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The discriminative stimulus effects of i.v. nicotine in rhesus monkeys: pharmacokinetics and apparent pA_2 analysis with dihydro- β -erythroidine

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Abstract

Quantitative analysis of antagonism is infrequently used to identify nAChRs mediating behavioral effects. Here, nicotine (0.032 mg/kg i.v.) was established as a discriminative stimulus in rhesus monkeys responding under a fixed ratio 5 schedule; pharmacokinetics and underlying nAChR mechanism(s) were examined. When measured up to 4 h in venous blood, the training dose resulted in the following mean pharmacokinetic parameters: nicotine $C_{max}=71.7$ ng/ml, $t_{1/2}=116$ min, and clearance=6.25 ml/min/kg; cotinine $C_{max}=191$ ng/ml; and 3OH-cotinine $C_{max}=63$ ng/ml. The ED_{50} value of nicotine to produce discriminative stimulus effects was 0.013 mg/kg. Epibatidine and varenicline increased drug-lever responding to 97% and 95%, respectively (ED_{50} values=0.00015 and 0.031 mg/kg, respectively), whereas cocaine, midazolam, and morphine produced no more than 28% drug-appropriate responding. Mecamylamine and dihydro- β -erythroidine (DH β E) dose-dependently attenuated the discriminative stimulus effects of the nicotine training dose, whereas methyllycaconitine (MLA) did not. DH β E (0.1 and 0.32) produced rightward shifts of the nicotine and varenicline dose-response functions; Schild plots fitted through individual data resulted in slopes that were not different from unity; the apparent pA_2 calculated for DH β E did not significantly differ in the presence of nicotine (6.68) or varenicline (6.65). Compared to human cigarette smoking, nicotine blood levels after 0.032 mg/kg nicotine i.v. took a similar time to reach maximal concentration, levels at C_{max} were similar to smoking 2-3

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Conflicts of interest

The authors declare no conflicts of interest.

cigarettes, while average nicotine levels were comparable to smoking 5-6 cigarettes. Apparent pA_2 analysis with DH β E under these conditions is consistent with nicotine and varenicline acting through the same nAChRs to produce discriminative stimulus effects.

1. Introduction

Tobacco use, predominantly in the form of cigarette smoking, remains the largest preventable cause of death in the United States (U.S. Department of Health and Human Services, 2014). Even with the assistance of tobacco cessation products, only 10% of quit attempts result in continuous abstinence for up to one year (U.S. Department of Health and Human Services, 2014). A more thorough understanding of the pharmacology of tobacco cessation products in whole animals is needed to identify new pharmacotherapeutics. The abuse-related effects of nicotine are mediated by $\alpha 4\beta 2^*$ nAChRs (* denotes the possible presence of additional subunits; Gotti et al., 2010) and current nAChR-based smoking cessation aids (e.g., varenicline) target $\alpha 4\beta 2^*$ nAChRs (Rollema et al., 2007).

Drug discrimination is used to identify *in vivo* pharmacological mechanism(s) that mediate the effects of centrally acting drugs. Discrimination of abused drugs, in particular, can be used to identify potential drug abuse treatments. For example, drugs that mimic or antagonize the discriminative stimulus effects of nicotine are considered possible candidates for the development of tobacco cessation products (Rollema et al., 2007; Smith and Stolerman, 2009). Varenicline has been shown to mimic the discriminative stimulus effects of nicotine in some studies (Rollema et al., 2007; Jutkiewicz et al., 2011) and to antagonize those effects in another study (LeSage et al., 2009). However, the pharmacological mechanism(s) by which varenicline produces discriminative stimulus effects, i.e., whether or not nicotine and varenicline share receptor mechanisms *in vivo*, has not been firmly established using quantitative analysis of antagonism. Rightward shifts in the dose response functions of these compounds with nAChR subtype-specific antagonists can be subjected to *in vivo* apparent pA_2 analysis, providing a measure of antagonist potency and allowing for hypotheses and/or conclusions to be drawn about the involvement of nicotinic receptor subtypes in mediating the discriminative stimulus effects of these drugs. In the current study, a nicotine discrimination assay in rhesus monkeys was developed with the following objectives in mind. First, nicotine was administered i.v. to mimic the rapid delivery of nicotine to the brain associated with inhalation of nicotine from smoking. The feasibility of training i.v. nicotine as a discriminative stimulus has been demonstrated using squirrel monkeys (Takada et al., 1988). Pharmacokinetics were assessed by measuring nicotine and its metabolites, cotinine and 3OH-cotinine, in whole blood at various times after administration of the training dose. Second, a relatively small training dose was selected to increase the selectivity of nicotine for $\alpha 4\beta 2^*$ nAChRs. The results of previous studies suggest that selectivity for $\beta 2$ -containing nAChRs decreases as nicotine training dose increases (Jutkiewicz et al., 2011). Receptor subtypes were assessed by testing nAChR agonists (epibatidine and varenicline), nAChR antagonists (mecamylamine, dihydro- β -erythroidine [DH β E], and MLA), and drugs whose primary sites of action do not include nAChRs (midazolam, cocaine, and morphine). A third objective was to gain adequate sensitivity and experimental space so that multiple rightward shifts in nicotine dose-response

functions could be generated in the presence of an antagonist. The abuse-related effects of nicotine are mediated by $\alpha 4\beta 2^*$ nAChRs; however, additional subtypes (e.g., $\alpha 7$) have been implicated (Brunzell and McIntosh, 2012; Harenza et al., 2014). Quantitative analysis of surmountable antagonism in the presence of two doses of DH β E was used to gain insight into whether or not the discriminative stimulus effects of nicotine and varenicline are mediated by the same nAChR subtypes.

2. Methods

2.1. Subjects

Four adult rhesus monkeys (*Macaca mulatta*), consisting of two males and two females, discriminated 0.032 mg/kg nicotine i.v. from saline. Monkeys were experimentally and pharmacologically naïve prior to the current study. Monkeys weighed 7.5-10.8 kg and were fed primate chow (Harlan Teklad High Protein Monkey Diet; Madison, WI), fresh fruit and peanuts. They were housed individually in stainless steel cages and maintained under controlled humidity and temperature on a 14/10-h light-dark cycle with continuous access to water in the home cage. The maintenance and experimental use of animals was carried out in accordance with the 2011 Guide for Care and Use of Laboratory Animals. All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at The University of Texas Health Science Center at San Antonio.

2.2. Surgery

Monkeys were anesthetized with ketamine hydrochloride (10 mg/kg i.m.; Butler Pharmaceuticals, Dublin, OH), followed by isoflurane inhaled via face mask (1.5-3.0%). A catheter (heparin-coated polyurethane i.d.=1.02 mm, o.d.=1.68 mm; Instech Laboratories, Plymouth Meeting, PA) was inserted into a femoral, jugular or subclavian vein. Suture silk (coated vicryl; Ethicon Inc., Somerville, NJ) was used to secure the catheter to the vessel. The catheter extended from the vessel and was attached to a vascular access port (Mida-cbas-c50; Instech Laboratories) located subcutaneously at the midscapular region of the back.

2.3. Apparatus

When outside of the home cages, monkeys were seated in commercially available chairs (Model R001; Primate Products, Miami, FL) which provided restraint at the neck and arms. Feet were restrained by a pair of shoes mounted on the front of the chair. Shoes were fitted with brass electrodes able to deliver a brief electric shock (3 mA, 250 ms) from an a/c generator (Coulbourn Instruments, Allentown, PA). Training and testing were conducted in sound-attenuating, ventilated operant chambers containing an operant panel below which was mounted a food receptacle. The operant panel consisted of a row of three lights and a second row of two levers; levers were mounted beneath the leftmost and rightmost lights. All operant behavior was controlled and recorded by a computer connected to a commercially available interface and Med-PC software (MedAssociates, Inc., St. Albans, VT).

2.4. Discrimination Training

Monkeys discriminated 0.032 mg/kg nicotine from saline i.v. administered via chronic indwelling catheter during experimental sessions conducted once per day at 7:00 am, 7 days per week. For two monkeys the left lever was correct after nicotine and the right lever was correct after saline; in the other two monkeys, the lever assignments were reversed. Two training conditions were possible: when monkeys received an injection of the training dose of nicotine, only responses on the drug lever (i.e., correct lever) were reinforced; when monkeys received an injection of saline, only responses on the saline lever (i.e., correct lever) were reinforced. The method of reinforcement was stimulus-shock termination (SST) under a fixed ratio 5 (FR5) schedule. Stimulus-shock termination was used in the current study to be consistent with previously published studies examining the acute and chronic effects of nicotine, including nicotine withdrawal, in rhesus monkeys (Cunningham and McMahon, 2014). Stimulus-shock termination has the advantage of being more resistant than food presentation to the disruptive effects of drugs (McMahon and France, 2002). Following injection was a one min pretreatment interval spent in the operant chamber during which no lights were illuminated and pressing the levers had no scheduled consequences. Following the pretreatment interval, red lights were illuminated above each lever and shock was scheduled to occur every 10 s. Completion of the FR on the correct lever caused the lights to extinguish and postponed the shock schedule for 30 s. Responses on the incorrect lever reset the FR requirement. During the 30-s timeout, lever pressing had no scheduled consequences. After the timeout, the red lights were re-illuminated, the levers became active, and the shock schedule resumed (i.e., a shock scheduled to occur every 10 s). The duration of SST responding was 10 min initially; it was shortened to 5 min once discrimination time course data became available. If four shocks were delivered in a session, then the session was immediately terminated. Nicotine and saline training alternated daily or every other day; a training condition (i.e. nicotine or saline) was not repeated for more than two days consecutively. After five consecutive training sessions in which greater than 80% of the total session responses were made on the correct lever and less than five responses were made on the incorrect lever prior to delivery of the first reinforcer, monkeys were tested.

2.5. Discrimination Testing

Test days were scheduled so that approximately half followed a training day on which the monkey received nicotine and half followed a training day on which the monkey received saline. On test days, completion of the FR5 on either lever was reinforced. Test days were always followed by training days, and monkeys were required to meet the discrimination training criteria (i.e., greater than 80% responses made on the correct lever and fewer than five responses made on the incorrect lever prior to delivery of the first reinforcer) for three consecutive days before another test day was substituted into the training schedule; this included at least one nicotine-training day and one saline-training day.

To examine the effects of nicotine over time, a single pretreatment interval was studied per test session conducted on different days and in non-systematic order. The 1-min pretreatment was determined under standard test conditions. Test sessions were conducted at 3, 10 and 30 min after administration of the training dose; during these tests, saline was administered i.v. 1 min before the session. Dose-response functions for nicotine, epibatidine,

varenicline, midazolam, cocaine, and morphine were determined by administering a dose i.v. 1 min before the 5-min test session. A dose of mecamylamine, DH β E, or MLA was administered s.c. 30 min before tests followed by saline or a dose of nicotine 1 min before the response period. DH β E was also administered 30 min before tests followed by a dose of varenicline. Dose-response functions included ineffective doses, i.e., a dose producing less than 20% of the maximum effect, up to a dose producing greater than 80% of the maximum effect or until shocks were delivered.

2.6. LC/MS/MS Measurement of Nicotine, Cotinine, and 3OH-Cotinine in Monkey Blood

Blood was collected through the indwelling catheter while monkeys were seated in chairs. The first 2 ml of blood were discarded and an additional 2 ml were transferred to a 3-ml Vacutainer tube containing 5.4 mg EDTA (Becton Dickinson, Franklin Lakes, NJ) and frozen at -80°C . Immediately following blood collection, the catheters were flushed with 3 ml of heparinized saline. A baseline sample was obtained before administration of 0.032 mg/kg nicotine i.v. into the saphenous vein. Subsequent samples were collected at various time points up to 120 min post-injection. Pharmacokinetic assessments of nicotine and its metabolites were performed at least 48 h after any other nicotine exposure.

Nicotine, cotinine, trans-3OH-cotinine, and d4-nicotine (internal standard) were obtained from Sigma-Aldrich (St. Louis, MO) as 1 mg/ml methanol stock solutions. HPLC grade methanol and acetonitrile were purchased from Fisher (Fair Lawn, NJ). All other reagents were purchased from Sigma-Aldrich. Milli-Q water was used for preparation of all solutions. Stock solutions were stored as 100 μl aliquots at -80°C until the day of the experiment. Working stock solutions were prepared each day from the 1 mg/ml stock solutions at a concentration of 10 $\mu\text{g}/\text{ml}$ to spike the calibrators. The HPLC system consisted of a CBM 20A Controller (Shimadzu Kyoto, Japan), two 20A pumps, a SIL 20AC-HT autosampler, and an API4000 Q-trap tandem mass spectrophotometer with turbo ion spray (ABSciex, Framingham, MA). The analytical column was an ACE-C18-PFP (3.0×75 mm, 3 μ ; MacMod, Chadds Ford, PA) which was maintained at 23°C during the chromatographic runs using a Shimadzu CTO-20AC column oven. Mobile phase A was 6.5 mM ammonium acetate in Milli-Q water. Mobile phase B was HPLC grade acetonitrile. The flow rate was 0.2 ml/min. Nicotine eluted at 5.8 min, cotinine at 2.7 min, and 3-hydroxy cotinine at 2.5 min during the following step gradient (0% B from 0-6 min, 100% B from 6.1 to 15 min, 0% B from 15.1 to 20 min). The transitions used to detect and quantify the compounds were 162.9 129.9 (nicotine), 176.9 80.1 (cotinine), 193.0 80.0 (3OH-cotinine), and 166.9 136.1 (d4-nicotine; internal standard).

On the day of the assay, samples were thawed at room temperature, protected from light. Nicotine, cotinine, and 3OH-cotinine were quantified using 100 μl of uncoagulated (EDTA) whole blood sample according to the following protocol. Equal volumes of calibrator and unknown samples were added to 10 μl of 10 $\mu\text{g}/\text{ml}$ d4-nicotine (internal standard) and 500 μl of acetonitrile. The samples were vortexed vigorously for 2 min, shaken for 30 min and then centrifuged at 3,200 g for 30 min at 23°C (subsequent centrifugations were performed under the same conditions). Supernatants were transferred to 1.8 ml microfilterfuge tubes and centrifuged. Twenty μl of the eluants were injected into the LC/MS/MS. The ratio of the

peak area of each analyte to that of the internal standard d4-nicotine (response ratio) for each unknown sample was compared against a linear regression of calibrator response ratios at 0, 7.8, 15.6, 31.3, 62.5, 125, 250, 500, 1000 and 2000 ng/ml to quantify nicotine, cotinine, and 3OH-cotinine. Concentrations were expressed as ng/ml of whole blood.

2.7. Drugs

Drugs were delivered s.c. in the midscapular region of the back or i.v. via chronic indwelling catheter in a volume of 0.1-1 ml/kg. Drug doses are expressed as the weight, in mg/kg, of the forms listed below, except in the case of nicotine, which is expressed as the weight of the free base. (-)-Nicotine hydrogen tartrate salt (Sigma-Aldrich), epibatidine dihydrochloride (Sigma-Aldrich), varenicline dihydrochloride (Research Technology Branch of the National Institute on Drug Abuse, Rockville, MD), cocaine hydrochloride (Sigma-Aldrich), midazolam hydrochloride (Bedford Laboratories, Bedford, OH), morphine sulfate (Research Technology Branch of the National Institute on Drug Abuse), mecamylamine (Waterstone Technology, Carmel, IN), dihydro- β -erythroidine hydrobromide (Tocris Bioscience, Bristol, UK), and methyllycaconitine citrate (Research Technology Branch of the National Institute on Drug Abuse) were dissolved in physiological saline. For nicotine, the amount of sodium hydroxide (Sigma-Aldrich) needed to adjust the pH of the solution to 7 was determined and figured into the calculation of volume for a desired concentration.

2.8. Data Analyses

Pharmacokinetic parameters for nicotine, cotinine and 3OH-cotinine were estimated from blood concentrations by a model-independent method as described previously (Perez-Stable et al., 1998). AUC_{0-240} was determined by the trapezoidal rule. The terminal half-life of nicotine was determined and extrapolated using regression analyses. For each animal, the regression analysis contained data from at least 4 different time points in the terminal phase and as many data points as possible, consistent with the visual assessment of a straight line on the log-transformed scale. Total clearance was computed as dose divided by $AUC_{0-\infty}$. All pharmacokinetic calculations were performed using Phoenix WINNONLIN 6.4 (Certara, Princeton, NJ).

The percentage of responses on the nicotine-associated lever was calculated by dividing the number of responses on that lever by the sum of responses made on the nicotine- and saline-associated levers. Rate of responding was measured in responses/s. Responding during timeouts was not included. Due to overlap of the 5-min response periods in the experiments determining the time courses of the training drugs (e.g., 1, 3, and 10 min), the primary measure recorded in these experiments (% Nicotine Lever) was the total number of responses made on the drug lever prior to delivery of the first reinforcer, divided by the total number of responses made on both levers prior to delivery of the first reinforcer. The mean of the rate of responding from the five most recent saline training days for which the subject met the training criteria was considered the control response rate for a given test. Test data for response rate were calculated for each subject as a percentage of their control response rate (% Control Response Rate). Both measures (i.e., % Nicotine Lever and % Control Response Rate) were averaged across subjects and expressed as a mean $\pm$ 1 standard error of the mean. The percentage of responses on the nicotine lever was only plotted and included in

the analysis if the response rate was greater than or equal to 20% of the control response rate. Response rate data were always included. These two measures were plotted as a function of dose or a function of time using GraphPad Prism (v. 6.0; GraphPad Scientific, San Diego, CA). Linear regression of the values that defined the linear portion of the dose-response function for each subject were included in the same analysis to calculate the slope, ED₅₀ value, and 95% confidence limits. *F*-ratio tests were used to compare slopes of the dose-response functions; if the slopes were not significantly different from one another, a common slope was used to calculate potency ratios and 95% confidence limits (Tallarida, 2000). The ED₅₀ values were considered significantly different from one another when the 95% confidence limits of the potency ratio did not include 1. There was a significant effect on response rate as a function of dose if the slope of the line fit by linear regression through the 3 largest doses tested was significantly different from 0. Effects as a function of time were determined to be significant by repeated measures ANOVA ($p < 0.05$).

Schild plots were constructed by expressing the logarithm of the dose ratio-1 on the ordinate and the negative logarithm of the molar dose of DH β E on the abscissa (Arunlakshana and Schild, 1959). Straight lines were fit to individual Schild plots simultaneously using the following equation: $\log(\text{dose ratio}-1) = -\log(\text{molar dose of DH}\beta\text{E}) \times \text{slope} + \text{intercept}$. Two mathematical models were used to examine the Schild plot for DH β E: a simpler model (i.e., slope constrained to unity or -1) and a more complex model that allowed slope to vary. The two models were compared *F*-ratio tests. If the calculated *F*-value comparing the slopes of the two lines was not significant ($p < 0.05$), i.e., the unconstrained slopes of the individual lines were not significantly different from unity, then the pA₂ value was calculated with both the constrained and unconstrained slope.

3. Results

3.1. Control performance

Nicotine dose-dependently increased responding on the drug lever (Fig. 1A), whereas saline resulted in 1% responding on the nicotine lever. The discrimination dose-response function ranged from 0.0056 mg/kg up to the training dose. Responding at the training dose was 100% on the nicotine lever. The ED₅₀ value (95% confidence limits) of nicotine for producing discriminative stimulus effects was 0.013 (0.0055-0.029) mg/kg. Drug-lever responding following administration of 0.032 mg/kg nicotine i.v. significantly varied as a function of time ($F_{3,9}=7.87$, $p < 0.05$). The onset to the maximum effect of the training dose was 1 min or less and the duration of the effect was less than 30 min (Fig. 1B). When calculated as an average from 10 consecutive saline training sessions, excluding drug training sessions, rate of responding in responses per s for each respective monkey was 3.47, 2.24, 2.08, and 1.54 responses per s. Nicotine did not significantly modify the rate of responding up to the training dose.

3.2. Nicotine, cotinine, and 3OH-cotinine pharmacokinetics

Mean and standard error of the mean plasma concentrations of nicotine, cotinine, and 3OH-cotinine are shown in Fig. 2. Individual and mean pharmacokinetic parameters are summarized in Table 1. Following administration of 0.032 mg/kg nicotine i.v., plasma

nicotine concentration increased rapidly and reached maximum concentration within 9 minutes; the average maximum nicotine concentration (C_{max}) observed was 71.7 ng/ml. An average exposure (AUC_{0-inf}) of 8610 minute*ng/ml was observed after the i.v. training dose.

3.3. Effects of nAChR agonists and non-nAChR drugs

The nAChR agonists epibatidine and varenicline dose-dependently increased nicotine-lever responding to a maximum of 97% at a dose of 0.00032 mg/kg epibatidine and 95% at a dose of 0.1 mg/kg varenicline (Fig. 3A, diamonds and squares, respectively). The slopes of the nicotine, epibatidine and varenicline dose-response functions were not significantly different from each other ($F_{2,22} = 2.19$, $p = 0.14$). The ED_{50} values (95% confidence limits) of epibatidine and varenicline were 0.00015 (0.00011-0.00022) and 0.031 (0.018 - 0.053) mg/kg, respectively. Epibatidine was 80-fold more potent than nicotine ($p < 0.0001$); nicotine was 2.4-fold more potent than varenicline ($p < 0.5$). Epibatidine and varenicline did not significantly alter response rate up to the largest doses tested (Fig. 3B).

Midazolam (i.v.) produced a maximum of 26% nicotine-lever responding at 0.032 mg/kg and significantly decreased response rate ($F_{1,10} = 10.7$, $p < 0.01$) to 38% of control at 0.1 mg/kg (Fig. 3C and 3D, gray triangles). Cocaine (i.v.) produced a maximum of 28% nicotine-lever responding and decreased response rate to 76% (Fig. 3C and 3D, gray circles): while the effects of cocaine on response rate were not significant, doses larger than 0.32 mg/kg were not tested due to concerns over toxicity. Morphine (i.v.) produced a maximum of 26% responding on the nicotine lever at 3.2 mg/kg (Fig. 3C and 3D, gray inverted triangles). Larger doses of morphine were not tested due to concerns over toxicity.

3.4. Effects of mecamylamine, DH β E and MLA in combination with the nicotine training dose

When combined with the training dose of nicotine (0.032 mg/kg), mecamylamine dose-dependently decreased the percentage of nicotine-lever responding (Fig. 4A, triangles). The ED_{50} value (95% confidence limits) of mecamylamine to attenuate the effects of 0.032 mg/kg nicotine was 0.13 (0.07-0.23) mg/kg. Mecamylamine (1 mg/kg) alone produced 0% responding on the nicotine lever and in combination with nicotine produced a downward shift of the nicotine dose-response function, i.e., responding did not exceed 1% on the nicotine lever when 1 mg/kg mecamylamine was studied in combination with doses of nicotine up to 0.056 mg/kg (data not shown).

DH β E, when combined with 0.032 mg/kg nicotine, dose-dependently decreased discriminative stimulus effects; the ED_{50} value (95% confidence limits) of DH β E to attenuate the effects of the training dose of nicotine was 0.18 (0.067-0.51) mg/kg (Fig. 4A, inverted triangles).

MLA up to a dose of 10 mg/kg did not significantly modify the discriminative stimulus effects of either 0.032 mg/kg nicotine (Fig. 4A, diamonds) or the next smaller dose of nicotine (0.0178 mg/kg), which alone produced 79% nicotine-lever responding (data not shown). Rate of responding was not significantly altered at any dose of mecamylamine, DH β E, or MLA alone or in combination with any dose of nicotine (Fig. 4B).

3.5. DH β E in combination with nicotine and varenicline

DH β E dose-dependently antagonized the discriminative stimulus effects of nicotine and varenicline (Fig. 5A and 5C, respectively). None of these dose combinations significantly altered response rate (Fig. 5B and 5D). For discriminative stimulus effects, the dose ratios of the ED₅₀ value (95% confidence limits) of nicotine determined in the presence of 0.1 mg/kg DH β E expressed over the control nicotine ED₅₀ value per individual monkey were 1.7, 1.8, 1.8, and 3.3 (mean=2.2-fold rightward shift in the nicotine dose-response function). The corresponding dose ratios calculated for nicotine in the presence of 0.32 mg/kg DH β E versus control were 7.2, 3.2, 4.4, and 4.3 (mean=4.7). The dose ratios of the ED₅₀ value (95% confidence limits) of varenicline determined in the presence of 0.1 mg/kg DH β E expressed over the control varenicline ED₅₀ value per individual monkey were 1.4, 1.7, 2.1, and 3.2 (mean=2.1-fold rightward shift in the varenicline dose-response function). The corresponding dose ratios calculated for varenicline in the presence of 0.32 mg/kg DH β E versus control were 2.5, 2.9, 3.7, and 5.7 (mean=3.7).

The Schild plots for antagonism of the discriminative stimulus effects of nicotine and varenicline are shown in Fig. 6; the coefficients of determination (r^2) were 0.67 and 0.50, respectively, and the unconstrained slopes (95% confidence limits) were -1.05 (-1.78 to -0.32) and -0.89 (-1.77 to -0.01), respectively. Neither slope was significantly different from unity (i.e., -1; $p=0.87$ and 0.76 , respectively) nor from each other ($p=0.73$). The apparent pA₂ values (95% confidence limits) of DH β E calculated from the constrained slopes were 6.58 (6.41–6.74) in the presence of nicotine and 6.45 (6.25–6.65) in the presence of varenicline. The corresponding pA₂ values calculated from the unconstrained slopes were 6.56 and 6.47.

4. Discussion

Nicotine was established as a discriminative stimulus in rhesus monkeys at a dose of 0.032 mg/kg expressed as the weight of the free base administered i.v. The training dose resulted in a C_{max} equivalent to the expected nicotine concentration in a smoker after 2-3 cigarettes (Hukkanen et al., 2005; Benowitz et al., 2009); the average exposure (AUC_{0-inf}) was comparable to that after smoking 5-6 cigarettes in humans, assuming each cigarette delivers 1.5 mg of nicotine and a clearance of 1110 mL/min (Hukkanen et al., 2005). Overall, nicotine plasma levels were longer lasting compared to the time course of discriminative stimulus effects (compare Fig. 1 right to Fig. 2 top), suggesting that tolerance and receptor desensitization might be responsible for the loss of discriminative stimulus effects despite persistently high nicotine levels. The apparent acute tolerance observed in the current study is consistent with that previously reported for the subjective and cardiovascular effects of cigarette smoking in humans (Perkins et al., 1994).

Several results reported here are consistent with the discriminative stimulus effects of 0.032 mg/kg nicotine i.v. being selectively mediated by nAChRs. First, epibatidine and varenicline fully substituted for the nicotine discriminative stimulus with relative potencies similar to those reported previously under conditions presumably reflecting nAChR agonism (Rodriguez et al., 2014; Desai and Bergman, 2015). Second, drugs whose primary mechanisms of action do not involve nAChR agonism, such as cocaine, midazolam, and

morphine, did not fully substitute for the nicotine discriminative stimulus up the largest doses that did not disrupt behavior (current results; Li et al., 2008; Gould et al., 2011). Similar results have been obtained previously with cocaine and morphine in squirrel monkeys discriminating nicotine i.v. (Takada et al., 1988). Third, mecamylamine and DH β E dose-dependently antagonized the discriminative stimulus effects of nicotine.

This study represents one of the few attempts to apply quantitative analytic approaches to nicotine antagonism in whole animals (see also Jutkiewicz et al., 2011). Apparent pA₂ (i.e., *in vivo* Schild) analysis is a useful quantitative method for evaluating the extent to which the same receptor subtype(s) mediates an effect that is shared among agonists, especially when more than one receptor subtype can mediate a given effect. For example, *in vivo* Schild analysis has been used to examine the relative contribution of different opioid receptor subtypes to the discriminative stimulus effects of different opioid agonist training drugs (Bertalmio and Woods, 1987). In contrast, *in vivo* Schild analysis has been infrequently used to calculate the potency of nicotine antagonists and to compare nAChR mechanism(s) of action among agonists, and this is likely due to the relative lack of appropriate nAChR antagonists available for *in vivo* studies. The validity of pA₂ analysis depends upon the availability of simple, competitive, reversible antagonists.

Here, *in vivo* Schild analysis demonstrated that nicotine and varenilcine acted at the same DH β E-sensitive nAChRs, which are likely α 4 β 2. In a previous study (Jutkiewicz et al., 2011), Schild analysis was used to quantify antagonism of the discriminative stimulus effects of nicotine by DH β E in rats discriminating a relatively small dose of nicotine s.c. The current study confirms and extends these findings to another species, route of administration, and a second nAChR agonist. The Schild plot, which defines the relationship between the dose of antagonist and the magnitude of antagonism, was constructed through two doses of antagonist, rather than at least three, which is typical. Linear regression through only two doses of antagonist was possible because a dose ratio was calculated at both doses for very subject (n=4) resulting in eight values for the regression. A third, larger dose of DH β E was not studied due to concerns over other, adverse effects (i.e., seizures) of nicotine i.v. that might not be antagonized as effectively as discriminative stimulus effects. The unconstrained slopes did not differ from unity (i.e., -1) and the coefficients of determination were reasonable given the relatively small number of observations. Previous studies have demonstrated that the discriminative stimulus effects of nicotine are antagonized by DH β E (Gommans et al., 2000; Shoaib et al., 2000; Stolerman et al., 1997) and mecamylamine (Morrison and Stephenson, 1969; Stolerman et al., 1999). The current study demonstrates the feasibility of using *in vivo* Schild analysis to not only calculate the apparent affinity of DH β E (i.e., 0.094-0.13 mg/kg in rhesus monkeys), but also compare the nAChR site of action of different agonists.

MLA did not antagonize the nicotine discriminative stimulus suggesting lack of involvement of α 7 nAChRs. The current results are consistent with the results of previous studies showing that α 7 knockout mice readily acquire the discrimination of nicotine (Stolerman et al., 2004), and that MLA does not antagonize the nicotine discriminative stimulus in rats (Zaniewska et al., 2006) or mice (Gommans et al., 2000), even when MLA is administered by the intracerebroventricular route (Brioni et al., 1996). Collectively, these results suggest

that the discriminative stimulus effects of nicotine are not mediated by $\alpha 7$ nAChRs. However, this interpretation likely depends on the nicotine training dose. The discriminative stimulus effects of nicotine at relatively large training doses appear to be mediated by different nAChR subtypes than smaller training doses (Jutkiewicz et al., 2011). In rhesus monkeys discriminating a 1.78 mg/kg nicotine s.c. expressed as the weight of the free base, mecamylamine but not DH β E antagonized the discriminative stimulus effects of nicotine and varenilcine (Cunningham et al., 2012), suggesting that non-DH β E sensitive nAChRs mediate the discriminative stimulus effects of nicotine established at relatively large doses.

In summary, a dose of 0.032 mg/kg nicotine delivered i.v., trained as a discriminative stimulus in rhesus monkeys, exhibits pharmacokinetics (i.e., C_{max}) similar to that in humans smoking 2-3 cigarettes (Hukkanen et al., 2005). Moreover, the pharmacology underlying this discrimination appears to be selective for actions at DH β E-sensitive receptors; these are likely $\alpha 4\beta 2$ nAChRs based on the binding profile of DH β E (Williams and Robinson, 1984; Harvey and Luetje, 1996; Harvey et al., 1996; Papke et al., 2008). Because $\alpha 4\beta 2$ nAChRs are a major target for the development of smoking cessations aids, the present discrimination assay might be useful for identifying novel drugs for this particular indication. In this experimental assay, nicotine and varenilcine exert strikingly similar discriminative stimulus effects that appear to be mediated by the same nAChRs, likely $\alpha 4\beta 2$, suggesting that the therapeutic effects of varenicline are due, at least in part, to its ability to mimic the effects of nicotine.

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Abbreviations

DHβE	dihydro- β -erythroidine
FR	fixed ratio
nAChR	nicotinic acetylcholine receptor
MLA	methyllaconitine

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Fig. 1. The discriminative stimulus effects of nicotine in monkeys trained to discriminate 0.032 mg/kg i.v. nicotine as a function of dose (A) ($n=4$) and time (B) ($n=4$)

Left abscissae: Saline (S) or dose of nicotine expressed as free base weight per kilogram body weight (mg/kg). Right abscissae: time in minutes. Ordinates: mean ($\pm$ S.E.M.) percentage of responding on the nicotine lever.

Fig. 2. Blood concentrations of nicotine (A), cotinine (B), and 3OH-cotinine (C) following administration of 0.032 mg/kg nicotine i.v. expressed as mean and standard error of the mean Abscissae: time in minutes. Ordinates: ng of nicotine, cotinine, or 3OH-cotinine per ml blood.

Fig. 3. Effects of nAChR agonists (A and B) ($n=4$) and non-nAChR drugs (C and D) ($n=4$) in monkeys discriminating 0.032 mg/kg nicotine i.v.

Abscissae: Saline (S) or dose in mg/kg body weight administered i.v. Ordinates: mean ($\pm$ S.E.M.) percentage of responding on the nicotine lever (top) and mean ($\pm$ S.E.M.) rate of responding expressed as a percentage of control (bottom). The nicotine data are re-plotted from Fig. 1.

Fig. 4. Discriminative stimulus (A) and rate effects (B) of the nicotine training dose (0.032 mg/kg i.v.) in combination with mecamylamine, DHβE, or MLA ($n=4$)

Abcissae: The training dose of nicotine (Nic 0.032) or dose of antagonist in mg/kg body weight administered s.c. Ordinates: mean ($\pm$ S.E.M.) percentage of responding on the nicotine lever (top) and mean ($\pm$ S.E.M.) rate of responding expressed as a percentage of control (bottom).

Fig. 5. Effects of nicotine (A and B) and varenicline (C and D) in combination with DHβE (0.1 and 0.32 mg/kg) in monkeys discriminating 0.032 mg/kg nicotine i.v. ($n=4$)

Abscissae: Saline (S) or dose of nicotine in mg/kg body weight administered i.v. Ordinates: mean ($\pm$ S.E.M.) percentage of responding on the nicotine lever (top) and mean ($\pm$ S.E.M.) rate of responding expressed as a percentage of control (bottom).

Fig. 6. Schild plots for DHβE in the presence of nicotine (A) or varenicline (B) constructed from the mean data shown in Fig. 5

Abscissae: negative logarithm of DHβE dose in moles per kilogram. Ordinates: mean ($\pm$ S.E.M.) logarithm of the dose ratio-1. Schild plots were constructed from the unconstrained slopes (dashed lines) and by constraining the slopes to -1 (solid lines).

Table 1

Individual and mean pharmacokinetic parameters for nicotine, cotinine, and 3OH-cotinine in blood after 0.032 mg/kg nicotine i.v. in rhesus monkeys. Results were derived from data plotted in Fig. 2.

Nicotine						
Monkey	AUC ₀₋₂₄₀	AUC _{0-infinity}	T _{max}	C _{max}	Half-life	Clearance
	(minute*ng/ml)	(minute*ng/ml)	(ng/ml)	(ng/ml)	(minute)	(ml/minute/kg)
M1	9680	19900	30	91.1	195	1.61
M2	2780	3030	2	40.5	71.1	10.6
M3	2510	3660	2	38.1	114	8.75
M4	6650	7850	2	117	85.5	4.08
Mean	5400	8610	9	71.7	116	6.25
SD	3420	7830	14	38.9	55.4	4.13

Cotinine			3OH-Cotinine	
Monkey	AUC ₀₋₂₄₀	C _{max}	AUC ₀₋₂₄₀	C _{max}
	(minute*ng/ml)	(ng/ml)	(minute*ng/ml)	(ng/ml)
M1	59500	410	19000	170
M2	15600	146	3340	31.1
M3	18100	90.6	1490	10.1
M4	18500	119	5010	40.6
Mean	28000	191	7200	63
SD	21100	147	7970	72.5