UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
F. EDWARD HEBERT SCHOOL OF MEDICINE
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799
FAX: (301/295-6772

APPROVAL SHEET

Title of Dissertation: "Nicotine-induced Antinociception in Male and Female Sprague-Dawley Rats"

Name of Candidate: MAJ Nathaniel Apatov
Doctor of Philosophy Degree
21 July 1999

Dissertation and Abstract Approved:

Brian Cox, Ph.D.
Dept of Pharmacology and Neuroscience Program
Committee Chairperson
Date: 8/4/99

Neil Grunberg, Ph.D.
Department of Medical & Clinical Psychology
and Neuroscience Program
Committee Member
Date: 8/4/99

Ann Jerse, Ph.D.
Neuroscience Program
Committee Member
Date: 8/4/99

Donald Newman, Ph.D.
Neuroscience Program
Committee Member
Date: 8/4/99

Paul Pudimat, M.D.
Neuroscience Program
Committee Member
Date: 8/4/99

CAPT Jane McCarthy, USPHS
Committee Member
Date: 8/4/99

Printed on Recycled Paper

The author hereby certifies that the use of any copyrighted material in the dissertation entitled:

"Nicotine-induced Antinociception in Male and Female Sprague-Dawley Rats"

beyond brief excerpts is with the permission of the copyright owner, and will save and hold harmless the Uniformed Services University of the Health Sciences from any damage which may arise from such copyright violations.

Nathaniel M. Apatov
Neuroscience Program
Uniformed Services University
of the Health Sciences

Abstract

Title of Dissertation: Nicotine-induced Antinociception in Male and Female Sprague-Dawley Rats

Nathaniel Michael Apatov, Doctor of Philosophy, 1999

Dissertation Directed by: Neil E. Grunberg, Ph.D.
Professor
Department of Medical and Clinical Psychology
and Neuroscience Program

Nicotine is a key pharmacologically-active ingredient in tobacco that has cognitive and behavioral effects, including antinociception. The present research examined effects of nicotine as an antinociceptive agent in male and female Sprague-Dawley rats.

Nicotine was administered subcutaneously (SC) to 145 male and female Sprague-Dawley rats. Behavioral measures of nociception included tail-flick, hot-plate, and cold-flick. The Formalin test, a model for persistent pain, was used to examine nicotine-induced antinociception. After antinociceptive testing, animals were sacrificed and blood was collected and assayed for plasma 17-β-estradiol (in females), testosterone (in males), plasma nicotine and cotinine,

brain and spinal cord nicotine and cotinine levels.

On the tail-flick measure, 8 and 12 minutes after drug injection, males increased latencies to respond after receiving 0.1 or 1.0 mg/kg nicotine, whereas females increased latencies to respond only after receiving 1.0 mg/kg nicotine. Gender differences in pain responses in the absence of nicotine may have contributed to the gender differences in nicotine-induced antinociception. Nicotine was antinociceptive for both sexes on hot-plate and on cold-flick tests.

On the Formalin Test, 20 and 25 minute after nicotine injection, 1.0 mg/kg nicotine was significantly antinociceptive for females but not for males. Thirty minutes after nicotine injection, 1.0 mg/kg nicotine was significantly antinociceptive for males but not females.

Estrus cycle stage was significantly correlated with the cold-flick measure such that females in diestrus stage had longer latencies than females in other estrus stages. 17-β-estradiol and testosterone did not significantly correlate with nicotine-induced antinociception.

Plasma, brain, and spinal cord nicotine levels significantly correlated with acute and persistent pain measures. Cotinine levels were a poor predictor of antinociception.

Nicotine was antinociceptive in both acute and persistent pain models. Males responded with increased sensitivity to nicotine-induced antinociception on the tail-flick measure. Plasma, brain, and spinal cord levels of nicotine were good predictors of antinociception in the rat. Mechanisms underlying the gender

differences in nicotine-induced antinociception may involve estrous stage for females but this possibility requires further examination. The neuroanatomy of males and females should be examined with regard to the differential effects of nicotine on antinociception.

Nicotine-Induced Antinociception in Male and Female Sprague-Dawley Rats

by

Nathaniel Michael Apatov

Dissertation submitted to the Faculty of the

Neuroscience Graduate Program

Uniformed Services University of the Health Sciences

in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy, 1999

Acknowledgments

This scholarly work is the culmination of a long-standing dream. That dream was to take my clinical knowledge and experience and blend it with my love of science. Realizing that dream would take me from being a good clinician to becoming an excellent scientist practitioner. This blending of my "former life" and my "present life" would allow me to formulate interesting and important questions *of clinical relevance*, investigate those questions and, finally, to communicate those findings in a succinct and articulate manner. Although I will continue to grow as both a scientist and a clinician, I believe that I have contributed significantly to realizing my dream with this dissertation.

There are many people who have supported and guided me through this process. I thank a most excellent mentor and friend, Neil E. Grunberg. Neil has taught me many valuable lessons over the last four years, not the least of which is how to "love my science." For this lesson, I am grateful. Neil was fortunate in his career to have been mentored by two great men, Joshua Lederberg and Stanley Schachter. He was very lucky indeed; almost as lucky as I was, having been mentored by Neil.

I also thank my other committee members, Dr. Brian Cox, Dr. Donald Newman, Dr. Paul Pudimat, Dr. Ann Jerse, and Dr. Jane McCarthy. Their input and insights were extremely valuable and they provided careful scrutiny of this document. This scrutiny helped me ensure that the dissertation was both comprehensive and correct. Also, the time spent with Don Newman (a man with the patience of a saint) poring over slides and slices was particularly helpful.

Don Newman is a wonderfully talented teacher.

I must also thank my friends and colleagues, Bruce Schoneboom, Martha Faraday, Peter Schefeule, Matt Rahman, Stephanie Nespor, and Hirsch Davis. I must also offer a special thanks to my dear friend and collaborator, LTC Tom Miller. There were many times that Tom's support kept me going...especially when I had very little energy left. Thank you, Tom.

I am grateful to Dr. Neal Benowitz, Dr. Peyton Jacob III, and Lisa Yu for all of their help and advice in performing the pharmacokinetic assays. This was a key component of my dissertation that I could only perform reliably with their guidance.

I want to thank Walter Reed Army Medical Center and the Department of Clinical Investigations for their financial support. Grant G1 72CW made this work possible.

I thank the U.S. Army Nurse Corps for giving me the opportunity to pursue a doctoral degree and work on this project. I hope to repay this gift with many years of productivity as an Army Nurse Corps officer.

All of these individuals were important in my journey. However, I must acknowledge the support and love from the two most important people in my life, my children, Emily and Erica Apatov.

Erica and Emily, you are the source of all of my sunshine and inspiration. You give me hope and allow me to dream of a bright tomorrow. My life has been made incredibly richer because you two are a part of it. I thank you with all my heart.

Table of Contents

Approval Sheet i

Abstract iii

Acknowledgments vii

Table of Contents ix

List of Tables xi

List of Figures xii

Introduction 1

Pain 4

Why Treat Pain? 7

Pain Theory 8

Neuroanatomy of Pain Transmission 9

Gender Differences and Pain 13

Animal Models of Pain 14

Persistent Pain Model 16

Rat Strains 18

Pharmacologic Treatment of Pain 19

Nicotine 22

Nicotinic Receptors in the Brain 24

Nicotine as an Antinociceptive Drug 24

Human Research and Nicotine-induced Analgesia 25

Animal Research and Nicotine-induced Analgesia 28

Nicotine Metabolism 30

Cotinine 31
Mechanisms of Nicotine-induced Antinociception 32
Nicotine and Locomotion 35
Nicotine Tolerance 36
Analgesia and Gender Differences 39
Mechanisms Underlying Gender Differences 41
Sex Hormones as a Source of Gender Differences 42
Nicotine Pharmacokinetics as a Source of Gender Differences 44
Summary 46
Hypotheses 48
Methods 50
Experiment I 50
Experiment II 54
Experiment III 57
Results 65
Experiment I 65
Experiment II 80
Experiment III 92
Discussion 111
Tables 132
References 148

List of Tables

Table 1. Means of Tail-Flick Latencies, Females and Males 133

Table 2. Means of Hot-Plate Latencies, Females and Males 134

Table 3. Means of Cold-Flick Latencies, Females and Males. 135

Table 4. Means of Total Locomotor Distance 136

Table 5. Means of Total Formalin Scores 137

Table 6. Means of Formalin Scores at 5 Minutes 138

Table 7. Means of Formalin Scores at 10 Minutes 139

Table 8. Means of Formalin Scores at 15 Minutes 140

Table 9. Means of Formalin Scores at 20 Minutes 141

Table 10. Means of Formalin Scores at 25 Minutes 142

Table 11. Means of Formalin Scores at 30 Minutes 143

Table 12. Means of Plasma Nicotine and Cotinine Levels 144

Table 13. Means of Brain Nicotine Levels 145

Table 14. Means of Spinal Cord Nicotine Levels 146

Table 15. Means of Plasma Levels of Testosterone and Estradiol 147

List of Figures

Figure 1. Nicotine's Structure 22

Figure 2. Metabolic Pathway of Nicotine 31

Figure 3. Tail-Flick Latencies, Females and Males at 8 Minutes 67

Figure 4. Tail-Flick Latencies, Females and Males at 12 Minutes 69

Figure 5. Hot-Plate Latencies, Females and Males at 8 Minutes 71

Figure 6. Hot-Plate Latencies, Females and Males at 12 Minutes 72

Figure 7. Cold-Flick Latencies, Females and Males at 8 Minutes 74

Figure 8. Cold-Flick Latencies, Females and Males at 12 Minutes 75

Figure 9. Locomotor Activity, Females and Males 77

Figure 10. Locomotor Activity at 2-minute Intervals, Females 78

Figure 11. Locomotor Activity at 2-minute Intervals, Males 79

Figure 12. Total Formalin Scores, Females and Males 82

Figure 13. Formalin Scores at 5 Minutes, Females and Males 84

Figure 14. Formalin Scores at 10 Minutes, Females and Males 85

Figure 15. Formalin Scores at 15 Minutes, Females and Males 86

Figure 16. Formalin Scores at 20 Minutes, Females and Males 88

Figure 17. Formalin Scores at 25 Minutes, Females and Males 89

Figure 18. Formalin Scores at 30 Minutes, Females and Males 91

Figure 19. Regression Correlation, Brain Nicotine Levels and Tail-Flick . . . 95

Figure 20. Regression Correlation, Spinal Cord Nicotine Levels and Tail-Flick 96

Figure 21. Regression Correlation, Brain Nicotine Levels and Hot-Plate . . . 98

Figure 22. Regression Correlation, Spinal Cord Nicotine Levels and Hot-Plate 99

Figure 23. Regression Correlation, Brain Nicotine Levels and Cold-Flick . 102

Figure 24. Regression Correlation, Spinal Cord Nicotine Levels and Cold-Flick 103

Figure 25. Cold-Flick Estrous Cycle Testing 108

Figure 26. Formalin at 5 Minutes and Estrous Cycle Stage 109

CHAPTER I

Introduction

Pain is a universal experience which provides an organism useful information about the *internal milieu* or about an external threat to homeostasis. Pain is often defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage" (Bonica, 1979, p. 6). In the past, opioid analgesics have been the primary pharmacologic agents used in treating pain. Unfortunately, these drugs have side effects that limit their usefulness. This limitation of the opioid drugs has stimulated interest in analgesic actions of other drugs. Nicotine, the pharmacologically active drug in tobacco smoke, has been reported to have analgesic properties (Aceto, Awaya, Martin, & May, 1983; Apatov, 1998; Damaj, Welch, & Martin, 1994; Iwamoto, 1989; Jamner, Girdler, Shapiro, & Jarvik, 1998; Pomerleau, 1986). These reports, however, include studies that lack proper controls and have other methodologic weaknesses. With regard to the human literature, studies have produced inconsistent results reporting no effect of nicotine on pain as well as positive analgesia from nicotine. These problems prevent a definitive conclusion regarding nicotine's antinociceptive properties. Additionally, the one human study that examined gender differences and nicotine-induced antinociception did not use body weight to calculate nicotine dose and used an electrocutaneous stimulator (Jamner et al., 1998). Body weight could affect transmission and perception of a transcutaneous pain stimulus. There is a single animal study addressing sex differences in nicotine-induced antinociception (Apatov, 1998).

That study used a chronic infusion model and only examined acute measures of nociception. A single, controlled study examining nicotine-induced antinociception using acute, nicotine injections in male and female rats is needed. The present research is designed to accomplish this goal.

The current project explores the effects of nicotine as an analgesic agent in a rat model. Male and female rats are included to determine whether sex differences exist in nicotine-induced antinociception. A review of the research literature on this topic reveals that most analgesic testing uses subjects, human and animal, of a single sex, usually males. However, evidence exists that males and females differ in their responses to analgesics (Berkley, 1997; D'Amour & Smith, 1941; Feine, Bushnell, Miron, & Duncan, 1991; Woolfe & MacDonald, 1944) with males usually more sensitive to analgesics.

Although males and females differ in their responses to classic analgesics, the mechanism underlying these differences has been elusive. Two of the most likely explanations for these sex-related differences in antinociception are: 1) pharmacokinetic differences, and 2) sex hormone-related differences. The experiments included in this doctoral research examined whether there are gender differences in nicotine-induced antinociception and examined whether the pharmacokinetics and sex hormones may account for these differences.

Experiment I used an acute subcutaneous (SC) nicotine administration paradigm to assess antinociceptive responses to acutely noxious stimuli in male and female Sprague-Dawley rats. The two classic nociceptive tests were used:

hot-plate and tail-flick. In addition to these two tests, a cold-flick test was used to determine if nicotine provides analgesia to noxious cold stimuli as well as to noxious hot stimuli. Changes in locomotor activity in response to an acute injection of nicotine also were assessed. This measure was added to determine whether any changes in the antinociceptive responses were a result of changes in the animal's ability to respond to a motor task instead of changes in antinociceptive thresholds.

Experiment II investigated nicotine's antinociceptive effects also following an acute injection, but used a tonically painful stimulus: an SC injection of 2% formalin into a hind paw in Sprague-Dawley rats of both sexes. Injecting a dilute formalin solution into a rat's paw elicits stereotypical behaviors reported to be an index of pain (Dubuisson & Dennis, 1977; Tjolsen, Berge, Hunskaar, Rosland, & Hole, 1992).

During Experiments I and II, estrus cycle staging was assessed in female rats. This information was used to determine if the subject's position in the estrus cycle affects nicotine-induced antinociception. Plasma testosterone was assayed in male rats, and plasma 17-β-estradiol was assayed in female rats, to determine if sex hormone levels correlate with nicotine-induced antinociception.

Experiment III used the same subjects from the previous two experiments to explore the mechanisms underlying gender differences in nicotine-induced antinociception. Following an additional, acute, subcutaneous (SC) injection of nicotine, all subjects were sacrificed and blood, brain, and spinal cord samples were obtained to determine whether nicotine-induced antinociception correlated

with plasma and tissue levels of nicotine and cotinine (the primary metabolite). If males and females have discrepant nicotine levels in plasma, brain, or spinal cord, these data may help explain gender differences in nicotine-induced antinociception. In addition, differences in nicotine and cotinine levels in brain and spinal cord may account for discrepancies between spinally-mediated antinociceptive responses from supraspinally-mediated antinociceptive responses.

This project explored the potential role of nicotine as an analgesic agent and how gender may modulate nicotine's analgesic properties. To set this research project in context, the relevant literature is reviewed. First, a review of pain is presented that includes pain theory, neuroanatomy of pain, animal models, and pharmacology to treat pain. Following these sections, a review of nicotine and nicotine's effects is provided. Finally, there is a review of gender differences in analgesia and possible mechanisms underlying gender differences in nicotine-induced antinociception. These sections provide the background for the research presented.

Pain

Pain is a subjective symptom and is often difficult to directly verify by physicians and other health-care professionals. Most of us experience pain at one point or another in our lives. As we age we are more likely to experience an acutely, painful injury such as banging a finger with a hammer or a more chronic pain, perhaps one associated with chronic joint pain. The enormity and complexity of this issue is considerable.

Humans experience both acute and chronic or persistent pain. Acute injuries occur at home, at the work place, and in automobiles. Most, if not all, of these injuries are associated with pain. Chronic pain is also a significant consideration and significantly affects quality of life. A major contributor to the pain population are those afflicted by low back pain. Low back pain is a common occupational injury and is the most frequent cause of activity limitation in people below the age of 45 years, the second most frequent reason for physicians' visits, the fifth most frequent reason for hospitalization, and third ranked reason for surgical procedures (Wall & Melzack, 1993). The cost to society in terms of days lost and claims paid involves billions of dollars. In one recent large-scale Canadian study (Cassidy, Carroll, & Cote, 1998), 28% of the population reported experiencing low back pain at the time of the study and 84% had experienced low back pain during their lifetimes.

Pain is a common entity in hospitalized patients as well. A random sampling of inpatients found that 67% of patients had experienced pain within prior 24 hours. Although post-surgical patients were more likely to report pain, 21% of non-surgical patients reported moderate to severe pain (Abbott et al., 1992).

Among hospitalized patients, a common pain is the pain associated with a malignancy. Pain is a source of fear and frustration in cancer patients. In fact, in one study over 70% of patients with cancer report moderate to severe pain and many report that they **fear pain more than death** (Grossman, 1993). Only a small percentage of these patients were adequately medicated for pain.

A unique population of individuals who have considerable experience with pain is the military. Soldiers, sailors, and marines have a history of experiencing pain. This pain may be foot pain associated with a ten-mile road march with full gear or, alternatively, the pain associated with a high velocity, projectile disrupting body integrity. In either scenario, the pain may be severe enough to affect the way the individual is able to perform his or her duties. If that duty is to capture an enemy's stronghold, then the distraction caused by the pain may be sizable and may cost the person his life. Because pain in the military is an occupational hazard it is critical to determine how to treat pain effectively.

Another population that may have unique analgesic requirements is women. There are increasing reports in the literature that men and women differ in the frequency with which they experience pain and in their analgesic requirements (Unruh, 1996). Usually, analgesic medication is prescribed for patients based on the intensity of the pain, patient age, patient weight, and pre-existing diseases. These parameters are important, but do not take into account relevant information regarding individual genetic differences including gender.

Although pain may be a universal experience, pain relief is not. Several studies examining pain and analgesia report that pain goes largely unrelieved. A report examining the incidence of pain in medical and surgical in inpatients indicated that 58% of patients reported experiencing "excruciating pain" during their hospitalization (Donovan, Dillon, & McGuire, 1987). Another study conducted in 1990 examining pain in postoperative surgical patients reported a 9% incidence of "unbearable pain," and a 24% incidence of "severe pain" (Owen,

McMillan, & Rogowski, 1990). Patients may be under-medicated for two primary reasons; physicians do not deeply understand the pharmacology of analgesics and they often exaggerate the dangers of patient addiction (Marks & Sachar, 1973).

Why treat pain?

If pain has a role in alerting the animal or person that injury has occurred, then is there a reason to intervene and attempt to attenuate the pain experience? Indeed, pain is considered noxious and most people would agree that pain adds an unpleasant dimension to their conscious states. Therefore, relieving pain may be considered humane and ethical. There are, however, other biologic consequences of pain that add stress and that may contribute to the disruption of homeostasis. These consequences do not always assist the individual to cope with or protect the injury site. In fact, some of these consequences may have a deleterious effect. Therefore, the amelioration of pain is a worthwhile endeavor and any mechanism, psychological, physical, or pharmacological, is worth exploring.

The pathophysiologic changes that occur after acute tissue injury may result in moderate to severe pain. In addition, there is a sympathoadrenal activation which may lead to hypertension, increased risk of hemorrhage, or stroke. This increase in sympathetic tone can cause tachycardia, arrhythmias, and potentially, congestive heart failure. Metabolic changes, including hyperglycemia, hypernatremia, and hypokalemia, also may have a negative effect on the individual (Sinatra, Hord, Ginsberg, & Preble, 1992). These

alterations related to tissue damage and the accompanying pain response make the alleviation of pain more than just a humane endeavor, but rather an important adjunct to return people to optimal health.

Pain Theory

Many theories have been advanced to try to explain the phenomenon of pain. These theories include the Specificity Theory (Descartes, 1641) and Pattern Theory (Nafe, 1934). These theories are inconsistent with data gathered in the 20th Century. The theory that best fits our knowledge of pain is the Gate Control Theory of Pain (Melzack & Wall, 1965). This theory suggests that small afferents, relaying pain information, enter the spinal cord and synapse in the superficial laminae (substantia gelatinosa). Neuronal activity in other large and small afferent fibers also can synapse here and, in doing so, modulate the pain response. Therefore, the substantia gelatinosa is hypothesized to act as a gating site where afferent pain information is modulated prior to ascending rostrally. The implications of this theory are that cognitive or biologic factors can influence pain transmission at this gating area.

This neuroanatomical and neurophysiological information can be used to help develop drugs that ameliorate pain. Drugs applied along the pain pathway are able to block pain impulses from ascending rostrally, e.g., local anesthetic agents. These agents keep us from perceiving the pain. Another strategy is to administer drugs that affect the way we feel about the pain. Drugs with central actions may elicit analgesia by activating centrally located, descending-inhibitory pathways and, in doing so, attenuate pain transmission. Such drugs are the

mainstay of analgesic therapies. Morphine acts in this way to diminish pain. Patients receiving morphine analgesia report that they can still feel the pain, but it doesn't bother them anymore. The pain is still perceived, but it is no longer distressing (Gilman, Rall, Nies, & Taylor, 1993).

Nicotine has been reported to have analgesic properties, but It is unclear how nicotine may be modulating pain (Apatov, 1998; Caggiula, Epstein, Perkins, & Saylor, 1995). Is there an interruption in the transmission of the noxious stimulus or, like morphine, does nicotine modulate the way pain is perceived? Nicotine may be analgesic in animals and humans, but it is not known whether nicotine is analgesic for heat-induced pain only or if it is analgesic for both acute and chronic pain. Further, it is important to investigate if genetic differences, specifically gender differences, modulate analgesia. This information may help health professionals: 1) develop analgesics that are better tailored to the individual, and 2) to assist those individuals who may smoke to attenuate pain to quit smoking.

Neuroanatomy of Pain Transmission

In order to understand how drugs may attenuate pain, it is relevant to consider how pain is communicated from the injury site to the brain where the noxious information is processed. Information regarding noxious stimuli is transmitted via nociceptors from the periphery to the neuraxis by way of thinly-myelinated, small-diameter Aδ fibers and unmyelinated, small diameter C-fibers. Aδ fibers carrying sharp, fast, pain information relay these signals to the spinal cord and terminate within laminae I, V, and X (Meyer, Campbell, & Raja, 1993).

C-fibers relay information about slow, burning, pain and terminate in Lamina II (substantia gelatinosa) (Jones, 1992). These afferent fibers then synapse in the spinal cord and ascend rostrally in several important ascending pathways. These pathways, traveling in the anterolateral, white matter of the cord, include the spinothalamic (STT), spinoreticular, and spinomesencephalic that are collectively referred to as the spinal lemniscus (Sinatra et al., 1992).

Spinothalamic Tract

In humans, the spinothalamic tract (STT) carries information regarding pain, temperature, and touch to thalamus. The cell bodies originate in laminae I, IV-VI, and X (Willis, Kenshalo, & Leonard, 1979). Most of the cells project to the contralateral thalamus decussating in the ventral white commissure. The spinothalamic tract has been described as comprising two separate tracts. There exists a more medial tract which courses through the brainstem terminating in medial thalamic nuclei, and there also exists a more lateral tract that terminates in the more lateral thalamic nuclei. The axons of the STT are arranged somatotopically. At cervical levels, axons representing lower extremities and caudal body are situated more laterally in the spinal cord, while axons representing upper extremities and rostral body are more anteromedially placed (Walker, 1940).

The medial pathway is considered to be a phylogenetically older pathway (Mehler, 1962) and sends collaterals to higher structures and terminates in medial thalamic nuclei, e.g., intralaminar nucleus (IL) and ventralmedial posterior nucleus (VMpo) (nucleus submedius in cat). These nuclei are believed to be

involved in the emotional aspects of pain, and ultimately terminate in limbic structures specifically: insula, prefrontal, and cingulate cortices.

The lateral tract is thought to be a phylogenetically newer pathway (Mehler, 1962), does not collateralize to higher structures, and terminates in lateral thalamic nuclei, e.g., ventral posterior lateral caudal (VPLc) and the posterior complex (PO). This pathway is believed to be involved in the sensory-discriminative aspects of pain. Further, these neurons project to SI (primary somatosensory) cortex which is also involved in discriminating noxious stimuli (Willis & Westlund, 1997).

Spinomesencephalic Tract

Spinomesencephalic neurons emanate primarily from laminae I and IV-VI (although some originate in the ventral horn and lamina X) and ascend rostrally to the contralateral midbrain. This tract projects to the periaqueductal gray (PAG), nucleus cuniformis, intercolliculus nucleus, anterior and pretectal nuclei and the interstitial nucleus of Cajal (Willis, 1985). The tract is roughly, somatotopically organized. Spinomesencephalic projections from caudal body parts terminate more caudally in the midbrain, whereas projections from the more rostral parts terminate more rostrally in the midbrain.

Projections from spinomesencephalic neurons to PAG are involved with aversive behavioral responses to pain (vocalization and autonomic responses) (Skultety, 1963). In addition to these behavioral responses, activation of PAG initiates a descending inhibitory, analgesic pathway. Projections from spinomesencephalic neurons to nucleus cuniformis activate the midbrain

locomotor center (Brooks, 1986) and the reticular activating system (RAS). There are also projections via the spinocollicular tract that may play a role in visual orienting, searching, and tracking.

Spinoreticular Tract

Cells of the spinoreticular tract originate in the deep layers of the dorsal horn and in laminae VII and VIII of the ventral horn (Willis, 1985). The spinoreticular ascends with the STT in ventrolateral spinal cord and brainstem (Mehler, Feferman, & Nauta, 1960) and projects primarily to the pontomedullary reticular formation which is involved in alerting and arousal and may turn on descending analgesic pathways. In addition, the spinoreticular tract projects to the intermediate reticular formation, including parabrachial nucleus. These pathways mediate the cardiovascular and respiratory responses to pain.

Spinolimbic Tract

Bishop (1959) described a multisynaptic pathway from the periphery, relayed through the medial thalamus, to the limbic system (Bernard & Besson, 1990; Scheibel & Scheibel, 1958). This information is thought to travel in the spinoreticular tract and project to medial thalamus (Nauta & Kuypers, 1958), hypothalamus (Burstein, Cliffer, & Geisler, 1990), and amygdala (Bernard & Besson, 1990).

Alternate Pathways

The above tracts are the classically described pathways that carry information regarding noxious stimuli to the brain. However, there are two other lesser known pathways that also are involved in pain processing: the

spinocervicothalamic pathway and the dorsal column pathways.

The cells of the spinocervicothalamic pathway originate in the dorsal horn of the spinal cord and relay neurons in the lateral cervical nucleus in segments C1 and C2 (Willis, 1985). These axons ascend in the lateral funiculus and terminate in the lateral cervical nucleus. Projections from the lateral cervical nucleus go to the contralateral VPL nucleus and PO complex (Berkley, 1980).

The dorsal column system carries information subserving two-point touch, graphesthesia, and kinesthesia. It has been reported that when the tract is cut by a limited, midline myelotomy, patients with intractable pelvic pain receive relief suggesting that this pathway plays a role in the relay of visceral pain (Hirshberg, Al-Chaer, Lawand, Westlund, & Willis, 1996). The cells of the postsynaptic dorsal column pathway appear to relay visceral and epicritic information to the thalamus (Willis & Westlund, 1997).

Gender Differences and Pain

Many people believe that men and women are differentially sensitive to pain (Bendelow, 1993). Opinions regarding which gender is more or less sensitive to pain and analgesia differ and currently no consensus exists. Evidence does exist in human and animal research suggesting that sex differences exist in response to pain. These differences are evidenced in human prevalence studies for certain painful syndromes, such as low back pain and myofascial pain. Women report experiencing more clinically painful syndromes, such as migraines and temporomandibular joint disorders, than do men (Unruh, 1996). Women report more temporary and persistent pains (Crook, Rideout, &

Browne, 1984) and more severe pain (Reisbord & Greenland, 1985). This body of research suggests that men and women have distinctly different pain experiences and that women may be more sensitive to noxious stimuli than are men. There are several hypotheses for these gender-related differences in response to noxious stimuli. These hypotheses include: differences in structure and function of sensory afferents, gender-associated differences in processing of noxious stimuli, and differential modulation of efferent, inhibitory pathways (Fillingim & Maixner, 1995). Whether these differences account for what is observed clinically, that is, that men and women differ in their perceptions and in the frequency that they report pain, remains unknown.

Animal Models of Pain

The study of pain and analgesia is important. It is both ethical and physiologically sound to achieve a scientific understanding of pain and learn how to treat it effectively. However, pain is a complex phenomenon. It is highly subjective and it difficult to assess and measure across individuals. Although studying pain in humans has face-validity, there are many constraints because of the intrinsic unpleasantness of pain. Because pain is such a complex perceptual construct, it is difficult to break this complex construct down to its constituent components in order to study it. Therefore, animals research in pain is crucial because it allows researchers to use specific noxious stimuli and quantify the effect of these stimuli on objective, discrete, observable behaviors. Additionally, it is possible to manipulate experimental variables while keeping constant extraneous variables that could impact pain perception.

For these reasons, animals play an important role in the study of pain. The goals of animal research in pain is to perform research that "cannot or should not be performed in humans" (Stanley & Paice, 1997, p.1). Chapman and colleagues (1985) outline reasons animals play an important role in the study of pain. The use of animals is critical because: 1) it permits manipulation of experimental variables that can be studied at the cellular and subcellular levels, and 2) animal models may be used to model certain human pathological conditions while manipulating physiologic or pharmacologic variables that are not possible or ethical in human subjects.

There are several different paradigms to study nociception in rodents. One measure involves monitoring escape and avoidance behaviors after presentation of a painful stimulus. Another example is a motivational choice paradigm where animals must choose between either a reward or noxious stimulus (Chapman et al., 1985; Feldman, Meyer, & Quenzer, 1997). In the present research, two classic tests of nociception are used: the hot-plate and tail-flick tests (D'Amour & Smith, 1941; Woolfe & MacDonald, 1944). These two measures are reported to test nociceptive processing at two levels: spinal and supraspinal (Caggiula et al., 1995). Most investigators use one measure or the other in order to study pain in a rat model. Although these two measures assess antinociception on two levels of processing (spinal and supraspinal), they are both acute measures and do not address the processing of noxious impulses that may persist over time.

The hot-plate and tail-flick tests utilize heat as the noxious stimulus.

Although thermal (temperature) sensitivity is a useful way to measure the antinociceptive properties of drugs, heat has been the most widely used. However, there are data suggesting that using noxious cold as the painful stimulus may be a more sensitive method of detecting antinociception when using drugs with moderate analgesic profiles (Pizziketti, Pressman, Geller, Cowan, & Adler, 1985). In that report, opioid analgesics and mixed agonist-antagonists analgesics produced a dose-related effect using the cold-flick method. In later studies, the cold-flick test proved useful in assessing μ-opioid agonists (Adams, Geller, & Adler, 1994) as well as κ and δ-opioid agonists (Briggs, Rech, & Sawyer, 1998; Tiseo, Geller, & Adler, 1988).

In the studies cited above, the temperature of the noxious cold stimulus ranged from -3° C to -10° C. However, the optimal temperature for this measure is not known. To address this issue, Wang, Ho, Hu, and Chu (1995) used a series of cold water/ethanol baths to determine an optimal cold test. After testing solutions ranging between -5° and -30° these investigators concluded that -20° C was optimal. In that report the authors present a scatter plot of their latency data. At -15° C the tail-flick latencies range between approximately 10 seconds and 65 seconds. At -20° C the tail-flick latencies range between approximately 3 and 22 seconds. In the current study, cold-flick temperature was held at -17° C ± 1° C to: 1) avoid latencies greater than 60 seconds (to prevent tissue damage) and 2) avoid latencies shorter than 10 seconds (minimizing a floor effect).

Persistent Pain Model

The tail-flick, hot-plate, and cold-flick tests elicit a behavioral response to

an acutely, noxious stimulus. This is one way to determine whether nicotine has antinociceptive properties and may be analogous to the sensation experienced when one places his hand on a hot stove and gets a transiently, painful sensation. Acute pain models are useful and widely used, but used alone capture only one aspect of pain and it has been argued that these acute measures do not bear close resemblance to clinical pain (Sternbach, 1976). Acute measures capture the quick, sharp, pain associated with Aδ fiber activation, but to capture the slower, burning type pain associated with C-fiber activation and more fully characterize nicotine-induced antinociception the inclusion of a persistent pain model was needed. A persistent noxious stimulus may be produced by the injection of a physiologic irritant into an animals paw. Formalin has been used to cause inflammation and to produce a model of chronic pain (Dubuisson & Dennis, 1977). This model is an attempt to simulate the human experience of having a continuous pain as might be caused by trauma or disease. Over the last 20 years the Formalin Test has been employed extensively as a model of injury-induced pain in rodents (Dubuisson & Dennis, 1977; Franklin & Abbott, 1989; Tjolsen et al., 1992; Wheeler-Aceto & Cowan, 1991). The Formalin Test has another advantage. Weak and moderate analgesic agents have been reported to have clear antinociceptive effects using this measure (Hunskaar, Fasmer, & Hole, 1985). Sensitivity may be further increased by using low concentrations of formalin (Tjolsen et al., 1992) (see Chapter II, Methods).

The Formalin Test differs from most other pain models because it allows

assessment of an animal's reaction to actual tissue injury and therefore is considered a more valid model for clinical pain than other models (Tjolsen et al., 1992). It allows the investigator to assess an animal's response to a moderate, continuous, noxious sensation generated by injured tissue and may be analogous to human postoperative pain (Abbott & Franklin, 1986).

Although the Formalin Test is a excellent model of a tonically painful stimulus, the duration is limited and beyond one hour the discomfort is minimal. In addition, low concentrations of formalin do not cause macroscopically visible tissue damage in mice (Rosland, Tjolsen, Maehle, & Hole, 1990). Two hours after injection the paw appears to be used normally; the animal eats, grooms, moves freely (Dubuisson & Dennis, 1977).

By using hot-plate, tail-flick, and cold-flick, as well as the Formalin Test, one can explore multi-level pain processing, and both transient and persistent pain. These tests when administered as a battery can fully characterize the antinociceptive activity of mild to moderate analgesics. Nicotine, if analgesic, may be a mild or moderate analgesic drug necessitating multi-dimensional testing.

Rat Strains

The current research examined the effects of nicotine on antinociception in male and female Sprague-Dawley rats using both acute and persistent models of nociception. The Sprague-Dawley is the classic, albino laboratory rat used for scientific research in multiple disciplines. These rats have been bred for genetic homogeneity for multiple generations. As a result of these breeding techniques,

animals are almost genetically identical to each other.

This strain of rat was used for three reasons. First, Sprague-Dawley rats are the most common laboratory animals used in analgesic testing. In order to compare results of nicotine-induced analgesia with other analgesics, it was necessary to use the same strain of rat. Second, Sprague-Dawley rats were used in a previous report (Apatov, 1998) of sex-related differences with nicotine-induced analgesia. Using the same animal strain allows a direct comparison between the chronic, nicotine administration used in that report and the acute, administration model used in the present research.

Lastly, as stated earlier Sprague-Dawley rats are bred for genetically similarity. These experiments investigate gender differences therefore, by holding other genetic variables constant, one can attribute differences in nicotine-induced antinociception to the gender manipulation and not to other extraneous genetic variables.

Pharmacologic Treatment of Pain

Pharmacologic intervention has been the mainstay of pain management. Drugs used to treat pain include: opioids, local anesthetics, and non-steriodal anti-inflammatory drugs (Gilman et al., 1993). One can use drugs to treat pain by: applying the drug locally to the injury site, applying drugs somewhere along the pain pathway (e.g., spinal cord), or administering the drug systemically, where the drug may act at a single or multiple sites. Each of these modalities has its individual strengths and weaknesses. Local anesthetic agents can relieve pain completely. In small doses there is almost no effect on level of

consciousness. The difficulty is that local anesthetic agents must be applied either directly to the site or somewhere along the neuraxis. Therefore, their usefulness is limited by the logistics of delivering the drug to an appropriate site of action. In addition, if the moderate to high dosages of the drugs get into the systemic circulation, central nervous system depression, convulsions, and death can ensue (Gilman et al., 1993).

Another class of drugs that are useful in treating pain are the non-steroidal antiinflammatory drugs, e.g., ibuprofen, naproxen, and fenbufen. These agents provide analgesia and also may act peripherally to decrease the inflammatory cascade (Wall & Melzack, 1993). However, as with most drugs, their usefulness is limited by their side effects which include gastrointestinal upset and blood dyscrasias (Gilman et al., 1993). As a result, the most common way to modulate moderate to severe pain is to administer centrally acting analgesic drugs.

Classically, pain has been treated with opioid analgesics (Feldman et al., 1997). Drugs like morphine and fentanyl are fairly effective in treating pain, but they have some important limitations. These limitations are a result of the side effects associated with these agents. While some of these limitations are merely troublesome for patients (e.g., pruritus, constipation, nausea), other side effects, like respiratory depression, can be life-threatening (Gilman et al., 1993). Another important side effect of these agents is sedation. Many of the powerful opioids (morphine, fentanyl, meperidine, etc.) depress higher order CNS processing (Gilman et al., 1993). These drugs make the patient somnolent and hamper normal cognitive functioning. If the patient is lying in a hospital bed with side-

rails raised being monitored by nurses and doctors, then sedation is not a problem. If however, the person is supposed to be operating a crane, using a power saw, or driving a school bus, then there is a danger. There is another occupation that be at risk for similar concerns. This population is the military. These individuals may be operating a bazooka instead of a crane, using a semi-automatic rifle instead of a power saw, or perhaps driving a tank instead of a bus. For members of the armed forces, slowed cognition or impaired judgement may not just be harmful to the individual, but may pose a threat to multiple people. While at increased risk for a painful injury, people in the military are not good candidates for conventional, analgesic agents.

For individuals experiencing moderate to severe pain, analgesia without sedation is problematic. Although opioids are potent analgesics and are easily administered, the impairment of cognitive functioning may not allow individuals performing critical duties to continue to do so. The problem of being able to deliver analgesia while not sedating is an important issue meriting further exploration.

Nicotine, a centrally-acting drug that does not have sedative effects and is a sympathomimetic agent may be a good candidate to provide analgesia under these circumstances. Therefore, nicotine may be useful in specific situations, such as in wartime. Nicotine also might prove useful as an analgesic adjuvant. Analgesic adjuvants are drugs that may not be considered classic analgesics, but may be useful in combination with other analgesic agents. Drugs such as baclofen, methotrimeprazine, and cortisol have been used for this purpose

(Fagerström & Schneider, 1989; Fromm, 1994; Patt, Proper, & Reddy, 1994; Watanabe & Bruera, 1994). By administering a small dose of nicotine with another analgesic (opioid), it may be possible to decrease the dosage of the opiate thus sparing the depressant effects of the opiate on cognitive functioning. Additionally, because nicotine is a sympathomimetic drug, it may balance the vagotonic effects of the opioids. Therefore, nicotine may be an excellent choice to explore as an alternative analgesic.

Nicotine

Nicotine [-3-(1-methyl-2-pyrrolidinyl)-pyridine] is a naturally occurring alkaloid found in tobacco products; specifically *nicotiana tabacum*, *nicotiana rustica* and related species. It is a tertiary amine composed of a pyrrolamine and pyridine ring (see Figure 1). Nicotine has a pKa of 8.0 (aqueous solution, 25° C). There are several stereoisomeric forms, but the form primarily self-administered is the (S)-Nicotine form. Nicotine base is a colorless to pale, yellow, oily, liquid that will turn brown upon exposure to air.

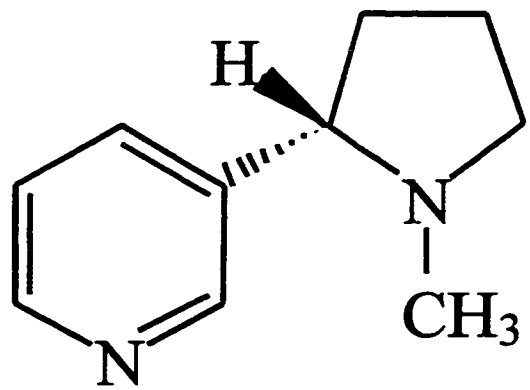

Figure 1: Nicotine

Nicotine is defined by Goodman and Gilman (1993) as a ganglionic stimulant. Although it does stimulate autonomic ganglia initially, it also can cause a persistent depression and blockage of the ganglia. In addition to this peripheral effect, nicotine has other peripheral and central actions. Nicotine can stimulate adrenal medullary cells to discharge catecholamines. It is an agonist at the cholinergic receptors at the neuromuscular junction and is known to have an excitatory effect on cardiovascular and respiratory systems. It also can stimulate sensors in the skin, tongue, and stomach.

Nicotine's ability to agonize cholinergic receptors centrally is profound. Nicotine is a powerful psychoactive drug (Balfour, 1984) and can stimulate the CNS markedly and cause tremors, convulsions and, in large enough dosages, nicotine can cause death.

Nicotine also may have an effect on cognition. Smokers report that smoking helps calm them and alleviate anxiety (Pomerleau, Turk, & Fertig, 1984). Other cognitive effects include stress modulation, affect modulation, and improvement in attention and perhaps memory (USDHHS, 1988). Another central effect may be analgesia (Apatov, 1998; Pomerleau et al., 1984; Yang, Wu, & Zbuzek, 1992). It is conceivable that these cognitive effects are reinforcing enough to cause people to continue to smoke and may be part of the reason as to why smoking is such a persistent behavior.

Although nicotine use may be reinforced through its actions on cognition, there is also the drug's effects on the classic reward pathway. This model proposes that addictive drugs cause an increase in dopamine release from the

nucleus accumbens (NAcc) which then binds to receptors upstream in the ventral tegmental area (VTA) (area A10) (Bozarth, 1994; Koob, 1992).

Nicotinic Receptors in the Brain

Nicotine is a ligand at a subclass of acetylcholine receptors in the brain. The receptors are composed of pentameric protein units. The receptor consists of two agonist-binding subunits (α units) and three structural subunits (β units). The α units include $\alpha2$, $\alpha3$, $\alpha4$, $\alpha5$, and $\alpha6$. The β units include $\beta2$, $\beta3$, and $\beta4$. In addition, there is a subfamily of nicotinic, acetylcholine receptors (nAChRs) that are able to form homo-oligomers. These are the $\alpha7$-$\alpha8$ subfamily (Picciotto, 1999). The most widely expressed nicotinic receptors in the brain are the $(\alpha4)_3(\beta2)_2$ receptor and the $\alpha7$ receptor (Wada, Wada, & Boulter, 1989; Zoli, Le Novere, Hill, & Changeux, 1995). Nicotine reinforcement occurs via binding of nicotinic receptors in the mesolimbic dopamine pathway between the VTA and the nucleus accumbens. These regions are thought to be involved in the reward pathway of addictive drugs.

Nicotine as an Antinociceptive Drug

Of the 4000 chemicals found in tobacco smoke, nicotine is the pharmacologically active agent and is known to possess psychoactive properties (USDHHS, 1988). It was suggested over 75 years ago that one of these psychoactive properties may be analgesia. It was reported in 1921 that smokers, when allowed to smoke, have increased pain thresholds (Mildenhall, 1921). In an uncontrolled study, Mildenhall used the "cold pressor apparatus" to elicit pain and examine the connection between smoking (not nicotine) and

analgesia. This procedure involved a subject simply immersing his arm into an ice-water bath (4° C) until the subject could no longer stand the pain. The subsequent analgesic or antinociceptive effect was attributed to the peripheral activation of nicotinic, cholinergic, receptors. It was believed that by causing vasoconstriction and cutting down blood flow to peripheral tissues, that nicotine was rendering either the peripheral receptors or peripheral nerves insensitive to the noxious stimulus (ice-cold water), and in doing so was providing analgesia. Therefore, nicotine may have been modulating pain, but it was considered a known mechanism (peripheral vasoconstriction) and perhaps, not very interesting. This explanation of nicotine's analgesic properties was accepted until the late 1970s when renewed interest in nicotine as an analgesic emerged.

There are reports in the anesthesia literature that cholinergic agonists (not nicotine *per se*) have central analgesic properties in animals and humans (Naguib & Yaksh, 1994; Prado & Goncalves, 1997). It is known that pain stimulates release of acetylcholine in the spinal cord (Eisenach, Detweiler, Tong, D'Angelo, & Hood, 1996) and that spinal cholinergic receptors have antinociceptive actions that can be mimicked by intrathecal administration of cholinesterase inhibitors (Naguib & Yaksh, 1994). These reports suggest at least one mechanism by which nicotine may be inducing antinociception.

Human Research and Nicotine-induced Analgesia

There are a number of studies examining the effects of smoking or nicotine and antinociception. Nesbitt (1973) reported that smokers had greater endurance to electric shock than did non-smokers. This finding was replicated

by Silverstein (1982). Pomerleau (1986) examined the effects of either high-nicotine cigarettes or tobacco-snuff on pain, using the cold pressor paradigm. Both high-nicotine cigarettes and tobacco snuff increased endurance to ice-water pain as compared with control subjects. Similar results were reported by Fertig, Pomerleau, and Sanders (1986). In contrast, Sult and Moss (1986) reported no effect of cigarette smoking on endurance of either electric shock or cold pressor pain. Other investigators have also reported that nicotine had little or no analgesic effect on an electrical pain stimulus (Knott, 1990; Shiffman & Jarvik, 1984). These early studies are difficult to evaluate as the investigators were not studying pain *per se*, but rather they were studying pain as a subset of anxiety or stress. Therefore, these studies may not have been accurately examining the effects of pain as much as stress or anxiety. A more recent series of studies by Perkins and colleagues (1994) used nicotine administered by nasal spray to determine the effects of nicotine on antinociception in smokers and non-smoking individuals. Nicotine had a significant, although modest effect on increasing pain detection latencies in both smokers and non-smokers. This is an important finding because it suggests that nicotine's antinociceptive effects are not merely the result of nicotine withdrawal relief as has been suggested by Hughes (1991). Hughes posited that nicotine is not analgesic, but rather abstinence from smoking lowers pain thresholds. Therefore, nicotine may not be analgesic, but withdrawal from nicotine may sensitize people to noxious stimuli. If this hypothesis is correct, then smoking (or nicotine self-administration) merely raises pain thresholds back to baseline. This experiment by Perkins is the first

NOTE TO USERS

Page(s) not included in the original manuscript are unavailable from the author or university. The manuscript was microfilmed as received.

27

This reproduction is the best copy available.

UMI

Animal Research and Nicotine-induced Antinociception

In 1973, the issue of nicotine as an antinociceptive in animals was addressed by Phan, Dóda, Bite, and György (1973). These researchers used several models to assess nicotine-induced antinociception in mice. This group injected 1% acetic acid intraperitoneally and observed writhing behavior as well as using the more classic hot-plate and tail-flick methods to measure nociception. Nicotine was administered to the mice either intraperitoneally or intraventricularly. Nicotine had antinociceptive effects that were not antagonized by central nicotinic blockade with mecamylamine, suggesting that this antinociceptive effect was peripherally mediated.

The question of a central versus a peripheral antinociceptive action of nicotine was further examined by Sahley and Berntson (1979). They injected nicotine subcutaneously (SC) or intracerebroventricularly (ICV) and observed the effects of peripheral (hexamethonium), central (mecamylamine), or mixed (scopolamine) cholinergic antagonist on tail-flick latencies in male rats. Subcutaneous injection of nicotine resulted in antinociception that was blocked by centrally active, nicotinic or cholinergic blocking agents, but not by peripherally-acting agents. Further, small doses of nicotine (25 μg) injected ICV was effective at antinociception. These results support a central, not peripheral, antinociceptive effect.

Aceto, Bagley, Dewey, Fu, and Martin (1986) went on to ask the question, "Where in the CNS is nicotine acting to produce antinociception?" Using [^{3}H]nicotine this group tried to correlate antinociceptive activity with site of

injection, and with levels of nicotine in specific areas of the brain or spinal cord in male Sprague-Dawley rats. Although levels of [^{3}H]nicotine in spinal cord was approximately half of that found in the brain, antinociception was greatest when [^{3}H]nicotine was injected directly into the subarachnoid space. Additionally, [^{3}H]nicotine was more potent (by 40-fold) when injected subarachnoid versus the intracerebroventricular route. These data suggest that nicotine-induced antinociception may be mediated at the level of the spinal cord. These findings have profound implications. First, nicotine and nicotine analogs may be considered for use in certain pain syndromes to ameliorate pain. Unlike the potent opioids which are sympatholytics, nicotine has sympathomimetic properties that may be useful in certain disease states (e.g., asthma). Second, cigarette smokers who present for surgical procedures and are prohibited from smoking may have increased analgesic requirements. These individuals may need supplemental analgesia all through their hospital course. Lastly, and perhaps most importantly, nicotine-induced analgesia may be a reason why people smoke. While it is unlikely that people initiate tobacco use because of its analgesic effect, smokers may continue to smoke because nicotine helps them cope with certain types of pain. Conversely, smoking cessation may make people more aware of their pain. Nicotine-induced analgesia may promote tobacco use and therefore strategies aimed at providing smokers alternate means of obtaining analgesia may aid in smoking cessation therapy.

Nicotine is self-administered in tobacco products by millions of people in the United States. While it is well-documented that cigarette and tobacco use

has many deleterious health hazards, there is little evidence to suggest that nicotine *per se* is a dangerous drug (in small dosages). Indeed, nicotine may be seen someday as a therapeutic drug. There is research suggesting that nicotine may be useful in treating diseases such as Alzheimer's and Parkinson's disease (Westman, Levin, & Rose, 1995). Further, there appears to be therapeutic potential for nicotine in the treatment of ulcerative colitis.

The present research is designed to determine whether an acute bolus of nicotine (as one might receive after smoking a cigarette) is analgesic in both males and females. Further, whether nicotine is analgesic for acute and persistent, noxious stimuli has never been examined in a single study. There is one study examining nicotine-induced antinociception using a persistent pain model (Zarrindast, Pazouki, & Nassiri-Rad, 1997). The subjects in this study were male, Swiss, albino mice, but had serious methodological flaws preventing the study from providing useful information about nicotine-induced antinociception (e.g., the timing of the nicotine injection was incorrect; nicotine would not be significantly antinociceptive at the time of the Formalin Test).

Nicotine Metabolism

Nicotine undergoes extensive hepatic metabolism and has several metabolites that include cotinine, nicotine-*N*-1'-oxide, nornicotine, and norcotinine. Figure 2 presents the major metabolic pathways of nicotine. Of these metabolites, cotinine and nicotine-*N*-1'-oxide are the major ones with nicotine-*N*-1'-oxide being quantitatively less important. Cotinine is formed in the liver in a two-step process requiring the cytochrome P-450 enzyme system

(Petersen, Norris, & Thompson, 1984). Cotinine itself undergoes metabolism such that only 17 percent is excreted unchanged in the urine (Benowitz, Kuyt, Jacob, Jones, & Osman, 1983). The major metabolite of cotinine appears to be trans-3'-hydroxycotinine, however the importance of this metabolite has not been established (Jacob, Benowitz, & Shulgin, 1988).

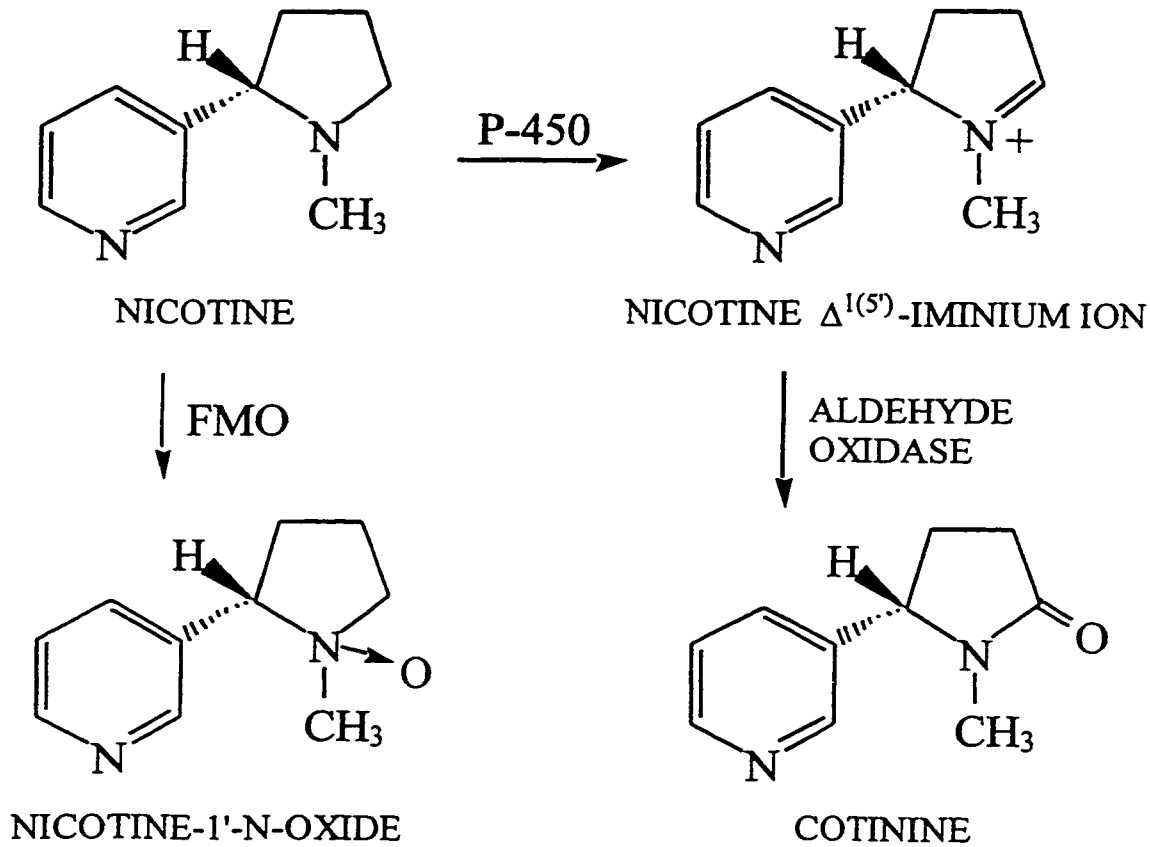

Figure 2: Metabolic Pathway of Nicotine

Cotinine

Cotinine is formed in the liver as nicotine is metabolized in a two-step process involving oxidation of the pyrrolidine ring and subsequent reaction by a cytoplasmic oxidase in which the iminium ion is metabolized (USDHHS, 1988). Cotinine is excreted largely unchanged in urine (Benowitz et al., 1983).

Cotinine may be important because it has been reported to have both central and peripheral effects, with and without nicotine present (Dwoskin, Teng, Buxton, & Crooks, 1999; Hatsukami et al., 1998; Sastry & Hemontolor, 1998). In addition, early work by Applegren, Hansson, and Schmiterlöw (1962) and later by Deutsch and colleagues (Deutsch, Hegedus, Greig, Rapoport, & Soncrant, 1992) reported that cotinine was found in rat and mouse brain after systemic injection of [^{14}C]-methylnicotine. Further, cotinine may possess analgesic properties (Erenmemisoglu & Tekol, 1994). These investigators reported that 5, 10, or 20 mg/kg cotinine injected into mice produced an increase in tail-flick latencies in a dose-dependent response.

When cotinine has been administered peripherally, it can be found in brain tissue suggesting that cotinine crosses the blood brain barrier (Crooks, Li, & Dwoskin, 1997). Also, because cotinine has a longer elimination half-life than nicotine it may significantly contribute to the pharmacologic effects of nicotine.

Mechanisms of Nicotine-Induced Antinociception

Nicotine produces a wide range of effects on the central nervous system (Martin, Tripathi, Aceto, & May, 1983). One of these centrally-mediated effects may be an effect on nociception. Presumably, these effects would be mediated through nAChRs receptors in the brain and spinal cord. The precise mechanism and location of nicotine-induced antinociception is not known, however there are several theories. The following is a review of what is known regarding how nicotine might be acting to modulate nociception.

One of the earliest reports of nicotine-induced antinociception using an

animal model proposed that the effect was centrally mediated because hexamethonium (a peripheral blocking agent) was unable to reverse the antinociceptive effect, whereas mecamylamine (a central and peripheral block agent) did block antinociception (Matilla, Ahtee, & Saarnivaara, 1968). These investigators attributed the analgesic effect to a post-stimulatory blockage of the reticular formation. Nicotine-induced antinociception was confirmed in 1973 (Phan et al., 1973), but these investigators found that DMMP (1,1,-dimethyl-4-phenyl-piperizine) iodide, a specific ganglionic stimulant with no blocking characteristics, also induced antinociception. These investigators therefore attributed nicotine's analgesic affects to cholinergic stimulation. In addition, when researchers injected acetylcholine intraventricularly (Pedigo, Dewey, & Harris, 1975), the resulting antinociception was blocked by a muscarinic antagonist, but not by a nicotinic agonist. This finding suggested that muscarinic receptors were at least partially responsible for nicotine-inducted antinociception. This conclusion is further supported by the fact that there is substantial evidence that systemic or spinal administration of acetylcholinesterase inhibitors and muscarinic receptor agonists increase nociceptive thresholds (Hartvig, Gillberg, Torsten, & Post, 1989).

Other investigators have hypothesized that nicotine's antinociceptive effect is a result of the presynaptic release of acetylcholine and that nicotine-induced antinociception appeared to act independent of the opioid receptor system as naloxone failed to block the effect (Sahley & Berntson, 1979), although these findings have not been supported by other research.

Several investigators have implicated the opioid system in nicotine-induced antinociception. Davenport, Houdi, and Van Loon (1990) reported nicotine-induced release of endogenous opioid peptides in rat brain. Zarrindast, Pazouki, and Nassari-Rad (1997) reported that coadministration of nicotine and morphine produced greater antinociception than that produced by either drug alone. These investigators argued for an interaction between opioid and cholinergic receptors.

Whether nicotine acts solely on central nAChRs or whether there is an interaction with the opioid system is unclear. It is also unclear where in the brain these receptors may be stimulated to produce antinociception. Iwamoto (1989) attempted to localize the receptors involved in antinociception. Based on a immunohistochemical study showing appreciable level of nicotine binding into the mesopontine tegmentum (Swanson, Simmons, Whiting, & Lindstrom, 1987), Iwamoto implanted a cannula into the pedunculopontine tegmental nucleus (PPTg). By injecting nicotine into this area, he was able to induce antinociception on hot-plate and tail-flick measures. In a later study, Iwamoto (1991) injected nicotine into 185 sites in forebrain, midbrain, and hindbrain. In this report, Iwamoto found that injection into pedunculopontine tegmental nucleus and the nucleus raphe magus (NRM) of the ventral medulla elicited antinociception on hot-plate and tail-flick. He hypothesized a modulatory antinociceptive pathway from PPTg to NRM.

If nicotine is found to definitively have antinociceptive properties, then this effect is likely to occur by binding with central nAChRs. These receptors may

themselves modulate nociception. While It is possible that nicotinic stimulation may activate the opioid system, there is insufficient evidence to support this hypothesis. Where and how this system operates remains unknown. The connection between pedunculopontine tegmentum and nucleus raphe magus make these areas likely sites of nicotine-induced antinociception. However, whether nicotine is antinociceptive after acute administration has not been determined. Further, if nicotine is antinociceptive it is important to clarify under which conditions it demonstrates this property.

Nicotine and Locomotion

Nicotine is a stimulant that exerts variable effects on locomotor activity (Reavill & Stolerman, 1990). This change in locomotor activity may be a result of stimulation of nicotinic receptors through activation of the mesolimbic dopaminergic (DA) neurons in the ventral tegmental area of the brain (Corrigall, Franklin, Coen, & Clarke, 1992). Because most measures of nociception in rats involve movement, and because nicotine affects movement, it is also important to examine locomotion when studying nicotine-induced antinociception.

The effects of nicotine on locomotor activity are complex. These effects include both a stimulatory effect and a depressant effect (Stolerman, 1990). These effects depend on dosage, time of administration, and previous drug history (Stolerman, Garcha, & Mirza, 1995). In addition, constant nicotine infusions may elicit different results than repeated boluses (Benwell, Balfour, & Khadra, 1994). The administration of the nicotinic antagonist, mecamylamine, inhibits nicotine-induced increases in locomotor activity (Reavill & Stolerman,

1990). Because behavioral measures of nociception have a motor component, the above data suggest that it may be important to try to tease out nicotine's effect on gross locomotor activity. Therefore, the present research included a measure of locomotor activity to ensure that differences in nicotine-induced antinociception were a result of decreased sensitivity to a noxious stimulus and not because of alterations in the ability of the animal to move.

Nicotine Tolerance

Tolerance may be defined as a "diminished response to the administration of a drug after repeated exposure to that drug" (Feldman et al., 1997). This definition suggests that when tolerance exists, larger doses of a drug must be administered to get the same effect as that which occurred with the original dose. Tolerance may the result of: 1) pharmacokinetics (increased metabolism and/or decreased bioavailability), 2) a reduction in the number or activity of receptors, 3) pharmacodynamics (receptors are exposed to the same amount or concentration of drug, but do not respond as expected) or 4) a behavioral tolerance (the animal is able to compensate based on previous experience with the drug). There is a considerable body of literature that suggests that repeated dosages of nicotine result in tolerance (Clarke & Kumar, 1983; Hakan & Ksir, 1991; Rosecrans, Wiley, Bass, & Karan, 1995; Saah, Raygada, & Grunberg, 1994; Stolerman, Fink, & Jarvik, 1973). This issue of nicotine tolerance is important consideration in light of the present research. In this research, rats received five SC injections of nicotine over a six week period. If tolerance to nicotine was operating, then it might be possible that as animals progressed through the battery of behavioral

measures there would be a diminished response to nicotine's antinociceptive properties. This section reviews the pertinent literature.

Studies of nicotine tolerance in animals dates back to Dixon and Lee (1912). However, many of these early studies used very large dosages and were hampered by the technology and methods of the period. Later, as techniques improved nicotine tolerance was studied by Domino (1965) on learned, pole-climbing, avoidance behavior. This investigator found mild tolerance effects, even after a single previous nicotine pretreatment. Stolerman, Fink and Jarvik (1973) studied nicotine tolerance in rats using spontaneous locomotor activity as the assessment tool. In their assessment of chronic nicotine tolerance, animals received repeated intraperitoneal (i.p.) injections of nicotine acid tartrate, three times a day for 8 days prior to testing. Using this dosing paradigm, animals exhibited tolerance that persisted 90 days later.

Other investigators examined nicotine tolerance by administering nicotine in drinking water (Falkeborn, Larsson, & Nordberg, 1981). In this paradigm, animals consumed approximately 4 mg/kg/day and were then tested for motor activity in a maze. Later, tolerance was assessed after an acute injection of 0.3 mg nicotine base/kg 24 hours after drug withdrawal. This effect was not present following 28 days of nicotine withdrawal. Clark and Kumar (1983) administered SC nicotine 0.4 mg/kg daily to induce a state of nicotine tolerance. Rats were then given acute SC nicotine injections with dosages ranging from 0.4 - 1.6 mg/kg. With daily nicotine administrations, tolerance to locomotor activity was seen with acute injections of 0.8 mg/kg nicotine. In this experiment, tolerance to

nicotine resulted in a stimulation of locomotion. This stimulatory effect on locomotion has been confirmed by other investigators (Ksir, Hakan, Hall, & Kellar, 1985; Morrison & Stephenson, 1972). Collins, Romm, and Wehner (1988) injected nicotine SC 1.6 mg/kg twice daily and reported tolerance to nicotine on locomotor activity 2-4 days after initiation of treatment.

Chronic exposure to nicotine appears to affect locomotion. This effect may be manifested as inhibition or as a stimulation. In the present experiments, if repeated nicotine administration results in an increase in motor activity, then animals in the present research might be hyper-reactive to noxious stimuli. As a result, the data would be misinterpreted as nicotine lacking antinociceptive properties. Conversely, if repeated nicotine administration results in a decrease in motor activity, then animals in the present research might be hypo-reactive to noxious stimuli. These data would be misinterpreted as nicotine having substantial antinociceptive properties. Therefore, to correctly interpret nicotine-induced antinociception, animals must have: 1) limited exposure to drug, 2) substantial drug washout periods, or 3) a comparison group added to the experiment that has had limited nicotine exposure, so that the effects of tolerance can be factored out statistically.

Whether subjects become tolerant to the behavioral effects will depend on the length of time that nicotine is administered prior to the behavioral measure, dosage of the drug, route of administration, nicotine formulation, and the duration of nicotine administration. In the present experiment, animals received a single nicotine injection prior to each behavioral measure. Although

there was a total of five behavioral measures and five nicotine injections, there was a wash-out period of one week between each measure. The purpose of this wash-out period was to try to prevent tolerance from operating during antinociceptive testing.

Analgesia and Gender Differences

The literature supports the hypothesis that gender differences in pain and nociception exist (Keefe, 1986; Maixner & Humphrey, 1993; Morris, 1991; Unruh, 1996). These differences exist in the absence of any intervening attempts to attenuate the noxious stimulus. This phenomenon is important in order to understand the biological mechanisms underlying pain and pain perception. It also is important to inquire whether or not gender affects one's ability to treat pain pharmacologically. It does appear that gender plays a role in analgesic efficacy as well.

It has been reported that men and women respond differently to a number of psychoactive drugs, including analgesics (Griffin, Weiss, Mirin, & Lange, 1989; Lex, 1991). Although women report more pain than do men, it is not clear which sex receives better pain relief from analgesic agents. Some research literature reports that women request analgesic medication more often than men do (Eggen, 1993). Other studies (De Kock, Eisenach, Tong, Schmitz, & Scholtes, 1997) report that female patients require significantly less self-administered, morphine sulfate via patient-controlled, analgesia pump (PCA) than do male patients after abdominal surgery. Recently, Gear and colleagues (1996) investigated the analgesic effects of the kappa-opiate agonist pentazocine on

postoperative male and female dental patients. Females receiving pentazocine had better analgesia than did males receiving similar treatment. Conversely, Walker and Carmody (1998) reported that although a single dose of ibuprofen (a non-steroidal analgesic) was an effective analgesic against electrically-induced experimental pain in male subjects, this dosage was ineffective in female subjects.

This finding of sex-specific pain responses is paralleled in animal studies. It is known that male and female rats respond differently to opioid-induced antinociception. Specifically, Bartok and Craft (1997) reported that peak analgesic effects of the kappa agonist, U69,593 and the delta agonist, [D-Pen2, D-Pen5]enkephalin (DPDPE) occurred earlier in females than males on the hot-plate measure. This research is, in contrast, to findings by Cicero, Nock, and Meyer (1997) who reported that male rats were more sensitive to another opioid analgesic, morphine on hot-plate and tail-flick measure. In another rodent study, male and female Swiss-Webster mice showed no difference in antinociception when females receive almost twice the morphine dosage that was administered to males (Candido, Maldonado, Megias, & Catena, 1992). A variety of other studies using numerous procedural and methodological differences suggest that males and females differ in response to analgesic drugs and that females may be more sensitive to pain and less sensitive to some analgesics.

The reasons that males and females differ in response to pain and analgesia are complex. Human studies suggest many factors may contribute to gender-related pain discrepancies including the gender of the experimenter

(Levine & De Simone, 1991). Another proposed mechanism for gender differences in pain responses is body size (Larkin, Reilly, & Kittler, 1986). It may be that differences in the tissue density or distribution of fat affects the transmission of painful stimuli. These hypotheses are novel, but do not always replicate and do not seem to account for sex differences reported in the animal literature. These facts suggest that there may be other factors that are modulating the differences in analgesic responses.

These studies suggest that gender differences in analgesic responses are substantial and that they span across drugs working through at least two distinctly different mechanisms. It seems that it is difficult to predict which gender is more sensitive to the analgesic effects of a particular drug. Whether one gender or another will report greater sensitivity depends upon the particular drug, dosage, and the means by which analgesia is measured. The current research was designed to define nicotine-induced antinociception. In characterizing this phenomenon it is important to determine whether sex plays a significant role in mediating the antinociceptive response.

Mechanisms Underlying Gender Differences

To treat pain effectively, it is important to understand the biological mechanisms underlying pain and pain perception. It also is important to inquire how gender affects pharmacologic treatment of pain. It does appear that gender plays a role in analgesic efficacy, but the mechanism for this phenomenon is unclear.

Two plausible explanations for gender differences in pain and analgesia

perception are: 1) central neurobiologic differences in pain and analgesic processing, and 2) pharmacokinetic gender-specific drug processing variations. These two possibilities are the focus of the present research.

Central neurobiologic differences may be responsible for variations in pain and analgesia in males and females. Males and females may differ in the number or density of analgesic receptors. Analgesic drugs may have different binding affinities in males and females and this could be a result of receptor subunit composition. Although differences in receptor density or composition may be important, they were not investigated here. Instead, another possible mediator of the differences observed in response to analgesic agents was investigated. That potential mediator is sex hormones.

Sex Hormones as a Source of Gender Differences

In the past, analgesic testing in animals have utilized young, male rodents. Males were used to prevent the confound of estrus effects on antinociception. While these studies may have been valid for the population tested, the information gathered may not have been valid for females.

It is unclear whether sex hormones may play a significant role in sex-specific antinociceptive responses to both pain and analgesia. It has been shown that sex steroids modulate pain sensitivity in rats (Frye, Cuevas, & Kanarek, 1993). It also appears that both estradiol and progesterone act separately to influence pain sensitivity (Kepler, Kest, Kiefel, Cooper, & Bodnar, 1989). Further, androgenized, female rats respond similarly to males and castrated, male rats exhibit pain thresholds similar to females (Beatty & Fessler,

1976; Beatty & Fessler, 1977). Human studies also suggest that gonadal hormones may influence pain sensitivity, but the results are widely discrepant. Within the experimental literature there are a number of body areas stimulated, as well as a variety of pain induction and assessment methodologies used, making it difficult to draw definitive conclusions (Fillingim et al., 1997; Tedford, Warren, & Flynn, 1977; Veith, Anderson, & Slade, 1984). Another issue equally unclear is whether sex hormones modulate analgesic responses.

While there may be information regarding gender-related differences to pain, there are few studies examining gender-related differences to acute administration of psychoactive drugs. It has been suggested that sex hormones may directly influence analgesia (Kepler et al., 1989). This phenomenon is seen during pregnancy when rising progesterone and 17-β-estradiol levels increase nociceptive thresholds (Dawson-Basoa & Gintzler, 1993). Additionally, estrogen presence also may be necessary for the integrity of the endogenous opioid system (Ryan & Maier, 1988) and may vary with estrus cycle stage (Ryan, Goodale, & Maier, 1987).

The existing studies examining sex hormones and antinociception use opioids as the analgesic agents. Dawson-Basoa and Gintzler (1996) found that estrogen and progesterone modulate kappa-opioid analgesia at the level of the spinal cord. Further, Pinsky, Koven, and LaBella (1975) found that testosterone was permissive in morphine-induced analgesia. These findings were later confirmed by Rao and Saifi (1985). Sex steroids may play a role in endogenous and/or exogenous opioid-induced analgesia. Whether sex steroids modulate the

effects of other analgesics, specifically nicotine, is unknown.

Nicotine Pharmacokinetics as a Source of Gender Differences

A second possible source of sex-related differences in antinociception is pharmacokinetics. It is possible that there are differences in drug distribution or metabolism and that those differences may be responsible wholly or in part for differences observed in antinociception.

This question of pharmacokinetics as the source of sex differences in antinociception has been addressed using an opioid analgesic. It has been reported that sex differences exist in opioid-induced antinociception (Kavaliers & Innes, 1987; Romero & Bodnar, 1986). There is a single study addressing the question of pharmacokinetics and that sex differences in morphine-induced analgesia (Cicero, Nock, & Meyer, 1996). In this study, male and female Sprague-Dawley rats were given SC morphine sulfate and tested on a number of antinociceptive tests. Following testing, animals were sacrificed and blood was assayed for serum morphine levels. These investigators reported that differences in morphine-induced antinociception did not seem to be dependent on pharmacokinetic differences as equivalent blood levels occur after morphine administration (Cicero et al., 1996). Therefore, they hypothesized that behavioral differences observed were a result of intrinsic gender-related differences in receptor sensitivity.

It has been reported that women metabolize drugs more rapidly than men (Kato, 1974). Unlike morphine, nicotine may be differentially metabolized in males and females. More than 60 years ago, it was observed that female rats

were more likely than males to die after a single SC nicotine injection (Holck, Kanan, Mills, & Smith, 1937). Also, there are data supporting sexual dimorphism in nicotine metabolism and distribution in rat (Kyerematen, Owens, Chattopadhyay, de Bethizy, & Vesell, 1988a; Nwosu & Crooks, 1988). Kyerematen and colleagues (1988b) examined nicotine's pharmacokinetics in male and female Sprague-Dawley rats. This group reported that, although females had a larger volume of distribution, male rats appeared to have a higher rate of nicotine metabolism. As a result, plasma levels of nicotine did not differ in male and female rats.

The research cited above suggests that there may be differences in nicotine metabolism in males and females. The relationship has between and levels of nicotine and cotinine in blood and neural tissue and nicotine-induced nocifensive behaviors has not been studied. Rosecrans and Schecter (1972) tried to relate nicotine drug levels in male and female rat brains to activity level. These researchers reported that female rats accumulated higher nicotine levels than did males. Additionally, female rats were more sensitive to nicotine-induced increases in activity levels than were male rats. These researchers did not sample tissue levels of nicotine or any metabolites. It is not known whether differences in plasma and tissue levels of nicotine or cotinine correlate with their antinociceptive effects.

Males and females metabolize nicotine at different rates. If nicotine is antinociceptive, and gender differences exist in nicotine-induced antinociception, then it may be these pharmacokinetic differences that are responsible for sex-

specific antinociceptive responses. Serum nicotine levels may be different in males or females. Alternatively, serum levels may be equivalent in males and females, but CNS tissue levels of nicotine may differ in males and females. The present research explores whether differences in plasma, brain, and spinal cord levels of nicotine account for any differences seen in male and female rats experiencing nicotine-induced antinociception.

Summary

Physical pain is a part of life and over a lifetime, it is almost unavoidable. Pain has an important role in alarming the individual of impending tissue damage. However, if the pain persists over time the value of the alarm diminishes and pain becomes a pathologic entity. Therefore, providing pain relief is desirable. In fact, patients have better outcomes when pain is treated effectively (Buckley, MacIntosh, & Beattie, 1990).

All people do not perceive pain similarly, as pain is a highly subjective experience. However, individual genetic differences may affect pain perception. One of these genetic differences, gender, seems to affect both pain perception and pain relief. Understanding the influence of gender on analgesia may help us to develop better ways of providing analgesia to both genders.

The mainstay of analgesics for moderate to severe pain are the opioid analgesics. Opioid analgesics are very effective in modulating pain, but they are associated with several undesirable side effects. These side effects make the search for alternate analgesic agents attractive. One such alternative drug, may be nicotine. Although there are conflicting reports, nicotine may have analgesic

properties (Pomerleau et al., 1984) and may have therapeutic value as an analgesic or as an analgesic adjuvant.

Smoking is a huge problem affecting millions of people worldwide. Of the thousands of chemicals in tobacco smoke, nicotine is generally accepted to be the pharmacologically active agent. Nicotine is an addictive drug and this addiction operates through the classic reward pathways in the brain. In addition to this mechanism, there may be other rewarding effects of nicotine that may play a role in nicotine addiction. One of these "other rewards" may be analgesia. People may smoke to alleviate acute or chronic pain.

The purpose of the present research was to establish nicotine as an antinociceptive agent in males and females and to define conditions under which the phenomenon occurs. By using different paradigms of antinociceptive testing and by using both acute and persistent models, it may be possible to characterize the conditions under which nicotine is antinociceptive in both sexes. Additionally, if gender differences exist in nicotine-induced antinociception, then the present research seeks to explore two possible mechanisms.

Hypotheses

Hypothesis 1: Nicotine administration will increase hot-plate, tail-flick, and cold-flick latencies in a positive dose-dependent manner **(Replication)**.

Rationale: Previous studies have determined that nicotine may be antinociceptive in rats (Apatov, 1998; Caggiula et al., 1995; Sahley & Berntson, 1979; Yang et al., 1992).

Hypothesis 2: Nicotine administration will stimulate activity in low dosages and decrease locomotor activity in high dosages **(Replication)**.

Rationale: Acute administration of nicotine depresses locomotor activity in rats (Stolerman et al., 1995).

Hypothesis 3: Nicotine administration will enhance antinociception in male rats more than in female rats **(Original Hyp.)**.

Rationale: Female rats are less sensitive to the effects of analgesics (Bodnar, Romero, & Kramer, 1988) and will have decreased latencies on acute measures of nociception and increased Formalin pain scores.

Hypothesis 4: Nicotine administration will decrease formalin-induced pain scores in a dose-dependent manner **(Original Hyp.)**.

Rationale: Previous studies have determined that nicotine has some antinociceptive properties in rats on acute measures of nociception, e.g., hot-plate and tail-flick. A decrease in the Formalin pain score is associated with greater antinociception and is an index of a drug's analgesic properties (Abbott, Franklin, & Westbrook, 1995; Tjolsen et al., 1992).

Hypothesis 5: Nicotine-induced antinociception will be reduced in estrus

and metestrus in female Sprague-Dawley rats (**Original Hyp.**).

Rationale: Previous research in Wistar rats report that pain latencies may decrease during the estrus and metestrus phases of the reproductive cycle (Martínez-Gómez, 1994). Women in luteal stage report lower pain thresholds (Jamner et al., 1998), therefore rats in this estrus stage may exhibit similarly decreased latencies on acute pain measures and higher Formalin pain scores.

Hypothesis 6: Pain thresholds will be increased by elevated testosterone levels in male Sprague-Dawley rats **(Original Hyp.)**.

Rationale: Studies in gonadectomized male rats support the hypothesis that the absence of testosterone decreases nociceptive latencies and that the replacement of these hormones reinstates latencies to baseline levels (Forman, Tingle, Estilow, & Cater, 1989). Other reports suggest that testosterone may be negatively correlated with pain thresholds (Rao & Saifi, 1985).

Hypothesis 7: Brain and spinal cord nicotine levels will correlate positively with nicotine-induced antinociception **(Original Hyp.)**.

Rationale: Nicotine is a known antinociceptive agent. Higher tissue levels should increase antinociception (Apatov, 1998; Yang et al., 1992)

Hypothesis 8: Brain and spinal cord cotinine levels will correlate positively with increases in antinociception **(Original Hyp.)**.

Rational: Cotinine has been reported to have antinociceptive properties (Erenmemisoglu & Tekol, 1994).

CHAPTER II

Methods

Experiment I

Subjects

Subjects were 120 Sprague-Dawley male and female rats (Charles River Laboratories, Wilmington, MA) with 12 rats of each sex in each treatment group. This sample size was determined based on a power analysis and previous studies. Rats were approximately 7 weeks old at the start of the experiment. Animals were individually housed in 35.6 cm x 15.2 cm x 20.3 cm plastic cages with absorbent Pine-Dri, wood chip bedding. Animals were maintained under a 12 h reverse light/dark cycle (lights off at 0700 hours) at approximately 23° C and 50% relative humidity. Rodents are nocturnal animals and would normally be asleep during the day. Reverse light cycling allows the rats to undergo nociceptive testing during daylight hours. It is unknown whether light cycle affects antinociceptive testing, but to avoid interrupting sleep cycles testing was done in this manner. Tap water and rodent chow (Harlan Teklad 4% Mouse/Rat Diet 7001) were made available continuously.

Drug

Nicotine (0.001 mg/kg, 0.01 mg/kg, 0.1 mg/kg, 1.0 mg/kg) or saline (n=12 subjects per treatment group) was administered by subcutaneous (SC) injection on the animal's dorsal surface between the withers, immediately prior to antinociceptive testing. Physiologic saline (0.9% NaCl) was used to prepare the drug solutions from nicotine dihydrochloride. Nicotine dosages are expressed as

nicotine base and administered in total volumes of 0.25 - 1 ml. These nicotine dosages were based on previous research employing nicotine SC as an analgesic in rats (Mousa, Aloyo, & Van Loon, 1988; Rogers & Iwamoto, 1993; Sahley & Berntson, 1979). Exact dosages were determined by pilot studies. These pilots were used to determine the lowest dosage of nicotine that would elicit antinociception and the highest dosage of nicotine that would elicit antinociception without causing significant respiratory distress and seizure activity.

Hot-Plate

Hot-plate latencies were measured with the Omnitech Hot Plate analgesiometer (Omnitech Electronics, Inc.). The hot-plate apparatus consists of a metal plate heated to 51° C and the apparatus is enclosed by plexiglass on all sides and top. The rat is placed on this apparatus until it either licks one of its hind paws or 60 seconds elapses. When either criterion was met the rat was quickly removed and returned to its cage (Cicero et al., 1996; Woolfe & MacDonald, 1944). Two trials were performed on each subject at 8 minutes and 12 minutes after injection. These times were chosen based on prior reports examining nicotine-induced antinociception using SC dosing (Matilla et al., 1968; Rogers & Iwamoto, 1993; Sahley & Berntson, 1979). In these reports, nicotine had its peak effect on antinociception after SC injection between 7.5 and 15 minutes. Based on these reports and convenience at testing, 8 and 12 minutes post-injection were chosen for acute nociceptive measures.

Tail-Flick

Tail-Flick latencies were measured using the Omnitech Tail-Flick Analgesia Monitor Model TF (Omnitech Electronics, Inc.). The tail-flick apparatus is a platform with a recessed channel to hold each animal's tail based on the procedures of D'Amour and Smith (1941). Near the end of the channel is a radiant heating coil and a photoelectric cell. Rats are placed on a platform so that the tip of the tail is extended approximately 2.5 cm beyond the radiant heating coil that was heated to 52° C. When the rat flicks its tail out of the channel, the photoelectric beam is broken, the trial is ended, and the apparatus records the latency to respond. Two trials were performed on each subject at 8 minutes and 12 minutes after injection.

Cold-Flick Test

A solution of water and 95% ethanol were cooled and stored in a freezer until the temperature reached -15°C ± 1° C (Fischer Scientific, Pittsburgh, PA). The use of a water/ethanol solution is based on a report by Wang, Ho, Hu, and Chu (1995). This solution was removed from the freezer prior to testing and the temperature was maintained in a Dewar's flask for the length of the test. The rat was held firmly above the cold bath so that the distal one third of the tail was submerged into the solution. The time from initial submersion of the tail until the tail was moved or flicked out of the solution was designated as the nociceptive threshold. This measurement was made with a stopwatch and measured to the nearest tenth of a second. The trial was terminated when the animal flicked its tail out of the bath or 60 seconds elapsed (to avoid tissue damage). Two trials

were performed on each subject at 8 minutes and 12 minutes after injection.

Locomotor Activity

Locomotor activity was assessed using an Omnitech Digiscan infrared photocell system (Model RXYZCM [16 TAO]); Omnitech Electronics, Columbus, Ohio (Stolerman et al., 1995; Zubrycki, Giordano, & Sanberg, 1990). Animals were placed in a clear Plexiglas chamber (40 cm x 40 cm x 30 cm) for a 30 minute period. Fifteen pairs of infrared photocells located 2 cm above the floor measure horizontal movements made by the rats. An additional fifteen pairs of infrared photocells located 10.5 cm above the floor of the chamber measure vertical movement. The dependent variable measured was total distance. Total distance traveled is automatically calculated from beam breaks in a two minute period and transferred to a computer via an Omnitech analyzer (Model DCM-8-BBU). Analyses were performed on total distance and was calculated by adding together the scores recorded every two minutes for the 30 minute assessment period. Animals were acclimated twice several days prior to the test periods by placing the animals in the locomotor boxes and allowing them to remain in the boxes with overhead lights turned off in a manner identical to the testing procedure.

Estrus Cycle

To determine estrus cycle staging for female rats, vaginal smears were performed to determine whether rats were in: estrus, metestrus, diestrus, or proestrus. Estrus staging was performed after measures of nociception were completed for that day. The tip of a sterile, Dacron tipped swab was inserted

into 1 mm into the rat's vagina. Cells from the vaginal epithelium were then removed from the vagina and transferred to a labeled glass, microscope slide (Emery & Schwabe, 1936; Jerse, 1998). This slide was then viewed under a light microscope at 40x magnification. Consistent with the criteria described by Hafez (1970), estrus cycle staging were determined using the following criteria: estrus, presence of cornified epithelial cells only; metestrus, presence of 50 percent cornified epithelial cells and 50 percent leukocytes; diestrus, presence of leukocytes only; and proestrus, presence of round epithelial cells only. A slide was rated as being representative of a particular stage when 50% of the cells seen were characteristic of that particular stage.

Experiment II

Experiment II began one week after the completion of Experiment I. This waiting period allowed for wash-out of nicotine and cotinine (nicotine and cotinine have elimination half-lives of approximately 2 hours and 20 hours, respectively).

Subjects

Subjects were the same animals as those used in Experiment I; i.e., 120 Sprague-Dawley male and female rats (Charles River Laboratories, Wilmington, MA). An additional 25 Sprague-Dawley male and female rats (Charles River Laboratories, Wilmington, MA) were tested to evaluate potential of behavioral tolerance to repeated nicotine administration. It is known that repeated exposure to a drug may induce either a pharmacokinetic or pharmacodynamic tolerance. Because of the experimental design, these animals were to receive multiple SC nicotine injections over the course of 5-6 weeks. To evaluate whether tolerance

to nicotine affected nociceptive behaviors, this additional group of 25 animals was added. These animals received nicotine once at the beginning of the experiment during locomotor testing and then, 5 weeks later, they again received nicotine during formalin testing. Rats were approximately 10 weeks old at the start of the experiment. Animals were individually housed in 35.6 cm x 15.2 cm x 20.3 cm plastic cages with absorbent Pine-Dri, wood chip bedding. Animals were maintained under a 12 h reverse light/dark cycle (lights off at 0700 hours) at approximately 23° C and 50% relative humidity. Tap water and rodent chow (Harlan Teklad 4% Mouse/Rat Diet 7001) were made available continuously.

Drug

The same dosages of nicotine used in Experiment I were used in Experiment II. Animals received the same dosages in Experiment II that they had received in Experiment I. Nicotine was administered by SC injection and rats were then tested.

Formalin Test

A tonic (persistent) pain model was induced by injecting an inflammatory agent (formalin) into the hind paw of the rat (Tjolsen et al., 1992). Each rat was first acclimated to the 30 x 30 x 30 plastic cage in which observation of the formalin-induced behavior was monitored. Below this cage was a mirror to aid in the observation of the animal's paw. The rat was wrapped up in a towel to immobilize the animal and a subcutaneous injection of 50 µl 2% formalin using a 300 µl insulin syringe with a 30 gauge needle was made into the dorsal surface of the right hind paw. This injection took approximately 3 seconds. Ten minutes

post-injection the animal was given an SC nicotine injection and the inflamed paw was subjected to antinociceptive testing. Immediately following the paw injection, the rats were placed in a clear glass chamber (approx. 18 cm x 29 cm x 12.5 cm) with a glass floor and allowed to acclimate to this environment before testing. This acclimation period was to allow the animals to become used to the novel environment of a cage without bedding. Subsequent to the formalin injection, nociceptive behaviors were rated in 5 minute intervals. Four distinct behaviors, as described by Dubuisson and Dennis (1977), were counted and timed. The following rating system was then applied: 0= normal weight bearing on the affected paw; 1=resting the paw lightly on the floor or limping; 2= elevating the affected paw off the floor; and 3= licking, biting, or grooming the affected paw. Each animal was observed by one of three trained observers. These observers had inter-rater reliabilities with each other of +0.98, based on the observation of four animals.

Estrus Cycle

Estrus cycle staging was performed as described above under Experiment I.

Timetable for Testing

For Experiments I and II, all drug treatments and nociceptive testing occurred between 0800 hrs and 1500 hrs. Testing was done at the same time of day to ensure consistent testing and avoid variance associated with diurnal hormone patterns. Estrus cycle staging was performed after analgesic testing, between 1400 hrs and 1600 hrs to maintain consistency.

Experiment III

Experiment III began one week after the completion of Experiment II. This waiting period allowed for the wash-out of nicotine and cotinine based on elimination half-lives of 2 and 20 hours, respectively.

Subjects

Subjects were the same animals as those used in Experiments I and II: 145 Sprague-Dawley male and female rats (Charles River Laboratories, Wilmington, MA). Rats were approximately 11 weeks old at the start of the experiment. Animals were individually housed in 35.6 cm x 15.2 cm x 20.3 cm plastic cages with absorbent Pine-Dri, wood chip bedding. Animals were maintained under a 12 h reverse light/dark cycle (lights off at 0700 hours) at approximately 23° C and 50% relative humidity. Tap water and rodent chow (Harlan Teklad 4% Mouse/Rat Diet 7001) were made available continuously.

Drug

The same animals received the same dosages of nicotine used in Experiments I and II in Experiment III. Nicotine was administered by SC injection 12 minutes prior to sacrifice.

Sacrifice

To harvest blood and tissue specimens for Experiment III all subjects were sacrificed by decapitation. Animals were decapitated to allow for rapid collection of blood and tissue and to minimize stress-induced hormonal changes. Rats were given a final SC injection of nicotine and 12 minutes later the rats were sacrificed. Trunk blood was collected in 12 x 75 mm, 5 ml polypropylene

tubes. Brain and spinal cord tissue were collected into 30 ml polypropylene tubes for assay.

Nicotine and Cotinine Levels

Nicotine and its primary metabolite cotinine were measured in serum, brain, and spinal cord. These measurements were used to: 1) verify that the animals that had received different dosages of nicotine (0.001 mg/kg, 0.01 mg/kg, 0.1 mg/kg, 1.0 mg/kg, or 0 mg/kg) via SC administration had plasma and tissue levels of drug, and 2) whether differences in nicotine and/or cotinine levels contribute to differences in nicotine-induced antinociception in male and female rats. At the end of the experiment all subjects were sacrificed by decapitation. Following decapitation, 5-7 ml of trunk blood was collected in a 7 ml polypropylene tube containing 100 units of lithium heparin (anticoagulant). The tubes were then capped, inverted gently several times, and placed in a tray of wet ice. Within 60 minutes the tubes were removed from the ice and placed into a centrifuge and spun at 1000 X G for 20 minutes to separate plasma from the other blood constituents. Once separated, 2.3 ml of plasma was then aliquotted into 12 x 75 mm, 5 ml polypropylene tubes and then transferred to a freezer for storage at -70° C for later nicotine and cotinine assays.

Whole brains were removed for nicotine assay. Whole brains were placed in prelabelled 30 ml polypropylene tubes that contained 2ml 5N NaOH. This strong alkali solution dissolved the brain tissue for subsequent assay. Further, this alkali solution stabilized nicotine and cotinine into their free base forms making them more suitable for transport and storage. These tubes

containing brain tissue and alkali solution were left at room temperature for 24 hours to allow the tissue to completely dissolve. Upon complete dissolution of the tissue specimens the tubes were placed in a -70° C freezer for storage until later assay. This preparation procedure is based on methods developed by Benowitz and Jacob (1999).

Spinal cords were removed following decapitation in the manner described by Yaksh and Harty (1981). Following decapitation, a small incision were made through superficial tissues at the caudal end of the rat. Then, a scissors is used to cut through the spinal canal at the level of the fifth lumbar vertebra. A 3 ml luer-lock syringe with 0.9% saline was loaded with a 25 gauge needle and is inserted caudal end of the vertebral canal. Pressure was exerted on the plunger so that the spinal cord was extruded through the cervical end of the vertebral canal. Each specimen of spinal cord was placed in a pre-labeled tube for subsequent analysis and treated similar to brain tissue specimens described above.

Determination of Nicotine and Cotinine in Blood, Brain, and Spinal Cord

The methods for determining nicotine and cotinine in blood has been modified from the method described by Jacob, Wilson, and Benowitz (1981). Brain and spinal cord tissue underwent a preliminary extraction step (described below) prior to the extraction described for blood specimens. Internal standards are added to each tissue sample prior to extraction. Following thawing blood, brain, and spinal cord specimens were aliquotted into 0.3 ml (brain and spinal cord) or 0.5 ml (blood) samples. Then 30 µl (brain) or 50 µl (brain and spinal

cord) of an internal standard solution containing 20 ng Ortho-nicotine perchlorate and 200 ng Ortho-cotinine perchlorate were added to serve as internal standards.

Preliminary Extraction for Brain and Spinal Cord

Following addition of internal standards, 0.5 ml 4N H_2SO_4 and 3.0 ml toluene:butanol (70:30) were added to brain and spinal cord specimens to trap nicotine and cotinine in an aqueous layer. Samples were then vortexed, centrifuged, and the toluene/butanol layer was then aspirated. The remaining acid layer was frozen in an acetone dry bath. The organic residue was then discarded and 0.5 ml NaOH was added to return each sample to an alkali medium necessary for the subsequent extraction step. The samples were then extracted according to the procedures described for blood specimens. Extraction of tissue was performed based on personal communications from Benowitz and Jacob (1996).

Treatment of Blood Specimens

After the brain and spinal cord specimens were treated as above, 0.5 ml 2N NaOH in 0.2N NH_3 were added to all brain, spinal cord, and plasma samples. To each specimen, 3.0 ml toluene:butanol (70:30) was added. These samples then were vortexed, centrifuged, and frozen in an acetone dry bath. The aqueous layer was discarded and the organic layer which contains nicotine and cotinine was then transferred to tubes containing 0.5 ml 1M H_2SO_4. These tubes were vortexed, centrifuged, and frozen. Once frozen, the organic layer was discarded, the aqueous layer containing nicotine and cotinine was poured into

new, pre-labeled tubes and 0.5 ml 50% K_2CO_3 in 0.2N NH_3 and 0.4 ml toluene:butanol (90:10) are added. This mixture was vortexed, centrifuged, and refrozen. Aliquots (1-5 µl) of the organic layer was transferred into autosampler vials and analyzed by gas chromatography on 1.8 m x 2 mm I.D. Carbowax-KOH or SP-2250 DB columns at 145° C as described in Jacob, Wilson, and Benowitz (1981). Quantification was achieved by calculating peak height ratios of nicotine to the internal standard and referencing the standard curve.

17-β-Estradiol

Following sacrifice, approximately 2 ml of blood were collected into pre-labeled, 5 ml polypropylene tubes and placed in an ice bucket. These samples were centrifuged at 3000 rpm (1500 g) at 4° C in a tabletop refrigerated centrifuge (IEC Centra, Model GP8R). A 50 µl sample of serum was removed from each tube using a plastic pipette and transferred to Eppendorf tubes. These samples were stored at -80° C until removed for later assay.

Serum 17-β-estradiol levels were analyzed using a radioimmunoassay (RIA) 17-β-estradiol kit manufactured by ICN Biomedicals, Inc. Performance characteristics of the assay include specificity of the antiserum and percent coefficient of variation (C.V.). This assay is 100% specific for 17-β-estradiol, 20% specific for estrone, and 1.51% specific for estriol. The antiserum has less than 1% specificity for all other steroid hormones. The reliability of this assay is presented as inter-assay and intra-assay variability. ICN Biomedicals reports that inter-assay variability ranged from 5.9 - 11.9% C.V. Intra-assay variability ranged from 4.7 - 10.6% C.V.

Radioimmunoassays rely on the binding of an antibody to a specific antigen of interest (17-β-estradiol). To quantify the amount of antigen, known concentrations of radioactive and non-radioactive antibody were added to unknown concentrations of antigen. The radioactive and non-radioactive antibodies then compete for binding with the antigen. Following an incubation period, the radioactive species was counted using a gamma radiation counter.

Standard 17-β-estradiol concentrations (0, 10, 30, 300, 1000, & 3000 pg/ml) and unknown samples were added to consecutively numbered, coated tubes. 17-β-estradiol labeled with I^{125} was added to each tube and vortexed briefly. Samples were incubated for 90 minutes at 37°C. The contents of each tube were aspirated leaving a pellet adhering to the bottom of the tube. The tubes were placed in the gamma counter (RackGamma II, Model number 1270-004) for counting. The gamma counter counts radioactive I^{125} and extrapolates the amount of radioactive antigen bound to antibody based on the standard curve.

Testosterone

Following sacrifice, approximately 2 ml of blood were collected into pre-labeled, 5 ml polypropylene tubes and placed in an ice bucket. These samples were centrifuged at 3000 rpm (1500 g), 4° C in a tabletop refrigerated centrifuge (IEC Centra, Model GP8R). A 50 µl serum sample was removed from each tube using a plastic pipette and transferred to Eppendorf tubes. These samples were stored at -80° C until removed for later assay.

Serum testosterone levels were analyzed using a radioimmunoassay

(RIA) testosterone kit manufactured by ICN Biomedicals, Inc. Performance characteristics of the assay include specificity of the antiserum and % C.V. This assay is 100% specific for testosterone, 7.80% specific for 5-β–Dihydrotestosterone, and 2% specific for 11-Oxotestosterone. The antiserum has less than 1% specificity for all other steroid hormones. The reliability of this assay is presented as inter-assay and intra-assay variability. ICN Biomedicals reports that inter-assay variability ranged from 6.81 - 15.16% C.V. Intra-assay variability ranged from 9.57 - 13.0% C.V.

Radioimmunoassays rely on the binding of an antibody to a specific antigen of interest (testosterone). To quantify the amount of antigen, known concentrations of radioactive and non-radioactive antibody were added to unknown concentrations of antigen. The radioactive and non-radioactive antibodies then compete for binding with the antigen. Following an incubation period, the radioactive species were counted using a gamma radiation counter.

Standard testosterone concentrations (0, 0.2, 0.6, 2.0, 6.0, & 20 ng/ml) and unknown samples were added to consecutively numbered, coated tubes. Testosterone labeled with I^{125} was added to each tube and vortexed briefly. Samples were incubated for 2 hours at 37°C. The contents of each tube was aspirated leaving a pellet adhering to the bottom of the tube. The tubes were placed in the gamma counter (RackGamma II, Model number 1270-004) for counting. The gamma counter counts radioactive I^{125} and extrapolates the amount of radioactive antigen bound to antibody based on the standard curve.

Data Analytic Strategy

The three experiments used a 5 (0, 0.001, 0.01, 0.1, 1.0 mg/kg nicotine) x 2 (male, female) between-subjects experimental design to examine the effects of nicotine and sex on analgesia and potentially-relevant mechanisms. Data were analyzed using the computer software package Statistical Product and Service Solutions (SPSS), Version 9.0 (Prentice-Hall, Chicago). Analyses were performed using analysis of variance (ANOVA) or analysis of covariance (ANCOVA). That is, ANOVA was used when baseline values did not differ among treatment groups. ANCOVA was used when there were differences among treatment groups at baseline. ANOVA also was used to analyze the Formalin Test pain behavioral data (Experiment II) following standard practices in the animal pain research literature (F.V. Abbott, personal communication). The Tukey Honestly Significant Difference (HSD) test was used to determine the *a posteriori* statistically significant differences among treatment groups. This *post hoc* test is a relatively conservative test to determine statistical significance. Levels of serum, brain, and spinal cord nicotine and cotinine levels were expressed as ng/ml or ng/g tissue. These data were analyzed using a series of regression correlations to determine if nicotine or cotinine levels were related to antinociception. All tests were two-tailed and with an alpha level set at 0.05 or better.

CHAPTER III

Results

This section presents the results of three experiments. Experiment I examined the effects of nicotine on acute measures of nociception. Experiment II examined the effects of nicotine in a persistent model of nociception. Experiment III examined the pharmacokinetics of nicotine; the effects of nicotine on plasma levels of sex hormones; and analyzed the relationship between these measures and behavioral measures of nociception. The text includes the findings with supporting statistical analyses. The figures present the major findings. Tables (pp. 132-147) present additional information.

Experiment I

Experiment I examined the effects of SC administration of nicotine (0.001 mg/kg, 0.01 mg/kg, 0.1 mg/kr or 1 mg/kg) or saline on three acute nociceptive measures (i.e., tail-flick, hot-plate, and cold-flick). All of these behavioral measures were tested at two time-points (i.e., at 8 and 12 minutes post-injection) after no treatment and, on another day, after SC injection of one of the drug treatments. The results for tail-flick, hot-plate, and cold-flick latencies to respond are presented in this section.

Tail-Flick Latency

Tail-flick latency is a spinally-mediated measure of analgesia. The rat's tail is placed in a channel over a radiant heat source. The latency for the rat to flick its tail out of the channel is a behavioral measure of nociception (see Chapter II, Methods for details).

There was a significant difference in no-treatment responses between males and females at Time 1 [$F(1, 111) = 8.445$, $p < 0.01$] and at Time 2 [$F(1, 111) = 10.280$, $p < 0.01$] with males having longer latencies than females. Subsequent analyses of tail-flick responses to the treatment conditions were analyzed by analysis of covariance (ANCOVA) with no-treatment latencies to respond used as covariates.

Time-point 1 (8 Minutes Post-Injection)

There was a significant difference among treatment groups [$F(4, 105) = 19.783$, $p < 0.001$] at Time-point 1 with the 1 mg/kg and 0.1 mg/kg nicotine treatment groups increasing latency significantly. In addition, there was a significant drug by sex interaction [$F(4, 105) = 2.843$, $p < 0.05$]. Separate ANCOVAs for each sex revealed significant differences among treatment groups for male rats [$F(4, 53) = 11.946$, $p < 0.001$] and for female rats [$F(4,50) = 11.014$, $p < 0.001$). *Post hoc* analyses revealed that the latencies for males increased significantly in response to 0.1 and 1.0 mg/kg nicotine and the latencies for females increased significantly in response to 1.0 mg/kg nicotine. Figure 3 presents these findings.

Time-point 2 (12 Minutes Post-Injection)

There was a significant difference among treatment groups [$F(4, 105) = 11.880$, $p < 0.001$] at Time-point 2 with nicotine (1 mg/kg and 0.1 mg/kg) significantly increasing tail-flick latencies compared with controls. In addition, there was a significant drug by sex interaction [$F(4, 105) = 2.542$, $p < 0.05$]. Separate ANOVAs for each sex revealed significant differences among

Tail-Flick at 8 Minutes Post Injection

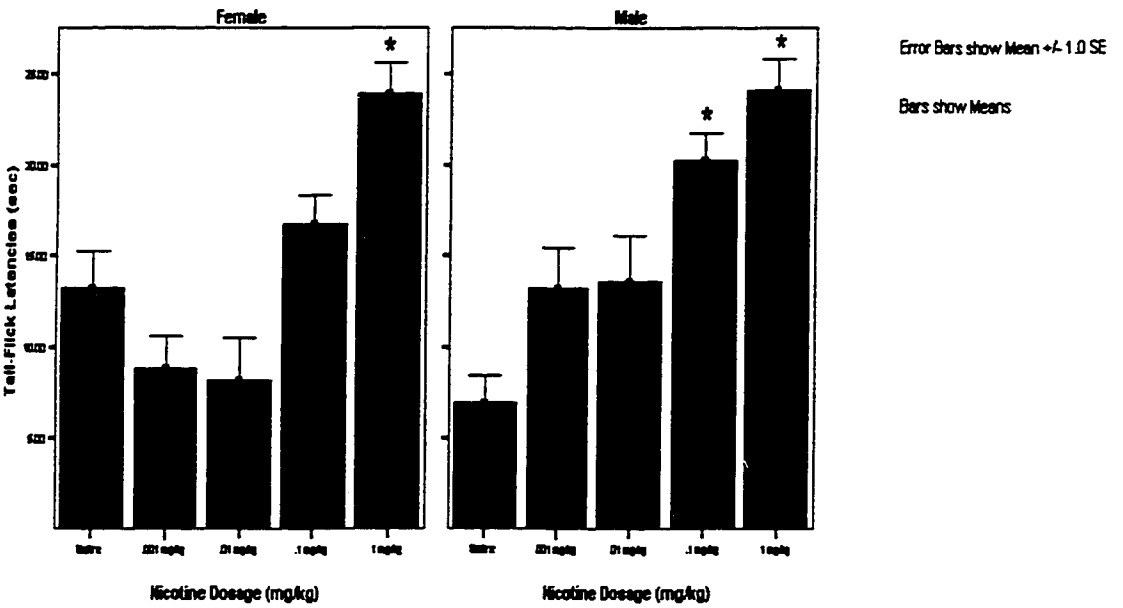

Figure 3. Effects of Nicotine on Tail-Flick 8 Minutes Post Injection.
Nicotine 1 mg/kg increased latencies (compared with control) for females at 1.0 mg/kg, whereas nicotine 0.1 mg/kg and 1.0 increased latencies for males (compared with control) (Asterisks indicate statistically significant findings, $p < 0.05$).

treatment groups for male rats [$F(4, 53) = 4.699$, $p = 0.01$] and for female rats [$F(4,51) = 9.699$, $p < 0.001$). For male rats 0.1 mg/kg and 1.0 mg/kg nicotine were significantly different from saline, whereas for females the 1.0 mg/kg significantly differed from saline. The latencies for males and females increased with increasing dosages of nicotine, in addition, males appeared to have a leftward shift of the dose-response curve. Figure 4 presents these findings.

Hot Plate Latency

Hot-Plate latency is a supraspinally-mediated measure of analgesia. The rat is placed on a heated metal plate which is enclosed on all sides by clear plexiglass. The latency for the rat lift and lick its hind paw is a behavioral measure of nociception (see Chapter II, Methods for details).

There were no significant differences in no-treatment responses between males and females at Time-points 1 or 2. In addition, there were no significant differences among males or females that were assigned to different treatment groups. Therefore, subsequent analyses were performed by ANOVA.

Time-point 1 (8 Minutes Post-Injection)

There was a significant difference among treatment groups [$F(4, 108) = 11.384$, $p < 0.001$] at Time-point 1 with nicotine 1 mg/kg significantly increasing hot-plate latencies over controls. Separate ANOVAs for each sex revealed significant differences among treatment groups for male rats [$F(4, 56) = 8.309$, $p < 0.001$] and for female rats [$F(4, 53) = 3.656$, $p < 0.05$]. For males and for females, there was a significant difference among treatment groups with the 1 mg/kg nicotine treatment groups increasing latency significantly over saline

Tail-Flick at 12 Minutes Post Injection

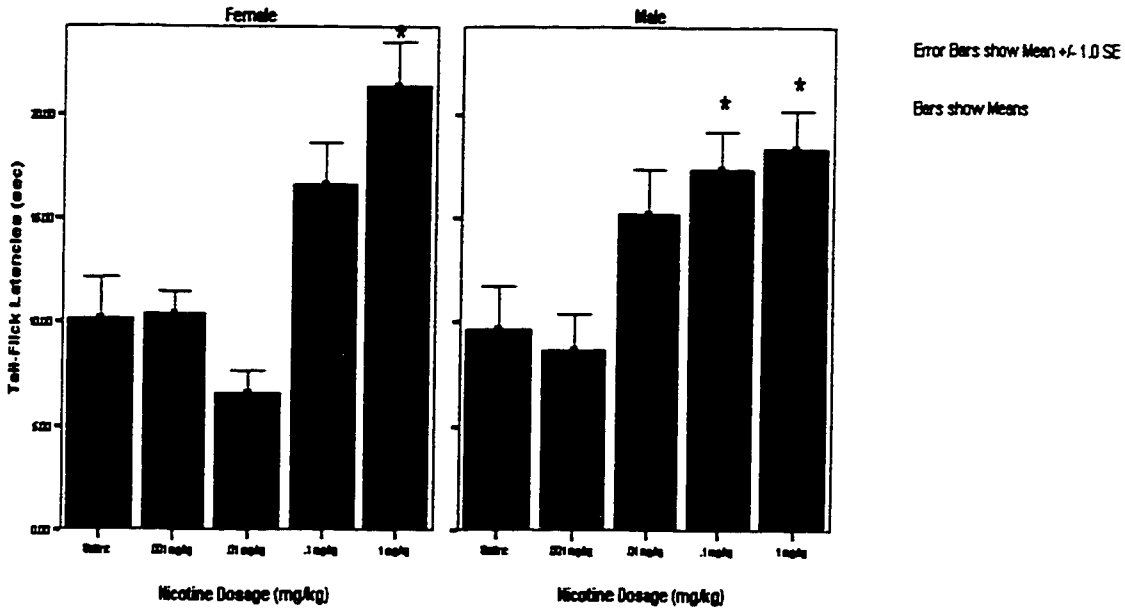

Figure 4. Effects of Nicotine on Tail-Flick 12 Minutes Post Injection.
Nicotine 1 mg/kg increased latencies (compared with control) for females at 1.0 mg/kg, whereas nicotine 0.1 mg/kg and 1.0 increased latencies for males (compared with controls). (Asterisks indicate statistically significant findings, $p < 0.05$).

controls. Figure 5 presents these findings.

Time-point 2 (12 Minutes Post-Injection)

There was a significant difference among treatment groups [$F(4, 108) = 12.378$, $p < 0.001$] at Time-point 2 with nicotine 1 mg/kg significantly increasing hot-plate latencies over controls. Separate ANOVAs for each sex revealed significant differences among treatment groups for male rats [$F(4, 55) = 8.675$, $p < 0.001$] and for female rats [$F(4, 53) = 4.284$, $p < 0.005$]. For males and for females, there was a significant difference among treatment groups with the 1 mg/kg nicotine treatment groups increasing latency significantly over saline controls. Figure 6 presents these findings.

Cold-Flick Latency

Cold-Flick latency is a spinally-mediated measure of analgesia. The rat is held over a Dewar's flask containing an iced solution of ethanol and water. The rat's tail is then submerged into the ethanol/water bath. The latency for the rat to flick its tail out of the liquid is a behavioral measure of nociception (see Chapter II, Methods for details).

At Time-point 1 there was a significant difference in the no-treatment responses among the groups that were to receive different treatments [$F(4, 111) = 2.474$, $p<0.05$]. Therefore, subsequent analyses of cold-flick latency responses to the treatment conditions were analyzed by analysis of covariance (ANCOVA) with no-treatment latencies to respond used as covariates.

Time-point 1 (8 Minutes Post-Injection)

There was a significant difference among treatment groups [$F(4, 107) =$

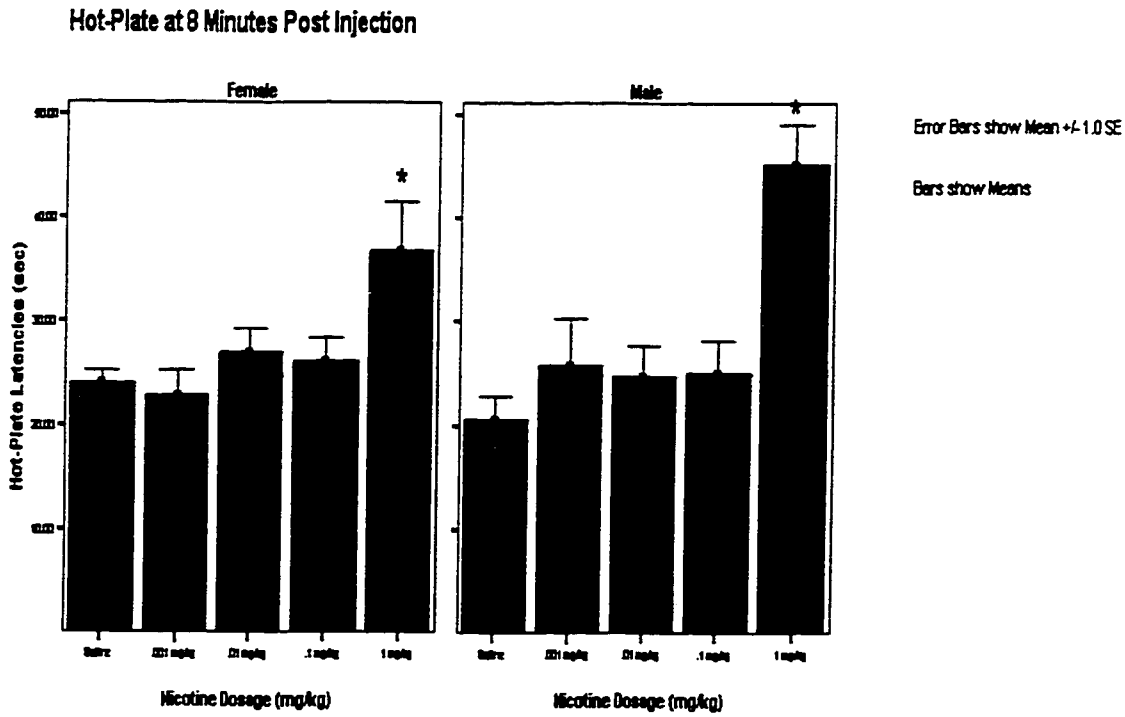

Figure 5. Effects of Nicotine on Hot-Plate 8 Minutes Post Injection. Nicotine 1 mg/kg increased latencies (compared with control) for females and males (Asterisks indicate statistically significant findings, $p < 0.05$).

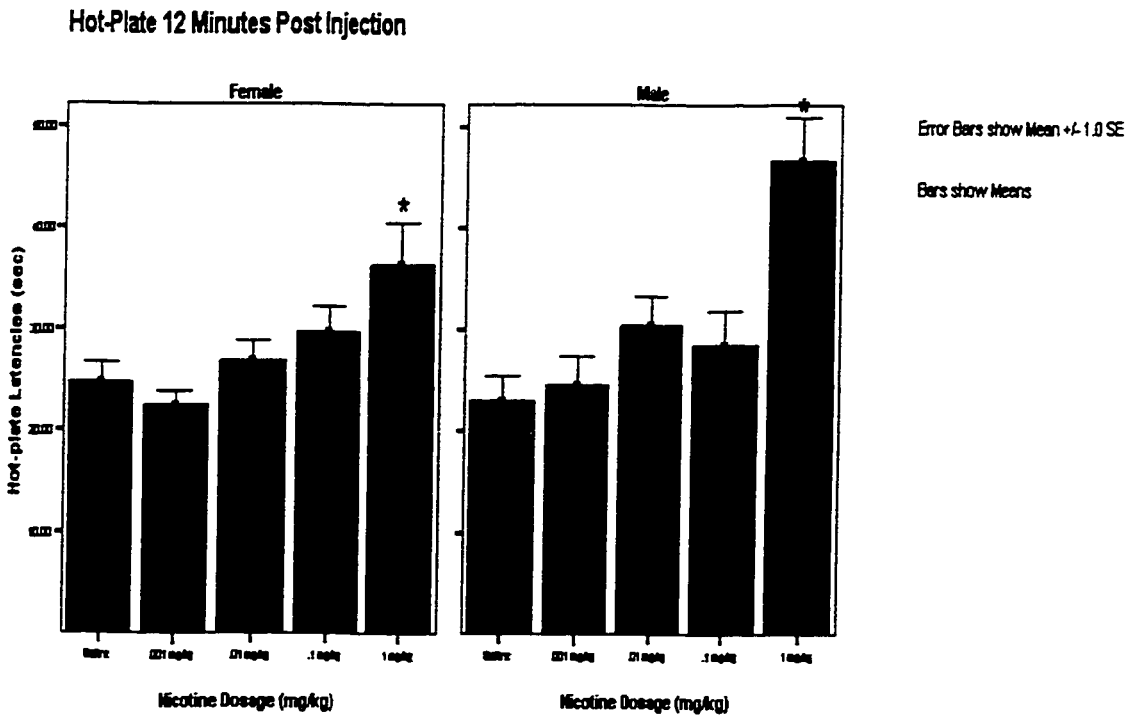

Figure 6. Effects of Nicotine on Hot-Plate 12 Minutes Post Injection. Nicotine 1 mg/kg increased latencies (compared with control) for females and males (Asterisks indicate statistically significant findings, $p < 0.05$).

21.536, $p < 0.001$] at Time-point 1 with the 1 mg/kg nicotine treatment groups increasing latency significantly. Separate ANCOVAs for each sex revealed significant differences among treatment groups for male rats [$F(4, 55) = 22.862$, $p < 0.001$] and for female rats [$F(4, 51) = 5.175$, $p = 0.001$]. For males and for females, there was a significant difference among treatment groups with the 1 mg/kg nicotine treatment groups increasing latency significantly over saline controls. Figure 7 presents these findings.

Time-point 2 (12 Minutes Post-Injection)

There was a significant difference among treatment groups [$F(4, 108) = 17.484$, $p < 0.001$] at Time-point 2 with all nicotine treatment groups (0.001 mg/kg, 0.01 mg/kg, 0.1 mg/kg, or 1 mg/kg) increasing latency significantly over saline controls.

Separate ANCOVAs for each sex revealed significant differences among treatment groups for male rats [$F(4, 55) = 10.377$, $p < 0.001$] and for female rats [$F(4, 51) = 7.619$, $p < 0.001$]. For males and females, there was a significant difference among treatment groups with the 1 mg/kg nicotine treatment groups increasing latencies significantly over saline controls. Figure 8 presents these findings.

Locomotor

Nicotine is known to affect locomotion. Because the acute nociceptive measures involve movement, it was important to determine whether effects of nicotine on locomotion were responsible for effects of nicotine on antinociception. Therefore, a separate experiment was performed prior to the antinociceptive testing in the same rats.

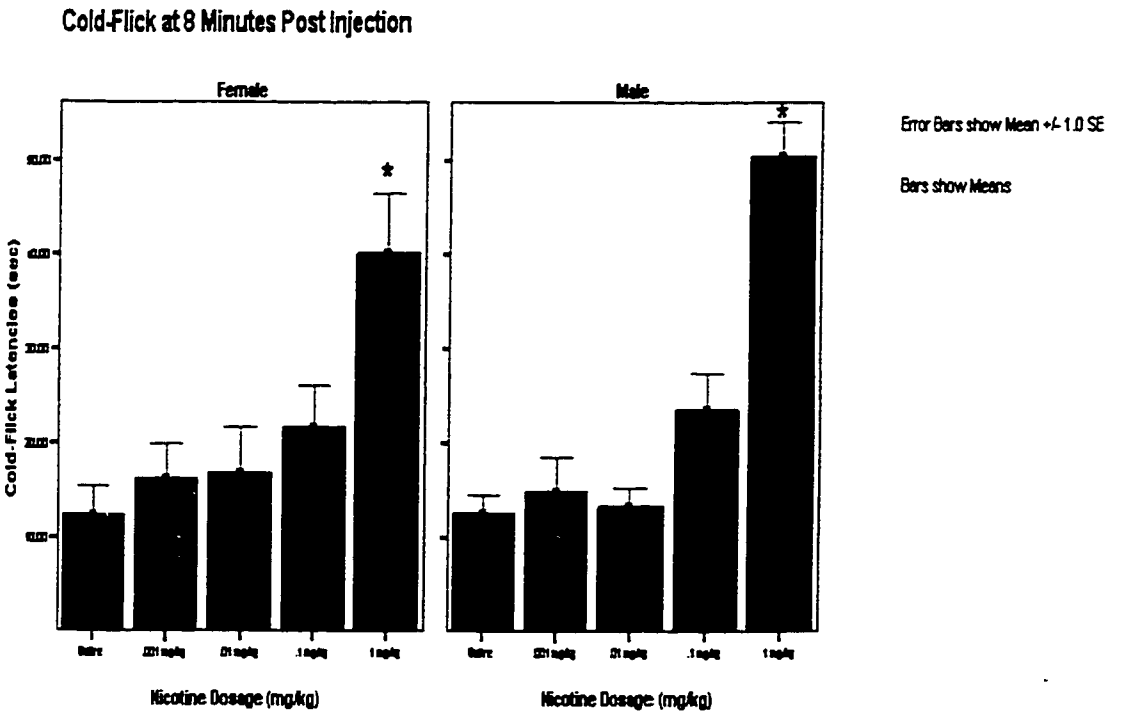

Figure 7. Effects of Nicotine on Cold-Flick 8 Minutes Post Injection.
Nicotine 1 mg/kg signifcantly increased latencies for females and males (compared with control)
(Asterisks indicate statistically significant findings, $p < 0.05$).

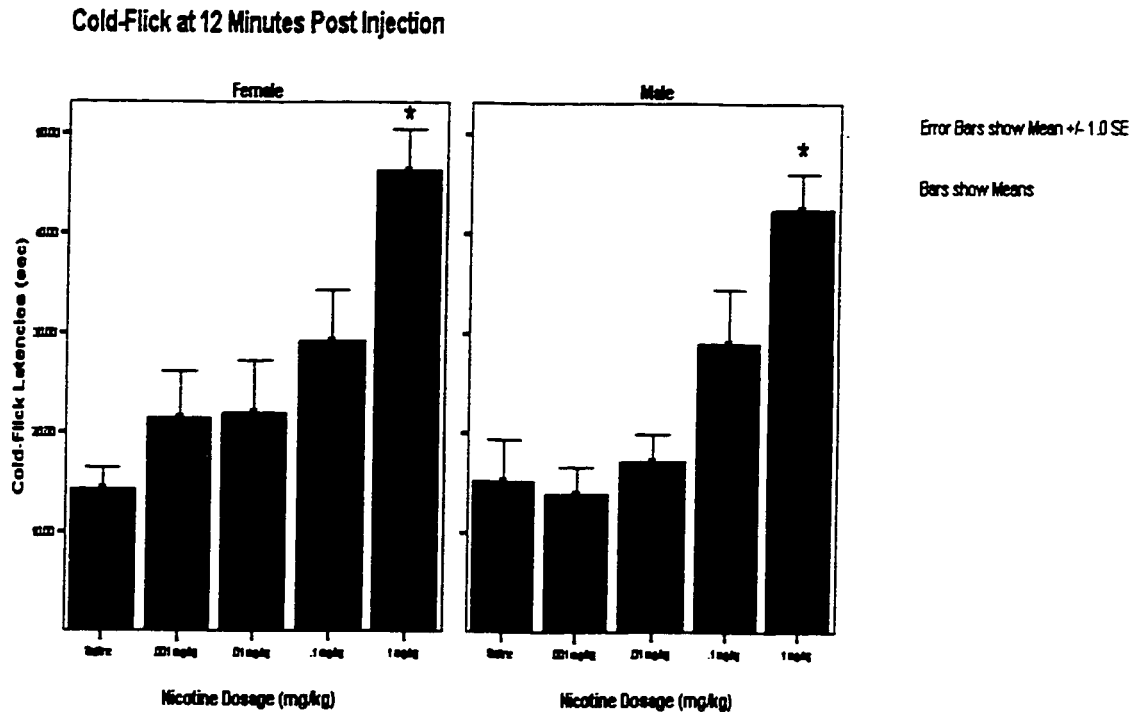

Figure 8. Effects of Nicotine on Cold-Flick 12 Minutes Post Injection.
Nicotine 1 mg/kg signifcantly increased latencies for females and males (compared with control)
(Asterisks indicate statistically significant findings, $p < 0.05$).

For this purpose, subjects were given an acute SC injection of the dosages of nicotine (either 0, 0.001, 0.01, 0.1, or 1.0 mg/kg) that they would receive for the antinociceptive testing but, for this experiment, locomotion was measured.

One of the most frequent measures of locomotor activity used is total distance (Chuang & Lin, 1994; Mead, Hargreaves, Ossenkopp, & Kavaliers, 1995; Morse, Erwin, & Jones, 1993; Schreur & Nichols, 1986). One week prior to nicotine or saline treatment testing, rats were subjected to a no-treatment baseline testing. Baseline testing revealed that there were no significant differences among drug groups [$F(5, 133) = .561$, p = n.s.]. Baseline differences were found between sexes with females moving a greater total distance than did males. Because of this difference subsequent locomotor analyses used baseline values as covariates.

There was a significant difference among treatment groups [$F(4, 110) = 9.093$, $p < 0.001$] with 0.1 mg/kg and 1.0 mg/kg nicotine decreasing total distance locomotor activity. In addition, there were differences in total distance depending on sex [$F(1, 110) = 6.615$, $p < 0.05$] with males moving less total distance than did females. Analyses also revealed a sex by drug interaction [$F(4, 110) = 8.864$, $p < 0.001$]. Separate ANCOVAs for each sex revealed significant differences among treatment groups for male rats [$F(5, 55) = 8.565$, $p < 0.001$] and for female rats [$F(5, 54) = 11.654$, $p < 0.001$]. For males, there was a significant difference among treatment groups with the 1 mg/kg and the 0.1 mg/kg nicotine treatment groups decreasing movement significantly over saline controls. For females, there was a similar significant difference among treatment groups with the 1 mg/kg nicotine treatment groups decreasing movement significantly. Figure 9 presents these findings for males and females separately. Figures 10 and 11 present locomotor activity for males and females at each 2-minute time-point.

Total Distance Travelled Over 30 Minute Period in Response to Nicotine or Saline

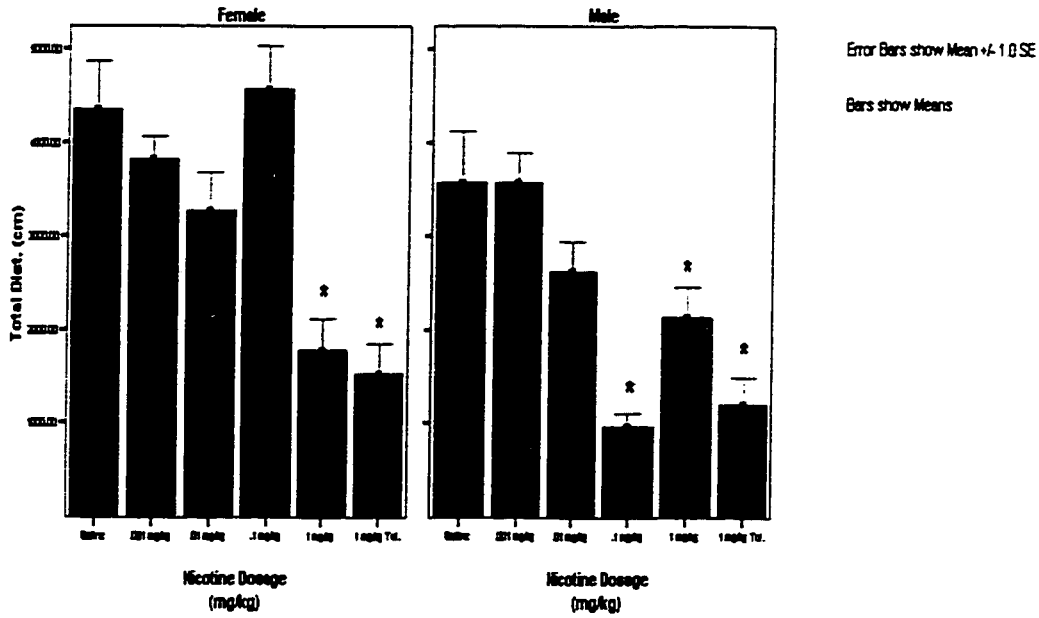

Figure 9. Total Distance Travelled by Males and Females Over 30 Minute Period in Response to Nicotine or Saline. Nicotine 0.1 mg/kg and 1 mg/kg significantly decreased locomotor activity for males, whereas 1 mg/kg significantly decreased locomotor activity in females (Asterisks indicate statistically significant findings, $p < 0.05$).

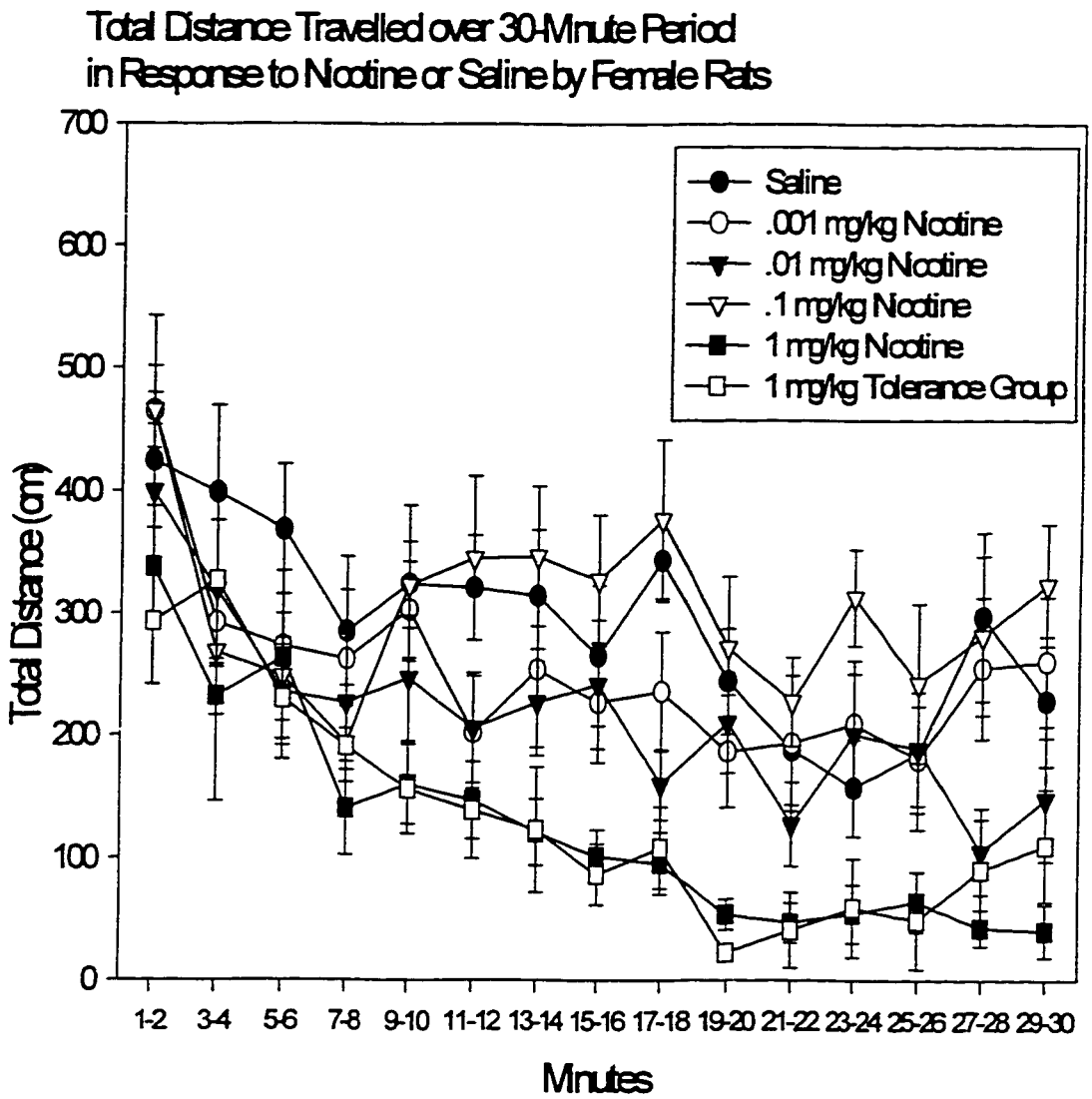

Figure 10. Total Distance Traveled (cm) During 30-Minute Period in Locomotor Chambers in Response to Nicotine or Saline. Nicotine (1.0 mg/kg) significantly decreased locomotor activity.

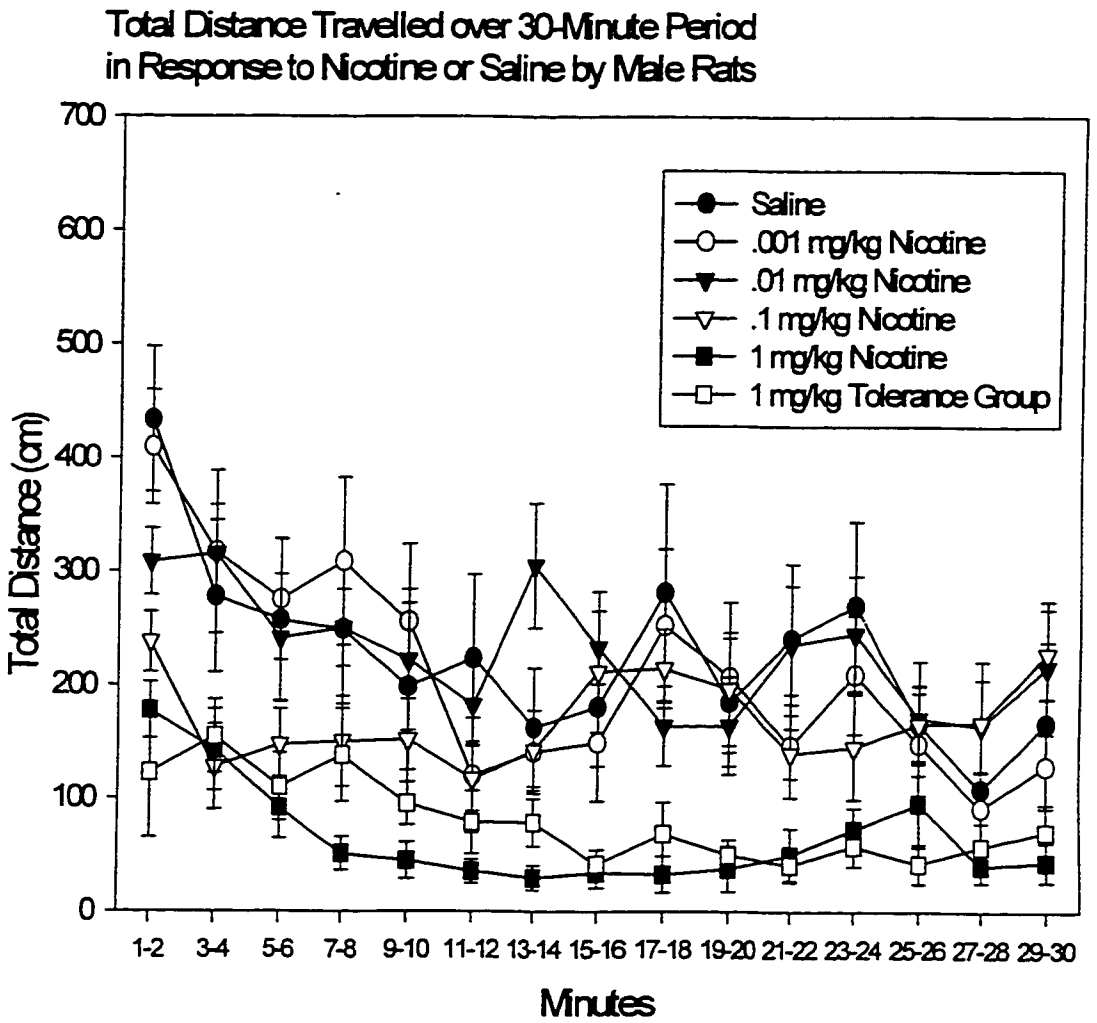

Figure 11. Total Distance (cm) Traveled During 30-Minute Period in Locomotor Chambers in Response to Nicotine or Saline. Nicotine (0.1 and 1.0 mg/kg) significantly decreased locomotor activity.

Acute Nociceptive Measures and Locomotion

Based on these effects of the highest dosages of nicotine to decrease locomotion and pain responses, it was important to analyze effects of nicotine on the acute nociceptive measures using locomotion as a covariate. Therefore, all acute measures of antinociception were analyzed using locomotor data as a covariate. When locomotor data are used as a covariate, the statistical significance of the acute nociceptive findings **did not change**.

Summary of Experiment I

Experiment I examined the effects of nicotine or saline on three acute, nociceptive (pain) measures. Nicotine clearly had an antinociceptive effect on the three measures. On the tail-flick measure, males appear to be more sensitive to nicotine's antinociceptive effects. On the hot-plate and cold-flick measures, nicotine was antinociceptive but there were no apparent sex differences. Nicotine reduced gross body movement, but these effects did not account for the effects of nicotine-induced antinociception.

Experiment II

Formalin Testing

The Formalin Test is a model of a persistent or chronic pain (Aloisi, Albonetti, & Carli, 1994). To perform the Formalin Test, a physiologic irritant is injected into the rat's hind paw. Ten minutes after the formalin injection, the animal is given an SC injection of either nicotine or saline. The animal then is observed for stereotypic pain behaviors for the next 30 minutes (minutes 10 through 40 after the formalin injection). These stereotypic pain behaviors are summed for each 5-minute period (i.e., minutes 0-5 post nicotine or saline

injection, minutes 6–10 post nicotine or saline injection, and so on). The Total Formalin score is calculated as the cumulative score over the six 5-minute periods. Pain scores can range from 0 to 4 such that the greater the numeric value, the greater the nociceptive responses. A detailed description of these methods is presented in Chapter II, Methods.

This section presents the results of the Formalin Test. The first section presents the effects of nicotine on the Total Formalin score during the entire 30 minute observation period. Next, the results for each of the six, 5-minute observation periods are presented.

Total Formalin Score

When males and females were considered together, there was a significant effect of drug treatment [$F(4, 110) = 4.041$, $p < 0.005$] with *post hoc* tests indicating that the 1 mg/kg nicotine dosage groups decreased the Total Formalin score significantly. Separate ANOVAs for each sex revealed significant differences among treatment groups for female rats [$F(4,55) = 2.607$, $p < 0.05$)], with the 1.0 mg/kg nicotine dosage significantly decreasing Total Formalin score as compared with 0.001 mg/kg nicotine, but not when compared with saline. There was a trend for treatment effects among male rats [$F(4, 55) = 5.158$, $p = 0.063$]. *Post hoc* tests however, did not reveal any differences among dosage groups. Figure 12 presents these findings for males and females separately.

Formalin Testing - 5 minutes

When males and females were considered together, there were no significant differences among drug groups at the 5-minute testing point

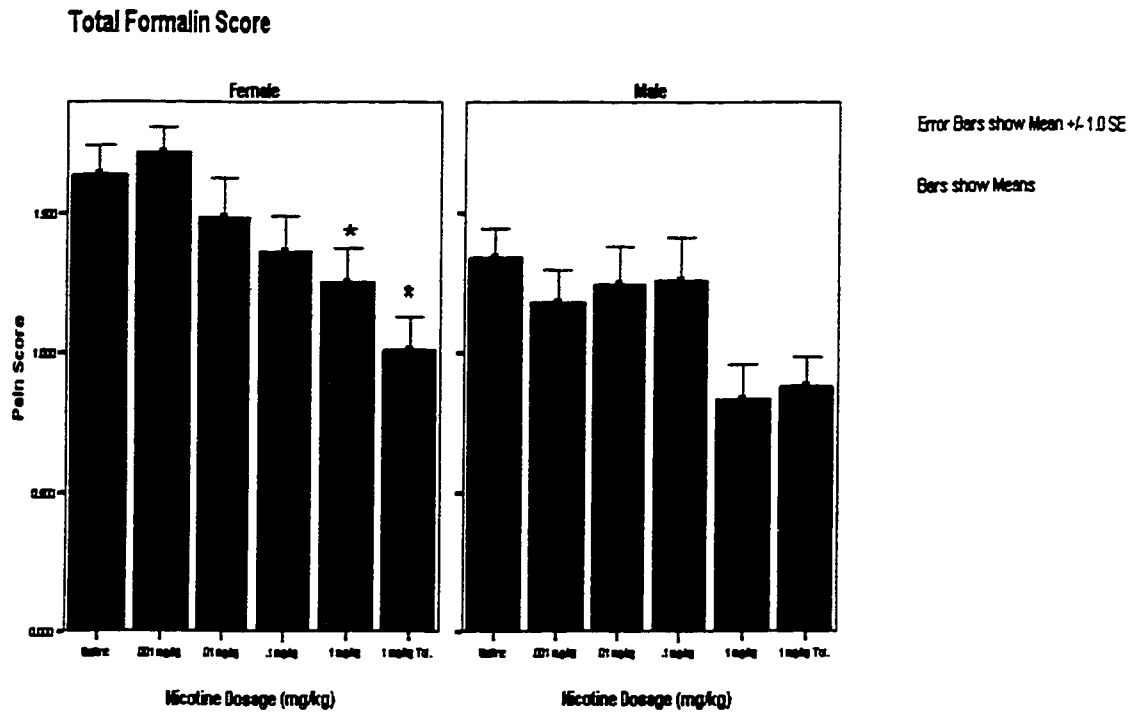

Figure 12. Effects of Nicotine on Formalin Score (cumulative score for six 5-minute periods). Nicotine 1 mg/kg decreased pain scores (compared with control) for females, but not for males (Asterisks indicate statistically significant findings, $p < 0.05$).

[$F(4, 110) = 1.575$, n.s.]. There was, however, a significant difference between sexes [$F(1, 110) = 8.755$, $p < 0.005$] with males exhibiting significantly lower Formalin Testing scores (greater antinociception) than did females. Separate ANOVAs for each sex did not reveal any significant differences based on drug treatment condition. Figure 13 presents these findings for males and females separately.

Formalin Testing - 10 minutes

When males and females were considered together, there was no effect of drug among treatment groups at this time-point [$F(4, 110) = .716$, n.s.]. There was a significant difference between sexes [$F(1, 110) = 8.883$, $p < 0.005$] with males exhibiting lower pain scores (greater antinociception) than females. Separate ANOVAs for each sex did not reveal any significant differences based on drug treatment condition. Figure 14 presents these findings for males and females separately.

Formalin Testing - 15 minutes

When males and females were considered together, there was a significant effect of drug treatment [$F(4, 110) = 2.901$, $p < 0.05$] with the 1.0 mg/kg nicotine dosage decreasing Formalin scores significantly compared with saline controls. In addition, there was a difference in Formalin behavioral responses based on sex [$F(1, 110) = 8.123$, $p = 0.005$] such that males exhibited significantly lower Formalin Testing scores (i.e., greater antinociception) than did females. Separate ANOVAs for each sex revealed a trend for differences among treatment groups for female rats [$F(4, 55) = 2.465$, $p = 0.056$], but not for male rats [$F(4,55) = 1.743$, n.s.). Figure 15 presents these findings for males

Formalin Test at 5 Minutes

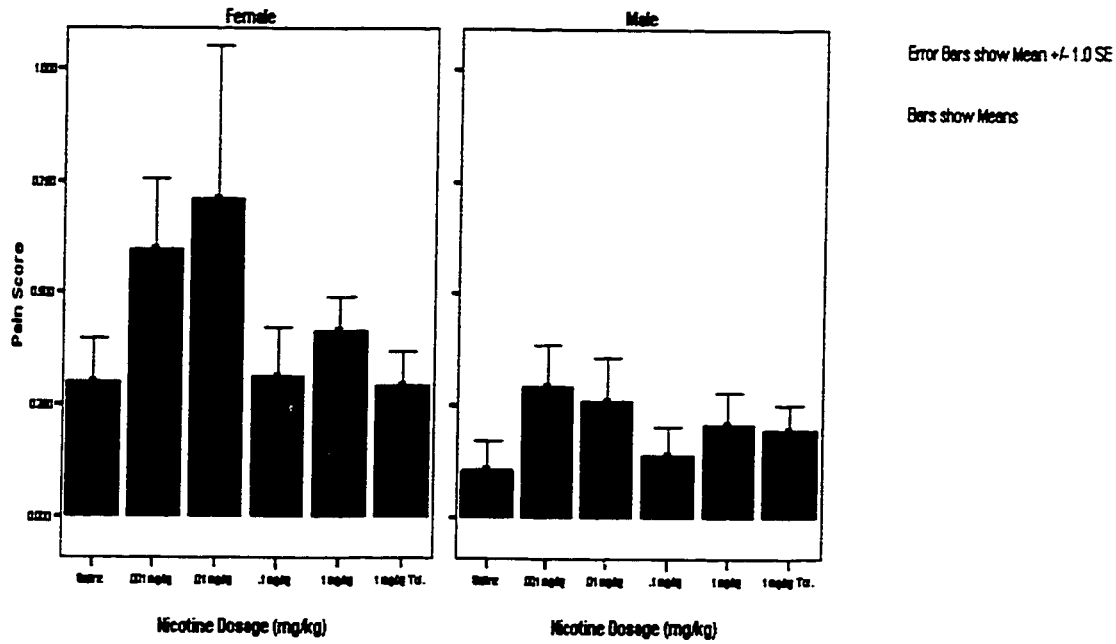

Figure 13. Effects of Nicotine on Formalin 5 Minutes Post Injection.
Nicotine did not significantly decrease Formalin Pain Scores.

Formalin Test at 10 Minutes

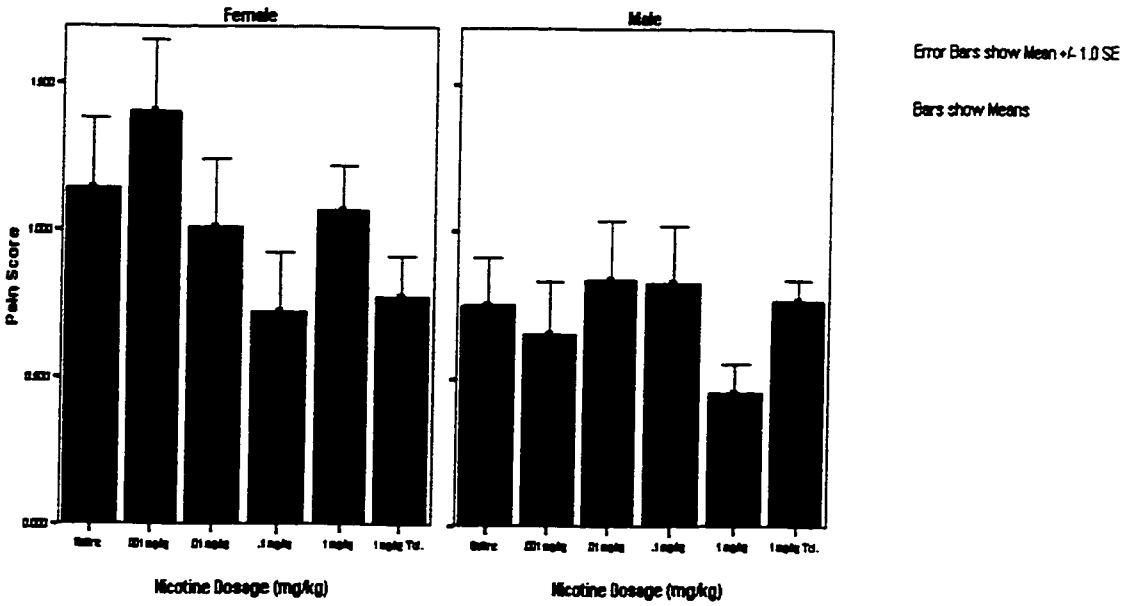

Figure 14. Effects of Nicotine on Formalin 10 Minutes Post Injection.
Nicotine did not significantly decrease Formalin Pain Scores.

Formalin Test at 15 Minutes

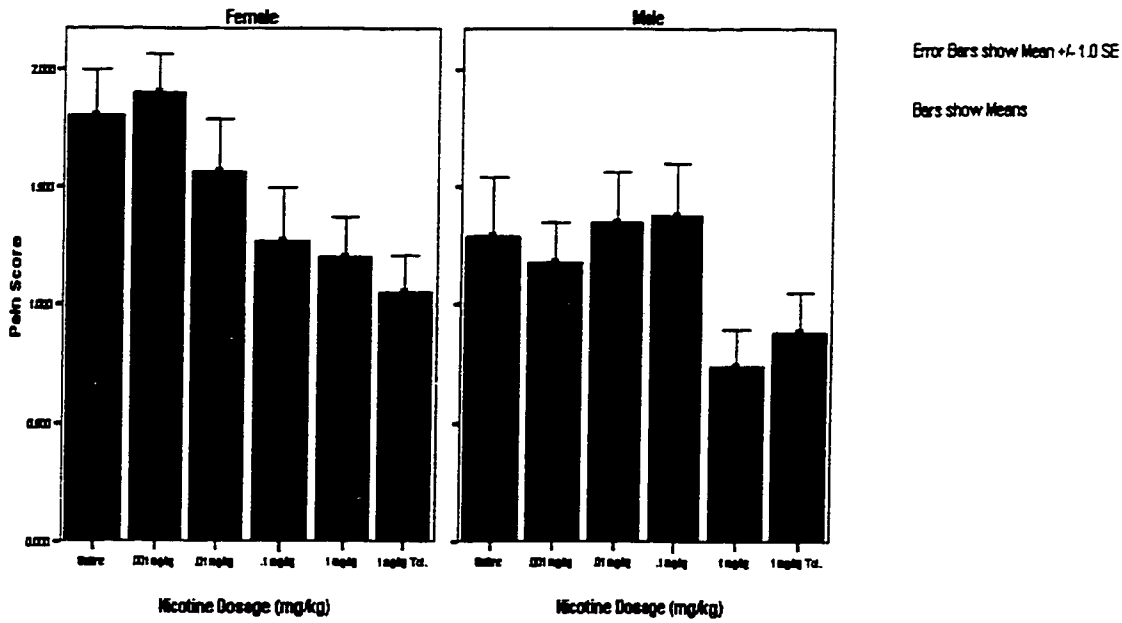

Figure 15. Effects of Nicotine on Formalin 15 Minutes Post Injection. Nicotine did not signifcantly decrease Formalin Pain Scores.

and females separately.

Formalin Testing - 20 minutes

When males and females were considered together, there was a significant effect of drug treatment [$F(4, 110) = 4.790$, $p = 0.001$] with the 1.0 mg/kg nicotine dosage decreasing Formalin scores significantly compared with saline controls. In addition, there was a difference in Formalin behavioral responses based on sex [$F(1, 110) = 9.740$, $p = 0.005$] such that males exhibited significantly lower Formalin Testing scores than did females. Separate ANOVAs for each sex revealed significant differences among treatment groups for female rats [$F(4, 55) = 4.227$, $p = 0.005$] such that the 1.0 mg/kg treatment group exhibited decreased nociception as compared with saline-treated subjects. There was not a significant treatment effect of nicotine for male rats [$F(4,55) = 2.154$, n.s.). Figure 16 presents these findings for males and females separately.

Formalin Testing - 25 minutes

When males and females were considered together, there was a significant effect of drug treatment [$F(4, 110) = 4.928$, $p = 0.001$] with the 1 mg/kg nicotine dosage decreasing Formalin scores significantly compared with saline controls. In addition, there was a difference in Formalin behavioral responses based on sex [$F(1, 110) = 9.628$, $p < 0.005$] such that males exhibited significantly lower Formalin Testing scores than did females. Separate ANOVAs for each sex revealed significant differences among treatment groups for female rats [$F(4, 55) = 3.451$, $p < 0.05$] but not for male rats [$F(4,55) = 2.202$, n.s.]. For females, the 1 mg/kg dosage was significantly different from saline controls. Figure 17 presents these findings for males and females separately.

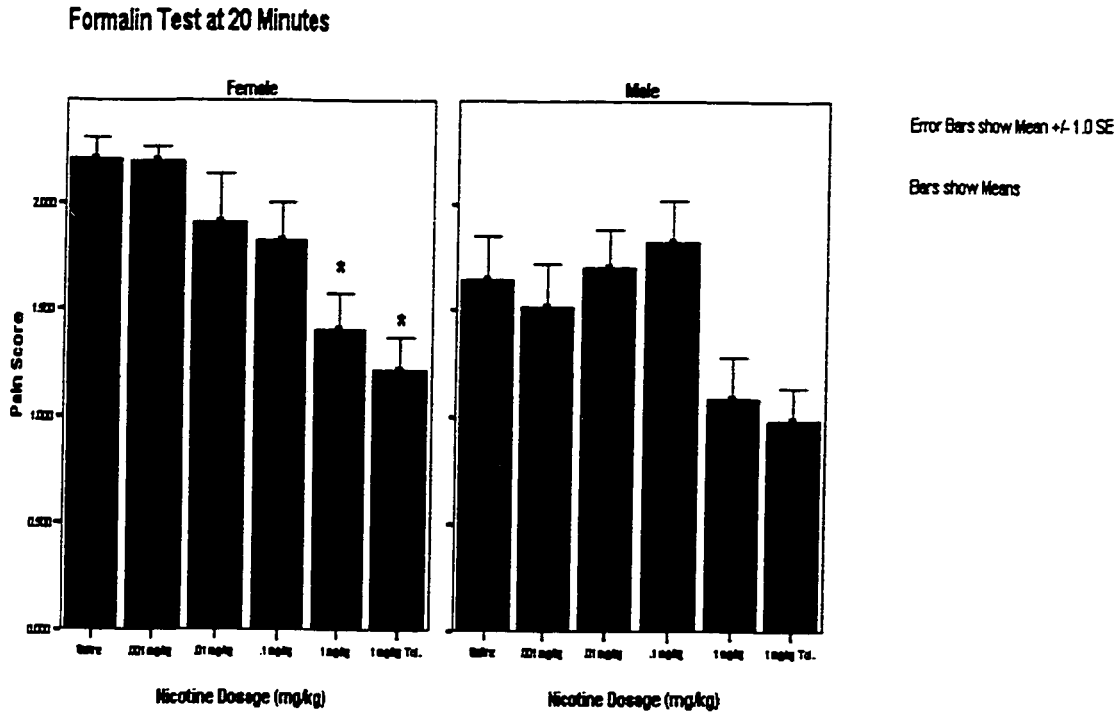

Figure 16. Effects of Nicotine on Formalin 20 Minutes Post Injection.
Nicotine 1 mg/kg significantly decreased Formalin Pain Scores for females, but not for males (compared with controls) (Asterisks indicate statistically significant findings, $p < 0.05$).

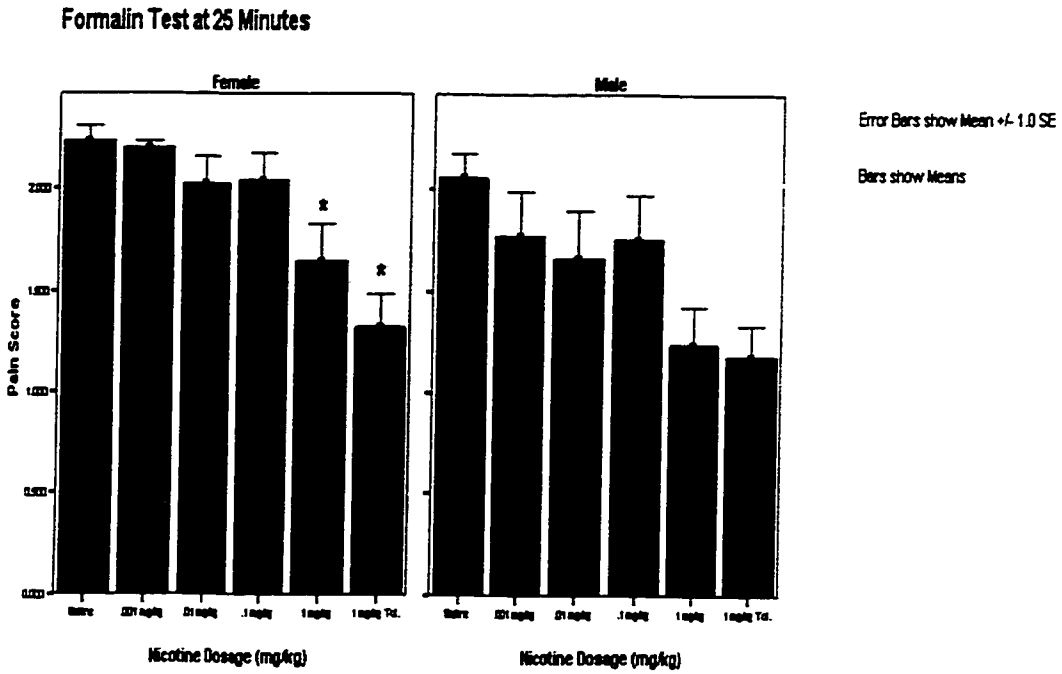

Figure 17. Effects of Nicotine on Formalin 25 Minutes Post Injection.
Nicotine 1 mg/kg significantly decreased Formalin Pain Scores for females, but not for males (compared with controls) (Asterisks indicate statistically significant findings, $p < 0.05$).

Formalin Testing - 30 minutes

When males and females were considered together, there was a significant effect of drug treatment [$F(4, 110) = 3.150$, $p < 0.05$] with the 1 mg/kg nicotine treatment groups decreasing the Formalin score significantly compared with saline controls. In addition, there was a difference in Formalin behavioral responses based on sex [$F(1, 110) = 10.023$, $p < 0.005$] such that males exhibited significantly lower Formalin Testing scores than did females. Separate ANOVAs for each sex revealed significant differences among treatment groups for male rats such that the 1 mg/kg dosage decreased Formalin Scores when compared with saline controls [$F(4, 55) = 2.519$, $p = 0.051$]. There were no differences among treatment groups for female rats [$F(4,55) = .922$, n.s.]. Figure 18 presents these findings for males and females separately.

Summary of Experiment II

Experiment II examined the effects of nicotine or saline on a persistent antinociceptive (pain) model. Significant differences among treatment groups became evident 15 minutes after nicotine administration and continued for the remainder of the 30 minute observation period. For the 15, 20, 25, and 30 minute time-points there were sex differences such that males had lower scores (greater antinociception) than did females. Separate ANOVAs for each sex revealed significant differences for females at the 15, 20, and 25 minute time-points as a result of drug treatment condition and significant differences for males as a result of drug treatment condition at the 30 minute time-point.

Formalin Test at 30 Minutes

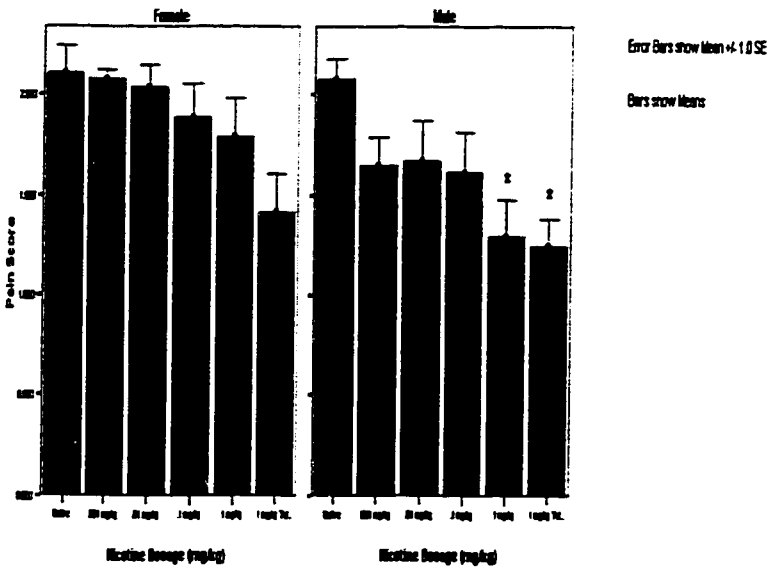

Figure 18. Effects of Nicotine on Formalin 30 Minutes Post Injection. Nicotine 1 mg/kg significantly decreased Formalin Pain Scores for males, but not for females (compared with controls) (Asterisks indicate statistically significant findings, $p < 0.05$).

Experiment III

Experiment III examined the pharmacokinetics of an acute, SC nicotine injection. Following an injection of either nicotine or saline, animals were sacrificed and trunk blood was collected for plasma nicotine and cotinine levels. Whole brains and spinal cords were removed from each animal and these tissues also were assayed for nicotine and cotinine. The purpose of this experiment was to determine if there are sex-related pharmacokinetic differences following an acute, SC nicotine injection.

This experiment also examined the contribution of sex hormones to nicotine-induced antinociception. At sacrifice, plasma was collected for sex hormone assays. Testosterone levels in males and 17-β-estradiol in females were assayed using a radioimmunoassay technique. Analyses were then performed to correlate sex hormone levels to individual nociceptive testing data to determine whether sex hormone levels could predict antinociceptive responses.

Nicotine and Cotinine Levels and Nociceptive Measures

This section presents the results of a series of regression correlations performed on nicotine or cotinine and the nociceptive behavioral testing. The results are presented in the same order as presented in Experiments I and II: tail-flick, hot-plate, cold-flick, Total Formalin score, Formalin 5 minute time-point, Formalin 10 minute time-point, and so on.

Tail-flick

Time-point 1 (8 Minutes Post Injection)

Plasma nicotine levels correlated significantly with tail flick latencies at

time-point 1 [r = .527, p < .001]. Plasma nicotine was more highly correlated with tail-flick latencies at this time-point 1 for females [r = .622, p <.001] than for males [r = .431, p < .001].

There was a significant correlation between **plasma cotinine** and tail-flick latencies at time-point 1 [r = .438, p <.05]. At this time-point plasma nicotine was significantly correlated for females [r = .481, p < .05], but not for males [r = .147, p = n.s.].

There was a significant correlation between **brain nicotine** levels and tail flick latencies at time-point 1 [r = .548, p <. 001]. The correlations were significant for male [r = .261, p<. 001] and female [r = .352, p < .001] rats.

Spinal cord nicotine levels also correlated with tail-flick latencies [r = .554, p < .001]. The correlations were significant for male [r = .503, p < .001] and female rats [r = .611, p < .001].

Time-point 2 (12 Minutes Post-Injection)

Plasma nicotine levels correlated significantly with tail-flick latencies at time-point 2 [r = .365, p < .005]. Plasma nicotine was significantly correlated with tail-flick latencies at this time-point for females [r = .570, p < .001], but not for males [r = .120, p < n.s.].

There was a significant correlation between **plasma cotinine** and tail-flick latencies at time-point 2 [r = .377, p <.05]. At this time-point plasma cotinine was significantly correlated with tail-flick latencies for females [r = .489, p < .05], but not for males [r = .222, p = n.s.].

There was a significant correlation between **brain nicotine** levels and tail-flick latencies at time-point 2 [r = .445, p < . 001]. The correlation was greater for

females [r = .577, p < .001] than for males [r = .306, p < .05]. Figure 19 presents these findings for males and females separately.

Spinal cord nicotine levels also correlated with tail-flick latencies [r = .444, p < .001]. The correlation was greater for females [r = .595, p < .001] than for male rats [r = .303, p < .05]. Figure 20 presents these findings for males and females separately.

Hot-plate

Time-point 1 (8 Minutes Post-Injection)

Plasma nicotine levels significantly correlated with hot-plate latencies at time-point 1 [r = .482, p < .001]. The correlation was significant for males [r = .637, p < .001], but not for females [r = .287, p < n.s.].

There was no correlation between plasma cotinine and hot-plate latencies at time-point 1 [r = .256, p = n.s.]. There were no sex specific differences.

Brain nicotine significantly correlated with hot-plate latencies at time-point 1 [r = .467, p < .001]. This correlation was greater for male [r = .588, p < .001] than for female rats [r = .334, p < .05].

Spinal cord nicotine levels correlated significantly with hot-plate latencies at time-point 1 [r = .497, p < .001]. This correlation was greater for male [r = .599, p < .001] than for female rats [r = .359, p < .01].

Brain Nicotine Levels Correlated with Tail-Flick Latencies

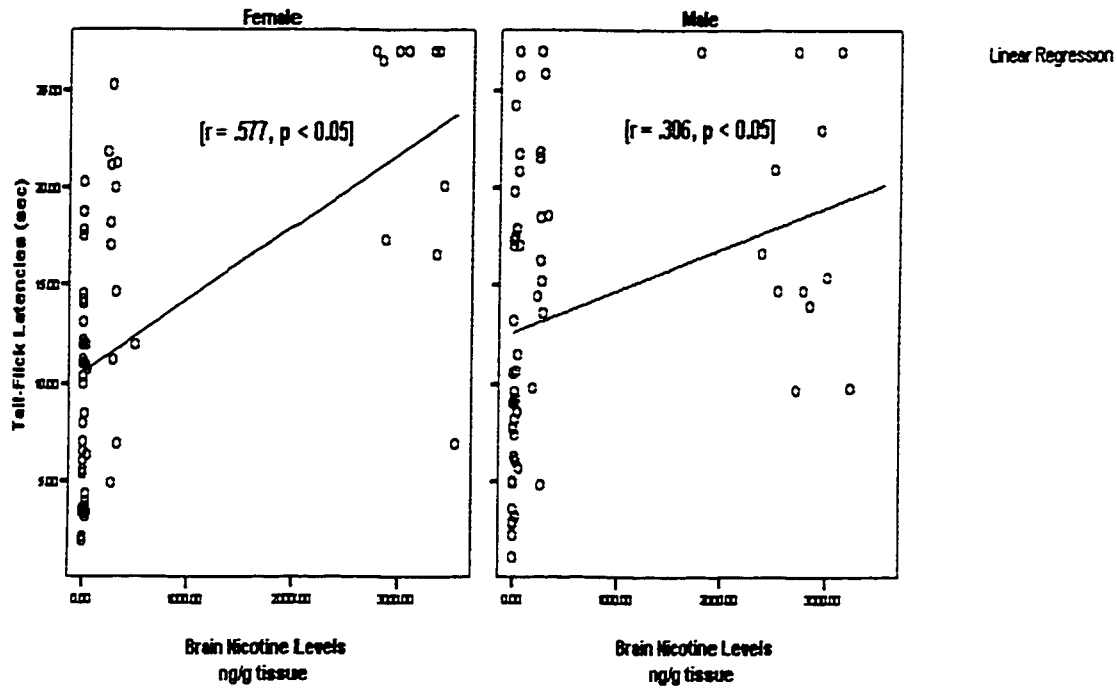

Figure 19. Brain Nicotine Levels 12 Minutes Post Injection
Correlated significantly with Tail-Flick Latencies (sec).
These values correlated significantly for females [r = .577, p < 0.05] and for males [r = .306, p < 0.05].

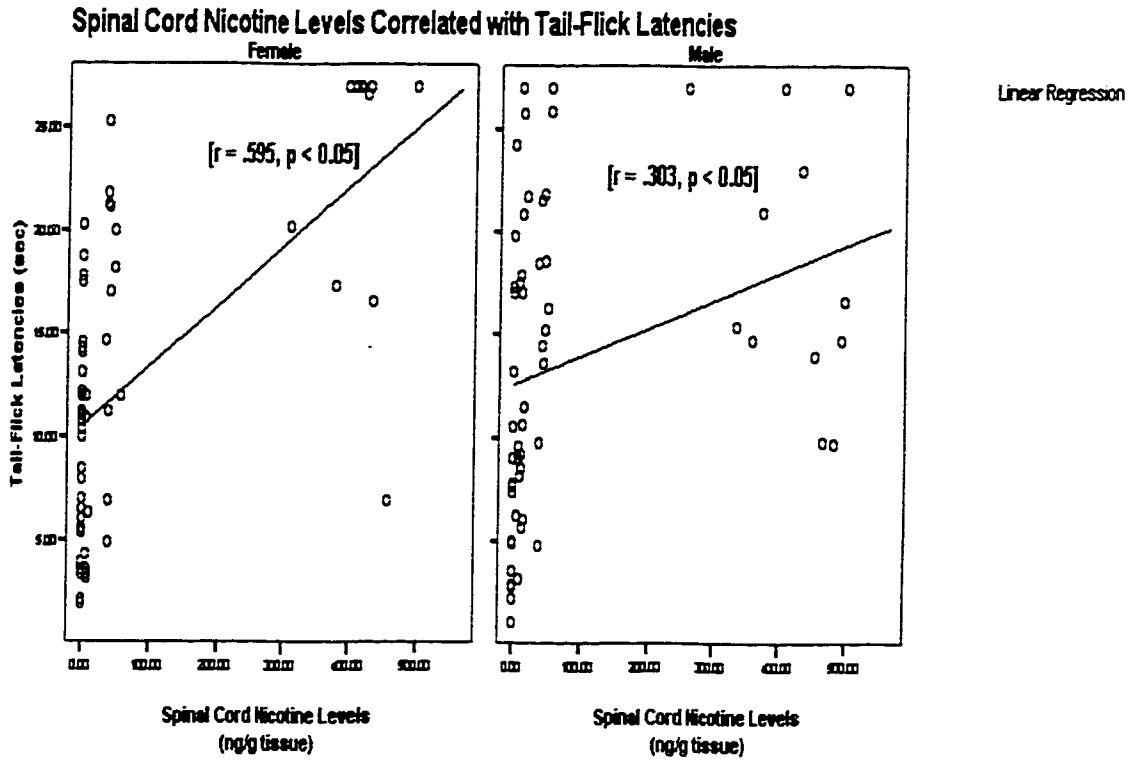

Figure 20. Spinal Cord Nicotine Levels 12 Minutes Post Injection Correlated with Tail-Flick Latencies (sec). These values correlated significantly for females [r = .595, p < 0.05] and for males [r = .303, p < 0.05].

Time-point 2 (12 Minutes Post-Injection)

Plasma nicotine levels significantly correlated with hot-plate latencies at time-point 2 [$r = .410$, $p < .001$]. The correlation was significant for males [$r = .536$, $p < .001$], but not for females [$r = .242$, $p <$ n.s.].

There was no correlation between **plasma cotinine** and hot-plate latencies at time-point 2 [$r = .256$, $p =$ n.s.]. There were no sex specific differences.

Brain nicotine significantly correlated with hot-plate latencies at time-point 2 [$r = .474$, $p < .001$]. This correlation was greater for male [$r = .593$, $p < .001$] than for female rats [$r = .351$, $p < .05$]. Figure 21 presents these findings for males and females separately.

Spinal cord nicotine levels correlated significantly with hot-plate latencies at time-point 2 [$r = .479$, $p < .001$]. This correlation was greater for male [$r = .604$, $p < .001$] than for female rats [$r = .304$, $p < .05$]. Figure 22 presents these findings for males and females separately.

Brain Nicotine Levels Correlated with Hot-Plate Latencies

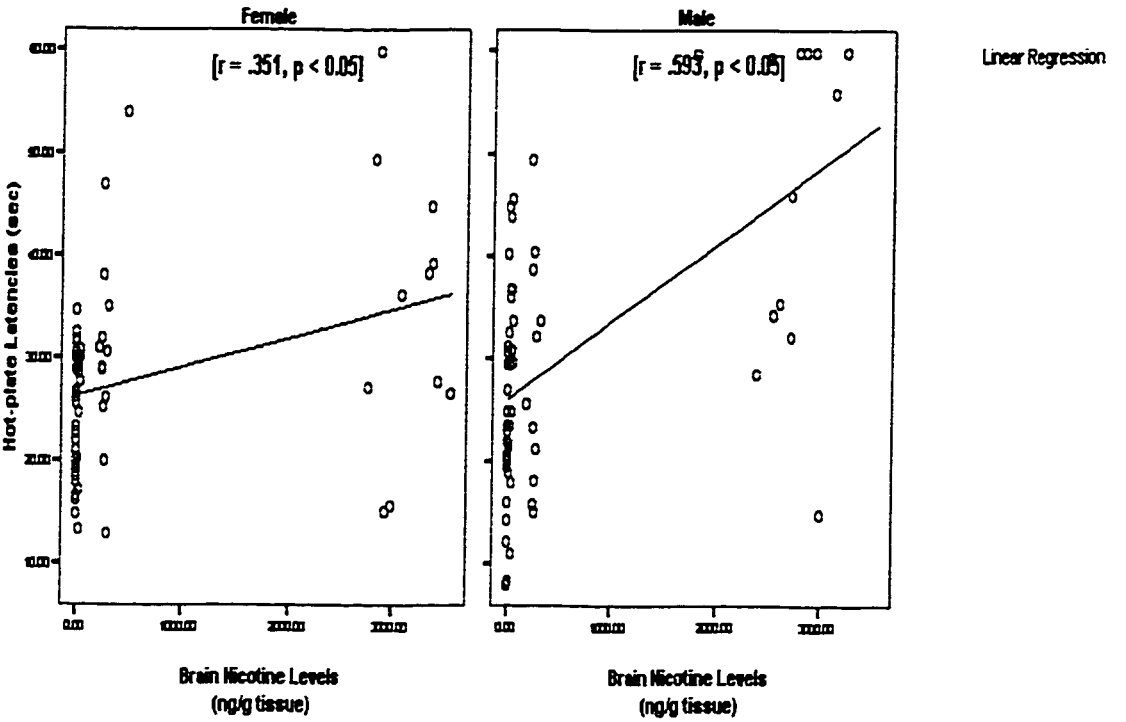

Figure 21. Brain Nicotine Levels and Hot-Plate Latencies 12 minutes Post injection Correlated with Hot-Plate Latencies (sec). These values correlated significantly for females [r = .351, p < 0.05] and for males [r = .593, p < 0.05].

Spinal Cord Nicotine Levels Correlated with Hot-Plate Latencies

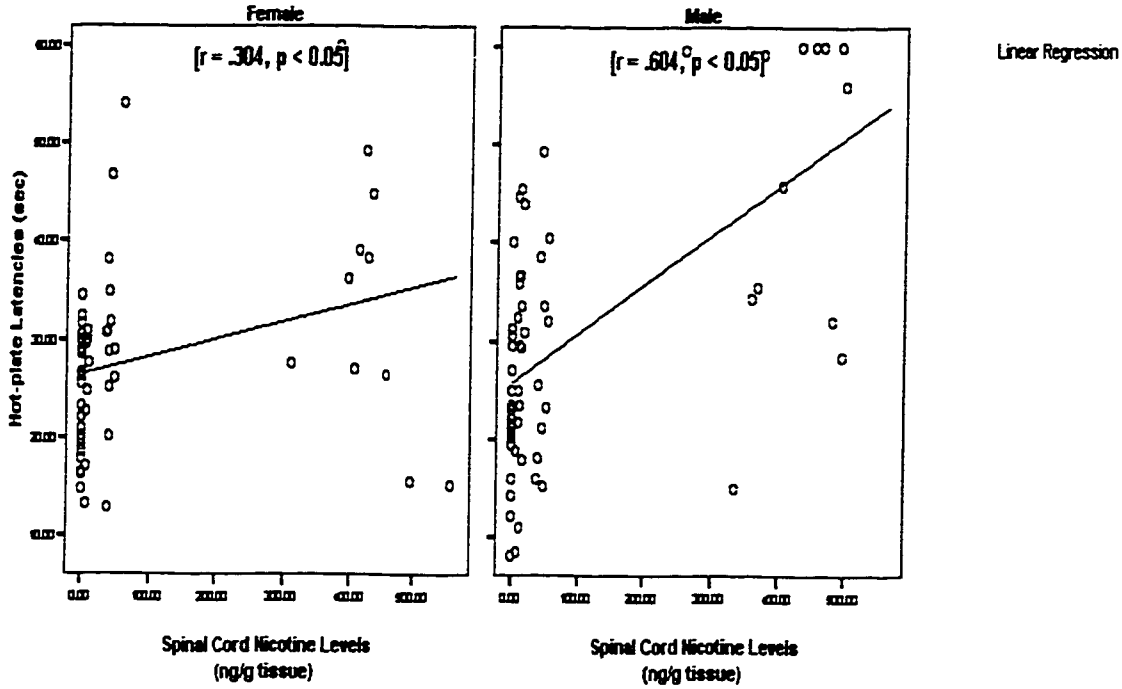

Figure 22. Spinal Cord Nicotine Levels 12 Minutes Post Injection Correlated with Hot-Plate Latencies (sec).
These values correlated significantly for females [r = .304, p < 0.05] and for males [r = .604, p < 0.05].

Cold-flick

Time-point 1 (8 Minutes Post-Injection)

Plasma nicotine levels significantly correlated with cold-flick latencies at time-point 1 [$r = .678$, $p < .001$]. This correlation was greater for male [$r = .671$, $p < .001$] than for female rats [$r = .284$, $p < .001$].

Plasma cotinine levels significantly correlated with cold-flick latencies at time-point 1 [$r = .400$, $p < .05$]. There were no sex specific differences.

Brain nicotine significantly correlated with cold-flick latencies at time-point 1 [$r = .670$, $p < .001$]. This correlation was greater for male [$r = .806$, $p < .001$] than for female rats [$r = .543$, $p < .001$].

Spinal cord nicotine levels correlated significantly with cold-flick latencies at time-point 1 [$r = .702$, $p < .001$]. This correlation was greater for male [$r = .823$, $p < .001$] than for female rats [$r = .568$, $p < .001$].

Time-point 2 (12 Minutes Post-Injection)

Plasma nicotine levels significantly correlated with cold-flick latencies at time-point 2 [$r = .544$, $p < .001$]. The correlation was significant for males [$r = .550$, $p < .001$] and females [$r = .547$, $p = .001$].

There was no correlation between **plasma cotinine** and cold-flick latencies at time-point 2 [$r = .266$, $p =$ n.s.]. There were no sex specific differences.

Brain nicotine significantly correlated with cold-flick latencies at time-point 2 [$r = .566$, $p < .001$]. The correlation was significant for male [$r = .332$, $p < .001$] and female rats [$r = .311$, $p < .001$]. Figure 23 presents these findings for males and females separately.

Spinal cord nicotine levels correlated significantly with cold-flick latencies at time-point 2 [$r = .580$, $p < .001$]. The correlation was significant for male [$r = .589$, $p < .001$] and female rats [$r = .581$, $p < .001$]. Figure 24 presents these findings for males and females separately.

Formalin Testing

This section presents a series of correlations performed on nicotine or cotinine levels in plasma and tissue and the Formalin Testing scores. Analyses begin with Total Formalin score and continue with the analyses for each 5 minute testing period.

Total Formalin

There were no significant correlations between Total Formalin scoring (collapsing responses over the entire 30 minute period) and plasma nicotine, plasma cotinine, brain nicotine, or spinal cord nicotine levels.

Formalin Testing - 5 minutes

There were no significant correlations at 5 minutes between Formalin Testing and plasma nicotine, plasma cotinine, brain nicotine, or spinal cord nicotine levels.

Formalin Testing - 10 minutes

There were no significant correlations at 10 minutes between Formalin Testing and plasma nicotine, plasma cotinine, brain nicotine, or spinal cord

Brain Nicotine Levels Correlated with Cold-Flick Latencies

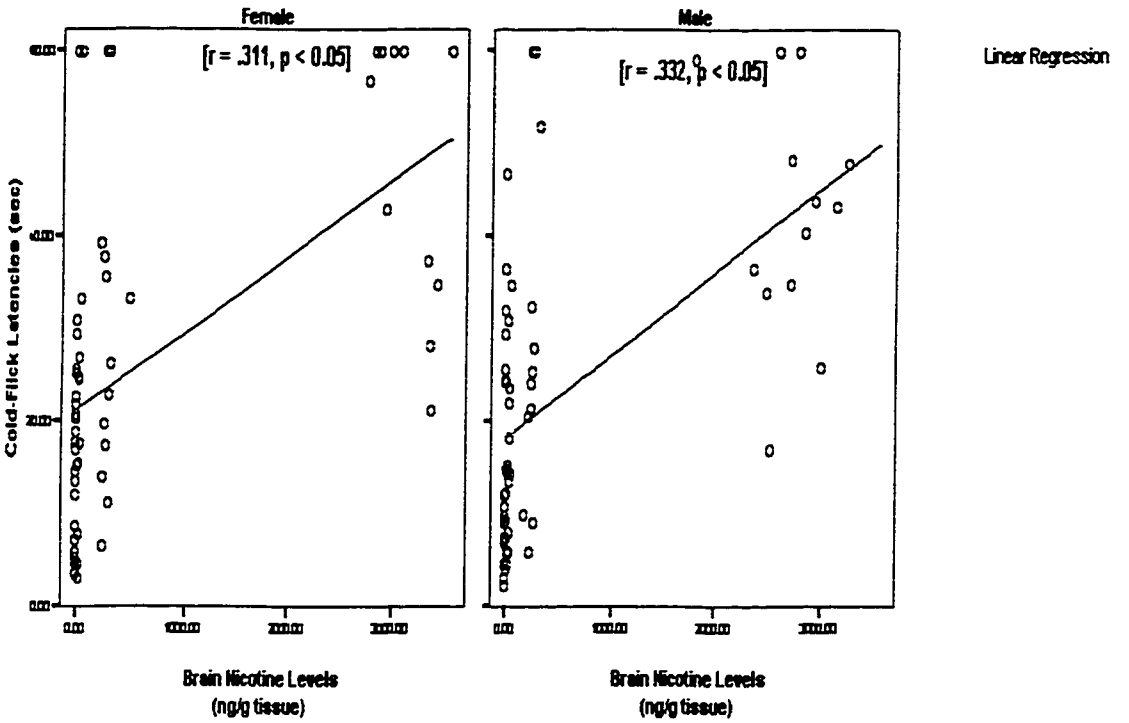

Figure 23. Brain Nicotine Levels 12 minutes Post Injection Correlated with Cold-Flick Latencies (sec). These values correlated significantly for females [r = .311, p < 0 .05] and for males [r = .332, p < 0 .05].

Spinal Cord Nicotine Levels Correlated with Cold-Flick Latencies

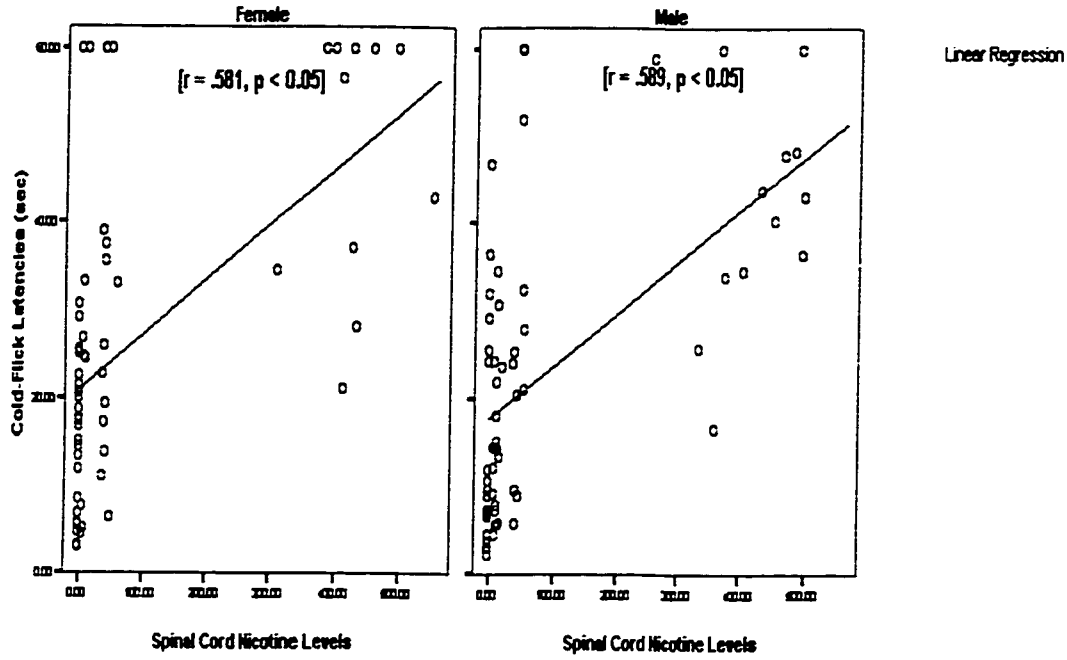

Figure 24. Spinal Cord Nicotine Levels 12 minutes Post Injection Correlated with Cold-Flick Latencies (sec). These values correlated significantly for females [r = .581, p < .05] and for males [r = .589, p < .05].

nicotine levels.

Formalin Testing - 15 minutes

At 15 minutes **plasma nicotine** levels correlated significantly with Formalin Testing [r = .290, p < .005]. The correlation was significant for **males** [r = .365, p < .05], but not for females [r = .224, p = 005]. There was no significant correlation between **plasma cotinine** and Formalin Testing at this time-point.

Brain nicotine correlated significantly with Formalin Testing at this time-point [r = .309, p < .001]. This correlation was significant for **male** [r = .310, p < .01] and **female** [r = .341, p < .005] rats.

Spinal cord nicotine levels correlated significantly with Formalin Testing at this time-point [r = .298, p < .001]. This correlation was significant for **male** [r = .283, p < .05] and **female** [r = .323, p < .01] rats.

Formalin Testing - 20 minutes

At 20 minutes **plasma nicotine** levels correlated significantly with Formalin Testing [r = .418, p < .001]. This correlation was significant for **male** [r = .453, p = .001] and for **female** [r = .393, p = .005] rats. There was no significant correlation between **plasma cotinine** and Formalin Testing at this time-point [r = .265, p = n.s.].

Brain nicotine correlated significantly with Formalin Testing at this time-point [r = .431, p < .001]. This correlation was greater for **female** [r = .528, p < .001] than for **male** rats [r = .384, p = .001].

Spinal cord nicotine levels correlated significantly with Formalin Testing at this time-point [r = .419, p < .001]. This correlation was greater for **female** [r =

.505, p < .001] than for **male** rats [r = .361, p = .001].

Formalin Testing - 25 minutes

At 25 minutes **plasma nicotine** levels significantly correlated with Formalin Testing [r = .355, p < .001]. This correlation was greater for **females** [r = .445, p = 001] than for **males** [r = .297, p = .05]. There was no significant correlation between **plasma cotinine** and Formalin Testing at this time-point [r = .265, p = n.s.].

Brain nicotine significantly correlated with Formalin Testing at this time-point [r = .428, p < .001]. This correlation was greater for **female** [r = .560, p < .000] than for **male** rats [r = .369, p = .001].

Spinal cord nicotine levels correlated significantly with Formalin Testing at this time-point [r = .4191, p < .001]. This correlation was greater for **female** [r = .536, p < .001] than for **male** rats [r = .339, p = .005].

Formalin Testing - 30 minutes

At 30 minutes **plasma nicotine** levels significantly correlated with Formalin Testing [r = .262, p < .01]. This correlation was significant for **female** [r = .299, p = .05], but not for male rats [r = .243, p = n.s].

Regression analysis revealed a no significant relationships between Formalin Testing at this time-point and plasma cotinine [r = .108, p = n.s.].

Brain nicotine significantly correlated with Formalin Testing at this time-point [r = .335, p < .001]. This correlation was significant for **male** [r = .336, p < .005] and **female** rats [r = .378, p = .005].

Spinal cord nicotine levels correlated significantly with Formalin Testing

at this time-point [$r = .321$, $p < .001$]. This correlation was significant for **male** [$r = .302$, $p < .05$] and **female** rats [$r = .359$, $p = .005$].

Testosterone

There was a trend for nicotine administration to affect testosterone levels [$F(4, 56) = 2.419$, $p = 0.059$] in male rats; 1 mg/kg nicotine appeared to raise testosterone levels. Brain nicotine levels [$r = .282$, $p < .05$] and spinal cord nicotine levels [$r = .280$, $p < .05$] correlated significantly with testosterone levels in males. However, plasma nicotine and plasma cotinine levels did not correlate with testosterone levels.

A series of correlations were performed to determine whether a relationship exists between plasma testosterone and behavioral measures of nociception (acute and persistent). These analyses did not reveal any significant findings.

Estrus Cycle Staging

Estrus cycle staging was performed on all females undergoing behavioral testing. Staging was performed immediately after completion of nociceptive testing. Animals were determined to be in proestrus, estrus, metestrus, or diestrus by vaginal swabbing (see Methods, Chapter II)

There was no significant relationship between estrus stage and tail-flick or hot-plate latencies. For **cold-flick,** there was a significant effect of estrus cycle

stage at time-point 1 such that females in diestrus had longer cold-flick latencies than did females in either proestrus or estrus [$F(2, 54) = 3.401$, $p = 0.05$] (see figure 25). There were no animals in the metestrus stage. There also was a significant effect of estrus cycle stage on Formalin testing. This effect was significant only at the 5 minute testing period [$F(2, 56) = 3.456$, $p = 0.05$] (see figure 26). Females in estrus stage exhibited higher pain scores than females in either proestrus or diestrus.

17-β-Estradiol

There was no effect of treatment group on 17-β-estradiol levels. A series of correlations were performed to determine whether a relationship exists between 17-β-estradiol levels and plasma or tissue levels of nicotine and cotinine. Analyses reveal that there were no significant relationships between 17-β-estradiol levels and plasma nicotine, brain nicotine, brain cotinine, or spinal cord nicotine levels.

Summary of Results of Experiment III

Regression analyses indicated that plasma nicotine, plasma cotinine, brain nicotine, and spinal cord nicotine levels were significantly correlated with acute measures of nociception. Internal analyses by sex reveal that these drug levels did not uniformly predict the behavioral responses of males and females. For the tail-flick measure, plasma and tissue levels were generally more predictive for females than for males. Conversely, on the hot-plate and cold-flick measures, plasma and tissue levels of nicotine and cotinine had a greater

Cold-Flick at 8 Minutes Post Injection Compared with Estrus Cycle Stage

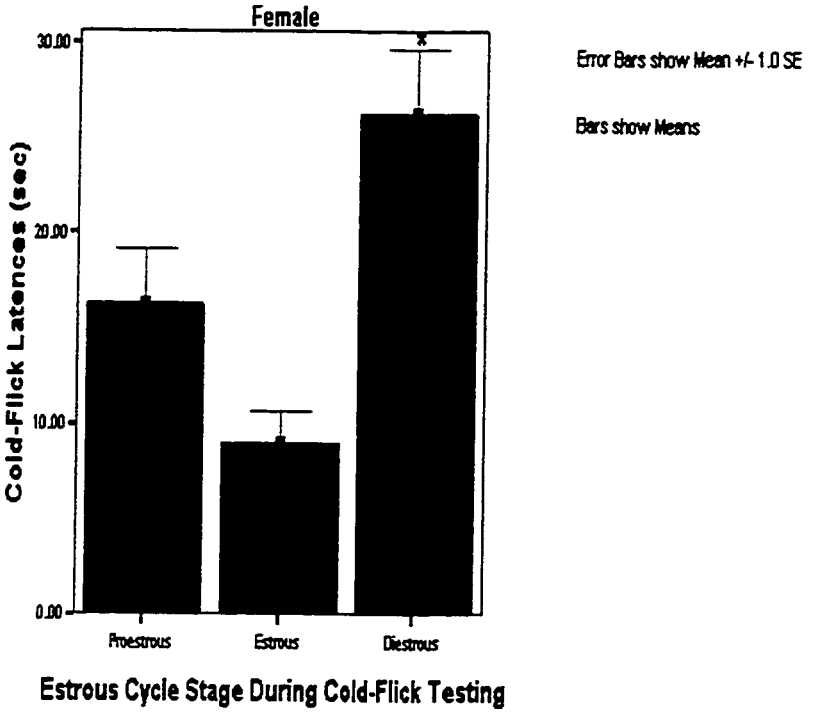

Figure 25. Relationship Between Estrus Cycle Stage and Cold-Flick 8 Minutes Post Injection. Females in diestus had signifcantly longer latencies than females in proestrus or in estrus stages (Asterisks indicate statistically significant findings, $p < 0.05$).

Formalin Testing at 5 Minutes Post Injection Compared with Estrus Cycle Stage

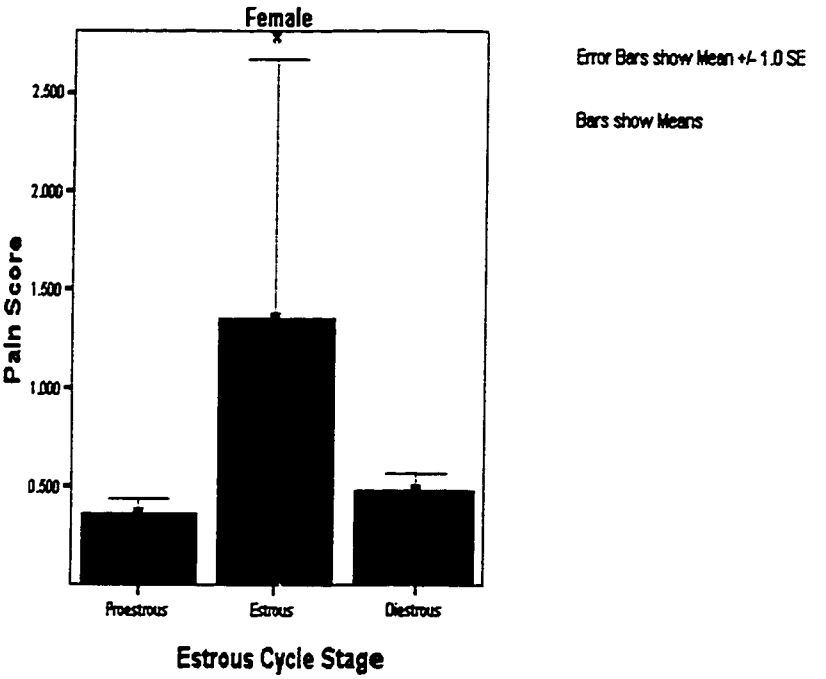

Figure 26. Relationship Between Estrus Cycle Stage and Formalin Test 5 Minutes Post Injection. Females in estrus stage had higher pain scores than females in proestrus or in diestrus (Asterisks indicate statistically significant findings, $p < 0.05$).

predictive value for males than for females.

With regard to the persistent pain model elicited by formalin, drug levels did not correlate with antinociception for the first 10 minutes of testing. After 15 minutes has elapsed, plasma and tissue levels do correlate with antinociception.

Internal analysis by sex reveals that initially plasma nicotine is predictive of antinociception in males and not females. This effect was reversed by the 25 minute time-point with plasma nicotine levels being more highly correlated for females than for males. In addition, brain and spinal cord nicotine levels were equally predictive of antinociceptive behavior initially, but then become more predictive for females than for males. By the time 30 minutes had elapsed, these differences in predictability were again similar.

Sex hormones played a small role in nicotine-induced antinociception. Estrus cycle stage was implicated as a factor in a single acute measure at one time-point (cold-flick time-point 1 and at a single time-point during Formalin Testing (5 minute time-point) estrus cycle modulated antinociception. Levels of 17-β-estradiol did not significantly affect antinociception. Although testosterone correlated with brain and spinal cord nicotine levels, there were no significant relationships between plasma testosterone levels and nociceptive behaviors.

CHAPTER IV

Discussion

Several human and animal studies have reported that nicotine is antinociceptive (Apatov, 1998; Maisonneuve, Mann, Deibel, & Glick, 1997; Mildenhall, 1921; Pomerleau, 1986; Sahley & Berntson, 1979). These studies, however, could only reach tentative conclusions because of methodologic limitations. For example, none of these studies ruled out nicotine's effects on gross motor movements as a potential confound in studies of responses to pain. Animal studies have used a maximum tail-flick latency of 10 seconds (instead of 25 or 30 seconds), thereby restricting the information gathered and artificially narrowing the variances of responses in nicotine-induced antinociception. In addition, previous reports in the animal literature have limited their measures to acute heat as the noxious, pain stimulus and have not examined responses to more persistent stimuli or to different types of noxious stimuli. Moreover, no animal and only one human study (Jamner et al., 1998) have examined individual differences (such as gender differences) in effects of nicotine on antinociception.

There are other reasons that reports regarding nicotine-induced antinociception in the animal literature remain tentative. The existing studies on nicotine-induced antinociception limit the dependent measures to one or two acute antinociceptive tests: hot-plate or tail-flick. Further, there are wide variations in behavioral testing procedures in rodents yet little detail is provided in methods sections to allow comparisons of one study to another. Because there is so much variability with regard to how the measure is conducted, whether

animals are acclimated to the apparatuses prior to testing, and at what point in the animal's light cycle testing is occurring, it is impossible to draw firm conclusions from previous research. Another problem with the research literature is that it does not usually address important issues such as light cycle or handling techniques. Frye and Duncan (1996, p. 28) do an excellent job describing their methods: "...all animals were tested during the dark phase of the light cycle beginning at 1100 h. Behavioral testing consisted of *gently* holding each rat in a towel while smoothing its tail into the tail groove of the tail-flick apparatus." This detailed description however, is the exception rather than the rule. Light cycle (Martínez-Gómez, Cruz, Salas, Hudson, & Pachecos, 1994) and stress (Yamada & Nabeshima, 1995) play important roles in analgesic responses and therefore these variables need to be addressed in studies of drugs and antinociception.

The goals of the present experiments were: 1) to examine the antinociceptive effects of nicotine in a tightly controlled experiment that included measures of locomotion and a means to determine whether multiple nicotine injections resulted in tolerance or sensitization, 2) to examine whether nicotine-induced antinociception attenuated both acute and chronic pain, 3) to determine whether antinociceptive effects of nicotine extend to a noxious cold stimulus, 4) to determine whether there are gender differences in these effects, and 5) to examine two possible mechanisms for gender differences in nicotine's analgesic effects (specifically, pharmacokinetics and sex hormone interactions).

Overall, nicotine had antinociceptive effects in acute (hot and cold) and persistent pain paradigms. These effects were not a result of changes in

locomotor activity and these findings held for both male and female rats. Sex differences were observed for the tail-flick measure, with males appearing more sensitive to nicotine-induced antinociception than females. Sex differences also were observed in the persistent pain paradigm, with females exhibiting higher pain scores and nicotine inducing antinociception sooner for females than for males. These differences in antinociceptive responses to nicotine could not be explained by sex hormones.

With regard to pharmacokinetics, plasma nicotine, brain nicotine levels, and spinal cord nicotine levels, all were significant statistical predictors of antinociception. These findings held true for acute and persistent, noxious stimuli. While there were sex differences in the pharmacokinetic findings, no clear pattern emerged with regard to the relationship between plasma/tissue levels of nicotine or cotinine and measures of nociception.

These findings are first discussed with regard to acute and persistent responses. Then, the gender differences and possible mechanisms are addressed. Next, basic science implications and clinical applications are discussed. Finally, future studies are described.

Acute Measures of Nociception

Three different, acute measures of nociception were used: hot-plate, cold-flick, and tail-flick (to a heat source). Three measures were used to more fully characterize nicotine's active putative antinociceptive actions to acutely noxious stimuli. Nicotine **(at 1.0 mg/kg)** was antinociceptive in response to all three acute, noxious, thermal stimuli. This finding confirms Hypothesis I and replicates previous reports (Apatov, 1998; Damaj et al., 1994). The present work

goes beyond the previous literature by including a noxious, cold stimulus. The consistent findings for hot and cold, noxious stimuli make it clear that nicotine's antinociceptive effects are not restricted to a single type of noxious thermal, stimulus. Instead, these effects are more general. The inclusion of the cold-flick test was important because the cold-flick test is reported to be sensitive to more moderate analgesics (Pizziketti et al., 1985). The finding that nicotine is antinociceptive for both hot and cold stimuli suggest that nicotine-induced antinociception is a broad phenomenon.

Males appeared to be more sensitive to nicotine-induced antinociception only on the tail-flick measure. This finding is a partial confirmation of Hypothesis 3.

Locomotor

Nicotine has marked effects on locomotor activity, even at relatively moderate dosages (0.4 mg/kg and higher) (Bowen, Eury, & Grunberg, 1986; Grunberg & Bowen, 1985). Immediately after animals receive an acute, nicotine injection they may be seen shaking, stumbling, or merely standing in place for a prolonged period of time. Previous research has not investigated whether these changes in locomotor patterns may be affecting the animal's ability to respond to a behavioral measure of nociception (all of which have a motor component). Locomotor testing was performed to determine if nicotine's ability to stimulate or inhibit an animal's ability to move affected antinociception. Unless this potential confound is controlled for, it is impossible to be certain that nicotine is inducing antinociception rather than perturbations in motor function that appear to be antinociceptive in motor-dependent responses.

In the present research, nicotine significantly decreased locomotor activity at 0.1 and 1.0 mg/kg. This finding is a partial confirmation of Hypothesis 2 (no stimulatory effect was seen at lower dosages). Also, males and females were different in locomotor activity with males appearing to be more sensitive to nicotine's impairment of locomotion. When these differences were used as covariates on measures of nociception, however, there were no statistically significant changes in the antinociceptive effects of nicotine. Neither latency to respond to an acutely noxious stimulus nor behavioral responses to the persistent pain paradigm was significantly affected by nicotine-induced motor changes. These findings indicate that nicotine has antinociceptive properties that are independent of its effects on motor function.

Tolerance to Nicotine

During the course of this experiment, most animals received five subcutaneous (SC) nicotine injections. One group of animals was included in the research that received one injection at the beginning of Experiment I immediately prior to locomotor testing and another single injection for immediately prior to Formalin testing in Experiment II. This group was included to evaluate whether six nicotine injections over a six week period would result in different effects (e.g., either tolerance or sensitization) to the painful stimuli than would fewer injections of nicotine. There were no significant differences between the 1 mg/kg animals that received 5 injections and the 1 mg/kg group that received one injection at the outset of the experiments and another injection 5 weeks later. Therefore, because of the one week wash-out period between injections, tolerance or sensitization to nicotine were not confounds in the current

research.

Formalin Testing: A Model of Persistent Pain

When first characterizing a drug believed to have antinociceptive effects, hot-plate and tail-flick are excellent tests for predicting analgesic activity (Dewey, Harris, Howes, & Nuite, 1970; Hunskaar, Berge, & Hole, 1986). Additionally, responses to these two tests strongly suggest whether brain or spinal cord sites are involved in the drugs' antinociceptive properties (Caggiula et al., 1995). While this information is critical to initially evaluate analgesic properties, it has been argued that these tests bear little resemblance to clinical pain (Sternbach, 1976). Therefore, to more fully characterize pain responses in an animal model, a persistent or chronic pain model should be employed. In the current research, the Formalin Test was used to address this issue. The Formalin Test also is useful to determine the time course of a drug's analgesic properties. The Formalin Test is able to generate pain scores every 5 minutes for the entire length of the test. In the current experiment, the Formalin Test generated six 5-minute pain scores. Nicotine antinociception has been reported to plateau after approximately 20 minutes (Matilla et al., 1968). Therefore, this experiment examined the ascent and descent of nicotine's antinociceptive time course. Because this test can generate 30 minutes of data in 5 minute increments, it is possible to establish an analgesic profile of the drug. This time course information may be useful to help determine how to dose nicotine in humans in order to relieve pain.

To date there is only one published study that examined nicotine-induced antinociception in a persistent pain model. Zarrindast and colleagues (1997)

examined nicotine's effects on Formalin Testing in mice injected with nicotine SC, 20 minutes prior to Formalin Testing. Nicotine-induced antinociception has been reported to peak 10 minutes after SC injection (Matilla et al., 1968). Therefore, the current research examined nicotine's effects on antinociception in a persistent pain model 10 minutes post-formalin injection.

Nicotine was antinociceptive in the current experiments using this model of persistent pain. Antinociception was not apparent until 15 minutes and then continued through the end of the testing period (i.e., 30 minutes). Only the 1.0 mg/kg dosage was an effective antinociceptive on this persistent pain test. This finding disconfirms Hypothesis 4.

It is noteworthy that there were sex differences in nicotine-induced antinociception on persistent pain. Nicotine was significantly antinociceptive for females at 15 minutes after injection. Later, after 30 minutes, nicotine was antinociceptive for males but not for females. These findings may be interpreted as: 1) females are more sensitive to nicotine-induced antinociception in a persistent pain model, or 2) that there is a different time course for antinociception in male subjects.

The interpretation that females may be more sensitive to the antinociceptive effects of nicotine is not likely because it was the males that were more responsive to the nicotine-induced antinociception in the tail-flick measure. It remains possible, however, that responses to acute and persistent pain models differ by sex. The present findings disconfirm Hypothesis 3 and deserve further study.

A second interpretation of these findings is that males and females are

equally sensitive to the drug, meaning that antinociception is equivalent, but that antinociception in females peaks several minutes earlier than in males. It is possible that for nicotine-induced antinociception, it takes males longer to get an antinociceptive effect from the drug. If this were true, then the 30 minutes of Formalin Testing may have been adequate in females, but not long enough to capture the antinociceptive profile for males. This possibility of females reacting sooner than males to nicotine-induced antinociception was not seen in the acute measures, where there were two time-points (i.e., 8 and 12 minutes post-injection). The Formalin Test allowed observation of an animal's reaction to a persistent noxious stimulus for a 30 minute time period. This test was an important addition to these experiments not only because the Formalin Test is a model for persistent pain, but also because it allows the investigator to make inferences as to time course of the analgesic. In the present experiment, following a formalin paw injection, nicotine was injected and animals were observed for a 30 minute period. This experimental design allowed the investigator to observe the onset of nicotine analgesia, the peak effect of the drug (assuming a 10 to 15 minute peak as hypothesized), followed by a decrease in analgesic efficacy. This procedure was an attempt to map the time-course of nicotine-induced antinociception.

Plasma and Tissue Nicotine and Cotinine

Experiment III examined the pharmacokinetics of an acute, SC nicotine injection in blood plasma and tissue in the central nervous system. In this experiment, trunk blood was collected at sacrifice to assay for plasma nicotine and cotinine levels. Additionally, whole brains and spinal cords were removed at

sacrifice and each specimen was digested for subsequent nicotine and cotinine assays. Specific areas were not dissected from either brain tissue or spinal cord tissue because: 1) it is unknown which areas of brain or spinal cord are specifically involved in mediating nicotine-induced antinociception, and 2) it was unknown whether sufficient levels of nicotine or cotinine for detection by GC assay would be present in a particular, discrete region of the brain.

Overall, plasma nicotine, plasma cotinine, and tissue levels of nicotine were significantly correlated with all three acute nociceptive measures. These findings confirm Hypotheses 7 and 8. Levels of cotinine in brain and spinal cord were not consistently high enough to be reliably detected by the GC assay, indicating that 12 minutes is not enough time for significant levels of cotinine to be formed in tissue. Plasma nicotine and cotinine levels were important as a confirmation of the drug manipulation. Rats received SC injections of nicotine (0, 0.001, 0.01, 0.1 and 1 mg/kg) and blood levels reflected these different dosages.

It was hoped that correlations between drug levels in tissue might help to determine whether nicotine was acting in the brain versus spinal cord to induce antinociception. However, the findings were unclear with regard to this matter. Brain and spinal cord nicotine and cotinine levels were similarly correlated for tail-flick and hot-plate measures. The tail-flick measure has been reported to be a spinally-mediated measure of nociception (Caggiula et al., 1995). It is presumed that the cold-flick measure would be working through a similar pathway. The hot-plate measure is a more complex measure of nociception requiring a higher level of processing. It is believed to be a supra-spinal measure of nociception (Caggiula et al., 1995). If only spinal cord nicotine or

cotinine levels were highly predictive of the tail-flick and cold-flick measures, then this finding would have supported the interpretation that nicotine is acting at the level of the spinal cord to elicit antinociception. Similarly, if on the hot-plate measure, only brain levels of nicotine were highly predictive of latency scores, then this finding would have supported the interpretation that nicotine is acting in the brain, because the hot-plate measure is a supra-spinal measure of nociception.

Sex Differences

Nicotine has antinociceptive effects and there were gender differences in tail-flick latencies and in response to Formalin Testing. Sex differences in nicotine's antinociceptive effects may have been influenced by sex differences that exist even in the absence of the drug. Two potential mechanisms for these gender differences were examined: hormonal contributions and pharmacokinetic differences.

There were no significant correlations between sex hormones and nicotine-induced antinociception. Neither 17-β-estradiol nor testosterone significantly correlated with behavioral measures of nociception. This finding disconfirms hypothesis 6. It is not known how sex hormones modulate nociception. Other studies using morphine as the analgesic agent reported sex hormone modulation of antinociception (Dawson-Basoa & Gintzler, 1993; Forman et al., 1989; Rao & Saifi, 1985). It is possible that morphine-induced antinociception is modulated by sex hormones, whereas nicotine-induced antinociception is not. The mechanism by which sex hormones may mediate antinociception is unknown, but there appears to be an interaction between sex

hormones and opioid receptors. This conclusion is based on a report by Rao (1985). In that report elevated testosterone levels prevented naloxone-reversal of morphine-induced antinociception.

Estrus cycle staging was performed on all females immediately after antinociceptive testing. Although there are four distinct stages that may be identified by cytological examination from the vaginal wall (Freeman, 1994; Young, Bloing, & Blandau, 1941), in the present subjects, cytological evidence of the metestrus stage was rarely seen. This may have been a result of the relative short amount of time that female rats spend in this phase of the estrus cycle (6-8 hours) or the time of day that specimens were taken (1300-1600 hrs.). As a result correlations were performed using three estrus stages instead of four.

Estrus cycle stage was significantly related to cold-flick at Time-Point 1 and to Formalin Testing at the 5-minute time-point. For cold-flick, the diestrus stage was associated with longer cold-flick latencies, whereas on the Formalin Test, the estrus stage was associated with higher pain scores than females in other estrus stages. This finding partially confirms Hypothesis 5. These data are difficult to interpret because there was no correlation between estrus cycle stage and 17-β-estradiol levels. It does not appear that estrus cycle stage plays a significant role in nicotine-induced antinociception.

Implications for Basic Science

Nicotine continues to be a compound worthy of further study. The U.S. Food and Drug Administration (FDA) has been scrutinizing nicotine to determine if further control of this addictive substance is necessary (Nowak, 1994) and the public continues to be bombarded by the health hazards of cigarette smoking

making nicotine, an important drug to understand. Multiple studies have examined nicotine's analgesic/antinociceptive effects, but the results often have been limited by the methodology used. While nicotine has been reported to act centrally to elicit analgesia (Aceto et al., 1986; Sahley & Berntson, 1979), precisely where in the central nervous system it is acting remains a mystery. Investigators injecting nicotine into the sub-arachnoid space report that the spinal cord is the likely site of action (Aceto et al., 1986; Tripathi, Martin, & Aceto, 1982). Others hypothesize the brain to be the site of action (Iwamoto, 1989; Rogers & Iwamoto, 1993). It may be that different methods of assessing nicotine-induced antinociception may actually engage different neural mechanisms (Caggiula et al., 1995). The current research attempted to address this issue by administering a battery of tests, acute and chronic, spinal and supraspinal. Unfortunately, no clear site of action for nicotine-induced antinociception was identified. Systemic injection of nicotine has not been useful to identify site of action. Therefore, identifying nicotinic receptors involved in nicotine-induced antinociception in brain and spinal cord, and infusing nicotine directly into discrete areas to establish the precise location of these receptors is indicated. The strategy employed by Iwamoto (1989) could help elucidate this issue.

Nicotine is antinociceptive depending upon the sex of subject and the type of noxious stimulus used. Nicotine may be analgesic under some conditions, but not others. Therefore, there may be other drugs, similar to nicotine that might be more effective at providing analgesia under a broader range of conditions and may warrant consideration as analgesics. Recent studies examining epibatidine,

a naturally occurring substance from the poison, dart frog has proven interesting to study nicotinic receptor activation in antinociception (Muller, 1996). Additionally, synthetic nicotinic agonists are being developed and tested for potential use as analgesics (Bannon et al., 1998). Some of these compounds are reported to be 200 times more potent than morphine (Donnelly-Roberts et al., 1998) and could have value to help elucidate the mechanism underlying nicotine-induced analgesia.

The present study found that nicotine was antinociceptive for males and for females, but that males and females responded differently depending on the noxious stimulus and the time course of the drug. Males and females do not respond identically to analgesics and it is likely that other genetic differences would cause animals (and people) to respond differently. This difference in response to drugs suggests that the "one size fits all" approach to drug development and prescription is inadequate. Drugs must be tested using males and females as well as in different animal strains or species to clearly delineate the conditions under which analgesia is achieved. It is not reasonable to develop drugs that are effective for one-third or one-half of the population and to market it as a general purpose analgesic. If a particular analgesic drug is much more effective in one population, then this information must be made known so that prescribers and patients understand the implications. Individual differences need to be investigated before drugs are allowed to proceed to clinical trials and during these trials there must be a mechanism to evaluate the impact of these differences.

Clinical Applications

The current research confirmed that nicotine has analgesic properties. There may be other drugs that are similar to nicotine that also may possess analgesic properties. Nicotine or nicotine analogs could be used as part of an analgesic regimen for both acute and chronically painful ailments. Nicotine agonists might be suitable to administer to patients in and out of the hospital to alleviate pain. Nicotine might not be best prescribed as a primary analgesic. It may find its role as an analgesic adjuvant. Like caffeine or codeine, nicotine might be packaged with acetaminophen or aspirin to augment analgesia. Nicotine might be used in conjunction with a potent opioid to increase analgesia, perhaps only in individuals of one sex.

People smoke cigarettes for a variety of reasons. Perhaps some people self-administer nicotine to obtain analgesia. Nicotine's analgesic properties may help to attenuate acute or chronic psychologic or somatic pain. There are a number of chronic diseases with concomitant painful syndromes (e.g., fibromyalgia, rheumatoid arthritis, degenerative joint disease) that may induce people to smoke to alleviate pain. One way to help these smokers to quit smoking may be to provide an alternate analgesic or to administer a nicotine patch along with Tylenol® or some other analgesic medication. Some smokers may be individuals in pain and have learned to attenuate this pain by self-administering tobacco. Therefore, nicotine's ability to produce analgesia may partially account for its self-administration.

Approximately 25% of adults in the United States smoke cigarettes (USDHHS, 1988). Therefore, it is logical to assume that a similar percentage of

patients hospitalized for surgical procedures also smoke. Most hospitalized patients are not able to smoke cigarettes. This restriction may be because of respiratory concerns or because the patient is bedridden and unable to get to an area where smoking is permitted. Nicotine as a therapeutic drug may be especially beneficial to these patients. Small dosages of nicotine might be analgesic and may prevent nicotine withdrawal. Withdrawal from nicotine is associated with unpleasant cognitive effects, patients undergoing nicotine withdrawal and pain may reap the greatest benefits from nicotine analgesia. These patients may get a two-fold benefit from nicotine administration.

The fact that nicotine possesses analgesic properties may have important implications for anesthesia practice and nursing. Anesthesia as a discipline has several components to include amnesia, arreflexia, atonia, and analgesia. In anesthesia, analgesic dosages are primarily calculated based on age, weight, and coexisting disease. Patients using nicotine patches to quit smoking may have more modest anesthesia requirements. If these individuals were medicated with the routine dosages of opioid analgesic, then the opioid and nicotine act synergistically. In addition, this same mechanism may lead to delayed emergence from anesthesia. Further, patients who are acutely withdrawn from nicotine, as is done as a routine preoperative precaution, may become hyperalgesic as a result of the withdrawal state caused by nicotine abstinence. Tobacco smokers who have abstained from smoking may require more anesthesia and analgesia than a patient undergoing a similar procedure who is a non-smoker.

Relevance to Military Medicine

These clinical implications and implications of nicotine-induced analgesia are particularly relevant to military medicine because one third of all military members use tobacco products. Many of these soldiers, sailors, and marines are addicted to nicotine. In an attempt to reduce morbidity and mortality associated with tobacco use, the Department of Defense (DOD) has been aggressively promoting tobacco deglamorization and smoking cessation programs in an attempt to reduce tobacco use in the military. In addition, DOD is raising the price of tobacco products sold in military exchanges and commissaries because of the recognition of the high prevalence of smoking in the military.

These new policies also may have an unexpected impact in wartime. In the past, wounded soldiers on the battlefield had ready access to cigarettes and other tobacco products. Not only was smoking allowed on the battlefield, it was encouraged. Cigarettes were once dispensed in a package along with food rations. Although cigarettes are no longer packaged with food rations, smoking has been an accepted practice in the military until quite recently.

The battlefield of the future may be quite different. Military members may encounter a "tobaccoless" battlefield in the future. In light of the effects of nicotine as an analgesic, present and future troops may experience greater chronic and acute pain than did previous troops who self-administered nicotine. Acute battle injuries may require greater amounts of other analgesic agents and soldiers needing surgical procedures may require greater amounts of anesthesia and analgesia requirements than in previous conflicts. Military medics, nurses,

nurse anesthetists, and anesthesiologists may be treating troops who, as a result of being nicotine-free, need and respond differently to analgesic agents.

Limitations of Present Research

The current research was designed to determine whether nicotine was antinociceptive in male and female rats and to examine two possible mechanisms underlying gender differences in nicotine-induced antinociception: sex hormones or pharmacokinetic differences. The study was designed to manipulate drug levels and measure behavioral outcomes of nociception. At the conclusion of Experiment II, animals were sacrificed 12 minutes after an SC nicotine injection (this time point coincides with time-point 2 of the acute antinociceptive measures). Blood and tissue were harvested for biochemical and pharmacokinetic analysis. Behaviors were not measured at the time of sacrifice. Although the behaviors were correlated with the pharmacokinetic and biochemical data, these specimens were collected at a different time. Therefore, the hormone levels, and the plasma and tissue, nicotine levels may have been disparate from the same levels at the time of behavioral testing.

At the time of antinociceptive testing, estrus cycle staging was performed. This was another attempt to relate measures of antinociception to estrus stage in order to infer a relationship between levels of sex and antinociception. Data analysis did not reveal a significant correlation between estrus cycle stage and levels of 17-β-estradiol. The lack of a significant correlation limits the ability to make a definitive statement regarding the relationship between the estrus stage and antinociception.

In the present research, it was hypothesized that there was a relationship

between sex hormone levels and antinociception induced by nicotine. In males, testosterone is the primary sex hormone. In females, 17-β-estradiol is the primary sex hormone. It must be noted, however, that male rats have endogenous estrogens present and that females have circulating estrogens, androgens, and also have significant levels of progesterone. Estrogen levels were not measured in male rats and neither androgen levels nor progesterone levels were measured in female rats. It is possible that these sex hormones may modulate antinociception.

The human experience of pain is complex. Pain is often difficult to assess and quantify. To help study and understand pain, animal models have been used. Animal models use a number of strategies to produce a physiologic state that parallel the human experience of pain. Even the terminology suggests that there are intrinsic differences in the experiencing of noxious stimuli. In animals, we discuss nociception instead of pain. Antinociception is the term coined in the animal literature instead of discussing analgesia. While these experiments used several different measures to assess pain, one of which, the Formalin Test, is a clinical model of chronic pain, the generalizability of these findings to the human pain experience may be limited.

Future Directions

Nicotine may be useful as an analgesic agent. It appears that it is analgesic for one sex, but not the other depending on the dosage, measure of nociception used, and the time course. The current experiment examined plasma and tissue levels at a single time point. Future research should establish the time course for nicotine-induced antinociception and relate it to blood and

tissue levels in the CNS at those time points. This issue of analgesic time course should be explored with human subjects. Men and women should have nicotine administered to them via patches, gum, or nasal spray and then a series of noxious stimuli should be presented. This information would be helpful in the characterization of nicotine-induced analgesia.

Analgesia may be one reason that people smoke and may also play a role in smoking recidivism. People with chronic pain may "need" to self-administer nicotine to attenuate pain for chronic injury or disease. Human studies involving the administration of alternative analgesics to individuals that are trying to quit smoking should be conducted. Perhaps giving people aspirin or acetaminophen could increase the incidence of successful abstinence from using tobacco products.

Because analgesia plays such an important role in anesthesia and recovery from surgery, future studies should explore nicotine's analgesic properties in smokers and nonsmokers having surgery. How nicotine administration affects analgesic requirements is not presently known. Other stimulants, such as amphetamine, are known to increase anesthetic requirements (Rogers, Tinker, Covino, & Longnecker, 1993). It is possible that nicotine's sympathomimetic activity may increase analgesic/anesthetic requirements or its analgesic properties might decrease analgesic/anesthetic requirements. Administering a nicotine patch as a preoperative medication to smoker's undergoing smoking withdrawal as well as to non-smokers would be an interesting study.

Nicotine may have a role as an analgesic adjuvant. Because nicotine is a

drug with addictive liability it may not be useful to have a bottle of nicotine pills in the medicine cabinet next to the Tylenol®. The dosages necessary to provide analgesia may be enough to become addictive or the dosages may be effective as analgesics without inducing addiction. In addition, some analgesics are best when used in combination with other analgesics (e.g., acetaminophen and codeine). Animal and human studies combining small dosages of nicotine with other analgesics should be conducted to determine analgesic efficacy as well as addictive liability. It may be that nicotine and aspirin are more effective in providing analgesia than either substance alone and may also help people to decrease or quit nicotine self-administration.

There are many analgesic drugs on the pharmaceutical market. Some of these drugs are extremely potent and can only be used safely in a hospital (e.g., fentanyl). Other drugs are sold over-the-counter to ameliorate minor discomfort from illness or injury (e.g., ibuprofen). Although these two drugs have been used safely and effectively for many years, we still do not know how individual, genetic differences may account for differences in analgesic efficacy. A systematic series of controlled experiments examining genetic differences in analgesic efficacy needs to be conducted. One of the most obvious genetic differences is, of course, gender. It would be useful to study gender differences in analgesia (and anesthesia) over a broad range of drugs in both animals and humans. Then, the next step would be to expand this series of experiments to include other major, genetic differences such as animal strain or in the case of humans, ethnic background. This information not only would help clinicians provide better analgesia to patients using gender and ethnicity to assist them in prescribing

analgesics, but this information might be used to determine mechanisms underlying gender and other genetic differences. Further, it may be possible to map the genes involved in analgesia that could help to develop better analgesics or to manipulate those genes to increase analgesic responses to both endogenous and exogenous analgesics.

Conclusion

In summary, the present research examined nicotine-induced antinociception in male and female rats. Nicotine is antinociceptive for acute and persistent noxious stimuli and this effect was not a result of a decrement in motor functioning. Males and females respond differently to nicotine-induced antinociception on some measures and not on others. The mechanisms underlying sex differences remains unknown and future research should be aimed at elucidating these mechanisms.

Tables

Table 1 Means of Tail-Flick Latencies, Females and Males

Tail-Flick Latencies (seconds) 8 minutes Post Nicotine or Saline Injection

			N	Mean	
Sex	Nicotine Dosage		Statistic	Statistic	Std. Error
Female	Saline	Tail-Flick testing at 8 minutes	11	13.25	2.06
	.001 mg/kg	Tail-Flick testing at 8 minutes	12	8.78	1.75
	.01 mg/kg	Tail-Flick testing at 8 minutes	11	8.13	2.37
	.1 mg/kg	Tail-Flick testing at 8 minutes	11	16.78	1.57
	1 mg/kg	Tail-Flick testing at 8 minutes	11	23.94	1.66
Male	Saline	Tail-Flick testing at 8 minutes	12	6.93	1.45
	.001 mg/kg	Tail-Flick testing at 8 minutes	11	13.19	2.24
	.01 mg/kg	Tail-Flick testing at 8 minutes	12	13.59	2.52
	.1 mg/kg	Tail-Flick testing at 8 minutes	12	20.29	1.51
	1 mg/kg	Tail-Flick testing at 8 minutes	12	24.15	1.72

Tail-Flick Latencies (seconds)12 minutes Post Nicotine or Saline Injection

			N	Mean	
Sex	Nicotine Dosage		Statistic	Statistic	Std. Error
Female	Saline	Tail-Flick Testing at 12 minutes	12	10.15	1.96
	.001 mg/kg	Tail-Flick Testing at 12 minutes	12	10.38	1.13
	.01 mg/kg	Tail-Flick Testing at 12 minutes	11	6.59	1.08
	.1 mg/kg	Tail-Flick Testing at 12 minutes	11	16.65	1.96
	1 mg/kg	Tail-Flick Testing at 12 minutes	11	21.35	2.17
Male	Saline	Tail-Flick Testing at 12 minutes	12	9.67	2.04
	.001 mg/kg	Tail-Flick Testing at 12 minutes	11	8.69	1.69
	.01 mg/kg	Tail-Flick Testing at 12 minutes	12	15.22	2.17
	.1 mg/kg	Tail-Flick Testing at 12 minutes	12	17.33	1.84
	1 mg/kg	Tail-Flick Testing at 12 minutes	12	18.36	1.86

Table 2 Means of Hot-Plate Latencies, Females and Males

Hot-Plate Latencies (seconds) 8 minutes Post Nicotine or Saline Injection

			N	Mean	
Sex	Nicotine Dosage		Statistic	Statistic	Std. Error
Female	Saline	Hot-Plate testing at 8 minutes	12	24.06	1.22
	.001 mg/kg	Hot-Plate testing at 8 minutes	11	22.75	2.51
	.01 mg/kg	Hot-Plate testing at 8 minutes	11	26.94	2.29
	.1 mg/kg	Hot-Plate testing at 8 minutes	12	26.18	2.28
	1 mg/kg	Hot-Plate testing at 8 minutes	12	36.74	4.82
Male	Saline	Hot-Plate testing at 8 minutes	12	20.48	2.34
	.001 mg/kg	Hot-Plate testing at 8 minutes	12	25.70	4.60
	.01 mg/kg	Hot-Plate testing at 8 minutes	12	24.79	2.91
	.1 mg/kg	Hot-Plate testing at 8 minutes	12	25.09	3.18
	1 mg/kg	Hot-Plate testing at 8 minutes	13	45.28	3.73

Hot-Plate Latencies (seconds) 12 minutes Post Nicotine or Saline Injection

			N	Mean	
Sex	Nicotine Dosage		Statistic	Statistic	Std. Error
Female	Saline	Hot-plate testing at 12 minutes	12	24.78	1.80
	.001 mg/kg	Hot-plate testing at 12 minutes	11	22.47	1.36
	.01 mg/kg	Hot-plate testing at 12 minutes	11	26.92	1.89
	.1 mg/kg	Hot-plate testing at 12 minutes	12	29.71	2.47
	1 mg/kg	Hot-plate testing at 12 minutes	12	36.23	4.13
Male	Saline	Hot-plate testing at 12 minutes	12	22.87	2.49
	.001 mg/kg	Hot-plate testing at 12 minutes	12	24.51	2.88
	.01 mg/kg	Hot-plate testing at 12 minutes	12	30.36	2.90
	.1 mg/kg	Hot-plate testing at 12 minutes	11	28.55	3.38
	1 mg/kg	Hot-plate testing at 12 minutes	13	46.66	4.35

Table 3 Means of Cold-Flick Latencies, Females and Males

Cold-Flick Latencies (seconds) 8 minutes Post Nicotine or Saline Injection

			N	Mean	
Sex	Nicotine Dosage		Statistic	Statistic	Std. Error
Female	Saline	Cold-Flick Testing at 8 minutes	12	12.43	3.03
	.001 mg/kg	Cold-Flick Testing at 8 minutes	11	16.22	3.66
	.01 mg/kg	Cold-Flick Testing at 8 minutes	10	16.88	4.75
	.1 mg/kg	Cold-Flick Testing at 8 minutes	12	21.72	4.37
	1 mg/kg	Cold-Flick Testing at 8 minutes	12	40.13	6.31
Male	Saline	Cold-Flick Testing at 8 minutes	12	12.60	1.83
	.001 mg/kg	Cold-Flick Testing at 8 minutes	12	14.92	3.74
	.01 mg/kg	Cold-Flick Testing at 8 minutes	12	13.28	2.05
	.1 mg/kg	Cold-Flick Testing at 8 minutes	12	23.50	4.02
	1 mg/kg	Cold-Flick Testing at 8 minutes	13	50.59	3.59

Cold-Flick Latencies (seconds) 12 minutes Post Nicotine or Saline Injection

			N	Mean	
Sex	Nicotine Dosage		Statistic	Statistic	Std. Error
Female	Saline	Cold-Flick Testing at 12 minutes	12	14.25	2.18
	.001 mg/kg	Cold-Flick Testing at 12 minutes	11	21.34	4.71
	.01 mg/kg	Cold-Flick Testing at 12 minutes	10	21.78	5.31
	.1 mg/kg	Cold-Flick Testing at 12 minutes	12	29.18	5.12
	1 mg/kg	Cold-Flick Testing at 12 minutes	12	46.19	4.27
Male	Saline	Cold-Flick Testing at 12 minutes	12	15.18	4.23
	.001 mg/kg	Cold-Flick Testing at 12 minutes	12	13.83	2.77
	.01 mg/kg	Cold-Flick Testing at 12 minutes	12	17.21	2.67
	.1 mg/kg	Cold-Flick Testing at 12 minutes	12	29.03	5.46
	1 mg/kg	Cold-Flick Testing at 12 minutes	13	42.32	3.63

Table 4 Means of Total Locomotor Distance

Total Distance (cm) Over 30 Minute Period

			N	Mean	
Sex	Nicotine Dosage		Statistic	Statistic	Std. Error
Female	Saline	Total Dist. (cm) Testing	12	4346.8	517.1
	.001 mg/kg	Total Dist. (cm) Testing	12	3801.9	261.5
	.01 mg/kg	Total Dist. (cm) Testing	12	3244.2	419.8
	.1 mg/kg	Total Dist. (cm) Testing	12	4554.1	469.5
	1 mg/kg	Total Dist. (cm) Testing	12	1764.0	351.5
	1 mg/kg Tolerance	Total Dist. (cm) Testing	12	1092.6	251.8
Male	Saline	Total Dist. (cm) Testing	12	3547.8	558.8
	.001 mg/kg	Total Dist. (cm) Testing	12	3550.4	328.7
	.01 mg/kg	Total Dist. (cm) Testing	12	2605.3	324.5
	.1 mg/kg	Total Dist. (cm) Testing	12	969.4	138.7
	1 mg/kg	Total Dist. (cm) Testing	13	2119.7	322.3
	1 mg/kg Tolerance	Total Dist. (cm) Testing	12	1198.2	279.3

Table 5 Means of Total Formalin Scores, Females and Males

Formalin Test-Total Score Over 30 Minute Period
(0= normal weight bearing; 1=resting the paw lightly or limping; 2= elevating the affected paw; and 3= licking, biting, or grooming the affected paw)

			N	Mean	
Sex	Nicotine Dosage		Statistic	Statistic	Std. Error
Female	Saline	Formalin total score	12	1.64	0.11
	.001 mg/kg	Formalin total score	12	1.72	0.09
	.01 mg/kg	Formalin total score	12	1.49	0.14
	.1 mg/kg	Formalin total score	12	1.36	0.13
	1 mg/kg	Formalin total score	12	1.25	0.12
	1 mg/kg Tolerance Group	Formalin total score	12	1.01	0.12
Male	Saline	Formalin total score	11	1.34	0.10
	.001 mg/kg	Formalin total score	12	1.18	0.12
	.01 mg/kg	Formalin total score	12	1.24	0.14
	.1 mg/kg	Formalin total score	12	1.26	0.16
	1 mg/kg	Formalin total score	13	0.84	0.12
	1 mg/kg Tolerance Group	Formalin total score	12	0.88	0.10

Table 6 Means of Formalin Scores at 5 Minutes, Females and Males

Formalin Test - 5 Minutes
(0= normal weight bearing; 1=resting the paw lightly or limping; 2= elevating the affected paw; and 3= licking, biting, or grooming the affected paw)

			N	Mean	
Sex	Nicotine Dosage		Statistic	Statistic	Std. Error
Female	Saline	Formalin total score	12	0.30	0.09
	.001 mg/kg	Formalin total score	12	0.60	0.16
	.01 mg/kg	Formalin total score	12	0.71	0.34
	.1 mg/kg	Formalin total score	12	0.31	0.11
	1 mg/kg	Formalin total score	12	0.41	0.08
	1 mg/kg Tolerance Group	Formalin total score	12	0.29	0.08
Male	Saline	Formalin total score	11	0.10	0.07
	.001 mg/kg	Formalin total score	12	0.29	0.10
	.01 mg/kg	Formalin total score	12	0.26	0.10
	.1 mg/kg	Formalin total score	12	0.14	0.07
	1 mg/kg	Formalin total score	13	0.21	7.531-02
	1 mg/kg Tolerance Group	Formalin total score	12	0.20	0.06

Table 7 Means of Formalin Scores at 10 Minutes, Females and Males

Formalin Test -10 Minutes
(0= normal weight bearing; 1=resting the paw lightly or limping; 2= elevating the affected paw; and 3= licking, biting, or grooming the affected paw)

			N	Mean	
Sex	Nicotine Dosage		Statistic	Statistic	Std. Error
Female	Saline	Formalin 10 min	12	1.15	0.23
	.001 mg/kg	Formalin 10 min	12	1.40	0.25
	.01 mg/kg	Formalin 10 min	12	1.01	0.23
	.1 mg/kg	Formalin 10 min	12	0.72	0.20
	1 mg/kg	Formalin 10 min	12	1.07	0.15
	1 mg/kg Tolerance Group	Formalin 10 min	12	0.78	0.13
Male	Saline	Formalin 10 min	11	0.75	0.17
	.001 mg/kg	Formalin 10 min	12	0.65	0.18
	.01 mg/kg	Formalin 10 min	12	0.84	0.20
	.1 mg/kg	Formalin 10 min	12	0.83	0.19
	1 mg/kg	Formalin 10 min	13	0.45	0.10
	1 mg/kg Tolerance Group	Formalin 10 min	12	0.77	0.07

Table 8 Means of Formalin Scores at 15 Minutes, Females and Males

Formalin Test -15 Minutes
(0= normal weight bearing; 1=resting the paw lightly or limping; 2= elevating the affected paw; and 3= licking, biting, or grooming the affected paw)

			N	Mean	
Sex	Nicotine Dosage		Statistic	Statistic	Std. Error
Female	Saline	Formalin 15 min	12	1.80	0.19
	.001 mg/kg	Formalin 15 min	12	1.90	0.16
	.01 mg/kg	Formalin 15 min	12	1.56	0.23
	.1 mg/kg	Formalin 15 min	12	1.27	0.23
	1 mg/kg	Formalin 15 min	12	1.20	0.17
	1 mg/kg Tolerance Group	Formalin 15 min	12	1.05	0.15
Male	Saline	Formalin 15 min	11	1.29	0.25
	.001 mg/kg	Formalin 15 min	12	1.18	0.17
	.01 mg/kg	Formalin 15 min	12	1.35	0.22
	.1 mg/kg	Formalin 15 min	12	1.38	0.22
	1 mg/kg	Formalin 15 min	13	0.74	0.16
	1 mg/kg Tolerance Group	Formalin 15 min	12	0.88	0.17

Table 9 Means of Formalin Scores at 20 Minutes, Females and Males

Formalin Test - 20 Minutes
(0= normal weight bearing; 1=resting the paw lightly or limping; 2= elevating the affected paw; and 3= licking, biting, or grooming the affected paw)

			N	Mean	
Sex	Nicotine Dosage		Statistic	Statistic	Std. Error
Female	Saline	Formalin 20 min	12	2.21	0.10
	.001 mg/kg	Formalin 20 min	12	2.19	0.07
	.01 mg/kg	Formalin 20 min	12	1.91	0.23
	.1 mg/kg	Formalin 20 min	12	1.82	0.18
	1 mg/kg	Formalin 20 min	12	1.41	0.17
	1 mg/kg Tolerance Group	Formalin 20 min	12	1.22	0.15
Male	Saline	Formalin 20 min	11	1.64	0.20
	.001 mg/kg	Formalin 20 min	12	1.52	0.20
	.01 mg/kg	Formalin 20 min	12	1.70	0.18
	.1 mg/kg	Formalin 20 min	12	1.82	0.20
	1 mg/kg	Formalin 20 min	13	1.09	0.19
	1 mg/kg Tolerance Group	Formalin 20 min	12	0.99	0.16

Table 10 Means of Formalin Scores at 25 Minutes, Females and Males

Formalin Test - 25 Minutes
(0= normal weight bearing; 1=resting the paw lightly or limping; 2= elevating the affected paw; and 3= licking, biting, or grooming the affected paw)

			N	Mean	
Sex	Nicotine Dosage		Statistic	Statistic	Std. Error
Female	Saline	Formalin 25 min	12	2.23	0.08
	.001 mg/kg	Formalin 25 min	12	2.20	0.03
	.01 mg/kg	Formalin 25 min	12	2.02	0.13
	.1 mg/kg	Formalin 25 min	12	2.04	0.14
	1 mg/kg	Formalin 25 min	12	1.64	0.18
	1 mg/kg Tolerance Group	Formalin 25 min	12	1.32	0.16
Male	Saline	Formalin 25 min	11	2.06	0.12
	.001 mg/kg	Formalin 25 min	12	1.77	0.21
	.01 mg/kg	Formalin 25 min	12	1.66	0.23
	.1 mg/kg	Formalin 25 min	12	1.76	0.22
	1 mg/kg	Formalin 25 min	13	1.24	0.19
	1 mg/kg Tolerance Group	Formalin 25 min	12	1.18	0.15

Table 11 Means of Formalin Scores at 30 Minutes, Females and Males

Formalin Test - 30 Minutes
(0= normal weight bearing; 1=resting the paw lightly or limping; 2= elevating the affected paw; and 3= licking, biting, or grooming the affected paw)

			N	Mean	
Sex	Nicotine Dosage		Statistic	Statistic	Std. Error
Female	Saline	Formalin 30 min	12	2.11	0.14
	.001 mg/kg	Formalin 30 min	12	2.08	0.04
	.01 mg/kg	Formalin 30 min	12	2.04	0.11
	.1 mg/kg	Formalin 30 min	12	1.89	0.17
	1 mg/kg	Formalin 30 min	12	1.79	0.20
	1 mg/kg Tolerance Group	Formalin 30 min	12	1.41	0.19
Male	Saline	Formalin 30 min	11	2.08	0.11
	.001 mg/kg	Formalin 30 min	12	1.65	0.14
	.01 mg/kg	Formalin 30 min	12	1.67	0.20
	.1 mg/kg	Formalin 30 min	12	1.61	0.20
	1 mg/kg	Formalin 30 min	13	1.29	0.19
	1 mg/kg Tolerance Group	Formalin 30 min	12	1.24	0.14

Table 12 Means of Plasma Nicotine and Cotinine Levels

Plasma Nicotine Levels (ng/ml)

			N	Mean	
Sex	Nicotine Dosage		Statistic	Statistic	Std. Error
Female	Saline	Plasma Nicotine ng/ml	12	1.07	
	.01 mg/kg	Plasma Nicotine ng/ml	12	2.68	0.25
	.1 mg/kg	Plasma Nicotine ng/ml	12	27.46	0.86
	1 mg/kg	Plasma Nicotine ng/ml	12	292.65	23.75
	1 mg/kg Tolerance	Plasma Nicotine ng/ml	12	312.83	7.55
Male	.01 mg/kg	Plasma Nicotine ng/ml	12	4.67	0.29
	.1 mg/kg	Plasma Nicotine ng/ml	12	36.72	1.80
	1 mg/kg	Plasma Nicotine ng/ml	13	305.69	11.18
	1 mg/kg Tolerance	Plasma Nicotine ng/ml	12	266.08	23.84

Plasma Cotinine Levels (ng/ml)

			N	Mean	
Sex	Nicotine Dosage		Statistic	Statistic	Std. Error
Female	.1 mg/kg	Plasma Cotinine ng/ml	7	12.49	0.64
	1 mg/kg	Plasma Cotinine ng/ml	12	72.78	7.16
	1 mg/kg Tolerance	Plasma Cotinine ng/ml	12	67.08	5.45
Male	1 mg/kg	Plasma Cotinine ng/ml	13	64.28	4.71
	1 mg/kg Tolerance	Plasma Cotinine ng/ml	11	56.52	2.78

Table 13 Means of Brain Nicotine

Brain Nicotine Levels (ng/ml) 12 Minutes Post Injection

			N	Mean	
Sex	Nicotine Dosage		Statistic	Statistic	Std. Error
Female	Saline	Brain Nicotine Levels ng/g tissue	12	0.00	0.00
	.001 mg/kg	Brain Nicotine Levels ng/g tissue	12	0.68	0.68
	.01 mg/kg	Brain Nicotine Levels ng/g tissue	12	29.13	3.34
	.1 mg/kg	Brain Nicotine Levels ng/g tissue	12	270.23	8.34
	1 mg/kg	Brain Nicotine Levels ng/g tissue	12	2919.30	233.83
	1 mg/kg Tolerance	Brain Nicotine Levels ng/g tissue	12	3060.47	82.92
Male	Saline	Brain Nicotine Levels ng/g tissue	12	1.06	1.06
	.001 mg/kg	Brain Nicotine Levels ng/g tissue	12	6.21	2.05
	.01 mg/kg	Brain Nicotine Levels ng/g tissue	12	38.06	2.56
	.1 mg/kg	Brain Nicotine Levels ng/g tissue	12	251.15	9.68
	1 mg/kg	Brain Nicotine Levels ng/g tissue	13	2707.15	103.72
	1 mg/kg Tolerance	Brain Nicotine Levels ng/g tissue	12	2488.30	237.90

Table 14 Means of Spinal Cord Nicotine Levels

Spinal Cord Nicotine Levels (ng/g tissue) 12 Minutes Post Injection

			N	Mean	
Sex	Nicotine Dosage		Statistic	Statistic	Std. Error
Female	Saline	Spinal Cord Nicotine Levels ng/g tissue	12	0.00	0.00
	.001 mg/kg	Spinal Cord Nicotine Levels ng/g tissue	12	0.58	0.58
	.01 mg/kg	Spinal Cord Nicotine Levels ng/g tissue	12	4.48	1.19
	.1 mg/kg	Spinal Cord Nicotine Levels ng/g tissue	12	40.35	1.12
	1 mg/kg	Spinal Cord Nicotine Levels ng/g tissue	12	396.83	35.22
	1 mg/kg Tolerance	Spinal Cord Nicotine Levels ng/g tissue	12	434.42	19.19
Male	Saline	Spinal Cord Nicotine Levels ng/g tissue	12	0.00	0.00
	.001 mg/kg	Spinal Cord Nicotine Levels ng/g tissue	12	4.35	1.34
	.01 mg/kg	Spinal Cord Nicotine Levels ng/g tissue	12	13.87	0.74
	.1 mg/kg	Spinal Cord Nicotine Levels ng/g tissue	12	45.49	1.61
	1 mg/kg	Spinal Cord Nicotine Levels ng/g tissue	13	416.63	20.69
	1 mg/kg Tolerance	Spinal Cord Nicotine Levels ng/g tissue	12	389.59	42.50

Table 15 Means of Levels of Testosterone and 17-β-Estradiol

Testosterone Levels (ng/ml) 12 Minutes Post Injection

			N	Mean	
Sex	Nicotine Dosage		Statistic	Statistic	Std. Error
Male	Saline	Testosterone ng/ml	12	1.25	0.23
	.001 mg/kg	Testosterone ng/ml	12	1.91	0.35
	.01 mg/kg	Testosterone ng/ml	12	2.24	0.37
	.1 mg/kg	Testosterone ng/ml	12	1.79	0.32
	1 mg/kg	Testosterone ng/ml	13	2.73	0.46
	1 mg/kg Tolerance Group	Testosterone ng/ml	12	1.06	0.17

17-β-Estradiol Levels (pg/ml) 12 Minutes Post Injection

			N	Mean	
Sex	Nicotine Dosage		Statistic	Statistic	Std. Error
Female	Saline	17-B-Estradiol pg/ml	11	151.05	23.72
	.001 mg/kg	17-B-Estradiol pg/ml	11	158.38	27.82
	.01 mg/kg	17-B-Estradiol pg/ml	11	119.75	13.43
	.1 mg/kg	17-B-Estradiol pg/ml	12	135.21	9.29
	1 mg/kg	17-B-Estradiol pg/ml	11	124.83	11.90
	1 mg/kg Tolerance Group	17-B-Estradiol pg/ml	12	141.07	9.79

References

Abbott, F. V. (1998). Personal Communication.

Abbott, F. V., & Franklin, K. B. (1986). Noncompetitive antagonism of morphine analgesia by diazepam in the formalin test. Pharmacol Biochem Behav, 24, 319-321.

Abbott, F. V., Franklin, K. B., & Westbrook, R. F. (1995). The formalin test: scoring properties of the first and second phases of the pain response in rats. Pain, 60(1), 91-102.

Abbott, F. V., Gray-Donald, K., Sewitch, M. J., Johnston, C. C., Edgar, L., & Jeans, M. E. (1992). The prevalence of pain in hospitalized patients and resolution over six months. Pain, 50(1), 15-28.

Aceto, M. D., Awaya, H., Martin, B. R., & May, E. L. (1983). Antinociceptive action of nicotine and its methiodide derivatives in mice and rats. Br J Pharmacol, 79(4), 869-76.

Aceto, M. D., Bagley, R. S., Dewey, W. L., Fu, T. C., & Martin, B. R. (1986). The spinal cord as a major site for the antinociceptive action of nicotine in the rat. Neuropharmacology, 25(9), 1031-6.

Adams, J. U., Geller, E. B., & Adler, M. W. (1994). Receptor selectivity of icv morphine in the rat cold water tail-flick test. Drug and Alcohol Dependence, 35, 197-202.

Apatov, N. M. (1998). The effects of nicotine on antinociception in male and female Sprague-Dawley and Long-Evans rats with and without stress. Unpublished Masters Thesis, Uniformed Services University of the Health Sciences, Bethesda.

Applegren, L. E., Hansson, E., & Schmiterlöw, C. G. (1962). The accumulation and metabolism of [^{14}C]-labeled nicotine in the brain of mice and cats. Acta Physiol Scand, 56, 249-257.

Balfour, D. J. K. (Ed.). (1984). Nicotine and the Tobacco Smoking Habit. (Vol. Section 114). New York: Pergamon Press.

Bannon, A. W., Decker, M. W., Curzon, P., Buckley, M. J., Kim, D. J., Radek, R. J., Lynch, J. K., Wasicak, J. T., Lin, N. H., Arnold, W. H., Holladay, M. W., Williams, M., & Arneric, S. P. (1998). ABT-594 [(R)-5-(2-azetidinylmethoxy)-2-chloropyridine]: a novel, orally effective antinociceptive agent acting via neuronal nicotinic acetylcholine receptors: II. In vivo characterization. J Pharmacol Exp Ther, 285(2), 787-94.

Bartok, R. E., & Craft, R. M. (1997). Sex differences in opioid antinociception. J Pharmacol Exp Ther, 282(2), 769-78.

Beatty, W. W., & Fessler, R. G. (1976). Ontogeny of sex differences in open-field behavior and sensitivity to electric shock in the rat. Physiol Behav, 16, 413-417.

Beatty, W. W., & Fessler, R. G. (1977). Gonadectomy and sensitivity to electric shock in the rat. Physiol Behav, 19, 1-6.

Bendelow, G. (1993). Pain perceptions, emotions, and gender. Sociology of Health and Illness, 15, 273-294.

Benowitz, N. L., & Jacob, P., 3rd. (1999). Personal Communication .

Benowitz, N. L., Kuyt, F., Jacob, P. I., Jones, R. T., & Osman, A. L. (1983). Cotinine disposition and effects. Clinical Pharmacology and Therapeutics, 309, 139-142.

Benwell, M. E., Balfour, D. J., & Khadra, L. F. (1994). Studies on the influence of nicotine infusions on mesolimbic dopamine and locomotor responses to nicotine. Clin Investig, 72(3), 233-9.

Berkley, K. J. (1980). Spatial relationships between the terminations of somatic sensory and motor pathways in the rostral brainstem of cats and monkeys I. Ascending somatic sensory inputs to lateral diencephalon. J Comp Neurol(193), 283-317.

Berkley, K. J. (1997). Sex differences in pain. Behavioral & Brain Sciences, 20(3), 371-380.

Bernard, J. F., & Besson, J. M. (1990). The spino(trigemino)pontoamygdaloid pathway: electrophysiological evidence for an involvement in pain processes. J Neurophysiol, 67, 67-186.

Bishop, G. H. (1959). The relationship between nerve fiber size and sensory modality: phylogenic implications of the afferent innervation of cortex. J Nerv Men Dis, 128, 89-114.

Bodnar, R. J., Romero, M. T., & Kramer, E. (1988). Organismic variables and pain inhibition: roles of gender and aging. Brain Res Bull, 21(6), 947-953.

Bonica, J. J. (1979). Pain terms: a list of definitions and notes on usage. Pain, 6, 247-252.

Bowen, D. J., Eury, S. E., & Grunberg, N. E. (1986). Nicotine's effects on female rats' body weight: caloric intake and physical activity. Pharmacol Biochem Behav, 25(6), 1131-1136.

Bozarth, M. A. (1994). Opiate reinforcement processes: re-assembling multiple mechanisms. Addiction, 89, 1425-1434.

Briggs, S. L., Rech, R. H., & Sawyer, D. C. (1998). Kappa antinociceptive activity of spiradoline in the cold-water tail-flick assay in rats. Pharmacol Biochem Behav, 60(2), 467-72.

Brooks, V. B. (1986). The Neural Basis of Motor Control. New York: Oxford University Press.

Buckley, D. N., MacIntosh, J., & Beattie, W. S. (1990). Epidural analgesia prevents loss of lung volume. Anesthesiology, 73, A764.

Burstein, R., Cliffer, K. D., & Geisler, G. J. J. (1990). Cells of origin of the spinohypothalamic tract. J Neurosci, 291, 329-344.

Caggiula, A. R., Epstein, L. H., Perkins, K. A., & Saylor, S. (1995). Different methods of assessing nicotine-induced antinociception may engage different neural mechanisms. Psychopharmacology (Berl), 122(3), 301-6.

Candido, A., Maldonado, A., Megias, J. L., & Catena, A. (1992). Successive negative contrast in one-way avoidance learning in rats. Q J Exp Psychol [B], 45(1), 15-32.

Cassidy, J. D., Carroll, L. J., & Cote, P. (1998). The Saskatchewan health and back pain survey. Spine, 23(17), 1860-1866.

Chapman, C. R., Casey, K. L., Dubner, R., Foley, K. M., Gracely, R. H., & Reading, A. E. (1985). Pain management: an overview. Pain, 22, 1-31.

Chuang, J. I., & Lin, M. T. (1994). Pharmacological effects of melatonin treatment on both locomotor activity and brain serotonin release in rats. J Pineal Res, 17(1), 11-16.

Cicero, T. J., Nock, B., & Meyer, E. R. (1996). Gender-related differences in the antinociceptive properties of morphine. J Pharmacol Exp Ther, 279(2), 767-773.

Cicero, T. J., Nock, B., & Meyer, E. R. (1997). Sex-related differences in morphine's antinociceptive activity: relationship to serum and brain morphine concentrations. J Pharmacol Exp Ther, 282(2), 939-944.

Clarke, P. B., & Kumar, R. (1983). The effects of nicotine on locomotor activity in non-tolerant and tolerant rats. Br J Pharmacol, 78(2), 329-37.

Collins, A. C., Romm, E., & Wehner, J. M. (1988). Nicotine tolerance: an analysis of the time course of its development and loss in the rat. Psychopharmacology (Berl), 96(1), 7-14.

Corrigall, W. A., Franklin, K. B., Coen, K. M., & Clarke, P. B. (1992). The mesolimbic dopaminergic system is implicated in the reinforcing effects of nicotine. Psychopharmacology (Berl, 107(2-3), 285-289.

Crook, J., Rideout, E., & Browne, G. (1984). The prevalence of pain complaints in a general population. Pain, 18(3), 299-314.

Crooks, P. A., Li, M., & Dwoskin, L. P. (1997). Metabolites of nicotine in rat brain after peripheral nicotine administration. Cotinine, nornicotine, and norcotinine. Drug Metab Dispos, 25(1), 47-54.

Damaj, M. I., Welch, S. P., & Martin, B. R. (1994). Nicotine-induced antinociception in mice: role of G-proteins and adenylate cyclase. Pharmacol Biochem Behav, 48(1), 37-42.

D'Amour, F. F., & Smith, D. L. (1941). A method for determining loss of pain sensation. J Pharmacol Exp Ther, 72, 74-79.

Davenport, K. E., Houdi, A. A., & Van Loon, G. R. (1990). Nicotine protects against mu-opioid receptor antagonism by beta-funaltrexamine: Evidence for nicotine-induced release of endogenous opioids in brain. Neurosci Lett, 113(1), 40-46.

Dawson-Basoa, M. B., & Gintzler, A. R. (1993). 17-Beta-estradiol and progesterone modulate an intrinsic opioid analgesic system. Brain Res, 601(1-2), 241-245.

Dawson-Basoa, M. B., & Gintzler, A. R. (1996). Estrogen and progesterone activate spinal kappa-opiate receptor analgesic mechanisms. Pain, 64, 169-177.

De Kock, M., Eisenach, J., Tong, C., Schmitz, A. L., & Scholtes, J. L. (1997). Analgesic doses of intrathecal but not intravenous clonidine increase acetylcholine in cerebrospinal fluid in humans. Anesth Analg, 84(4), 800-803.

Descartes, R. (1641). Meditation on First Philosophy. Paris.

Deutsch, J., Hegedus, L., Greig, N. H., Rapoport, S. I., & Soncrant, T. T. (1992). Electron-impact and chemical ionization detection of nicotine and cotinine by gas-chromatography-mass spectrometry in rat plasma and brain. J Chromatogr, 579, 93-98.

Dewey, W. L., Harris, L. S., Howes, J. F., & Nuite, J. A. (1970). The effect of various neurohumoral modulators on the activity of morphine and the narcotic antagonists in the tail-flick and phenylquinone tests. The Journal of Pharmacology and Experimental Therapeutics, 175(3), 435-442.

Dixon, W. E., & Lee, W. E. (1912). Tolerance to nicotine. Quarterly Journal of Experimental Physiology, 5, 373-383.

Domino, E. F. (1965). Some behavioral actions of nicotine. In U. S. von Euler (Ed.), Tobacco alkaloids and related compounds . Oxford: Pergamon.

Donnelly-Roberts, D. L., Puttfarcken, P. S., Kuntzweiler, T. A., Briggs, C. A., Anderson, D. J., Campbell, J. E., Piattoni-Kaplan, M., McKenna, D. G., Wasicak, J. T., Holladay, M. W., Williams, M., & Arneric, S. P. (1998). ABT-594 [(R)-5-(2-azetidinylmethoxy)-2-chloropyridine]: a novel, orally effective analgesic acting via neuronal nicotinic acetylcholine receptors: I. In vitro characterization. J Pharmacol Exp Ther, 285(2), 777-786.

Donovan, M., Dillon, P., & McGuire, L. (1987). Incidence and characteristics in a pain sample in a same of medical-surgical inpatients. Pain, 30, 69-78.

Dubuisson, D., & Dennis, S. G. (1977). The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. Pain, 4(2), 161-174.

Dwoskin, L. P., Teng, L., Buxton, S. T., & Crooks, P. A. (1999). (S)-(-)-Cotinine, the major brain metabolite of nicotine, stimulates nicotinic receptors to evoke [3H]dopamine release from rat striatal slices in a calcium-dependent manner. J Pharmacol Exp Ther, 288(3), p905-11.

Eggen, A. E. (1993). The Tromso study: frequency and predicting factors of analgesic drug use in a free-living population (12-56 years). J Clinical Epidemiology, 46, 1297-1304.

Eisenach, J. C., Detweiler, D. J., Tong, C., D'Angelo, R., & Hood, D. (1996). Cerebrospinal fluid norepinephrine and acetylcholine concentrations during acute pain. Regional Anesthesia and Pain Management, 82(3), 621-626.

Emery, F. E., & Schwabe, E. L. (1936). The vaginal smears of rats as influenced by frequent examinations. The Anatomical Record, 64(2), 147-154.

Erenmemisoglu, A., & Tekol, Y. (1994). Do nicotine metabolites have an effect on pain perception? Antinociceptive effect of cotinine in mice. Pharmazie, 49(5), 374-375.

Fagerström, K. O., & Schneider, N. G. (1989). Measuring nicotine dependence: a review of the Fagerstrom Tolerance Questionnaire. J Behav Med, 12(2), p159-82.

Falkeborn, Y., Larsson, C., & Nordberg, A. (1981). Chronic Nicotine Exposure in Rat: A behavioral and biochemical study of tolerance. Drug and Alcohol Dependence, 8, 51-60.

Feine, J. S., Bushnell, M. C., Miron, D., & Duncan, G. H. (1991). Sex differences in the perception of noxious heat stimuli. Pain, 44, 255-262.

Feldman, R. S., Meyer, J. S., & Quenzer, L. F. (1997). Principles of Neuropsychopharmacology. Sunderland, Mass.: Sinauer Associates, Inc.

Fertig, J. B., Pomerleau, O. F., & Sanders, B. (1986). Nicotine-produced antinociception in minimally deprived smokers and ex-smokers. Addict Behav, 11(3), 239-248.

Fillingim, R. B., & Maixner, W. (1995). Gender differences in the responses to noxious stimuli. Pain Forum, 4(4), 209-221.

Fillingim, R. B., Maixner, W., Girdler, S. S., Light, K. C., Harris, M. B., Sheps, D. S., & Mason, G. A. (1997). Ischemic but not thermal pain sensitivity varies across menstrual cycle. Psychosomatic Medicine, 59, 512-520.

Forman, L. J., Tingle, S., Estilow, S., & Cater, J. (1989). The response to analgesia testing is affected by gonadal steroids in the rat. Life Sciences, 45, 447-454.

Franklin, K. B. J., & Abbott, F. V. (1989). Techniques for assessing the effects of drugs on nociceptive responses. (Vol. 13): Humana Press.

Freeman, M. E. (1994). The neuroendocrine control of the ovarian cycle of the rat. In E. Knobil, J. D. Neill, G. S. Greenwald, C. L. Markert, & D. W. Pfaff (Eds.), The Physiology of Reproduction (pp. 613-658). New York: Raven Press.

Fromm, G. H. (1994). Baclofen as an adjuvant analgesic. J Pain Symptom Management, 9(8), p500-9.

Frye, C. A., Cuevas, A., & Kanarek, R. B. (1993). Diet and estrous cycle influence pain sensitivity in rats. Pharmacol Biochem Behav, 45, 255-260.

Frye, C. A., & Duncan, J. E. (1996). Estradiol benzoate potentiates steroids' effects on pain sensitivity. Pharmacology Biochemistry and Behavior, 53(1), 27-32.

Gear, R. W., Gordon, N. C., Heller, P. H., Paul, S., Miaskowski, C., & Levine, J. D. (1996). Gender difference in analgesic response to the kappa-opioid pentazocine. Neurosci Lett, 205(3), 207-209.

Gilman, A. G., Rall, T. W., Nies, A. S., & Taylor, P. (Eds.). (1993). Goodman and Gilman's: The Pharmacologic Basis of Therapeutics. New York: McGraw-Hill, Inc.

Griffin, M. L., Weiss, R. D., Mirin, S. M., & Lange, U. (1989). A comparison of male and female cocaine abusers. Arch Gen Psychiatry, 46(2), 122-126.

Grossman, S. A. (1993). Undertreatment of cancer pain: barriers and remedies. Support Care Cancer, 1(2), 74-78.

Grunberg, N. E., & Bowen, D. J. (1985). The role of physical activity in nicotine's effects on body weight. Pharmacol Biochem Behav, 23(5), 851-854.

Hafez, E. S. E. (Ed.). (1970). Reproduction and Breediing Techniques for Laboratory Animals. Philadelphia: Lea & Febiger.

Hakan, R. L., & Ksir, C. (1991). Acute tolerance to the locomotor stimulant effects of nicotine in the rat. Psychopharmacology (Berl, 104(3), 386-90.

Hartvig, P., Gillberg, P. G., Torsten, G. J., & Post, C. (1989). Cholinergic mechanisms in pain and analgesia. TiPS, December 1989 suppl., s49-s55.

Hatsukami, D., Pentel, P. R., Jensen, J., Nelson, D., Allen, S. S., Goldman, A., & Rafael, D. (1998). Cotinine: effects with and without nicotine. Psychopharmacology (Berl), 135(2), 141-150.

Hirshberg, R. M., Al-Chaer, E. D., Lawand, N. B., Westlund, K. N., & Willis, W. D. (1996). Is there a pathway in the posterior funiculus that signals visceral pain? Pain, 67, 291-305.

Holck, H. G. O., Kanan, M. A., Mills, R. M., & Smith, E. L. (1937). Studies upon the sex-dependence in rats to certain barbiturates and to nicotine. J Pharmacol Exp Ther, 60, 323-346.

Hughes, J. R. (1991). Distinguishing withdrawal relief and direct effects of smoking. Psychopharmacology, 104, 409-410.

Hunskaar, S., Berge, O. G., & Hole, K. (1986). A modified hot-plate test sensitive to mild analgesics. Behavioral Brain Research, 21, 101-108.

Hunskaar, S., Fasmer, O. B., & Hole, K. (1985). Formalin test in mice, a useful technique for evaluating mild analgesics. J Neurosci Methods, 14(1), 69-76.

Iwamoto, E. T. (1989). Antinociception after nicotine administration into the mesopontine tegmentum of rats: evidence for muscarinic actions. J Pharmacol Exp Ther, 251(2), 412-421.

Iwamoto, E. T. (1991). Characterization of the antinociception induced by nicotine in the pedunculopontine tegmental nucleus and the nucleus raphe magnus. J Pharmacol Exp Ther, 257(1), 120-133.

Jacob, P. I., Benowitz, N. L., & Shulgin, A. T. (1988). Recent studies of nicotine metabolism in humans. Pharmacol Biochem Behav, 30(1), 249-253.

Jacob, P. I., Wilson, M., & Benowitz, N. L. (1981). Improved gas chromographic method for the determination of nicotine and cotinine in biologic fluids. Journal of Chromatography, 222, 61-70.

Jamner, L. D., Girdler, S. S., Shapiro, D., & Jarvik, M. E. (1998). Pain inhibition, nicotine, and gender. Exp Clin Psychopharmacol, 6(1), 96-106.

Jerse, A. (1998). Personal Communication.

Jones, S. L. (1992). Anatomy of pain. In R. S. Sinatra, A. H. Hord, B. Ginsberg, & L. M. Preble (Eds.), Acute Pain: Mechanisms and Management (pp. 8-29). St Louis, MO: Mosby Year-Book.

Kato, R. (1974). Sex-related differences in drug metabolism. Drug Metab Rev, 3, 1-32.

Kavaliers, M., & Innes, D. G. L. (1987). Sex and day-night differences in opiate-induced responses of insular deer mice. *Peromyscus maniculatus triangularis*. Pharmacol Biochem Behav, 27, 477-482.

Keefe, F. (1986). Depression, pain, and pain behavior. J Consult Clin Psychol, 54, 665-669.

Kepler, K. L., Kest, B., Kiefel, J. M., Cooper, M. L., & Bodnar, R. J. (1989). Roles of gender, gonadectomy and estrous phase in the analgesic effects of intracerebroventricular morphine in rats. Pharmacol Biochem Behav, 34(1), 119-127.

Knott, V. J. (1990). Effects of cigarette smoking on brain evoked responses to electrical stimulation. Pharmacology Biochemistry and Behavior, 35, 341-346.

Koob, G. F. (1992). Drugs of abuse: Anatomy, pharmacology, and function of reward pathways. Trends in Pharmacologic Sciences, 13, 177-184.

Ksir, C., Hakan, R. L., Hall, D. P., & Kellar, K. J. (1985). Exposure to nicotine enhances the behavioral stimulant effect of nicotine and increases binding of [H^3]acetylcholine to nicotinic receptors. Neuropharmacology, 24, 527-531.

Kyerematen, G. A., Owens, G. F., Chattopadhyay, B., de Bethizy, J. D., & Vesell, E. S. (1988a). Sexual dimorphism of nicotine metabolism and distribution in the rat. Drug Metabolism and Disposition, 16(6), 823-828.

Kyerematen, G. A., Taylor, L. H., de Bethizy, J. D., & Vesell, E. S. (1988b). Pharmacokinetics of nicotine and 12 metabolites in the rat. Application of a new radiometric high performance liquid chromatography assay. Drug Metab Dispos, 16(1), 125-129.

Larkin, W. D., Reilly, J. P., & Kittler, L. B. (1986). Individual differences in sensitivity to transient electrocutaneous stimulation. IEEE Trans Biomed Eng, 33, 495-504.

Levine, F. M., & De Simone, L. L. (1991). The effects of experimenter gender on pain report in male and female subjects. Pain, 44(1), 69-72.

Lex, B. W. (1991). Some gender differences in alcohol and polysubstance users. Health Psychol, 10(2), 121-132.

Maisonneuve, I. M., Mann, G. L., Deibel, C. R., & Glick, S. D. (1997). Ibogaine and the dopaminergic response to nicotine. Psychopharmacology (Berl), 129(3), 249-56.

Maixner, W., & Humphrey, C. (1993). Gender differences in pain and cardiovascular responses to forearm ischemia. Clin J Pain, 9(1), 16-25.

Marks, R. M., & Sachar, E. J. (1973). Undertreatment of medical inpatients with narcotic analgesics. Ann Intern Med, 78, 173-181.

Martin, B. R., Tripathi, H. L., Aceto, M. D., & May, E. L. (1983). Relationship of the biodisposition of the stereoisomers of nicotine in the central nervous system to their pharmacological actions. J Pharmacol Exp Ther, 226(1), 157-163.

Martínez-Gómez, M., Cruz, Y., Salas, M., Hudson, R., & Pachecos, P. (1994). Assessing pain threshold in the rat: Changes with estrus and time of day. Physiology & Behavior, 55(4), 651-657.

Matilla, M. J., Ahtee, L., & Saarnivaara, L. (1968). The analgesic and sedative effects of nicotine in white, mice, rabbits, and golden hamsters. Ann Med Exp Fenn, 46, 78-84.

Mead, L. A., Hargreaves, E. L., Ossenkopp, K. P., & Kavaliers, M. (1995). A multivariate assessment of spontaneous locomotor activity in the Mongolian gerbil (Meriones unguiculatus): Influences of age and sex. Physiol Behav, 57(5), 893-899.

Mehler, W. R. (1962). The anatomy of the so called "pain tract" in man: An analysis of the course and distribution of the ascending fibers of the fasciculus anterolateralis. In J. D. French & R. W. Porter (Eds.), Basic Research in Paraplegia (pp. 26-55). Springfield: Charles C. Thomas.

Mehler, W. R., Feferman, M. E., & Nauta, W. J. H. (1960). Ascending axon degeneration following anterolateral cordotomy. An experimental study in the monkey. Brain, 83, 718-751.

Melzack, R., & Wall, P. D. (1965). Pain Mechanisms: a new theory. Science, 150, 971-979.

Meyer, R. A., Campbell, J. N., & Raja, S. N. (1993). Peripheral neural mechanisms of nociception. In P. D. Wall & R. Melzack (Eds.), Textbook of Pain . Edinburgh: Churchill Livingstone.

Mildenhall, W. L. (1921). Effect of tobacco smoking on sensory thresholds. J Pharmacol Exp Ther, 17, 333-334.

Morris, D. (1991). Hysteria, Pain, and Gender. Berkeley, CA: University of California Press.

Morrison, C. F., & Stephenson, J. A. (1972). The occurrence of tolerance to the central depressant effect of nicotine. Br J Pharmacol, 45, 151-156.

Morse, A. C., Erwin, V. G., & Jones, B. C. (1993). Strain and housing affect cocaine self-selection and open-field locomotor activity in mice. Pharmacol Biochem Behav, 45(4), 905-912.

Mousa, S. A., Aloyo, V. J., & Van Loon, G. R. (1988). Tolerance to tobacco smoke- and nicotine-induced analgesia in rats. Pharmacol Biochem Behav, 31(2), p265-268.

Muller, C. E. (1996). [Epibatidine--a nicotine-related, analgesic alkaloid from poison-dart frogs]. Pharm Unserer Zeit, 25(2), 85-92.

Nafe, J. P. (1934). . Worcester, Mass.: Clark University Press.

Naguib, M., & Yaksh, T. L. (1994). Antinociceptive effects of spinal cholinesterase inhibition and isobolographic analysis of the interaction with mu and alpha 2 receptor systems. Anesthesiology, 80(6), 1338-48.

Nauta, H. J. W., & Kuypers, H. G. J. M. (1958). Some ascending pathways in the brainstem reticular formation. In H. H. Jasper, L. D. Proctor, R. S. Knighton, W. C. Noshay, & R. C. Costello (Eds.), Reticular Formation of the Brain (pp. 3-30). Boston: Little, Brown.

Nesbitt, P. (1973). Smoking, physiological arousal, and emotional response. Journal of Personality and Social Psychology, 25, 137-144.

Nowak, R. (1994). Nicotine scrutinized as FDA seeks to regulate cigarettes. Science, 263, 1555-1556.

Nwosu, C. G., & Crooks, P. A. (1988). Species variation and stereoselectivity in the metabolism of nicotine enantiomers. Xenobiotica, 18(12), 1361-1372.

Owen, H., McMillan, V., & Rogowski, D. (1990). Postoperative pain therapy: a survey of patients' expectations and their experiences. Pain, 41, 303-307.

Patt, R. B., Proper, G., & Reddy, S. (1994). The neuroleptics as adjuvant analgesics. J Pain Symptom Management, 9(7), p446-53.

Pedigo, N. W., Dewey, W. L., & Harris, L. S. (1975). Determination and characterization of the antinociceptive activity of intraventricularly administered acetylcholine. J Pharmacol Exp Ther, 193, 845-852.

Perkins, K. A., Grobe, J. E., Fonte, C., Goettler, J., Caggiula, A. R., Reynolds, W. A., Stiller, R. L., Scierka, A., & Jacob, R. G. (1994). Chronic and acute tolerance to subjective, behavioral and cardiovascular effects of nicotine in humans. J Pharmacol Exp Ther, 270(2), 628-638.

Petersen, D. R., Norris, K. J., & Thompson, J. A. (1984). A comparative study of the dispostion of nicotine and its metabolites in three inbred strains of mice. Drug Metabolism and Disposition, 12(6), 725-731.

Phan, D. V., Doda, M., Bite, A., & Gyorgy, L. (1973). Antinociceptive activity of nicotine. Acta Physiol Acad Sci Hung, 44(1), 85-93.

Picciotto, M. (1999). Nicotine Addiction: from molecules to behavior. The Neuroscientist, 4(6), 391-393.

Pinsky, C., Koven, S. J., & LaBella, F. S. (1975). Evidence for role of endogenous sex steroids in morphine antinociception. Life Sciences(16), 1785-1786.

Pizziketti, R. J., Pressman, N. S., Geller, E. B., Cowan, A., & Adler, M. W. (1985). Rat cold water tail-flick: a novel analgesic test that distinguishes opioid agonists from mixed agonist-antagonists. Eur J Pharmacol, 119(1), 23-29.

Pomerleau, O. F. (1986). Nicotine as a psychoactive drug: anxiety and pain reduction. Psychopharmacol Bull, 22(3), 865-869.

Pomerleau, O. F., Turk, D. C., & Fertig, J. B. (1984). The effects of cigarette smoking on pain and anxiety. Addict Behav, 9(3), 265-271.

Prado, W. A., & Goncalves, A. S. (1997). Antinociceptive effect of intrathecal neostigmine evaluated in rats by two different pain models. Braz J Med Biol Res, 30(10), p1225-31.

Rao, S. S., & Saifi, A. Q. (1985). Influence of testosterone on morphine analgesia in albino rats. Ind J Physiol Pharmac, 29(2), 103-106.

Reavill, C., & Stolerman, I. P. (1990). Locomotor activity in rats after administration of nicotinic agonists intracerebrally. Br J Pharmacol, 99(2), 273-278.

Reisbord, L. S., & Greenland, S. (1985). Factors associated with self-reported back-pain prevalence: a population-based study. J Chronic Dis, 38(8), 691-702.

Rogers, D. T., & Iwamoto, E. T. (1993). Multiple spinal mediators in parenteral nicotine-induced antinociception. J Pharmacol Exp Ther, 267(1), 341-349.

Rogers, M. C., Tinker, J. H., Covino, B. G., & Longnecker, D. E. (Eds.). (1993). Principles and Practice of Anesthesiology. St. Louis: Mosby Year-Book, Inc.

Romero, M. T., & Bodnar, R. J. (1986). Gender differences in two forms of cold-water swim analgesia. Physiol Behav, 37(6), 893-897.

Rosecrans, J. A., Wiley, J. L., Bass, C. E., & Karan, L. D. (1995). Nicotine-induced acute tolerance: studies involving schedule-controlled behavior. Brain Res Bull, 37(4), 359-362.

Rosencrans, J. A., & Schechter, M. D. (1972). Brain area nicotine levels in male and female rats of two strains. *Archives Internationales de Pharmacodynamie et de Therapie*, 196, 46-54.

Rosland, J. H., Tjolsen, A., Maehle, B., & Hole, K. (1990). The formalin test in mice: Effect of formalin concentration. Pain, 42(2), 235-242.

Ryan, S., Goodale, H., & Maier, S. F. (1987). Stress-induced analgesia varies as a function of estrous cycle and sex steroid replacement therapy. Soc Neurosci Abstr, 11, 128.

Ryan, S. M., & Maier, S. F. (1988). The estrous cycle and estrogen modulate stress-induced analgesia. Behav Neurosci, 102(3), 371-380.

Saah, M. I., Raygada, M., & Grunberg, N. E. (1994). Effects of nicotine on body weight and plasma insulin in female and male rats. Life Sciences, 55(12), 925-931.

Sahley, T. L., & Berntson, G. G. (1979). Antinociceptive effects of central and systemic administrations of nicotine in the rat. Psychopharmacology (Berl), 65(3), 279-283.

Sastry, B. V., & Hemontolor, M. E. (1998). Influence of nicotine and cotinine on retinal phospholipase A2 and its significance to macular function. J Ocul Pharmacol Ther, 14(5), 447-458.

Scheibel, M. E., & Scheibel, A. B. (1958). Structural substrates for integrative patterns in the brain stem reticular core. In , H. H. Jasper, L. D. Proctor, R. S. Knighton, W. C. Noshay, & R. C. Costello (Eds.), Reticular Formation of the Brain (pp. 31-55). Boston: Little, Brown.

Schreur, P. J., & Nichols, N. F. (1986). Two automated locomotor activity tests for dopamine autoreceptor agonists. Pharmacol Biochem Behav, 25(1), p255-261.

Shiffman, S., & Jarvik, M. E. (1984). Cigarette smoking, physiological arousal, and emotional response: Nesbitt's paradox reexamined. Addictive Behaviors, 9, 95-98.

Silverstein, B. (1982). Cigarette smoking, nicotine addiction, and relaxation. Journal of Personality and Social Psychology, 42, 946-970.

Sinatra, R. S., Hord, A. H., Ginsberg, B., & Preble, L. M. (Eds.). (1992). Acute Pain: Mechanisms and Management. Saint Louis: Mosby Year Book.

Skultety, F. M. (1963). Stimulation of the periaqueductal grey and hypothalamus. Arch Neurol, 8, 608-620.

Stanley, K. L., & Paice, J. A. (1997). Animals in pain research. Seminars in Oncology Nursing, 13(1), 3-9.

Sternbach, R. A. (1976). The need for an animal model of chronic pain. Pain, 2, 2-4.

Stolerman, I. P. (1990). Behavioural pharmacology of nicotine: implications for multiple brain nicotinic receptors. Ciba Found Symp, 152, 3-16.

Stolerman, I. P., Fink, R., & Jarvik, M. E. (1973). Acute and chronic tolerance to nicotine measured by activity in rats. Psychopharmacologia, 30(4), 329-342.

Stolerman, I. P., Garcha, H. S., & Mirza, N. R. (1995). Dissociations between the locomotor stimulant and depressant effects of nicotinic agonists in rats. Psychopharmacology (Berl), 117(4), 430-437.

Sult, S. C., & Moss, R. A. (1986). The effects of cigarette smoking on the perception of electrical stimulation and cold pressor pain. Addictive Behaviors, 11, 447- 451.

Swanson, L. W., Simmons, D. M., Whiting, P. J., & Lindstrom, J. (1987). Immunohistochemical localization of neuronal nicotinic receptors in the rodent central nervous system. Journal Neuroscience, 7, 3334-3342.

Tedford, W. H., Warren, D. E. J., & Flynn, W. E. (1977). Alteration of shock aversion thresholds during menstrual cycle. Percept Psychophysiol, 21, 193-196.

Tiseo, P. J., Geller, E. B., & Adler, M. W. (1988). Antinociceptive action of intracerebroventricularly administered dynorphin and other opioid peptides in the rat. J Pharmacol Exp Ther, 246, 449-453.

Tjolsen, A., Berge, O. G., Hunskaar, S., Rosland, J. H., & Hole, K. (1992). The formalin test: an evaluation of the method. Pain, 51(1), 5-17.

Tripathi, H. L., Martin, B. R., & Aceto, M. D. (1982). Nicotine-induced antinociception in rats and mice: correlation with nicotine brain levels. J Pharmacol Exp Ther, 221(1), 91-96.

Unruh, A. M. (1996). Gender variations in clinical pain experience. Pain, 65(2-3), 123-167.

USDHHS. (1988). The health consequences of smoking: nicotine addiction. A report of the Surgeon General (Vol. DHHS Pub. No. (CDC) 88-8406, pp. 75-143, 375-456). Washington, D.C: U.S. Government Printing Office.

Veith, J. L., Anderson, J., & Slade, S. A. (1984). Plasma beta-endorphin, pain thresholds, and anxiety levels across the human menstrual cycle. Physiol Behavior, 32, 31-34.

Wada, E., Wada, K., & Boulter, J. (1989). Distribution of alpha 2, alpha 3, alpha 4, and beta 2 neuronal nicotinic subunit mRNAs in the central nervous system: A hybridization histochemical study in rat. J Comp Neurol, 284, 314-335.

Walker, A. E. (1940). The spinothalamic tract in man. Arch Neurol Psychiatry, 43, 284-298.

Walker, J. S., & Carmody, J. J. (1998). Experimental pain in healthy human subjects: gender differences in nociception and in response to ibuprofen. Anesth Analg, 86(6), 1257-1262.

Wall, P. D., & Melzack, R. (Eds.). (1993). Textbook of Pain. Edinburgh: Churchill Livingstone.

Wang, J., Ho, S., Hu, Y., & Chu, K. (1995). An innovative cold tail-flick test: The cold ethanol tail-flick test. Anesthesia and Analgesia, 80, 102-107.

Watanabe, S., & Bruera, E. (1994). Corticosteroids as adjuvant analgesics. J Pain Symptom Management, 9(7), p442-5.

Westman, E. C., Levin, E. D., & Rose, J. E. (1995). Nicotine as a therapeutic drug. NC Medical Journal, 56(1), 48-51.

Wheeler-Aceto, H., & Cowan, A. (1991). Standardization of the rat paw formalin test for the evaluation of analgesics. Psychopharmacology (Berl), 104(1), 35-44.

Willis, W. D. (1985). The Pain System. Basel: Karger.

Willis, W. D., Kenshalo, D. R., & Leonard, R. B. (1979). The cells of origin of the primate spinothalamic tract. J Comp Neurol, 188, 543-574.

Willis, W. D., & Westlund, K. N. (1997). Neuroanatomy of the pain system and of the pathways that modulate pain. J Clin Neurophysiol, 14(1), 2-31.

Woolfe, G., & MacDonald, A. D. (1944). The evaluation of the analgesic action of pethidine hydrochloride (demerol). J Pharmacol Exp Ther, 80, 300-307.

Yaksh, T. L., & Harty, G. J. (1981). Simple method for rapid removal of rat spinal cord. Brain Res Bull, 6(5), 449-450.

Yamada, K., & Nabeshima, T. (1995). Stress-induced behavioral responses and multiple opioid systems in the brain. Behav Brain Res, 67(2), 133-145.

Yang, C. Y., Wu, W. H., & Zbuzek, V. K. (1992). Antinociceptive effect of chronic nicotine and nociceptive effect of its withdrawal measured by hot-plate and tail-flick in rats. Psychopharmacology (Berl), 106(3), 417-420.

Young, W. C., Bloing, J. C., & Blandau, R. J. (1941). The vaginal smear picture, sexual receptivity, and time of ovulation in the albino rat. Anatomical Record, 80, 37-45.

Zarrindast, M. R., Pazouki, M., & Nassiri-Rad, S. (1997). Involvement of cholinergic and opioid receptor mechanisms in nicotine-induced antinociception. Pharmacol Toxicol, 81(5), 209-213.

Zoli, M., Le Novere, N., Hill, J. A. J., & Changeux, J. P. (1995). Developmental regulation of nicotinic receptor subunit mRNAs in the rat central and peripheral nervous systems. Journal of Neuroscience, 15, 1912-1939.

Zubrycki, E. M., Giordano, M., & Sanberg, P. R. (1990). The effects of cocaine on multivariate locomotor behavior and defecation. Behav Brain Res, 36(1-2), 155-159.