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THE EFFECT OF SAZETIDINE-A AND OTHER NICOTINIC LIGANDS ON NICOTINE CONTROLLED GOAL-TRACKING IN FEMALE AND MALE RATS

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Abstract

Nicotine is the primary addictive component of tobacco products and its complex stimulus effects are readily discriminated by humans and non-human animals. Previous preclinical research investigating directly the nature of the nicotine stimulus has been limited to male rodents. The current study began to address this significant gap in the literature by training female and male rats to discriminate 0.4 mg/kg nicotine from saline in the discriminated goal-tracking task. In this task, access to sucrose was intermittently available on nicotine session. On saline session, intermixed with nicotine sessions on separate days, sucrose was not available. Both sexes acquired the discrimination as evidenced by increased head entries into the sucrose receptacle (goal-tracking) evoked by nicotine. The nicotine generalization curves were also similar between females and males. The pharmacological profile of the nicotine stimulus was assessed using substitution and targeted combination tests with the following compounds: sazetidine-A, PHA-543613, PNU-120596, bupropion, nornicotine, and cytosine. For both sexes, nornicotine fully substituted for the nicotine stimulus, whereas sazetidine-A, bupropion, and cytosine produced partial substitution. Female and male rats also responded similarly in interaction tests where a combination of 1 mg/kg of sazetidine-A plus nicotine or nornicotine shifted the nicotine dose-effect curve to the left; however, combinations of sazetidine-A plus bupropion or cytosine failed to produce this effect. These findings begin to fill a significant gap in our understanding of sex differences with respect to the nicotine stimulus and response to therapeutically interesting combinations using a model that includes both sexes.

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DISCLOSURE:

The authors declare no conflict of interest.

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INTRODUCTION

Smoking is the leading preventable cause of death in the United States, responsible for approximately 443,000 deaths annually (CDC, 2008). In spite of well-known health risks, nearly 70 million Americans continue to use tobacco products (NIDA, 2012). Most smokers, almost 70%, report that they desire to quit smoking (CDC, 2011). Gender can play an important role in this tenacious addiction, as well as in the success of smoking cessation treatments. Though fewer women smoke than men, the quit attempts of women are less likely to be successful compared to male counterparts, and there is increased sensitivity to social and behavioral factors related to tobacco use among women [for reviews see Perkins (2009) and Schnoll, Patterson, & Lerman (2007)]. There is no doubt that socio-environmental factors are likely the cause of some of these sex differences. However, the potential import of biological factors cannot be ignored (Arnold, 2009; Beery and Zucker, 2011; Cahill, 2006; Wetherington, 2007). These include the widespread effect of gonadal hormones early in development on the organization of the physiology including the nervous system, the differential impact of circulating gonadal hormones that vary across the lifespan of the individual, and the differential expression of X and Y genes in women and men (i.e., sex chromosome effects).

Preclinical models with rodents have also identified sex differences in the effects of nicotine further suggesting a biological mechanism for at least some of the differences. For example, females were more sensitive than males to the acute locomotor effects of nicotine (Harrod *et al*, 2004; Kanýt *et al*, 1999). This sex difference in the stimulant effects of nicotine was reduced in gonadectomized females (Booze *et al*, 1999; Kanýt *et al*, 1999). Further, female rats have higher rates of nicotine self-administration in comparison to males (Donny *et al*, 2000). Adult female rats also appear to be more sensitive to the reward-enhancing effects of nicotine and they will take more nicotine than males when a weak sensory reinforcer also follows lever pressing (Chaudhri *et al*, 2005). Consistent with this observation, in a place conditioning tasks, adult female rats were more sensitive than male rats to the conditioned rewarding effects of nicotine; evidence of conditioned reward was abolished in ovariectomized females (Torres *et al*, 2009). In the present study, we sought to extend the work on sex differences by investigating whether the nature of the nicotine stimulus differed between female and male rats.

Over the years, a variety of tasks have been developed to study the interoceptive stimulus effects of drugs in rodents and primates (Murray *et al*, 2011; Stoleran *et al*, 2009; Wooters *et al*, 2009). These drug discrimination tasks have served as powerful tools for understanding the behavioral and neuropharmacological processes of psychoactive substances. For the present study, we used the drug discriminated goal-tracking (DGT) task (Besheer *et al*, 2004) to study the interoceptive stimulus effects of nicotine. In the DGT task, rats have intermittent access to non-contingent liquid sucrose deliveries when given nicotine before the session. On intermixed saline sessions, sucrose is not available. A discrimination between nicotine and saline develops as evidenced by nicotine differentially evoking goal-tracking [i.e., “anticipatory” head entries into the site where sucrose was delivered; see Boakes (1977) and Farwell and Ayres (1979)]. This discrimination is specific to the

pharmacological effect of nicotine and does not reflect a drug vs no drug discrimination (Besheer *et al*, 2004; Murray and Bevins, 2009).

Sex differences involving interoceptive conditioning with the nicotine stimulus have not been studied in the DGT task. Further, after an extensive search of the two-lever drug discrimination literature (cf. Stoleran *et al*, 2009; Wooters *et al*, 2009), we could not find a published paper with rodents that used nicotine as the training stimulus and examined potential sex differences (cf. Bevins and Charntikov, 2015). The only published sex difference involving the nicotine stimulus in rodents that we found was that reported in Jung *et al*. (2000). In that report, male and female rats were trained to discriminate pentylentetrazol from saline. In substitution tests, nicotine prompted less pentylentetrazol-like responding in females than males. Albeit limited, this outcome, combined with the work from Perkins and colleagues in humans (Perkins, 1999; Perkins *et al*, 1996), indicates the need to better understand the nature of the nicotine stimulus in both sexes.

With this need in mind, the goal of the present study was to begin to fill this gap in the scientific literature on nicotine drug discrimination. Table 1 lists the ligands we used to better understand the nature of the nicotine stimulus in female and male rats, as well as the purported mechanism(s) of each ligand. The ligands were selected because of their action on nicotinic acetylcholine receptors [nAChR (e.g., sazetidine-A)] and/or their use in smoking cessation (e.g., bupropion). As detailed in the Methods section, we investigated the substitution pattern of bupropion, cytisine, nornicotine, sazetidine-A, PNU-120596, and PHA-543613 in the DGT task. Based on the results of our substitution tests, we used interaction tests to further inform us about pharmacological profile of selected nicotine-like ligands. Because there is a significant gap in understanding pharmacology associated with sazetidine-A's nicotine-like stimulus effect, we focused our interaction tests on combinations including sazetidine-A with nicotine, bupropion, nornicotine, or cytisine. Interaction tests were also used to test the effect of PNU-120596, a positive allosteric modulator for the $\alpha 7$ subunit of nicotinic acetylcholine receptors, in a condition of elevated cholinergic tone induced by a low nicotine dose.

MATERIALS AND METHODS

Animals

Subjects were young adult male (n=24) and female (n=24) Sprague-Dawley rats from Harlan Laboratories (Indianapolis, IN, USA). Males weighed 271–312 g and females weighed 206–230 g upon arrival. Rats were divided and tested consecutively in two equal sets; 12 rats of each sex per replication. They were individually housed in clear polycarbonate cages (48.3 × 26.7 × 20.3 cm) lined with TEK-Fresh® cellulose bedding (Harlan Laboratories). The colony room was on a 12 h light:dark schedule; lights on at 0600. All experiments were conducted during the light cycle. Water was freely available in the home cage. Access to chow (Harlan Teklad Diet, Harlan Laboratories, KY, USA) was restricted to maintain rats at 85% of free-feeding body weight. This 85% target was determined by sampling free-feed weight for three days after a week of acclimation to our colony. This 85% target weight was increased by 2 grams every 4 weeks. Rats were handled for at least 2 min during the 3 days

before the start of the experiment. All protocols were approved by the University of Nebraska-Lincoln Institutional Animal Care and Use Committee.

Apparatus

Behavioral testing was conducted in commercially available chambers (ENV-008CT, Med Associates, St. Albans, VT, USA) measuring $30.5 \times 24.1 \times 21.0$ cm ($l \times w \times h$). Each chamber was enclosed in a sound- and light-attenuating cubicle equipped with an exhaust fan. Each conditioning chamber was constructed of aluminum sidewalls, polycarbonate front, back, and ceiling, with a floor constructed of metal rods. A recessed receptacle ($5.2 \times 5.2 \times 3.8$ cm; $l \times w \times d$) was centered on one of the sidewalls. The receptacle had a dipper arm, when raised, provided access to 0.1 ml of 26% (w/v) sucrose solution. Dipper access was monitored by an infrared beam placed 1.2 cm into the receptacle and 3 cm above the chamber floor. A second infrared beam that monitored chamber activity was located 4 cm above the floor and 14.5 cm from the side wall containing the receptacle. Experimental session and collection of beam breaks were controlled using Med Associates interface and software (Med-PC for Windows, version IV).

Drugs

(-)-Nicotine hydrogen tartrate and (-)-cytisine [CYT] were purchased from Sigma (St. Louis, MO, USA). Bupropion hydrochloride [BUP] was purchased from Toronto Research Chemicals (Toronto, ON, Canada). S-(-)-nornicotine fumarate [NOR] was provided by Girindus America, Inc. (Cincinnati, OH, USA). PHA-543613 [PHA], sazetidine-A [SAZ-A], and PNU-120596 [PNU] were gifts from the National Institute on Drug Abuse (Research Triangle Institute, Research Triangle Park, NC, USA). Nicotine doses are reported as free base equivalent, whereas all other compounds are reported in salt form. The vehicle, route of administration, injection volume, and injection-to-placement interval (IPI) for each compound are detailed in Table 1.

Behavioral Procedures

Discrimination Training—Rats were injected with nicotine (0.4 mg/kg, subcutaneous [SC]) in the home cage for 3 consecutive days before discrimination training commenced to reduce the initial locomotor-suppressant effects of nicotine (Besheer *et al*, 2004; Bevins *et al*, 2001). In each of 32 daily training sessions, rats were administered nicotine or saline 5 min before placement in the chamber for the 20-min session. Rats received a pseudorandom order of 16 nicotine and 16 saline sessions with the condition that no more than two of the same session type (nicotine vs. saline) occurred in a row. On nicotine sessions, there were 36 deliveries of sucrose with each access limited to 4 sec. The first sucrose delivery occurred between 124 and 152 sec from the start of the session with 4 possible start times pseudorandomized throughout the training phase. Time between subsequent sucrose deliveries ranged from 4 to 80 sec (mean = 25 sec). For intermixed saline sessions, sucrose was withheld. Discrimination training, and all remaining phase, was conducted in a dark chamber. On one of the training days, two females and ten males had an inappropriate program executed during training (i.e., saline day instead of nicotine, or vice versa). These rats had 3 extra nicotine and 3 extra saline sessions. To estimate the missing value for each

rat for graphing and analyses, the average of the day preceding and the day following the error was used.

Testing Protocol—Generalization and substitution testing phases started immediately following discrimination training. On the first 4 consecutive days of a 5-day test cycle, rats had 2 nicotine and 2 saline training sessions intermixed as described earlier. On day 5, a 4-min test session occurred in place of a training session; no sucrose was available during this test session (Besheer *et al*, 2004; Murray *et al*, 2009). Specifics regarding the ligands and order of testing are briefly detailed below and represented in Table 1 and Figure 1.

Nicotine Generalization—Across the 2 sets of rats, we determined 4 separate nicotine dose-effect curves. Three of the dose-effect curves used the testing protocol described earlier (see Table 1 and Figure 1 for timing and doses). Determination of the final nicotine dose-effect curve used a cumulative dosing regimen (Thompson *et al*, 1983). In this regimen, rats were administered saline, then after the 5-min IPI, there was a 4-min sucrose-free test. Rats were removed from the chamber and given 0.025 mg/kg of nicotine. Following the IPI, there was another 4-min sucrose-free test. This procedure was repeated with 0.025 (0.05 cumulative dose), 0.05 (0.1), 0.1 (0.2), and finally 0.2 (0.4) mg/kg nicotine.

Ligand Substitution and Combination Tests—Substitution tests employed the same 5-day cycle described in the testing protocol section. On the 5th day of the cycle, the assigned ligand was administered at the prescribed IPI (Table 1 and Figure 1 include the procedural details, doses, and order of testing). All doses were selected from previous studies (see Table 1). Testing combinations such as PNU-120596 plus nicotine was as described earlier, except on the test day there were two injections at the designated IPIs.

Dependent Measures

For acquisition, we used dipper entry rate per second before the first sucrose delivery, or equivalent time during saline sessions. This measure avoids any influence of sucrose access in nicotine sessions on our measure of learning. For testing, we used the rate of dipper entries per second across the 4-min test session; recall that no sucrose occurred in these tests. We kept a rate measure so that the reader could compare the outcome of testing with acquisition. To measure general chamber activity, we used the number of center beam brakes throughout all the sessions.

Statistical Analyses

To examine acquisition of drug discrimination responding (nicotine vs. saline), three-way ANOVAs were performed with Sex as a between-subjects factor and Drug and Session as within-subjects factors. Significant interactions were followed by separate ANOVAs and LSD tests (see supplementary Table 2). Data from the substitution and generalization tests were analyzed using a multilevel approach. Multilevel modeling for repeated measures provides a number of advantages when compared to ANOVAs. For example, multilevel modeling does not assume that the relation between the covariate and the outcome is the same across the groups and thus does not require meeting the assumption of homogeneity. Furthermore, unlike ANOVA, multilevel modeling does not assume that the different cases

of data were independent and hence can model relations between different outcomes which may be inter-related. Finally, multilevel modeling is more robust in dealing with missing data or unequal group sizes which is often the case in preclinical animal models.

With these advantages, dose-effect curves from substitution tests and generalization tests were analyzed by building a model with a maximum likelihood fit from a baseline that does not include any predictors other than an intercept. The model was built by first adding predictor 1 (Dose), then predictor 2 (Sex), then predictor 3 (Activity), and finally an interaction between predictor 1 and 2 (Dose $\times$ Sex). The predictor or an interaction was declared significant when its addition improved the model by accounting for significantly more variance. The summaries of the model were used to estimate and report contrasts. Contrasts were restricted to control comparisons only (i.e., compared to 0 mg/kg control dose). Outcome of model comparisons and contrasts for each analysis are shown in Supplemental Tables 3 through 5.

RESULTS

Discrimination Training

Set 1—Recall Figure 1 for the progression for each set of rats through the experiment. An omnibus ANOVA on acquisition training (see Figure 2A) revealed significant main effects of Drug and Session, as well as a significant Sex $\times$ Drug and Sex $\times$ Drug $\times$ Session interactions (see Supplemental Table 2 for output of all omnibus ANOVAs). The significant Sex $\times$ Drug $\times$ Session interaction was followed by two-way Sex $\times$ Session ANOVA, separately for nicotine and saline sessions. For nicotine sessions, there were significant main effects of Sex [$F(1,22)=5.2$, $p<0.05$] and Session [$F(18,384)=14.1$, $p<0.001$], but no Sex $\times$ Session interaction [$F(18,384)=1.1$, $p=0.31$]. On average, male rats had overall higher responding than females. However, this difference dissipated by the end of the training (see sessions 15–19; Figure 2A). For saline sessions, there was a main effect of Session [$F(18,384)=9.8$, $p<0.001$]; the main effect of Sex and the Sex $\times$ Session interaction were not significant (F_s 1.2, p_s 0.5). A one-way ANOVA within each Sex revealed that among females, nicotine evoked higher conditioned responding than saline for sessions 5 to 16 [Drug, $F(1,395)=324.2$, $p<0.001$; Session, $F(18,395)=2.8$, $p<0.001$; Drug $\times$ Session, $F(18,395)=8.4$, $p<0.001$; LSD tests]. Likewise, among males, nicotine evoked higher responding on sessions 5 to 16 [Drug, $F(1,395)=457.9$, $p<0.001$; Session, $F(18,395)=4.2$, $p<0.001$; Drug $\times$ Session, $F(18,395)=10.7$, $p<0.001$; LSD tests]. In sum, by the end of the acquisition phase, all rats reliably discriminated nicotine from saline and the magnitude of responding between female and males was similar.

Set 2—An omnibus ANOVA revealed significant main effects of Drug and Session, as well as significant Sex $\times$ Session and Drug $\times$ Session interactions (see Table 2); no other interactions were significant. The Sex $\times$ Session interaction was not further investigated because a comparison that aggregates Drug (nicotine and saline) is not meaningful across sessions. Responding on the first nicotine session was lower than saline; this switched, with the nicotine stimulus evoking higher conditioned responding than saline for sessions 5 to 16 (LSD tests on combined means from females and males; Figure 2B; for consistency, data

shown for females and males separately). The transient difference between females and males in Set 1 was not seen in Set 2 and is consistent with other unpublished data in our laboratory. Regardless, the performance of Set 2 at the end of training was comparable to Set 1 with robust discrimination and similar responding in females and males.

Nicotine Generalization

DEC-1—Conditioned responding significantly differed depending on the Dose [$\chi^2(5)=99.63$, $p<.0001$], but not Sex or Activity (for all analyses in this section see Supplemental Table 3). There was no Dose $\times$ Sex interaction. Contrasts revealed that 0.05, 0.1, 0.2, and 0.4 mg/kg nicotine evoked higher conditioned responding than saline (Figure 3A).

DEC-2—Conditioned responding significantly differed depending on the Dose [$\chi^2(4)=75.40$, $p<.0001$], but not Sex or Activity. There was no Dose $\times$ Sex interaction. Contrasts revealed that 0.1 and 0.4 mg/kg nicotine evoked more conditioned responding than saline (Figure 3B).

DEC-3—Conditioned responding significantly differed depending on the Dose [$\chi^2(4)=86.27$, $p<.0001$], but not Sex or Activity. There was no Dose $\times$ Sex interaction. Contrasts revealed that 0.05, 0.1, and 0.4 mg/kg nicotine evoked more conditioned responding than saline (Figure 3C).

Cumulative DEC—Responding using the cumulative dose-effect method resembled the pattern observed with the standard across session approach used to determine DEC-1 to 3. Conditioned responding significantly differed depending on the Dose [$\chi^2(4)=71.50$, $p<.0001$]. The addition of the Sex factor to the model did not meet the criterion for significance ($p=0.07$). There was also no effect of Activity or Dose $\times$ Sex interaction. Contrasts revealed that 0.1 and 0.4 mg/kg cumulative nicotine doses evoked greater conditioned responding than saline (Figure 3D).

DECs comparison—To compare DEC's (excluding 0.2 mg/kg dose from the DEC-1), the data from DEC-1 to 3 were aggregated and an Order factor with three levels for each DEC was appended to the data. There was no differences in conditioned responding between three determinations of the nicotine DEC (no effect of Order, $p=0.42$). In addition, there was no interaction with Order as a factor, nor a Dose $\times$ Sex interaction. This outcome suggests relative stability of responding across experiments and time of determination.

Substitution for the Nicotine Stimulus

Sazetidine-A—Conditioned responding significantly differed depending on the Dose [$\chi^2(3)=56.48$, $p<.0001$; for all analytical output in this section see Supplemental Table 4]. There was no effect of Sex or Activity, nor was there a Dose $\times$ Sex interaction. Contrasts revealed that 1.0 and 3.0 mg/kg sazetidine-A evoked higher conditioned responding than saline (Figure 4A).

PHA—Conditioned responding significantly differed depending on the Dose [$\chi^2(3)=9.84$, $p<.05$]. There was no effect of Sex or Activity, nor was there a Dose $\times$ Sex interaction. Contrasts revealed that responding evoked by 3.0 mg/kg PHA was lower than saline (Figure 4B).

PNU—There was no effect of Dose, Sex, or Activity. There was no significant Dose $\times$ Sex interaction. Neither dose of PNU evoked responding that differed from saline (Figure 4C).

Bupropion—Conditioned responding significantly differed depending on the Dose [$\chi^2(5)=14.73$, $p<.05$]. There was no effect of Sex or Activity, nor was there a Dose $\times$ Sex interaction. Contrasts revealed that responding evoked by 10, 20, 30, and 60 mg/kg evoked greater conditioned responding than saline (Figure 4D).

Nornicotine—Conditioned responding significantly differed depending on the Dose [$\chi^2(4)=92.39$, $p<.0001$]. There was no effect of Sex or Activity, nor was there a Dose $\times$ Sex interaction. Contrasts revealed that responding evoked by 3.0 and 5.6 mg/kg was higher than saline (Figure 4E).

Cytisine—Conditioned responding significantly differed depending on the Dose [$\chi^2(3)=14.21$, $p<.01$] and Sex [$\chi^2(4)=6.49$, $p<.05$]. There was no Dose $\times$ Sex interaction and there was no effect of Activity. Contrasts revealed that responding for females [$b=0.05$, $t(33)=2.78$, $p=0.008$] and males [$b=0.02$, $t(33)=3.11$, $p=0.003$] controlled by 1.0 mg/kg cytisine was higher than responding evoked by saline (Figure 4F).

Overall comparison of ligands tested for substitution—To determine whether a compound partially or fully substituted for the nicotine stimulus, we compared responding evoked by the training dose of nicotine (0.4 mg/kg nicotine; DEC-1 to 3 combined) to the dose of each ligand that evoked the highest mean level of responding: 5.6 mg/kg nornicotine; 1.0 mg/kg sazetidine-A; 10 mg/kg bupropion; 1.0 mg/kg cytisine; 1.0 mg/kg PHA; 1.0 mg/kg of PNU. Because this comparison includes rats from both sets, we used a one-way GLM ANOVA with Drug as a between-subjects factor to analyze the data. There was a main effect of Drug [$F(6, 118)=27.16$, $p<0.0001$]. Only nornicotine fully substituted for the nicotine stimulus as responding evoked by the 5.6 mg/kg dose did not differ from that evoked by nicotine (Figure 5; Tukey HSD tests). Sazetidine-A, bupropion, and cytisine partially substituted for the nicotine stimulus as responding evoked by each of these ligands was higher than saline (recall Figure 4), but lower than that controlled by the training dose of nicotine (Figure 5; Tukey HSD tests). Responding evoked by PHA or PNU did not substitute for the nicotine stimulus as their responding was significantly lower than responding evoked by nicotine and that responding did not differ from responding evoked by saline in their respective substitution tests (Tukey HSD tests).

Combination Tests

Sazetidine-A (1 mg/kg) + Nicotine—Responding significantly differed depending on the Dose [$\chi^2(4)=16.25$, $p<.01$; for all statistical output in this section see Supplemental Table 5]. There was no effect of Sex or Activity, nor was there a Dose $\times$ Sex interaction.

Contrasts revealed that responding evoked by a combination of 1 mg/kg of sazetidine-A and 0.05, 0.1, or 0.4 mg/kg of nicotine evoked higher conditioned responding than sazetidine-A alone (Figure 6A).

Sazetidine-A (1 mg/kg) + Bupropion—Responding significantly differed depending on the Dose [$\chi^2(5)=57.15$, $p<.0001$] and Activity [$\chi^2(1)=3.98$, $p=0.046$]. There was no effect of Sex, nor was there a Dose $\times$ Sex interaction. Contrasts revealed that this combination did not enhance conditioned responding. Conversely, responding evoked by a combination of 1 mg/kg of sazetidine-A and 20, 30, or 60 mg/kg of bupropion evoked lower conditioned responding than sazetidine-A alone (Figure 6B). Because it appears that higher doses coincide with the decrease in goal-tracking responding, we conducted additional multivariate analyses investigating whether there was a Dose $\times$ Activity interaction and whether there was difference in Activity between each bupropion dose and sazetidine-A alone (0 mg/kg bupropion dose; mean comparisons on Activity alone measure). We found that, although there was significant Dose $\times$ Activity interaction [$\chi^2(1)=12.03$, $p=0.034$], Activity alone at any dose from 5–60 mg/kg did not differ from 0 (sazetidine-A 1 mg/kg). Therefore, the decrease in goal-tracking at higher doses likely reflects the interaction of pharmacological stimulus effect and hyper-locomotor effects at 20 mg/kg dose or hypo-locomotor effects at 30 and 60 mg/kg (see point size representing activity in Figure 6B).

Sazetidine-A (1 mg/kg) + Nornicotine—Responding significantly differed depending on the Dose [$\chi^2(4)=12.35$, $p<.05$]. There was no effect of Sex or Activity, nor was there a Dose $\times$ Sex interaction. Contrasts revealed that responding evoked by a combination of 1 mg/kg of sazetidine-A and 3 mg/kg of nornicotine was higher than sazetidine-A alone (Figure 6C).

Sazetidine-A (1 mg/kg) + Cytisine—There was no effect of Dose or Sex on responding. There was also no Dose $\times$ Sex interaction. Although Dose comparisons did not reveal any significant differences (Figure 6D), addition of the Activity variable to the statistical model indicated a significant effect of Activity on responding [$\chi^2(1)=15.12$, $p<.001$].

Nicotine (0.05 mg/kg) + PNU—There was no effect of Dose or Sex on responding. There was also no Dose $\times$ Sex interaction. Contrasts did not reveal any significant differences (Figure 6E).

DISCUSSION

Since the publication of Bevins and Charntikov (2015), there still remains not a single published paper that we can find that has nicotine as the training stimulus and investigates potential sex differences. Given this scientific gap, the main purpose of this study was to examine the nature of the nicotine stimulus in female and male rats using ligands selected for their action on nAChRs and/or their use in smoking cessation (recall Table 1). Overall, females and males acquired the discrimination between 0.4 mg/kg nicotine and saline at a similar rate. Further, the between session nicotine dose-effect curves were also comparable between sexes. Substitution and combination tests overall revealed similar patterns with the exception of cytisine. Cytisine evoked nicotine-like responding that was slightly, but

significantly, higher for females. Although we did not discover any major differences between females and males, this study reflects an important step in understanding the nature of the nicotine stimulus in both sexes.

Nicotine primarily exerts its stimulus effects by activating nicotinic acetylcholine receptors located in the central nervous system [see Wooters et al (2009) for a review]. Centrally and peripherally active nAChR antagonists like mecamylamine block responding controlled by the nicotine stimulus (Besheer *et al*, 2004; Dwoskin *et al*, 2008; Struthers *et al*, 2009). In contrast, nAChR antagonists that do not readily cross the blood brain barrier (e.g., hexamethonium) do not affect responding to the nicotine stimulus (Besheer *et al*, 2004; Kumar *et al*, 1987). The $\alpha 4\beta 2$ -containing nAChRs seem especially important for the stimulus effects of nicotine. nAChR agonists with varying specificity for the $\alpha 4\beta 2$ -containing receptors like TC-2559, ABT-594, ABT-418, and A-85380 evoke responding comparable to nicotine in substitution tests (Damaj *et al*, 1995; Goldberg *et al*, 1989; Jutkiewicz *et al*, 2011; Papke *et al*, 2007; Reichel *et al*, 2010). Partial agonists for $\alpha 4\beta 2$ -containing nAChRs like varenicline and cytisine prompt partial to full substitution depending on the study (LeSage *et al*, 2009; Reichel *et al*, 2010; Smith *et al*, 2007).

In the present study, we found that that cytisine and sazetidine-A, both ligands with high affinity for the $\alpha 4\beta 2$ -containing receptors, met our definition of partial substitution for the nicotine stimulus. However, the mean level of responding evoked by the cytisine was less than half that of sazetidine-A, and less than a third of what was controlled by the training dose of nicotine (see Figure 5). Sazetidine-A, is one of the promising drugs for the treatment of nicotine dependence. A conference abstract from the Society for Neuroscience is the only report known to us of sazetidine-A substitution for the nicotine stimulus (Xiao *et al*, 2007).

In vitro electrophysiological assays show that sazetidine-A potently evoked dopamine release from neurons expressing nAChRs (Zwart *et al*, 2008); this effect was blocked by dihydro- β -erythroidine and by mecamylamine. Because dopamine release from striatal slices is mediated by $\alpha 4\beta 2$ - and $\alpha 6\beta 2$ -containing receptors (Grady *et al*, 2007; Mogg *et al*, 2004; Salminen, 2004; Smith *et al*, 2007), these electrophysiological findings suggest the involvement of these receptors. Furthermore, Zwart *et al* (2008) found that the $\alpha 6$ nAChR selective antagonist α -CtxMII reduced striatal cell response evoked by sazetidine-A by approximately 48%. This effect indicated that sazetidine-A induced dopamine release was almost equally mediated by $\alpha 4\beta 2$ - and by $\alpha 6$ -containing nAChRs. Along with some other promising preclinical reports (Johnson *et al*, 2012; Levin *et al*, 2010; Rezvani *et al*, 2010) our findings further confirm the treatment potential of sazetidine-A.

In contrast to the heteromeric nAChRs just discussed, the homomeric $\alpha 7$ nAChR does not seem to play a role in generation of the stimulus effects of nicotine. For example, $\alpha 7$ nAChR agonists GTS-21 and WO 01/60821A1 do not substitute for the nicotine stimulus (Smith *et al*, 2007; Stolerman *et al*, 2004). Furthermore, pretreatment with the $\alpha 7$ nAChR antagonist GTS-21 or methyllycaconitine (MLA) does not alter responding controlled by the nicotine stimulus (Brioni *et al*, 1996; Van Haaren *et al*, 1999; Struthers *et al*, 2009; Zaniewska *et al*, 2006). Corroborating and extending this past research, we found that neither PNU-120596, a positive allosteric modulator for the $\alpha 7$ nAChR, nor PHA-543613, a selective agonist for the $\alpha 7$ nAChRs, evoked nicotine-like responding (see later discussion).

Previous studies report mixed findings with bupropion substitution for the nicotine stimulus: full substitution (Wiley *et al*, 2002; Wilkinson *et al*, 2009, 2010; Young and Glennon, 2002); partial substitution (Desai *et al*, 2003); no substitution (Shoaib *et al*, 2003). At this point, unidentified procedural differences across studies are likely responsible for different outcomes. Our study adds partial substitution in females and males to this list. Recall that nicotine-like responding was highest at the 10, 20, 30, and 60 mg/kg doses of bupropion (see Figure 4D). Responding was somewhat lower and more variable at the higher doses (20–60 mg/kg). A likely factor to be considered in this effect is bupropion evoked hyperlocomotion that has been previously well documented (Cooper *et al*, 1980; Nielsen *et al*, 1986; Wilkinson & Bevins, 2007, Wilkinson *et al*, 2006). In fact, previous exposure to nicotine can enhance the subsequent locomotor stimulant effects of bupropion (Wilkinson *et al*, 2006). However, our multivariate analysis does not show a significant contribution of the locomotor activity to the dose-dependent variability in our dependent measure – dipper entries. This outcome suggests that, at least in the experimental paradigm presented in this study, bupropion's locomotor effects do not interfere with its nicotine-like stimulus effects.

Nornicotine, is an alkaloid found in tobacco products, as well as a minor metabolite of nicotine in humans, non-human primates, and rodents (Benowitz, 1991; Ghosheh *et al*, 1999). In the brain, nornicotine binds to various combinations of nAChR subunits among which it has highest affinity for $\alpha 6/3\beta 2\beta 3$, $\alpha 7$, and $\alpha 4\beta 2$ subunits ($\alpha 6/3\beta 2\beta 3 > \alpha 7 > \alpha 4\beta 2 > \alpha 3\beta 4 > \alpha 3\beta 2\alpha 5 > \alpha 3\beta 2\beta 3 > \alpha 3\beta 2$; Papke *et al*, 2007). In the present study, we replicated past work showing that nornicotine (3.0 and 5.6 mg/kg) evoked nicotine-like responding in male rats (Desai *et al*, 1999; Goldberg *et al*, 1989; Reichel *et al*, 2010) and extended this observation to female rats. This substitution was comparable between sexes and at the highest dose tested (5.6 mg/kg), responding was statistically similar to the 0.4 mg/kg training dose of nicotine (i.e., full substitution). In fact, nornicotine was the only compound tested that fully substituted for the nicotine stimulus (see Figure 5).

Similar to substitution tests, combination tests can be used to elucidate receptor mechanisms contributing to the stimulus effects of nicotine. In the present study, findings from the substitution tests, along with the known pharmacological profiles of the ligands, guided our selection of drug interaction tests (recall Table 1). Because there is a limited understanding of the nicotine-like effects of sazetidine-A, we elected to perform a number of interaction tests with sazetidine-A and ligands with known nicotine-like stimulus effects. These types of tests are critical to start understanding receptor mechanism underlying sazetidine-A's substitution for the nicotine stimulus and further considering sazetidine-A as a possible therapeutic agent. To this end, we tested in females and males the effect of combining sazetidine-A with nicotine, bupropion, nornicotine, or cytisine. Notably, only combinations of 1 mg/kg sazetidine-A plus nicotine or nornicotine increased nicotine-like responding relative to sazetidine-A alone. Enhancement by the low dose of nicotine (0.05 mg/kg) likely reflects a summative action of the two compounds on $\alpha 4\beta 2$ -containing nAChRs. Importantly, we included the higher doses of nicotine (0.1 to 0.4 mg/kg) in the combination tests to determine if the partial agonist effect of sazetidine-A would decrease conditioned responding evoked by the nicotine stimulus. LeSage and colleagues (2009) found that the $\alpha 4\beta 2$ partial agonist varenicline blunted responding to their training dose of nicotine. We, however, did not find a similar effect. Rather, these higher doses increased responding

relative to sazetidine-A alone. Although the combination tests did not include a nicotine benchmark, examination of the nicotine dose-effect curves in the present study indicate that nicotine at 0.1 mg/kg or higher prompted responding comparable to the 0.4 mg/kg training dose. Future research will need to examine whether a higher dose of sazetidine-A can attenuate responding to these higher doses of nicotine.

The combination of 3 mg/kg nornicotine with sazetidine-A enhanced nicotine-like responding above that controlled by sazetidine-A alone. This effect was not seen at the higher dose of nornicotine (5.6 mg/kg) that prompted full substitution for nicotine when tested alone. Perhaps this decrease with 5.6 mg/kg nornicotine in the combination reflects partial antagonism by sazetidine-A. Our enthusiasm for this possibility is diminished by the lack of such an effect when the training dose of nicotine was used in the interaction test. Alternatively, when two compounds are tested together, the combination may produce stimulus effects that are distinct from either of the two drugs alone. These distinct stimulus elements may differ sufficiently from the training dose of nicotine, thus reducing responding in the test. The present study was not designed to examine this possibility. Future research will be needed to parse out potential neuropharmacological mechanisms contributing to the reduction in nicotine-like responding with the sazetidine-A plus 5.6 mg/kg nornicotine combination. Indeed, a similar possibility may explain the loss of conditioned responding at the higher doses of bupropion in combination of sazetidine-A; see also earlier discussion regarding locomotor effects of bupropion.

PNU-120596 is a selective positive allosteric modulator for the $\alpha 7$ nAChRs. As detailed earlier, PNU-120596 alone did not substitute for the nicotine stimulus. To determine if this lack of effect reflected insufficient ACh tone in key brain areas, we examined whether PNU-120596 would enhance partial generalization evoked by a low dose of nicotine (0.05 mg/kg). Enhancement of conditioned responding was not evident at either dose of PNU-120596 tested in combination with nicotine. This outcome is consistent with the present work and published studies indicating that the $\alpha 7$ nAChR does not play a role in generating the nicotine stimulus (Brioni *et al*, 1996; Van Haaren *et al*, 1999; Struthers *et al*, 2009; Zaniowska *et al*, 2006).

Women on average take less time than men to become dependent, make fewer attempts to quit smoking, remain abstinent for less time, have a higher relapse rate, and show less benefit from nicotine replacement therapy (Okene, 1993; Perkins and Scott, 2008; Perkins, 2001; Schnoll *et al*, 2007). Research from animal models often corroborate these effects providing support for the assertion that some sex differences are rooted in biology (Harrod *et al*, 2004; Torres and O'Dell, 2016). As described in the Introduction and reviewed in Bevins and Charntikov (Bevins and Charntikov, 2015), until now, there has not been a single published study that we could find that used nicotine as the training stimulus and explicitly investigated sex differences. Overall, female rats, like their male counterparts, readily learn a discrimination between nicotine and saline, display a dose-dependent nicotine generalization curve, show substitution patterns as expected from published studies, and, importantly, do not show excessive variability as lab lore might suggest (cf. Prendergast *et al*, 2014). Clearly much more research is needed in this area and the present research provides investigators

with a strong beginning regarding the nature of the nicotine stimulus and the import of sex as a biological variable.

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Highlights

- Stimulus effects of nicotine were assessed in female and male rats
- Nicotine-saline discrimination trained using the discriminated goal-tracking task
- Substitution ligands were potential pharmacotherapies or nAChR-binding compounds
- Overall, substitution was similar in female and male rats across the tests

Fig. 1.
A graphical representation of the testing order for each Set of rats used in this study.

Fig. 2. Data from the discrimination training phase represented as a mean ($\pm$ SEM) number of dipper entries per second during 2 min prior to initial sucrose or equivalent in timing during no reward presentations. (A) Acquisition of discrimination from Set 1 and (B) Set 2. *Denotes sessions with significant differences between saline and nicotine evoked responding. #Denotes overall differences in responding during discrimination training phase.

Fig. 3.

A mean ($\pm$ SEM) number of total dipper entries for each nicotine dose-effect curves. (A, B, and C) Data from dose-effect curves 1–3. (D) Data from cumulative dose-effect curve.

*Denotes significant difference of combined female and male responding from 0 mg/kg dose (saline).

Fig. 4.

A mean ($\pm$ SEM) number of total dipper entries for substitution tests with (A) sazetidine-A, (B) PHA, (C) PNU, (D) bupropion, (E) nornicotine, and (F) cytisine. *Denotes significant difference of combined female and male responding from 0 mg/kg dose (saline).

Fig. 5.

A box-plot showing a magnitude of responding from the selected doses of each substitution ligand that evoked the highest responding during substitution tests. A black dot within the boundaries of the box represents a mean responding for each particular ligand with the actual mean number indicated above that dot. Scattered grey dots and open triangles represent individual responding of females and males (respectively) for each ligand tested. ##Denotes full substitution for the nicotine stimulus. #Denotes partial substitution for the nicotine stimulus.

Fig. 6.

A mean ($\pm$ SEM) number of total dipper entries from combination tests with (A) sazetidine-A and nicotine, (B) sazetidine-A and bupropion, (C) sazetidine-A and nornicotine, (D) sazetidine-A and cytisine, (E) nicotine and PNU. Point size plotted on panels B and D represents general chamber activity for each tested combination and does not represent proportional change in locomotor activity.

TABLE 1

Drug Information

Test Type	Drug	Doses (mg/kg)	IPI	Route	Mechanism
Generalization-1 ¹	Nicotine	0, 0.025, 0.05 , 0.1, 0.2, 0.4	5 min	SC	$\alpha_4\beta_2 > \alpha_3\beta_4 > \alpha_3\beta_2 > \alpha_7$ (Wooters et al, 2009)
Generalization- (2 ¹ , 3 ² , 4 ²)	Nicotine	0, 0.025, 0.05, 0.1, 0.4			
Substitution ¹ Interaction ^{1,2}	Sazetidine-A	0, 0.3, 1 , 3	10 min	SC	$\alpha_4\beta_2$ partial agonist (Xiao et al, 2006)
Substitution ¹ Interaction ²	PHA-543613	0, 1, 3, 10	10 min	SC	α_7 nAChR agonist (Wishka et al, 2006)
Substitution ¹ Interaction ²	PNU-120596 [†]	0, 10, 30	10 min	SC	α_7 nAChR positive allosteric modulator (Hurst et al, 2005)
Substitution ² Interaction ²	Bupropion	0, 5, 10, 20, 30, 60	15 min	IP	DAT and NET inhibitor, nAChR antagonist (Dwoskin et al, 2006)
Substitution ² Interaction ²	Nornicotine	0, 0.3, 1, 3, 5.6	15 min	SC	$\alpha_6/3\beta_2\beta_3 > \alpha_7 > \alpha_4\beta_2 > \alpha_3\beta_4 > \alpha_3\beta_2\alpha_5 > \alpha_3\beta_2\beta_3 > \alpha_3\beta_2$ (Papke et al, 2007)
Substitution ² Interaction ²	Cytisine	0, 0.1, 0.3, 1	15 min	SC	$\alpha_4\beta_2 > \alpha_3\beta_4 > \alpha_7$ (Smith et al, 2007)

[†] All drugs, except PNU-120596, were administered at 1 mg/ml volume and were diluted in saline; PNU-120596 was administered at 0.5 mg/ml and was dissolved in 10% Tween prepared in H₂O.

¹ Denotes tests on the first subset of rats.

² Denotes tests using the second subset of rats. Interaction tests are indicated by the brackets (interaction **doses** in bold; also cf. Figure 1).

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TABLE 2

Omnibus ANOVA Effects and Interactions for Discrimination Training.

	Sex ME			Drug ME			Session ME			Sex × Drug			Sex × Session			Drug × Session			Sex×Drug×Session		
	df	F	P	df	F	P	df	F	P	df	F	P	df	F	P	df	F	P	df	F	P
Nicotine CS Training																					
Set 1	1,22	3.8	0.06	1,790	781.9	***	18,790	6.3	***	1,790	19.3	***	18,790	1	0.5	18,790	18.2	***	18,790	1.2	0.2
Set 2	1,22	0.5	0.48	1,682	543.1	***	15,682	10.8	***	1,682	0.8	0.37	15,682	1.7	*	15,682	18.9	***	15,682	1.1	0.33

Significance:

*
p < 0.05,

**
p < 0.01,

p < 0.001

TABLE 3

Summary of multivariate analysis performed on nicotine dose effect curves.

Summary of Multivariate Analysis										Dose Comparison (Compared to Intercept - 0 dose)			
Model	df	AIC	BIC	logLik	Test Models	L.Ratio	p-value	Dose	Value	Std.Error	df	t-value	p-value
Nicotine DEC-1													
Baseline Model	1	6	-181.2694	-163.4506	96.6347			(Intercept)	0.0618	0.0193	115	3.1951	0.0018
Adding Dose Factor	2	11	-270.9047	-238.2368	146.4524	1 vs 2	99.6353	<.0001	0.025	0.0451	115	1.9252	0.0567
Adding Sex Factor	3	12	-268.9432	-233.3054	146.4716	2 vs 3	0.0384	0.8446	0.05	0.0781	115	3.3329	<0.01
Adding Activity Variable	4	13	-266.9855	-228.3779	146.4928	3 vs 4	0.0423	0.8370	0.1	0.1965	115	8.3852	<.0001
Adding Dose × Sex	5	18	-258.3436	-204.8870	147.1718	4 vs 5	1.3581	0.9288	0.2	0.2142	115	9.1413	<.0001
								0.4	0.1861	0.0234	115	7.9409	<.0001
Nicotine DEC-2													
Baseline Model	1	6	-142.6495	-125.9246	77.3248			(Intercept)	0.0677	0.0194	92	3.4852	0.0008
Adding Dose Factor	2	10	-210.0592	-182.1842	115.0296	1 vs 2	75.4096	<.0001	0.025	0.0045	92	0.1702	0.8652
Adding Sex Factor	3	11	-208.2875	-177.6251	115.1438	2 vs 3	0.2284	0.6327	0.05	0.0490	92	1.8469	0.0680
Adding Activity Variable	4	12	-209.4995	-176.0496	116.7498	3 vs 4	3.2120	0.0731	0.1	0.1566	92	5.9068	<.0001
Adding Dose × Sex	5	16	-202.6564	-158.0565	117.3282	4 vs 5	1.1569	0.8851	0.4	0.2160	92	8.1470	<.0001
Nicotine DEC-3													
Baseline Model	1	6	-127.4968	-110.7718	69.7484			(Intercept)	0.0493	0.0207	92	2.3759	0.0196
Adding Dose Factor	2	10	-205.7724	-177.8975	112.8862	1 vs 2	86.2757	<.0001	0.025	0.0143	92	0.5679	0.5715
Adding Sex Factor	3	11	-204.7676	-174.1052	113.3838	2 vs 3	0.9952	0.3185	0.05	0.0642	92	2.5600	<0.05
Adding Activity Variable	4	12	-203.0978	-169.6479	113.5489	3 vs 4	0.3302	0.5655	0.1	0.1861	92	7.4170	<.0001
Adding Dose × Sex	5	16	-196.7432	-152.1433	114.3716	4 vs 5	1.6454	0.8006	0.4	0.2271	92	9.0497	<.0001
Cumulative Nicotine DEC													
Baseline Model	1	6	-180.1846	-163.4596	96.0923			(Intercept)	0.0375	0.0173	92	2.1657	0.0329
Adding Dose Factor	2	10	-243.6858	-215.8109	131.8429	1 vs 2	71.5012	<.0001	0.025	-0.0011	92	-0.0478	0.9620
Adding Sex Factor	3	11	-244.8765	-214.2141	133.4383	2 vs 3	3.1907	0.0741	0.05	0.0378	92	1.7228	0.0883
Adding Activity Variable	4	12	-244.4537	-211.0038	134.2268	3 vs 4	1.5772	0.2092	0.1	0.0771	92	3.5088	<0.001
Adding Dose × Sex	5	16	-239.7040	-195.1042	135.8520	4 vs 5	3.2504	0.5168	0.4	0.1882	92	8.5686	<.0001
DECs Comparison													
Baseline Model	1	4	516.7894	501.24502	62.3947								

Summary of Multivariate Analysis														
								Dose Comparison (Compared to Intercept - 0 dose)						
	Model	df	AIC	BIC	logLik	Test Models	L.Ratio	p-value	Dose	Value	Std.Error	df	t-value	p-value
	4	11	-714.3466	-671.5995	368.1733	3 vs 4	1.7228	0.4226						
Adding Order Factor														
	5	19	-706.8614	-633.0254	372.4307	4 vs 5	8.5147	0.3849						
Adding Order × Dose														
	6	21	-703.2787	-621.6705	372.6394	5 vs 6	0.4174	0.8117						
Adding Order × Sex														
	7	33	-686.2856	-558.0441	376.1428	6 vs 7	7.0069	0.8572						
Adding Order × Sex × Dose														

Significance: $p < 0.05$

TABLE 4

Summary of multivariate analysis performed on substitution tests.

Summary of Multivariate Analysis										Dose Comparison (Compared to Intercept – 0 dose)			
Model	df	AIC	BIC	logLik	Test Models	L:Ratio	p-value	Dose	Value	Std.Error	df	t-value	p-value
Sazetidine-A													
Baseline Model	1	6	-161.9674	-146.5813	86.9837			(Intercept)	0.0611	0.0161	69	3.7882	0.0003
Adding Dose Factor	2	9	-212.4521	-189.3729	115.2260	1 vs 2	56.4847	0.3	0.0040	0.0188	69	0.2120	0.8327
Adding Sex Factor	3	10	-210.7146	-185.0711	115.3573	2 vs 3	0.2625	1	0.1248	0.0188	69	6.6284	<0001
Adding Activity Variable	4	11	-209.0284	-180.8205	115.5142	3 vs 4	0.3138	3	0.1205	0.0188	69	6.3980	<0001
Adding Dose × Sex	5	14	-203.4283	-167.5275	115.7142	4 vs 5	0.4000						
PHA													
Baseline Model	1	6	-190.6206	-179.6488	101.3103			(Intercept)	0.0559	0.0053	23	10.6363	0.0000
Adding Dose Factor	2	9	-194.4687	-178.0110	106.2344	1 vs 2	9.8481	1	-0.0056	0.0107	19	-0.5228	0.6072
Adding Sex Factor	3	10	-195.9384	-177.6520	107.9692	2 vs 3	3.4697	3	-0.0251	0.0080	19	-3.1519	0.0052
Adding Activity Variable	4	11	-196.7946	-176.6796	109.3973	3 vs 4	2.8562	10	-0.0103	0.0128	19	-0.8024	0.4322
Adding Dose × Sex	5	14	-197.5796	-171.9786	112.7898	4 vs 5	6.7850						
PNU													
Baseline Model	1	6	-275.8900	-262.5730	143.9450			(Intercept)	0.0403	0.0062	42	6.4589	0.0000
Adding Dose Factor	2	8	-275.0130	-257.2570	145.5065	1 vs 2	3.1230	10	0.0078	0.0074	42	1.0585	0.2959
Adding Sex Factor	3	9	-275.4817	-255.5062	146.7409	2 vs 3	2.4687	30	-0.0055	0.0074	42	-0.7490	0.4581
Adding Activity Variable	4	10	-273.5311	-251.3360	146.7655	3 vs 4	0.0493						
Adding Dose × Sex	5	12	-272.8289	-246.1948	148.4144	4 vs 5	3.2978						
Bupropion													
Baseline Model	1	6	-168.5782	-150.7593	90.2891			(Intercept)	0.0528	0.0257	115	2.0551	0.0421
Adding Dose Factor	2	11	-173.3117	-140.6438	97.6559	1 vs 2	14.7335	5	0.0563	0.0354	115	1.5897	0.1147
Adding Sex Factor	3	12	-171.6037	-135.9660	97.8019	2 vs 3	0.2920	10	0.1278	0.0354	115	3.6111	0.0005
Adding Activity Variable	4	13	-170.0661	-131.4585	98.0330	3 vs 4	0.4623	20	0.0872	0.0354	115	2.4630	0.0153
Adding Dose × Sex	5	18	-164.7649	-111.3082	100.3824	4 vs 5	4.6988	30	0.0974	0.0354	115	2.7525	0.0069
								60	0.0752	0.0354	115	2.1245	0.0358
Nor													
Baseline Model	1	6	-152.0848	-135.4607	82.0424			(Intercept)	0.0361	0.0189	90	1.9038	0.0601

Summary of Multivariate Analysis											Dose Comparison (Compared to Intercept – 0 dose)				
Model		df	AIC	BIC	logLik	Test Models	L.Ratio	p-value	Dose	Value	Std.Error	df	t-value	p-value	
Adding Dose Factor		2	10	-236.4837	-208.7769	128.2419	1 vs 2	92.3989	<.0001	0.3	0.0210	0.0219	90	0.9633	0.3380
Adding Sex Factor		3	11	-235.4891	-205.0116	128.7446	2 vs 3	1.0054	0.3160	1	0.0285	0.0219	90	1.3049	0.1952
Adding Activity Variable		4	12	-236.5127	-203.2645	130.2564	3 vs 4	3.0236	0.0821	3	0.1587	0.0219	90	7.2638	<.0001
Adding Dose × Sex		5	16	-232.3416	-188.0106	132.1708	4 vs 5	3.8289	0.4297	5.6	0.2058	0.0219	90	9.4170	<.0001
Cytisine															
Baseline Model		1	6	-316.1732	-300.7871	164.0866									
Adding Dose Factor		2	9	-324.3837	-301.3046	171.1918	1 vs 2	14.2105	0.0026	(Intercept)	0.0351	0.0093	69	3.7816	0.0003
Adding Sex Factor		3	10	-328.8797	-303.2362	174.4398	2 vs 3	6.4960	0.0108	0.1	0.0111	0.0102	69	1.0864	0.2811
Adding Activity Variable		4	11	-327.4096	-299.2018	174.7048	3 vs 4	0.5299	0.4666	0.3	0.0160	0.0102	69	1.5617	0.1229
Adding Dose × Sex		5	14	-323.8509	-287.9500	175.9254	4 vs 5	2.4413	0.4860	1	0.0385	0.0102	69	3.7685	0.0003

Significance: p < 0.05

TABLE 5

Summary of multivariate analysis performed on interaction tests.

Summary of Multivariate Analysis										Dose Comparison (Compared to Intercept - 0 dose)				
	Model	df	AIC	BIC	logLik	Test Models	L.Ratio	p-value	Dose	Value	Std.Error	df	t-value	p-value
Sazetidine-A (1 mg/kg) + Nicotine														
	Baseline Model	1	6	-203.6288	-186.9039	107.8144			(Intercept)	0.1477	0.0215	86	6.8593	0.0000
	Adding Dose Factor	2	10	-211.8859	-184.0110	115.9430	1	vs 2	0.025	0.0456	0.0234	86	1.9450	0.0550
	Adding Sex Factor	3	11	-210.0676	-179.4052	116.0338	2	vs 3	0.05	0.0738	0.0234	86	3.1507	0.0022
	Adding Activity Variable	4	12	-209.3410	-175.8911	116.6705	3	vs 4	0.1	0.0897	0.0235	86	3.8147	0.0003
	Adding Dose × Sex	5	16	-207.8024	-163.2025	119.9012	4	vs 5	0.4	0.0501	0.0235	86	2.1293	0.0361
Sazetidine-A (1 mg/kg) + Bupropion														
	Baseline Model	1	6	-175.3424	-157.5235	93.6712			(Intercept)	0.2047	0.0242	115	8.4436	0.0000
	Adding Dose Factor	2	11	-222.4964	-189.8285	122.2482	1	vs 2	5	0.0175	0.0268	115	0.6550	0.5138
	Adding Sex Factor	3	12	-220.6418	-185.0040	122.3209	2	vs 3	10	0.0076	0.0268	115	0.2854	0.7759
	Adding Activity Variable	4	13	-222.6250	-184.0175	124.3125	3	vs 4	20	-0.0875	0.0268	115	-3.2686	0.0014
	Adding Dose × Sex	5	18	-222.1560	-168.6993	129.0780	4	vs 5	30	-0.1043	0.0268	115	-3.8977	0.0002
									60	-0.1523	0.0268	115	-5.6876	<.0001
Sazetidine-A (1 mg/kg) + Normicotine														
	Baseline Model	1	6	-191.4700	-174.7450	101.7350			(Intercept)	0.1488	0.0232	92	6.4261	0.0000
	Adding Dose Factor	2	10	-195.8254	-167.9505	107.9127	1	vs 2	0.3	0.0080	0.0249	92	0.3208	0.7491
	Adding Sex Factor	3	11	-194.5819	-163.9195	108.2909	2	vs 3	1	0.0399	0.0249	92	1.6039	0.1122
	Adding Activity Variable	4	12	-194.0579	-160.6080	109.0289	3	vs 4	3	0.0771	0.0249	92	3.0962	0.0026
	Adding Dose × Sex	5	16	-193.5860	-148.9861	112.7930	4	vs 5	5.6	0.0146	0.0249	92	0.5858	0.5595
Sazetidine-A (1 mg/kg) + Cytisine														
	Baseline Model	1	6	-188.7135	-173.3274	100.3567			(Intercept)	0.1102	0.0200	69	5.5227	0.0000
	Adding Dose Factor	2	9	-186.4404	-163.3613	102.2202	1	vs 2	0.1	0.0274	0.0202	69	1.3562	0.1795
	Adding Sex Factor	3	10	-186.1612	-160.5177	103.0806	2	vs 3	0.3	0.0365	0.0202	69	1.8025	0.0758
	Adding Activity Variable	4	11	-199.2826	-171.0747	110.6413	3	vs 4	1	0.0151	0.0202	69	0.7468	0.4577
	Adding Dose × Sex	5	14	-196.0664	-160.1655	112.0332	4	vs 5						
														0.4262

Summary of Multivariate Analysis										Dose Comparison (Compared to Intercept - 0 dose)				
Model	df	AIC	BIC	logLik	Test Models	L Ratio	p-value	Dose	Value	Std.Error	df	t-value	p-value	
Nicotine (0.05 mg/kg) + PNU														
Baseline Model	1	6	-154.0586	-140.3986	83.0293			(Intercept)	0.1443	0.0209	46	6.8933	0.0000	
Adding Dose Factor	2	8	-150.2239	-132.0106	83.1120	1 vs 2	0.1654	10	-0.0063	0.0157	46	-0.3982	0.6923	
Adding Sex Factor	3	9	-151.2968	-130.8068	84.6484	2 vs 3	3.0729	30	-0.0030	0.0157	46	-0.1880	0.8517	
Adding Activity Variable	4	10	-149.2971	-126.5304	84.6485	3 vs 4	0.0003							
Adding Dose × Sex	5	12	-149.5242	-122.2042	86.7621	4 vs 5	4.227075							

Significance: $p < 0.05$