

EXAMINATION OF THE GATEWAY HYPOTHESIS IN A RAT MODEL

by

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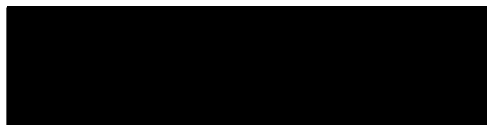
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ABSTRACT

Title of Thesis: Examination of the Gateway Hypothesis in a Rat Model

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The Gateway Hypothesis is based on epidemiological data and states there is a progression of drug use from use of a softer drug (e.g., nicotine) to use of a harder drug (e.g., morphine). It has been suggested that this sequence is causal and is relevant to drug prevention policies and programs. The present experiment used an animal model to investigate whether the Gateway Hypothesis involves a causal progression. The subjects were 16 female and 16 male Sprague Dawley rats with ages comparable to late adolescence/emerging adulthood in humans. Subjects received nicotine (6 mg/kg/day) or saline for 21 days subcutaneously via osmotic minipump and were subsequently allowed to self-administer morphine intravenously for 10 days. Results did not confirm the Gateway Hypothesis. In fact, rats pre-exposed to nicotine self-administered significantly less morphine than did rats pre-exposed to saline. These findings may be relevant to future drug use prevention policies and programs.

TABLE OF CONTENTS

LIST OF TABLES.....	ix
LIST OF FIGURES	x
CHAPTER 1: Introduction	1
Background	2
Specific Drugs.....	5
Opioids	6
Tobacco	7
Nicotine	9
The Value of Animal Models.....	11
Sex differences	13
Opioids	13
Tobacco	14
CHAPTER 2: Overview and Specific Aims	15
CHAPTER 3: Methods	17
Subjects	18
Housing	18
Independent Variables.....	19
Nicotine	19
Sex.....	20
Time	21
Dependent Variables	21
Morphine Self-Administration (MSA)	21
Operant Conditioning Chamber	22
Inactive Lever Presses	22
Horizontal Activity (HA)	23
Body Weight	23
Equipment And Procedures	23
Open Field Activity (OFA)	23
Minipump Surgery	25
Catheter Surgery	26
Procedure	28
Baseline (BL)	28
Groups.....	28
Nicotine or Saline Exposure	29
Recovery from Catheter Surgery	29
Morphine Self-administration	29
CHAPTER 4: Data Analytic Strategy	31

Statistical Analyses	31
CHAPTER 5: Results	32
Missing Data	32
Morphine Self-Administration	32
Morphine Self-Administration Repeated-measures Analysis of Variance	32
Morphine Self-Administration Repeated-measures Analysis of Variance Split by Sex	33
Females	33
Males	34
Summary of Morphine Self-Administration	34
Inactive Lever Repeated-measures Analysis of Variance	35
Inactive Lever Repeated-measures Analysis of Variance Split by Sex	35
Females	35
Males	36
Summary of Inactive Lever Activity	36
Horizontal Activity	36
Horizontal Activity Repeated-measures Analysis of Variance	36
Horizontal Activity Repeated-measures Analysis of Variance Split by MSA	37
Horizontal Activity Repeated-measures Analysis of Variance Split by Sex	38
Females	38
Males	39
Horizontal Activity Repeated-measures Analysis of Variance Split by Sex and MSA	39
Females	39
Males	40
Summary of Horizontal Data	40
Body Weight	41
Body Weight Repeated-measures Analysis of Variance	41
Body Weight Repeated-measures Analysis of Variance Split by Sex	42
Females	42
Males	42
Body Weight Repeated-measures Analysis of Variance Split by Phase	43
Pre-Nicotine/Saline Exposure	43
During Nicotine/Saline Exposure	43
Post-Nicotine/Saline Exposure/Pre-Morphine Self-Administration	44
During Morphine Self-Administration	45
Body Weight Repeated-measures Analysis of Variance Split by Sex and Phase	45
Females	45
Pre-Nicotine/Saline Exposure	45
During Nicotine/Saline Exposure	45
Post Nicotine/Saline Exposure/Pre-Morphine Self-Administration	46
During Morphine Self-Administration	46
Males	46
Pre-Nicotine/Saline Exposure	46
During Nicotine/Saline Exposure	47

Post-Nicotine/Saline Exposure/Pre-Morphine Self-Administration	47
During Morphine Self-Administration	47
CHAPTER 6: Evaluation of Hypotheses	49
CHAPTER 7: Discussion.....	50
Limitations	52
Missing Data	52
Independent Variables.....	53
Methodology	54
Clinical Implications	54
Future Directions.....	55
Independent Variables.....	55
Methodology	56
CHAPTER 8: Summary.....	58
CHAPTER 9: Conclusions	59
APPENDIX A: Figures.....	60
APPENDIX B: Tables	75
REFERENCES	95

LIST OF TABLES

Table 1: Treatment Cell Breakdown.....	75
Table 2: Overall rANOVA of MSA.....	75
Table 3: rANOVA of female MSA.....	76
Table 4: rANOVA of male MSA.....	76
Table 5: Overall rANOVA of inactive lever.....	77
Table 6: rANOVA of female inactive lever.....	78
Table 7: rANOVA of male inactive lever.....	78
Table 8: Independent sample's t-test of HA baseline.....	79
Table 9: Overall rANOVA of HA.....	79
Table 10: Overall rANOVA of HA pre-MSA.....	80
Table 11: Overall rANOVA of HA during-MSA.....	81
Table 12: Independent sample's t-test of female HA baseline.....	82
Table 13: rANOVA of female HA.....	82
Table 14: Independent sample's t-test of male HA baseline.....	82
Table 15: rANOVA of male HA.....	83
Table 16: rANOVA of female pre-MSA HA.....	83
Table 17: rANOVA of female during-MSA HA.....	84
Table 18: rANOVA of male pre-MSA HA.....	84
Table 19: rANOVA of male during-MSA HA.....	85
Table 20: Independent sample's t-test of BW baseline.....	85
Table 21: Overall rANOVA of BW.....	85
Table 22: Overall rANOVA of female vs. male BW.....	86
Table 23: Independent sample's t-test of female BW baseline.....	86
Table 24: Independent sample's t-test of male BW baseline.....	87
Table 25: rANOVA of female overall BW.....	87
Table 26: rANOVA of male overall BW.....	87
Table 27: rANOVA of pre-nicotine/saline exposure females vs. males BW.....	88
Table 28: rANOVA of BW during nicotine/saline exposure females vs. males.....	89
Table 29: rANOVA of post-nicotine/saline exposure, pre-MSA females vs. males BW.....	89
Table 30: rANOVA of BW during MSA female vs. male.....	90
Table 31: rANOVA of female pre-nicotine/saline exposure BW.....	91
Table 32: rANOVA of female nicotine/saline exposure BW.....	91
Table 33: rANOVA of female post-nicotine/saline exposure, pre-MSA BW.....	92
Table 34: rANOVA of female during MSA BW.....	92
Table 35: rANOVA of male pre-nicotine/saline exposure BW.....	93
Table 36: rANOVA of male nicotine/saline exposure BW.....	93
Table 37: rANOVA of male post-nicotine/saline exposure, pre-MSA BW.....	94
Table 38: rANOVA of male during MSA BW.....	94

LIST OF FIGURES

Figure 1: IACUC Approval of Protocol.....	60
Figure 2: Experimental Timeline	61
Figure 3: Sample Nicotine Bitartrate Calculation (Male Cohort 2).....	62
Figure 4: Surgery Procedure Guidance Form	63
Figure 5: Subcutaneous Location of Minipump Implant in the Rat	63
Figure 6: Cross-section Illustration of the ALZET Model 2002 Minipump	64
Figure 7: Illustration of Catheter.....	64
Figure 8: Catheter Implantation	65
Figure 9: Morphine Self-Administration (MSA), Overall.....	66
Figure 10: Morphine Self-Administration, Females	66
Figure 11: Morphine Self-Administration, Males	67
Figure 12: Inactive Lever, Presses, Overall	67
Figure 13: Inactive Lever Presses, Females.....	68
Figure 14: Inactive Lever Presses, Males	68
Figure 15: Horizontal Activity, Overall.....	69
Figure 16: Horizontal Activity, Females	69
Figure 18: Body Weight, Overall.....	71
Figure 19: Body Weight, Females	71
Figure 20: Body Weight, Males.....	72
Figure 21: Body Weight, Most Representative Day, Overall.....	72
Figure 22: Body Weight, Most Representative Day, Females.....	73
Figure 23: Body Weight, Most Representative Day, Males	73

CHAPTER 1: Introduction

The Gateway Hypothesis proposed there is a developmental progression in the use of drugs such that certain drugs serve as a “gateway” for the use of other drugs in a specific, causal, temporal sequence (73; 74; 76). According to this hypothesis, involvement with drugs is not opportunistic but follows a definite pathway in which an individual who self-administers specific, “softer or lower” drugs (e.g., alcohol, nicotine) is at increased risk to progress to use of another, “harder or higher” drug (e.g., cocaine, heroin) (74). The Gateway Hypothesis is defined as the sequencing of initiation between drug classes, and association in the use of drugs, such that use of a lower drug (e.g., nicotine) in the sequence increases the risk of using drugs higher in the sequence (e.g., morphine) (74). The Gateway Hypothesis implies three interrelated propositions about sequencing, association of initiation, and causation (75). Sequencing implies there is a fixed relationship between two drug substances, such that use of one substance is regularly initiated before the other. Association implies that the initiation of one substance increases the likelihood of initiation of the second substance. Causation implies that use of the first substance actually causes use of the second substance (75). Additionally, Kandel (75) defined lower order drugs as nicotine, alcohol, or marijuana and higher order drugs as any other illicit drug or their prescription counterpart (e.g., heroin and morphine) (76). Finally, Kandel (75) stated that this developmental sequence of involvement in drugs is one of the most replicated findings in the epidemiology of drug use.

The purpose of the present experiment was to examine whether this progression is, in fact, causal. Specifically, the present experiment examined if rats pre-exposed to

nicotine, subsequently self-administered more morphine compared to rats that were not pre-exposed to nicotine. If the Gateway Hypothesis progression of drug use is causal, then it is particularly important to prevent exposure to “soft” drugs. If the Gateway Hypothesis does not involve causal progression, then drug prevention and treatment should focus on drug use which is particularly harmful. This master’s thesis first presents relevant background information about the Gateway Hypothesis; information about the drugs that are the focus of study in this project (nicotine and morphine); and a brief review about the value of animal models. Next, specific aims and hypotheses are presented followed by methods. Then, the results and discussion are presented and finally a summary, conclusion, appendices, and references appear.

BACKGROUND

The Gateway Hypothesis was not the first to propose a developmental progression of drug use. In the 1930s, the Stepping Stone Hypothesis was proposed by A. E. Fossier in the *New Orleans Medical and Surgical Journal* (118) which stated that the use of “softer or lower” drugs (e.g., nicotine, alcohol) invariably led to the use of “harder or higher” drugs (e.g., cocaine, heroin) without exception (74; 138). The Stepping Stone Hypothesis was based on clinical populations of heroin users who were asked about prior drug use. Individuals reported prior use of marijuana (alcohol and nicotine were not considered drugs at the time) and, therefore, the Stepping Stone Hypothesis indicated that all individuals who use marijuana would later progress to the use of heroin (106). However, the Stepping Stone Hypothesis lacks evidence. For instance, although heroin users indicated they used marijuana prior to heroin, no evidence existed that all marijuana

users progressed to heroin use. The causal progression of drug use purported by the Stepping Stone Hypothesis has been rejected (132).

The Gateway Hypothesis does not purport the inevitability of moving from a softer drug to a harder drug, but instead indicates that use of soft drugs increases the likelihood of subsequent use of hard drugs. Although explicit statements regarding causality were denied by Kandel when she stated “although the data show a clear sequence in drug use, a particular drug does not invariably lead to other drugs higher up in the sequence” (73), causation is implicit in such statements as “one licit drug is required to make the progression to marijuana use” (77) or “the use of a drug at a lower stage is necessary but not sufficient for progression to a higher stage indicating involvement with more serious drugs” (77). Further, the Gateway Hypothesis and the Stepping Stone Hypothesis are often referred to interchangeably as if they are identical (11; 138). Although the original intention of the Gateway Hypothesis was not to imply causality, since its introduction more than 40 years ago, the Gateway Hypothesis has become almost synonymous with the Stepping Stone Hypothesis, resulting in the common interpretation that the developmental progression of drug use is, in fact, inevitable.

With this causal understanding (or misunderstanding) about the developmental progression of drug use, the Gateway Hypothesis has influenced public policy and drug prevention programs for the past 40 years. For example, President Richard Nixon initiated the “War on Drugs” in the early 1970s. With this “War,” President Nixon drastically increased the size and presence of federal drug control agencies, initiated measures such as mandatory sentencing and no-knock warrants, and increased resources

for the research of prevention and treatment of any “drug” use (excluding nicotine and alcohol) (28; 103). Some historians have suggested that President Nixon’s “War on Drugs” and focus on marijuana was, in fact, a means to arrest anti-Vietnam War demonstrators on college campuses (25). The “War on Drugs” saw an unprecedented expansion in 1981 during the Reagan administration with the number of people behind bars for nonviolent drug law violations increasing from 50,000 in 1980 to more than 400,000 by 1997 because of President Reagan’s “zero tolerance” program for possession and use of drugs (101). The average annual amount of funding for eradication and prohibition programs increased from an average of \$437 million annually during the Nixon and Carter presidencies to \$1.4 billion annually during President Reagan’s first term. In contrast, funding for education, prevention, and rehabilitation programs was cut from an average of \$386 million to \$362 million (114).

During this same time period, First Lady Nancy Reagan started her “Just Say No” campaign that was directed toward adolescents and children to dissuade them from engaging in drug use at an early age, with the goal to prevent the use of any drugs including nicotine and alcohol (33). Additionally, in 1984, the drinking age in the United States was raised from 18 to 21 and in 1987 the tobacco smoking age was raised from 16 to 18 (5; 98). The support for these changes in legislation grew out of the Gateway Hypothesis in that alcohol and tobacco were now categorized as “Gateway Drugs” (131). Additional restrictions and legislation were enacted with two goals in mind: (a) limit the use of these lower/softer drugs, and (b) by limiting use of the lower/softer drugs, the use of higher/harder drugs also will be limited.

These legislative and public policy changes during the past 40 years are largely the result of the Gateway Hypothesis. Validity of the Gateway Hypothesis is based on two criteria: (a) sequencing of initiation of use between drug classes and (b) association in the use of the drugs, such that the use of the lower (e.g., nicotine, alcohol) drug in the sequence increases the risk of using the higher (e.g., cocaine, heroin) drug in the sequence (73; 74). These criteria were established based on epidemiological data with adolescents that is correlational, not causal.

Although, public policy has been shaped by this Hypothesis, this Hypothesis has not been thoroughly examined in an experimental way. If the Gateway Hypothesis is true, then it follows that it is an important concept that needs continued experimental research and has significant public health and policy implications. However, if the Gateway Hypothesis is false, 40 years of public health initiatives and policy changes have been enacted based on inaccurate information. The following sections discuss the basis for the current experiment which examined whether rats pre-exposed to nicotine subsequently self-administered greater amounts of morphine compared to rats not pre-exposed to nicotine.

SPECIFIC DRUGS

The Gateway Hypothesis indicated that certain drugs are considered “lower or softer” drugs which precede use of “higher or harder” drugs (73; 74). The lower drugs include licit substances such as tobacco and alcohol and the higher drugs include illicit substances such as cocaine and heroin and their prescription counterparts (e.g., morphine) (73; 74).

Opioids

Opioid use (specifically morphine) is increasing in the United States and worldwide (97; 116; 134). To illustrate, the number of opioid pain relievers prescribed in the United States has dramatically increased over the past 25 years. The number of prescriptions for opioids (e.g., hydrocodone and oxycodone) have escalated from around 76 million in 1991 to nearly 207 million in 2013, with the United States the biggest consumer globally, accounting for almost 100% of the world total for hydrocodone (e.g., Vicodin) and 81% for oxycodone (e.g., Percocet) (133).

This greater availability of prescription opioids has been followed by alarming increases in the negative consequences associated with their abuse. For example, more than 1.2 million emergency room visits involved nonmedical use of over-the-counter medications, prescription drugs, or other types of pharmaceuticals in 2011 (12). At 46%, opioid pain relievers were the most common type of drugs involved in medical emergencies related to nonmedical use of prescription medications; narcotic pain relievers were involved in 29% of medical emergencies. Overall, medical emergencies resulting from nonmedical use of pharmaceuticals increased 132% from 2004 to 2011, with use of opioids increasing 183% (12). The treatment admission rate for opiates other than heroin has more than doubled in ten years. Admissions rates were 167% higher in 2013, at 57 per 100,000 adolescents and adults, than in 2003, at 21 per 100,000 (127); and overdose deaths due to prescription opioid pain relievers have more than tripled in the past 20 years, estimated at 16,651 deaths in the United States in 2010 (88). Additional drug overdose, particularly opioid overdose, is the leading cause of accidental death in the United States, with over 52,000 lethal overdoses in 2015 (13).

Similar to civilian populations, opioid use in the military also has increased. Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF) veterans have a morphine use prevalence rate of 15% compared to the general population prevalence rate of 4% (130). This increase in opioid use has led to a significant increase in overdose-related hospitalizations and deaths (130). The risk factors for morphine abuse in military populations are relatively unknown (45; 65), therefore, research is needed to determine who is more likely to abuse opioids, in general, and after combat injuries, specifically. Given the higher rates of tobacco use in the military (see below) as well as the increased rates of opioid use, the purpose of the present experiment was to investigate the effects of pre-exposure to nicotine on subsequent morphine self-administration. If, according to the Gateway Hypothesis, the use of nicotine leads to use of morphine, then more focus should be directed toward the prevention of tobacco use. However, if the use of nicotine does not lead to use of morphine, in a causal fashion, as implied by the Gateway Hypothesis, focus should be shifted to the prevention and treatment of harder drugs, in this case, morphine.

Tobacco

Worldwide approximately 1.1 billion people smoke cigarettes, with increases in prevalence rates occurring in multiple parts of the Eastern Mediterranean and Africa, and almost half will die prematurely from smoking-related illnesses (145-149). More than 7 million people are killed each year from tobacco and more than 6 million of these deaths are the direct result of tobacco use while the other 1 million are the result of non-tobacco users being exposed to second-hand smoke (149). In the United States, tobacco is the leading preventable cause of death (50). From 2005-2009, cigarette smoking and

exposure to tobacco smoke led to 480,000 premature deaths annually in the United States (137). Another 16 million people live with serious illnesses caused by tobacco use (17). Chronic health diseases resulting from tobacco use accounts for 75% of American healthcare spending (136) which equates to roughly \$170 billion per year (17). Cigarette smoking is the predominant form of tobacco use and in the United States, almost 20% of American adults use tobacco; is more common in males than females; is more frequent among people with less education and lower socio-economic status (16; 148; 149). Of critical importance for young Americans is the fact that nearly all tobacco use begins in childhood and adolescence. Among adults who had ever tried a cigarette, more than 80% reported trying their first cigarette before 18 years of age (136).

With concern toward the young Americans tasked with defending the United States, it is relevant to note that tobacco use is more prevalent in the military as compared to gender- and age-matched civilians (70). According to the 2011 Department of Defense (DoD) Health Related Behaviors Survey of Active Duty Military Personnel, total DoD cigarette smoking prevalence was 24%. However, other research has indicated a higher rate of tobacco smoking for military service members, with prevalence estimates being around 40% (100), compared to 15.1% of the entire U.S. adult population (15). The mean prevalence rate for smokeless tobacco users in the military is about 9.4%, and the majority of smokeless tobacco users in the military are enlisted white males (10).

In the military, tobacco use has been identified as a significant threat to mission and unit readiness, and the number one cause of preventable mortality and premature death in the United States (100). Given the harmful, and often deadly, effects of smoking on health and the concern about smoking contributing to the use of harder substances,

there have been efforts at the policy level to reduce smoking in the military. Each branch of the armed forces has launched individual initiatives to curb smoking, and the DoD has implemented policy banning smoking from all DoD workplaces (70). Significant resources are spent on smoking cessation, raising awareness of the risks of use, and enacting policies to discourage use; yet, tobacco use among military members continues (100). Because of the disproportionately high usage of tobacco products in the military, it is important to examine the addictive component of tobacco and explore possible implications of this high rate of use on subsequent use of harder drugs.

Nicotine

Experimental research indicates that nicotine (3-(1-methyl-2-pyrrolidinyl)-pyridine) is the most important, highly addictive, naturally occurring component in tobacco (43; 47; 56; 66; 71; 81; 108; 109; 117; 124; 135; 142; 150). Nicotine is a highly toxic liquid alkaloid found in several plants and the half-life of nicotine is approximately 2 hours (50; 56). Biological theories have emphasized the positively and negatively reinforcing properties of nicotine in accounting for addiction (9; 42; 62; 135; 139). The resulting maintenance of tobacco use is especially driven by the psychopharmacological mechanisms involved with nicotine (50). Furthermore, there is substantial evidence that psychoactive effects of nicotine contribute to long-term tobacco use and nicotine addiction (24; 46; 52; 59; 63; 119; 124).

The location of nicotine, in the form of cigarettes, chew, and/or smokeless tobacco, in the drug sequence (i.e., as a lower order drug) (74); has been particularly troublesome because tobacco use often precedes use of other drugs, such as alcohol, marijuana, and harder drugs (e.g., opioids) (38; 95). According to the Gateway

Hypothesis, initiation of nicotine use (e.g., cigarettes, chew) precedes and may cause initiation of harder drug use (e.g., opioids). However, despite extensive correlational data, there is little direct empirical evidence that addresses potential causal mechanisms of the Gateway Hypothesis as it relates to nicotine use.

Possible explanations for initial nicotine use and subsequent use or abuse of other drugs include psychological, sociological, economic, behavioral, and/or biological variables (54). It is difficult, if not impossible, to examine or manipulate these variables in human children, adolescents, or adults; therefore, reasons for initiation of use and maintenance of use are restricted to self-report measures. For example, human studies of the Gateway Hypothesis are unable to determine causality between lower drugs and higher drugs because of multiple variables that cannot be controlled (e.g., availability, legal issues, cultural factors). Further, in naturalistic population studies, it is not possible to control for all potentially relevant factors. Additionally, it is unethical to experimentally study the Gateway Hypothesis in humans because the manipulation of exposure to lower and higher drug classes is not permissible (e.g., it is unethical to manipulate the use of tobacco and morphine use in humans). By utilizing a rat model, potential confounding variables are controlled that may influence the relationship between nicotine and subsequent morphine use, such as economic factors, social pressure, and/or availability. A rat model provides the opportunity to examine whether prior use of one drug (i.e., nicotine) increases subsequent use of another drug (i.e., morphine), which is fundamental to understand the Gateway Hypothesis.

THE VALUE OF ANIMAL MODELS

Animal models are able to contribute meaningfully to the study of the Gateway Hypothesis and allow for testing of causal mechanisms regarding drug effects (54). In studying multicausal phenomena, such as reasons why people use drugs, all variables may be potentially relevant and animal models allow the separation and control of variables so that individual causal contributions can be assessed (54). Because of the wide array of reasons people use nicotine and other, higher drugs, animal models are a method to remove psychological, sociological and economic factors and focus on the behavioral and biological factors that may be important determinants in the Gateway Hypothesis. Although animal models cannot mimic the variety of cultural, social, and psychological factors that determine drug behavior in humans, animal models can examine whether prior use of one drug increases the subsequent use of another drug, which is a fundamental concept relevant to the Gateway Hypothesis.

Also, there are ethical limitations in conducting true experiments with adolescent humans to investigate whether or not pre-exposure to nicotine affects subsequent morphine self-administration. An animal model provides a direct and ethical means to investigate causality, minimize confounding factors, and isolate variables of interest. Researchers have advocated for such animal models to help ascertain these important relationships (78). An animal model allows experimental control of key independent variables (i.e., nicotine/saline, male/female), and dependent variables (i.e., morphine self-administration).

The Gateway Hypothesis only has been addressed in three controlled experiments (39; 80; 86). The results of these studies do not provide a clear answer regarding the Gateway Hypothesis and each of these studies has methodological limitations. Klein (80)

examined effects of 19 days of saline, 6 mg/kg/day nicotine, or 12 mg/kg/day nicotine administration via osmotic minipump on subsequent oral fentanyl (50 µg/ml) self-administration by adolescent female and male Wistar rats for 4 weeks. Results revealed that only 6 mg/kg/day of nicotine exposure increased subsequent fentanyl oral self-administration in non-stressed male rats; 12 mg/kg/day dosage did not result in increased fentanyl self-administration for male rats; neither dosage of nicotine changed fentanyl self-administration in female rats; and immobilization stress prior to fentanyl access attenuated or reversed the effects of 6 mg nicotine/kg/day on subsequent fentanyl self-administration in male rats. Klein (80) concluded that the findings partially supported the Gateway Hypothesis but the results were not conclusive. Levine et al (86) examined 1 day and 7 days of nicotine self-administration (50 µg/ml) on subsequent effects of 4 days of one intraperitoneal cocaine administration (20 mg/kg) on locomotor activity and conditioned place preference of male C57BL/6J mice. Levine et al (86) reported that 7 days of nicotine exposure (but not 1 day of nicotine exposure) increased locomotor response to subsequent cocaine administration and these mice showed an increase in conditioned place preference for the cocaine-coupled chamber compared to mice exposed only to cocaine for 7 days. This priming of the cocaine response by nicotine occurred after more than 1 day of exposure to nicotine and concurrent exposure to nicotine and the first exposure to cocaine. The authors argued that these findings support the Gateway Hypothesis in that exposure of one drug increased the effects of the second, subsequently administered drug. However, it is important to note a co-author of this study was the creator of the Gateway Hypothesis (i.e., Denise Kandel); the drugs were all sympathomimetics that generally increase activity; and no experimental condition self-

administered the higher order drug, cocaine. Frederiksson et al (39) examined effects of alcohol exposure on subsequent cocaine self-administration and reinstatement. The authors exposed Wistar or alcohol-preferring rats to 20% alcohol or water in home cages for 7 weeks (three, 24-hour sessions/week) or exposed Wistar rats to intoxicating levels of alcohol in vapor chambers for 14 hours/day for 7 weeks. Following alcohol exposure, acquisition of cocaine intravenous self-administration was tested using 0.125, 0.25, 0.5, and 1.0 mg/kg cocaine. Subsequently, lever pressing was extinguished and reinstatement of cocaine-seeking was induced by cocaine-paired cues and priming (0, 2.5, 5, 10 mg/kg intraperitoneal injections of cocaine) (39). Results indicated that pre-exposure to alcohol had no effect on cocaine self-administration or relapse. The results did not support the Gateway Hypothesis.

SEX DIFFERENCES

Opioids

Research indicates that women are more sensitive to pain than men (113) and more likely to report chronic pain (41), which could contribute to the high rates of opioid prescription use among women (3). In addition, women may be more likely to take prescription opioids without a prescription to cope with pain, even when men and women report similar pain levels. Research also suggests that women are more likely to misuse prescription opioids to self-treat for other problems such as anxiety or tension (92).

A possible consequence of prescription opioid misuse is fatal overdose, which can occur because opioids suppress respiration. Although men are more likely to die from prescription drug overdoses, women are closing the gap on drug overdose deaths. From 1999 to 2010, deaths from prescription opioid pain reliever overdoses increased more for

women (400%) than for men (265%) (14). In 2010, about 18 women per day (14) compared to about 27 men (88) died from overdosing on prescription pain relievers. Middle-aged women (ages 45-54) are more likely than women of other age groups to die from a prescription pain reliever overdose (14).

Tobacco

The 2014 Surgeon General Report tragically reported that over the past 50 years, the disease risks from tobacco smoking by women have risen to the point where they are now equal with men for lung cancer, chronic obstructive pulmonary disease, and cardiovascular diseases (137). While this finding is of critical importance, previous research has suggested that there are sex differences with regard to smoking behavior (58). Specifically, it has been reported that females score higher on negative affect reduction from smoking; and that under stress, females are more likely to try additional cigarettes after the initial smoking experience (40; 68; 69; 121). In light of these reported sex differences with regard to smoking behavior as well as numerous other research questions, it comes as no surprise that the National Institutes of Health now require the inclusion of females and males in all cell and animal studies (19). Because of potential sex differences in tobacco and opioid abuse, the current study included females and males to examine potential sex differences with regard to the Gateway Hypothesis.

CHAPTER 2: Overview and Specific Aims

The purpose of the current experiment was to investigate the Gateway Hypothesis by examining the effects of nicotine pre-exposure on subsequent morphine self-administration in a rat model. Because of the continued high rates of tobacco (cigarettes, smokeless tobacco, hookah, and chew/dip) use in the general population as well as the increased prevalence of opioid use and abuse among 18-45 year olds, this experiment is important to help understand whether there is a sequential causal relationship between nicotine and opioid use.

If pre-exposure to nicotine does, in fact, cause an increase in subsequent morphine self-administration, the focus in public health initiatives and policy should remain and intensify on preventing the use of nicotine. If, however, the results of the present experiment do not support that pre-exposure to nicotine leads to an increase in subsequent morphine self-administration, then public health and policy initiatives should focus more on the prevention and treatment of harder drugs (e.g., morphine, cocaine, heroin). There are two specific aims of the present experiment:

Specific Aim 1: To examine the effects of nicotine pre-exposure on subsequent morphine self-administration in a rat model.

Hypothesis 1: Rats previously exposed to nicotine will self-administer greater amounts of morphine compared to rats previously exposed to saline.

Rationale: The Gateway Hypothesis implies that there is a fixed relationship between two drug substances, such that one drug is regularly initiated before the other (sequencing); that the initiation of one drug increases the likelihood of initiation of the

second drug (association); that use of the first drug may cause use of the second drug (73-75). This research experimentally examined these possibilities.

Specific Aim 2: To examine the effects of nicotine pre-exposure on subsequent morphine self-administration in female and male rats.

Hypothesis 2: Morphine self-administration will be greater in male rats compared to morphine self-administration in female rats.

Rationale: Previous research has indicated that males have significantly higher rates of lifetime and past year prescription opioid nonmedical use compared to females (126) and males are more likely to die from opioid overdose compared to females (14). This experiment provided experimental findings that compared morphine self-administration rates between male and female rats.

Hypothesis 3: Male rats previously exposed to nicotine will self-administer greater amounts of morphine compared to female rats.

Rationale: Research has indicated that tobacco and opioid use is more common among males than females (14; 126; 137). Additionally, because males are more likely to use tobacco products, they might also be more likely to engage in use of harder drugs such as morphine (74).

CHAPTER 3: Methods

To address the above hypotheses, the current experiment was conducted as a 2 (saline, 6 mg/kg/day nicotine) x 2 (female, male), repeated measures (morphine self-administration days 1-10) full factorial, mixed design. This experimental design resulted in four conditions with eight subjects in each treatment condition (see Appendix B, Table 1). The number of subjects per condition was based on previous research that used similar subjects, independent and dependent variables, and methods (2; 4; 31; 34; 48; 53; 59; 84; 96; 102; 151; 152) that established 5-12 subjects per cell was sufficient to detect effects of nicotine and/or morphine drug exposure on naïve rats.

Additionally, a repeated-measures Analysis of Variance (rANOVA) power analysis was conducted using G*Power 3.1 (36; 37) and resulting treatment cell sizes were based on the power analyses and previous studies (2; 4; 31; 34; 48; 53; 59; 84; 96; 102; 151; 152). The rANOVA, within-between interaction, power analysis indicated a total sample size requirement of 24 subjects (for four groups [nicotine/saline and female/male] and ten repeated measures [morphine self-administration days 1-10]) for power of 0.95. Effect size (f) was estimated to be 0.3 based on previous literature on morphine self-administration in rats that found effect sizes ranging from 0.24 – 0.44 (7; 83). In addition, previous literature regarding behavioral measures and nicotine administration have yielded moderate to large effect sizes, $\eta^2 = 0.06-0.14$ (2; 4; 31; 34; 48; 53; 59).

Because of ethical and logistical limitations (e.g., availability of 8 operant conditioning chambers for morphine self-administration) of running 24 animals at one time, multiple cohorts of this study were run to obtain necessary power. Additionally,

because rats underwent surgery to implant catheters in their right jugular vein, it was anticipated that some rats would die during the surgery or the catheters would become clogged at some point during the morphine self-administration phase. Therefore, an additional 8 rats were included in order maintain sufficient power.

There was a total of 32 subjects (8 per condition) which were investigated in four separate, counter-balanced within-sex cohorts of eight subjects (see Appendix B, Figure 1 for treatment cell breakdown). Experimental procedures and environmental conditions were identical across the four cohorts (see Appendix A, Figure 2 for experimental timeline). Animal husbandry conditions, independent and dependent variables, experimental timeline, and data analytic strategy are explained below.

SUBJECTS

The subjects of the experiment were 16 female and 16 male Sprague Dawley (SD) rats from Charles River Laboratories (Wilmington, Massachusetts). The adolescence of SD rats ends around 42 and 55 days for females and males, respectively (104; 123), and adulthood begins around 60 days (87). The rats were approximately 54 days old upon arrival to model emerging adulthood in humans and to be comparable in age to young adult combat veterans (152). To investigate sex differences, the experiment included an equal number of female and male rats.

HOUSING

Subjects were individually housed in standard polycarbonate shoebox cages (42.5 x 20.5 x 20 cm) with hardwood chip bedding (Pine-Dri). Subjects were single housed to prevent the effects of social and environmental enrichment on activity or drug self-administration (31; 110; 115). Cage bedding was changed two times each week by

husbandry personnel in the Uniformed Services University (USUHS) Laboratory of Animal Medicine for animal health and to minimize any effects of unclean housing conditions. The subjects had free and continuous access to standard, bland laboratory chow (Harlan Teklad 4% Mouse/Rat Diet 7001) and water. The temperature of the housing rooms was kept at approximately 23°C with a relative humidity of approximately 40%. The lighting in the housing rooms was programmed to a 12-hour reverse light cycle (0600 lights off, 1800 lights on) to synchronize the active part of the rats' circadian cycle and measurement of the rats' behaviors with the human work day because rats are nocturnal creatures (110). Prior to data collection, rats were numbered and gentled by experimenters to allow for the animals to become accustomed to the touch, sounds, and odors of the experimenters. The gentling procedures occurred 7 days prior to behavioral testing (110). The experiment was conducted under an approved protocol by USUHS Institutional Animal Care and Use Committee (MPS-14-898; see Appendix A, Figure 1) and in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (99).

INDEPENDENT VARIABLES

There were three independent variables for this study: nicotine, sex, and time. Each variable is discussed below.

Nicotine

Nicotine bitartrate (Sigma Pharmaceuticals; dissolved in physiological saline) solution was administered via osmotic mini-pump (Alzet Model 2002, Lot numbers 10269-12 and 10317-13, Durect Corporation). Minipumps were filled with either a nicotine solution (6 mg/kg/day) or saline (0 mg/kg/day) with a delivery rate of 0.42

μl/hour (128). Nicotine dosages were prepared based on the average weight of subjects within each of the two nicotine conditions (see Appendix A, Figure 3 for sample nicotine bitartrate calculation). The 0 mg and 6 mg nicotine bitartrate/kg/day dosages represent a non-smoker and a ½- to 1-pack per day smoker, respectively (48; 52; 53; 59; 144) and was selected on the basis of previous studies of nicotine and body weight, food consumption, behavioral responses and biochemical responses (51; 55; 57).

This drug administration paradigm and dosage have been used extensively in rats and has reported results that are comparable with effects of tobacco smoking by humans (1; 2; 48; 49; 52; 143). (112)Richardson and Tizabi (1994) indicated that 6 mg nicotine/kg/day minipump administration, in male rats, yields plasma nicotine levels of approximately 100 ng/ml, which is similar to the average (i.e., ½ to 1 pack of cigarettes per day) human smoker. The inclusion of 0 mg/kg/day nicotine (i.e., saline) and 6 mg/kg/day nicotine dosages and not higher or lower dosages were based on recent findings that suggested activity differences in rats were mostly limited to moderate amounts of nicotine (Moosey, 2014).

Sex

Females and males were included in the present experiment because sex differences are present in both nicotine and opioid use among females and males, humans and animals (3; 14; 41; 96; 137; 141; 151; 152). Additionally, because of these reported sex differences with regard to nicotine and opioid use as well as differences in other research domains, the National Institutes of Health now require the inclusion of females and males in all animal studies (19).

Time

Time was included as a within-subject independent variable to allow for: observation of changes in dependent variables over time (i.e., morphine self-administration, inactive lever presses, horizontal activity as measured via the Open Field Activity [OFA]) and verification of nicotine delivery to the rat through body weight assessments. Behavioral measures were collected at six time points: baseline (Day -2), time 1 (Day 11), time 2 (Day 20), baseline 2 (Day 30), time 3 (Day 36), and time 4 (Day 43). Prior to formal data collection at baseline, a 60-minute acclimation period was conducted to allow the rats to acclimate to the OFA environment and thereby prevent any potential stress effects on horizontal activity measures (Day -4) (110). Please refer to Appendix A, Figure 2 for the experimental timeline.

DEPENDENT VARIABLES

There were four dependent variables for this study: morphine self-administration, inactive lever presses, horizontal activity, and body weight. Descriptions of equipment, procedures, and each measurement are provided in this section.

Morphine Self-Administration (MSA)

Morphine hydrochloride was administered intravenously at a rate of 0.5 mg/kg/0.1 ml/5 s infusion. Previous literature reported that male rats will self-administrator this concentration by lever press or nose poke (129; 153; 154). By measuring the amount of lever presses, intravenous MSA was assessed. Amount of morphine consumed for all subjects was measured as a continuous dependent variable measured by volume of morphine self-administered by the subjects over a period of 10 days (3 hour sessions, 5 days per week, Monday – Friday, for 2 weeks).

Operant Conditioning Chamber

Eight operant conditioning chambers (Med Associates Inc, St. Albans, VT) were used for the MSA phase. Each chamber is equipped with two levers, an infusion pump, and a 10-ml glass syringe connected to a fluid swivel (Instech, Plymouth Meeting, PA) by Teflon tubing (84; 102). After 10 days of recovery from the catheter surgery (see below), animals were placed in the operant conditioning chambers and allowed to self-administer morphine (0.5 mg/kg/infusion, 0.1 ml across 5 s) on one lever press/injection (fixed ratio 1) reinforcement schedule in a daily 3-hour session (5 days/week, Monday – Friday) for 2 weeks. During each drug infusion, a cue light above the active lever was illuminated, and the house light was extinguished for an additional 20-s (time-out) period during which the cue light above the active lever was turned off. The lever press responses on the inactive lever and the time-out period were recorded but have no programmed consequences (84; 102); however, inactive lever press data were utilized to assess differences in lever presses between the active and inactive levers. Drug intake was assessed while rats were in the operant conditioning chambers 5 days/week (Monday – Friday) for 2 weeks (Days 31- 42) and body weight was measured on each MSA day at 0800.

Inactive Lever Presses

Inactive lever presses were recorded and analyzed to assess whether MSA as measured by the active lever presses, was the result of drug-seeking behavior or changes in locomotor activity or exploratory behavior (84; 102).

Horizontal Activity (HA)

HA is a measurement of the animal's general health and gross motor movement (31; 59; 60; 85; 151). The measurement is based on the number of times the animal has broken an infrared photoelectrical beam on the lower half of the Open Field Activity chamber. HA was utilized to assess the rat's overall health and locomotor activity. Additionally, HA was measured to determine whether MSA was affected by overall levels of activity, in general, and more specifically to determine if there were differences in activity that might contribute to MSA.

Body Weight

Body weight of all rats was measured throughout the experiment (see Appendix A, Figure 2 for timeline). Body weight was utilized as a measure of overall general health and as a manipulation check during the nicotine pre-exposure phase to ensure rats were appropriately receiving either nicotine or saline depending on their drug pre-exposure condition. Previous research has reported that rats exposed to nicotine do not gain weight as quickly as rats not exposed to nicotine (52; 53; 57; 143); therefore, body weight was utilized to ensure that rats were receiving nicotine via the osmotic minipumps. Additionally, body weight was utilized to determine the appropriate amount of morphine hydrochloride rats were to receive during the morphine self-administration phase.

EQUIPMENT AND PROCEDURES

Open Field Activity (OFA)

OFA is a measurement of an animal's natural and unconditioned locomotor activity when placed into an environment and left undisturbed for a period of 60 minutes. OFA has been used in investigations of nicotine, morphine, stress, and affect with rats

(31; 32; 35; 59; 152) and data obtained offer indices of overall health and locomotor activity.

OFA was measured using Accuscan Superflex Sensor Version 2.2 infrared photocell system in Accuscan Instruments Standard Animal Cages (measuring 40 x 40 x 30 cm; Accuscan Instruments Incorporated, Columbus, OH) located in a separate room from the animal housing room. The OFA room is designed and constructed with materials to decrease external acoustic disruptions that may create unwanted variance. The Standard Animal Cage is constructed of polycarbonate with a ventilated, removable Plexiglas lid that allows sufficient airflow while preventing the animal from escaping during the trial. The animal's movements are tracked by three paired 16-photocell Superflex Sensors which continuously transmit the location data to the Accuscan Superflex Node located on the upper-rear of the Standard Animal Cage. The Superflex Node transmits the OFA data to a central computer through a universal serial bus connector. The data from the eight chambers were processed and aggregated by Accuscan Fusion Software (Version 3.4) for further data interpretation and analysis (96; 141; 151; 152).

Data collection was conducted during the rats' active cycle for a period of 60 minutes for each measurement time point (see Appendix A, Figure 2 for experimental timeline). The OFA equipment begins recording data immediately after the rats are placed into their respective Standard Animal Cage. The experimenter then exited the testing room and turned off the testing room's overhead red light leaving the animal in complete darkness during the trial. After the 60 minute testing trial concludes, the animals were retrieved from the Standard Animal Cage and returned to their home cages

in the housing rooms. The experimenter then cleaned and deodorized each Standard Animal Cage with Clidox (Pharmacal, Naugatuck, CT) solution between each subject to ensure proper maintenance as well as to prevent undue stress from residual feces and urine.

Minipump Surgery

On day -1, rats were assigned to either the nicotine group or saline control group based on body weights to ensure that all group members had a similar starting average body weight within approximately 10 grams of each other. Rats that were more than two standard deviations from the mean weight of their respective sex (female, male) were assigned to the saline group. On day 0, all rats were anesthetized individually in a plastic chamber using a 5% isoflurane/oxygen mixture (flow rate: 0.5 to 1.0 L/min) and were maintained in their anesthesia-induced unconscious state throughout the surgical implantation of the minipumps via a nose cone and tube that delivered a combination of 0.25 – 3% isoflurane and oxygen from the induction chamber. One dose of buprenorphine (0.05 - 0.1 mg/kg) was given subcutaneously for pain relief. The minipump equations and implantation procedures were based on Grunberg (48). Please see Appendix A, Figure 4 for surgery procedure form.

To implant the Alzet osmotic minipumps (type 2002, Durect Corporation, Palo Alto, CA), a small 3 cm x 5 cm area below the scapula and between the withers was shaved and the skin was swabbed with a Betadine solution. A 1 cm mid-scapular transverse incision was made and a “pocket” was created for the minipump by inserting blunt-nosed scissors into the incision and spreading the subcutaneous tissue. The minipump was inserted and the incision was closed with two or three 9 mm stainless steel

wound clips and the rat was returned to its home cage for monitoring until recovery (96; 141; 151; 152). Rats in both the nicotine condition and saline condition received the minipump surgery. Those rats in the nicotine condition received minipumps filled with a nicotine solution and those in the saline condition received minipumps filled with saline. Recovery from surgery was determined to occur when the rats were fully ambulatory. Please see Appendix A, Figures 5 and 6 for location of Alzet minipump implantation and cross-sectional illustration of Alzet Model 2002 (Durect Corporation) minipump.

Catheter Surgery

On day 22 (see Appendix A, Figure 2 for experimental timeline), all rats underwent a surgery to implant catheters for subsequent morphine self-administration. Rats were anesthetized with a ketamine/xylazine (100mg/kg and 10mg/kg) intraperitoneal solution. The back of the animal, from thighs to neck, and the right side of the throat and chest down were shaved and sterilized with Betadine. A 4 cm vertical incision was made in the midscapular region beginning about 1 cm below the scapula and the minipump was removed prior to the implantation of the catheter. Blunt-nosed surgical scissors were used to create a larger subcutaneous (SC) pocket for the catheter base. The animal was then positioned on its back, the jugular vein was located and using a scalpel, a 1.5 – 2 cm incision was made over the vein, penetrating the dermis and epidermis. A second SC space was made with blunt-nosed surgical scissors for the catheter to run from the jugular vein over the right shoulder and to the base of the catheter. The catheter base was put into place before the catheter was inserted into the vein to prevent tugging at the vein and/or catheter assembly. Two fine forceps with curved tips were used to gently tease apart the tissue that overlaps the vein. With the vein exposed, a 1 mm thick, 5 cm

long steel bar was slid under the vein to maintain its exposure. The vein was cleaned with a saline solution and then cut halfway through with artery scissors and a needle was inserted. A silastic catheter (Dow Corning, Midland, MI; 0.02" inner diameter x 0.037" outer diameter) was then inserted into the jugular vein and secured to the neck muscles with Bard Marlex mesh (Ethicon Inc., Somerville, NJ) to the surrounding tissue. Correct placement was checked by flushing the catheter with saline. The catheter was sutured to the vein and the incisions closed with stitches (129). Please refer to Appendix A, Figure 7 for illustration of catheter.

The other end of the catheter was threaded SC over the right shoulder to the rat's back to exit the skin and exposed to the outside through the back using a 22-gauge guide cannula (Plastics One, Roanoke, VA) imbedded in dental cement on Bard Marlex mesh (Ethicon Inc., Somerville, NJ) through the 2-cm opening near the midscapular region (83; 84; 94). Both incisions were secured with 9 mm wound clips and antibiotic ointment (Neosporin) was applied. Then 0.2 ml of saline containing heparin and gentamycin (30 U/mL) was injected into the catheter. See Appendix A, Figure 8 for illustration of catheter implantation (129). Rats were observed during their recovery from the surgery until the anesthesia wore off and they were ambulatory. Rats were allowed 10 days to recover (refer to Appendix A, Figure 2 for experimental timeline) from catheter surgery prior to morphine self-administration. During this 10 day period, the catheters were flushed with saline/heparin solution, one time per day, and Neosporin was applied to the incisions on each rat's back and chest.

PROCEDURE

The experiment was conducted in four phases: (a) baseline; (b) nicotine or saline exposure; (c) recovery from catheter surgery/cessation of nicotine or saline; and (d) morphine self-administration. Please refer to Appendix A, Figure 2 for the experimental timeline. Prior to the baseline phase, animals were numbered and then gentled for approximately 5 minutes per day for 3 days.

Baseline (BL)

Rats were handled for 10 minutes per day and body weight was measured on days (-8), (-6), (-4), (-2) and (-1). Days were determined based on the day of the minipump surgery, which is recorded as Day 0. On day (-4) rats were placed into the OFA chamber for acclimation, with BL OFA measured on day (-2). After the baseline period (days -9 to -1), animals were assigned to experimental (nicotine) and control (saline) groups based on body weight to ensure that the treatment groups did not differ significantly from one another.

Groups

Body weights from rats were assessed for two consecutive days (-2 and -1) to ensure rats were adequately gaining weight. Rats were rank ordered from lightest to heaviest and separated into experimental (nicotine) and control (saline) groups by selecting every other rat to be in the experimental group beginning with the first rat (52). Rats that were more than two standard deviations from the mean weight, in either direction, of their respective sex (female, male) were assigned to the saline group. This movement of rats from the nicotine to the saline condition was done because nicotine calculations were based on the mean weight of rats in the nicotine group (Please see

Appendix A, Figure 3 for sample nicotine calculation). Rats that were too heavy or too light would either have received too little nicotine or too much nicotine, respectively, to adequately model an average human smoker. Additionally, without this adjustment, results regarding effects of nicotine pre-exposure on subsequent morphine self-administration would be more difficult to interpret with an increased variance of nicotine exposure in rats assigned to the nicotine condition.

Nicotine or Saline Exposure

Following BL, osmotic minipumps, containing either saline or nicotine, were implanted (day 0) into all subjects based on procedures reported in the literature and described above (1; 2; 48; 52; 55; 57). Nicotine exposure lasted a total of 21 days (days 0-21) with OFA trials (i.e., HA) measured on day 11 (Time 1 [T1]), and day 20 (Time 2 [T2]).

Recovery from Catheter Surgery

Following catheter surgery (see above) animals were kept in their home cages for 10 additional days (days 23-29) to allow for recovery from the catheter surgery prior to the morphine self-administration phase of the experiment. OFA was measured on day 30 (Baseline 2 [BL2]).

Morphine Self-administration

Following 10 days of recovery, animals were placed in the operant conditioning chambers and allowed to self-administer morphine (0.5 mg/kg/infusion, 0.1 ml across 5 s) on one lever press/injection (fixed ratio 1) reinforcement schedule in a daily 3-hour session (5 days/week, Monday – Friday) for 2 weeks (days 31-42) See above for

morphine self-administration paradigm. OFA was measured on day 36 (Time 3 [T3]) and day 43 (time 4 [T4]). Refer to Appendix A, Figure 2 for the experimental timeline.

CHAPTER 4: Data Analytic Strategy

STATISTICAL ANALYSES

All data were analyzed using SPSS 20.0. Tests were two-tailed using $\alpha = .05$. The analyses consisted of several steps for each dependent variable (morphine self-administration [MSA], horizontal activity [HA], inactive lever press, and body weight): independent samples t-tests to examine baseline differences; omnibus repeated-measures Analysis of Variance (rANOVA) to measure change over time (baseline [BL], time 1 [T1], time 2 [T2], baseline 2 [BL2], time 3 [T3], time 4 [T4]) for each group for each dependent variable to evaluate Hypothesis 1; rANOVAs comparing females vs. males to evaluate Hypothesis 2; and rANOVAs which split for sex (female, male) to evaluate Hypothesis 3. Greenhouse-Geisser corrections were applied if a violation of sphericity was detected. Planned-comparisons of nicotine groups are presented as pairwise comparisons with mean differences between the two significant values. *Post-hoc* analyses were not conducted given that each variable did not involve more than two levels. As all analyses are rANOVAs, raw values (means, standard deviations) are reported. Data presented in the text include only significant results. Figures, tables, and all data (significant and non-significant) are presented in Appendices A and B, respectively.

CHAPTER 5: Results

MISSING DATA

Thirty-two rats were included in the analyses for the nicotine/saline exposure phase. Two female rats assigned to the saline condition died during the catheter surgery. A total of 14 female rats (eight assigned to the nicotine condition and six assigned to the saline condition) were included in the analyses following catheter surgery (i.e., morphine self-administration [MSA], inactive lever presses, horizontal activity, and body weight). During MSA, three male rats assigned to the saline condition experienced clogged catheters (MSA day 5, day 6, and day 7; respectively). Following the clogged catheters, each rat was unable to self-administer morphine from the day of the clog to the end of the MSA days. A total of thirteen male rats (eight assigned to the nicotine condition and five assigned to the saline condition) were included in the analyses during MSA. The missing data are assumed to have occurred at random; therefore, no threat to internal validity is likely to have occurred.

MORPHINE SELF-ADMINISTRATION

Morphine Self-Administration Repeated-measures Analysis of Variance

Please refer to Appendix A, Figure 9 for morphine self-administration (MSA) results for all subjects. An omnibus repeated-measures Analysis of Variance (rANOVA) was performed on MSA data. Mauchly's Test of Sphericity was significant at $p < .001$; therefore, the Greenhouse-Geisser correction was applied. The overall rANOVA revealed a significant main effect of drug ($F[1, 23] = 10.634$, $p = .003$, $\eta_p^2 = .298$), such that rats assigned to the saline condition ($M = 10.682$, $SEM = 1.425$) self-administered greater

amounts of morphine compared to rats assigned to the nicotine condition ($M = 4.644$, $SEM = 1.182$). MSA increased significantly over time ($F[3.406, 85.138] = 7.631$, $p < .001$, $\eta_p^2 = .234$). There was no significant drug x time interaction.

A rANOVA was performed on the MSA data to compare females and males. Please refer to Appendix A, Figures 10 and 11 for MSA for females and males, respectively. Mauchly's Test of Sphericity was significant at $p < .001$; therefore, the Greenhouse-Geisser correction was applied. The overall rANOVA reveal a significant main effect of drug ($F[1,23] = 9.630$, $p = .005$, $\eta_p^2 = .295$, such that rats assigned to the saline condition ($M = 10.622$, $SEM = 1.485$) self-administered greater amounts of morphine compared to rats assigned to the nicotine condition ($M = 4.644$, $SEM = 1.227$). There was no main effect of sex. MSA increased significantly over time ($F[3.238, 74.473] = 7.321$, $p < .001$, $\eta_p^2 = .241$). There were no drug x sex, drug x time, sex x time, or drug x sex x time interactions. Details of these analyses appear in Appendix B, Table 2.

Morphine Self-Administration Repeated-measures Analysis of Variance Split by Sex

Data were split by sex to examine main effects for drug, time, and all interactions for females and males, separately. Please refer to Appendix A, Figures 10 and 11 for MSA results for female and male rats, respectively.

Females

For female rats, Mauchly's Test of Sphericity was significant at $p < .001$; therefore, the Greenhouse-Geisser correction was applied. The rANOVA revealed a significant main effect of drug ($F[1, 12] = 6.052$, $p = .030$, $\eta_p^2 = .335$), such that the rats

assigned to the saline condition ($M = 11.283$, $SEM = 2.000$) self-administered more morphine than the rats assigned to the nicotine condition ($M = 4.775$, $SEM = 1.732$). MSA significantly increased over time ($F[2.951, 35.418] = 7.283$, $p = .001$, $\eta_p^2 = .378$). There was no drug x time interaction. See Appendix B, Table 3 for more detailed information.

Males

For male rats, Mauchly's Test of Sphericity was significant at $p < .001$; therefore, the Greenhouse-Geisser correction was applied. The rats assigned to the saline condition self-administered greater amounts of morphine compared to the rats assigned to nicotine condition; this difference approached significance ($F[1, 11] = 3.780$, $p = .078$, $\eta_p^2 = .256$). There was no significant main effect of time and no significant drug x time interaction. See Appendix B, Table 4 for more detailed information.

Summary of Morphine Self-Administration

There was a significant main effect for drug and time, such that rats assigned to the saline condition self-administered significantly more morphine compared to rats assigned to the nicotine condition. In addition, morphine self-administration increased significantly over time, controlling for both drug and sex. For female and male rats, there was a significant, or approaching significant, effect of drug and time such that morphine self-administration increased over time and rats assigned to the saline condition self-administered greater amounts of morphine compared to rats assigned to the nicotine condition.

Inactive Lever Repeated-measures Analysis of Variance

Please refer to Appendix A, Figure 12 for inactive lever results for all subjects. An omnibus rANOVA was performed on the inactive lever data. Mauchly's Test of Sphericity was significant at $p < .001$; therefore, the Greenhouse-Geisser correction was applied. There was no significant main effect of drug. The rANOVA revealed a significant main effect of time ($F[4.505, 108.129] = 7.029, p < .001, \eta_p^2 = .227$), such that inactive lever presses decreased significantly over time. There was no significant drug x time interaction.

A rANOVA was performed on the inactive lever data to compare females and males. Please refer to Appendix A, Figures 13 and 14 for inactive lever data for females and males, respectively. Mauchly's Test of Sphericity was significant at $p < .001$; therefore, the Greenhouse-Geisser correction was applied. There was no main effect of drug or sex. Inactive lever presses decreased significantly over time ($F[4.292, 94.418] = 7.106, p < .001, \eta_p^2 = .244$). There were no significant drug x sex, drug x time, sex x time, or drug x sex x time interactions. Details of these analyses appear in Appendix B, Table 5.

Inactive Lever Repeated-measures Analysis of Variance Split by Sex

Data were split by sex to examine main effects for drug, time and all interactions for females and males, separately. Please refer to Appendix A, Figures 13 and 14 for inactive lever results for female and male rats, respectively.

Females

For female rats, Mauchly's Test of Sphericity was significant at $p < .001$; therefore, the Greenhouse-Geisser correction was applied. There was no significant main

effect of drug; however, the rANOVA did reveal a significant effect of time ($F[3.383, 40.599] = 4.844, p = .004, \eta_p^2 = .288$), such that inactive lever presses decreased over time. There was no drug x time interaction. See Appendix B, Table 6 for more detailed information.

Males

For male rats, Mauchly's Test of Sphericity was significant at $p < .001$; therefore, the Greenhouse-Geisser correction was applied. There was no significant main effect of drug. Inactive lever presses decreased significantly over time ($F[3.259, 32.594] = 3.478, p = .024, \eta_p^2 = .258$). There was no drug x time interaction. See Appendix B, Table 7 for more detailed information.

Summary of Inactive Lever Activity

For both female and male rats, there was no significant effect of drug or sex; however, inactive lever presses decreased significantly over time.

HORIZONTAL ACTIVITY

Horizontal Activity Repeated-measures Analysis of Variance

Please refer to Appendix A, Figure 15 for horizontal activity (HA) for all subjects. An independent sample's t-test was conducted to assess whether significant differences were present at baseline. There were no significant differences in baseline HA for either females or males (See Appendix B, Table 8). An omnibus rANOVA was performed on all HA timepoints (BL, T1, T2, BL2, T3, and T4). Mauchly's Test of Sphericity was significant at $p < .001$; therefore, the Greenhouse-Geisser correction was applied. The overall rANOVA revealed a main effect of drug ($F[1, 26] = 5.904, p = .022, \eta_p^2 = .185$),

such that overall, HA, as measured over the duration the experiment, was greater for the rats assigned to the saline condition compared to rats assigned to the nicotine condition. There was no main effect of time or drug x time interaction.

A rANOVA was performed on the HA data to compare females and males. Please refer to Appendix A, Figures 16 and 17 for HA data for females and males, respectively. Mauchly's Test of Sphericity was significant at $p < .001$; therefore, the Greenhouse-Geisser correction was applied. The overall rANOVA revealed a significant main effect of drug ($F[1, 24] = 5.650, p = .026, \eta_p^2 = .191$), such that rats assigned to the saline condition exhibited greater horizontal activity compared to rats assigned to the nicotine condition. There was no main effect of sex or time. There was a significant sex x time interaction ($F[2.586, 62.056] = 4.986, p = .005, \eta_p^2 = .172$). There was no significant drug x time or drug x sex x time interaction. See Appendix B, Table 8 for more detailed information.

Horizontal Activity Repeated-measures Analysis of Variance Split by MSA

Please refer to Appendix A, Figure 15 for HA for pre-MSA and during-MSA HA. Data were split by pre-MSA (BL, T1, T2, BL2) and during MSA (T3, T4) to examine main effects for drug, time, sex, and all interactions.

For HA pre-MSA, Mauchly's Test of Sphericity was not significant at $p = .115$; therefore, sphericity is assumed. There were no significant interactions (drug x sex x time, drug x sex, drug x time, or sex x time) nor were there any significant main effects of drug, sex, or time. See Appendix B, Table 10 for more detailed information.

For HA during-MSA, sphericity is assumed because only two time points are being analyzed. The overall rANOVA revealed a significant sex x time interaction ($F[1,$

24] = 12.764, $p = .002$, $\eta_p^2 = .347$). There was a significant main effect of drug ($F[1,24] = 5.045$, $p = .034$, $\eta_p^2 = .174$), such that rats assigned to the saline condition ($M = 14415.035$, $SEM = 1643.894$) exhibited greater HA compared to rats assigned to the nicotine condition ($M = 9372.192$, $SEM = 1529.250$). There also was a significant main effect of time ($F[1, 24] = 6.618$, $p = .017$, $\eta_p^2 = .216$), such that HA was significantly greater at T3 ($M = 12484.366$, $SEM = 1353.174$) compared to T2 ($M = 10302.861$, $SEM = 1205.866$). There was no significant main effect of sex and no significant drug x sex x time, drug x sex, or drug x time interactions. See Appendix B, Table 11 for more detailed information.

Horizontal Activity Repeated-measures Analysis of Variance Split by Sex

Data were split by sex to examine main effects for drug, time, and all interactions for females and males, separately. Please refer to Appendix A, Figures 16 and 17 for HA of female and male rats, respectively. An independent sample's t-test was conducted to assess whether or not significant differences were present at baseline for females and males, separately. There were no significant differences in baseline HA for either females or males (See Appendix B, Table 12 and 14, respectively).

Females

For female rats, Mauchly's Test of Sphericity was significant at $p < .001$; therefore, the Greenhouse-Geisser correction was applied. There was no main effect of drug. There was a main effect of time ($F[2.000, 26.002] = 5.181$, $p = .013$, $\eta_p^2 = .285$), such that activity at T3 (6602.210 , $SEM = 1249.851$) was significantly less than all other time points (BL [$M = 10806.339$, $SEM = 749.291$], T1 [$M = 11539.625$, $SEM =$

909.074], T2 [M = 11264.634, SEM = 811.642], BL2 [M = 10412.786, SEM = 745.936], and T4 [M = 14201.982, SEM = 2073.247]). There was no drug x time interaction. See Appendix B, Table 13 for more detailed information.

Males

For male rats, Mauchly's Test of Sphericity was significant at $p = .050$; therefore, the Greenhouse-Geisser correction was applied. There was no main effect for drug or time. There was no drug x time interaction. See Appendix B, Table 15 for more detailed information.

Horizontal Activity Repeated-measures Analysis of Variance Split by Sex and MSA

Data were split by sex and MSA to examine main effects for drug, time, and all interactions for females and males, separately. Please refer to Appendix A, Figures 16 and 17 for HA of female and male rats pre- and during MSA, respectively.

Females

For female rats pre-MSA, Mauchly's Test of Sphericity was significant at $p = .007$; therefore, the Greenhouse-Geisser correction was applied. There was no significant drug x time interaction or significant main effects of drug or time. See Appendix B, Table 16 for more detailed information.

For female rats during MSA, sphericity is assumed because only two time points are being analyzed. There was a significant main effect of time ($F[1, 13] = 15.889$, $p = .002$, $\eta_p^2 = .550$) such that HA at T3 (M = 14201.982, SEM = 2073.247) was significantly greater than horizontal activity at T2 (M = 6602.210, SEM = 1249.851). There was no

significant main effect of drug or significant drug x time interaction. See Appendix B, Table 17 for more detailed information.

Males

For male rats pre-MSA, Mauchly's Test of Sphericity was not significant; therefore, sphericity is assumed. There was no significant drug x time interaction or significant main effects of drug or time. See Appendix B, Table 18 for more detailed information.

For male rats during MSA, sphericity is assumed because only two time-points are being analyzed. There was no significant drug x time interaction and significant main effects of drug or time. See Appendix B, Table 19 for more detailed information.

Summary of Horizontal Data

There was a significant sex x time interaction, such that female rats increased in HA over time, but male rats did not. Additionally, the overall rANOVA revealed a significant main effect of drug, such that rats assigned to the saline condition exhibited greater HA compared to rats assigned to the nicotine condition controlling for sex and across all phases of the experiment. For female rats, there was a significant main effect of time, such that HA at T3 was significantly less than all other time points (BL, T1, T2, BL2, T4). Rats assigned to the nicotine condition and rats assigned to the saline condition did not significantly differ on HA during the pre-MSA phase when they were receiving nicotine or saline. Rats assigned to the saline condition exhibited greater HA compared to rats assigned to the nicotine condition during MSA when they no longer were receiving nicotine or saline.

BODY WEIGHT

Body Weight Repeated-measures Analysis of Variance

Please refer to Appendix A, Figure 18 for body weight for all subjects. An independent sample's t-test was conducted to assess whether or not significant differences were present at baseline. There were no significant differences in baseline body weight for either females or males (See Appendix B, Table 20). An omnibus rANOVA was performed on all body weight time points (see Appendix A, Figure 2 for timeline). Mauchly's Test of Sphericity was not significant; therefore, sphericity is assumed. The overall rANOVA revealed a significant main effect of time ($F[37, 1036] = 107.291, p < .001, \eta_p^2 = .793$), such that overall, body weight increased over time. There was no main effect of drug or drug x time interaction. See Appendix B, Table 21 for more detailed information.

A rANOVA was performed on the body data to compare females and males. Please refer to Appendix A, Figures 19 and 20 for body weight data for females and males, respectively. Mauchly's Test of Sphericity was not significant; therefore, sphericity is assumed. The overall rANOVA revealed a significant main effect of sex ($F[1, 26] = 268.031, p < .001, \eta_p^2 = .912$), such that males ($M = 368.947, SEM = 5.485$) weighed significantly more than females ($M = 241.963, SEM = 5.485$); a significant main effect of time ($F[37, 962] = 573.465, p < .001, \eta_p^2 = .957$), such that body weight increased over time; a significant drug x time interaction ($F[37, 962] = 2.832, p < .001, \eta_p^2 = .098$), such that rats assigned to the saline group gained significantly more weight over time compared to rats assigned to the nicotine group; and a significant sex x time interaction ($F[37, 962] = 121.888, p < .001, \eta_p^2 = .824$), such that males gained

significantly more weight over time compared to females. There was no significant drug x sex x time interaction. See Appendix B, Table 22 for more detailed information.

Body Weight Repeated-measures Analysis of Variance Split by Sex

Data were split by sex to examine main effects for time, drug, and all interactions for females and males, separately. Please refer to Appendix A, Figures 19 and 20 for body of female and male rats, respectively. An independent sample's t-test was conducted to assess whether or not significant differences were present at baseline for females and males, separately. There were no significant differences in baseline body weight for either females or males (See Appendix B, Table 23 and Table 24, respectively).

Females

For female rats, Mauchly's Test of Sphericity was not significant; therefore, sphericity is assumed. There was no main effect of drug. The rANOVA revealed a significant main effect of time ($F[37, 481] = 187.323, p < .001, \eta_p^2 = .935$), such that body weight increased significantly over time. There was no drug x time interaction. See Appendix B, Table 25 for more detailed information.

Males

For male rats, Mauchly's Test of Sphericity was not significant; therefore, sphericity is assumed. There was no main effect for drug. There was a main effect of time ($F[37, 481] = 396.802, p < .001, \eta_p^2 = .968$), such that body weight increased significantly over time. There was a significant drug x time interaction ($F[37, 481] = 2.311, p < .001, \eta_p^2 = .151$), such that rats assigned to the saline condition gained significantly more

weight over time compared to rats assigned to the nicotine condition. See Appendix B, Table 26 for more detailed information.

Body Weight Repeated-measures Analysis of Variance Split by Phase

Data were split by phase to examine main effects of drug, sex, and time as well as interactions pre-nicotine/saline exposure, during nicotine/saline exposure, post-nicotine/saline exposure, pre-morphine self-administration and during morphine self-administration. Please refer to Appendix A, Figure 18 for body weight split by phases.

Pre-Nicotine/Saline Exposure

Mauchley's Test of Sphericity was significant at $p < .001$; therefore, the Greenhouse-Geisser correction was applied. The rANOVA revealed a significant main effect of sex ($F[1, 28] = 148.347, p < .001, \eta_p^2 = .841$), such that males weighed significantly more than females, and a significant main effect of time ($F[1.511, 42.314] = 139.294, p < .001, \eta_p^2 = .833$), such that weight significantly increased over time. There was also a significant sex x time interaction ($F[1.511, 42.314] = 62.921, P < .001, \eta_p^2 = .692$), such that male rats gained significantly more weight over time compared to the female rats. There was no main effect of drug and no significant drug x sex, drug x time, or drug x sex x time interactions. See Appendix B, Table 27 for more detailed information.

During Nicotine/Saline Exposure

Mauchley's Test of Sphericity was significant at $p < .001$; therefore, the Greenhouse-Geisser correction was applied. The rANOVA revealed a significant main effect of sex ($F[1, 28] = 260.288, p < .001, \eta_p^2 = .903$), such that males weighed

significantly more than females, and a significant main effect of time ($F[2.541, 71.156] = 359.931, p < .001, \eta_p^2 = .928$), such that weight significantly increased over time. There was also a significant sex x time interaction ($F[2.541, 71.156] = 85.284, P < .001, \eta_p^2 = .753$), such that male rats gained significantly more weight over time compared to the female rats. There was no main effect of drug and no significant drug x sex, drug x time, or drug x sex x time interactions. See Appendix B, Table 28 for more detailed information.

Post-Nicotine/Saline Exposure/Pre-Morphine Self-Administration

Mauchley's Test of Sphericity was significant at $p < .001$; therefore, the Greenhouse-Geisser correction was applied. The rANOVA revealed a significant main effect of sex ($F[1, 27] = 282.936, p < .001, \eta_p^2 = .913$), such that males weighed significantly more than females, and a significant main effect of time ($F[3.432, 92.654] = 58.486, p < .001, \eta_p^2 = .684$), such that weight significantly increased over time. The rANOVA also revealed a significant drug x time interaction ($F[3.432, 92.654] = 8.647, p < .001, \eta_p^2 = .243$), such that rats assigned to the saline condition gained significantly more weight over time compared to rats assigned to the nicotine condition, and a significant sex x time interaction ($F[3.432, 92.654] = 3.696, p = .011, \eta_p^2 = .120$), such that male rats gained significantly more weight over time compared to the female rats. There was no main effect of drug and no significant drug x sex, or drug x sex x time interactions. See Appendix B, Table 29 for more detailed information.

During Morphine Self-Administration

Mauchley's Test of Sphericity was significant at $p < .001$; therefore, the Greenhouse-Geisser correction was applied. The rANOVA revealed a significant main effect of sex ($F[1, 26] = 294.751, p < .001, \eta_p^2 = .919$), such that males weighed significantly more than females, and a significant main effect of time ($F[3.606, 93.763] = 21.366, p < .001, \eta_p^2 = .451$), such that weight significantly increased over time. The rANOVA also revealed a significant sex x time interaction ($F[3.606, 93.763] = 3.164, p = .021, \eta_p^2 = .108$), such that male rats gained significantly more weight over time compared to the female rats. There was no main effect of drug and no significant drug x sex, drug x time, or drug x sex x time interactions. See Appendix B, Table 30 for more detailed information.

Body Weight Repeated-measures Analysis of Variance Split by Sex and Phase

Females

Pre-Nicotine/Saline Exposure

Mauchley's Test of Sphericity was significant at $p = .003$; therefore, the Greenhouse-Geisser correction was applied. There was no main effect of drug. The rANOVA did reveal a significant main effect of time ($F[2.405, 33.676] = 24.763, p < .001, \eta_p^2 = .639$) such that body weight increased significantly over time. There was no drug x time interaction. See Appendix B, Table 31 for more detailed information.

During Nicotine/Saline Exposure

Mauchley's Test of Sphericity was significant at $p < .001$; therefore, the Greenhouse-Geisser correction was applied. There was no main effect of drug. The rANOVA did reveal a significant main effect of time ($F[3.857, 53.997] = 80.244, p <$

.001, $\eta_p^2 = .851$), such that body weight increased significantly over time. There was no drug x time interaction. See Appendix B, Table 32 for more detailed information.

Post Nicotine/Saline Exposure/Pre-Morphine Self-Administration

Mauchley's Test of Sphericity was not significant; therefore, sphericity is assumed. There was no main effect of drug. The rANOVA did reveal a significant main effect of time ($F[9, 117] = 21.557, p < .001, \eta_p^2 = .624$), such that body weight increased significantly over time. There was no drug x time interaction. See Appendix B, Table 33 for more detailed information.

During Morphine Self-Administration

Mauchley's Test of Sphericity was significant at $p < .001$; therefore, the Greenhouse-Geisser correction was applied. There was no main effect of drug. The rANOVA did reveal a significant main effect of time ($F[4.088, 53.140] = 9.548, p < .001, \eta_p^2 = .423$), such that body weight increased significantly over time. There was no drug x time interaction. See Appendix B, Table 24 or more detailed information.

Males

Pre-Nicotine/Saline Exposure

Mauchley's Test of Sphericity was significant at $p < .001$; therefore, the Greenhouse-Geisser correction was applied. There was no main effect of drug. The rANOVA did reveal a significant main effect of time ($F[1.314, 18.397] = 116.275, p < .001, \eta_p^2 = .893$), such that body weight increased significantly over time. There was no drug x time interaction. See Appendix B, Table 35 or more detailed information.

During Nicotine/Saline Exposure

Mauchley's Test of Sphericity was significant at $p < .001$; therefore, the Greenhouse-Geisser correction was applied. There was no main effect of drug; however, it did approach significance ($F[1, 14] = 4.046, p = .064, \eta_p^2 = .224$); such that male rats assigned to the saline conditioned weighed more than male rats assigned to the nicotine condition. The rANOVA did reveal a significant main effect of time ($F[1.702, 23.828] = 288.418, p < .001, \eta_p^2 = .954$), such that body weight increased significantly over time. There was no drug x time interaction. See Appendix B, Table 36 for more detailed information.

Post-Nicotine/Saline Exposure/Pre-Morphine Self-Administration

Mauchley's Test of Sphericity was significant at $p < .001$; therefore, the Greenhouse-Geisser correction was applied. There was no main effect of drug. The rANOVA did reveal a significant main effect of time ($F[1.364, 19.094] = 45.025, p < .001, \eta_p^2 = .763$), such that body weight increased significantly over time. Additionally, there was a significant drug x time interaction ($F[1.364, 19.094] = 11.037, p = .002, \eta_p^2 = .441$), such that male rats assigned to the nicotine condition gained significantly more weight over time compared to male rats assigned to the saline condition. See Appendix B, Table 37 for more detailed information.

During Morphine Self-Administration

Mauchley's Test of Sphericity was significant at $p < .001$; therefore, the Greenhouse-Geisser correction was applied. There was no main effect of drug. The rANOVA did reveal a significant main effect of time ($F[2.763, 35.923] = 13.993, p <$

.001, $\eta_p^2 = .518$), such that body weight increased significantly over time. There was no drug x time interaction. See Appendix B, Table 38 for more detailed information.

CHAPTER 6: Evaluation of Hypotheses

The purpose of the present experiment was to examine the effects of nicotine pre-exposure on subsequent morphine self-administration in female and male rats. Each hypothesis is evaluated in this section. Specific aims and hypotheses are re-stated for reference.

Specific Aim 1: To examine the effects of nicotine pre-exposure on subsequent morphine self-administration in a rat model.

Hypothesis 1: The hypothesis that rats previously exposed to nicotine will self-administer greater amounts of morphine compared to rats previously exposed to saline **was not confirmed**. In fact, the rats previously exposed to nicotine self-administered significantly less morphine compared to rats previously exposed to saline.

Specific Aim 2: To examine the effects of nicotine pre-exposure on subsequent morphine self-administration in female and male rats.

Hypothesis 2: The hypothesis that male rats will self-administer greater amounts of morphine compared to female rats **was not confirmed**. Self-administration of morphine did not differ significantly depending on sex.

Hypothesis 3: The hypothesis that male rats pre-exposed to nicotine will self-administer greater amounts of morphine compared to female rats **was not confirmed**. Self-administration of morphine did not differ significantly depending on a sex x drug interaction.

CHAPTER 7: Discussion

The purpose of the present experiment was to experimentally examine the Gateway Hypothesis. Since its inception in 1975 (73), the Gateway Hypothesis has been presumed to be true and utilized to enact various policy decisions regarding substance use. The definition of the Gateway Hypothesis is the sequencing of initiation between drug classes and association in the use of drugs, such that use of a lower drug (e.g., nicotine) in the sequence increases the risk of using drugs higher in the sequence (e.g., morphine). Kandel (74); (75) implies three interrelated propositions about sequencing, association of initiation, and causation. Sequencing implies that there is a fixed relationship between two drug substances, such that one substance is initiated before the other. Association implies that the initiation of one substance increases the likelihood of initiation of the second substance. Causation implies that use of the first substance actually causes use of the second substance (75). However, research regarding the Gateway Hypothesis has been epidemiological and causation cannot be assumed without a true experimental design, which was accomplished by the use of a rat model.

Contrary to hypotheses following from the Gateway Hypothesis, the findings of the present experiment did not confirm that pre-exposure to nicotine increased subsequent morphine self-administration (MSA). In fact, the present experiment found significant results in the opposite direction, that pre-exposure to nicotine lowered subsequent MSA for both females and males. These findings are consistent with recent findings that indicate pre-exposure to moderate, high, or intoxicating levels of alcohol had no effect on subsequent cocaine self-administration or relapse to cocaine seeking (39).

MSA was utilized as a measure of higher order drug intake with nicotine pre-exposure utilized as a lower order drug. Lever presses on an active lever (i.e., the lever that administered the morphine) were operationalized as morphine intake. In addition to measuring active lever MSA, inactive lever presses (i.e., presses on a lever that did not result in MSA) and horizontal activity (HA), measured within an open field apparatus, were measured to determine whether MSA was the result of increased activity or the result of intentional self-administration of morphine.

For females and males, across four separate cohorts, MSA was significantly less (or approached significance) for rats assigned to the nicotine condition than for rats assigned to the saline condition. These results are in direct contradiction with the Gateway Hypothesis which hypothesizes that rats assigned to the nicotine condition should self-administer significantly more morphine than rats assigned to the saline condition. Because nicotine may alter activity, HA was measured to determine if MSA may have been the result of nicotine-induced changes in activity. The two conditions did not differ significantly on HA during the pre-MSA phase when rats were receiving nicotine or saline. During MSA (when rats were self-administering morphine and were no longer receiving nicotine or saline), rats that had previously received saline exhibited significantly greater HA compared to rats that had previously received nicotine. The rats assigned to the saline condition may have increased HA as a result of their greater MSA because higher levels of morphine, compared to levels of morphine self-administered by rats assigned to the nicotine condition, are associated with increased psychomotor activity (6). Additionally, inactive lever presses were measured to determine if MSA may have

been the result of increased activity or haphazard lever pressing. Again, the two groups did not differ significantly on inactive lever presses.

It is noteworthy that there are several elements in the methodology to indicate that these findings are stable. First, MSA cannot be explained by activity level and secondly, there were no significant difference between the nicotine and saline group on inactive lever presses. Therefore, any changes in MSA were not due to general activity level or lever presses. In addition, body weight was evaluated for two reasons: (a) to evaluate general health and ensure the rats were appropriately gaining weight, and (b) to confirm whether or not the rats received nicotine. Based on the slower rate of weight gain for rats assigned to the nicotine condition, it can be assumed that the rats did, in fact, receive nicotine and the rats assigned to the saline condition did not. Additionally, the experiment was conducted across four separate cohorts which found the same results each time. The replication of the findings further increase the reliability.

LIMITATIONS

Missing Data

Two female rats assigned to the saline condition and three male rats assigned to the saline condition were unable to self-administer morphine for the entire duration of the morphine self-administration because of death during surgery (females) or for a portion of morphine self-administration because of clogged catheters (males). Although the missing data are assumed to have occurred at random, the total sample size was decreased to 27, during the morphine self-administration phase, resulting in a decrease in power.

Independent Variables

There are some limitations with the experiment's independent variables. With regard to the nicotine paradigm, an osmotic minipump (non-contingent exposure) was used instead of self-administration (contingent exposure) of nicotine. Use of a minipump is a valid, reliable, and effective way of administering nicotine; however, it is different from the self-administration of tobacco or nicotine (e.g., smoking tobacco). Use of a minipump to administer nicotine is an efficient way to induce nicotine dependence in animals and has resulted in findings regarding the possible neurobiological mechanisms of reward, dependence, and withdrawal (89; 90). However, contingent drug exposure (i.e., cigarette smoking and nicotine self-administration) and non-contingent exposure have different psychological and physiological effects (29; 91). Nicotine absorption through subcutaneous administration is slower than that achieved through inhalation, is more similar to the use of a transdermal nicotine patch, and the speed of administration has been shown to critically influence the reinforcing effects of drugs of abuse (23; 105; 122). While minipumps deliver nicotine continuously over 24 hours per day, this administration paradigm differs from cigarette smoking in humans because humans smoke nicotine-containing tobacco products intermittently and not during sleep.

Additionally, nicotine was administered to the rats; whether or not some other aspect of tobacco (e.g., one of the other 7,000 chemicals) contributes to differences in self-administration of morphine was not assessed. Utilization of the minipump paradigm has produced results that parallel cigarette smoking in humans (1; 2; 48; 49; 52; 143). Richardson and Tizabi (112) indicated that 6 mg nicotine/kg/day minipump administration, in male rats, yields plasma nicotine levels of approximately 100 ng/ml, which is similar to the average human smoker (i.e., $\frac{1}{2}$ to 1 pack of cigarettes per day).

Methodology

There were some limitations with the experiment's methodology. With regard to the duration of exposure to nicotine and morphine, rats were pre-exposed to either nicotine or saline for 21 consecutive days and to morphine for 10 non-consecutive days. These are relatively short time durations of exposure. However, rats, on average, live about three years (111), and several studies have identified a correspondence of 30 days of human life to 1 day in a rat's life (44; 67; 79; 107). Therefore, 2 weeks of nicotine exposure in a rat is roughly equivalent to a year and a half of nicotine use in a human. This exposure period was chosen to parallel nicotine use by adolescents/emerging adults because the Gateway Hypothesis was based on studies of younger people (73).

Notably, the exposure of nicotine or saline and morphine did not overlap in the present experiment. Following pre-exposure to either nicotine or saline, there was a 10-day period in which the rats were not exposed to nicotine, saline, or morphine. Following this 10-day period, rats were allowed to self-administer morphine for 3 hours per day, Monday through Friday, for 2 weeks. In humans, it is assumed that individuals typically do not stop using a lower drug when they begin using a harder drug. When individuals progress to use of a higher drug, such as morphine, individuals are still typically using cigarettes or alcohol, rather than stopping use and switching to another substance.

CLINICAL IMPLICATIONS

If the present findings are generalizable to the human condition, then the present findings indicate that treatment and prevention should target opioid use and abuse rather than focusing on decreasing tobacco use to subsequently decrease opioid use. Focus on the prevention of tobacco use is important in its own right because of the harm associated

with its use rather than focusing on prevention of tobacco use as a prevention of opioid use.

In fact, results indicated that nicotine exposure actually decreased opioid use compared to saline exposure. Counterintuitively, nicotine exposure, of some sort, may actually be associated with decreased morphine use. This is not to say that individuals should smoke tobacco; however, nicotine replacement therapy or nicotine agonists (e.g., Chantix) or other nicotinic acetylcholine, dopaminergic, or endogenous nicotinic agonists might be used as a morphine self-administration prevention strategy. Recent literature (27; 72) has examined the effects of anti-opiate medications for smoking cessation, but nicotine, in some form, to treat opioid use and abuse has not been explored.

FUTURE DIRECTIONS

Independent Variables

To coincide with the aforementioned limitations, future research might build upon the independent variables included in this experiment. Regarding nicotine administration, self-administration of nicotine should be considered in future studies to more adequately mirror nicotine use by humans. Nicotine can be self-administered in an animal model (usually intravenously) contingent on an operant response such as a nose poke or lever press (93). However, although self-administration of nicotine has been reported in the literature since the 1980s (21; 22; 26; 140), stable and consistent rates of self-administration of nicotine are difficult to establish compared to other drugs of abuse such as morphine, and require careful control of other experimental variables such as drug infusion duration, food training, diet restriction, and need for cued infusions of nicotine (18; 20; 64; 82; 125). Some of the difficulties of self-administration of nicotine may be

due to the aversive properties of the drug (8). Specifically, the difference between rewarding and punishing doses of nicotine appears to be relatively small in that rats will self-administer dosages of 0.01-0.06 mg/kg nicotine (22; 26; 120); however, dosages of 0.1 mg/kg nicotine or greater have been reported to cause seizures (22; 61).

An alternative to self-administration of nicotine could be the use of programmable and refillable osmotic minipumps. The ALZET iPRECIO programmable infusion pump allows non-contingent delivery of nicotine to rats at a flow rate of 0.0-30 μ l/hr for a maximum duration of 6 months (Durect Corporation, 2017). Additionally, the programmable features enable a variety of dosing applications such as constant versus variable delivery, multiple bolus dosing, escalating drug administration, surgical recovery and washout periods, and circadian rhythm infusions (Durect Corporation, 2017). Use of this device could enable researchers to program dosages of nicotine on a schedule similar to what we would expect in humans; however, the cost of these devices (\$250 per minipump) may limit their wide-spread use.

Methodology

Future research might build upon the methodology included in the present experiment. In the current experiment, the administration of nicotine and morphine did not overlap. In humans, it is assumed that use of nicotine often does not cease once an individual begins to use a harder drug, such as morphine. Future experiments should examine an overlap in nicotine administration and subsequent MSA to better mirror use in humans. Preliminary data from a follow-up study examining whether continuation of nicotine exposure during MSA had similar or different results to the current experiment found that rats assigned to the nicotine condition and rats assigned to the saline condition

did not differ significantly in regard to their morphine intake (30). This finding may indicate that overlap of the nicotine and morphine is important and still reject a causal interpretation of the Gateway Hypothesis.

Finally, if the findings of the present experiment are true, then future research should be conducted with humans. For example, could a nicotine agonist be utilized in the treatment of opioid use? Because of the opioid epidemic and the high use of opioids in military populations, should military service members be required to utilize nicotine gum especially given the short term cognitive benefits of nicotine and potential long term positive effects of decreased opioid use?

CHAPTER 8: Summary

The purpose of the present experiment was to examine the effects of nicotine pre-exposure on subsequent morphine self-administration in female and male rats. The experiment did not confirm the *a priori* hypotheses based on the Gateway Hypothesis that rats previously exposed to nicotine will self-administer greater amounts of morphine compared to rats previously exposed to saline; nor did the experiment confirm that male rats will self-administer greater amounts of morphine compared to female rats. In fact, the experiment found significant results in the opposite direction. That is, rats previously exposed to nicotine self-administered significantly less morphine compared to rats previously exposed to saline. The use of animal models provided the ability to experimentally evaluate the Gateway Hypothesis and future research should seek to replicate the current findings, vary the method of nicotine administration, and overlap the nicotine and morphine exposures to determine whether or not means of administration or overlap of drugs are important considerations in the Gateway Hypothesis.

CHAPTER 9: Conclusions

The hypothesis that rats pre-exposed to nicotine will self-administer greater amounts of morphine compared to rats pre-exposed to saline was not confirmed. Results indicated significant differences in the opposite direction, such that rats pre-exposed to nicotine self-administered significantly less morphine compared to rats pre-exposed to saline. These findings do not support the Gateway Hypothesis and instead the results indicated that nicotine exposure reduced subsequent morphine self-administration. If the present findings are true and generalize to the human condition, then a nicotine agonist might be useful to prevent or to attenuate morphine use.

APPENDIX A: Figures

Figure 1: IACUC Approval of Protocol

	UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES 4301 JONES BRIDGE ROAD BETHESDA, MARYLAND 20814-4799	
April 2, 2014		
MEMORANDUM FOR DR. NEIL GRUNBERG, DEPARTMENT OF MEDICAL AND CLINICAL PSYCHOLOGY		
SUBJECT: IACUC Approval of Protocol – Initial Review		
The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on April 2, 2014:		
<u>Title of Application:</u> "Behavioral investigations of nicotine and caffeine in rats (<i>Rattus norvegicus</i>)"		
<u>USUHS Protocol Number:</u> MPS-14-898		
<u>Expiration Date:</u> April 1, 2017		
<u>Supporting Grant(s) Number:</u> E072194414		
<u>Name of Principal Investigator:</u> Dr. Neil Grunberg		
The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is A3448-01. The IACUC approved the above referenced application as submitted.		
An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.		
Prior to placing your first animal order, please contact MAJ. Amanda Christy to schedule a pre-protocol planning meeting (295-3708). This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.		
 Brian M. Cox, Ph.D. Chair, Institutional Animal Care and Use Committee		
cc: Office of Research		

Figure 2: Experimental Timeline

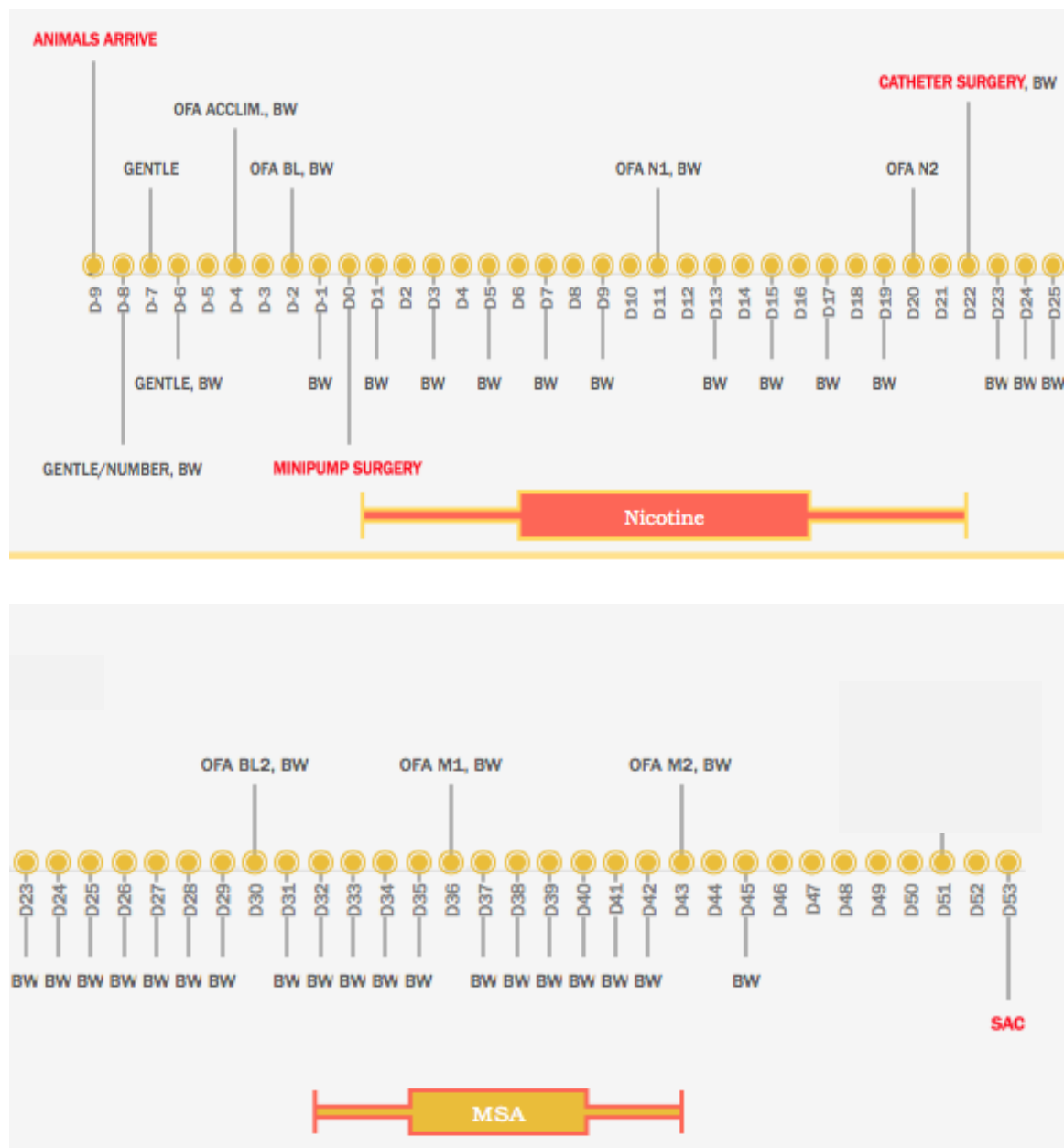


Figure 3: Sample Nicotine Bitartrate Calculation (Male Cohort 2)

OSMOTIC PUMP SPECIFICATIONS:

The following information can be found in the package insert:

Model # 2002

Lot # 10307-13

Mean *in vitro* pump rate = 0.52 $\mu\text{l/hr}$

Mean *in vitro* pump duration = 22 day(s)

Mean pump fill volume = 239.3

Mean pump fill volume = (*in vitro* pump rate) x (24 $\text{hr}/1 \text{ day}$) x (*in vitro* pump duration) = (0.52 $\mu\text{l/hr}$) x (24 $\text{hr}/1 \text{ day}$) x (22 day(s)) = 274.56 μl

In vivo pump rate conversion factor (CF) = 0.95*

(*Note: CF is constant for all ALZET osmotic pumps)

Daily *in vivo* pump rate = (CF) x (*in vitro* pump rate) x (24 $\text{hr}/1 \text{ day}$) = (0.95) x (0.52 $\mu\text{l/hr}$) x (24 $\text{hr}/1 \text{ day}$) = 11.856 $\mu\text{l/day}$

Pump operation duration = (pump fill volume) / (daily *in vivo* pump rate) = (239.3 μl) / (11.856 $\mu\text{l/day}$) = 20.18 day(s)*

(*Note: Duration is calculated to confirm sufficient number of days for the experiment)

NICOTINE BASE DOSAGE SOLUTION (Males):

Dose concentration = (dosage choice) / [(daily *in vivo* pump rate) x (1 ml/1,000 μl)] = (6 mg/kg/ day) / [(11.856 $\mu\text{l/day}$) x (1 ml/1,000 μl)] = 506.07 mg Nic. base/kg/ml

Nicotine Base concentration = (mean animal body weight) x (dose concentration) = (0.28507 kg) x (506.07 mg Nic. base/kg/ml) = 144.27 mg Nic. Base./ml

Nicotine base to Nicotine Bitartrate conversion factor (CF) = 2.850*

(*Note: CF is constant for all Nic. Bit. conversions)

- CF= Formula weight of nicotine

Bitartrate/Molecular Weight Nicotine

Nicotine Bitartrate conversion = (Nic. Base. concentration) x (2.850) = (144.27 mg Nic. Base./ml) x (2.850) = 411.17 mg Nic. Bit./ml

TOTAL SALINE VOLUME:

Total Saline solution volume = (pump fill volume) x (1 ml/1,000 μl) x (number of animals) = (239.3 μl) x (1 ml/1,000 μl) x (4 rats) = 0.96 ml (minimum total volume)*
(*Note: You may increase the total Nicotine solution volume in order to ensure adequate solution volume for the experiment)

TOTAL AMOUNT OF NICOTINE BITARTRATE:

Nicotine Bitartrate = (total Saline volume) x (Nicotine concentration) = (0.96 ml) x (411.17 mg Nic. Bit./ml) x (1 g/1,000 mg) = 0.395 g Nic. Bit.

ACTUAL PROPORTION CALCULATIONS:

(A) Actual amount of Nicotine Bitartrate measured = 0.947 g Nic. Bit.

(B) Actual amount of Nicotine Bitartrate needed = 0.395 g Nic.

(C) Conversion factor (CF) = (A) / (B) = (0.947 g Nic. Bit.) / (0.395 g Nic. Bit.) = 2.40

ACTUAL SALINE VOLUME:

Actual Saline volume = (total Saline volume) x (CF) = (0.96 ml) x (2.40) = 2.30 ml Saline to add

Nicotine Lot Number: N5260-25G

Figure 4: Surgery Procedure Guidance Form

- ❑ Note: pump preparation and labeling should be complete at this point
- ❑ Reserve Surgery prep room, isofluorane, knock-out box, 2 x isofluorane masks, O2, heating pads for cages, scissors (at least 2 x 6 inch short/curved nosed to tent; and 2 x long/straight nose to cut), biohazard bags, scavengers, trash bin, and LAM personnel support by completing form and also notifying LAM vet personnel in person
- ❑ Establish accountability (go/no roster) and identify roles/responsibilities for day of surgery. Disseminate this a week prior to date. Specific roles may include
 - Supervision/General assistance
 - Anesthesia & Rat shaver supervision
 - Surgeons x 2
 - Recorder
 - Animal transport and post-operative care
 - Animal transport, clean-up, general assistance
- ❑ At least day before ensure everything set up in surgery room
- ❑ Ensure all equipment has been inspected, cleaned, organized, and consolidated in the wet lab (at least 2 days prior to sacrifice). Items may include (but see specific list and diagram, separate document):
 - 3-4 rolls of paper towels
 - Scissors
 - Stapler, staples, stapler removal tool (2 sets)
- ❑ Conduct mock run-through morning of surgery with all required personnel. Ensure room is set up the way you need it. Practice oral communication procedures (e.g., calling out rat numbers, and pump # confirmation)
 - Ensure Vet techs are provided education on how to shave (this is very important as it will impact the surgery cut)
- ❑ Post-operative: use betadyne ointment after rats transported back to housing room

Figure 5: Subcutaneous Location of Minipump Implant in the Rat



Image by ALZET (2012)

Figure 6: Cross-section Illustration of the ALZET Model 2002 Minipump

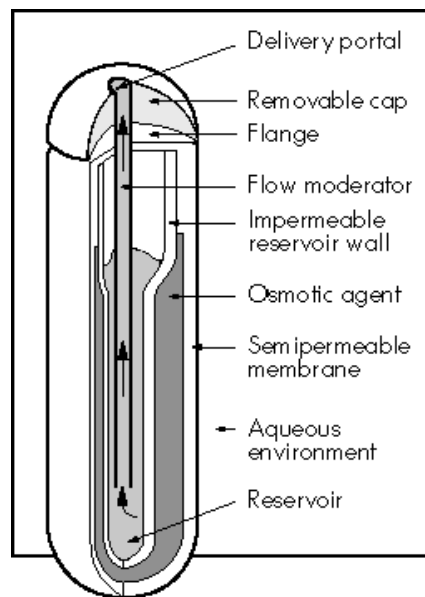


Image by ALZET (2012)

Figure 7: Illustration of Catheter

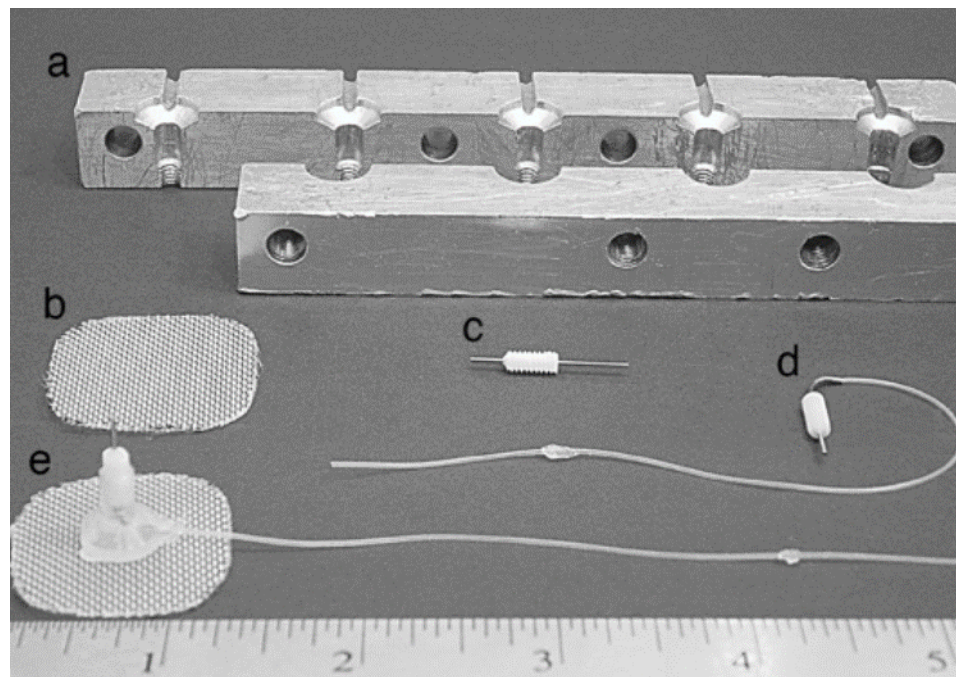


Figure 8: Catheter Implantation

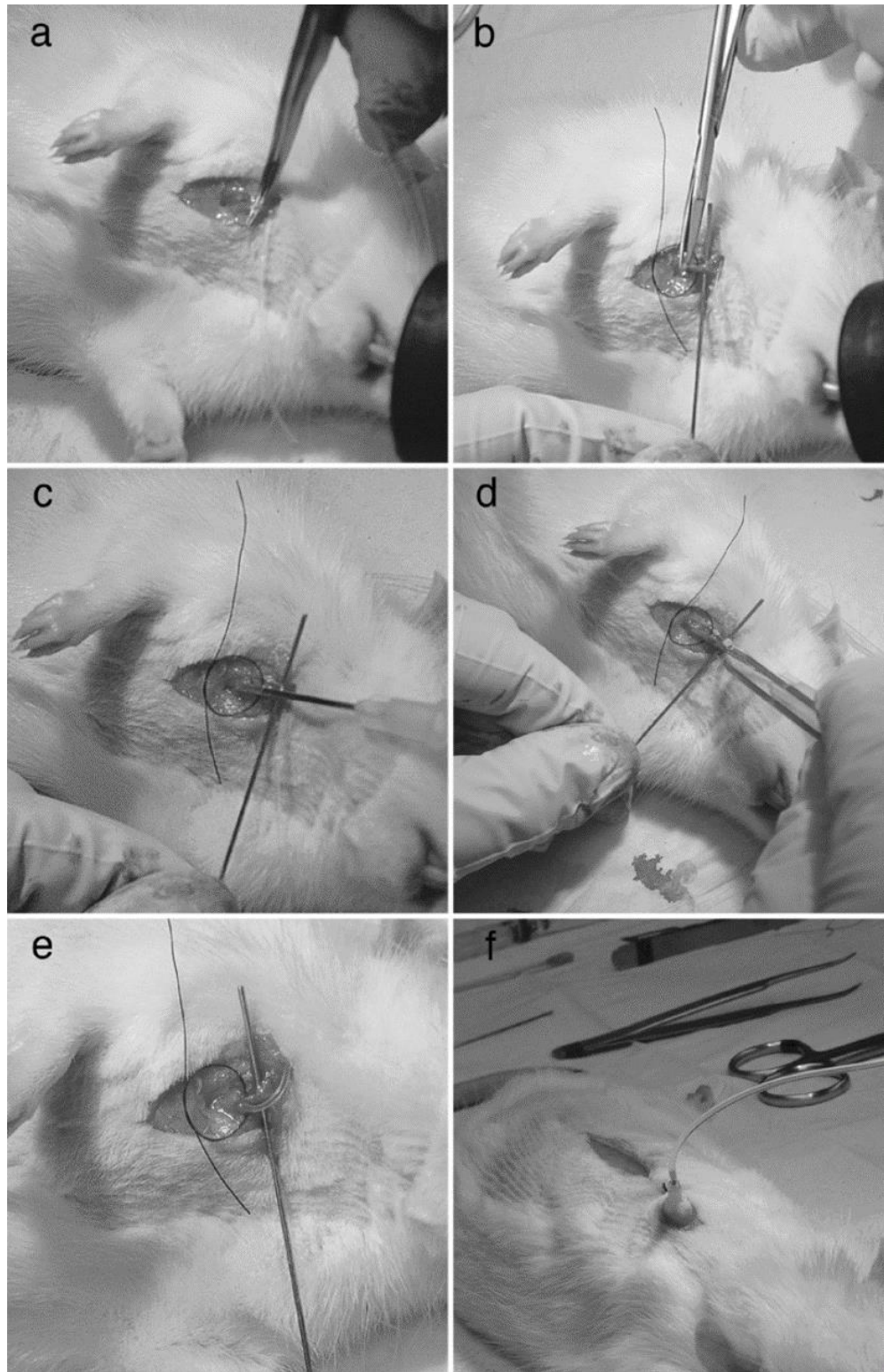


Figure 9: Morphine Self-Administration (MSA), Overall

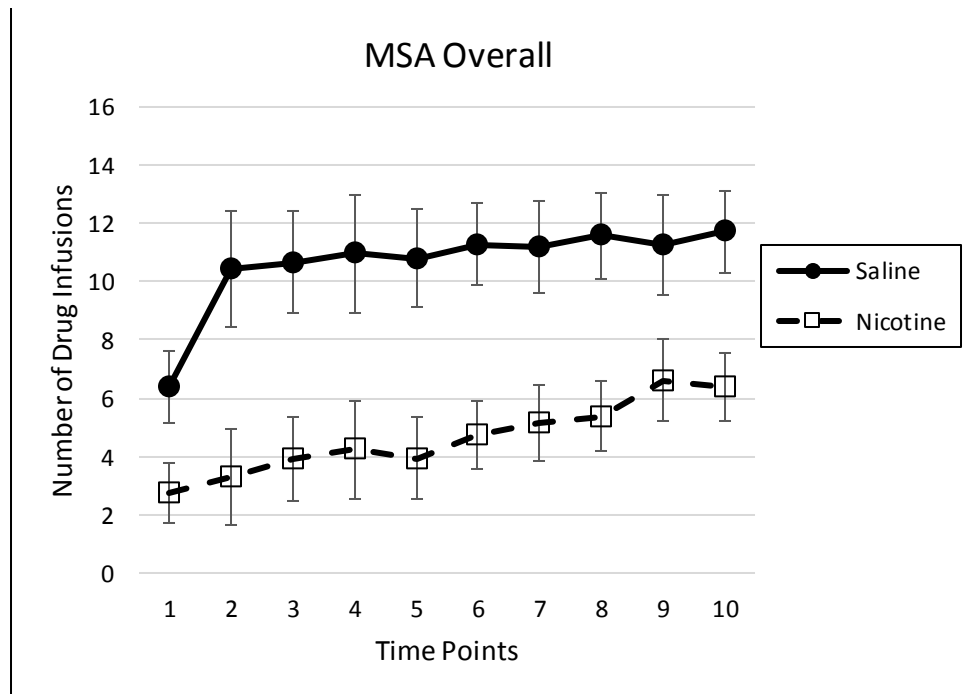


Figure 10: Morphine Self-Administration, Females

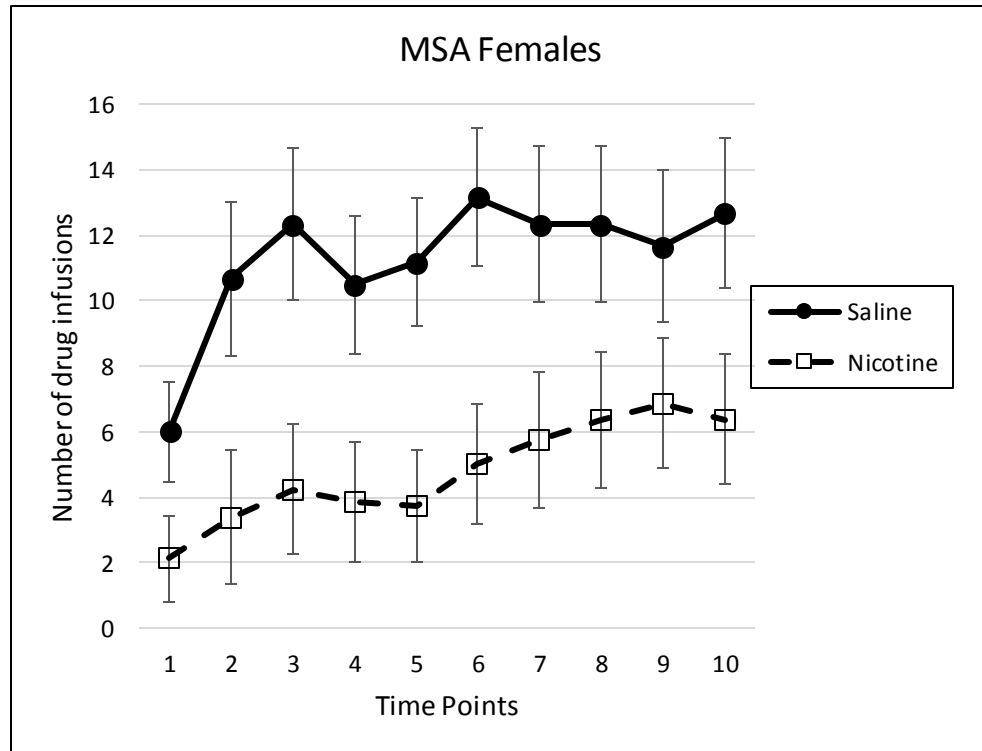


Figure 11: Morphine Self-Administration, Males

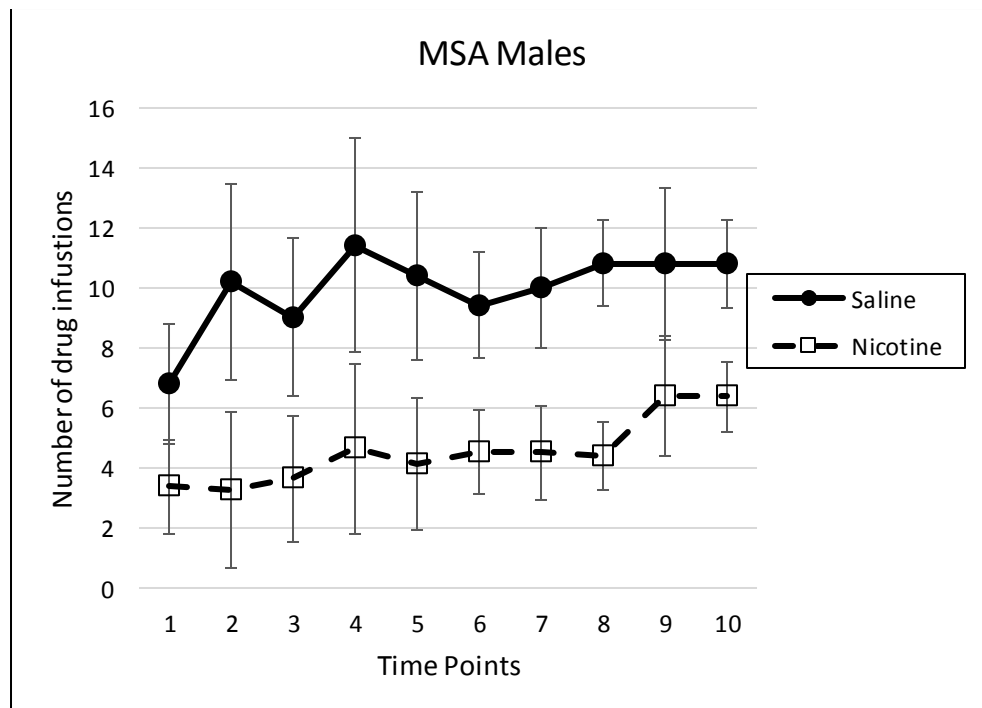


Figure 12: Inactive Lever, Presses, Overall

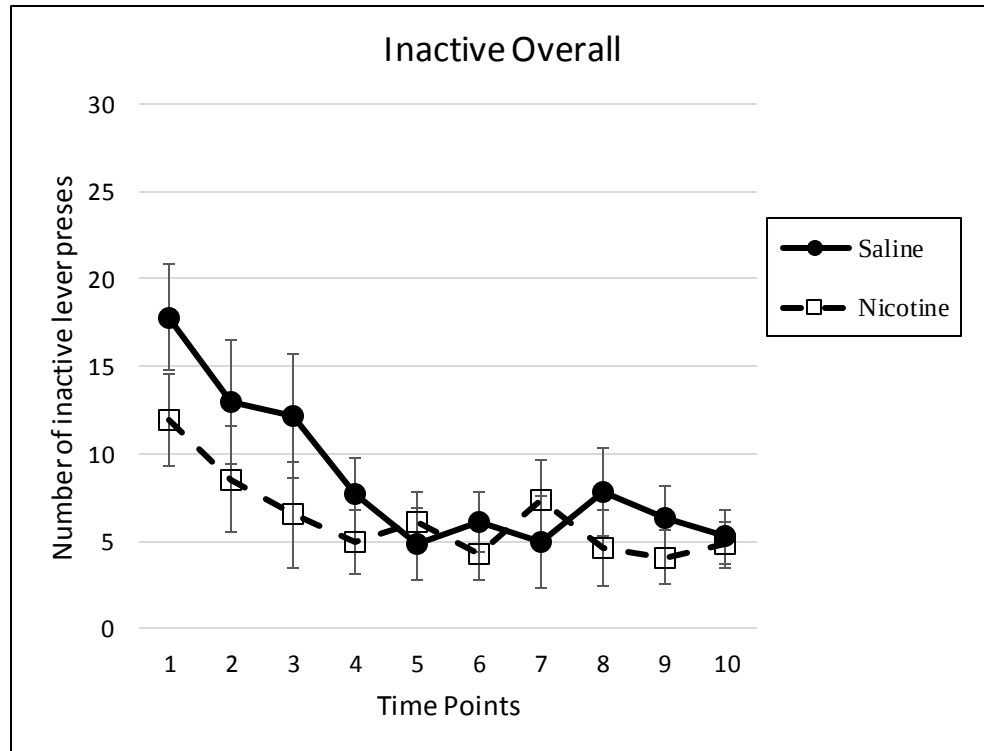


Figure 13: Inactive Lever Presses, Females

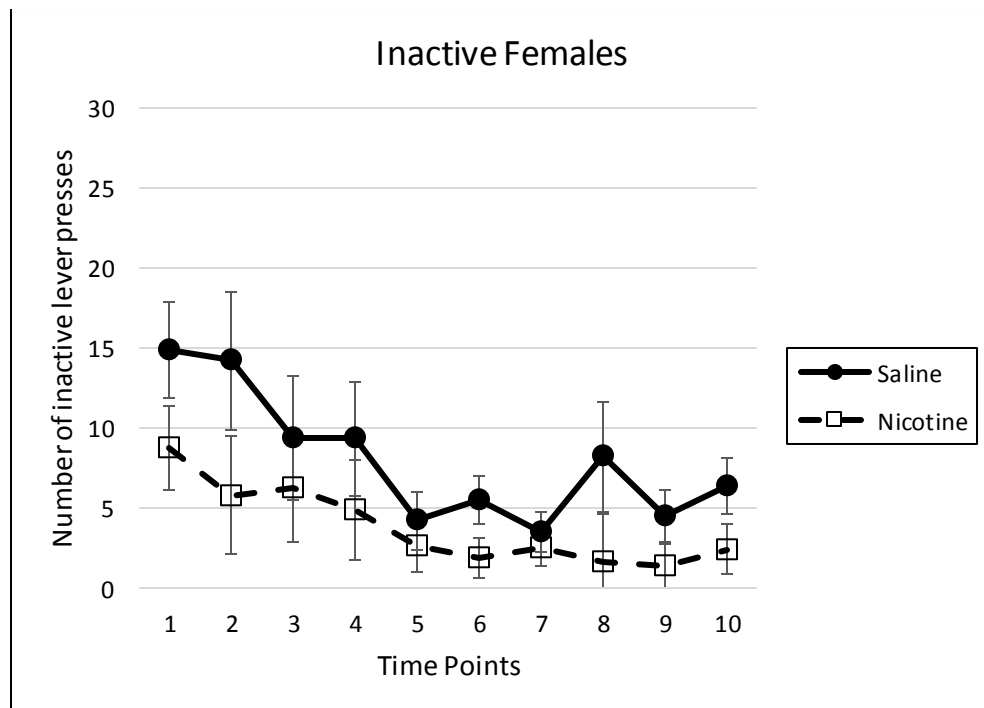


Figure 14: Inactive Lever Presses, Males

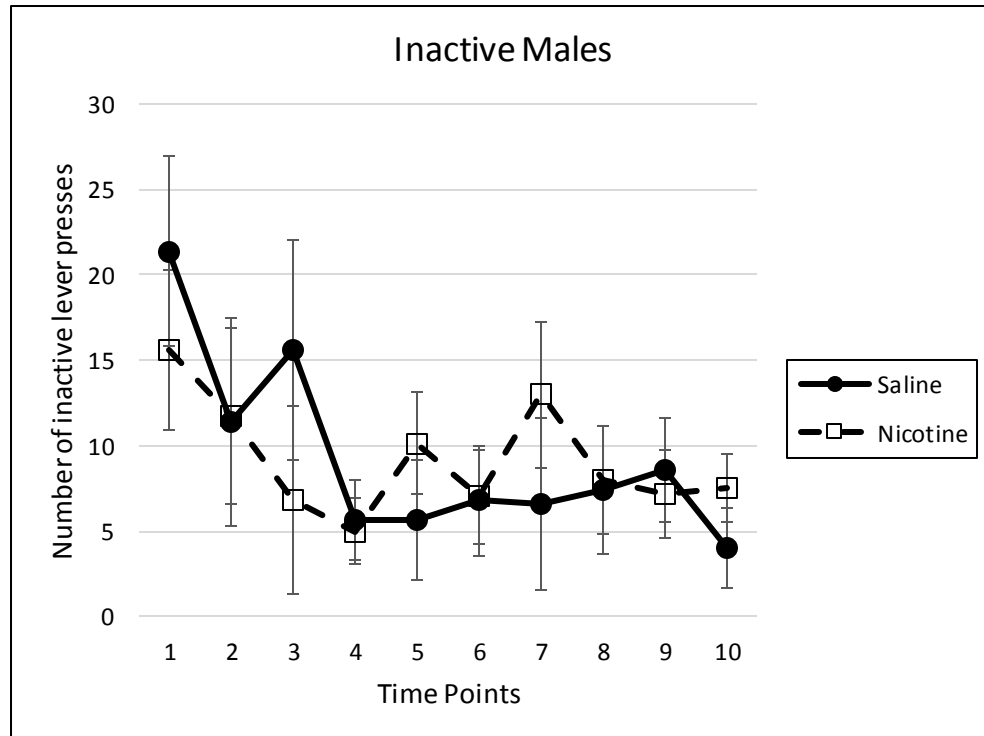


Figure 15: Horizontal Activity, Overall

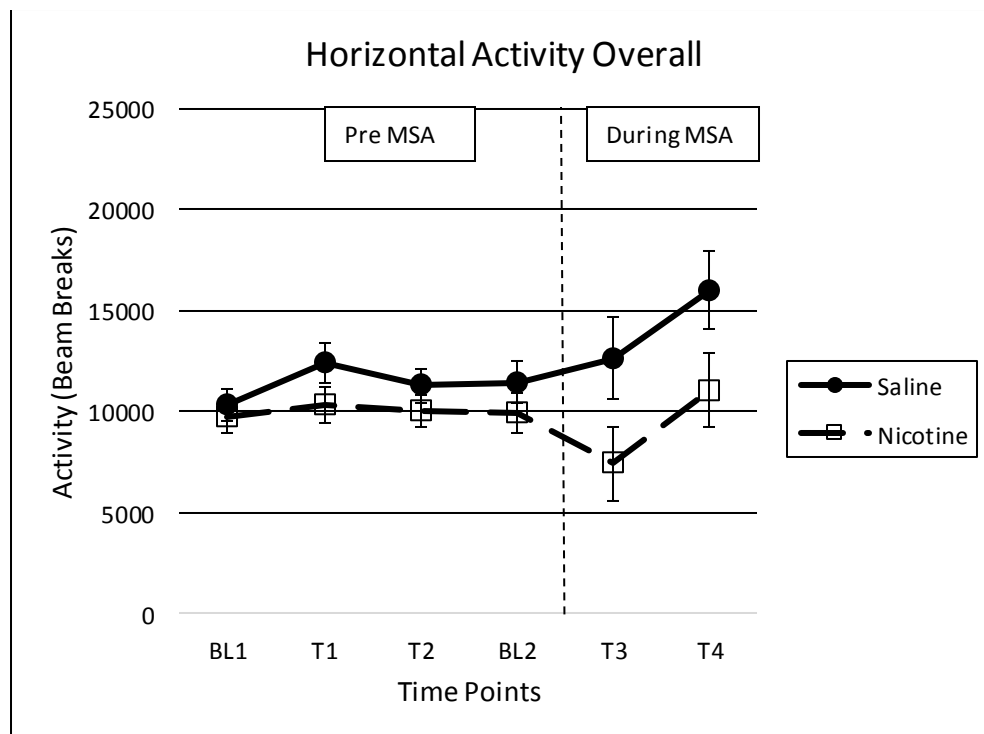


Figure 16: Horizontal Activity, Females

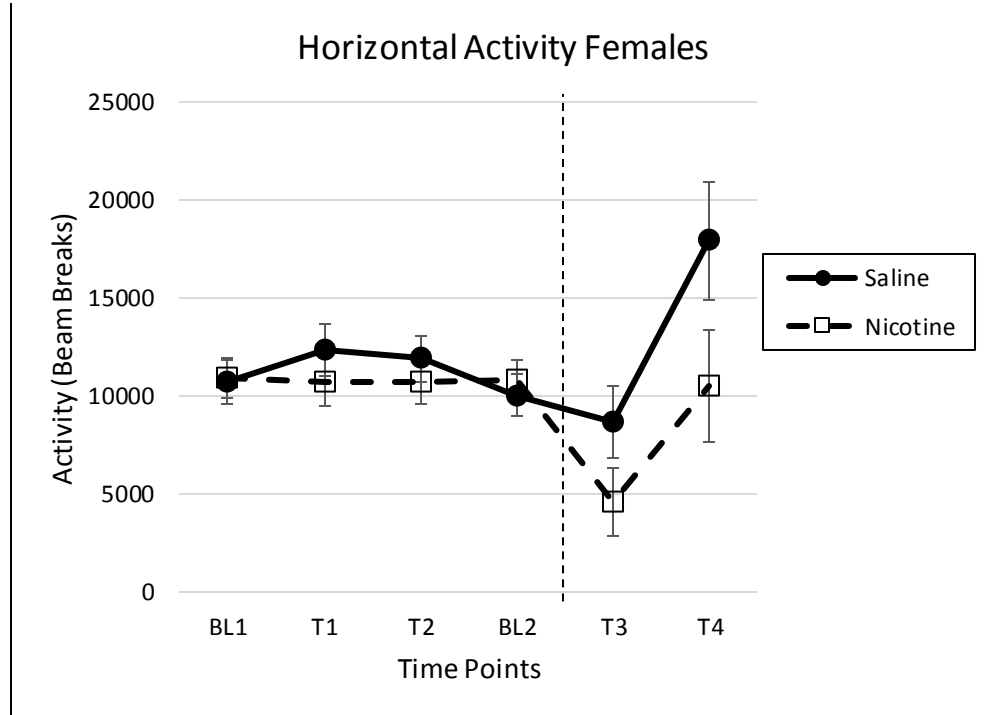


Figure 17: Horizontal Activity, Males

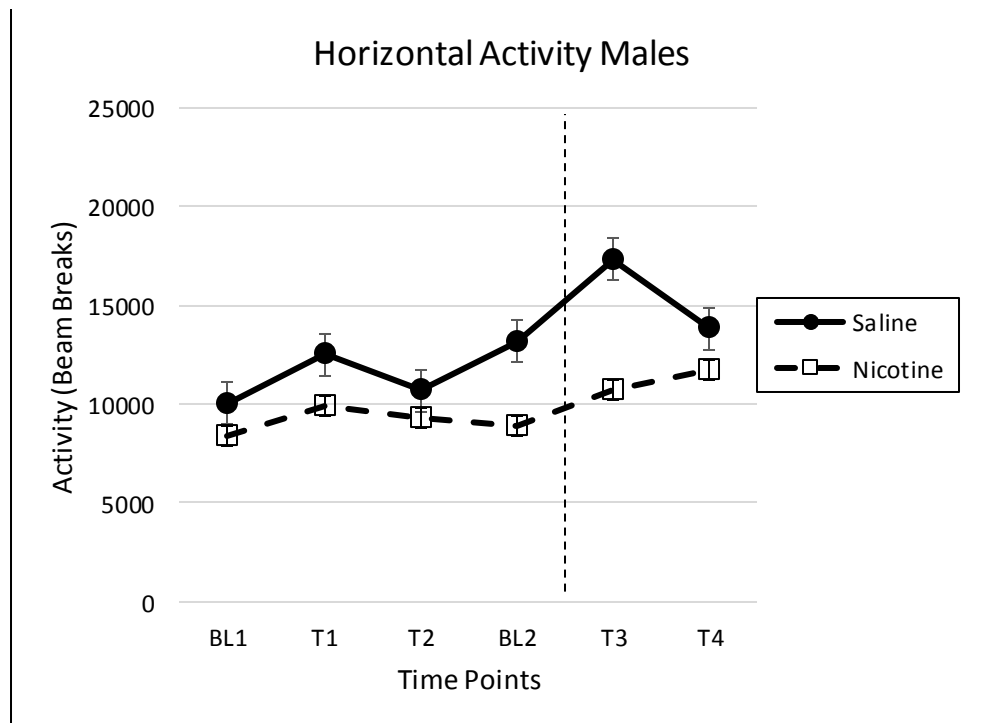


Figure 18: Body Weight, Overall

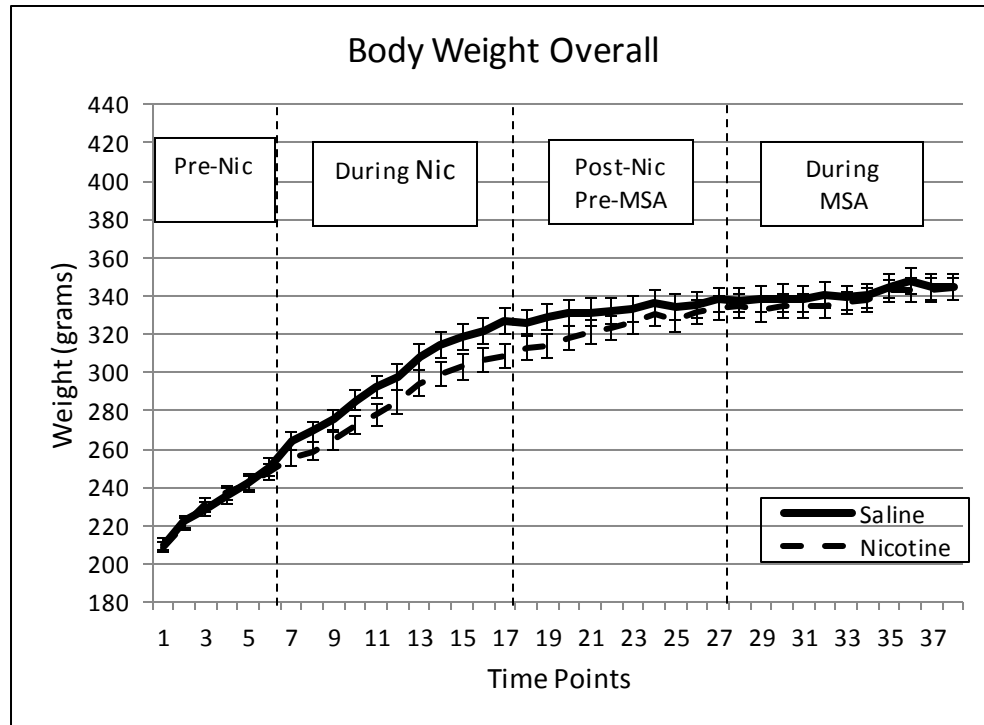


Figure 19: Body Weight, Females

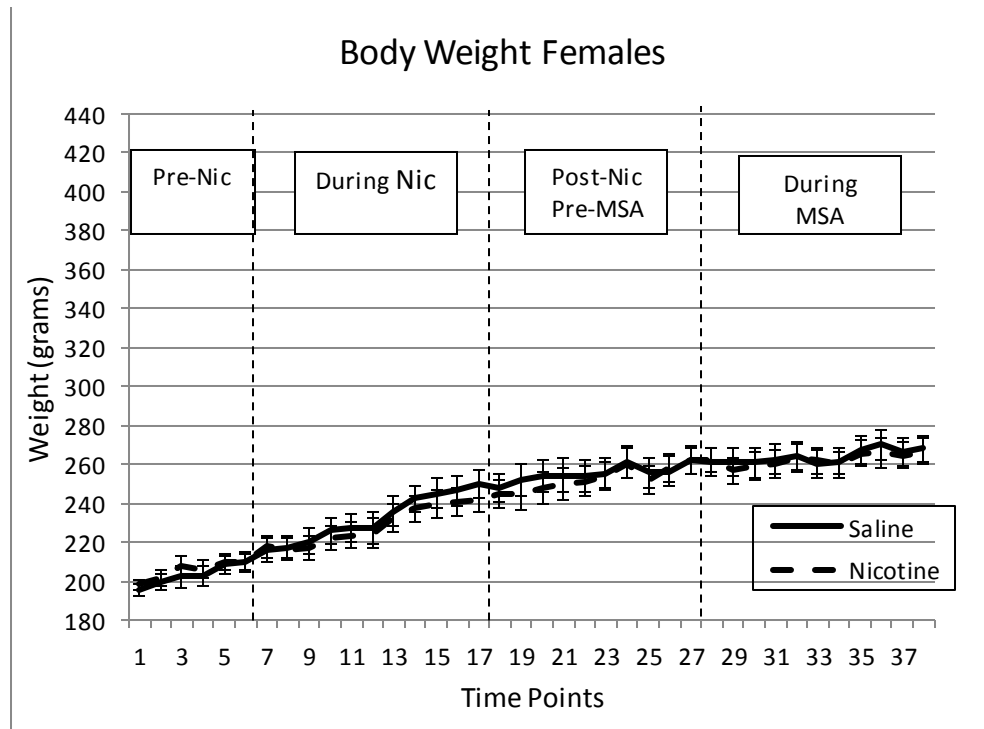


Figure 20: Body Weight, Males

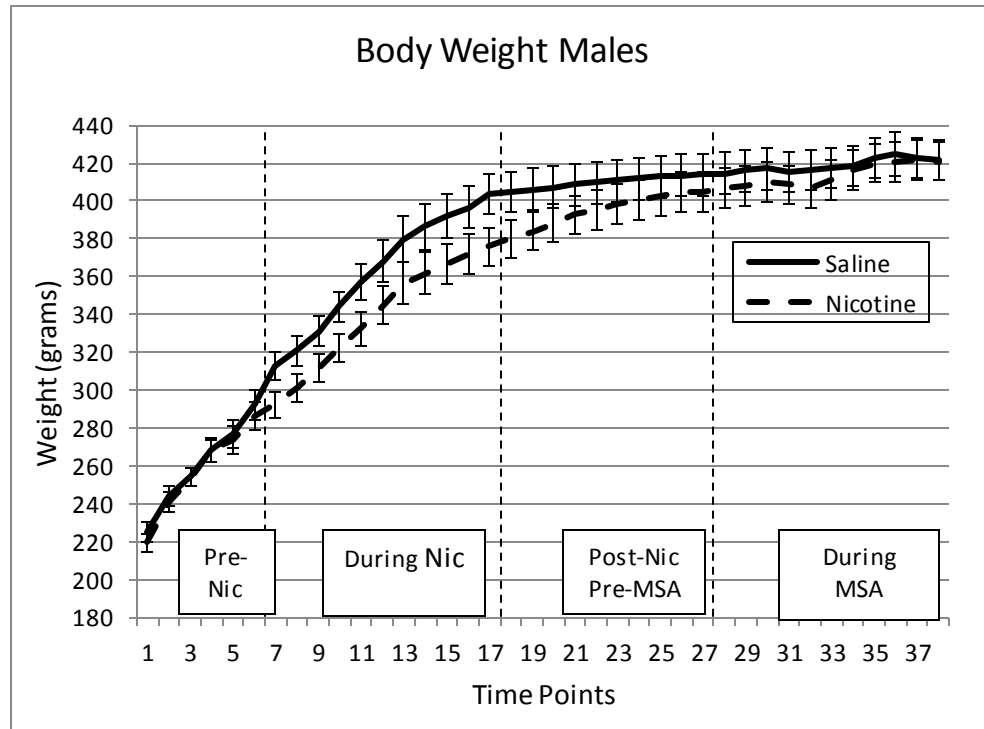


Figure 21: Body Weight, Most Representative Day, Overall

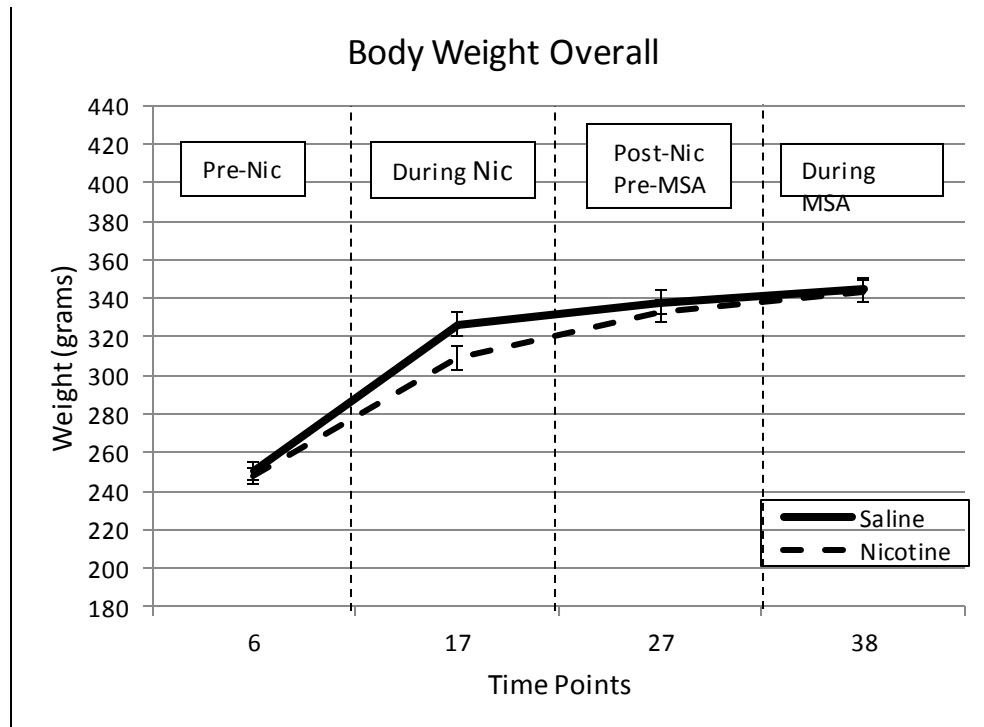


Figure 22: Body Weight, Most Representative Day, Females

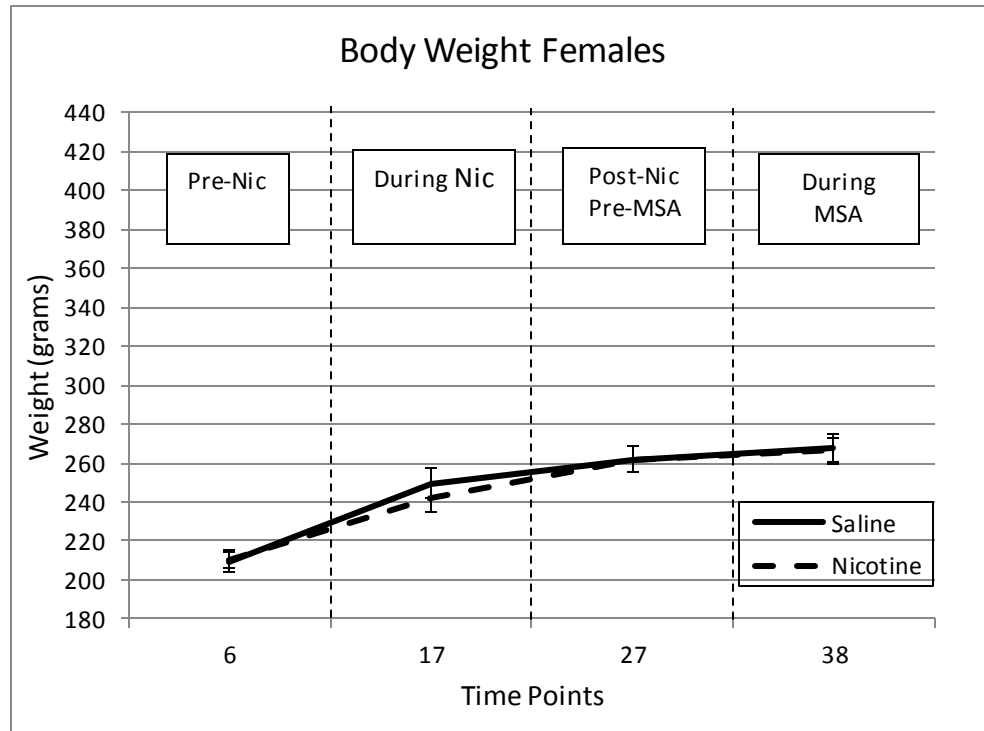
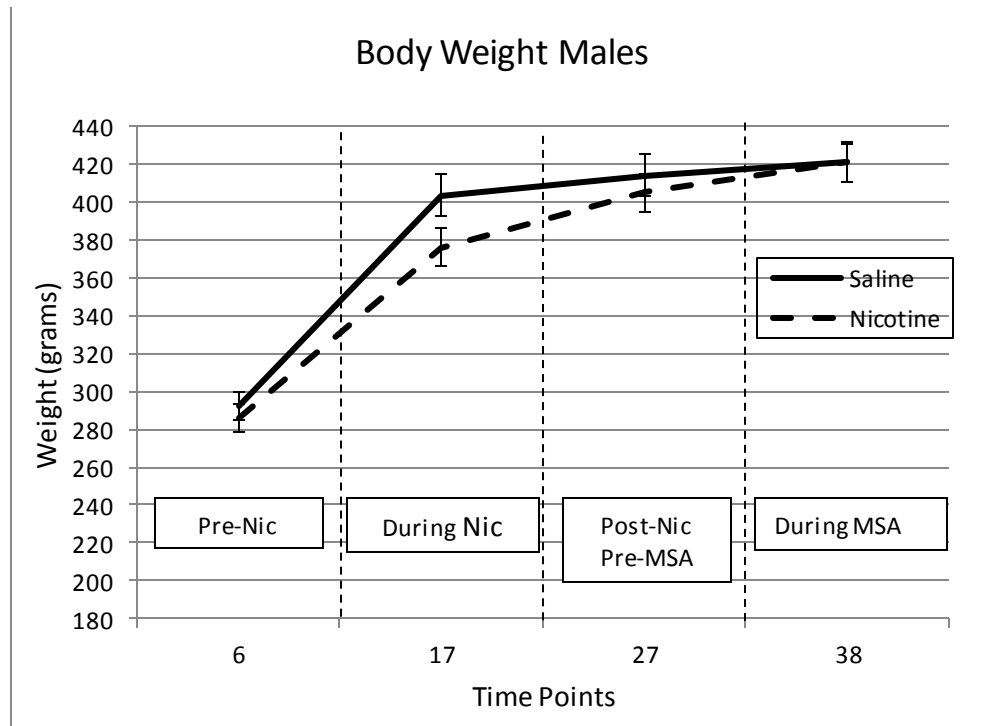


Figure 23: Body Weight, Most Representative Day, Males



APPENDIX B: Tables

Table 1: Treatment Cell Breakdown

Sex	Condition	
	Nicotine	No Nicotine
Male	n=8	n=8
Female	n=8	n=8

Table 2: Overall rANOVA of MSA

Overall rANOVA of MSA within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	399.569	3.406	117.329	7.631	.000	.234	.991
Time*Drug	76.236	3.406	22.386	1.456	.229	.055	.399
Error (time)	1309.082	85.138	15.376				

Overall rANOVA of MSA between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	15310.243	1	15310.243	68.509	.000	.733	1.000
Drug	2376.539	1	2376.539	10.634	.003	.298	.880
Error	5586.957	23	223.478				

Overall rANOVA of female vs. male MSA within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	387.267	3.238	119.602	7.321	.000	.241	.985
Time*Drug	72.534	3.238	22.401	1.371	.257	.056	.365
Time*Sex	75.940	3.238	23.453	1.436	.237	.059	.381
Time*Drug*Sex	21.743	3.238	6.715	.411	.760	.018	.131
Error (time)	1216.701	74.473	16.338				

Overall rANOVA of female vs. male MSA between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	15115.651	1	15115.651	62.795	.000	.732	1.000
Drug	2317.978	1	2317.978	9.630	.005	.295	.844
Sex	40.782	1	40.782	.169	.684	.007	.068
Drug*Sex	18.249	1	18.249	.076	.786	.003	.058
Error	5536.441	23	240.715				

Table 3: rANOVA of female MSA

rANOVA of female MSA within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	349.895	2.951	118.549	7.283	.001	.378	.970
Time*Drug	58.010	2.951	19.654	1.208	.321	.091	.293
Error(time)	576.483	35.418	16.277				

rANOVA of female MSA between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	8841.260	1	8841.260	36.846	.000	.754	1.000
Drug	1452.288	1	1452.288	6.052	.030	.335	.618
Error	2879.433	12	239.953				

Table 4: rANOVA of male MSA

rANOVA of male MSA within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	125.444	2.022	62.027	2.155	.139	.163	.396
Time*Drug	37.382	2.022	18.484	.642	.537	.055	.144
Error(time)	640.218	22.247	28.778				

rANOVA of male MSA between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	6444.716	1	6444.716	26.681	.000	.708	.997
Drug	913.085	1	913.085	3.780	.078	.256	.427
Error	2657.008	11	241.546				

Table 5: Overall rANOVA of inactive lever

Overall rANOVA of inactive lever within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	2384.463	4.505	529.248	7.029	.000	.227	.997
Time*Drug	437.571	4.505	97.122	1.290	.276	.051	.418
Error (time)	8141.075	108.129	75.290				

Overall rANOVA of inactive lever between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	14092.535	1	14092.535	37.356	.000	.609	1.000
Drug	324.658	1	324.658	.861	.363	.035	.145
Error	9054.004	24	377.250				

Overall rANOVA of female vs. male inactive lever within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	2419.194	4.292	563.686	7.106	.000	.244	.996
Time*Drug	466.279	4.292	108.646	1.370	.248	.059	.428
Time*Sex	381.371	4.292	88.861	1.120	.353	.048	.353
Time*Drug*Sex	273.228	4.292	63.664	.803	.534	.035	.257
Error (time)	7489.329	94.418	79.321				

Overall rANOVA of female vs. male inactive lever between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	14453.048	1	14453.048	40.157	.000	.646	1.000
Drug	289.145	1	289.145	.803	.380	.035	.138
Sex	710.984	1	710.984	1.975	.174	.082	.270
Drug*Sex	262.774	1	262.774	.730	.402	.032	.129
Error	7918.083	22	359.913				

Table 6: rANOVA of female inactive lever

rANOVA of female inactive lever within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	1206.307	3.383	356.554	4.844	.004	.288	.902
Time*Drug	161.050	3.383	47.602	.647	.607	.051	.181
Error(time)	2988.300	40.599	73.605				

rANOVA of female inactive lever between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	4760.467	1	4760.467	20.510	.001	.631	.985
Drug	600.010	1	600.010	2.585	.134	.177	.316
Error	2785.283	12	232.107				

Table 7: rANOVA of male inactive lever

rANOVA of male inactive lever within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	1565.296	3.259	480.237	3.478	.024	.258	.748
Time*Drug	547.296	3.259	167.912	1.216	.321	.108	.306
Error(time)	4501.029	32.594	138.093				

rANOVA of male inactive lever between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	9982.292	1	9982.292	19.448	.001	.660	.977
Drug	.292	1	.292	.001	.981	.000	.050
Error	5132.800	10	513.280				

Table 8: Independent sample's t-test of HA baseline

	Levene's Test for Equality of Variances					t-test for Equality of Means	
	F	Sig.	t	df	Sig.	Mean Difference	Std. Error Difference
Equal variances assumed	.675	.418	.358	29	.723	363.579	1015.373

Table 9: Overall rANOVA of HA

Overall rANOVA of HA within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	240261313.4	2.739	87714076.94	2.497	.072	.088	.569
Time* Drug	134323192.3	2.739	49038418.43	1.396	.253	.051	.339
Error (time)	2502108325	71.218	35133240.65				

Overall rANOVA of HA between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	2.045E+10	1	2.045E+10	421.416	.000	.942	1.000
Drug	286466164.5	1	286466164.5	5.904	.022	.185	.648
Error	1261477889	26	48518380.36				

Overall rANOVA of female vs. male HA within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	221187479.3	2.586	85543156.95	2.665	.064	.100	.580
Time* Drug	127790448.0	2.586	49422319.85	1.540	.217	.060	.358
Time* Sex	413791718.6	2.586	160031888.0	4.986	.005	.172	.863
Time* Drug* Sex	104942952.0	2.586	40586164.47	1.264	.293	.050	.299
Error (time)	1991893339	62.056	32098118.21				

Overall rANOVA of female vs. male HA between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	2.042E+10	1	2.042E+10	394.454	.000	.943	1.000
Drug	292437075.2	1	2924370775.2	5.650	.026	.191	.626
Sex	12722753.74	1	12722753.74	.246	.625	.010	.076
Drug*Sex	7880145.702	1	7880145.702	.152	.700	.006	.066
Error	12422119852	24	51759160.49				

Table 10: Overall rANOVA of HA pre-MSA

Overall rANOVA of pre-MSA HA within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	25486178.76	3	8495392.920	1.203	.314	.043	.312
Time*Drug	1713486.036	3	571162.012	.081	.970	.003	.064
Time*Sex	21723513.53	3	7241171.177	1.026	.386	.037	.269
Time* Drug*Sex	34436853.22	3	11478951.07	1.626	.190	.057	.412
Error (time)	571809877.4	81	7059381.202				

Overall rANOVA of pre-MSA HA between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	1.455E+10	1	1.455E+10	445.341	.000	.943	1.000
Drug	2938452.217	1	2938452.217	.090	.767	.003	.090
Sex	16990454.56	1	16998454.56	.520	.477	.019	.107
Drug*Sex	2750317.872	1	2750317.872	.084	.774	.003	.059
Error	882332940.7	27	32678997.80				

Table 11: Overall rANOVA of HA during-MSA

Overall rANOVA of during-MSA HA within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	140246751.8	1	140246751.8	6.618	.017	.216	.695
Time*Drug	1326037.387	1	1326037.387	.063	.805	.003	.057
Time*Sex	270477703.9	1	270477703.9	12.764	.002	.347	.929
Time*Drug*Sex	52391814.19	1	52391814.19	2.472	.129	.093	.327
Error (time)	508578009.6	24	21190750.40				

Overall rANOVA of during-MSA HA between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	7839983815	1	7839983815	112.246	.000	.824	1.000
Drug	352353245.8	1	352353245.8	5.045	.034	.174	.578
Sex	123294647.6	1	123294647.6	1.765	.196	.069	.248
Drug*Sex	7211486.275	1	7211486.275	.103	.751	.004	.061
Error	1676312287	24	69846345.28				

Table 12: Independent sample's t-test of female HA baseline

	Levene's Test for Equality of Variances					t-test for Equality of Means	
	F	Sig.	t	df	Sig.	Mean Difference	Std. Error Difference
Equal variances assumed	.360	.559	-.164	13	.872	-245.821	1498.582

Table 13: rANOVA of female HA

rANOVA of female HA within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	449608365.7	2.000	224789719.9	5.181	.013	.285	.781
Time* Drug	176102653.3	2.000	88045661.78	2.029	.152	.135	.380
Error (time)	1128036911	26.002	43383243.75				

rANOVA of female HA between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	1.046E+10	1	1.046E+10	264.057	.000	.953	1.000
Drug	110099264.7	1	110099264.7	2.779	.119	.176	.339
Error	514957408.6	13	39612108.35				

Table 14: Independent sample's t-test of male HA baseline

	Levene's Test for Equality of Variances					t-test for Equality of Means	
	F	Sig.	t	df	Sig.	Mean Difference	Std. Error Difference
Equal variances assumed	.210	.654	.739	14	.472	1017.250	1375.661

Table 15: rANOVA of male HA

rANOVA of male HA within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	203156050.7	2.419	83973701.34	2.587	.085	.190	.515
Time* Drug	64672125.03	2.419	26731951.59	.824	.470	.070	.188
Error (time)	863856428.5	26.612	32461039.18				

rANOVA of male HA between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	1.000E+10	1	1.000E+10	151.290	.000	.932	1.000
Drug	184825352.0	1	184825352.0	2.796	.123	.203	.333
Error	727262443.2	11	66114767.56				

Table 16: rANOVA of female pre-MSA HA

rANOVA of female pre-MSA HA within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	11101658.69	1.944	5710886.580	.815	.451	.059	.172
Time* Drug	15207331.42	1.944	7822916.140	1.117	.342	.079	.222
Error (time)	177057394.5	25.271	7006262.109				

rANOVA of female pre-MSA HA between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	7235417775	1	7235417775	286.575	.000	.957	1.000
Drug	2935674.550	1	2935674.550	.116	.739	.009	.062
Error	328222257.7	13	25247865.98				

Table 17: rANOVA of female during-MSA HA

rANOVA of female during-MSA HA within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	431248827.1	1	431248827.1	15.889	.002	.550	.957
Time*Drug	19964592.86	1	19964592.86	.736	.407	.054	.125
Error(time)	352834613.5	13	27141124.12				

rANOVA of female during-MSA HA between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	3231680878	1	3231680878	53.526	.000	.805	1.000
Drug	248094319.1	1	248094319.1	4.109	.064	.240	.467
Error	784880053.7	13	60375388.75				

Table 18: rANOVA of male pre-MSA HA

rANOVA of male pre-MSA HA within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	37001118.42	3	12333706.14	1.312	.283	.086	.324
Time*Drug	21147853.42	3	7049284.474	.750	.528	.051	.197
Error(time)	394752482.9	42	9398868.641				

rANOVA of male pre-MSA HA between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	7323872215	1	7323872215	185.043	.000	.930	1.000
Drug	17308720.14	1	17308720.14	.437	.519	.030	.095
Error	554110683.0	14	39579334.50				

Table 19: rANOVA of male during-MSA HA

rANOVA of male during-MSA HA within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	9883440.059	1	9883440.059	.698	.421	.060	.119
Time*Drug	32825175.44	1	32825175.44	2.318	.156	.174	.285
Error(time)	155743396.1	11	14158490.55				

rANOVA of male during-MSA HA between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	4630641653	1	4630641653	57.141	.000	57.141	1.000
Drug	120666252.8	1	120666252.8	1.489	.248	.119	.200
Error	891432233.1	11	81039293.92				

Table 20: Independent sample's t-test of BW baseline

	Levene's Test for Equality of Variances					t-test for Equality of Means	
	F	Sig.	t	df	Sig.	Mean Difference	Std. Error Difference
Equal variances assumed	.678	.417	.009	30	.993	.05500	6.11926

Table 21: Overall rANOVA of BW

Overall rANOVA of BW within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	1765086.484	37	47705.040	107.291	.000	.793	1.000
Time*Drug	8717.183	37	235.600	.530	.991	.019	.602
Error (time)	460637.995	1036	444.631				

Overall rANOVA of BW between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	105892483.7	1	105892483.7	590.427	.000	.955	1.000
Drug	14771.237	1	14771.237	.082	.776	.003	.059
Error	5021770.385	28	179348.942				

Table 22: Overall rANOVA of female vs. male BW

Overall rANOVA of female vs. male BW within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	1765086.484	37	47705.040	573.465	.000	.957	1.000
Time*Drug	8717.183	37	235.600	2.832	.000	.098	1.000
Time*Sex	375163.405	37	10139.551	121.888	.000	.824	1.000
Time*Drug*Sex	3541.322	37	95.711	1.151	.249	.042	.967
Error (time)	80026.281	962	83.187				

Overall rANOVA of female vs. male BW between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	105892483.7	1	105892483.7	6203.516	.000	.996	1.000
Drug	14771.237	1	14771.237	.865	.361	.032	.146
Sex	4575222.041	1	4575222.041	268.031	.000	.912	1.000
Drug*Sex	8175.689	1	8175.689	.479	.495	.018	.102
Error	443813.548	26	17069.752				

Table 23: Independent sample's t-test of female BW baseline

	Levene's Test for Equality of Variances					t-test for Equality of Means	
	F	Sig.	t	df	Sig.	Mean Difference	Std. Error Difference
Equal variances assumed	.002	.967	-1.043	14	.314	-4.37000	4.18854

Table 24: Independent sample's t-test of male BW baseline

	Levene's Test for Equality of Variances					t-test for Equality of Means	
	F	Sig.	t	df	Sig.	Mean Difference	Std. Error Difference
Equal variances assumed	.298	.594	.668	14	.515	4.4800	6.7089

Table 25: rANOVA of female overall BW

rANOVA of female overall BW within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	270426.020	37	7308.811	187.323	.000	.935	1.000
Time*Drug	1370.837	37	37.050	.950	.557	.068	.905
Error(time)	18767.237	481	39.017				

rANOVA of female overall BW between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	33222909.54	1	33222909.54	2795.306	.000	.995	1.000
Drug	484.148	1	484.148	.041	.843	.003	.054
Error	154508.246	13	11885.250				

Table 26: rANOVA of male overall BW

rANOVA of male overall BW within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	1869823.869	37	50535.780	396.802	.000	.968	1.000
Time*Drug	10887.667	37	294.261	2.311	.000	.151	1.000
Error(time)	61259.045	481	127.358				

rANOVA of male overall BW between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	77244796.15	1	77244796.15	3471.013	.000	.996	1.000
Drug	22462.777	1	22462.777	1.009	.333	.072	.154
Error	289305.301	13	22254.254				

Table 27: rANOVA of pre-nicotine/saline exposure females vs. males BW

rANOVA of pre-nicotine/saline exposure females vs. males BW within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	35323.360	1.511	23373.896	139.294	.000	.833	1.000
Time* Drug	192.685	1.511	127.502	.760	.440	.026	.155
Time* Sex	15956.077	1.511	10558.329	62.921	.000	.692	1.000
Time* Drug* Sex	24.575	1.511	16.261	.097	.855	.003	.062
Error (Time)	7100.470	42.314	167.802				

rANOVA of pre-nicotine/saline exposure females vs. males BW between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	10285620.88	1	10285620.88	10278.278	.000	.997	1.000
Drug	1.976	1	1.976	.002	.965	.000	.050
Sex	148452.953	1	148452.953	148.347	.000	.841	1.000
Drug*Sex	844.131	1	844.131	.844	.366	.029	.144
Error	28020.004	28	1000.714				

Table 28: rANOVA of BW during nicotine/saline exposure females vs. males

Overall rANOVA of BW during nicotine/saline exposure female vs. male within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	137270.531	2.541	54016.034	359.931	.000	.928	1.000
Time*Drug	449.973	2.541	177.065	1.180	.320	.040	.280
Time*Sex	32525.641	2.541	12798.858	85.284	.000	.753	1.000
Time*Drug*Sex	69.971	2.541	27.534	.183	.879	.007	.080
Error (time)	10678.653	71.156	150.073				

Overall rANOVA of BW during nicotine/saline exposure female vs. male between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	29769975.43	1	29769975.43	5846.167	.000	.995	1.000
Drug	15835.603	1	15835.603	3.110	.089	.100	.399
Sex	1325442.273	1	1325442.273	260.288	.000	.903	1.000
Drug*Sex	11613.633	1	11613.633	2.281	.142	.075	.308
Error	142582.190	28	5092.211				

Table 29: rANOVA of post-nicotine/saline exposure, pre-MSA females vs. males BW

Overall rANOVA of BW post-nicotine/saline exposure, pre-MSA female vs. male within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	7720.340	3.432	2249.747	58.486	.000	.684	1.000
Time*Drug	1141.382	3.432	332.604	8.647	.000	.243	.997
Time*Sex	487.873	3.432	142.169	3.696	.011	.120	.827
Time*Drug*Sex	292.135	3.432	85.130	2.213	.083	.076	.584
Error (time)	3564.058	92.654	38.466				

Overall rANOVA of BW post-nicotine/saline exposure, pre-MSA female vs. male between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	33301500.66	1	33301500.66	5518.731	.000	.995	1.000
Drug	6206.433	1	6206.433	1.029	.320	.037	.165
Sex	1707309.117	1	1707309.117	282.936	.000	.913	1.000
Drug*Sex	2882.812	1	2882.812	.478	.495	.017	.102
Error	162925.218	27	6034.267				

Table 30: rANOVA of BW during MSA female vs. male

Overall rANOVA of BW during MSA female vs. male within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	4349.532	3.606	1206.103	21.366	.000	.451	1.000
Time*Drug	223.823	3.606	62.065	1.099	.359	.041	.317
Time*Sex	644.106	3.606	178.607	3.164	.021	.108	.773
Time*Drug*Sex	279.273	3.606	77.441	1.372	.252	.050	.390
Error (time)	5292.993	93.763	56.451				

Overall rANOVA of BW during MSA female vs. male between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	37915840.28	1	37915840.28	5812.606	.000	.996	1.000
Drug	903.957	1	903.957	.139	.713	.005	.065
Sex	1922672.868	1	1922672.868	294.751	.000	.919	1.000
Drug*Sex	254.146	1	254.146	.039	.845	.001	.054
Error	169598.944	26	6523.036				

Table 31: rANOVA of female pre-nicotine/saline exposure BW

rANOVA of female pre-nicotine/saline exposure BW within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	2081.544	2.405	865.352	24.763	.000	.639	1.000
Time*Drug	49.448	2.405	20.557	.588	.591	.040	.148
Error(time)	1176.815	33.676	34.945				

rANOVA of female pre-nicotine/saline exposure BW between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	3981346.503	1	3981346.503	5000.070	.000	.997	1.000
Drug	382.214	1	382.214	.480	.500	.033	.099
Error	11147.614	14	796.258				

Table 32: rANOVA of female nicotine/saline exposure BW

rANOVA of female nicotine/saline exposure BW within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	19349.792	3.857	5016.858	80.244	.000	.851	1.000
Time*Drug	249.868	3.857	64.784	1.036	.396	.069	.300
Error(time)	3375.897	53.997	62.520				

rANOVA of female nicotine/saline exposure BW between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	9266117.979	1	9266117.979	2692.632	.000	.995	1.000
Drug	163.317	1	163.317	.047	.831	.003	.003
Error	48178.012	14	3441.287				

Table 33: rANOVA of female post-nicotine/saline exposure, pre-MSA BW

rANOVA of female post-nicotine/saline exposure, pre-MSA BW within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	3411.241	9	379.027	21.557	.000	.624	1.000
Time*Drug	276.935	9	30.771	1.750	.085	.119	.768
Error(time)	2057.117	117	17.582				

rANOVA of female post-nicotine/saline exposure, pre-MSA BW between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	9631978.855	1	9631978.855	2268.794	.000	.994	1.000
Drug	304.240	1	304.240	.072	.793	.005	.057
Error	55190.443	13	4245.419				

Table 34: rANOVA of female during MSA BW

rANOVA of female during MSA BW within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	1511.608	4.088	369.797	9.548	.000	.423	.999
Time*Drug	118.605	4.088	29.015	.749	.566	.054	.227
Error(time)	2058.026	53.140	38.729				

rANOVA of female during MSA BW between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	11381121.10	1	11381121.10	2802.259	.000	.995	1.000
Drug	99.742	1	99.742	.025	.878	.002	.052
Error	52798.319	13	4061.409				

Table 35: rANOVA of male pre-nicotine/saline exposure BW

rANOVA of male pre-nicotine/saline exposure BW within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	49197.893	1.314	37439.634	116.275	.000	.893	1.000
Time*Drug	167.812	1.314	127.705	.397	.593	.028	.096
Error(time)	5923.655	18.397	321.993				

rANOVA of male pre-nicotine/saline exposure BW between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	6452727.325	1	6452727.325	5354.202	.000	.997	1.000
Drug	463.892	1	463.892	.385	.545	.027	.089
Error	16872.390	14	1205.171				

Table 36: rANOVA of male nicotine/saline exposure BW

rANOVA of male nicotine/saline exposure BW within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	150446.380	1.702	88392.915	288.418	.000	.954	1.000
Time*Drug	270.077	1.702	158.680	.518	.574	.036	.120
Error(time)	7302.756	23.828	306.475				

rANOVA of male nicotine/saline exposure BW between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	21829299.71	1	21828299.72	3237.253	.000	.996	1.000
Drug	27285.918	1	27285.918	4.046	.064	.224	.466
Error	94404.178	14	6743.156				

Table 37: rANOVA of male post-nicotine/saline exposure, pre-MSA BW

rANOVA of male post-nicotine/saline exposure, pre-MSA BW within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	4846.462	1.364	3553.498	45.025	.000	.763	1.000
Time*Drug	1187.998	1.364	871.058	11.037	.002	.441	.938
Error(time)	1506.940	19.094	78.922				

rANOVA of male post-nicotine/saline exposure, pre-MSA BW between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	25939147.07	1	25939147.07	3370.760	.000	.996	1.000
Drug	9087.889	1	9087.889	1.181	.296	.078	.173
Error	107734.776	14	7695.341				

Table 38: rANOVA of male during MSA BW

rANOVA of male during MSA BW within subject

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Time	3482.030	2.763	1260.097	13.993	.000	.518	1.000
Time*Drug	384.491	2.763	139.142	1.545	.222	.106	.357
Error(time)	3234.968	35.923	90.053				

rANOVA of male during MSA BW between subjects

Source	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Observed Power
Intercept	28457392.05	1	28457392.05	3167.330	.000	.996	1.000
Drug	1058.361	1	1058.361	.118	.737	.009	.062
Error	116800.625	13	8984.663				

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