2015

Effects of Nicotinic Acetylcholine Receptor Agonists in Assays of Pain-Stimulated and Pain-Depressed Behavior in Rats

Kelen Freitas

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Effects of Nicotinic Acetylcholine Receptor Agonists
in Assays of Pain-Stimulated and Pain-Depressed Behavior in Rats

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

by

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Richmond, Virginia
September, 2015
Acknowledgements

“The will of God will never take us where the Grace of God cannot sustain us.” (Billy Graham). The work contained within this dissertation would not have been possible without the endless support of multiple individuals. I would like to offer my most heartfelt appreciation and gratitude to my mother, who supported me throughout my graduate school career. She is an example of pure, unconditional love. She has never asked for anything but that I follow my heart. Thank you so much mom. I would like also to thank my father and brother for their love, enthusiasm, and encouragement throughout my studies. I also extend a special thanks to my stepfather for being my sponsor and principal enabler for my time in the U.S.A. I would like to express my sincere gratitude to my advisor, Dr. Steve Negus, for his guidance, support, patience, and encouragement throughout my graduate school career. His patience and guidance at every stage of my work was remarkable. I have learned a great deal from observing him tackle difficult challenges with intelligence, patience and perseverance. It is not often that one finds an advisor that even though is very busy have never denied me help when asked. I could not have imagined a better advisor for my doctoral experience. Thank you for being an amazing advisor, Dr. Negus. I would like also to gratefully acknowledge my committee members. Dr. Damaj, Dr. Acevedo, Dr. Nicholson and Dr. Porter, for their insightful comments, advice and crucial contribution in this dissertation. I also thank Dr. Matt Banks for his constructive comments on my research. I would also like to thank all of my labmates, who created a convivial place to work. In particular, I would like to thank Julie Bonano and Mike Leitl for their support in and out of the lab. To my friends Eduardo, Ashok, Bethi, Pretal and Kellianne, thank you for supporting me throughout this process. Finally, I would like to thank my boyfriend Vikram for standing by my side, supporting me and always finding a way to make me smile.
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iv
List of Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>nAChRs</td>
<td>Nicotinic acetylcholine receptors</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglia</td>
</tr>
<tr>
<td>MFB</td>
<td>Medial forebrain bundle</td>
</tr>
<tr>
<td>NAcc</td>
<td>Nucleus accumbens</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
</tr>
<tr>
<td>GABA</td>
<td>Glutamate and γ-aminobutyric acid</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenously</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>i.pl.</td>
<td>Intraplantar</td>
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<tr>
<td>s.c.</td>
<td>Subcutaneously</td>
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<tr>
<td>mg/kg</td>
<td>Milligrams per kilogram</td>
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<tr>
<td>FR</td>
<td>Fixed ratio</td>
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<tr>
<td>ICSS</td>
<td>Intracranial self-stimulation</td>
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<tr>
<td>MCR</td>
<td>Maximum control rate</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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List of Compounds

Chapter II:
(-)-Nicotine hydrogen tartrate
Mecamylamine
Dihydro-beta-erythroidine (DHβE)
5-Iodo-A-85380 (5-I-A-85380)

Chapter III:
Lactic acid
Mecamylamine
5-Iodo-A-85380 (5-I-A-85380)
PNU 282987

Chapter IV:
Scopolamine
Lactic acid
Formalin
CFA
Abstract

Effects of Nicotinic Acetylcholine Receptor Agonists in Assays of Pain-Stimulated and Pain-Depressed Behavior

By Kelen Freitas

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

Virginia Commonwealth University, 2015

Advisor: S. Stevens Negus, Ph.D.

Though a host of analgesics have been developed to alleviate pain, especially acute pain, significant side effects and a lack of long-term efficacy have encouraged research attempts to pursue novel targets that may be associated with fewer side effects or a more sustained efficacy. Among these new targets are members of the nicotinic family of acetylcholine receptors (nAChRs). The non-selective nAChR agonists nicotine and epibatidine have been shown to function as potent antinociceptive drugs in many acute and chronic preclinical pain models, while nicotine has produced analgesic effects in humans. However, these non-selective nAChRs agonists also produce various side effects, including gastrointestinal and cardiovascular complications that limit clinical utility. To reduce these side effects, recent research has focused on evaluating the potential role of specific nAChR subtypes in the modulation of nociception. Traditionally, assays of pain-stimulated behaviors, or behaviors that increase in rate, frequency or intensity after presentation of a noxious stimulus, have been used to evaluate nAChR agonists and other classes of candidate analgesics pre-clinically. However, clinically relevant pain states are often associated with the depression of behavior; for example in humans, pain is often accompanied by impaired function in daily activities and depression of mood. To address these depressant manifestations of pain, novel preclinical assays have been developed to assess the
expression and pharmacological modulation of pain-depressed behaviors, or behaviors that decrease in rate, frequency or intensity after presentation of a noxious stimulus. Additionally, the effects of nAChR agonists in preclinical assays of pain-depressed behavior are unknown. In assays of pain-stimulated behavior, agonism of α4β2* receptors appears to play a prominent role in antinociception produced by drugs that target nAChRs. Recent research suggests that α7 nAChR subtype might be an alternative target. Accordingly, the primary goal of this dissertation was to compare antinociceptive effects of the nAChR agonist nicotine and more selective nicotinic agonists in assays of pain-stimulated and pain-depressed behavior. Results from this body of work show that both nicotine and the more selective α4β2* agonist 5-I-A-85380 produced antinociception in both types of assays, whereas an α7 agonist did not. Taken together, these results suggest that α4β2* nAChR agonists may be especially effective to treat signs of pain-related behavioral depression; however nonselective behavioral effects of these compounds may contribute to apparent antinociception. Studies of nAChR agonist effects on pain-depressed behavior were conducted using an assay of intracranial self-stimulation (ICSS) as a baseline behavior that is depressed by noxious pain stimuli, and pain-related depression of ICSS can be selectively alleviated by clinically effective analgesics. As a prelude to studies of nAChR agonist effects on pain-related depression of ICSS, a preliminary study was conducted to assess effects of nicotine and 5-I-A-85380 on ICSS in the absence of a noxious stimulus. These studies indicated that selective α4β2* agonists may have higher abuse potential than nicotine. Additionally, cognitive function is one domain of behavior that may be impaired by pain, and nAChR agonists are used to treat cognitive impairment produced by other non-pain pathologies. Accordingly, a final goal of this project was to develop an assay of pain-related cognitive impairment in rats that could be used to evaluate effects of nAChR agonists. Although results of
this study did provide evidence for pain-related impairment of cognition, the effects of the pain stimuli were sufficiently variable and transient to make this procedure impracticable for use in studies with nAChR agonists.
Chapter I

Introduction

1. The Clinical Problem of Pain

According to the International Association for the Study of Pain, pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage (IASP, 2008). This definition acknowledges that pain has several dimensions. Melzack and Wall (1965) suggest three distinct dimensions: (1) – the sensory-discriminative dimension, which indicates that the subject can detect and discriminate between different modalities of noxious stimuli (e.g. mechanical and thermal), different intensities of innocuous and noxious stimuli, and different anatomical locations of stimulus delivery; (2) – the motivational-affective dimension, which indicates pain-related changes in motivated behavior (e.g. increased motivation to escape, decreased motivation to engage in normally rewarding activities) and mood (e.g. depression and anxiety); and (3) – the cognitive dimension, which refers to disruption in cognitive functions such as attention, memory, or decision-making. The personal experience of pain is often problematic to describe due to its complex nature. Below, are two examples of personal quotes from people describing their pain:

“\ I had an abdominal hysterectomy 05/07/14 almost a month ago. My stomach is still sore and strangely it is peeling; like sunburn! I'm tired and want to be back to being me! Okay, so reality is I'm thinking another month and I may be able to sit, lay down or get out of a car without wincing. My incision isn't as
sore as the ridge of skin that sits above it! I hope that shrinks! Good news is I lost about 7 lb. Who knows what's ahead of me without all my lady parts! Wishing all my fellow hysterectomy sisters a speedy recovery!” Patient comments: hysterectomy – describe your experience (2014, June 5). Retrieved from http://www.medicinenet.com/hysterectomy/patient-comments-274.htm

“I have had idiopathic neuropathy for 9 years (starting when I was 35). The burning in my feet and legs feels like I am being burned alive. I have tried everything available, natural and prescribed, without much help. I can no longer work, shower for very long, exercise, or walk more than a few steps at a time. The doctors have given up trying to help me. The chronic pain rules my life.” Patient comments: hysterectomy – describe your experience (2014, March 19). Retrieved from http://www.medicinenet.com/peripheral_neuropathy/patient-comments-188.htm

Pain is a uniquely individual and subjective experience, and its existence in humans is typically assessed using verbal reports. In the clinic, measurement of pain intensity is the primary measure of pain. There are several variations of verbal reports scaling pain intensity, including visual analogue scales (VAS), numerical rating scales (NRS), and verbal rating scales (VRS). In VAS instruments, for example, a single horizontal line is used to represent a one-dimensional continuum of pain intensity. One end of the line represents no pain and the other end represents the worst possible pain one can imagine, and patients are asked to locate their own pain intensity with a hashmark drawn along the line (Cleeland and Ryan, 1994; Dworkin et al., 2005). NRS instruments measure pain intensity in a similar fashion except that the numbers 0 and 10 are used as anchors for “no pain” and “worst possible pain one can imagine,” respectively, and patients select a digit along the 0-10 continuum to represent their pain intensity (Cleeland and Ryan, 1994; Dworkin et al., 2005). VRS instruments permit more sophisticated interrogation of the pain experience by presenting patients with a list of adjectives that could potentially describe
sensory-discriminative components of pain (e.g. stabbing, burning) or affective-motivational components of pain (e.g. exhausting, terrifying), and then asking patients to endorse a rating (e.g. none, mild, moderate and severe) to specify the degree to which that adjective contributes to their pain experience (Cleeland and Ryan, 1994; Dworkin et al., 2005). For example, the McGill Pain Questionnaire was designed to provide quantitative measures of clinical pain. It is a self-report questionnaire that consists of a pain rating scale allowing patients to give their doctor a description of the quality and intensity of their pain (Melzack, 1975). In addition, pain diagnosis can also include assessment of changes in nonverbal behavior using tools such as the Brief Pain Inventory (BPI) (Cleeland and Ryan, 1994). The BPI measures both the intensity of pain (such as the sensory dimension) and interference of pain in a patient’s activities of daily living as shown below in Figure 1.0. As implied by the questions in the BPI, pain states are often associated with functional impairment and depression of behavior. For example, pain can interfere with (or depress) activities such as walking, climbing stairs, and work-related activities (Fisher et al., 2004). Moreover, relief of pain is usually accompanied by improvement in function, supporting the importance of including measures of functional impairment and behavioral depression in pain clinical trials (Dworkin et al., 2005).
Figure I.1

**Brief Pain Inventory**

Date / / Time: __________

Name: __________________

1) Throughout our lives, most of us have had pain from time to time (such as minor headaches, sprains, and toothaches). Have you had pain other than these everyday kinds of pain today? 
1. Yes 2. No

2) On the diagram shade in the areas where you feel pain. Put an X on the area that hurts the most.

3) Please rate your pain by circling the one number that best describes your pain at its worst in the past 24 hours.

<table>
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<tr>
<th>0</th>
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<th>8</th>
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<tbody>
<tr>
<td>No pain</td>
<td>you can imagine</td>
<td></td>
<td></td>
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4) Please rate your pain by circling the one number that best describes your pain at its least in the past 24 hours.

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<tr>
<td>No pain</td>
<td>you can imagine</td>
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5) Please rate your pain by circling the one number that best describes your pain on the average.

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<tbody>
<tr>
<td>No pain</td>
<td>you can imagine</td>
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6) Please rate your pain by circling the one number that tells how much pain you have right now.

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<tbody>
<tr>
<td>No pain</td>
<td>you can imagine</td>
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7) What treatments or medications are you receiving for your pain?

8) In the Past 24 hours, how much relief have you received from pain treatments or medications provided? Please circle the one percentage that most shows how much relief you have received.

<table>
<thead>
<tr>
<th>0%</th>
<th>10%</th>
<th>20%</th>
<th>30%</th>
<th>40%</th>
<th>50%</th>
<th>60%</th>
<th>70%</th>
<th>80%</th>
<th>90%</th>
<th>100%</th>
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<tr>
<td>No complete relief</td>
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9) Circle the number that describes how, during the past 24 hours, pain has interfered with your:

A. General activity

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<td>Does not interfere</td>
<td>Completely interferes</td>
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B. Mood

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C. Walking ability

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D. Normal work (includes both work outside the home and housework)

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<td>Does not interfere</td>
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E. Relations with other people

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F. Sleep

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<td>Does not interfere</td>
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G. Enjoyment of life

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<td>Does not interfere</td>
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</table>

*Brief Pain Inventory Short Form. Reprinted with permission from Charles S. Cleeland, PhD, Pain Research Group. Copyright 1991.*
In addition to classifying pain by its intensity and quality, pain can also be classified temporally as acute or chronic, and these temporal classifications of pain are associated with different clinical challenges. Generally, acute pain results from a discernable cause, like tissue damage, that can be triggered by a specific disease or injury. Acute pain is associated with skeletal muscle spasm and sympathetic nervous system activation, and it is self-limited. For example, acute pain is the type of pain experienced during a paper cut, needle prick, touching a hot stove, or after skin incision for surgery. When the injury has healed, the correlating pain subsides. In acute pain, pain serves an adaptive purpose; it motivates injured individuals to make decisions and take actions that will prevent them from reinjuring the damaged site until their tissues heal. It also corresponds to a condition inside the body whereby physiological processes promote healing and produce positive adaptation to the pain.

In general, with acute pain, the severity of pain directly correlates to the level of tissue damage. With chronic pain states, the relationship between the experience of pain and the extent of tissue damage may be more difficult to detect. For example, long-term health conditions such as cancer, arthritis, peripheral or central nerve damage, and psychological disorders all cause chronic pain conditions. Other common chronic pain conditions include some headache disorders (e.g. cluster headache) and low back pain. Chronic pain differs from acute pain in its onset, duration, and underlying biological mechanisms (Attal and Bouhassira, 1999). The distinction between acute and chronic pain has traditionally been determined by an arbitrary interval of time since onset. For example, acute pain lasts for less than 2-3 months, while chronic pain lasts longer than 3-6 months after onset and can last up to several years (Zhuo, 2007).

A final approach to pain classification is by its etiology as nociceptive, inflammatory or neuropathic (Blackburn-Munro, 2004). The distinction between these pain etiologies can be
described by reference to the neural circuitry of nociception shown in Figure 1.1. Briefly, noxious stimuli are defined as thermal, mechanical, chemical or electrical stimuli with potential to cause tissue damage, and these stimuli activate nociceptors that are present in any area of the body and can sense noxious stimuli either externally or internally. External examples include: skin, cornea and mucosa. Internal examples include: muscle, joint, bladder, and digestive tract. The nociceptors’ cell bodies are located in either the dorsal root ganglia or trigeminal ganglia (Jessell et al., 1991). The axons extend into the spinal cord where they form synapses in the dorsal horn of the spinal cord. There are two major groups of primary nociceptors. The first are the unmyelinated axons, also called C-fibers, which are slow conducters. These axons allow an action potential to travel at a rate of about 2 meters/second towards the CNS (Williams and Purves, 2001). The second are thin myelinated axons, also called A-delta fibers, which conduct more rapidly than do C-fibers. The A-delta axons allow an action potential to travel at a rate of about 20 meters/second towards the CNS (Williams and Purves, 2001). These nociceptors are also called first-order neurons. After reaching the spinal cord, the first-order neurons synapse onto second-order neurons that cross the midline of the spinal cord. The second-order neurons then send their information via the spinothalamic tract to the thalamus where the information is processed and subsequently sent by third-order neurons to the cerebral cortex.
Pain pathway showing multiple parts of the nervous system involved in processing nociception and pain. Adapted from https://etherpedia.wikispaces.com/Post-Operative+Pain
Nociceptive pain is caused by thermal, mechanical or chemical stimuli that have potential to cause tissue damage. These intense stimuli can directly activate primary nociceptors, which generally have higher thresholds than the sensory afferents that signal innocuous touch or temperature information (Walker et al., 1999). For example, the initial pain produced by tissue incision with a scalpel during surgery or by tissue burn after contact with a stove is associated with activation of primary nociceptors sensitive to mechanical stimulation (in the case of incision) or thermal stimulation (in the case of burn).

Inflammatory pain is caused by an insult to the integrity of tissues to produce inflammation and local release of a multitude of inflammatory mediators that include reactive cytokines such as cytokine gamma interferon (IFN-γ), tumor necrosis factor-α (TNF-α), histamine, and prostaglandins among others (Kidd and Urban, 2001). These chemical signals can directly activate nociceptors, but more importantly, they sensitize nociceptors to other mechanical or thermal stimuli, even some distance from the inflammatory field (Kidd and Urban, 2001). Moreover, one inflammatory mediator may sensitize distant nociceptors to another inflammatory mediator (Feghali and Wright, 1997). “Allodynia” and “hyperalgesia” are two terms used to describe behavioral responses associated with this nociceptor sensitization. Allodynia is defined as pain produced by a normally innocuous stimulus, and hyperalgesia is defined as the exaggeration of pain produced by a normally painful stimulus (Calmels et al., 2009; Vranken, 2009). Pain, allodynia and hyperalgesia associated with sunburn are examples of acute inflammatory pain produced by skin inflammation due to ultraviolet radiation. Pain associated with arthritis is a classic example of disease-induced chronic inflammatory pain.

Neuropathic pain is caused by a clinically diverse group of disorders that originate from damage to either peripheral nociceptors or to higher order neurons in nociceptive pathways
This neuronal damage can then produce abnormal activity in nociceptive circuits in one of several ways (Campbell and Meyer, 2006). For example, damaged primary nociceptors may fire spontaneously in the absence of other stimulation (resulting in spontaneous pain, which is pain the absence of an apparent eliciting stimulus), or higher order nociceptors may become sensitized not only to nociceptive input (resulting in hyperalgesia) but also to non-nociceptive input (resulting in allodynia) (Serra, 1999). Phantom limb pain after amputation of a limb is one example of neuropathic pain that involves readily observable damage to primary afferent neurons. However, the location and cause of nerve damage leading to neuropathic pain can often be more difficult to detect, and other types of neuropathic pain are associated with nerve damage produced by chemical agents (e.g. chemotherapy-induced peripheral neuropathy), disease (e.g. diabetic neuropathy), or traumatic events less severe that full amputation (e.g. brachial plexus avulsion).

Acute and chronic pain are among the most common reasons for physician visits, among the most common reasons for taking medications, and a major cause of work disability. According to Relieving Pain in America: A Blueprint for Transforming Prevention, Care, Education, and Research (Institute of Medicine, 2011), pain is a significant public health problem with a total annual incremental cost to the society at least $560-635 billion dollars in 2010 in the United States. This includes the total incremental cost of health care due to pain ranging between $262 to $300 billion dollars and $297-336 billion dollars due to lost productivity based on days of work missed, hours of work lost, and lower wages. According to a 2006 survey conducted by the National Institute of Mental Health, over one-half of adults aged 20 years and older report feeling pain at one or more body location including joints, back, neck, head, mouth, or face/jaw (Chartbook on Trends in the Health of Americans, 2006). In this survey
back pain was shown to be the leading cause of disability in Americans younger than 45 years old. Another survey conducted by the American Pain Foundation in 2006 evaluated 303 patients who suffered from chronic pain and were currently taking an opioid to treat their pain (Voices of Chronic Pain, 2006). Regarding impact on quality of life, this survey found that 59% of the patients reported an impact on their overall enjoyment of life, 77% reported feeling depressed, 70% reported having trouble concentrating and 86% reported inability to sleep well.

II. Strengths and Weaknesses of Existing Analgesic Drugs

Opioids and nonsteroidal anti-inflammatory drugs (NSAIDs) remain the most commonly prescribed pain medications (Kissin, 2010). Opioids exert their analgesic effect by binding to opioid receptors, which constitute a group of G protein-coupled receptors distributed broadly in the brain, spinal cord and digestive tract (Janecka et al., 2004). There are four main subtypes of opioid receptors including mu, delta, kappa, and nociceptin receptors, and effects of clinically available opioid analgesics such morphine, fentanyl, hydrocodone and oxycodone are mediated primarily by the mu subtype of opioid receptor (Yekkirala et al., 2010; Altarifi et al., 2015; Bokoch et al., 2015). Opioids are the most commonly prescribed class of medication in the United States (Kuehn, 2007). In a 2000 to 2001 representative survey of households in the United States, approximately 2 percent of the respondents reported opioid use for at least one month; arthritis and back pain were the most prevalent conditions for which opioids were prescribed (Hudson et al., 2008). Opioids are also commonly used for acute cancer pain, but opioid administration in chronic noncancer pain remains controversial because evidence of benefit with long-term treatment of chronic pain with opioids is lacking. For example, a 2014
systematic review of 39 studies in patients with chronic pain treated with opioids found no
evidence of long-term benefit, but found increased risk of serious harm (e.g., increased risk of
overdose) that was dose dependent (Retrieved from
http://www.ahrq.gov/research/findings/evidence-based-reports/opoidstp.html). The side effects
of opioids include potentially lethal respiratory depression as well as nausea and vomiting,
delayed gastric emptying causing constipation, bradycardia, sedation, and factors contributing to
high abuse liability including reinforcing effects and physical dependence (Campbell et al., 2015;
Chou et al., 2015; Krashin et al., 2015).

NSAIDs comprise a heterogeneous group of pharmacological agents. Traditional
NSAIDs act as nonselective inhibitors of the enzyme cyclooxygenase (COX), inhibiting both
COX-1 and COX-2 isoenzymes (Patrano, 1994). COX is responsible for formation of
prostanoids, including prostaglandins, prostacyclin and thromboxane (Bacchi et al., 2012).
Inhibition of COX can provide relief from the symptoms of pain and inflammation (Bjordal et
al., 2004). Examples of commonly used NSAIDs include acetylsalicylic acid (aspirin), ibuprofen
and ketoprofen. Aspirin, ibuprofen and ketoprofen are indicated for mild to moderate pain,
particularly of somatic origin. For example, they are frequently used for soft tissue injury,
strains, sprains, headaches, and arthritis (Singh, 2000). They also exert a slight but statistically
significant advantage when paired with opioids (McNicol et al., 2005). The main adverse effects
of NSAIDs are inhibition of blood clotting, gastrointestinal insult including bleeding and ulcer
formation, renal insult including acute renal failure, and adverse cardiovascular effects
(Patrignani and Patrono, 2015). NSAIDs have significant potential for interaction with drugs
commonly prescribed to patients with heart disease, most notably antihypertensive drugs, blood
thinners and low-dose aspirin (Shionoiri, 1993)
Paracetamol, known as acetaminophen in the United States and marketed under brand names such as Tylenol®, is the most commonly administered over-the-counter oral analgesic with both analgesic and antipyretic properties (Aghababian, 2010; Jawad, 2010). The mechanism of action of acetaminophen remains uncertain; however, it is suggested to inhibit COX being more selective to COX-2 (Hinz et al., 2008). Because of its selectivity for COX-2, acetaminophen does not significantly inhibit the production of the pro-clotting thromboxanes, and although acetaminophen is used for the treatment of inflammatory pain, it is not usually classified as an NSAID because it displays weak effects on some non-pain components of inflammation, such as tissue swelling (Simmons et al., 2000). Furthermore, acetaminophen is commonly combined with opioid medications to reduce the amount of opioid needed. However, such combination products may be difficult to titrate as the opioid dose is limited by the toxicities of acetaminophen at higher doses (McNicol et al., 2005). Acetaminophen overdose can lead to severe hepatotoxicity and is the most common cause of acute liver failure in the United States (Khashab et al., 2007). Concerns about hepatotoxicity also exist even at therapeutic doses of acetaminophen, especially in patients with chronic alcohol use or liver disease.

Monoamine uptake inhibitors are a heterogeneous group of medications used primarily to treat major depressive disorders (Finkel et al., 2008). There are two main classes of drugs in this class that are used to treat pain. First are the tricyclic antidepressants (TCAs), which act primarily as serotonin-norepinephrine reuptake inhibitors to produce an elevation of the synaptic concentrations of these neurotransmitters, and therefore an enhancement of neurotransmission (Finkel et al., 2008). Amitriptyline is one example of a TCA used to treat pain. The mechanism of TCA-induced analgesia is still uncertain; it has been hypothesized that the analgesic properties are associated with their indirect modulation of brain opioid systems downstream of serotonergic
and noradrenergic systems (Botney and Fields, 1983; de Gandarias et al., 1998; Benbouzid et al., 2008). TCAs have been prescribed for treatment of migraines, post-herpetic neuralgia and fibromyalgia (McQuay et al., 1996; Micó et al., 2006). Although TCAs are effective to treat some types of pain, they are also associated with multiple undesirable adverse effects that vary depending on the individual agent. Their adverse effects include anticholinergic effects such as dry mouth, orthostatic hypotension, constipation, and urinary retention; antihistaminergic effects, alpha-1 adrenergic receptor blockade, and cardiac effects (increasing intraventricular conduction, prolonged QT interval, prolonged conduction through the atrioventricular node) (Furukawa et al., 2002; Miskovic, 2015). The second main class of monoamine uptake inhibitors consists of drugs that also block norepinephrine and either serotonin or dopamine transporters but have fewer off target effects than TCAs. This group includes duloxetine, milnacipran and bupropion. For example, a recent study on bupropion (norepinephrine and dopamine uptake inhibitor) showed a high therapeutic effect of this drug in peripheral neuropathic pain (Sindrup et al., 2005). Common adverse effects include dry mouth, drowsiness, nausea, loss of appetite, constipation (Retrieved from , http://www.nlm.nih.gov/medlineplus/druginfo/meds/a609016.html , http://www.nlm.nih.gov/medlineplus/druginfo/meds/a695033.html).

Gabapentin and pregabalin are two examples of anticonvulsant drugs that are currently being recommended for the treatment of neuropathic pain (Attal et al., 2010). The mechanism of analgesic action for gabapentin and pregabalin has not been fully described. Similar in structure to the endogenous neurotransmitter GABA, gabapentin was found in humans and rats to increase GABA biosynthesis (Taylor, 1997), and to increase non-synaptic GABA neurotransmission in vitro (Taylor et al., 2014). Gabapentin also binds to the voltage-dependent calcium channel at the alpha 2-delta subunit and inhibits release of neurotransmitters including glutamate,
norepinephrine, substance P and calcitonin gene-related peptide in the CNS (Micheva et al., 2006). Pregabalin also binds to the voltage-gated calcium channels at the alpha 2-delta subunit and inhibits neurotransmitter release (Micheva et al., 2006). Gabapentin and pregabalin have been used for the treatment of pain associated with diabetic neuropathy, post-herpetic neuralgia, and central neuropathic pain (Attal et al., 2010). Pregabalin has been reported to cause euphoria, and it is classified as a Schedule V controlled substance in the United States. Other side effects of gabapentin and pregabalin include dizziness, bloating, confusion, clumsiness, continuous, uncontrolled, back-and-forth, or rolling eye movements (Retrieved from , http://www.nlm.nih.gov/medlineplus/druginfo/meds/a605045.html).

Overall, current treatments offer some relief for various types of pain; however, adverse effects and low efficacy are still the major drawbacks for the treatment of acute and chronic pain conditions. Therefore, safer and more efficacious analgesic agents are needed.

III. Preclinical Strategies for Analgesic Drug Development

Over the past several decades, the mechanisms of nociception and analgesia have been studied clinically and in animal models using biochemical, pharmacological, electrophysiological and behavioral approaches (Aceto et al., 1986; Brennan, 1999; Aicher et al., 2004; Sukhtankar et al., 2014). The use of animal models has been particularly important in identifying and understanding analgesic agents and their mechanisms. The process of nociceptive behavior has been shown to involve similar brain structures in humans and animals (Chang and Shyu, 2001), supporting the use of animal models to study pain. Given the need for novel analgesic agents with improved efficacy and safety, the use of preclinical tests using animals to
identify and validate novel molecular targets and approaches for managing pain, across its different modalities, remains a primary focus.

In preclinical studies, the evaluation of analgesics has predominantly relied on behavioral studies in rodents. These studies typically include at least two components: (1) a pain manipulation (e.g. delivery of a noxious stimulus) with the intent to produce pain state as an independent variable, and (2) the measurement of some stimulus-induced change in behavior interpreted as evidence of pain as the dependent variable. Pain manipulations can be categorized as acute noxious stimuli, inflammatory pain stimuli, and neuropathic pain stimuli. Acute noxious stimuli consist primarily of noxious thermal and mechanical stimuli. Inflammatory manipulations include treatments intended to produce or mimic inflammation, and examples include treatment with inflammatory mediators such as dilute acid or with antigenic substances such as complete Freund’s adjuvant (CFA). Neuropathic stimuli include treatments that damage neurons either by mechanical insult (e.g. chronic constriction injury of the sciatic nerve) or chemically (e.g. formalin or chemotherapeutics like paclitaxel). Pain behaviors can be categorized as the measurement of behaviors presumably indicative of that pain state, and drugs can then be evaluated for their ability to attenuate pain-related behaviors.

**III.1 Assays of Pain-Stimulated Behavior**

Here are some of the most common preclinical tests that have been developed to assess nociceptive, inflammatory and neuropathic pain states. For example, thermal nociception is commonly evaluated using the “tail flick” and “hot plate” tests (Fialip et al., 1986; Dauge et al., 1987). In the tail flick test, the independent variable is a light beam focused on the animal’s tail.
as a means of delivering a noxious thermal stimulus, and the dependent variable is latency in seconds before the animal moves (or “flicks”) its tail away from the light beam (Dauge et al., 1987). Note that this test is named for the behavior that is measured rather than for the stimulus that is delivered. A more complete name for this test, which identifies both the independent and dependent variable, is the “radiant heat tail flick test” (Fialip et al., 1986). Conversely, in the hot plate test, the independent variable is the heated surface of a plate as a source of the noxious stimulus, and the dependent variable is latency to paw licking or jumping. In this case, the test is commonly named for the device used to deliver the noxious stimulus rather than for the behavior that is elicited, and a more complete name might be “hot plate paw licking/withdrawal” test.

Tests of inflammatory pain are generally conducted using one of two approaches. In the first approach, subjects may be treated with an inflammatory mediator, and behavioral consequences of that treatment can be measured directly. For example, in tests of acid-induced abdominal stretching (or “writhing”) (Porreca et al., 1987; Cao et al., 2014), the independent variable is an intraperitoneal (IP) injection of a dilute acid solution (e.g. dilute acetic or lactic acid to produce a low pH similar to that at sites of tissue inflammation), and the independent variable is the number of abdominal stretches (or “writhes”) counted during an observation period. In the second approach, subjects are treated with an antigenic substance such as Complete Freund’s Adjuvant (CFA) or carrageenan to elicit a more sustained inflammatory response lasting for several days, and subjects are then tested for hypersensitive withdrawal responses to thermal or mechanical stimuli applied to the inflamed tissue (Ren and Dubner, 1999; Radhakrishnan et al., 2003, 2004; Chang et al., 2010). A common site for treatment with antigenic substances in this type of test is the rear paw, because this facilitates later probing of the inflamed tissue with thermal or mechanical stimuli and evaluation of paw-withdrawal responses. Finally, in common
tests of neuropathic pain, subjects are initially treated in ways intended to produce nerve damage either by physically damaging the nerve (e.g. by constriction or transection) or by treating the subject with chemical agents (e.g. formalin or chemotherapeutic drugs) that produce neuropathy (Hunskaar and Hole, 1987; Polomano et al., 2001; Lynch et al., 2004; Schiene et al., 2011; Austin et al., 2012). Subsequently, subjects are tested for hypersensitive withdrawal responses to thermal or mechanical stimuli applied to the tissue innervated by the damaged nerve. As in models of inflammatory pain, a common goal is to test hypersensitive withdrawal responses of a rear paw because of its accessibility, and as a result, a common target of nerve damage is the sciatic nerve that innervates the rear paw (Austin et al., 2012).

All of the conventional preclinical tests described above rely on dependent measures of either withdrawal responses from stimuli that can be escaped (e.g. paw withdrawal from a thermal or mechanical stimulus) or pseudo-withdrawal responses from stimuli that cannot be escaped (e.g. stretching/writhing after IP injection of dilute acid). We have categorized these types of behaviors as “pain-stimulated behaviors,” which can be defined as behaviors that increase in rate, frequency, duration or intensity in response to a noxious stimulus (Negus et al., 2006; Negus, Bilsky, et al., 2010; Pereira Do Carmo et al., 2009). Although these behaviors are widely used in pre-clinical pain research, an exclusive reliance on these measures is problematic for at least two reasons. Firstly, there is, difficulty in separating a drug’s motor impairment effects from its putative analgesic effects. For example, drugs that decrease noxious stimulus-induced behaviors by producing sedation and/or motor impairment may produce a false positive analgesic effect in assays of pain-stimulated behaviors. Secondly, the depression of behavior is commonly associated with pain in both humans and animals (Melzack, 1975; National Research Council, 1996). Therefore, it is reasonable to infer that only measuring pain-stimulated behaviors
is not sufficient for research evaluating underlying mechanisms and treatment of all clinically relevant pain behaviors. These problems associated with the use of pain-stimulated behaviors as dependent measures in preclinical research may contribute to the relatively poor preclinical-to-clinical translation of testing on candidate analgesic drugs (Seguin et al., 1995; Hill, 2000; Blackburn-Munro, 2004; Negus et al., 2006; Berge, 2011; Cobos and Portillo-Salido, 2013; Henderson et al., 2013; Světlík et al., 2013).

III.2 Assays of Pain-Depressed Behavior

In the effort to find alternatives to the use of pain-stimulated behaviors as dependent measures, we have investigated the potential usefulness of “pain-depressed behaviors,” which can be defined as behaviors that decrease in rate, frequency, duration or intensity in response to a noxious stimulus. For example, pain in humans is known to depress normally adaptive behaviors, such as walking, feeding, social interactions and affective behavior (Melzack, 1975; Ostelo and de Vet, 2005), suggesting that pain-depressed behaviors may serve as useful endpoints. In support of this notion, Stevenson et al (2006) found that IP administration of dilute acid to mice stimulated an abdominal stretching response (a pain-stimulated behavior) and depressed consumption of a liquid food (a pain-depressed behavior). The mu opioid analgesic morphine attenuated both acid-stimulated stretching and acid-induced depression of feeding, consistent with the clinical effectiveness of morphine to treat pain. Haloperidol also attenuated acid-stimulated stretching, but unlike morphine, it only exacerbated acid-induced depression of feeding. These findings were interpreted to suggest that haloperidol reduced acid-stimulated stretching by producing motor impairment rather than by producing analgesia. This is one
example that assays of pain-depressed behavior may contribute to improve translational efficiency in preclinical studies. More generally, the use of pain-depressed behaviors as dependent variables may be associated with at least two advantages relative to the use of pain-stimulated behaviors: 1) Antinociception is indicated by increases in the target behavior, and as a result, assays of pain-depressed behavior are not vulnerable to false-positive effects caused by nonselective behavioral depression or motor impairment (Negus, Bilsky, et al., 2010; Negus, Morrissey, et al., 2010; Kwilasz and Negus, 2012). 2) Assays of pain-depressed behavior may model pain-related functional impairment and/or depressed mood in humans and animals (Cleeland and Ryan, 1994; Dworkin et al., 2005), and thus may provide insight into the functional and affective dimensions of pain and the impact of drugs on these clinically relevant dimensions of pain. Consequently, one step toward achieving a closer alignment between preclinical and clinical measures of pain and analgesia has been to include measures of pain-related behavioral depression in studies to evaluate novel candidate analgesics. Studies for this dissertation included parallel assays of pain-stimulated and pain-depressed behavior to assess potential antinociceptive effects of nicotinic acetylcholine receptor agonists.

One assay that has been used to generate baseline behavior for studies of pain-depressed behavior is intracranial self-stimulation (ICSS). ICSS is an operant behavioral procedure in which experimental subjects (usually rats) are equipped with an electrode that targets a component of the brain reward system, and subjects are trained to emit an operant response (e.g. pressing a lever) to receive pulses of brain stimulation delivered via the electrode (Negus and Miller, 2014). The magnitude of brain stimulation can be varied by manipulating stimulation frequency, and increasing frequencies of brain stimulation maintain a frequency-dependent increase in response rates. Thus, low brain stimulation frequencies maintain low rates of
responding, whereas high rates of responding maintain high rates of responding. The graph that relates brain stimulation frequency (on the X-axis) to response rate (on the Y-axis) is called a frequency-rate curve, and a hypothetical example is shown in Figure 1.2. Historically, the most common use of ICSS in preclinical behavioral pharmacology has been to evaluate neurobiology of brain reward systems and pharmacology of abused drugs. For example, most drugs of abuse (e.g. cocaine) increase low rates of ICSS maintained by low brain-stimulation frequencies and produce leftward shifts in ICSS frequency-rate curves (Negus and Miller, 2014; Figure 1.2). Conversely, many aversive stimuli, including noxious stimuli that presumably produce aversive pain states, decrease high ICSS rates maintained by high brain-stimulation frequencies and produce rightward and/or downward shifts in ICSS frequency-rate curves (Negus, 2013; Figure 1.2). Decreases in ICSS rates produced by noxious stimuli provide one example of pain-related depression of behavior, and drugs can be evaluated for their effectiveness to block or reverse pain-related depression of ICSS (Negus, 2013; Negus and Altarifi, 2013). For example, we have found in previous studies that ICSS can be depressed by intraperitoneal injection of dilute lactic acid as an acute visceral noxious stimulus, and acid-induced depression of ICSS can be blocked by clinically effective analgesic drugs including both NSAIDs and mu opioid agonists. Conversely, acid-induced depression of ICSS is not blocked by drugs (e.g. centrally acting kappa opioid receptor agonists; dopamine receptor antagonists) that fail to produce clinically effective analgesia in humans but that do produce “false-positive” antinociception in conventional assays of pain-stimulated behavior. Studies for this dissertation evaluated effects of nicotinic acetylcholine receptor agonists on ICSS in the absence or presence of the acid noxious stimulus.
Figure I.3

Hypothetical ICSS frequency-rate curves to show the relationship between brain stimulation frequency and response rate under baseline conditions or after treatment with an abuse drug (e.g. cocaine) or a noxious stimulus. Abscissa, frequency of electrical brain stimulation in hertz. Ordinate, response rate of ICSS in arbitrary units.
III.4 Assays of Pain-Related Cognitive Impairment

As noted above, pain also has cognitive dimensions. For example, patients with chronic pain often report cognitive deficits including attention and memory deficits (Mao et al., 2014; Schmidt-Wilcke et al., 2014) and the performance of cognitive tasks can be impaired during pain (Crombez et al., 1997; Lorenz and Bromm, 1997; Lorenz et al., 1997; Tamburin et al., 2014). However, there has been relatively little effort in preclinical studies to assess either the impact of pain states on cognitive performance or the effects of candidate analgesics on pain-related changes in cognitive performance. In preclinical studies, operant tasks have been used to address this issue. For example, the 5-choice serial reaction time is an operant task commonly used to investigate sustained attention in rats (Boyette-Davis et al., 2008; Pais-Vieira et al., 2009). In this task, rats are trained to monitor multiple windows over 5 seconds, one of which lights up when a food reward can be earned (Bari et al., 2008). Boyette-Davis et al (2008) reported an increased number of trials in which the animals failed to attend to the task, but no impairment in accuracy (i.e the number of correct responses) was observed after formalin-induced acute inflammatory pain. Insofar as indirect and direct nicotinic acetylcholine agonists are used to treat cognitive impairment from some other disorders (e.g. use of to treat Alzheimer’s Disease) (Deardorff et al., 2015; Hahn, 2015; Keefe et al., 2015), we hypothesized that these drugs might also be useful for treatment of pain-related cognitive impairment. Accordingly, studies were undertaken in this dissertation with the goal of developing an assay of pain-related cognitive impairment that could be used in subsequent studies with nicotinic drugs and other classes of candidate analgesics.
IV. Nicotinic Acetylcholine Receptor Agonists as Candidate Analgesics

Nicotinic acetylcholine receptors (nAChRs) are known to play a significant role in pain transmission. This was first evidenced by reports from animal studies that found nicotine and epibatidine, both nonselective nicotinic agonists, to be antinociceptive drugs in both acute and chronic pain models (Damaj et al., 1998; Nishiyama, 2009; Dulu et al., 2014). However, high doses of nicotine were required to produce antinociception, and its effect was relatively modest with a short duration in rodents (Sahley and Berntson, 1979; Aceto et al., 1986). Epibatidine, a nAChR agonist isolated from the skin of an Ecuadorian frog, was about 100-fold more potent than morphine in rodents (Yogeeswari et al., 2006; Carroll, 2009). Nonetheless, epibatidine has a narrow therapeutic window because the dosage to produce antinociception is close to the dose that can cause seizures and death (Sullivan et al., 1994). The adverse effects of epibatidine are due to its non-selective actions on a broad range of nAChR subtypes, in particular those localized to peripheral ganglionic junctions. Therefore, the use of these nonselective nicotinic receptor agonists is limited by deleterious side effects (Bannon et al., 1995; Kesingland et al., 2000; Rowbotham et al., 2009). In order to develop safe and effective analgesic nicotinic agonists, selective ligands for nAChR subtypes implicated in modulating nociceptive transmission are required.

Pain transmission studies have implicated at least three nicotinic receptor subtypes: \(\alpha 4\beta 2^*\) (asterisk indicates assembly with other nAChR subunits), \(\alpha 6\beta 2^*\) and \(\alpha 7\). Due to its abundance in the central nervous system (CNS; see figure 1.3), the most commonly researched nAChR subtype is the \(\alpha 4\beta 2^*\). Appreciation of the involvement of this receptor in pain has come from selective agonist and antagonist studies as well as the use of genetic knockouts animals (Bitner et al., 1998; Marubio et al., 1999; Decker et al., 2004; Jackson et al., 2010). However,
another major subtype also with evidence suggesting a role in mediating nicotinic antinociception is the $\alpha_7$-homomer nicotinic subtype (Damaj et al., 2000) and recent studies have also provided evidence for a role of $\alpha_6\beta_2^*$ receptors (Wieskopf et al., 2015). Text below provides an overview of nAChR structure, with a particular focus on those subtypes thought to be play a role in antinociception.
Figure I.3

Brain expression of the nicotinic receptors. The various assemblies of nicotinic acetylcholine receptor subtypes are broadly distributed in the brain (Taly et al., 2009).
IV.1 Subunit Composition, Stoichiometry and Distribution

nAChRs are pentameric structures composed of five different subunits that form a central ion-conducting pore, allowing the permeability of cations, such as sodium, potassium, and calcium (Hurst et al., 2013). nAChRs are normally activated by the endogenous neurotransmitter acetylcholine, but these receptors can also be activated by nicotine and other exogenous drugs. Binding of acetylcholine, nicotine or other agonists to the ligand-gated ion channels causes different conformational states in the nAChR: activation (open) and subsequent desensitization (closed and incapable of re-activation), then a basal resting state (closed and capable of activation). nAChRs are present in both the peripheral nervous system (PNS) (at the skeletal neuromuscular junction and in the autonomic nervous system) and the CNS. nAChRs have distinct homomeric or heteromeric subunit compositions (Hurst et al., 2013). Homomeric nAChRs are made up of α7, α8 or α9 subunits, while heteromeric nAChRs comprise various combinations of α2-α6 with β2-β4 or α9 with α10 subunits (McGehee, 1999; Dani, 2001).

The α4β2* nAChR is abundant in the CNS and constitutes over 90% of high affinity [3H]-cystine binding in the rat brain (Flores et al., 1992). The heteromeric α4β2* receptors are characterized by their function as mediators of synaptic signaling, so that when a population of these receptors is stimulated by an increase in ACh concentration at a synapse, each ACh-bound receptor has an 80% probability of opening during a large synchronized current (Li and Steinbach, 2010) that then decays due to receptor desensitization in parallel with the rapid hydrolysis of ACh. The typical open times of heteromeric neuronal nAChRs are several milliseconds and frequently occur in bursts. Furthermore, prolonged presence of low concentrations of ACh produce high levels of heteromeric receptor desensitization. Agonists, antagonists or modulators can affect nAChRs, and each of these drug categories can be
subdivided based on their relative efficacies compared to the reference agonist ACh. For example, molecules that activate the nAChRs as effectively or more effectively than ACh may be classified as full agonists, and a key factor is whether they have selectivity for one type or class of nAChRs compared to others. Nicotine is a nAChR agonist with varying efficacy for all nAChR subtypes. α4β2* nAChRs have high affinity for nicotine, and nicotine activates them with high efficacy but with potency lower than the binding affinity (Papke et al., 2007). Therefore, these receptors are considered as primary targets for actions of nicotine.

On the other hand, the homopentameric α7 receptor is characterized by its high calcium permeability, its blockade by selective antagonists such as α-bungarotoxin and methyllycaconitine (MLA), and its rapid desensitization after agonist stimulation (Feuerbach et al., 2009). The α7 nAChR has a high permeability to calcium, especially when compared to other nAChRs and NMDA receptors (Séguéla et al., 1993; Castro and Albuquerque, 1995). When a population of these receptors is stimulated by an increase in ACh concentration at a synapse, each receptor has only 0.3% probability of opening during a large synchronized current, and under steady-state conditions, the probability of any single receptor being open is less than one in a million (Papke, 2014). The typical open times of the α7 receptors are less than 100 microseconds and frequently occur in isolation. There are several essential characteristics for the α7 subtype that differentiate it from the heteromeric nAChRs, specifically citing higher energy barriers for entering the open state and the low open state stability, a unique desensitized state, and a relatively small energy difference between the resting and desensitized states (Williams et al., 2011). It has been demonstrated that the α7 nAChR can change conformation from the desensitized to the resting state without passing through the active state. For example, upon desensitization, α7 nAChRs do not convert to a state with high affinity for ACh. Once ACh has
been removed or metabolized, the receptor rapidly desensitizes and returns to a state of rest, which means that it is closed and sensitive to ACh (Papke et al., 2009). However α7 nAChRs do not readily return to a functional state after the application of nicotine or the α7 agonist anabaseine (Papke et al., 2000). Interestingly, α7 nAChRs are not effectively opened by low-efficacy α7 partial agonists that have been shown to have functional activity in assays of signal transduction both in vivo and in vitro. For example, the α7 partial agonist GTS-21 (3-(2,4-dimethoxybenzylidene) anabaseine, which has low efficacy for human α7 receptors, nonetheless had positive effects in several human trials for different indications (Kox et al., 2009; Tregellas et al., 2010). It has been proposed that this α7 partial agonist may function as a pro-drug in humans (Meyer et al., 1998). Alternatively, these results may suggest that not all signaling by a ligand-gated ion channel receptor has to rely strictly on ion channel activation. For example, GTS-21 can stabilize desensitized conformations, which may be capable of mediating channel-independent signal transduction (Papke, 2014).

For some time, the α6 subunit was believed to not form functional receptors when expressed alone or when expressed in combination with other α or β subunit in heteromeric channels (Gerzanich et al., 1997). However, immunoprecipitation studies have demonstrated that α4, α6 and β2 are the most abundant nAChR subunits in the striatum and also that heteromeric α6* nAChRs such as α6β2 and α4α6β2 nAChRs are expressed in mouse mesolimbic dopaminergic neurons (Champtiaux et al., 2003). Endogenous ACh and nicotine can activate α6* nAChRs expressed on dopaminergic neurons (le Novère et al., 1999; Larsson et al., 2004). Although α6* nAChRs are abundant in the midbrain, they have been studied to a lesser extent than α4β2 or α7 nAChRs (Yang et al., 2009). It is difficult to express functional α6* nAChRs in vitro. For example, Kuryatov et al (2000) reported that is difficult to express the α6β2 nAChRs
in heterologous systems. This group has tested complex mixtures of α6 with several other nAChR subunits and found that the coexpression of α6, β3 and β4 subunit can produce the most efficient α6* nAChRs. As a result of the complex subunit combinations of naturally expressed α6* nAChRs and poor function in heterologous expression systems, it is still a challenge to develop selective compounds for α6* nAChRs.

**IV.2 Nicotinic Receptors in Nociceptive Systems**

A simplified schematic of the neural circuits involved in nociception was presented above in Figure 1.1, and the presence of nAChRs appears to not be homogeneous across these pain conduction pathways (Khan et al., 2003). Nicotinic receptors are found on peripheral terminals, cell bodies, and central terminals of primary afferent nociceptors (Genzen and McGehee, 2003) and on both inhibitory and excitatory interneurons in the spinal dorsal horn (Cordero-Erausquin et al., 2004). Furthermore, nAChRs are found on descending inhibitory noradrenergic neurons (Rowley et al., 2005) and serotonergic neurons (Cordero-Erausquin and Changeux, 2001) that modulate nociceptive signaling in the spinal dorsal horn. Lastly, nAChRs are found in many supraspinal areas, some of which may also be involved in nociception and pain (see Figure 1.3). The nAChRs have been the target of analgesic drug discovery for many years, with attention largely on α4β2* and α7 nAChRs. The α6* nAChRs recently started receiving attention on this area. Prevailing evidence suggests that α4β2* and α7 nAChRs and nicotinic receptors containing the α6 subunit are probably responsible for antinociceptive responses while α3β4* may be responsible for the adverse effects of nicotine (Rueter et al., 2000; Khan et al., 2001). Notably, antinociceptive responses and adverse effects can be attributed to different nAChR subtypes.
IV.3 Pre-Clinical and Clinical Evidence

The antinociceptive effects of nicotine, a relatively nonselective nAChR agonist, have been demonstrated in preclinical and clinical studies, although the therapeutic window is narrow. Prevailing evidence suggests that nicotine antinociception is mediated at least in part by α4β2* nAChRs. For example, nicotine, epibatidine and the synthetic nAChR agonist ABT-594, all of which produce antinociception in preclinical models, have also been shown to be potent and efficacious agonists of α4β2* receptors (Donnelly-Roberts et al., 1998). In randomized, double-blind, placebo-controlled phase II clinical trials, ABT-594 has produced promising results in patients with diabetic peripheral neuropathic pain. ABT-594 improved pain, though adverse effect levels were frequently reported (Rowbotham et al., 2009; Dutta and Awni, 2012). These side effects are probably mediated by activation of α3β4* nAChRs peripheral receptors. Indeed, ABT-594 is functionally only 2.4 times more selective for α4β2* over α3β4* nicotinic subtypes (Donnelly-Roberts et al., 1998). Additional evidence for the importance of α4β2* receptors in nAChR antinociception and analgesia has come from antagonism and gene-knockout experiments. For example, nicotine antinociception in rodents can be attenuated by the nonselective nAChR antagonist mecamylamine as well as by the more selective α4β2* antagonist dihydro-β-erythroidine (Cooley et al., 1990; Iwamoto, 1991; Damaj et al., 1995; Abdin et al., 2006) and nicotine has no antinociceptive effect in mice missing either the α4 or the β2 nAChR subunit (Marubio et al., 1999).

In addition to α4β2*, the α7 nAChR subtype may also contribute to antinociceptive effects of nicotine or other nAChR agonists. In vitro studies of both receptor binding (Kem et al., 1997; Jensen et al., 2003) and functional activity (Gerzanich et al., 1997; Eaton et al., 2003)
suggest that nicotine is approximately 100-fold selective for α4β2* vs. α7 nAChRs, and the selective α7 antagonist methyllycaconitine (MLA) failed to block thermal antinociception by nicotine in rats (Rao et al., 1996). Nonetheless, MLA did antagonize the antinociceptive effects of intrathecal nicotine in spinal nerve-ligated rats (Young et al., 2008), and a negative allosteric modulator of α7 nAChRs (meta-chlorophenylguanidine; MD-354) antagonized nicotine antinociception in a rat tail-flick procedure (Dukat et al., 2010). Moreover, α7 nAChR selective agonists produced antinociception in some rodent models of acute and inflammatory pain (Damaj et al., 1998; Wang et al., 2005; Gao et al., 2010) and α7 positive allosteric modulators also produced antinociception in mouse models of inflammatory and neuropathic pain (Freitas, Carroll, et al., 2013; Freitas, Ghosh, et al., 2013).

Studies to evaluate the role of α6* nAChRs as potential targets for antinociceptive drugs are just beginning to emerge. For example, the putative α6* agonist +PHT did not produce antinociception in rodents (Carroll et al., 2015), and central administration of an α6* antagonist did not block antinociceptive effects of nicotine in mice (Jackson et al., 2009). However, knockout of the α6* receptor exacerbated basal mechanical allodynia produced by inflammatory or neuropathic stimuli in mice, reduced the antinociceptive effects of centrally administered nicotine, and eliminated the antinociceptive effects of spinally or peripherally administered nicotine (Wieskopf et al., 2015). These results have been interpreted to suggest that agonists selective for a6 nAChRs may be more effective and safer as candidate analgesics than α4β2* agonists.

V. Effects of nAChR agonists on novel endpoints of pain-depressed behavior and pain-depressed cognition
Although the studies summarized above have provided evidence to suggest therapeutic potential of nAChR agonists for treatment of pain, virtually all of the preclinical studies have been conducted using assays of pain-stimulated behavior, and the weaknesses of these procedures were also discussed above. The effects of nicotine and nicotinic AChR agonists in preclinical assays of pain-depressed behaviors and pain-depressed cognition are unknown. Since no studies have assessed nicotine and nicotinic AChR agonists using assays of pain-depressed behavior or pain-depressed cognition, this project investigated the effects of nicotine and nicotinic AChR agonists using assays of pain-stimulated and pain-depressed behavior. The integration of findings from studies of nicotine and nAChR agonist effectiveness in preclinical assays of pain-stimulated and pain-depressed behavior and cognition may provide a more complete and translational profile of nAChR agonists as analgesics.

**VI. Overview of Dissertation Studies**

The goal of this project consisted of three specific aims ultimately intended to compare effects of nicotine and nicotinic AChR agonists in preclinical assays of pain-stimulated and pain-depressed behaviors. More specifically, Aim 1 compared effects of the non-selective nAChR agonist nicotine and the α4β2*-selective nAChR agonist 5-I-A-85380 on ICSS in the absence of any noxious stimulus. Because ICSS is often used to assess abuse-related effects of drugs, Aim 1 studies permitted a comparison of abuse-related effects produced by nicotine and 5-I-A-85380; however, these studies also provided preliminary data for Aim 2 studies, which compared effects of nicotine, 5-I-A-85380, and the selective α7 agonist PNU 282987 in the assay of pain-depressed ICSS and in a complementary assay of pain-stimulated behavior. Aim 3 focused on
the investigation of the effects of acute and chronic pain manipulations in a visual-signal detection task of attention in rats. The goal of Aim 3 was to determine if acute and chronic pain manipulations would impair attention-related aspects of cognitive function so that we could then test the ability of nicotine and nicotinic drugs to block pain-induced impairment of attention in this task. Our working hypothesis at the start of these studies was that nicotine and nAChR agonists selective for α4β2* and α7 receptor subtypes would produce antinociception with comparable potency and efficacy in assays of pain-stimulated and pain-depressed behavior in rats.
CHAPTER II

Comparison of Effects Produced by Nicotine and the $\alpha_4\beta_2^*$-Selective Agonist 5-I-A-85380

On Intracranial Self-Stimulation in Rats

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Overview of Aim I

Intracranial self-stimulation (ICSS) is one type of preclinical procedure for research on pharmacological mechanisms that mediate abuse potential of drugs acting at various targets including nicotinic acetylcholine receptors (nAChRs). This study compared effects of the non-selective nAChR agonist nicotine (0.032-1.0 mg/kg) and the $\alpha_4\beta_2^*$-selective nAChR agonist 5-I-A-85380 (0.01-1.0 mg/kg) on ICSS in male Sprague-Dawley rats. Subjects were implanted with electrodes targeting the medial forebrain bundle at the level of the lateral hypothalamus and trained to respond under a fixed-ratio 1 schedule for a range of brain stimulation frequencies (158-56 Hz). A broad range of 5-I-A-85380 doses produced an abuse-related increase (or "facilitation") of low ICSS rates maintained by low brain-stimulation frequencies, and this effect was blocked by both the nonselective nAChR antagonist mecamylamine and the selective $\alpha_4\beta_2^*$ antagonist dihyrdo-$\beta$-erythroidine (DH$\beta$E). Conversely, nicotine produced weaker ICSS facilitation across a narrower range of doses, and higher nicotine doses decreased high rates of ICSS maintained by high brain-stimulation frequencies. The rate-decreasing effects of a high
nicotine dose were blocked by mecamylamine but not DHβE. Chronic nicotine treatment produced selective tolerance to rate-decreasing effects of nicotine but did not alter ICSS rate-increasing effects of nicotine. These results suggest that α4β2* receptors are sufficient to mediate abuse-related rate-increasing effects of nAChR agonists in this ICSS procedure. Conversely, nicotine effects at non-α4β2* nAChRs appear to oppose and limit abuse-related effects mediated by α4β2* receptors, although tolerance can develop to these non-α4β2* effects. Selective α4β2* agonists may have higher abuse liability than nicotine.

**Chapter II Introduction**

Nicotine is the primary psychoactive constituent of tobacco that produces abuse-related behavioral effects such as reinforcing effects in assays of drug self-administration (Hanson et al., 1979; Corrigall and Coen, 1989; Shoaib et al., 1997). Preclinical research on determinants of abuse-related nicotine effects may guide strategies for prevention or treatment of tobacco abuse (Le Foll and Goldberg, 2005; Tuesta et al., 2011). Intracranial self-stimulation (ICSS) is another type of preclinical procedure that has been used to evaluate determinants of the abuse liability of nicotine and other drugs (Huston-Lyons and Kornetsky, 1992; Wise et al., 1992; Negus and Miller, 2014). In ICSS, operant responding is maintained by pulses of electrical stimulation delivered via electrodes implanted in brain reward areas (Kornetsky and Esposito, 1979; Wise, 1996; Carlezon and Chartoff, 2007; Vlachou et al., 2011). ICSS procedures commonly use different “doses” (i.e. different frequencies or intensities) of brain stimulation to maintain different rates of operant responding, and drug effects on low vs. high rates of ICSS can be used to draw inferences regarding abuse liability (Carlezon and Chartoff, 2007; Bauer et al., 2013; Negus and Miller, 2014). For example, “frequency-rate” ICSS procedures use a range of low to
high brain-stimulation frequencies to maintain a range of low to high rates of operant responding during daily experimental sessions. Many drugs with high abuse liability (e.g. cocaine, amphetamine) dose-dependently increase (or “facilitate”) low rates of ICSS maintained by low brain-stimulation frequencies while having little effect across a broad dose range on high rates of ICSS maintained by high brain-stimulation frequencies. Conversely, drugs with lesser or no abuse liability typically facilitate ICSS to a lesser degree across a narrower range of doses, or fail to facilitate ICSS, before recruiting effects at higher doses that reduce high ICSS rates maintained by high brain-stimulation frequencies. Thus, ICSS can be used to reveal and dissociate mechanisms that contribute to both ICSS rate-increasing effects, which correlate with abuse liability by other metrics, and ICSS rate-decreasing effects, which may limit and oppose abuse-related effects (Negus and Miller, 2014).

Nicotine produces biphasic effects on ICSS such that low doses produce relatively weak ICSS facilitation, and higher doses recruit rate-decreasing effects (Bauco and Wise, 1994). ICSS facilitation by low nicotine doses can be blocked by the non-selective nAChR antagonist mecamylamine or by selective antagonists of the α4β2* subtype of nAChR such as dihydro-β-erythroidine (DHβE) (Huston-Lyons and Kornetsky, 1992; Sagara et al., 2008; Tobey et al., 2012). This agrees with the finding that nicotine produces biphasic dose-related effects in other procedures used for assessment of abuse liability in animals, such as drug self-administration (Lau et al., 1994; Valentine et al., 1997; Rasmussen and Swedberg, 1998; Le Foll et al., 2007) and place-conditioning procedures (Risinger and Oakes, 1995; Le Foll and Goldberg, 2005), and also produces biphasic effects on measures of subjective effects in humans (Lundahl et al., 2000; Goodwin et al., 2015). Moreover, evidence from these other procedures also suggests a key role for α4β2* nAChRs in mediating abuse-related effects of nicotine (Tuesta et al., 2011). However,
less is known about the mechanisms that mediate effects associated with decreases in ICSS rates or with the descending limb of nicotine dose-effect curves in self-administration or place conditioning procedures. One possibility is that different populations of α4β2* in different brain areas mediate both rate-increasing and rate decreasing effects, but higher doses are required to produce sufficient activation of the latter population. This would parallel evidence that pharmacologically similar but anatomically distinct populations of mu opioid receptors mediate ICSS rate-increasing and rate-decreasing effects of mu opioid receptor agonists like morphine (Broekkamp et al., 1976; Altarifi et al., 2012). Alternatively, ICSS rate-decreasing effects of nicotine could be mediated by non-α4β2* receptors. For example, evidence from studies using genetic manipulations in mice and rats suggests that nAChRs containing an α5 subunit (designated α5* nAChRs) mediate rate-decreasing effects of nicotine in ICSS procedures and contribute to the descending limb of the nicotine dose-effect curves in drug self-administration and place conditioning procedures (Jackson et al., 2010; Fowler et al., 2011, 2013; Fowler and Kenny, 2014).

As one approach to further address these possibilities, the present study compared effects on ICSS in rats produced by nicotine and by the α4β2*-selective agonist 5-Iodo-A-85380 (5-I-A-85380) (Mukhin et al., 2000; Sihver et al., 2000; Liu et al., 2003; Liu, 2013). We hypothesized that, if α4β2* receptors mediate rate-increasing but not rate-decreasing effects of nicotine, then a selective α4β2* agonist like 5-I-A-85380 should produce rate-increasing effects across a broader range of doses than nicotine before recruiting rate-decreasing effects. This hypothesis also predicts that blockade of α4β2* receptors with DHβE should block rate-increasing effects of 5-I-A-85380 but not rate-decreasing effects of high-dose nicotine. Finally, this study evaluated changes in nicotine effects during chronic nicotine exposure to assess the degree to which that
exposure might differentially alter the rate-increasing effects of lower nicotine doses in comparison to the rate-decreasing effects of higher nicotine doses.

**Materials and Methods**

**Subjects**

Male Sprague-Dawley rats (Harlan, Fredrick, MD, USA) weighing 310-350 g at the time of surgery were individually housed and maintained on a 12-h light/dark cycle with lights on from 6:00 a.m. to 6:00 p.m. Rats had free access to food and water except during testing. Animal maintenance and research were in compliance with National Institutes of Health guidelines on the care and use of animal subjects in research, and all animal use protocols were approved by the Virginia Commonwealth University Institutional Care and Use Committee.

**Drugs**

(-)-Nicotine hydrogen tartrate, mecamylamine HCl and DHβE HBr were purchased from Sigma-Aldrich (St. Louis, MO). 5-I-A-85380 2HCl was synthesized at Research Triangle Institute and generously provided by Dr. Ivy Carroll. Nicotine doses are expressed as the free base of the drug, whereas mecamylamine, DHβE and 5-I-A-85380 doses are expressed as the salt forms. All solutions were prepared in saline for intraperitoneal (i.p.) injection in a volume of 1ml/kg.

**Intracranial Self-Stimulation (ICSS) Procedure**

**Surgery.** Rats were anesthetized with isoflurane gas (2.5-3% in oxygen; Webster Veterinary, Phoenix, AZ) for the implantation of stainless steel electrodes. The cathode of each
electrode was implanted in the left medial forebrain bundle at the level of the lateral hypothalamus (2.8 mm posterior and 1.7 mm lateral from bregma, and 8.8 mm below the skull). The anode was wrapped around one of three skull screws to serve as the ground, and the skull screws and electrode assembly were secured with orthodontic resin. Animals were allowed to recover for at least seven days prior to commencing ICSS training.

**Apparatus.** Experiments were conducted in sound attenuating chambers that contained modular acrylic test chambers (29.2 × 30.5 × 24.1) equipped with a response lever (4.5 cm wide, extended 2.0 cm through the center of one wall, 3 cm off the floor), stimulus lights (three lights colored red, yellow and green positioned 7.6 cm directly above the lever), a 2-W white house light, and an ICSS stimulator (Med Associates, St. Albans, VT). Electrodes were connected to the stimulator via bipolar cables and a commutator (Model SL2C, Plastics One, Roanoke, VA). A computer and software program (Med Associates, St. Albans, VT) controlled the stimulator, programming parameters and data collection.

**Training Procedure.** Rats were trained under a fixed-ratio 1 (FR 1) schedule of brain stimulation using procedures similar to those described previously for studies of acute and repeated morphine (Altarifi and Negus, 2011; Altarifi et al., 2013). Each lever press resulted in the delivery of a 0.5-s train of square wave cathodal pulses (0.1-ms pulse duration), and stimulation was accompanied by illumination of the stimulus lights above the lever. Responses during the 0.5-s stimulation period did not result in additional stimulation. During the initial phase of training, sessions lasted 30 to 60 min, the frequency of stimulation was held constant at 158 Hz, and the stimulation intensity was adjusted to the lowest value that would sustain ICSS rates of at least 30 stimulations per minute. Frequency manipulations were then introduced during sessions that consisted of sequential 10-min components. During each component, a
descending series of 10 current frequencies (158-56 Hz in 0.05 log increments) was presented, with a 60-s trial at each frequency. A frequency trial began with a 5-s time out followed by a 5-s “priming” phase, during which five non-contingent stimulations were delivered at a rate of one per second. This non-contingent stimulation was followed by a 50-s “response” phase, during which responding produced electrical stimulation under a FR 1 schedule. Training continued with three to 12 sequential components per day, and the current intensity was adjusted until rats reliably responded during the first three to four frequency trials of all components for at least three consecutive days. This intensity (range: 110-230 µA) was held constant for the remainder of the study.

**Testing Procedures.** Testing was conducted with acute and chronic dosing procedures. In the acute-dosing procedure, drugs were administered only on Tuesdays and Fridays to examine (a) nicotine and 5-I-A-85380 dose-effect curves, (b) nicotine and 5-I-A-85380 time courses, and (c) antagonism of nicotine and 5-I-A-85380 effects by mecamylamine and dihydro-β-erythroidine (DHβE). For dose-effect studies, test sessions consisted of three sequential “baseline” components followed first by a 10-min time out and then by three sequential “test” components. Nicotine (vehicle, 0.032-1.0 mg/kg) or 5-I-A-85380 (vehicle, 0.01-1.0 mg/kg) was administered at the beginning of the time out. For time-course studies, test sessions consisted of three consecutive baseline components followed by injection of nicotine (0.32 or 1.0 mg/kg) or 5-I-A-85380 (0.032 or 0.32 mg/kg) and then by pairs of consecutive test components beginning after 10, 30, 100 and 300 min. For antagonism studies, test sessions consisted of three sequential baseline components followed first by a 25-min time out and then by three sequential test components. Mecamylamine (vehicle, 1.0 mg/kg) or DHβE (vehicle, 2.0 mg/kg) was
administered 10-15min before nicotine (vehicle, 1.0 mg/kg) or 5-I-A-85380 (vehicle, 0.032 mg/kg).

One group of six drug-naïve rats (Group 1) was used for nicotine dose-effect, time-course and mecamylamine antagonism studies. A second group of five drug-naïve rats (Group 2) was used for 5-I-A-85380 dose-effect, time-course and mecamylamine antagonism studies. A third group of six rats (Group 3) was used for the DHβE-nicotine antagonism studies (two rats from Group 2 plus four drug-naïve rats). Finally a fourth group of six rats (Group 4) was used for the DHβE/5-I-A-85380 antagonism studies (two rats from Group 2 plus three rats came from Group 3 plus one drug-naïve rat). Treatment order was randomized with a Latin-square design. Three-component training sessions were conducted on Mondays, Wednesdays and Thursdays.

The chronic dosing procedure was conducted in a drug-naïve group of eight rats over a period of 32 days. During this period, “predrug baseline” ICSS was evaluated before initiation of chronic dosing (Days -3 to -1), cumulative nicotine dose-effect curves (0.1-1.0 mg/kg) were determined at weekly intervals (Days 0, 7, 14, 21 and 28), and regimens of repeated daily nicotine dosing or withdrawal were implemented between dose-effect curves (Days 1-6, 0.32 mg/kg/day; Days 8-13, 1.0 mg/kg/day; Days 15-20, 2.0 mg/kg/day administered in two 1.0 mg/kg injections at 9am and 5pm; Days 22-27, nicotine withdrawal). Predrug baseline ICSS (Days -3 to -1) was evaluated with three-component test sessions each day. Cumulative nicotine dose-effect sessions (Days 0, 7, 14, 21, 28) began with three “daily baseline” ICSS components followed by three 30-min testing periods, each comprised of a 10-min time out and two 10-min test components. A dose of nicotine was administered at the beginning of each time out, such that each dose increased the total cumulative dose by 0.5 log units from 0.1 to 1.0 mg/kg. Repeated daily nicotine treatments between determination of dose-effect curves were usually
administered in the context of ICSS sessions comprised of three daily baseline components followed first by a 10-min time out during which nicotine was administered and then by three test components. However, on some weekend days, the daily nicotine dose was administered in each rat’s home cage, and ICSS sessions were omitted. During the withdrawal period (Days 22-27), rats remained in their home cages and did not receive nicotine or ICSS sessions.

Data Analysis. The first baseline component of each session was considered to be an acclimation component, and data were discarded. The primary dependent variable for all remaining components was the reinforcement rate in stimulations/trial during each frequency trial. To normalize these raw data, reinforcement rates from each trial in each rat were converted to Percent Maximum Control Rate (%MCR) for that rat. For acute-dosing studies, MCR was defined as the mean of the maximal rates observed during the second and third “baseline” components for that day. For chronic dosing studies, MCR was defined as the mean of the maximal rates observed during the second and third components of the three predrug baseline sessions (six total components) before initiation of repeated dosing. For all studies, %MCR = [(rate during a frequency trial) / (MCR)] x 100. Normalized ICSS rates at each frequency were averaged across test components within each rat and then across rats to yield a “frequency-rate” curve for each experimental manipulation. Two-way ANOVA was used to compare frequency-rate curves, with ICSS frequency as one variable and dose or time as the second variable. A significant ANOVA was followed by a Holm-Sidak post hoc test, and the criterion for significance was set at p < 0.05.

To provide an additional summary measure of ICSS performance for antagonism studies, the total number of stimulations per component was calculated as the average of the total stimulations delivered across all 10-frequency trials of each component. Data were expressed as
a percentage of the total stimulations per component earned during the daily baseline. Thus, % Baseline Total Stimulations was calculated as $(\text{Mean Total Stimulations During Test Components} \div \text{Mean Total Stimulations During Baseline Components}) \times 100$. Data for antagonism treatment conditions were compared by one-way ANOVA, and a significant ANOVA was followed by the Tukey’s post hoc test. The criterion for significance was set at $p<0.05$.

**Results**

**Potency and time course of acute nicotine and 5-I-A-85380**

Under baseline conditions, electrical brain stimulation maintained a frequency-dependent increase in ICSS rates. For the 16 rats used in acute-dosing studies, the average $\pm$ SEM baseline MCR was $56.22 \pm 1.86$ stimulations per trial, and the average $\pm$ SEM number of total baseline stimulations was $249.55 \pm 19.07$ stimulations per component. Figure II.1 shows dose-effect and time-course data for nicotine effects on ICSS, and statistical results are reported in the figure legend. The low dose of 0.032 mg/kg nicotine did not significantly alter ICSS. A higher dose of 0.1 mg/kg nicotine facilitated ICSS at one frequency (89 Hz), and 0.32 mg/kg increased ICSS at one low frequency (71 Hz) and decreased ICSS at one higher frequency (126 Hz). The high dose of 1.0 mg/kg nicotine only depressed ICSS across a broad range of seven frequencies (79-158 Hz). In time-course studies, 0.32 mg/kg nicotine only decreased ICSS after 10 min at two frequencies (126 and 158 Hz), and then only facilitated ICSS after 30 min at one frequency (100 Hz). This nicotine dose did not alter ICSS at later time points. The 1.0 mg/kg nicotine dose
produced only rate-decreasing effects in the time course study. ICSS depression peaked at the earliest time point (10 min) and was no longer significant after 300 min.

Figure II.2 shows that the selective α4β2* agonist 5-I-A-85380 generally produced only facilitation of ICSS across a broad range of doses. Specifically, in dose-effect studies, the lowest dose of 0.01 mg/kg significantly depressed ICSS at one frequency (100Hz), but 0.032-1.0 mg/kg facilitated ICSS across a broad range of six frequencies (56-100 Hz). A higher dose of 3.2 mg/kg 5-I-A-85380 caused lethality in some rats during pilot studies, and it was not studied further in this group of rats. In the time-course study, 0.032 mg/kg 5-I-A-85380 produced maximal facilitation of ICSS at the earliest time points (10 and 30 min), and this effect waned after 100 min and was no longer apparent after 300 min. A higher dose of 0.32 mg/kg 5-I-A-85380 maintained maximal ICSS facilitation from 10-100 min, and significant ICSS facilitation was no longer apparent after 300 min.
Figure II.1

Dose-response (A) and time-course (B-C) of acute nicotine effects on ICSS. Abscissae: stimulation frequency in Hertz (Hz). Ordinates: ICSS rate expressed as percent maximum control rate (%MCR). Drug doses in units of milligram per kilogram (A) or pretreatment time in minutes after 0.32 mg/kg nicotine (B) or 1.0 mg/kg nicotine (C) are indicated in the legend. Filled points show frequencies at which reinforcement rates were statistically different from vehicle rates (A) or baseline rates (B,C) as determined by two-way ANOVA followed by Holm-Sidak post hoc test, p < .05. Two-way ANOVA results were as follows: (A) significant main effects of frequency $[F(9, 45) = 62.19, p < .001]$ and dose $[F(4, 20) = 32.97, p < .001]$ and a significant interaction $[F(36, 180) = 9.947, p < .001]$. (B) significant main effect of frequency $[F(9, 45) = 63.77, p < .001]$ but not of time $[F(4, 20) = 1.729, p = .1831]$; the interaction was significant $[F(36, 180) = 2.048, p = .0012]$. (C) significant main effects of frequency $[F(9, 45) = 59.00, p < .0001]$ and time $[F(4, 20) = 20.90, p < .0001]$ and a significant interaction $[F(36, 180) = 8.714, p < .0001]$. All data show mean ± SEM for six rats.
Figure II.2

Dose-response (A) and time course (B,C) of 5-I-A-85380 effects on ICSS. Abscissae: stimulation frequency in Hertz (Hz). Ordinates: ICSS rate expressed as percent maximum control rate (%MCR). Drug doses in units of milligram per kilogram (A) or pretreatment time in minutes after 0.032 mg/kg 5-I-A-85380 (B) or 0.32 mg/kg 5-I-A-85380 (C) are indicated in the legend. Filled points show frequencies at which reinforcement rates were statistically different from vehicle rates (A) or baseline rates (B,C) as determined by two-way ANOVA followed by Holm-Sidak post hoc test, p < .05. Two-way ANOVA results were as follows: (A) significant main effect of frequency [F(9, 36) = 81.79, p < .0001] and dose [F(5, 20) = 6.144, p = .0013] and a significant interaction [F(45, 180) = 6.111, p < .0001]. (B) significant main effect of frequency [F(9, 36) = 73.17, p < .0001] and time [F(4, 16) = 4.232, p = .0159] and a significant interaction [F(36, 144) = 3.443, p < .0001]. (C) significant main effect of frequency [F(9, 36) = 33.52, p < .0001] and time [F(4, 20) = 20.29, p < .0001] and a significant interaction [F(36, 144) = 4.695, p < .0001]. All data show mean ± SEM for five rats.
Antagonism effects of mecamylamine and DHBE

Figure II.3 shows effects of 1.0 mg/kg mecamylamine administered alone or as a pretreatment to 1.0 mg/kg nicotine or 0.032 mg/kg 5-I-A-85380. Mecamylamine alone had no effect on ICSS. Effects of mecamylamine pretreatment on ICSS facilitation produced by lower nicotine doses (0.1-0.32 mg/kg) were not studied because these nicotine effects were deemed too small to permit reliable detection of antagonism. However, as observed above in the dose-effect and time-course studies, 1.0 mg/kg nicotine decreased ICSS across a broad range of frequencies, and this nicotine effect was completely blocked by mecamylamine. Similarly, as observed in the dose-effect and time-course studies, 0.032 mg/kg 5-I-A-85380 facilitated ICSS across a broad range of frequencies, and this effect was significantly attenuated by mecamylamine.

Figure II.4 shows effects of 2.0 mg/kg DHßE administered as a pretreatment to 1.0 mg/kg nicotine or 0.032 mg/kg 5-I-A-85380. DHßE alone had no effect on ICSS. In contrast to mecamylamine, DHßE produced only a weak attenuation of nicotine-induced depression of ICSS, and nicotine still depressed ICSS across a broad range of frequencies. Conversely, DHßE fully blocked 5-I-A-85380-induced facilitation of ICSS.
Figure II.3

Effects of mecamylamine on rate-decreasing effects of 1.0 mg/kg nicotine (A,B) or on rate-increasing effects of 0.032 mg/kg 5-I-A-85380 (C,D). Full frequency-rate curves are shown in Panels A and C. Abscissae: stimulation frequency in Hertz (Hz). Ordinates: ICSS rate expressed as percent maximum control rate (%MCR). Filled points indicate a significant difference from treatment with “Vehicle+Vehicle” (Veh+Veh), and number signs (#) indicate significantly different from Veh+Nic. Two-way ANOVA results were as follows: (A) significant main effects of frequency \( [F(9, 45) = 109.5, p < .0001] \) and treatment \( [F(3, 15) = 51.34, p < .0001] \) and a significant interaction \( [F(27, 135) = 13.84, p < .0001] \); (C) significant main effects of frequency \( [F(9, 36) = 126.0, p < .0001] \) and treatment \( [F(3, 12) = 10.96, p = .0009] \) and a significant interaction \( [F(27, 108) = 3.610, p < .0001] \). Summary data for the total numbers of stimulations
per component on are shown in Panels B and D. Abscissae: Drug treatment. Ordinates: % baseline number of total stimulations per component. *, significantly different from Veh+Veh; #, significantly different from Veh+Nic. One-way ANOVA of data indicated a significant main effect of treatment in B (F3,15 = 59.49, p < .0001) and D (F3,12 = 9.26; p = .0019). All data show mean ± SEM for five-six rats.
Figure II.4

Effects of DHβE on rate-decreasing effects of 1.0 mg/kg nicotine (A,B) or on rate-increasing effects of 0.032 mg/kg 5-I-A-85380 (C,D). Full frequency-rate curves are shown in Panels A and C. Abscissae: stimulation frequency in Hertz (Hz). Ordinates: ICSS rate expressed as percent maximum control rate (%MCR). Filled points indicate a significant difference from treatment with “Vehicle+Vehicle” (Veh+Veh), and number signs (#) indicate significantly different from Veh+Nic. Two-way ANOVA results were as follows: (A) significant main effects of frequency \( [F(9, 45) = 44.12, p < .0001] \) and treatment \( [F(2, 10) = 37.67, p < .0001] \) and a significant interaction \( [F(18, 90) = 8.816, p < .0001] \); (C) significant main effects of frequency \( [F(9, 45) = 44.12, p < .0001] \) and treatment \( [F(2, 10) = 37.67, p < .0001] \) and a significant interaction \( [F(18, 90) = 8.816, p < .0001] \);
32.12, \( p < .0001 \) and treatment \( [F(3, 15) = 4.879, \ p = .0146] \) and a significant interaction \( [F(27, 135) = 1.762, \ p = .0189] \). Summary data for the total numbers of stimulations per component are shown in Panels B and D. Abscissae: Drug treatment. Ordinates: \% baseline number of total stimulations per component. *, significantly different from Veh+Veh; #, significantly different from Veh+Nic. One-way ANOVA of data indicated a significant main effect of treatment in B (\( F_{2,10} = 34.47, \ p < .0001 \)) and D (\( F_{3,15} = 13.70, \ p = .0001 \)). All data show mean ± SEM for six rats.
**Effects of repeated nicotine**

For the eight rats used in the repeated-dosing study, the average ± SEM baseline MCR was 58.84±5.52 stimulations per trial, and the average ± SEM number of total baseline stimulations was 263.71±39.71 stimulations per component. Figure II.5 shows changes in nicotine effects on ICSS produced by a regimen of repeated daily nicotine treatment. For this study, cumulative nicotine dose-effect curves were determined before, during and after repeated daily nicotine. On Day 0, before exposure to repeated nicotine, the effects of cumulative nicotine doses on ICSS were qualitatively similar to effects of acute nicotine described above. Thus, low doses of 0.1 and 0.32 mg/kg nicotine produced significant but modest ICSS facilitation, whereas a higher dose of 1.0 mg/kg nicotine only depressed ICSS. On Day 7, after one week of repeated exposure to 0.32 mg/kg/day nicotine, the lower nicotine doses continued to produce significant but modest ICSS facilitation. However, complete tolerance developed to the rate-decreasing effects of 1.0 mg/kg nicotine, and instead, this high nicotine dose facilitated ICSS at one frequency (79 Hz). A similar profile of nicotine effects was observed on Day 14 (after one week of 1.0 mg/kg/day nicotine), Day 21 (after one week of 2.0 mg/kg/day nicotine) and Day 28 (after one week of nicotine withdrawal). Figure II.6 shows that this regimen of repeated nicotine treatment did not alter daily baseline ICSS frequency-rate curves at any time. In particular, these ICSS data were collected approximately 24 hr after the last chronic nicotine dose of that week (approximately 16 hr when nicotine was administered twice per day), and there was no evidence of withdrawal-associated decreases in ICSS at this time. Figure II.7 compares ICSS frequency-rate curves after each nicotine dose on Day 0 (before repeated nicotine) and Day 21 (the last day of repeated nicotine). There was no significant difference across days in ICSS...
after cumulative dosing with 0.1 or 0.32 mg/kg nicotine, but there was a difference across days in effects of 1.0 mg/kg nicotine.
Figure II.5

Effects of cumulative nicotine on ICSS before, during, and after a regimen of repeated daily nicotine treatment. Cumulative nicotine dose-effect curves were determined before repeated daily nicotine administration (A, Day 0), after daily treatment with 0.32 mg/kg/day nicotine (B, Day 7), 1.0 mg/kg/day nicotine (C, Day 14), 2.0 mg/kg/day nicotine (D, Day 21), or after one week of nicotine withdrawal (E, Day 28). Effects of each cumulative nicotine dose on each day were compared to baseline ICSS rates on that day (Daily Baseline). Abscissae: stimulation frequency in Hertz (Hz). Ordinates: ICSS rate expressed as percent maximum control rate (%MCR). Filled points indicate a significant difference from the daily baseline. Two-way ANOVA results were as follows: Day 0: significant main effects of frequency \( [F(9, 63) = 68.37, p < .0001] \) and dose \( [F(3, 21) = 31.68, p < .0001] \) and a significant interaction \( [F(27, 189) = \)
5.444, p < .0001]. Day 7: significant main effects of frequency [F(9, 63) = 77.20, p < .0001] and dose [F(3, 21) = 11.26, p = .0001] and a significant interaction [F(27, 189) = 2.231, p = .0010]. Day 14: significant main effects of frequency [F(9, 63) = 74.67, p < .0001] and dose [F(3, 21) = 10.39, p = .0002] and a significant interaction [F(27, 189) = 2.513, p = .0002]. Day 21: significant main effects of frequency [F(9, 63) = 85.16, p < .0001] and dose [F(3, 21) = 7.643, p = .0012] and a significant interaction [F(27, 189) = 1.764, p = .0154]. Day 28: significant main effects of frequency [F(9, 63) = 52.45, p < .0001] and dose [F(3, 21) = 21.89, p < .0001] and a significant interaction [F(27, 189) = 3.425, p < .0001]. All data show mean ± SEM for eight rats.
Figure II.6

Effects of chronic nicotine on baseline ICSS performance. Abscissae: stimulation frequency in Hertz (Hz). Ordinates: ICSS rate expressed as percent maximum control rate (%MCR). Two-way ANOVA results were as follows: significant main effect of frequency \([F(9, 63) = 100.9, p < .0001]\) but not of nicotine treatment \([F(5, 35) = 1.47, p = .22]\); the interaction was also not significant \([F(45, 315) = 0.75, p = .8807]\). All data show mean ± SEM for eight rats.
Effects of repeated nicotine on ICSS. Each panel shows effects of a given cumulative nicotine dose administered before repeated nicotine and on the last day of repeated nicotine treatment. Abscissae: stimulation frequency in Hertz (Hz). Ordinates: ICSS rate expressed as percent maximum control rate (%MCR). Two-way ANOVA results were as follows: (A) significant main effect of frequency \[ F(9, 63) = 44.38, p < .0001 \] but not of dose \[ F(1, 7) = 0.15, p = .7069 \], and the interaction was not significant \[ F(9, 63) = 0.93, p = .5045 \]. (B) significant main effect of frequency \[ F(9, 63) = 64.44, p < .0001 \] but not of dose \[ F(1, 7) = 0.02, p = .8833 \], and the interaction was not significant \[ F(9, 63) = 0.77, p = .6488 \]. (C) significant main effects of frequency \[ F(9, 63) = 53.01, p < .0001 \] and dose \[ F(1, 7) = 22.24, p = .0022 \], and a significant interaction \[ F(9, 63) = 8.96, p < .0001 \]. All data show mean ± SEM for eight rats.
Discussion

Effects of Acute Nicotine and 5-I-A-85380

The present results are consistent with previous studies reporting a biphasic relationship between nicotine dose and the magnitude of ICSS facilitation in rats. Specifically, previous studies have reported modest but dose-dependent increases in ICSS facilitation by nicotine free base doses up to approximately 0.5 mg/kg, whereas higher nicotine doses produce less ICSS facilitation and may also begin to decrease maximal ICSS rates (Bauco & Wise, 1994; Huston-Lyons & Kornetsky, 1992; Spiller et al., 2009). Nicotine was slightly more potent to produce rate-increasing and rate-decreasing effects in the present study; however, the overall biphasic profile of nicotine effects was similar to that reported previously. This study extends on these earlier findings by showing the time course of acute nicotine effects. With 0.32 mg/kg nicotine, rate-decreasing effects predominated at the earliest time of testing (10 min), and these initial rate-decreasing effects dissipated rapidly and were followed by the later and transient emergence of exclusive rate-increasing effects at 30 min. Conversely, the higher dose of 1.0 mg/kg nicotine produced only rate-decreasing effects that peaked at 10 min and dissipated gradually over 300 min. This high nicotine dose failed to facilitate ICSS at any frequency at any time.

Like nicotine, the selective α4β2* agonist 5-I-A-85380 also facilitated ICSS. Insofar as drug-induced facilitation of ICSS is often predictive of drug-induced reinforcing effects in assays of drug self-administration (Negus & Miller, 2014), these results are consistent with evidence for self-administration of 5-I-A-85380 in rats (Liu et al., 2003). Moreover, the present study found that 5-I-A-85380 produced a higher degree of ICSS facilitation across a broader range of doses than nicotine, and 5-I-A-85380 failed to produce ICSS depression at any dose or time up to 5-I-
A-85380 doses that produced lethality. We interpret this finding to suggest two conclusions. First, these results are consistent with the hypothesis that α4β2* nAChRs are sufficient to mediate ICSS rate-increasing nicotine effects associated with other evidence for nicotine’s abuse potential (Liu, 2013; Sagara et al., 2008) but not ICSS rate-decreasing nicotine effects associated with other evidence for nicotine effects that may oppose or limit abuse potential (Fowler & Kenny, 2014; Fowler et al., 2011, 2013; Jackson et al., 2010). Second, these results suggest that 5-I-A-85380 may have higher abuse potential than nicotine. Indeed, the magnitude of ICSS facilitation produced by a 30-fold range of 5-I-A-85380 doses resembles the profile of effects produced on ICSS by some other drugs with very high abuse potential, such as cocaine and amphetamine (Bauer et al., 2013; Bonano, Runyon, Hassler, Glennon, & Stevens Negus, 2014). However, an unusual feature of 5-I-A-85380 effects was that all doses from 0.032-1.0 mg/kg produced profiles of ICSS facilitation that differed in duration but not in magnitude, suggesting a relatively quantal effect of 5-I-A-85380 on ICSS. This differs from the observation that drugs like cocaine or amphetamine produce more graded effects on ICSS, such that increasing doses produced progressively larger magnitudes of ICSS facilitation (Bauer et al., 2013; Bonano et al., 2014). Implications of this quantal effect for either underlying mechanisms of ICSS facilitation or for expression of abuse-related effects in other contexts (e.g. in drug self-administration procedures) remain to be determined.

**Mecamylamine and DHβE Antagonism**

Antagonism studies with mecamylamine and DHβE provide additional evidence to suggest differential mechanisms for rate-increasing and rate-decreasing effects of nicotine. Specifically, the rate-increasing effects of 5-I-A-85380 in the present study were blocked by both the non-selective nAChR antagonist mecamylamine and the α4β2*-selective antagonist DHβE.
Studies to antagonize nicotine-induced facilitation of ICSS were not attempted in the present study because this nicotine effect was deemed too small to permit reliable assessment of antagonism; however, our results with 5-I-A-85380 are consistent with previous reports that mecamylamine and DHßE also block nicotine-induced facilitation of ICSS (Huston-Lyons & Kornetsky, 1992; Sagara et al., 2008). Conversely, the rate-decreasing effects of nicotine were blocked by mecamylamine but not by DHßE. This finding is consistent with other evidence to suggest effectiveness of mecamylamine but not DHßE to block other signs of reduced motor activity by high nicotine doses. In rats, for example, mecamylamine but not DHßE blocked decreases in locomotion and decreases in food-maintained operant responding produced by high nicotine doses (Stolerman, Chandler, Garcha, & Newton, 1997). Taken together, these results are consistent with the conclusion that the rate-increasing effects of 5-I-A-85380 and low-to-intermediate nicotine doses are mediated by α4β2* receptors, whereas the rate-decreasing effects of higher nicotine doses are mediated primarily by non-α4β2* nAChRs.

Effects of Repeated Nicotine

Results produced by chronic nicotine provided a final source of evidence to suggest different mechanisms for ICSS rate-increasing vs. rate-decreasing effects of nicotine. Data summarized above indicated that the α4β2* antagonist DHßE selectively blocks the rate-increasing effects of nicotine, and ideally, complementary studies would be conducted with an antagonist to selectively block the rate-decreasing effects of nicotine. However, the nAChR subtype mediating these rate-decreasing effects has not been precisely determined, and selective pharmacological antagonists are not yet available for the α5* receptors implicated by studies using genetic manipulations (Fowler & Kenny, 2014; Fowler et al., 2013). As one approach to address this issue, this study evaluated effects of repeated nicotine as a strategy to produce
pharmacological tolerance rather than antagonism. The rationale for this approach was based on previous studies finding that regimens of repeated morphine could produce selective tolerance to the ICSS rate-decreasing but not the rate-increasing effects of morphine (Altarifi & Negus, 2011; Altarifi et al., 2013). Consistent with those studies with morphine, the present study found that repeated nicotine also produced selective tolerance to the ICSS rate-decreasing effects, but not to the rate-increasing effects, of nicotine. Moreover, the present results also agree with previous studies that showed selective tolerance to ICSS rate-decreasing effects of nicotine using other regimens of chronic nicotine exposure and testing (Bauco & Wise, 1994; Bozarth, Pudiak, & KuoLee, 1998).

Although this finding of selective tolerance supports the proposition that different nAChR populations mediate rate-increasing vs. rate-decreasing effects of nicotine, it does not identify the attributes of these receptor populations responsible for differential tolerance. One possibility is that nicotine dosing regimens used here selectively desensitized receptors that mediate rate-decreasing effects but not those mediating rate-increasing effects. However, two findings argue against selective desensitization as key mechanism for differential tolerance. First, tolerance was sustained for up to 1 week after termination of nicotine exposure. This agrees with other reports of sustained tolerance to other nicotine effects, such as locomotor depression and hypothermia, for periods up to 90 days after termination of nicotine treatment (Collins, Romm, & Wehner, 1988; Stolerman, Fink, & Jarvik, 1973). However, this long duration of tolerance after termination of nicotine exposure exceeds the usual time course of desensitization (typically on the order of minutes to hours), although longer durations of desensitization can be produced by long-term treatment with high nicotine doses (Quick & Lester, 2002). Second, evidence reviewed above suggests that rate-decreasing nicotine effects
are mediated at least in part by $\alpha_5^*$ receptors, but $\alpha_5^*$ receptors are more resistant than $\alpha_4\beta_2^*$ receptors to desensitization by nicotine and other nAChR agonists (Grady, Wageman, Patzlaff, & Marks, 2012; Wageman, Marks, & Grady, 2014). Regardless of the mechanism, the phenomenon of selective tolerance to nicotine’s rate-decreasing effects suggests that repeated exposure to nicotine may reduce nicotine effects that oppose and limit abuse-related nicotine effects in nicotine-naïve subjects, resulting in heightened vulnerability to abuse-related nicotine effects in nicotine-exposed subjects.

**Role for $\alpha_6^*$ receptors?**

$\alpha_6^*$ nAChRs are receptors that contain one or more $\alpha_6$ subunits instead of, or in addition to, $\alpha_4$ subunits, and a growing body of evidence suggests that $\alpha_6^*$ receptors also contribute to abuse-related effects of nicotine (Brunzell, 2012). For example, genetic knockout or pharmacologic antagonism of $\alpha_6^*$ nAChRs reduces nicotine self-administration and nicotine-induced place preference (Brunzell, Boschen, Hendrick, Beardsley, & McIntosh, 2010; Jackson, McIntosh, Brunzell, Sanjakdar, & Damaj, 2009; Pons et al., 2008), and a relatively selective $\alpha_6^*$ agonist was recently reported to produce conditioned place preference in mice (Carroll et al., 2015). Although 5-I-A-85380 is generally described as a selective $\alpha_4\beta_2^*$ agonist (Liu, 2013; Liu et al., 2003; Mukhin et al., 2000; Sihver et al., 2000), it also binds to $\alpha_6^*$ receptors (Kulak, Sum, Musachio, McIntosh, & Quik, 2002), leaving open the possibility that $\alpha_6^*$ receptors may contribute to effects of 5-I-A-85380 observed here. However, prevailing evidence suggests that DHβE is relatively selective for $\alpha_4^*$ vs. $\alpha_6^*$ nAChRs (Exley, Clements, Hartung, McIntosh, & Cragg, 2008; Papke et al., 2008; Yang et al., 2011), and it completely blocked ICSS facilitation by 5-I-A-85380. Although this result does not rule a role for $\alpha_6^*$ receptors, it suggests that $\alpha_4\beta_2^*$ receptors are necessary for 5-I-A-85380-induced ICSS facilitation.
This study compared effects of nicotine and the selective α4β2* agonist 5-I-A-85380 on ICSS in rats. Our results suggest that activation of α4β2* receptors is sufficient to produce ICSS rate-increasing effects associated with abuse potential, but α4β2* activation is neither sufficient nor necessary for nicotine-induced ICSS rate-decreasing effects. These results further suggest that α4β2* agonists may have higher abuse potential than nicotine in drug naïve subjects, and that repeated nicotine treatment can produce selective tolerance to nicotine-induced rate-decreasing effects, thereby increasing vulnerability to nicotine effects that contribute to abuse potential.
CHAPTER III

Effects of Nicotinic Acetylcholine Agonists in Assays of Acute Pain-Stimulated and Pain-Depressed Behavior in Rats

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Overview of Aim II

Agonists at nicotinic acetylcholine receptors (nAChRs) constitute one drug class being evaluated as candidate analgesics. Previous preclinical studies have implicated α4β2* and α7 nAChRs as potential mediators of the antinociceptive effects of nicotine and other nAChR agonists; however, these studies have relied exclusively on measures of pain-stimulated behavior, which can be defined as behaviors that increase in frequency, rate or intensity after presentation of a noxious stimulus. Pain is also associated with depression of many behaviors, and drug effects can differ in assays of pain-stimulated vs. pain-depressed behavior. Accordingly, this study compared effects of nicotine, the selective α4β2* agonist 5-I-A-85380, and the selective α7 agonist PNU 282987 in assays of pain-stimulated and pain-depressed behavior in male Sprague-Dawley rats. Intraperitoneal injection of dilute lactic acid served as an acute noxious stimulus to either stimulate a stretching response or depress operant responding maintained by electrical brain stimulation in an intracranial self-stimulation (ICSS) procedure. Nicotine produced a dose-dependent, time-dependent and mecamylamine-reversible blockade of
both acid-stimulated stretching and acid-induced depression of ICSS. 5-I-A-85380 also blocked both acid-stimulated stretching and acid-induced depression of ICSS, whereas PNU 282987 produced no effect in either procedure. Both nicotine and 5-I-A-85380 -were ≥10-fold more potent to block acid-induced depression of ICSS than to block acid-induced stimulation of stretching. These results suggest that stimulation of α4β2* nAChRs may be especially effective to alleviate signs of pain-related behavioral depression in rats; however, nonselective behavioral effects may contribute to apparent antinociception.

Chapter III Introduction

Agonists at nicotinic acetylcholine receptors (nAChRs) constitute one drug class being evaluated as a potential source of candidate analgesics for treatment of pain. The antinociceptive effects of nicotine, a relatively nonselective nAChR agonist, have been demonstrated in preclinical and clinical studies (Tripathi et al., 1982; Damaj et al., 1994; Ditre et al., 2011; Nirogi et al., 2013), although the therapeutic window is narrow (Greiff et al., 1993; Weingarten et al., 2008; Mishriky and Habib, 2014). Prevailing evidence suggests that nicotine antinociception is mediated at least in part by α4β2* nAChRs. For example, nicotine, epibatidine and ABT-594, all of which produce antinociception in preclinical models (Flores, 2000), have also been shown to be potent and efficacious agonists of α4β2* receptors (Donnelly-Roberts et al., 1998). Moreover, nicotine antinociception in rodents can be attenuated by the nonselective nAChR antagonist mecamylamine as well as by the more selective α4β2* antagonist dihydro-β-erythroidine (Cooley et al., 1990; Iwamoto, 1991; Damaj et al., 1995; Abdin et al., 2006), and
nicotine has no antinociceptive effect in mice missing either the α4 or the β2 nAChR subunit (Marubio et al., 1999).

In addition to α4β2*, the α7 nAChR subtype may also contribute to antinociceptive effects of nicotine or other nAChR agonists. In vitro studies of both receptor binding (Kem et al., 1997; Jensen et al., 2003) and functional activity (Gerzanich et al., 1995; Eaton et al., 2003) suggest that nicotine is approximately 100-fold selective for α4β2* vs. α7 nAChRs, and the selective α7 antagonist methyllycaconitine (MLA) failed to block thermal antinociception by nicotine in rats (Rao et al., 1996). Nonetheless, MLA did antagonize the antinociceptive effects of intrathecal nicotine in spinal nerve-ligated rats (Young et al., 2008), and a negative allosteric modulator of α7 nAChRs (meta-chlorophenylguanidine; MD-354) antagonized nicotine antinociception in a rat tail-flick procedure (Dukat et al., 2010). Moreover, α7 nAChR selective agonists produced antinociception in some rodent models of acute and inflammatory pain (Damaj et al., 1998, 2000; Wang et al., 2005; Gao et al., 2010), and α7 positive allosteric modulators also produced antinociception in mouse models of inflammatory and neuropathic pain (Freitas, Carroll, et al., 2013; Freitas, Ghosh, et al., 2013).

Preclinical studies to evaluate antinociceptive effects of nicotine and other nAChR agonists have relied exclusively on assays of pain-stimulated behavior, which measure behaviors (e.g. withdrawal reflexes) that increase in frequency, rate or intensity after presentation of a noxious and putatively painful stimulus. In assays of pain-stimulated behavior, antinociception is manifested by reduced expression of the target behavior; however, drug effects in assays of pain-stimulated behavior are often not predictive of clinical analgesia in humans (Negus et al., 2006; Whiteside et al., 2008; Mogil, 2009). In particular, drug-induced decreases in expression of pain-stimulated behaviors can reflect impaired ability to emit the motor response rather than
reduced sensitivity to the noxious stimulus. On the other hand, assays of pain-depressed behavior measure behaviors that decrease in frequency, rate or intensity after presentation of a pain stimulus (e.g. pain-related decreases in feeding, social interaction, or rates of positively reinforced operant behavior). Pain-depressed behaviors play a key role in pain diagnosis in both human and veterinary medicine (Cleeland and Ryan, 1994) and incorporation of procedures that measure pain-depressed behaviors may improve translational validity in tests of candidate analgesics (Negus et al., 2006; Negus, Bilsky, et al., 2010).

The effects of nAChR agonists have not been examined in assays of pain-depressed behavior. To address this issue, this study compared effects of nicotine and selective α4β2* and α7 nAChR agonists in assays of acute pain-stimulated and pain-depressed behavior that have been used previously to examine preclinical antinoceptive effects of other drugs including nonsteroidal anti-inflammatory drugs (Leitl et al., 2014), mu, delta and kappa opioids (Negus, Morrissey, et al., 2010; Negus et al., 2012; Altarifi et al., 2015), monoamine uptake inhibitors (Rosenberg et al., 2013; Miller et al., 2015) and cannabinoids (Kwilasz and Negus, 2012; Kwilaszt et al., 2014). Specifically, an intraperitoneal (i.p.) injection of dilute lactic acid was used as an acute chemical noxious stimulus to stimulate a stretching response and to depress operant responding in an intracranial self-stimulation (ICSS) procedure in rats. The antinociceptive effects of nicotine in both procedures were compared to effects of the α4β2*-selective agonist 5-I-A-85380 (Mukhin et al., 2000; Liu et al., 2003; Liu, 2013) and the α7-selective agonist PNU 282987 (Hajós et al., 2005; McLean et al., 2011).
Materials and Methods

Subjects

Male Sprague-Dawley rats (Harlan, Fredrick, MD, USA) weighing 310-350 g at the time of surgery were individually housed and maintained on a 12-h light/dark cycle with lights on from 6:00 a.m. to 6:00 p.m. Rats had free access to food and water except during testing. Animal maintenance and research were in compliance with National Institutes of Health guidelines on the care and use of animal subjects in research, and all animal use protocols were approved by the Virginia Commonwealth University Institutional Care and Use Committee.

Drugs

Lactic acid, (-)-nicotine hydrogen tartrate and mecamylamine HCl were purchased from Sigma-Aldrich (St. Louis, MO). 5-I-A-85380 and PNU 282987 were synthesized at Research Triangle Institute and generously provided by Dr. Ivy Carroll. Lactic acid was prepared in sterile water. Nicotine, mecamylamine, 5-I-A-85380 2HCl and PNU 282987 HCl were prepared in sterile saline. Nicotine doses are expressed as the free base of the drug, whereas mecamylamine, 5-I-A-85380 and PNU 282987 doses are expressed as the salt forms. All solutions were injected intraperitoneally in a volume of 1 ml/kg.

Intracranial Self-Stimulation (ICSS) Procedure

Surgery. Same as describe in chapter II.

Apparatus. Same as describe in chapter II.

Training Procedure. Same as describe in chapter II.

Testing Procedures. Once training was completed, ICSS testing began. For dose-effect testing with each drug, test sessions consisted of three sequential baseline components followed
first by a treatment interval, during which treatments were administered by intraperitoneal (i.p.) injection, and then by three sequential test components. The first component of each session was considered to be a “warm up” component, and data from this component were discarded. Data from the second and third components were used to calculate baseline parameters of frequency-rate curves for that session (see Data Analysis). During the treatment interval, rats were removed from the ICSS chambers, administered drug, and placed back into their home cages. After the designated pretreatment time had elapsed, 1.8% lactic acid or its vehicle (sterile water) was administered in a volume of 1 ml/kg, and rats were immediately placed back into their ICSS chambers for the three test components. This 30-minute test period was chosen to match the session length for stretching studies (see below) and because our previous studies demonstrated that lactic acid produced a sustained decrease in ICSS for up to 90 minutes (Pereira Do Carmo et al., 2009). The doses and pretreatment times for each test drug were based on preliminary data and previously published behavioral studies in rats (Liu et al., 2003; McLean et al., 2011; Liu, 2013) and were as follows: nicotine (vehicle, 0.01-0.1 mg/kg; 10 min pretreatment), 5-I-A-85380 (vehicle, 0.01-0.1 mg/kg; 30 min pretreatment) and PNU 282987 (vehicle, 3.2-32 mg/kg; 30 min pretreatment). Nicotine and 5-I-85380 were each tested in separate groups of six drug-naïve rats; PNU 282987 was tested in a group of four drug-naïve rats and one rat that had been tested previously with nicotine. Dose order was randomized across rats using a Latin-square design. Each week, a rat was tested with a given dose of test drug in combination with lactic acid vehicle on one test day and with 1.8% lactic acid on another test day. Test sessions were typically conducted on Tuesdays and Fridays, and three-component training sessions were conducted on Mondays, Wednesdays and Thursdays.
After completion of dose-effect studies, additional time-course and mecamylamine antagonism studies were conducted with nicotine. Time-course studies were conducted in a naïve group of six rats. Test sessions consisted of three baseline components, followed by a time out during which 0.1 mg/kg nicotine and 1.8% lactic acid were delivered, and then by two test components. Different test sessions were used to test different pretreatment time intervals between nicotine and 1.8% lactic acid (30, 100 and 300 min), and the order of pretreatment intervals was randomized across rats using a Latin-square design.

Antagonism studies were conducted in five naïve rats and two rats tested previously in nicotine dose-effect studies. Test sessions consisted of three baseline components, followed by a 25-min time out period and then by three test components. Mecamylamine (1.0 mg/kg) or its vehicle was delivered at the beginning of the time out, 15 min before nicotine (0.1mg/kg) or its vehicle, followed 10 min later by 1.8% lactic acid injection immediately before testing. Treatment order was randomized across rats using a Latin-square design.

**Data Analysis.** The primary dependent variable was the reinforcement rate in stimulations/trial during each frequency trial. To normalize these raw data, reinforcement rates from each trial in each rat were converted to Percent Maximum Control Rate (%MCR). MCR was defined as the mean of the maximal rates observed during the second and third “baseline” components for that day in that rat, and \[ \%MCR = \left( \frac{\text{rate during a frequency trial}}{\text{MCR}} \right) \times 100 \]. Normalized ICSS rates at each frequency were averaged across test components within each rat and then across rats to yield a “frequency-rate” curve for each experimental manipulation. Two-way ANOVA was used to compare frequency-rate curves, with ICSS frequency as one variable and dose or time as the second variable. A significant ANOVA was followed by a Holm-Sidak post hoc test, and the criterion for significance was set at \( p < 0.05 \).
To provide an additional summary measure of ICSS performance, the total number of stimulations per component was calculated as the average of the total stimulations delivered across all 10 frequency trials of each component. Data were expressed as a percentage of the baseline number of stimulations per component. Thus, % Baseline Total Stimulations was calculated as (Mean Total Stimulations During Test Components ÷ Mean Total Stimulations During Baseline Components) × 100. The average data across rats in each experimental condition were compared by paired t-test or one-way ANOVA as appropriate. A significant ANOVA was followed by a Dunnett post hoc test. The criterion for significance was set at p < 0.05. These data were also used to quantify blockade of acid-induced depression of ICSS as described previously (Rosenberg et al., 2013; Altarifi et al., 2015). Specifically, “percent acid blockade” was quantified using the equation ([test-acid]/baseline-acid)*100, where test was the total number of ICSS stimulations after treatment with drug + acid, “acid” was the total number of stimulations after acid alone, and “baseline” was the total number of stimulations in the absence of drug or acid. For all drugs producing greater than 50% acid blockade, linear regression was used to calculate an ED50 and 95% confidence limits, with ED50 defined as the effective dose producing 50% acid blockade.

Lactic Acid Stimulated Stretching Behavioral Procedure

During test sessions, a dose of test drug was administered i.p. prior to treatment with 1.8% lactic acid (i.p. in a volume of 1.0 ml/kg). Immediately after the acid injection, each rat was placed into an acrylic test chamber (31.0 X 20.1 X 20.0 cm) for a 30-minute observation period. A stretch was operationally defined as a contraction of the abdomen followed by extension of the hind limbs, and the number of stretches during the observation period was counted. The
following dose ranges were tested for each drug: nicotine (vehicle, 0.032-1.0 mg/kg), 5-I-A-85380 (vehicle, 0.32-3.2 mg/kg) and PNU 282987 (vehicle, 3.2-32 mg/kg). Each drug was
tested using the same pretreatment time as in the ICSS dose-effect studies, and each drug was
tested in a different group of six-eight rats. Dose order was randomized using a Latin-square
design, and testing was conducted once per week.

After completion of dose-effect studies, additional time-course and antagonism studies
were conducted in the nicotine group. For time course studies, test sessions consisted of an
injection of nicotine (1.0mg/kg) followed 10, 30, 100 or 300 min later by an acid injection
immediately before testing. A dose of 1.0 mg/kg nicotine was chosen for time-course studies
because it produced the greatest antinociceptive effect. Pretreatment times were randomized
across rats using a Latin-square design and test sessions were separated by 1 week. For
antagonism studies, 1.0 mg/kg mecamylamine or its vehicle was delivered 15 min before 1.0
mg/kg nicotine or its vehicle, and acid was delivered 10 min after nicotine. Treatment order was
randomized across rats using a Latin-square design, and test sessions were separated by 1 week.

**Data Analysis.** The primary dependent variable was the number of stretches counted
during each observation period in each rat. Data were averaged across rats and evaluated by one-
way ANOVA. A significant ANOVA was followed by a Tukey post hoc test, and the criterion
for significance was set at p < 0.05. For drugs producing greater than 50% reduction in stretching
relative to vehicle treatment, linear regression was used to calculate an ED50 and 95%
confidence limits, with ED50 defined as the effective dose to reduce stretching to 50% of vehicle
control. ED50 values were considered to be significantly different if 95% confidence limits did
not overlap.
Results

**Effects of nicotine, 5-I-A-85380 and PNU 282987 on acid-stimulated stretching**

Across all 20 rats used for studies of acid-stimulated stretching, i.p. administration of 1.8% lactic acid (1.0 ml/kg) after drug vehicle pretreatment elicited a mean ± S.E.M. of 14.75 ± 3.0 stretches. Figure 1 shows that nicotine produced dose-dependent, time-dependent, and mecamylamine-reversible antinociception in the assay of acid-stimulated stretching. Figure III.1A shows that stretching was significantly lower 10 min after administration of 0.1, 0.32 and 1.0 mg/kg nicotine than after nicotine vehicle, and the nicotine ED50 value is reported in Table III.1. Figure III.1B shows that 1.0 mg/kg nicotine produced a significant reduction in acid-stimulated stretching from 10 to 100 min after its administration. Figure III.1C shows that 1.0 mg/kg of mecamylamine significantly blocked the antinociceptive effect of 1.0 mg/kg nicotine.

Figure III.2 shows that the α4β2*-selective agonist 5-I-A-85380, but not the α7-selective agonist PNU 282987, also produced a dose-dependent decrease in acid-stimulated stretching. Figure III.2A shows that stretching was significantly lower 30 min after administration of 1.0 and 3.2 mg/kg 5-I-A-85380 than after 5-I-A-85380 vehicle, and the ED50 value is shown in Table III.1. 5-I-A-85380 was significantly less potent than nicotine. Conversely, Figure III.2B shows no significant reduction in stretching 30 min after administration of PNU 282987 doses up to 32 mg/kg.
Nicotine effects on acid-stimulated stretching. (A) Effects of nicotine (0.032-1.0 mg/kg) or its vehicle administered 10 min before acid. Abscissa, dose of nicotine in milligrams per kilogram. Ordinate for all panels, number of stretches observed during a 30-min observation period. (B) Effects of nicotine (1.0 mg/kg) administered 10 to 300 min before acid. Effects of vehicle administered 10 min before acid are included for comparison. Abscissa, time after nicotine administration in min. (C) Effects of mecamylamine (Mec) (1.0 mg/kg) or its vehicle on antinociceptive effects of nicotine (1.0 mg/kg, 10 min pretreatment). Effects of mecamylamine vehicle + nicotine vehicle before acid are included for comparison. Abscissa, dose of nicotine (Nic) and mecamylamine in milligrams per kilogram. One-way ANOVA indicated significant main effects of nicotine dose in (A) ($F_{4,20} = 26.97; p < 0.0001$), time in (B) ($F_{4,20} = 28.84; p < 0.0001$), and mecamylamine dose in (C) ($F_{2,10} = 48.64; p < 0.0001$). *, significantly different from Veh (A,B) or Veh+Veh (C), and $\$, significantly different from 1.0 Nic + Veh (C) as determined by Dunnett post hoc test in (A,B) or Tukey post hoc test (C); $p < 0.05$. All bars show mean ± S.E.M. in six rats.
Figure III.2

Effects of 5-I-A-85380 and PNU 282987 on acid-stimulated stretching. Abscissae, dose of 5-I-A-85380 (A) or PNU 282987 (B) in milligrams per kilogram administered 30 min before acid. Ordinates, number of stretches observed during a 30-min observation period. One-way ANOVA indicated a significant effect of 5-I-A-85380 treatment in (A) (F\(_{3,21} = 14.12; p < 0.0001\)) and no significant effect of PNU 282987 treatment in (B) (F\(_{3,15} = 2.14; p = 0.14\)). *, significantly different from Veh as determined by Dunnett post hoc test; p < 0.05. All bars show mean ± S.E.M. in eight rats (5-I-A-85380) or six rats (PNU 282987).
Table III.1.

ED50 values in mg/kg (95% confidence limits) for nicotinic drugs to produce antinociception in the assays of acid-stimulated stretching or acid-induced depression of ICSS

<table>
<thead>
<tr>
<th></th>
<th>Acid-Stimulated Stretching</th>
<th>Acid-Depressed ICSS</th>
</tr>
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<tbody>
<tr>
<td>Nicotine</td>
<td>0.14 (0.09-0.20)</td>
<td>0.011 (0.006-0.023)†</td>
</tr>
<tr>
<td>5-I-A-85380</td>
<td>1.19 (0.48-2.97)*</td>
<td>0.012 (0.006-0.021)†</td>
</tr>
<tr>
<td>PNU 282987</td>
<td>Inactive</td>
<td>Inactive</td>
</tr>
</tbody>
</table>

* Significantly different from nicotine ED50 within a given assay.

† Significantly different from the ED50 for that drug in the assay of acid-stimulated stretching.
Effects of nicotine, 5-I-A-85380 and PNU 282987 on acid-induced depression of ICSS

For the 27 rats used in these studies, the average ± S.E.M baseline MCR was 57.02 ± 2.69 stimulations per trial, and the average ± S.E.M number of total baseline stimulations per component was 258.55 ± 24.07. Figure 3 shows effects of treatment with acid vehicle or 1.8% lactic acid on ICSS for all 27 rats. After vehicle treatment, maximum reinforcement rates were usually observed at the highest stimulation frequencies (112-158 Hz), and responding decreased in a frequency-dependent manner. Administration of 1.8% lactic acid depressed ICSS, producing a rightward shift in the frequency-rate curve (Figure III.3A) and a decrease in the total number of stimulations per component (Figure III.3B). This acid-induced depression of ICSS provided a measure of pain-related behavioral depression, and nicotinic compounds were evaluated for their ability to block this acid-induced depression of ICSS.

Figure III.4 shows that 10-min pretreatment with nicotine produced a dose-dependent and complete blockade of acid-induced depression of ICSS and also facilitated control ICSS in the absence of the noxious acid stimulus. When administered as a pretreatment to acid vehicle, 0.01 mg/kg nicotine had no effect on ICSS, but higher nicotine doses of 0.032 and 0.1 mg/kg nicotine produced leftward shifts in the ICSS frequency-rate curve and significant facilitation of ICSS at intermediate frequencies of brain stimulation (79-100 Hz) (Figure III.4A). When administered as a pretreatment to 1.8% lactic acid, all nicotine doses from 0.01-0.1 mg/kg significantly ameliorated acid-induced depression of ICSS across a broad range of frequencies ranging from 79 to 141 Hz (Figure III.4B). Thus, nicotine was slightly more potent to produce an antinociceptive blockade of acid-induced depression of ICSS than to facilitate control ICSS. Summary data are shown in Figure III.4C, and the nicotine ED50 value is reported in Table. Nicotine was significantly more potent to block acid-induced depression of ICSS than to block
acid-stimulated stretching. Nicotine doses higher than 0.1 mg/kg were tested in pilot studies in some rats; however, these doses decreased rats of ICSS, and systematic studies with higher doses were not pursued.

Figure III.5 shows that nicotine produced a time-dependent and mecamylamine-reversible antinociception in the procedure of acid-induced depression of ICSS. A dose of 0.1 mg/kg nicotine blocked acid-induced depression of ICSS after 10 min (Figure III.4), and Figure III.5A shows that 0.1 mg/kg nicotine also significantly attenuated acid-induced depression of ICSS after 30 min but not after later pretreatment times. Figure III.5B shows that effects of 0.1 mg/kg nicotine were blocked by a dose of 1.0 mg/kg mecamylamine, which did not alter acid-induced depression of ICSS when it was given without nicotine.

Figure III.6 shows effects of 5-I-A-85380 and PNU 282987 on ICSS in the absence or presence of the acid noxious stimulus. Like nicotine, 5-I-A-85380 (0.01-0.1 mg/kg) produced a dose-dependent and complete blockade of acid-induced depression of ICSS, and it was slightly more potent to block acid-induced depression of ICSS than to facilitate control ICSS in the absence of the acid noxious stimulus (Figure III.6A-C). The ED50 value for 5-I-A-85380 is shown in Table III.1. There was no difference in the potencies of nicotine and 5-I-A-85380 to block acid-induced depression of ICSS, and like nicotine, 5-I-A-85380 was significantly more potent to block acid-induced depression of ICSS than acid-stimulated stretching. 5-I-A-85380 doses higher than 0.1 mg/kg were also tested in pilot studies in some rats; however, the high dose of 3.2 mg/kg caused death in some rats, and as a result, further studies with higher doses were not pursued. In contrast to nicotine and 5-I-A-85380, PNU 282987 (3.2-32 mg/kg) failed to alter control ICSS in the absence of the noxious stimulus and also failed to alleviate acid-induced depression of ICSS (Figure III.6D-F).
Acid-induced depression of ICSS. (A) ICSS frequency-rate curves determined after pretreatment with drug vehicle + lactic acid vehicle (Veh + LA Veh) or drug vehicle + 1.8% lactic acid (Veh+1.8% LA) for all rats in this study. Abscissa, frequency of electrical brain stimulation in hertz (log scale). Ordinate, rate of ICSS expressed as percent maximum control rate (%MCR). Two-way ANOVA indicated significant main effects of frequency ($F_{9,234} = 195.4; p < 0.0001$) and acid treatment ($F_{1,26} = 105.2; p < 0.0001$), and a significant interaction ($F_{9,234} = 15.23; p < 0.0001$). Filled symbols indicate a significant difference from Veh + LA Veh as determined by Holm-Sidak post hoc test; $p < 0.05$. (B) Summary data for 1.8% lactic acid effects on the total number of stimulations per component. Abscissa: pretreatment condition. Ordinate: percent baseline number of stimulations per component. *, Veh + 1.8% lactic acid significantly depressed ICSS compared with Veh + LA Veh as determined by paired t-test ($t_{26} = 9.14; p < 0.0001$). All data show mean ± S.E.M. in 27 rats.
Figure III.4

Effects of nicotine on control and acid-depressed ICSS. Left and center panels show ICSS frequency-rate curves determined when nicotine (0.01-0.1 mg/kg) was administered as a pretreatment to lactic acid vehicle (LA Veh, Panel A) or 1.8% lactic acid (1.8% LA, Panel B). Abscissae, frequency of electrical brain stimulation in hertz (log scale). Ordinates, %MCR. Filled symbols indicate a significant difference from Nic Veh+LA Veh in (A) or Nic Veh+1.8% LA in (B) as determined by Holm-Sidak post hoc test; *p < 0.05. All points show mean data from six rats, and error bars are omitted for clarity. Two-way ANOVA results were as follows: (A) significant main effects of frequency (F\textsubscript{9,45} = 117.5; p < 0.0001) and nicotine dose (F\textsubscript{3,15} = 7.67; p = 0.002) but not a significant interaction (F\textsubscript{27,135} = 1.54; p = 0.06); (B) significant main effects of frequency (F\textsubscript{9,45} = 44.16; p < 0.0001) and nicotine dose (F\textsubscript{3,15} = 7.83; p = 0.002) and a significant interaction (F\textsubscript{27,135} = 1.71; p = 0.02). (C) Summary data for nicotine effects on the total number of stimulations per component when nicotine was administered as a pretreatment to acid vehicle (open bars) or 1.8% lactic acid (filled bars). Abscissae: dose of nicotine in milligrams per kilogram. Ordinate: percent baseline number of stimulations per component. *, Significantly different from Nic Veh + LA Veh as determined by paired t-test, p<0.05. Upward arrows indicate that nicotine produced a significant increase in ICSS at one or more frequencies.
in analysis of the full frequency-rate curves in Panels A and B. All bars show mean ± S.E.M. in six rats.
Figure III.5

**Time course and mecamylamine antagonism of 0.1 mg/kg nicotine antinociception in the assay of acid-depressed ICSS.** (A) Abscissa, pretreatment interval between administration of 0.1 mg/kg nicotine and 1.8% lactic acid. Effects of nicotine vehicle + lactic acid vehicle (Veh+Veh) and nicotine vehicle + 1.8% lactic acid (Veh+1.8% LA) are also shown for comparison, with nicotine vehicle administered 10 min before acid vehicle or acid. Ordinate, percentage of baseline total number of stimulations per component. *, significantly different from Veh-Veh as determined by paired t test ($t_5 = 3.33; p < 0.02$). One-way ANOVA of data in filled bars indicated a significant effect of time ($F_{3,15} = 3.90; p = 0.03$). $\$, significantly different from Veh+1.8% LA as determined by Dunnett post hoc test; $p < 0.05$. All bars show mean ± S.E.M. in six rats. (B) Effects of 15-min pretreatment with mecamylamine (1.0 mg/kg) or its vehicle and 10 min pretreatment with nicotine (0.1 mg/kg) or its vehicle before acid treatment. Effects of mecamylamine vehicle + nicotine vehicle + acid vehicle are included for comparison. Abscissa, treatment with 1.0 mg/kg mecamylamine (1.0 Mec), 0.1 mg/kg nicotine (0.1 Nic), 1.8% lactic acid (1.8% LA) or their respective vehicles (Veh). Ordinate, percentage of baseline total number.
of stimulations per component. *, significantly different from Veh+Veh+Veh as determined by paired t test (t_6 = 4.17; p = 0.006). One-way ANOVA of data in filled bars indicated a significant main effect of treatment (F_{3,18} = 14.18; p < 0.0001). $, significantly different from Veh+Veh+1.8% LA as determined by Dunnett post hoc test; p < 0.05. All bars show mean ± S.E.M. in seven rats.
Figure III.6

Effects of 5-I-A-85380 (Panels A-C) and PNU 282987 (Panels D-F) on control and acid-depressed ICSS. Left and center panels show ICSS frequency-rate curves determined when 5-I-A-85380 (0.01-0.1 mg/kg) or PNU 282987 (3.2-32 mg/kg) was administered as a pretreatment to lactic acid vehicle (LA Veh, Panels A,D) or 1.8% lactic acid (1.8% LA, Panels B,E). Abscissae, frequency of electrical brain stimulation in hertz (log scale). Ordinates, %MCR. Filled symbols indicate a significant difference from Veh+LA Veh in (A) or Veh+1.8% LA in (B) as determined by Holm-Sidak post hoc test; p < 0.05. All points show mean data in six rats (5-I-A-85380) or five rats (PNU 282987), and error bars are omitted for clarity. Two-way ANOVA results for each panel were as follows: (A) significant main effects of frequency ($F_{9,45} = 29.84; p < 0.0001$)
and 5-I-A-85380 dose \( (F_{3,15} = 8.88; p = 0.001) \), and a significant interaction \( (F_{27,135} = 3.63; p < 0.0001) \); (B) significant main effects of frequency \( (F_{9,45} = 76.18; p < 0.0001) \) and 5-I-A-85380 dose \( (F_{3,15} = 6.17; p = 0.006) \), and a significant interaction \( (F_{27,135} = 2.52; p = 0.0003) \); (D) significant main effect of frequency \( (F_{9,36} = 150.5; p < 0.0001) \) but not PNU 282987 dose \( (F_{3,12} = 0.88; p = 0.47) \), and no significant interaction \( (F_{27,108} = 0.78; p = 0.77) \); (E) significant main effect of frequency \( (F_{9,36} = 51.75; p < 0.0001) \) but not PNU 282987 dose \( (F_{3,12} = 0.68; p = 0.58) \), and no significant interaction \( (F_{27,108} = 0.49; p = 0.98) \). Right panels show summary data for effects of 5-I-A-85380 (C) and PNU 282987 (F) on the total number of stimulations per component when drugs were administered as a pretreatment to acid vehicle (open bars) or 1.8% lactic acid (filled bars). Abscissae: drug dose in milligrams per kilogram. Ordinates: percent baseline number of stimulations per component. *, Significantly different from Veh + LA Veh as determined by paired t-test, \( p<0.05 \). Upward arrows indicate that 5-I-A-85380 produced a significant increase in ICSS at one or more frequencies in analysis of the full frequency-rate curves in Panels A and B. All bars show mean ± S.E.M. in six rats (5-I-A-85380 ) or five rats (PNU 282987).
Discussion

This study compared effects of nicotine with effects of selective α4β2* and α7 nAChR agonists in preclinical assays of pain-stimulated and pain-depressed behavior in rats. There were three main findings. First, both nicotine and the more selective α4β2* agonist 5-I-A-85380 produced antinociception in both assays, whereas the α7 agonist PNU 282987 did not. Second, both nicotine and 5-I-A-85380 were >10-fold more potent to produce antinociception in the assay of acid-depressed ICSS than in the assay of acid-stimulated stretching. Lastly, both nicotine and 5-I-A-85380 were also more potent to alleviate acid-induced depression of ICSS than to facilitate ICSS in the absence of the noxious stimulus. Taken together, these results suggest that α4β2* nAChR agonists may be especially effective to treat signs of pain-related behavioral depression; however, as discussed further below, nonselective behavioral effects of these compounds may contribute to apparent antinociception.

nAChR Agonist Effects on Acid-Stimulated Stretching. The potency, time course and mecamylamine antagonism of nicotine antinociception in the assay of acid-stimulated stretching is consistent with previous studies of nicotine in preclinical assays of pain-stimulated behavior. For example, previous studies in mice found that nicotine dose dependently decreased stretching elicited by intraperitoneal acid administration (Han et al., 2005; Kwon et al., 2008). Nicotine also blocked other pain-stimulated behaviors, such as tail- and paw-withdrawal responses from noxious thermal stimuli (Tripathi et al., 1982; Aceto et al., 1983; Rogers and Iwamoto, 1993) and withdrawal responses in subjects rendered hypersensitive to thermal or mechanical stimuli by inflammatory or neuropathic manipulations (Damaj et al., 1999; Abdin et al., 2006; Saika et al., 2015). As in the present study, nicotine antinociception is often shown to be dose- and/or time-dependent and sensitive to mecamylamine antagonism (Sahley and Berntson, 1979;
The effectiveness of 5-I-A-853805 to block acid-stimulated stretching is also consistent with previous reports of antinociception by A-85380 and other α4β2* agonists in assays of pain-stimulated behavior. 5-I-A-85380 was created by introduction of an iodine substituent onto the pyridine ring of A-85380 to generate an iodinated compound suitable for imaging studies (Mukhin et al., 2000; Rueter et al., 2006), and the parent compound A-85380 has a broad-spectrum antinociception profile with efficacy in acute, inflammatory and neuropathic pain models that rely on pain-stimulated behaviors (Curzon et al., 1998; Rueter et al., 2003). Likewise, other α4β2* agonists, such as NS3956 (Rode et al., 2012) and A-366833 (Ji et al., 2007; Nirogi et al., 2011), also produced antinociception in a broad range of pain-stimulated behavior assays in mice and rats.

Some evidence has accumulated to suggest that activation of α7 nAChRs may also be sufficient to produce antinociception in rodents. For example, intrathecal or intracerebroventricular administration of the α7 agonist choline produced MLA-reversible antinociception in a tail-flick assay in mice, and intravenous choline produced MLA-reversible antinociception in a formalin test in mice (Damaj et al., 2000; Wang et al., 2005). However, other studies failed to observe antinociception in rodents after treatment with drugs characterized as α7 agonists. For example, intravenous choline was not effective in a hot-plate assay in mice, and although the other α7 agonist SSR-180711 reduced formalin-induced licking and flinching in rats, the effect was attributed to nonspecific reduction of movement (Wang et al., 2005; Gao et al., 2010). In the present study, PNU 282987 failed to produce antinociception at doses up to 32 mg/kg. This lack of effectiveness is probably not due to inadequate dosing, because PNU 282987 was tested up to doses that did reverse MK-801-induced deficits on measures of cognitive performance in rats (Jones et al., 2014). Overall, the present results agree with previous
studies that failed to observe antinociception in assays of pain-stimulated behavior with α7 agonists in rats.

**nAChR Agonist Effects on Acid-Depressed ICSS.** This is the first study to examine effects of nAChR agonists in an assay of pain-depressed behavior, and drug effects in the assay of acid-depressed ICSS were qualitatively similar to effects in the assay of acid-stimulated stretching. Thus, nicotine produced dose-dependent, time-dependent and mecamylamine-reversible blockade of acid-induced depression of ICSS, and 5-I-A-85380 also produced dose-dependent antinociception, whereas PNU 282987 did not. The effects of nicotine and 5-I-A-85380 are also qualitatively similar to effects in this procedure produced by clinically effective analgesics including the NSAID ketoprofen and mu opioid receptor agonists like morphine (Pereira Do Carmo et al., 2009; Leitl et al., 2014a; Altarifi et al., 2015). Moreover, effects of nicotine and 5-I-A-85380 differed from effects of some other drugs, including kappa opioid receptor agonists (Negus, Morrissey, *et al.*, 2010; Negus *et al.*, 2012; Leitl *et al.*, 2014b) and cannabinoid receptor agonists (Kwilasz and Negus, 2012; Kwilasz et al., 2014), that produce antinociception in many assays of pain-stimulated behavior but fail to produce antinociception in assays of pain-depressed behavior. Taken together, the effectiveness of nicotine and 5-I-A-85380 to block both pain-stimulated and pain-depressed behaviors in rats supports further consideration of these and related compounds as candidate analgesics. Conversely, the failure of PNU 282987 to produce antinociception in either procedure does not support further consideration of α7 agonists.

Although effects of nicotine and 5-I-A-85380 were qualitatively similar in assays of acid-stimulated stretching and acid-depressed ICSS, both compounds were much more potent to block acid-induced depression of ICSS. Specifically, nicotine was 10-times more potent, and 5-I-A-
85380 was 100-times more potent, to block acid-induced depression of ICSS than to block acid-stimulated stretching. This differs from effects of morphine and most other mu opioid analgesics, which display similar potencies in these two assays (Periera Do Carmo, 2009; Altarifi et al., 2015). In the present study, the low doses of nicotine and 5-I-A-85380 that blocked acid-induced depression of ICSS have also been shown to increase other behaviors, whereas the high doses of these compounds that reduced acid-stimulated stretching have also been shown to depress other behaviors. For example, nicotine at doses up to 0.1-0.32 mg/kg produced dose-dependent increases in spontaneous locomotor activity, rates of food-maintained operant responding, and rates of ICSS, whereas doses ≥ 0.1-0.32 mg/kg decreased rates of all these behaviors (Clarke and Kumar, 1983; Goldberg et al., 1989; Cohen et al., 1991; Huston-Lyons and Kornetsky, 1992; Bauco and Wise, 1994; Whiteaker et al., 1995; Spiller et al., 2009). Effects of 5-I-A-85380 have not been examined in these other procedures in rats; however, like nicotine in the present study, 5-I-A-85380 was only slightly more potent to block acid-induced depression of ICSS than to increase control ICSS rates in the absence of the acid noxious stimulus, and 5-I-A-85380 decreased acid-stimulated stretching only at high doses ≥ 1.0 mg/kg that also produced audible and labored breathing (1.0 mg/kg) or lethality in some animals (3.2 mg/kg).

Taken together, these results suggest that non-selective behavioral activation may have contributed to apparent nicotine and 5-I-A-85380 antinociception in the assay of acid-depressed ICSS, and non-selective behavioral impairment may have contributed to apparent antinociception by these compounds in the assay of acid-stimulated stretching. This evidence for non-selective behavioral stimulation/impairment in apparent antinociception by nicotinic agonists may also be consistent with the narrow therapeutic window and emergence of
undesirable effects at analgesic doses for nAChR agonists in studies of acute pain in humans (Greiff et al., 1993; Weingarten et al., 2008; Mishriky and Habib, 2014).

**nAChR Agonist Effects on ICSS in the Absence of Acid.** This study focused primarily on effectiveness of nAChR agonists to block acid-induced depression of ICSS; however, all drugs were also tested for their effects on ICSS in the absence of noxious stimulation, and results are consistent with previous studies of nAChR agonist effects on ICSS. For example, previous studies have also shown that ICSS was facilitated after treatment with low but not high nicotine doses or with the α4β2* agonist SIB-1765F, whereas α7 agonists such as ARR-17779 did not alter ICSS (Bauco and Wise, 1994; Panagis et al., 2000; Spiller et al., 2009). Insofar as drug-induced facilitation of ICSS is often interpreted as evidence of abuse liability (Negus and Miller, 2014), the present results are consistent with the conclusion that abuse liability of α4β2* agonists may be one factor that limits their utility as candidate analgesics.

**Role for α6* receptors?** 5-I-A-85380 is commonly described as an α4β2*-selective agonist (Mukhin et al., 2000; Sihver et al., 2000; Liu et al., 2003; Liu, 2013) and results in this study with 5-I-A-85380 were interpreted to suggest a key role for the α4β2* subtype of nAChR in mediating blockade of acid-induced depression of ICSS. However, 5-I-A-85380 also binds to α6* receptors, which are nAChRs that contain the α6 subunit instead of, or in addition to, α4 subunits (Kulak et al., 2002; Capelli et al., 2011). Moreover, α6* nAChRs are located both in primary sensory neurons and in components of the mesolimbic dopamine system, and activation of these receptors is associated both with antinociception in assays of pain-stimulated behavior and with neurochemical and behavioral evidence for stimulation of the mesolimbic dopamine system (Brunzell, 2012; Wieskopf et al., 2015). In view of these considerations, the present results do not exclude a role for α6* nAChRs in mediating effects of nAChR agonists on pain-
depressed ICSS in rats.
CHAPTER IV

Effects of Acute and Sustained Pain Manipulations on Performance in a Visual-Signal Detection Task of Attention in Rats


Overview of Aim III

Patients with pain often display cognitive impairment including deficits in attention. The visual signal detection task (VSDT) is a behavioral procedure for assessment of attention in rodents. Male Sprague Dawley rats were trained in a VSDT and tested with three different noxious stimuli: (1) intraperitoneal injection of dilute lactic acid, (2) intraplantar injection of dilute formalin; and (3) intraplantar injection of complete Freund’s adjuvant (CFA). The muscarinic acetylcholine receptor antagonist scopolamine was also tested as a positive control. Scopolamine (0.01-1.0 mg/kg) dose-dependently reduced accuracy and increased response latencies during completed trials, and higher scopolamine doses increased omissions. Intraperitoneal lactic acid (0.56-5.6%) also increased response latencies and omissions, although it failed to alter measures of response accuracy. Formalin produced a significant but transient decrease in accuracy while also increasing both response latency and omissions. CFA failed to alter VSDT performance. Although VSDT effects were transient for formalin and absent for CFA, both treatments produced mechanical allodynia and paw edema for up to seven days. These results support the potential for noxious stimuli to produce a pain-related disruption of
attention in rats. However, relatively strong noxious stimulation appears necessary to disrupt performance in this version of the VSDT.

**Chapter IV Introduction**

The principal measures of pain in humans consist of verbal reports structured by instruments such as visual analog scales of pain severity (Melzack and Katz, 2006). However, noxious stimuli that elicit verbal reports of pain in humans also produce changes in nonverbal behavior in both humans and animals, and these nonverbal pain-related behaviors serve both as clinically relevant endpoints in pain assessment and as dependent measures in preclinical pain research (Negus et al., 2006; Mogil, 2009; Whiteside et al., 2013). One category of pain-related changes in behavior is impairment of cognitive function. Cognition embraces a range of processes that include attention, perception, working memory, long-term memory, executive function, language and social cognition (Keeler and Robbins, 2011), and pain has the potential to commandeer finite cognitive resources and reduce their availability for processing other stimuli. For example, chronic pain has been reported to reduce measures of attention in humans (Eccleston, 1994; Crombez et al., 1997; Lorenz et al., 1997; Eccleston and Crombez, 1999; Grace et al., 1999) and rodents (Boyette-Davis et al., 2008; Pais-Vieira et al., 2009).

The visual signal detection task (VSDT) is one type of procedure used to assess attention in rats [Parasuraman, 1984], and versions of the VSDT has been used to study effects of various manipulations on attention-related behavior [Bushnell et al., 1997; Rezvani and Levin, 2004; Hillhouse and Prus, 2013]. The goal of the present study was to assess changes in VSDT performance of rats produced by three different pain stimuli: (1) intraperitoneal injection of dilute lactic acid, (2) bilateral intraplantar injection of formalin, and (3) bilateral intraplantar injection of complete Freund’s adjuvant (CFA). Intraperitoneal injection of dilute acid is an acute
noxious stimulus that produces transient (~1 hr) stimulation of a stretching response as well as transient depression of positively reinforced operant responding in an assay of intracranial self-stimulation (ICSS) (Pereira Do Carmo et al., 2009). Intraplantar formalin also stimulates robust expression of acute pain behaviors (i.e. paw flinching and licking) for ~ 1 hr after its administration, and these initial effects are followed by a more sustained (≥2 weeks) period of paw inflammation, necrosis and neuropathy accompanied by hypersensitive withdrawal reflexes to thermal and mechanical stimuli and depression of ICSS (Grace et al., 2014; Leitl et al., 2014; Vierck et al., 2008). Intraplantar CFA elicits fewer acute pain behaviors immediately after its injection, but like formalin, it produces paw inflammation and sustained thermal/mechanical hypersensitivity, and it also produces significant but more transient (~1 day) depression of wheel running and ICSS in rats (Grace et al., 2014; Vierck et al., 2008). Thus, the noxious stimuli evaluated here differed in the intensity of acute pain behaviors (acid=formalin>CFA) and the duration of more sustained signs of pain such as mechanical hypersensitivity (formalin=CFA>acid). As a positive control, effects of these noxious stimuli were compared to effects produced by the muscarinic acetylcholine receptor antagonist scopolamine, which produces impairment attention in humans (Lenz et al., 2012) and VSDT performance in rats (Bushnell et al., 1997; Rezvani et al., 2009).

**Materials and Methods**

**Subjects**

Seventeen adult, male Sprague-Dawley rats (Harlan, Fredrick, MD, USA) were individually housed and maintained on a 12-h light/dark cycle with lights on from 6:00 a.m. to 6:00 p.m. All rats were given restricted access to food to maintain 85% of their ad libitum
weights. Rats had free access to water in their home cages. Animal maintenance and research were in compliance with National Institutes of Health guidelines on the care and use of animal subjects in research, and all animal use protocols were approved by the Virginia Commonwealth University Institutional Care and Use Committee.

**Drugs**

Scopolamine HCl (Sigma-Aldrich, St. Louis, MO) was dissolved in saline for intraperitoneal (i.p.) injection in a volume of 1 ml/kg, and doses are expressed as the salt. Lactic acid (Spectrum Chemical, Gardena, CA) was diluted in bacteriostatic water for i.p. injection in a volume of 1 ml/kg. Formalin (Fisher Scientific, Waltham, MA; diluted in saline to a 5% concentration) and CFA (Sigma-Aldrich) were administered in 100ul bilateral injections into the plantar aspect of the left and right hind paws using a 27 gauge needle.

**Visual Signal Detection Task Procedure**

**Apparatus.** Experiments were conducted in six identical operant chambers enclosed in sound attenuating cabinets equipped with a fan for ventilation and background noise (Med-Associates Inc., St. Albans. VT, USA). Each operant chamber was equipped with a round signal light (2.5 cm in diameter), a houselight, two retractable levers, and a food pellet dispenser. The signal light was positioned in the center of the front panel between the response levers and above the food receptacle. Signal light intensity was adjusted using a fader control that allowed for four different illumination levels (i.e. background illumination and 3 signal intensities) (ENV-226A, Med-Associates Inc.). Both background and signal illuminations were calibrated using a light
meter (LX1330B, HisGadget, Union City, CA). Equipment was operated and data were collected using Med PC version 4.1 (Med-Associates Inc.).

**Training Procedure.** After initial lever-press training, rats were trained according to procedures adapted from previously published studies (Bushnell, 1999; Rezvani and Levin, 2004; Rezvani et al., 2009; Hillhouse and Prus, 2013). Under the terminal schedule, daily sessions consisted of 180 trials divided into a randomized sequence of 90 “blank” trials and 90 “signal” trials. During blank trials, food was delivered only after responding on one lever (the “blank” lever), whereas during signal trials, food was delivered only after responding on the other lever (the “signal” lever). The assignment of left and right levers as the blank and signal levers was counterbalanced across rats. At the beginning of each trial, the levers were in the retracted position, the house light was on, and the signal light was illuminated at the low background intensity (0.6 lux). Each trial lasted 4.5-18.5 s and began with a pre-stimulus delay of 3, 6 or 12 s (30 blank trials and 30 signal trials with each delay, presented in randomized sequence). Subsequently, the “blank” or “signal” stimulus was delivered for 500 ms. During blank trials, there was no change in intensity of the signal light. During signal trials, the signal light intensity increased by 1.8 lux above background illumination. This stimulus was followed by a 1 sec post-stimulus delay, extension of the levers, and initiation of a response period. The trial ended and levers were retracted after a lever response was emitted or after 5 sec had elapsed, whichever occurred first. If a correct response occurred during the response period (i.e. response on the blank lever during a blank trial or on the signal lever during a signal trial), then lever retraction was accompanied by delivery of a food pellet. Conversely, if an incorrect response occurred, then lever retraction was accompanied by initiation of a 2 s time out, during which both the house light and signal light were turned off. Outcomes of each trial were designated as
follows: a correct response was called a “hit” during a signal trial and a “correct rejection” during a blank trial; an incorrect response was called a “miss” on a signal trial and a “false alarm” on a blank trial; and failure to emit a response within 5 s was considered an “omission.” Training was considered complete when a rat responded correctly for at least 70% of both blank and signal trials for 3 consecutive days.

Testing Procedures. Once training was complete, test sessions were initiated. Test sessions were identical to training sessions, with the exception that signal intensity during signal trials increased by one of three different values (0.6, 1.2, or 1.8 lux) rather than only the highest value of 1.8 lux. Thus, test sessions consisted of 90 blank trials, and 30 trials for each of the three signal intensities (equaling 90 total signal trials). Test sessions were used to evaluate effects of four different experimental manipulations. Scopolamine (0.01-1.0 mg/kg i.p.) was tested as a positive control to confirm sensitivity of the procedure to effects of a muscarinic acetylcholine receptor antagonist as reported in previous studies. Subsequently, three different noxious stimuli were tested: i.p. injection of dilute lactic acid (0.56-5.6% in distilled water and delivered in a volume of 1.0 ml/kg), bilateral intraplantar injection of formalin (5% in saline; 100 µl to each hind paw), or complete Freund’s adjuvant (CFA; 100 µl to each hind paw). Scopolamine (20 min pretreatment) was tested in a group of six experimentally naïve rats, and lactic acid (0 min pretreatment) was tested in a group of seven rats (six experimentally naïve, one from scopolamine group). Test sessions for scopolamine occurred twice a week (typically Tuesdays and Fridays) with at least 2 days separating each test, whereas tests with lactic acid occurred only once a week to minimize potential for tissue damage associated with closely spaced injections. A training session was always conducted on the day immediately preceding a test session, and the sequences of scopolamine and lactic acid doses were randomized across rats in a Latin square.
design. Formalin was tested in a group of five rats (one experimentally naïve and four from scopolamine group). Data for the naïve rat fell within the range of results obtained for the other animals on most endpoints, including endpoints that revealed significant effects, so text below reports combined results for all five rats. CFA was tested in a group of nine rats (four experimentally naïve and five from i.p. acid group). Results of statistical analyses were identical whether data from these two groups were analyzed separately or together, so text below reports combined results for all nine rats. In each group, rats were first treated with bilateral intraplantar saline and tested 15 min later to determine vehicle effects. At least two days later, rats were treated with bilateral formalin or CFA, and testing was conducted 15 min, 3 days and 7 days after treatment. The doses and pretreatment times for scopolamine were based on previous studies of scopolamine effects on performance of attention tasks in rats (Milar, 1981; Bushnell et al., 1997; Rezvani et al., 2009). The doses and pretreatment times for lactic acid, formalin and CFA were based on previous studies on pain-stimulated and pain-depressed behavior from our laboratory (Pereira Do Carmo et al., 2009; Leitl et al., 2014).

**Paw Swelling and Mechanical Allodynia**

Paw width and paw withdrawal threshold from von Frey filaments were measured before and seven days after formalin or CFA treatment to provide independent measures of inflammation-associated swelling and mechanical allodynia after these treatments. For paw width, dorsal-ventral thickness of the left hind paw was measured to the nearest 0.01 mm with electronic digital calipers (Traceable Calipers, Friendswood, TX). The von Frey filament test was used to measure sensitivity to a punctate pressure stimulus. Rats were placed on an elevated mesh galvanized steel platform in individual chambers with a hinged lid and allowed to
acclimate for at least 20 min. Subsequently, von Frey filaments (0.4 - 15 g in approximate 0.25 log increments; North Coast Medical, Morgan Hill, CA) were applied to the plantar aspect of the left hind paw using the “up-down” method to determine log median withdrawal threshold.\(^{25}\) Paw thickness and mechanical sensitivity were assessed for each rat on day 0 (before intraplantar formalin or CFA injection) and day 7 (after the last test in the visual signal detect task). Data for paw width and mechanical allodynia before and after formalin or CFA were compared by paired t-test, and the criterion for significance was \(p < 0.05.\)

**Data Analysis.** The following dependent variables were used: 1) percent hits for each signal intensity and for all signal trials combined, 2) percent correct rejections for blank trials, 3) response latency for signal and blank trials, and 4) response omissions for signal and blank trials. Percent hits for each signal intensity, and for all signal intensities combined, was calculated as
\[
\frac{\text{number of correct responses on signal trials}}{\text{number of signal trials completed}} \times 100
\]
Percent correct rejections was calculated as
\[
\frac{\text{number of correct responses on blank trials}}{\text{number of blank trials completed}} \times 100
\]
Response latency for completed trials was defined as the average time elapsed between lever extension and occurrence of a response during completed trials (determined separately for signal and blank trials). Omissions were defined as total number of trials during which no response occurred (determined separately for signal and blank trials). All data were reported as means +/- the standard error of the mean (SEM). Data were analyzed by one- or two-factor repeated-measures analysis of variance (ANOVA), and a significant ANOVA was followed by the Dunnett’s or Newman-Keuls post hoc test depending on the desired comparison. The criterion for significance was \(p < 0.05.\) All statistical analyses were conducted using the GraphPad Prism 6.0 for Windows (La Jolla, CA, USA).
Results

Baseline performance in the visual signal detection task

To provide an overview of baseline performance in the visual signal detection task, Figure IV.1 shows performance during signal trials after vehicle treatment for all 17 rats in the study. There was a significant main effect of signal intensity \( [F(2,32)=70.75, p<0.0001] \). Newman-Keuls post hoc testing indicated that accuracy of performance, quantified as percent hits, was intensity-dependent such that accuracy increased with each increase in signal intensity. When collapsed across all signal intensities, mean±SEM percent hit during signal trials was 72.04±11.51, and the mean±SEM percent correct rejections during blank trials was 88.68±5.71. Average response latencies during signal and blank trials were 0.38±0.13 sec and 0.42±0.13 sec, respectively, and the average numbers of response omissions was 0.11±0.33 for both signal and blank trials.
The effects of vehicle treatment on response accuracy during signal trials. Abscissa: Change in signal light intensity in lux. Ordinate: response accuracy quantified as percent hit. Statistically significant effects of light intensity are noted. *P<0.05 versus 0.6 (lux); # P<0.05 versus 1.2 (lux) as determined by one-way ANOVA followed by Newman-Keuls post hoc test. All data show mean ± SEM for 17 rats. Data are collapsed across all types of vehicle treatment used in this study: i.p. saline for scopolamine (N=6), i.p. water for lactic acid (N=6), bilateral intraplantar saline for both formalin and CFA (N=5). In cases where a rat was used to test two manipulations (e.g. scopolamine and formalin), then only the first vehicle test was included in this analysis. Vehicle control data for each group are shown in Figure IV.2.
Effects of scopolamine, lactic acid, formalin and CFA on response accuracy

Figure IV.2 shows effects of scopolamine (0.01-1.0 mg/kg) on accuracy of performance at each signal intensity during signal trials. Two-way ANOVA indicated main effects of dose [F(5, 25) = 13.98; p<0.0001] and signal intensity [F(2, 10) = 40.58; p<0.0001], and a significant interaction [F(10, 50) = 11.58; p<0.0001]. Scopolamine dose-dependently reduced response accuracy during signal trials (i.e. percent hit), and this effect was strongest for higher response accuracies maintained by higher signal intensities.

Figure IV.2 also shows effects of all three noxious stimuli on accuracy of performance at each signal intensity. Two-way ANOVAs indicated main effects of signal intensity (p<0.0001 for all groups), but there was not a main effect for acid concentration [F(5, 30) = 1.344; p=0.2729], time after formalin [F(3, 12) = 0.6816; p=0.5801], or time after CFA [F(3, 24) = 0.1043; p=0.9568]. The interaction was also not significant between signal intensity and either acid concentration [F(5, 30) = 1.344; p=0.2729] or time after CFA [F(6, 48) = 0.452; p=0.8586]. There was a significant interaction between signal intensity and time after formalin [F(6, 24) = 3.780; p=0.0086], and Dunnett’s post hoc test indicated a significant decrease in accuracy after 15 min.

Figure IV.3 shows effects of scopolamine and noxious stimuli on response accuracy for all signal trials combined and for blank trials, and one-way ANOVA results for each trial type are shown in Table I. Scopolamine reduced response accuracy during both signal trials and blank trials. Conversely, neither lactic acid nor CFA altered accuracy during either signal trials or blank trials. Formalin also failed to alter response accuracy during signal trials when data were collapsed across all signal intensities; however, formalin did reduce accuracy during blank trials, and Dunnett’s post hoc test indicated a significant decrease in accuracy after 15 min.
Figure IV.2

The effects of (A) scopolamine, (B) lactic acid, (C) formalin and (D) CFA on response accuracy during signal trials. Abscissae: Change in signal light intensity in lux. Ordinates: response accuracy quantified as percent hits. Filled points show doses at which percent hits were statistically different from vehicle as determined by two-way ANOVA followed by Dunnett’s post hoc test, p<0.05. All data show mean ± SEM for five-nine rats.
Figure IV.3

The effects of (A) scopolamine, (B) lactic acid, (C) formalin and (D) CFA on response accuracy for signal and blank trials. Abscissae: dose, concentration or pretreatment time of the compounds. Ordinates: response accuracy quantified as % correct across all signal trials or all blank trials. Filled points show doses, concentrations or times at which % correct were statistically different from vehicle as determined by one-way ANOVA followed by the Dunnett’s post hoc test, p<0.05. All data show mean ± SEM for five-nine rats.
Effects of scopolamine and noxious stimuli on response latencies and omissions

Figure IV.4 shows effects of scopolamine and noxious stimuli on measures of response latency and omissions, and one-way ANOVA results for each trial type are shown in Table IV.1. Both scopolamine and lactic acid dose-dependently increased response latencies and omissions during both signal and blank trials. Formalin also increased mean response latencies and omissions after 15 min, but this effect was significant only during blank trials, and formalin had no effect after 3 or 7 days. CFA had no effect at any time on either response latencies or omissions during either signal or blank trials.
Figure IV.4

The effects of (A,E) scopolamine, (B,F) lactic acid, (C,G) formalin and (D,H) CFA on response latencies (upper panels) and omissions (lower panels). Abscissae: dose, concentration or pretreatment time of the compounds. Ordinates: response latency (sec, panels A-D) or number of omissions (panels E-H). Filled points show doses, concentrations or times at which response latency or number of omissions were statistically different from vehicle as determined by one-way ANOVA followed by the Dunnett’s post hoc test, \(p<0.05\). All data show mean ± SEM for five-nine rats.
Table IV.1

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<th>Scopolamine</th>
<th>Lactic Acid</th>
<th>Formalin</th>
<th>CFA</th>
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<tr>
<td><strong>% Correct</strong></td>
<td></td>
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<td></td>
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<td>$F(5, 30) = 1.795$</td>
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<tr>
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<td>$F(5, 25) = 12.63$</td>
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One-way ANOVA results for data shown in figures IV.3 and IV.4.
Paw swelling and mechanical allodynia after formalin and CFA

Figure IV.5 shows that both CFA and formalin treatments produced significant paw swelling and mechanical allodynia seven days after treatment.
The effects of (A, C) formalin and (B, D) CFA on paw swelling and mechanical sensitivity. Abscissae: time before (baseline; BL) or seven days after formalin or CFA treatment. Ordinates: paw width in mm (A, B) or paw withdrawal threshold in log grams (C, D). Asterisks indicate that both formalin and CFA produced paw swelling [t (4) = 45.09, p<0.0001 and t (8) = 8.632, p<0.0001, respectively] and mechanical allodynia [t (4) = 4.785 p=0.0087 and t (8) = 3.933 p=0.0043 respectively] as assessed seven days after formalin or CFA treatment. All data show mean ± SEM for five-nine rats.
Discussion

**Effects of signal intensity and scopolamine treatment.** The present results agree with previous reports that response accuracy in VSDT procedures is dependent on signal intensity and impaired by scopolamine pretreatment (Bushnell et al., 1997; Rezvani et al., 2009). For example, in the present study, scopolamine displayed similar potencies to significantly reduce accuracy and increase response latency during both signal and blank trials, and it had three-fold weaker potency to increase response omissions. An identical profile of scopolamine effects was reported by Bushnell et al (1997) in a VSDT procedure using slightly different parameters for background and signal light intensities. Scopolamine also impaired accuracy of performance in other assays of attention in rodents, including a 5-choice serial reaction time task (Mishima et al., 2002), and it has also been found to impair measures of attention in human tests (Ellis et al., 2006). These findings confirm sensitivity of VSDT performance in the present study to an established positive control, and these scopolamine effects also provide a context for evaluating effects of noxious stimuli.

**Effects of noxious stimuli.** Two of the three noxious stimuli tested in this study also altered VSDT performance. Each noxious stimulus will be discussed in turn. First, i.p. lactic acid administration significantly increased both response latencies and omissions in this study, but unlike scopolamine, i.p. acid failed to significantly alter response accuracy during either signal or blank trials. Similar concentrations of i.p. acid in rats have also been reported to depress operant responding for electrical brain stimulation in an intracranial self-stimulation (ICSS) procedure and to stimulate a stretching response (Pereira Do Carmo et al., 2009). Moreover, both acid-induced depression of ICSS and stimulation of stretching appear to be related to pain, because both effects are blocked by clinically effective analgesics including the mu opioid
agonist morphine and the nonsteroidal anti-inflammatory drug ketoprofen (Pereira Do Carmo et al., 2009; Negus, 2013; Rosenberg et al., 2013). Overall, these results suggest that pain-like effects produced by i.p. acid administration in rats are sufficient to produce a nonselective decrease in operant behavior but not to produce a disruption in response-accuracy measures of attention in the VSDT procedure.

Of the three noxious stimuli tested here, intraplantar formalin produced a transient disruption of VSDT performance that most closely resembled the effects of scopolamine. Thus, when tested 15 min after administration, intraplantar formalin produced a small but significant decrease in response accuracy during both signal and blank trials as well as a significant increase in response latency during blank trials. However, four caveats warrant mention. First, the effects of formalin on response accuracy were relatively small insofar as their magnitude was less than or equal to the magnitude of effects produced by the intermediate dose of 0.1 mg/kg scopolamine. In particular, during signal trials, formalin-induced disruption was significant only when evaluated at the highest two signal intensities (Figure 2), and this effect did not achieve statistical significance when collapsed across all signal intensities (Figure 3). Second, in contrast to the effects of the intermediate scopolamine dose, the formalin-induced decrease in accuracy was accompanied by a significant increase in omissions during blank trials. Third, the formalin effects were transient, and were not evident three or seven days after formalin administration. This corresponds to the period of the “first and second phases” of the formalin response (1-2 hr after formalin administration) during which rats display vigorous paw flinching and licking responses (Tjølsen et al., 1992; Abbot et al., 2002; Fu et al., 2001). However, disruptions in VSDT performance were not evident at later times when paw edema, mechanical allodynia, and depression of ICSS are present (present study; Fu et al., 2001; Grace et al., 2014).
Finally, the present results agree with a previous report that intraplantar formalin disrupted performance in a 5-choice serial reaction time task in rats when testing occurred immediately after formalin injection (Boyette-Davis et al., 2008). However, in that study, formalin increased omissions but did not affect either response accuracy or response latency. Taken together, these results suggest that intraplantar formalin is sufficient to produce a transient and scopolamine-like disruption in performance, but the more sustained manifestations of pain-like behavior produced by formalin do not produce sustained disruption of any measure of VSDT performance.

Lastly, intraplantar CFA failed to alter any measure of VSDT performance. Again, this lack of effect cannot be attributed to inadequate CFA dosing, because this CFA treatment did produce sustained paw edema and mechanical allodynia, and similar CFA treatments have also been shown to produce transient decreases in ICSS and more sustained decreases in wheel running in rats (present study; Grace et al., 2014; Leitl et al., 2014). These results with intraplantar CFA differ from results of a previous study that found both decreased accuracy and increased omissions for 10 days after intra-articular administration of CFA in rats responding under a 5-choice serial reaction time task (Pais-Vieira et al., 2009). This difference may reflect greater sensitivity of the 5-choice serial reaction time task than the VSDT to sustained pain, greater intensity of pain after intra-articular than intraplantar CFA, or other procedural differences (e.g. strain of rat studied: Sprague-Dawley vs. Lister hooded). However, CFA effects in that study were not blocked by the clinically effective nonsteroidal anti-inflammatory drug carprofen that did block mechanical allodynia, suggesting that effects of CFA on performance may not have been related to pain. Results of the present study do not provide evidence for a CFA-induced disruption of attention.
In the present study, both i.p. lactic acid and bilateral intraplantar formalin functioned as noxious stimuli that acutely disrupted performance in a VSDT that has been used to assess modulators of attention in rats. These results may be related to clinical observations of pain-related disruption of attention in humans; however, the profiles of effects produced by i.p. acid and intraplantar formalin were transient and occurred during the time of maximal nociceptor activation and maximal stimulation of pain-related behaviors (i.e. the stretching response after i.p. acid or paw flinching/licking responses after i.pl. formalin). Notably, disruptions in VSDT performance were not evident at later times after formalin treatment, or at any time after i.pl. CFA treatment, despite the presence of both paw edema and mechanical hypersensitivity. As such, these results suggest that relatively strong activation of nociceptive pathways is required to disrupt performance in this version of the VSDT.

Several aspects of noxious stimulus effects on VSDT performance complicate the usefulness of this procedure for evaluation of drug effects on pain-related impairment of cognition. First, effects of all noxious stimuli tested here were transient, lasting no more than one session. Consequently, this procedure is not useful for studies of chronic pain-related depression of performance, and it would be impracticable to invest time and resources in training rats under this procedure if they can be tested only once with putative chronic pain stimuli like intraplantar CFA or formalin. Second, the noxious stimuli used here had little effect on the primary dependent variable of interest regarding attentional performance (i.e. accuracy of responding expressed as percent correct responses). Rather, the primary effect of noxious stimuli was an increase in response latencies and omissions, which suggest generalized depression of response rates rather than selective decreases in response accuracy. Lastly, we attempted to conduct studies with candidate analgesics such as ketoprofen on acid-induced
increases in response latency and omissions. In conducting these studies, we found that effects of acid alone became less reliable with repeated treatments, and although ketoprofen was effective to block acid effects in those rats that continued to show acid effects, other rats displayed apparent tolerance to acid effects and could not be used to evaluate blockade of those effects by analgesics or other drugs. In view of these complications, we determined that further studies to evaluate nAChR agonist effects on pain-related impairment of VSDT performance would not be practicable.
Nicotine is able to alleviate pain-depressed behavior. Previous preclinical studies have implicated $\alpha_4\beta_2^*$ and $\alpha_7$ nAChRs as potential mediators of the antinociceptive effects of nicotine and other nAChR agonists; however, these studies have relied exclusively on measures of pain-stimulated behavior (behaviors that increase in frequency, rate or intensity after presentation of a noxious stimulus). Pain is also associated with depression of many behaviors, and drug effects can differ in assays of pain-stimulated vs. pain-depressed behavior (behaviors that decrease in frequency, rate or intensity after presentation of a noxious stimulus). In our studies, nicotine produced a dose-dependent, time-dependent and mecamylamine-reversible blockade on both measures of pain-stimulated and pain-depressed behaviors. The selective $\alpha_4\beta_2^*$ agonist 5-I-A-85380 also blocked both measures of pain-stimulated and pain-depressed behaviors, whereas the selective $\alpha_7$ agonist PNU 282987 produced no effect in either procedure. This is the first study to examine effects of nAChR agonists in an assay of pain-depressed behavior, and drug effects in the assay of acid-depressed ICSS were qualitatively similar to effects in the assay of acid-stimulated stretching. Our results suggest that $\alpha_4\beta_2^*$ nAChR agonists may be especially effective to treat signs of pain-related behavioral depression; however, nonselective behavioral effects of these compounds may contribute to apparent antinociception.

Behavioral mechanisms. In the present study, the low doses of nicotine and 5-I-A-85380 that blocked acid-induced depression of ICSS have also been shown to increase other behaviors, whereas the high doses of these compounds that reduced acid-stimulated stretching have also
been shown to depress other behaviors. For example, nicotine at doses up to 0.1-0.32 mg/kg produced dose-dependent increases in spontaneous locomotor activity, rates of food-maintained operant responding, and rates of ICSS, whereas doses ≥ 0.1-0.32 mg/kg decreased rates of all these behaviors (Clarke and Kumar, 1983; Goldberg et al., 1989; Cohen et al., 1991; Huston-Lyons and Kornetsky, 1992; Bauco and Wise, 1994; Whiteaker et al., 1995; Spiller et al., 2009). Effects of 5-I-A-85380 have not been examined in these other procedures in rats; however, like nicotine in the present study, 5-I-A-85380 was only slightly more potent to block acid-induced depression of ICSS than to increase control ICSS rates in the absence of the acid noxious stimulus, and 5-I-A-85380 decreased acid-stimulated stretching only at high doses ≥ 1.0 mg/kg that also produced audible and labored breathing (1.0 mg/kg) or lethality in some animals (3.2 mg/kg). These results suggest that non-selective behavioral activation may have contributed to apparent nicotine and 5-I-A-85380 antinociception in the assay of acid-depressed ICSS, and non-selective behavioral impairment may have contributed to apparent antinociception by these compounds in the assay of acid-stimulated stretching.

**Pharmacological mechanisms.** In our studies, all drugs were also tested for their effects on ICSS in the absence of noxious stimulation, and results are consistent with previous studies of nAChR agonist effects on ICSS. For example, previous studies have also shown that ICSS was facilitated after treatment with low but not high nicotine doses or with the α4β2* agonist SIB-1765F, whereas α7 agonists such as ARR-17779 did not alter ICSS (Bauco and Wise, 1994; Panagis et al., 2000; Spiller et al., 2009). Insofar as drug-induced facilitation of ICSS is often interpreted as evidence of abuse liability (Negus and Miller, 2014), the present results are consistent with the conclusion that abuse liability of α4β2* agonists may be one factor that limits their utility as candidate analgesics. A broad range of 5-I-A-85380 doses produced facilitation
of low ICSS rates maintained by low brain-stimulation frequencies, and this effect was blocked by both the nonselective nAChR antagonist mecamylamine and the selective α4β2* antagonist dihyrdo-β-erythroidine (DHβE). Conversely, nicotine produced weaker ICSS facilitation across a narrower range of doses, and higher nicotine doses decreased high rates of ICSS maintained by high brain-stimulation frequencies. Studies to antagonize nicotine-induced facilitation of ICSS were not attempted in the present study because this nicotine effect was deemed too small to permit reliable assessment of antagonism; however, our results with 5-I-A-85380 are consistent with previous reports that mecamylamine and DHβE also block nicotine-induced facilitation of ICSS (Huston-Lyons & Kornetsky, 1992; Sagara et al., 2008). The rate-decreasing effects of a high nicotine dose were blocked by mecamylamine but not DHβE. This finding is consistent with other evidence to suggest effectiveness of mecamylamine but not DHβE to block other signs of reduced motor activity by high nicotine doses. In rats, for example, mecamylamine but not DHβE blocked decreases in locomotion and decreases in food-maintained operant responding produced by high nicotine doses (Stolerman, Chandler, Garcha, & Newton, 1997). Therefore our results are consistent with the conclusion that the rate-increasing effects of 5-I-A-85380 and low-to-intermediate nicotine doses are mediated by α4β2* receptors, whereas the rate-decreasing effects of higher nicotine doses are mediated primarily by non-α4β2* nAChRs. Ideally, complementary studies would be conducted with an antagonist to selectively block the rate-decreasing effects of nicotine. However, the nAChR subtype mediating these rate-decreasing effects has not been precisely determined, and selective pharmacological antagonists are not yet available for the α5* receptors implicated by studies using genetic manipulations (Fowler & Kenny, 2014; Fowler et al., 2013).
As mentioned above, nicotine produced a dose-dependent, time-dependent and mecamylamine-reversible blockade of both acid-stimulated stretching and acid-induced depression of ICSS. 5-I-A-85380 also blocked both acid-stimulated stretching and acid-induced depression of ICSS, whereas PNU 282987 produced no effect in either procedure. Our agonism studies suggest that activation of α4β2* nAChR is sufficient to produce antinociception of both acid-stimulated stretching and acid-induced depression of ICSS. However, we have not tested DHBE with nicotine or 5-I-A-85380 to investigate whether α4β2* nAChR agonism is necessary in producing antinociception in acid-stimulated stretching and acid-induced depression of ICSS. Cheng et al (2011), showed that DHBE blocked the antinociceptive effects of TC-2559, a selective α4β2* nAChR partial agonist, in models of pain-stimulated behavior. A parallel observation was made upon combined administration of another selective α4β2* nAChR partial agonist, sazetidine, with the nonselective nAChR antagonist mecamylamine (Cucchiaro et al., 2008; Zwart, 2008). Several selective agonists of α4β2* nAChR showed a broad-spectrum efficacy in preclinical models of pain-stimulated behaviors. Previous preclinical studies have shown that α4β2* nAChR is important for antinociception while a non-α4β2* nAChR may also be important. For example, an increased expression of the α5* subunit following spinal nerve ligation contributed to the maintenance of mechanical allodynia in rats (Vincler and Eisenach, 2005). Additionally, α6* nAChRs are located both in primary sensory neurons and in components of the mesolimbic dopamine system, and activation of these receptors is associated both with antinociception in assays of pain-stimulated behavior and with neurochemical and behavioral evidence for stimulation of the mesolimbic dopamine system (Brunzell, 2012; Wieskopf et al., 2015). 5-I-A-85380 is commonly described as an α4β2*-selective agonist (Mukhin et al., 2000; Sihver et al., 2000; Liu et al., 2003; Liu, 2013) and results in this study
with 5-I-A-85380 were interpreted to suggest a key role for the α4β2* subtype of nAChR in mediating blockade of acid-induced depression of ICSS. However, 5-I-A-85380 also binds to α6* receptors, which are nAChRs that contain the α6 subunit instead of, or in addition to, α4 subunits (Kulak et al., 2002; Capelli et al., 2011). In view of these considerations, the present results do not exclude a role for α6* nAChRs in mediating effects of nAChR agonists on pain-depressed ICSS in rats.

In clinical studies ABT-594, a selective α4β2* nAChR agonist produced promising results in a phase two clinical trial after seven weeks treatment for neuropathic pain. However, high occurrence of adverse effects like nausea and dizziness were observed with this compound (Rowbotham et al., 2009). Subsequently, it was observed that side effects with ABT-594 were mediated through ganglionic α3β4* nAChR (Zhu et al., 2011). ABT-894, a selective α4β2* nAChR agonist with an improved therapeutic window, failed to alleviate pain in two phase two studies of diabetic neuropathic pain (Rueter et al., 2008, Rowbotham et al., 2012). The clinical trials of α4β2* nAChR agonists raise the possibility of additional nicotinic receptors involved in pain. The α5* and α6* subunits may play an important role because these subunits can co-assemble with α4β2* nAChR to form functional heteromeric nAChR receptors, and various agonists may have different sensitivity towards these nicotinic receptors. Therefore, a better understanding of the nicotinic receptors composition and function is crucial for the development of novel α4β2* nAChR compounds for the treatment of pain.

**Attention Impairment.** Nicotinic compounds have a wide range of potential therapeutic applications ranging from pain to cognitive disorders. For example, the selective α4β2* nAChR agonists ABT-894 showed promising effects in adults with attention deficit-hyperactivity disorder (ADHD) (Bain et al., 2013). Gatto et al (2004) reported promising phase one safety and
pharmacokinetic profile for the selective α4β2* nAChR partial agonist ispronicline on behalf of cognitive performance. Chapter IV of this dissertation aimed to develop and validate the visual signal-detection task (VSDT) as a novel assay to assess pain-related depression of a cognitive performance. Bushnell et al. (1994) developed the VSDT as an animal model of human sustained attention (Parasuraman, 1984) for the purposes of quantifying specific effects of toxic chemicals on cognitive function (Bushnell et al., 1994, 2002, 2007). Bushnell et al (2003) measured the behavior of humans in a task formally homologous to the task for rats after varying the increments in the intensity of a lamp (magnitude of the signal). As with rats, the proportion of correct detection of the signal (P(hit)) in humans increased with increased signal intensity. This provides evidence that both humans and rats demonstrated similar performance trends by responding to changes in task in a similar manner. These studies were included in this dissertation because clinical studies indicate that pain can impair attention and because nAChR agonists alleviate cognitive impairment under various conditions. For example, the performance of cognitive tasks can be impaired during pain (Crombez et al., 1997; Lorenz and Bromm, 1997), and patients with chronic pain often report cognitive deficits including attention and memory deficits (Dick et al., 2002; Grace et al., 1999). We therefore decided to test whether the VSDT is an appropriate assay for initial studies to assess pain-depressed cognitive behavior.

The results presented in chapter IV agree with previous reports that response accuracy in VSDT procedures is dependent on signal intensity and impaired by scopolamine pretreatment (Bushnell et al., 1997; Rezvani et al., 2009). Two of the three noxious stimuli tested in this study also altered VSDT performance. Both i.p. lactic acid and bilateral intraplantar formalin functioned as noxious stimuli that disrupted performance in the VSDT. These results may be related to clinical observations of pain-related disruption of attention in humans; however, the
profiles of effects produced by i.p. acid and intraplantar formalin were transient and occurred
during the time of maximal nociceptor activation and maximal stimulation of pain-related
behaviors (i.e. the stretching response after i.p. acid or paw flinching/licking responses after i.pl.
formalin). Notably, disruptions in VSDT performance were not evident at later times after
formalin treatment, or at any time after i.pl. CFA treatment, despite the presence of both paw
edema and mechanical hypersensitivity. As such, these results suggest that relatively strong
activation of nociceptive pathways is required to disrupt performance in this version of the
VSDT. Because effects of noxious stimuli on VSDT performance were transient and variable,
we determined that this procedure was not useful for high-throughput evaluation of drug effects
on pain-related cognitive impairment. Accordingly, it will be necessary to develop other, more
sensitive procedures to evaluate effects of nAChR agonists or other drugs on pain-related
impairment of cognition.

**Future Directions.** Although these results suggest that α4β2* nAChR agonists may be
especially effective to treat signs of pain-related behavioral depression, more pharmacological
studies are needed. Antagonism studies of 5-I-A-85380 must be further studied. For example, 5-
I-A-85380 is commonly described as an α4β2*-selective agonist (Mukhin et al., 2000; Sihver et
al., 2000; Liu et al., 2003; Liu, 2013) and results in this study with 5-I-A-85380 were interpreted
to suggest a key role for the α4β2* subtype of nAChR in mediating blockade of acid-induced
depression of ICSS. Consequently, antagonism studies with the α4β2*-selective antagonist
DHßE would provide additional evidence to suggest a key role for the α4β2* subtype in
mediating antinociception in the assays of acid-induced depression of ICSS and stimulation of
stretching.

Additionally, 5-I-A-85380 also binds to α6* receptors (Kulak et al., 2002; Capelli et al.,
α6* nAChRs are located both in primary sensory neurons and in components of the mesolimbic dopamine system, and activation of these receptors is associated both with antinociception in assays of pain-stimulated behavior and with neurochemical and behavioral evidence for stimulation of the mesolimbic dopamine system (Brunzell, 2012; Wieskopf et al., 2015). In view of these considerations, studies with selective compounds for α6* receptors would also be important to investigate in acid-induced depression of ICSS. Selective α6* agonists are not currently available, however as one step toward distinguishing the roles of α4β2* and α6* nAChRs-mediated effects in antinociception, we tested effects of the novel compound +PHT in assays of pain-stimulated and pain-depressed behavior. +PHT is a low-efficacy ligand at α4β2* nAChRs and a high-efficacy ligand at α6* nAChRs and may produce agonist effects mediated primarily by α6* nAChRs in vivo (Carroll et al., 2015). In our studies, +PHT dose-dependently blocked acid-stimulated stretching, but unlike nicotine or 5-I-A-85380, it did not facilitate control ICSS or block acid-induced depression of ICSS. At this point, it is unclear if the poor effectiveness of +PHT to facilitate ICSS or block acid-induced depression of ICSS reflects the low efficacy of +PHT at α4β2* nAChRs, the high efficacy of +PHT at α6* nAChRs, or some other mechanism of +PHT effects. In general, though, these results do not support a role for α6* nAChRs in mediating antinociceptive effects of nicotine or 5-I-A-85380 in assays of pain-depressed behavior.

Lastly, the treatment of chronic pain is probably one of the most common and yet particularly difficult aspects of medical practice, and it would be useful to evaluate effects of nAChR agonists on chronic pain-related depression of behavior. In an effort to address this issue as part of this project, we conducted preliminary studies to evaluate effects of nicotine on sustained depression of ICSS produced by intraplantar formalin administration. Formalin is an
aqueous solution of formaldehyde (a cell toxin that cross links proteins to disrupt dynamic protein interactions critical to cell viability) that produces cell death including primary nociceptors and other sensory neurons associated with neuropathic pain. In our preliminary studies with intraplantar formalin we observed depression of ICSS, and formalin-induced depression of ICSS was dose-dependently blocked by nicotine (0.01-0.032 mg/kg). However, in a subsequent study, formalin failed to depress ICSS in enough rats to permit pharmacological assessment of nicotine effects, and further resources could not be devoted to this study. Our laboratory is currently studying different noxious stimuli in ICSS behavior that are commonly used to produce chronic pain in rodent models of pain-stimulated behaviors. After establishing chronic pain-depressed behavior using ICSS procedure, further studies exploring nicotinic drugs would be helpful in understanding precisely drug effects on chronic pain.
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