045, $p > 0.05$), the nicotine 0.15 mg/kg group (males, M = -96.65, +/- SEM = 32.36; females, M = -44.79, +/- SEM = 38.56; t (22) = 1.03, $p > 0.05$), the nicotine 0.30 mg/kg group (males, M = -69.07, +/- SEM = 50.43; females, M = -16.05, +/- SEM = 39.39; t (21) = 0.8181, $p > 0.05$), the norharmane 5.0 mg/kg group (males, M = -79.22, +/- SEM = 55.57; females, M = -48.39, +/- SEM = 52.74; t (22) = 0.4024, $p > 0.05$), the norharmane 10.0 mg/kg group (males, M = -43.77, +/- SEM = 41.98; females, M = -81.30, +/- SEM = 39.84; t (22) = 0.6485, $p > 0.05$), the vehicle-vehicle group (males, M = -44.17, +/- SEM = 22.17; females, M = -65.54, +/- SEM = 27.54; t (22) = 0.6046, $p > 0.05$), the vehicle-nicotine 0.30 mg/kg group (males, M = -9.07, +/- SEM = 36.14; females, M = -37.92, +/- SEM = 66.83; t (22) = 0.3797, $p > 0.05$), the norharmane 10.0 mg/kg-vehicle group (males, M = -116.2, +/- SEM = 112.5; females, M = 45.9, +/- SEM = 68.68; t (21) = 1.203, $p > 0.05$),

nor the e norharmane 10.0 mg/kg-nicotine 0.30 mg/kg (males, M = -37.00, +/- SEM = 42.94; females, M = -129.60, +/- SEM = 55.32; t (22) = 1.322, p > 0.05).

Because the results described above did not show any significant sex differences, male and female data was combined and analyzed using an unpaired t test for time (seconds) spent in the drug paired chamber versus the vehicle paired chamber for all vehicle or drug alone groups. The results from this statistical analyses are depicted in the graphs in Figure 10A (vehicle group), 10B (nicotine 0.15 mg/kg group), 10C (nicotine 0.30 mg/kg group), 10D (norharmane 5.0 mg/kg group), and 10E (norharmane 10.0 mg/kg group).

There was not a significant difference in time spent in the drug paired chamber versus the vehicle paired chamber in the vehicle group (non-preferred, M = 246.2, +/- SEM = 29.65; preferred, M = 291.4, +/- SEM = 291.4; t (22) = 0.9757, p > 0.05), the nicotine 0.15 mg/kg group (nicotine, M = 284.3, +/- SEM = 14.03; vehicle, M = 309.2, +/- SEM = 21.08; t (40) = 0.9816, p > 0.05), nor the nicotine 0.30 mg/kg group (nicotine, M = 261.0, +/- SEM = 17.25; vehicle, M = 304.7, +/- SEM = 20.21; t (44) = 1.645, p > 0.05). A significant place aversion was found in the norharmane 5.0 mg/kg group (norharmane, M = 236.8, +/- SEM = 19.6; vehicle, M = 300.6, +/- SEM = 21.18; t (46) = 2.211, p < 0.05) and the norharmane 10.0 mg/kg group (norharmane, M = 231.8, +/- SEM = 15.4; vehicle, M = 294.4, +/- SEM = 18.61; t (46) = 2.588, p < 0.05).

Figure 10. Mean Time (seconds) Spent Per Chamber

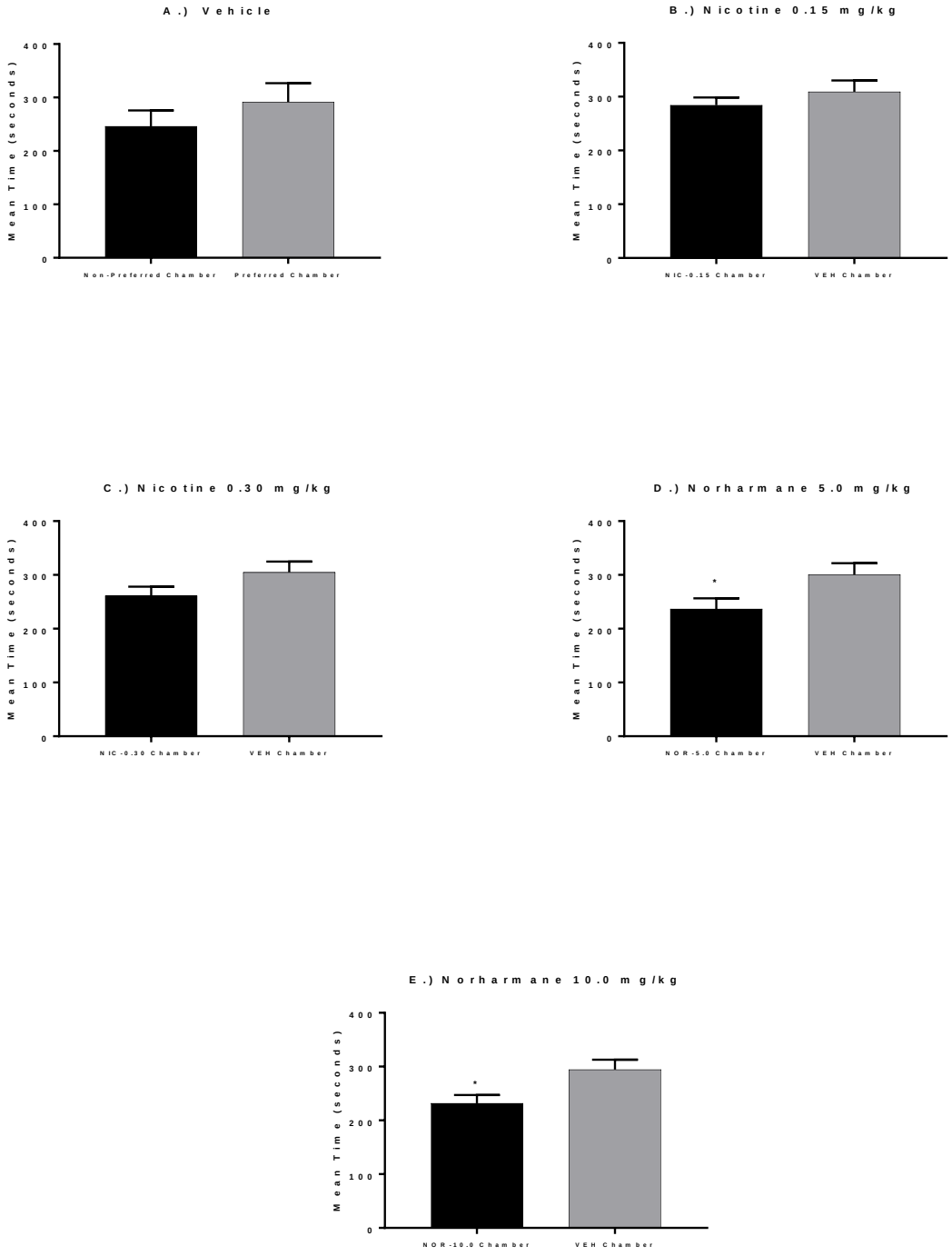

Figure 10. Combined male and female data calculated as time (seconds) spent in the non-preferred, or drug paired, chamber versus the preferred, or vehicle paired, chamber. A., vehicle group (N=12); B., nicotine 0.15 mg/kg group (N=21); C., nicotine 0.30 mg/kg group (N=23); D., norharmane 5.0 mg/kg group (N=24); E., norharmane 10.0 mg/kg group (N=24). (* denotes $p < 0.05$).

Additionally, a two factor between groups ANOVA (norharmane X nicotine) was used to determine if further analysis of the combined male and female place preference data for all combination groups showed a significant difference in time spent in the drug paired chamber versus the vehicle paired chamber. The results of the ANOVA are displayed in Table 4. Because there was no significant difference across groups, a covariate of percent of time spent in the drug paired chamber during the baseline pre-test trials was added and a two way factor between groups ANCOVA (norharmane X nicotine) was conducted. The results of the ANCOVA are displayed in Table 5. The ANOVA and ANCOVA were both analyzed using IBM SPSS Statistics Subscription (version 1.0.0.1012) due to the inability of Graph Pad Prism 7 to analyze data using an ANCOVA statistical test.

Table 4. Two way factor between groups ANOVA (norharmane X nicotine) results.

Tests of Between-Subjects Effects

Dependent Variable: Drug Paired Chamber

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	24040.032[a]	3	8013.344	.549	.650
Intercept	4875849.968	1	4875849.968	334.239	.000
Norharmane	13.911	1	13.911	.001	.975
Nicotine	2678.542	1	2678.542	.184	.669
Norharmane * Nicotine	21487.755	1	21487.755	1.473	.228
Error	1327500.853	91	14587.921		
Total	6220001.251	95			
Corrected Total	1351540.885	94			

a. R Squared = .018 (Adjusted R Squared = -.015)

Table 5. Two way factor between groups ANCOVA (norharmane X nicotine) results, with a covariate of the percent of time spent in the drug paired chamber during baseline pre-test trials.

Tests of Between-Subjects Effects

Dependent Variable: Drug Paired Chamber

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	26590.482[a]	4	6647.620	.452	.771
Intercept	1038915.693	1	1038915.693	70.570	.000
% Baseline Preference	2550.450	1	2550.450	.173	.678
Norharmane	135.845	1	135.845	.009	.924
Nicotine	3358.568	1	3358.568	.228	.634
Norharmane * Nicotine	19557.531	1	19557.531	1.328	.252
Error	1324950.403	90	14721.671		
Total	6220001.251	95			
Corrected Total	1351540.885	94			

a. R Squared = .020 (Adjusted R Squared = -.024)

An unpaired t test was also conducted to compare the males and females difference in percent of time spent in the drug paired chamber during the baseline trials versus test trials. (calculated as percent time spent in the drug paired chamber during the test trial – percent of time spent in the drug paired chamber during the baseline pre-test trials).

This statistical analysis did not indicate significant differences in the difference in the percent of time spent in the drug paired chamber during the baseline pre-test trials versus the test trial in males, nor females, for the vehicle group (males, M = 9.98, +/- SEM = 7.21; females, M = 13.49, +/- SEM = 8.56; $t(10) = 0.3139$, $p > 0.05$), the nicotine 0.15 mg/kg group (males, M = 12.43, +/- SEM = 3.74; females, M = 7.21, +/- SEM = 7.72; $t(15) = 0.6183$, $p > 0.05$), the nicotine 0.30 mg/kg group (males, M = 3.86, +/- SEM = 10.93; females, M = 10.93, +/- SEM = 5.07; $t(17) = 0.7602$, $p > 0.05$), the norharmane 5.0 mg/kg group (males, M = 1.24, +/- SEM = 6.01; females, M = 7.61, +/- SEM = 4.23; $t(22) = 0.8671$, $p > 0.05$), the norharmane 10.0 mg/kg group (males, M = 9.63, +/- SEM = 5.01; females, M = -5.82, +/- SEM = 6.07; $t(22) = 1.964$, $p > 0.05$), the vehicle-vehicle group (males, M = 4.05, +/- SEM = 4.37; females, M = -2.54, +/- SEM = 5.23; $t(22) = 0.9357$, $p > 0.05$), the vehicle-nicotine 0.50 mg/kg group (males, M = 6.55, +/- SEM = 5.67; females, M = 10.73, +/- SEM = 8.43; $t(22) = 0.4112$, $p > 0.05$), the norharmane 10.0 mg/kg-vehicle group (males, M = 4.39, +/- SEM = 10.29; females, M = 14.15, +/- SEM = 6.03; $t(21) = 0.7986$, $p > 0.05$), nor the norharmane 10.0 mg/kg-nicotine 0.30 mg/kg group (males, M = 9.59, +/- SEM = 3.44; females, M = 1.20, +/- SEM = 3.89; $t(22) = 1.616$, $p > 0.05$).

Because the results described above did not show any significant sex differences, male and female data was combined and analyzed using an unpaired t test for percent of time spent in the drug paired chamber during the baseline pre-test trials versus during the test trial for all vehicle or drug alone groups. The results from this statistical analyses are depicted in the graphs in Figure 11A (vehicle group), 11B (nicotine 0.15 mg/kg group), 11C (nicotine 0.30 mg/kg group), 11D (norharmane 5.0 mg/kg group), and 11E (norharmane 10.0 mg/kg group).

There was not a significant difference in the percent of time spent in the drug paired chamber during the baseline pre-test trials versus during the test trial in the vehicle group (males, M = 34.76, +/- SEM = 4.46; females, M = 46.48, +/- SEM = 5.01; $t(22) = 1.747$, $p > 0.05$), the nicotine 0.30 mg/kg group (males, M = 39.00, +/- SEM = 3.05; females, M = 46.2, +/- SEM = 3.18; $t(36) = 1.636$, $p > 0.05$), the norharmane 5.0 mg/kg group (baseline, M = 39.72, +/- SEM = 2.64; test trial, M = 44.15, +/- SEM = 3.32; $t(46) = 1.044$, $p > 0.05$), nor the norharmane 10.0 mg/kg group (baseline, M = 44.54, +/- SEM = 3.22; test trial, M = 44.28, +/- SEM = 2.61; $t(46) = 0.0635$, $p > 0.05$). The nicotine 0.15 mg/kg group did show a significant increase in the percent of time spent in the drug paired chamber during the test trial versus during the baseline pre-test trials (males, M = 36.67, +/- SEM = 3.28; females, M = 44.93, +/- SEM = 2.02; $t(34) = 2.144$, $p < 0.05$).

Figure 11. Mean Percent of Time in Drug Paired Chamber

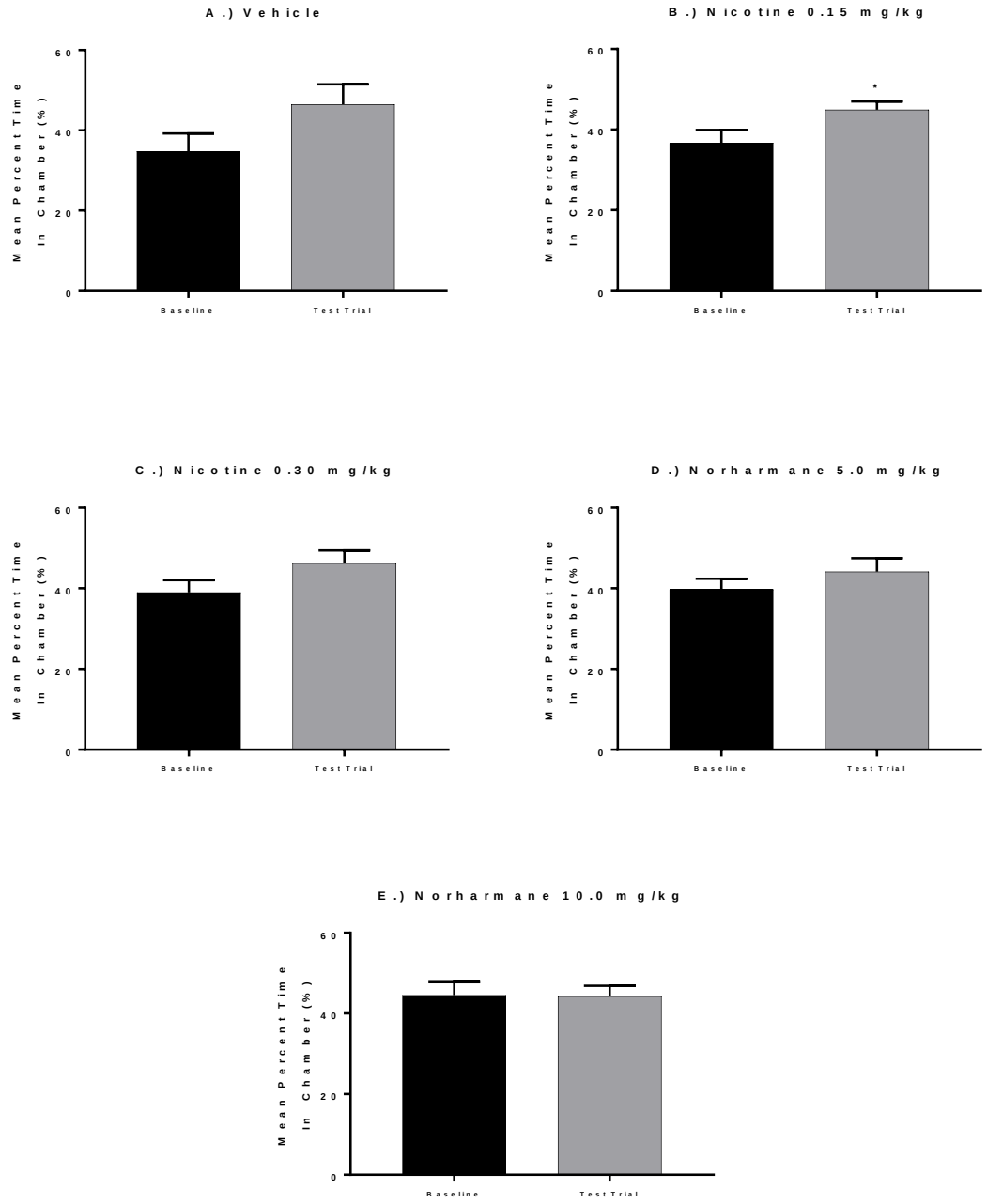

Figure 11. Combined male and female data calculated as percent of time spent in the drug paired chamber during the baseline pre-test trials versus the test trial. A., vehicle

group (N=12); B., nicotine 0.15 mg/kg group (N=21); C., nicotine 0.30 mg/kg group (N=23); D., norharmane 5.0 mg/kg group (N=24); E., norharmane 10.0 mg/kg group (N=24). (* denotes $p < 0.05$).

Additionally, a two factor between groups ANOVA (norharmane X nicotine) was used to determine if further analysis of the combined male and female percent of time spent in the drug paired chamber during the baseline pre-test trials versus the test trial, for all combination groups, showed a significant difference. The results of the ANOVA are displayed in Table 6. Because there was no significant difference across groups, a covariate of percent of time spent in the drug paired chamber during the baseline pre-test trials was added and a two way factor between groups ANCOVA (norharmane X nicotine) was conducted. The results of the ANCOVA are displayed in Table 7. As previously stated, the ANOVA and ANCOVA were both analyzed using IBM SPSS Statistics Subscription (version 1.0.0.1012), rather than Graph Pad Prism 7.

Table 6. Two way factor between groups ANOVA (norharmane X nicotine) results for comparing the percent of time spent in the drug paired chamber during the baseline pre-test trails versus the test trial.

Tests of Between-Subjects Effects

Dependent Variable: Drug Paired Chamber

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	24040.032[a]	3	8013.344	.549	.650
Intercept	4875849.968	1	4875849.968	334.239	.000
Norharmane	13.911	1	13.911	.001	.975
Nicotine	2678.542	1	2678.542	.184	.669
Norharmane * Nicotine	21487.755	1	21487.755	1.473	.228
Error	1327500.853	91	14587.921		
Total	6220001.251	95			
Corrected Total	1351540.885	94			

a. R Squared = .018 (Adjusted R Squared = -.015)

Table 7. Two way factor between groups ANCOVA (norharmane X nicotine) results, with a covariate of the difference of the percent of time spent in the drug paired chamber during baseline pre-test trials.

Tests of Between-Subjects Effects

Dependent Variable: Drug Paired Chamber

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	239677.306[a]	4	59919.326	4.850	.001
Intercept	3997475.928	1	3997475.928	323.576	.000
Percent Difference	215637.274	1	215637.274	17.455	.000
Norharmane	58.084	1	58.084	.005	.945
Nicotine	7353.433	1	7353.433	.595	.442
Norharmane * Nicotine	5376.634	1	5376.634	.435	.511
Error	1111863.579	90	12354.040		
Total	6220001.251	95			
Corrected Total	1351540.885	94			

a. R Squared = .177 (Adjusted R Squared = .141)

APPENDIX B

Institutional Animal Care and Use Committee Approval Form

The approval form from the Institutional Animal Care and Use Committee for use of animal subjects in the present study has been copied and attached.

SIGNATURE PAGE

IACUC #: 313 **PROPOSAL TITLE (From cover page):** Conditioned place preference with nicotine and an MAO inhibitor

X. ACKNOWLEDGEMENT BY PRINCIPAL INVESTIGATOR

I acknowledge responsibility for this project. I have read the Northern Michigan University Principles for the Care and Use of Laboratory Animals and certify that this project will be conducted in compliance with those principles. I assure that I will obtain Institutional Animal Care and Use Committee approval prior to significant changes in the protocol. I assure that this project does not unnecessarily duplicate previous research or instructional projects. I assure that students, staff and faculty on the project are qualified or will be trained to conduct the project in a humane, safe, and scientific manner.

Signature: _____ 08/11/2017

Principal Investigator Date

XI. APPROVAL OF SCIENTIFIC MERIT (to be completed by the Department Head)

Before the project is initiated, it must be reviewed and approved on the basis of its scientific merit.

☐ Review conducted by external agency.

☐ Governmental Agency: Please specify the reviewing agency or board Federal agency (e.g., NIH, NSF, USDA, etc.) and evidence of approval

☐ Nongovernmental agency (e.g., University review, specify if other):

☐ Departmental Review: I assure that this project has been reviewed and approved for scientific or instructional merit by:

☐ Expert reviewer (Name)

☐ Departmental Committee Review (Committee Name and Chairperson):

☐ Other (Describe):

Signature: _____ 08/12/2017

Department Head/Other Authorized Departmental Designee Date

XII. REVIEWED AND APPROVED BY THE IACUC

Signature: _____ 08/12/2017

Institutional Animal Care and Use Committee Chair Date

Signature: _____ 08/14/2017

Institutional Animal Care and Use Officer Date

Following action on this application, copies of approval or denial letters will be sent to the applicant, Department Head, and appropriate College Dean.