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

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ABSTRACT

Agonists at nicotinic acetylcholine receptors (nAChRs) constitute one drug class being evaluated as candidate analgesics. Previous preclinical studies have implicated \( \alpha_4\beta_2 \) and \( \alpha_7 \) nAChRs as potential mediators of the antinociceptive effects of \((-\)-nicotine hydrogen tartrate (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 versus pain-depressed behavior. Accordingly, this study compared the effects of nicotine, the selective \( \alpha_4\beta_2 \) agonist 5-(123I)iodo-3-[2(S)-2-azetidinylmethyl]pyridine (5-I-A-85380), and the selective \( \alpha_7 \) agonist N-(3R)-1-azabicyclo(2.2.2)oct-3-yl-4-chlorobenzamide 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 the operant responding, which is 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 N-(3R)-1-azabicyclo(2.2.2)oct-3-yl-4-chlorobenzamide produced no effect in either procedure. Both nicotine and 5-I-A-85380 were \( \geq 10 \)-fold more potent in blocking the acid-induced depression of ICSS than in blocking the acid-induced stimulation of stretching. These results suggest that stimulation of \( \alpha_4\beta_2 \) and/or \( \alpha_6\beta_2 \) nAChRs may be especially effective to alleviate the signs of pain-related behavioral depression in rats; however, non-selective behavioral effects may contribute to apparent antinociception.

Introduction

Agonists at nicotinic acetylcholine receptors (nAChRs) constitute one drug class being evaluated as candidate analgesics for the treatment of pain. Antinociceptive effects of \((-\)-nicotine hydrogen tartrate (nicotine), a relatively non-selective 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; Mishrity and Habib, 2014). Prevaling evidence suggests that nicotine antinociception is mediated at least in part by \( \alpha_4\beta_2 \) and/or \( \alpha_6\beta_2 \) nAChRs (i.e., nAChRs containing \( \alpha_4 \) and/or \( \alpha_6 \) subunits in addition to \( \beta_2 \) subunits, hereafter identified as \( \alpha_4\beta_2 \) nAChRs). For example, nicotine, epibatidine, and ABT-594 all produce antinociception in preclinical models (Flores, 2000) and function as potent and efficacious \( \alpha_4\beta_2 \) agonists (Donnelly-Roberts et al., 1998). Moreover, nicotine antinociception in rodents can be attenuated by the nonselective nAChR antagonist mecamylamine and the more selective \( \alpha_4\beta_2 \) antagonist dihydro-\( \beta \)-erythroidine (Cooley et al., 1990; Iwamoto, 1991; Damaj et al., 1995; Abdin et al., 2006) or by genetic deletion of \( \alpha_4 \) or \( \beta_2 \) nAChR subunits (Marubio et al., 1999). Lastly, a recent study presented evidence to suggest an important role for \( \alpha_6 \) receptors in mediating both nociception and nicotine antinociception in mice (Wieskopf et al., 2015).

In addition to \( \alpha_4\beta_2 \), the \( \alpha_7 \) nAChR subtype may also contribute to the 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 \( \alpha_4\beta_2 \) versus \( \alpha_7 \) nAChRs, and the selective \( \alpha_7 \) 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 \( \alpha_7 \) nAChRs \((\text{meta-chlorophenylglycine})\) antagonized nicotine antinociception in a rat tail-flick procedure (Dukat et al., 2010). Moreover, \( \alpha_7 \) nAChR selective agonists produced antinociception in some rodent models of acute and pain-related behaviors; however, non-selective behavioral effects may contribute to apparent antinociception.
inflammatory pain (Damaj et al., 1998, 2000; Wang et al., 2005; Gao et al., 2010), and α7 positive allosteric modulators produced antinociception in mouse models of inflammatory and neuropathic pain (Freitas et al., 2013a,b).

Preclinical studies to evaluate the 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, the 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 the expression of pain-stimulated behaviors can reflect the impaired ability to emit the motor response rather than reduce sensitivity to the noxious stimulus. In contrast, 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 or rates of positively reinforeced operant behavior). Pain-depressed behaviors play a key role in pain diagnosis in human and veterinary medicine (Cleeland and Ryan, 1994), and the incorporation of procedures that measure pain-depressed behaviors may improve the translational validity in tests of candidate analgesics (Negus et al., 2006, 2010a).

The effects of nAChR agonists have not been examined in assays of pain-depressed behavior. Accordingly, this study compared the effects of nicotine and selective α4/6β2 and α7 nAChR agonists in assays of acute pain-stimulated and pain-depressed behavior that have been used previously to examine the preclinical antinociceptive effects of other drugs, including nonsteroidal anti-inflammatory drugs (Leitl et al., 2014), mu-, delta-, and kappa-opioids (Negus et al., 2010b, 2012; Altarifi et al., 2015), monoamine uptake inhibitors (Rosenberg et al., 2013; Miller et al., 2015), and cannabinoids (Kwilsaz and Negus, 2012; Kwilsaz et al., 2014). Specifically, an i.p. injection of dilute lactic acid was used as an acute chemical noxious stimulus to stimulate a stretching response and depress the operant responding in an intracranial self-stimulation (ICSS) procedure in rats. The antinociceptive effects of nicotine in both procedures were compared with the effects of the α4/6β2-selective agonist 5-(123I)iodo-3-[2(S)-2-azetidinylmethoxy]pyridine (5-I-A-85380) (Kulak et al., 2002, Capelli et al., 2011, Mukhin et al., 2006; Liu et al., 2003; Liu, 2013) and the α7-selective agonist N-(3R)-1-azabicyclo(2.2.2)oct-3-yl-4-chlorobenzamide (PNU 282987) (Hajós et al., 2005; McLean et al., 2011).

Methods and Materials

Subjects

Male Sprague-Dawley rats (Harlan, Fredrick, MD) weighing 310–350 g at the time of surgery were individually housed and maintained on a 12-hour 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.

ICSS Behavioral Procedure

Surgery. Rats were anesthetized with isoflurane (2.5–3% in oxygen; Webster Veterinary, Phoenix, AZ) for 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 the 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 7 days prior to commencing ICSS training.

Apparatus. Experiments were conducted in sound attenuating chambers that contained modular acrylic test chambers (29.2 x 30.5 x 24.1) equipped with a response lever (4.5 cm wide, extended 2.0 cm through the center of one wall and 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 Associate controlled the stimulator, programming parameters, and data collection.

Training Procedure. Rats were trained under a fixed-ratio 1 schedule of brain stimulation using procedures similar to those described previously for studies with other drugs, including nonsteroidal anti-inflammatory drugs (Leitl et al., 2014), opioids (Negus et al., 2010b, 2012; Altarifi et al., 2015), monoamine uptake inhibitors (Rosenberg et al., 2013; Miller et al., 2015), and cannabinoids (Kwilsaz and Negus, 2012; Kwilsaz et al., 2014). Each lever press resulted in the delivery of a 0.5-second train of square wave cathodal pulses (0.1-millisecond pulse duration), and stimulation was accompanied by illumination of the stimulus lights above the lever. Responses during the 0.5-second stimulation period did not result in additional stimulation. During the initial phase of training, sessions lasted 30 to 60 minutes, the frequency of stimulation was held constant at 158 Hz, and the stimulation intensity was adjusted to the lowest value that would maintain reinforcement rates of at least 30 stimulations per minute. Frequency manipulations were then introduced during sessions that consisted of sequential 10-minute components. During each component, a descending series of 10 current frequencies (158–56 Hz in 0.05 log increments) was presented, with a 60-second trial at each frequency. A frequency trial began with a 5-second time out followed by a 5-second “priming” phase, during which five noncontingent stimulations were delivered at a rate of one per second. This noncontingent stimulation was followed by a 50-second “response” phase, during which the responding produced an electrical stimulation under a fixed-ratio 1 schedule. Training continued with 3–12 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 3 consecutive days. This intensity (range: 110–250 μA) was held constant for the remainder of the study.

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 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 the baseline parameters of the frequency-rate curves for that session (see Data Analysis). During the treatment interval, rats were removed from the ICSS chambers, administered the 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 the stretching studies (see below) and

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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; Freitas, et al., 2015) and were as follows: nicotine (vehicle, 0.01–0.1 mg/kg; 10-minute pretreatment), 5-L-A-85380 (vehicle, 0.01–0.1 mg/kg; 30-minute pretreatment), and PNU 282987 (vehicle, 3.2–32 mg/kg; 30-minute pretreatment). Nicotine and 5-L-A-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. The dose order was randomized across rats using a Latin-square design. Each week, a rat was tested with a given dose of the test drug in combination with the 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 the 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 the different pretreatment time intervals between nicotine and 1.8% lactic acid (30, 100, and 300 minutes), 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-minute 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 minutes before nicotine (0.1 mg/kg) or its vehicle, followed 10 minutes later by a 1.8% lactic acid injection immediately before testing. The 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 the 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 = [rate during a frequency trial/MCR] × 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 analysis of variance (ANOVA) was used to compare the 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, percent 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 a paired t test or one-way ANOVA as appropriate. A significant ANOVA was followed by Dunnett’s 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 the drug + acid, acid was the total number of stimulations after acid alone, and baseline was the total number of stimulations in the absence of the drug or acid. For all drugs producing greater than 50% acid blockade, linear regression was used to calculate ED_{50} and 95% confidence limits, with ED_{50} defined as the effective dose producing 50% acid blockade.

Lactic Acid–Stimulated Stretching Behavioral Procedure

During test sessions, a dose of the 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 × 20.1 × 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-L-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 to eight rats. The dose order was randomized using a Latin-square design, and testing was conducted once per week.

After completion of the dose-effect studies, additional time-course and antagonism studies were conducted in the nicotine group. For the time course studies, test sessions consisted of an injection of nicotine (1.0 mg/kg) followed 10, 30, 100, or 300 minutes later by an acid injection immediately before testing. A dose of 1.0 mg/kg nicotine was chosen for the 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 minutes before 1.0 mg/kg nicotine or its vehicle, and acid was delivered 10 minutes after nicotine. The 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 Tukey’s post hoc test, and the criterion for significance was set at P < 0.05. For drugs producing a greater than 50% reduction in stretching relative to vehicle treatment, linear regression was used to calculate ED_{50} and 95% confidence limits, with ED_{50} defined as the effective dose to reduce stretching to 50% of vehicle control. ED_{50} values were considered to be significantly different if 95% confidence limits did not overlap.

Drugs

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

Results

Effects of Nicotine, 5-L-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 1A shows that stretching
was significantly lower 10 minutes after administration of 0.1, 0.32, and 1.0 mg/kg nicotine than after nicotine vehicle, and the nicotine ED\textsubscript{50} value is reported in Table 1.

Figure 1B shows that 1.0 mg/kg nicotine produced a significant reduction in acid-stimulated stretching from 10 to 100 minutes after its administration. Figure 1C shows that 1.0 mg/kg of mecamylamine significantly blocked the antinociceptive effect of 1.0 mg/kg nicotine.

Figure 2 shows that the \(\alpha_4/\beta_2\)-selective agonist 5-I-A-85380, but not the \(\alpha_7\)-selective agonist PNU 282987, also produced a dose-dependent decrease in acid-stimulated stretching. Figure 2A shows that stretching was significantly lower 30 minutes after administration of 1.0 and 3.2 mg/kg 5-I-A-85380 than after the 5-I-A-85380 vehicle, and the ED\textsubscript{50} value is shown in Table 1. 5-I-A-85380 was significantly less potent than after the 5-I-A-85380 vehicle, and the ED\textsubscript{50} value is reported in Table 1. Because published data showed that nicotine was significantly more potent in blocking acid-induced depression of ICSS than in blocking acid-stimulated stretching, 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 Fig. 4C, and the nicotine ED\textsubscript{50} value is reported in Table 1. Nicotine was significantly more potent in blocking acid-induced depression of ICSS than in blocking acid-stimulated stretching. Nicotine doses higher than 0.1 mg/kg were tested in pilot studies in some rats; however, these doses decreased rates of ICSS, and systemic studies with higher doses were not pursued.

Figure 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 minutes (Fig. 4), and Fig. 5A shows that 0.1 mg/kg nicotine also significantly attenuated acid-induced depression of ICSS after 30 minutes but not after later pretreatment times. Figure 5B shows that the 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.

**TABLE 1**

<table>
<thead>
<tr>
<th>Class</th>
<th>Acid-Stimulated Stretching</th>
<th>Acid-Depressed ICSS</th>
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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)(^a)</td>
<td>0.012 (0.006–0.021)</td>
</tr>
<tr>
<td>PNU 282987</td>
<td>Inactive</td>
<td>Inactive</td>
</tr>
</tbody>
</table>

\(^a\)Significantly different from nicotine ED\textsubscript{50} within a given assay.

\(^x\)Significantly different from the ED\textsubscript{50} for that drug in the assay of acid-stimulated stretching.
Figure 6 shows the 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 in blocking acid-induced depression of ICSS than in facilitating control ICSS in the absence of the acid noxious stimulus (Fig. 6, A–C). The ED50 value for 5-I-A-85380 is shown in Table 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 in blocking acid-induced depression of ICSS than in 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 (Fig. 6, D–F).

Discussion

This study compared the effects of nicotine with the effects of selective α4/6β2 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 α4/6β2 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

Fig. 4. Effects of nicotine on control and acid-depressed ICSS. ICSS frequency-rate curves determined when nicotine (0.01–0.1 mg/kg) was administered as a pretreatment to lactic acid (LA) vehicle (Veh) (A) or 1.8% LA (B). The abscissa is the frequency of electrical brain stimulation in Hertz (log scale). The ordinates are %MCR. Filled symbols indicate a significant difference from nicotine Veh + LA Veh in (A) or nicotine Veh + 1.8% LA in (B) as determined by Holm-Sidak post hoc test; $P < 0.05$. All points show the 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 ($F_{9,45} = 117.5; P < 0.0001$) and nicotine dose ($F_{3,15} = 7.67; P = 0.002$) but not a significant interaction ($F_{27,135} = 1.54; P = 0.06$); (B) significant main effects of frequency ($F_{9,45} = 44.16; P < 0.0001$) and nicotine dose ($F_{3,15} = 7.83; P = 0.002$) and a significant interaction ($F_{27,135} = 1.71; P = 0.02$). (C) Summary data for the nicotine effects on the total number of stimulations per component when nicotine was administered as a pretreatment to the acid vehicle (open bars) or 1.8% lactic acid (filled bars). The abscissa are the dose of nicotine in milligrams per kilogram. The ordinate is the percent baseline number of stimulations per component. *$P < 0.05$ compared with Nic Veh + LA Veh as determined by paired t test. Upward arrows indicate that nicotine produced a significant increase in ICSS at one or more frequencies in the analysis of the full frequency-rate curves in (A and B). All bars show the mean ± S.E.M. in six rats.

Fig. 5. Time course and mecamylamine (Mec) antagonism of 0.1 mg/kg nicotine (Nic) antinociception in the assay of acid-depressed ICSS. (A) The abscissa is the pretreatment interval between the 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% LA (Veh + 1.8% LA) are also shown for comparison, with the nicotine vehicle administered 10 minutes before the acid vehicle or acid. The ordinate is the percentage of baseline total number of stimulations per component. *$P < 0.02$ compared with Veh + Veh as determined by paired t test ($t_5 = 3.33$). One-way ANOVA of data in the filled bars indicated a significant effect of time ($F_{3,15} = 3.90; P = 0.03$). $P < 0.05$ compared with Veh + 1.8% LA as determined by Dunnett’s post hoc test. All bars show the mean ± S.E.M. in six rats. (B) Effects of 15-minute pretreatment with Mec (1.0 mg/kg) or its vehicle and 10-minute pretreatment with Nic (0.1 mg/kg) or its vehicle before acid treatment. Effects of mecamylamine vehicle + nicotine vehicle + acid vehicle are included for comparison. The abscissa is treatment with 1.0 mg/kg Mec, 0.1 mg/kg Nic, 1.8% LA, or their respective vehicles. The ordinate is the percentage of baseline total number of stimulations per component. *$P = 0.006$ compared with Veh + Veh + Veh as determined by paired t test ($t_6 = 4.17$). One-way ANOVA of data in the filled bars indicated the significant main effect of treatment ($F_{3,18} = 14.18; P < 0.0001$). $P < 0.05$ compared with Veh + Veh + 1.8% LA as determined by Dunnett’s post hoc test. All bars show the mean ± S.E.M. in seven rats.
mecamylamine antagonism (Sahley and Berntson, 1979; AlSharari et al., 2012). The effectiveness of 5-I-A-85380 to block acid-stimulated stretching is also consistent with previous reports of antinociception by A-85380 and other $\alpha_{4/6}$ agonists in assays of pain-stimulated behavior. 5-I-A-85380 was created by the 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 agonists with selectivity for $\alpha_{4/6}$ receptors, 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 $\alpha_{7}$ nAChRs may also be sufficient to produce antinociception in rodents. For example, intrathecal or intracerebroventricular administration of the $\alpha_{7}$ 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 $\alpha_{7}$ agonists. For example, intravenous choline was not effective in a hot-plate assay in mice, and although the other $\alpha_{7}$ agonist SSR-180711 reduced formalin-induced licking and flinching in rats, the effect was attributed to a nonspecific reduction of movement (Wang et al., 2005; Gao et al., 2010). In the present study, PNU 282987 failed to produce antinociception at doses of 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 $\alpha_{7}$ agonists in rats.
nAChR Agonist Effects on Acid-Depressed ICSS. This is the first study to examine the effects of nAChR agonists in an assay of pain-depressed behavior, and the drug effects in the assay of acid-depressed ICSS were qualitatively similar to the 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 the 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., 2014; Altarifi et al., 2015). Moreover, the effects of nicotine and 5-I-A-85380 differed from the effects of some other drugs, including kappa-opioid receptor agonists (Negus, et al., 2010b, 2012; Leitl et al., 2014) and cannabinoid receptor agonists (Kwilas and Negus, 2012; Kwiklas et al., 2014), which 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 the 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 in blocking acid-induced depression of ICSS. Specifically, nicotine was 10 times more potent and 5-I-A-85380 was 100 times more potent in blocking acid-induced depression of ICSS than in blocking acid-stimulated stretching. This differs from the effects of morphine and most other mu-opioid analgesics, which display similar potencies in these two assays (Pereira Do Carmo et al., 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 of 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; Freitas et al., 2015). The effects of 5-I-A-85380 have not been examined extensively in these other procedures in rats, although we recently reported that 5-I-A-85380 doses of up to 1.0 mg/kg produced mecamylamine- and dihydro-β-erythroidine–reversible facilitation of ICSS in the absence of a noxious stimulus (Freitas et al., 2015). Moreover, like nicotine in the present study, 5-I-A-85380 was only slightly more potent in blocking acid-induced depression of ICSS than in increasing 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, which 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 nonselective behavioral activation may have contributed to apparent nicotine and 5-I-A-85380 antinociception in the assay of acid-depressed ICSS, and nonselective behavioral impairment may have contributed to apparent antinociception by these compounds in the assay of acid-stimulated stretching. This evidence for nonselective 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; Mishrikiy and Habib, 2014).

nAChR Agonist Effects on ICSS in the Absence of Acid. This study focused primarily on the effectiveness of nAChR agonists to block acid-induced depression of ICSS; however, all the drugs were also tested for their effects on ICSS in the absence of noxious stimulation and the 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 5-I-A-85380 or the other α4/6β2 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; Freitas et al., 2015). 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 α4/6β2 agonists may be one factor that limits their utility as candidate analgesics.

Role for α6* Receptors. 5-I-A-85380 is often described as an α4β2-selective agonist (Mukhin et al., 2000; Siiver et al., 2000; Liu et al., 2003; Liu, 2013), and the results of this study support previous evidence to implicate α4β2 receptors in antinociception by nAChR agonists. 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 in both primary sensory neurons and components of the mesolimbic dopamine system, and activation of these receptors is associated with both antinociception in assays of pain-stimulated behavior and 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 the effects of nAChR agonists on pain-depressed ICSS in rats.

Authorship Contributions
Participated in research design: Freitas, Carroll, Negus.
Conducted experiments: Freitas.
Performed data analysis: Freitas, Negus.
Wrote or contributed to the writing of the manuscript: Freitas, Carroll, Negus.

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