NICOTINE SELF-ADMINISTRATION IN ADOLESCENT MALE AND FEMALE RATS

by

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B.S. in Neuroscience, Bucknell University, 2009

Submitted to the Graduate Faculty of the

Kenneth P. Dietrich School of Arts and Sciences in partial fulfillment

of the requirements for the degree of

Master of Science

University of Pittsburgh

2014

UNIVERSITY OF PITTSBURGH

DIETRCH SCHOOL OF ARTS AND SCIENCES

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Tobacco product use is the leading preventable cause of death in the United States. Nearly 90% of current daily smokers initiated use during adolescence. Moreover, although national rates of tobacco use by adults have declined in recent years, adolescent initiation remains high. Despite this, little is known about adolescent initiation and use. The present study sought to determine the effects of sex and developmental stage in the acquisition of nicotine self-administration, across a range of doses paired with different nonpharmacological cues. Adolescent (postnatal day [P] 30) and adult (P90) male and female rats with unlimited access to food were allowed to nosepoke on a fixed ratio 2 schedule to intravenously self-administer nicotine (3, 10, 30, or 100 μg/kg/infusion) paired with a moderately reinforcing visual reinforcer (VS) or an initially neutral stimulus light presentation (conditioned stimulus; CS), during daily 1-h sessions. The lowest doses of nicotine (3 or 10 μg/kg) paired with CS presentations did not support acquisition of self-administration in adolescents; however, when paired with VS both low doses engendered acquisition. While adolescents did not acquire at 10 μg/kg + CS, both male and female adults acquired self-administration in this condition. All four sex and age groups acquired self-administration, and earned a similar number of infusions when responding for 30 μg/kg nicotine paired with CS presentations. When paired with VS, adolescents, particularly males, responded more for 30 μg/kg nicotine than adults. Cue condition did not affect adult acquisition for either

10 or 30 μg/kg. Finally, 100 μg/kg supported the acquisition of self-administration for male and female adolescents when paired with either CS or VS, at rates comparable to 30 μg/kg. These results demonstrate that adolescent rats will respond for low doses of nicotine when combined with a moderate reinforcer (VS), but do not respond more than adults for a moderate dose of nicotine. Our finding that a low nicotine dose may enhance responding for VS during adolescence suggests that combination of nicotine exposure with mild rewards may lead to increased exposure to nicotine, and result in nicotine self-administration.

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ACKNOWLEDGEMENTS

Thank you to my committee and other members of the lab, whose input was invaluable to the design and interpretation of this study, including: Alan F. Sved, Eric C. Donny, Edda Thiels, Deanne M. Buffalari, Tracy T. Smith, and Laura E. Rupprecht. Thank you to the technical assistants and undergraduates who assisted in data collection, including: Emily Pitzer, Josh Alberts, Samantha Cwalina, Alexandra Kenefake, Hangil Seo, Jessica Pelland, Corina Andriescu, Dora Danko, and Isha Vasudeva. I would also like to thank my family and partner Matthew for their encouragement and support throughout this process.

1.0 INTRODUCTION

Cigarette and smokeless tobacco product use is a major public health issue that impacts millions of people worldwide (WHO, 2012, May). Tobacco product use is the leading preventable cause of death and disease, including cancer and cardiovascular disease, in the United States (CDC, 2012a). Of the estimated 43.8 million current daily smokers in the US (CDC, 2012a), nearly 90% initiated tobacco use prior to the age of 18 (Levin, Rezvani, Montoya, Rose, & Swartzwelder, 2003; USDHHS, 2012). National surveys of middle and high school students reported that 7.1% of middle school and 23.2% of high school students reported current use (use on at least 1 of the last 30 days) of any type of tobacco product (CDC, 2012b). Generally, the earlier a smoker begins, the more they will smoke (Breslau, Fenn, & Peterson, 1993; Madden et al., 1997; Taioli & Wynder, 1991) and the greater difficulty they will have during cessation attempts (Breslau et al., 1993; Breslau & Peterson, 1996; Khuder, Dayal, & Mutgi, 1999). Furthermore, cessation periods that do occur will be shorter (Khuder et al., 1999), as long term or heavy smokers relapse more readily and frequently. Finally, despite laws prohibiting sale of tobacco products to minors, the extremely high prevalence of current smokers initiating use before age 18 suggests that this period should be studied in greater detail.

Adolescents are more vulnerable to nicotine use. The age of drug use initiation, and by extension, onset of drug dependence, is a risk factor for drug abuse (Anthony & Petronis, 1995). Although not all adult tobacco product users initiate use in adolescence, or necessarily early

adolescence, the great majority do (Corrigall, Zack, Eissenberg, Belsito, & Scher, 2001; USDHHS, 2012). Furthermore, heavier smokers tend to fall into the population that initiate use at an earlier age (Breslau et al., 1993; Chassin, Presson, Sherman, & Edwards, 1990; Taioli & Wynder, 1991). Adolescent smokers report use for many of the same reasons as adult smokers: feelings of pleasure or reward, control of body weight, and modulation of arousal and mood (USDHHS, 2012). Adolescent smokers also experience withdrawal symptoms like those experienced by adults (McNeill, West, Jarvis, Jackson, & Bryant, 1986; Rojas, Killen, Haydel, & Robinson, 1998), which is representative of physical dependence, and often leads to relapse after cessation attempts. Adolescent tobacco product users develop dependence at an accelerated rate (McNeill et al., 1986; Rojas et al., 1998; Spear, 2000), which has been replicated in rodent models comparing adolescent-exposed male rats to adult-exposed rats (Adriani et al., 2003). Moreover, adolescent rats acquire nicotine self-administration at relatively low doses, doses that do not engender drug-seeking behavior in adults (Li et al., 2012; Lynch, 2009). Altogether, these studies suggest an increased vulnerability for adolescents to experiment with and become dependent on nicotine.

There is evidence of a sex-difference in the susceptibility for initiation and continued tobacco product use (Benowitz & Hatsukami, 1998). Women are more susceptible to sustained drug use, including nicotine products (Carroll, Lynch, Roth, Morgan, & Cosgrove, 2004; Lynch, Roth, & Carroll, 2002), and develop nicotine dependence more readily than men (Li et al., 2012; Lynch, 2006, 2009). Associated with this difference in dependence vulnerability, women consistently record lower successful quit rates than men, and reduced rates of successful abstinence (Benowitz & Hatsukami, 1998; Perkins, Donny, & Caggiula, 1999). Relapse rates may be higher in women because they are more reactive to stress and depression, as well as cues

associated with nicotine use (Lynch, 2006). Smoking associated nonnicotine factors influence tobacco use by females much more than men (Perkins, 2009), which has been reproduced in an animal model (Chaudhri et al., 2005). In a test comparing the ability of nicotine to enhance responding for a moderately reinforcing visual stimulus in adolescent and adult males only, both age groups exhibited enhancement of the cue (Weaver et al., 2012). Although the role of nicotine-associated cues in supporting nicotine self-administration behavior of adults has been described in many instances, it is unclear if cues contribute to use by adolescents in a similar manner.

The sex-difference in adult use and relapse can be demonstrated in rat models of self-administration, suggesting a biological basis underlying the rapid transition to dependence and reactivity to cues and stress (Chaudhri et al., 2005; Donny et al., 2000; Donny et al., 2003; Rezvani et al., 2008). Donny et al. (2000) found that adult female rats self-administered more of low doses of nicotine than adult males, and exhibited greater motivation to respond for infusions. Although not supported in the study by Donny et al. (2000), it has been shown by other groups that reproductive and ovarian hormones contribute to the subjective effects of smoking experienced by women, and may underlie the withdrawal severity that leads to relapse (Becker & Hu, 2008; Carroll et al., 2004; Donny et al., 2000; Lynch, 2009; Lynch & Sofuoglu, 2010). Although adult men maintain higher nicotine intake and are marginally more likely to use tobacco products than women, the difference in usage rates disappears in adolescent populations.

The past two decades saw a marked rise in tobacco use among adolescent females (CDC, 2012b; Moolchan, Ernst, & Henningfield, 2000; USDHHS, 2012). The slight difference in smoking status among adult males and females disappears in adolescents (Lynch, 2009; SAMHSA, 2007), with both sexes reporting equal rates of current smoking. Additionally, the

developmental-difference in rates of tobacco product use is maintained within females. In studies looking solely at adolescent female nicotine self-administration compared to adult females, adolescent females self-administered significantly more nicotine than adults (Levin et al., 2003). These findings suggest that adolescent females may represent a particularly vulnerable population for initiation of tobacco product use, without much knowledge of how sex and age impact initiation.

The Family Smoking Prevention and Tobacco Control Act, passed in 2009, gives authority to the Food and Drug Administration (FDA) to dictate regulations on the manufacture, distribution, and marketing of tobacco products ("Family Smoking Prevention and Tobacco Control Act" of 2009). This jurisdiction includes mandating nicotine allowance levels for cigarettes; levels which may be reduced, but not banned (Congress *1256* §907(d)(3)(B), 2009). A goal of this act is to improve public health by promoting cessation of tobacco products (Congress *1256* §2(34), 2009), which may be mediated by lowering the amount of nicotine allowed in cigarettes to a threshold that does not sustain self-administration behavior. An analysis of threshold doses for adults and adolescents will be particularly helpful in contributing to deciding future regulation of tobacco product nicotine content, and while promoting cessation in current adult users is of predominant interest, it will be paramount to gain a better understanding of how adolescents and new initiates might respond to lower nicotine content cigarettes.

In sum, we hypothesize that adolescents will self-administer more nicotine than adults, particularly at low doses of nicotine. Furthermore, we hypothesize that female adolescents will be more reactive to cues paired with nicotine delivery. To test if there is a sex- and developmental-difference in nicotine self-administration, we directly compared the acquisition and maintenance of self-administration behavior of adolescent and adult male and female rats

across a range of nicotine doses. This work begins to systematically characterizes the acquisition of nicotine self-administration behavior of adolescent and adult male and female rats, and has begun to evaluate whether a reduction in the nicotine content of cigarettes will differentially impact adolescent (new initiates analogue) compared to adults (established smokers analogue).

2.0 METHODS

2.1 SUBJECTS

Male and female Sprague-Dawley rats (Harlan Farms, Indianapolis, IN) were used as subjects. Adolescent animals (*n* = 150; males, n = 75; females, n = 75) were weaned and shipped on postnatal day (P) 21, and arrived on P21 or 22. Adult rats (*n* = 64; n = 32 each males and females) arrived on P82, weighing between 168-357 g on the day after arrival. Studies were run in a series of multiple cohorts. Rats arrived from the supplier on the same date and experienced the same amount of time in our colony, as well as the same amount of time transitioning to the reversed light dark cycle prior to the start of self-administration. All rats were singly housed in hanging wire mesh cages, or in isolated tub cages on a ventilated rack, in a temperature and humidity controlled colony room. Rats were maintained on a reversed 12-h light/dark cycle (lights OFF 7 am), and all experimental procedures were carried out during the dark phase of the cycle. All animals had *ad libitum* access to food (Purina LabDiet 5000 or 5001) and water in their home cages throughout the experiment, except during 1-h self-administration sessions. All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

2.2 APPARATUS

All experimental sessions were conducted in 24 x 31 x 21 cm^3 (w x l x h) commercial operant chambers (Med Associates, St. Albans, VT). Chambers were enclosed in sound-attenuating cubicles with a ventilation fan. Operant chambers were outfitted with two nosepoke holes (2.5 cm in diameter), spaced 14 cm apart. White stimulus lights (3.5 cm in diameter) were located 6.25 cm above the top of each nosepoke portal. Only the stimulus light above the assigned active nosepoke was illuminated during the session. A houselight was located 1 cm below the ceiling of the chamber in the center of the wall containing both nosepoke portals and stimulus lights. The houselight was illuminated red for nicotine infusions paired with the conditional stimulus (CS), or white for infusions paired with the moderately rewarding visual stimulus (VS) (both described in depth below, in the section on *Self-Administration*). Intravenous (i.v.) infusions were delivered via an infusion pump through tubing connected to each animal's catheter. Tubing was protected by a metal casing connected to a swivel system that allowed for virtually unrestricted movement.

2.3 DRUGS

Nicotine hydrogen tartrate salt (Sigma, St. Louis, MO) was dissolved in 0.9% saline. The doses of nicotine available for self-administration were 3, 10, 30, or 100 μg/kg/infusion (reported as free base). All solutions were sterilized by being passed through a 0.22 μm filter. Drug infusion volume and duration were dependent on each animal's body weight. Infusions were approximately 4 times the lab standard 0.1 mL/kg/infusion, to ensure adolescent catheters were filled.

2.4 PROCEDURES

2.4.1 Catheter Construction

Adult-sized catheters were constructed from a 12.5 cm piece of Silastic tubing (0.30 mm ID, 0.64 mm OD, Dow Corning) and a threaded pedestal bolt (20 mm long, Plastics One) containing a stainless steel cannula (22-gauge, Plastics One) bent at a right angle. The Silastic tubing was enlarged and slipped over the base of the bolt. A 1 cm section of larger tubing (1.5 mm ID, 2.0 mm OD, Dow Corning) was placed over and attached to this juncture with silicone. This assembly was secured to a 2 x 3 cm piece of surgical mesh (Bard Davol, Warwick, RI) with dental cement. A bead of silicone was placed on the Silastic tubing 8 cm from the edge of the surgical mesh, used to anchor the tubing to the jugular vein with silk suture. Adolescent animals are approximately one fifth the weight of adult animals at the time of surgery (34-62 g compared to 200-350 g), necessitating the use of smaller catheters and in particular, shorter length of tubing to be inserted into the jugular vein. Adolescent catheters were constructed in the same manner as adult catheters, however all individual elements were decreased in size. An 11 cm piece of Silastic tubing was secured to a 14 mm pedestal bolt and 1 x 2 cm piece of surgical mesh. The silicone bead was placed 8 cm from the edge of the mesh, as in the adult catheters. This distance was maintained so as to provide plenty of tubing to extend as the adolescent animals grew in size, preventing restriction of movement and damage to the catheter. An effort was made to minimize the amount of dental cement used to complete the catheter, with a result of reducing the overall weight of the catheter, again to limit any restriction of the animals' movement. The final difference between adult and adolescent catheters and the implantation surgery was the length of tubing inserted into the jugular vein, ending in the right atrium of the heart. In adult

animals, the length of tubing below the silicone bead, inserted into the heart, was 3.6 cm. In adolescent animals, the length was adjusted based on the body weight of each animal. The tubing lengths were between 1.1-1.6 cm for animals weighing between 34-62 g.

2.4.2 Surgery

Rats were implanted with chronic indwelling catheters into the right external jugular vein on day P24-25 (adolescent) or P84-85 (adult) (figure 1). Rats were anesthetized with isoflurane (2-3% in 100% O_2). Incision sites on the dorsal surface between the scapulae, and on the ventral surface between the neck and right clavicle were shaved and swabbed with betadine solution. A longitudinal incision (approximately 10-15 mm) was made superior to the right clavicle, over the right external jugular vein, just above when the vein passes under the pectoral muscle. Blunt dissection was used to expose and gently separate the underlying muscle to locate the jugular vein. Two pieces of silk suture were placed under the vein. The incision site was covered and the animal was turned over to expose the dorsal surface. An incision that serves as the exit site for the catheter bolt was made on the dorsal surface between the scapulae, approximately 15-20 mm in length. The skin and underlying tissue were separated by blunt dissection, creating a subcutaneous pocket for the surgical mesh portion of the catheter. Hemostat forceps were used to subcutaneously pass the catheter tubing from the dorsal surface, over the right shoulder, and exited at the ventral incision. The jugular vein was again located using the sutures previously placed underneath, and a small incision was made in the jugular vein. The catheter tubing was then inserted into the vein, stopping at the silicone bead. Proper placement of the catheter was tested by attempting to draw blood back through the catheter using heparinized saline (described

in the following section). If blood was not drawn out, the tubing was adjusted in the vein until blood could be drawn. Once blood flow was apparent and without restriction, the two pieces of silk suture previously placed under the vein were tied on either side of the silicone bead, anchoring the catheter tubing in place. The ventral skin incision was then sutured closed. On the dorsal surface, the mesh serving as the catheter base and anchor was sutured to the underlying muscle, and the incision was sutured closed. Catheters were flushed with a solution containing an antibiotic and anticoagulant (see following section), and the catheter was sealed with a cap.

Figure 1. Experimental timeline

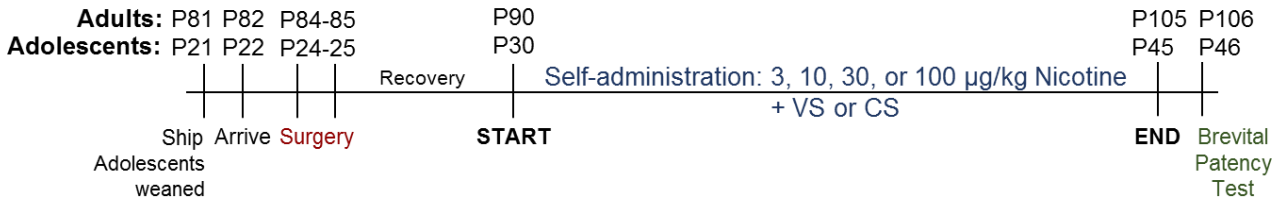

Figure 1. Timeline of key experimental events. Age is described in days postnatal (P), with ages of adults at each event listed above age of adolescents at the same events.

2.4.3 Catheter Maintenance and Patency Tests

After surgery, animals recovered in their home cage for a minimum of five days. For the first five days following surgery, for all animals catheters were flushed once daily with 0.1 mL sterile saline containing heparin (30 U/mL), timentin (66.67 mg/mL), and streptokinase (833 U/mL) to prevent infection and to maintain catheter patency. After this postsurgical period, catheters were flushed daily with solutions containing heparin and timentin. Catheter patency was tested throughout the experimental period twice weekly by attempting to draw blood back through the

catheter, and at the end of the experimental period, following the final self-administration session. In this test, a solution of Brevital (5 mg/kg) was infused into the catheter. Animals that did not show signs of ataxia were considered to have failed the patency test and were excluded from data analyses.

2.4.4 Self-Administration

Within age and sex groups, animals were assigned to self-administer one dose of nicotine: 3, 10, 30, or 100 μg/kg (adolescents), 10 or 30 μg/kg (adults); paired with a visual stimulus (VS) or conditional stimulus (CS) throughout the experiment. These doses were chosen to include a standard dose of nicotine that is at the peak of the dose-response curve and supports robust self-administration in adult animals (30 μg/kg/infusion) (Donny et al., 2003; Donny et al., 2012; Matta et al., 2007), as well as a range of both lower and higher doses of nicotine to capture the ascending limb and fully describe the dose-response curve for adolescents (Lynch, 2009; Torres, Tejeda, Natividad, & O'Dell, 2008). Table 1 summarizes the final sample sizes for each dose and cue light (VS or CS) pairing condition for all groups, after animals who failed tests of catheter patency were removed.

Table 1. Final sample sizes for nicotine and cue light pairing conditions for each group

	VS Paired			
Nicotine Dose (μg/kg)	Adolescent Male	Adolescent Female	Adult Male	Adult Female
3	8	6	--	--
10	9	6	8	8
30	14	12	8	7
100	4	4	--	--

	CS Paired			
Nicotine Dose (μg/kg)	Adolescent Male	Adolescent Female	Adult Male	Adult Female
3	3	4	--	--
10	9	14	8	8
30	11	10	8	8
100	4	4	--	--

All animals began on a fixed ratio (FR) 2 schedule of reinforcement. An FR2 schedule, in combination with the natural exploratory behavior and cued protocols, did not require prior operant training and was rapidly learned. Fulfilling the required number of responses in the randomly assigned active nosepoke portal resulted in a nicotine infusion paired with a VS or CS presentation. The VS was composed of the illumination of a white stimulus light above the active nosepoke hole for 1-s followed by a 60-s extinction of the white houselight. CS presentations were composed of a 15-s stimulus light presentation and a 60-s timeout (the remaining 45-s were unsignaled). The VS and CS represent external cues that are paired with nicotine (e.g., appearance of a cigarette, context in which smoking occurs), that may contribute to the maintenance of smoking behavior. The CS is an initially neutral stimulus that gains incentive value and becomes a conditioned reinforcer through the pairing with nicotine. The VS is a

moderately reinforcing nonpharmacological stimulus that supports modest rates of responding. When nicotine is paired with the VS, the reinforcing properties of the VS are enhanced by nicotine, and synergistically produces robust levels of responding (Donny et al., 2003). Acquisition criterion was defined as self-administering at least 5 infusions for three consecutive sessions, and maintaining that level of responding for at least half of the remaining number of sessions. The first day of this three day period is considered the first day of acquisition. Inactive responses were recorded but had no scheduled consequences. Self-administration sessions were 1-h in length and were conducted 7 days a week. Measures of behavior included: mean active and inactive nosepoke responses, total infusions earned, rate to acquire nicotine self-administration, and proportion of animals fulfilling acquisition criteria.

2.5 DATA ANALYSIS

Data analyses focused on assessing 1) discrimination between active and inactive nosepoke portals by adolescents, 2) the number of reinforcers earned, 3) the rate to acquire nicotine self-administration, and 4) the number of rats meeting criterion for acquisition of self-administration. The average number of infusions earned across the last three sessions of self-administration (sessions 14-16) was the primary dependent variable (expressed as mean ± SEM). Comparisons between conditions were analyzed by independent samples t-tests, one-, two-, or three-way tests of analysis of variance (ANOVA). The rate to meet the acquisition criterion was compared using the first of the three consecutive sessions meeting criterion (log-transformed values due to positive skew). Statistically significant main effects and interactions were explored *post hoc*

using the Bonferroni correction criterion to control for family-wise Type I error rate when appropriate. The proportion of animals in each sex and age group to meet acquisition criterion for the dose and cue conditions in which all four groups were tested (10 μg/kg and 30 μg/kg, CS and VS) was compared using the Fisher Exact Test. This was followed up with comparisons between groups ($\alpha = 0.0125$). An alpha level of $p < 0.05$ was used as the cutoff for statistical significance, unless noted otherwise. Statistical analyses were performed using SPSS (version 21).

3.0 RESULTS

Acquisition of Self-administration

Adolescent Males and Females

A main goal of these experiments was to determine if adolescent male and female rats would self-administer nicotine, tested across a range of doses, and cue conditions. Figures 2 and 3 show active and inactive nosepoke responses across sessions for adolescent females (Figure 2) and adolescent males (Figure 3) when paired with VS presentations (panel A in both figures) or with CS presentations (Figure 2B and 3B). When nicotine was paired with VS presentations, both adolescent females and males rapidly increased their active nosepoke responses across sessions, while inactive responses remained low. Both 30 and 100 μg/kg produced stable behavior by the final session for both groups. The two lowest doses produced more variability in responding. Comparison of active to inactive responses with paired sample t-tests within each adolescent group for each dose tested showed significant differences between active and inactive responses ($p < 0.05$, all comparisons), demonstrating that both adolescent females and males reliably self-administer a range of nicotine doses between 3-100 μg/kg when paired with VS presentations.

When nicotine was paired with the initially neutral CS stimulus, adolescent males and females increased active nosepoke responses only at 30 and 100 μg/kg nicotine. Adolescent males made significantly more active responses than inactive when responding for 30 μg/kg (t (10) = 3.564, $p < 0.01$), as well as 100 μg/kg (t (3) = 5.228, $p < 0.05$). Adolescent females

responded significantly more in the active nosepoke at 30 μg/kg nicotine (t (9) = 5.306, $p < 0.001$), and showed a non-significant trend for more active responses at 100 μg/kg (t (3) = 2.483, $p = 0.089$). Adolescent males and females showed a trend for a difference between active and inactive responses at 3 μg/kg (t (2) = 3.951, $p = 0.058$; t (3) = 2.494, $p = 0.088$, respectively), and only adolescent males showed a trend towards a difference at 10 μg/kg (t (8) = 2.243, $p = 0.055$). This suggests that the primary reinforcement of low doses of nicotine do not reliably support self-administration in adolescents.

Independent samples t-tests testing the effect of sex on active responses at each dose and cue condition, showed no significant effect ($p > 0.05$, all comparisons), with both adolescent females and males making a similar number of responses. Although both adolescent males and females had periods of heightened inactive responding in various dose and cue conditions, by the final three sessions of self-administration, there was no difference in the number of inactive responses made by adolescent females compared to adolescent males in all dose and cue conditions, as determined by independent samples t-tests ($p > 0.05$, all comparisons).

Comparison of the number of active responses made by each adolescent group, analyzed in separate ANOVAs for each sex with dose and cue as between-subjects factors, revealed a significant main effect of cue condition for both females ($F_{1,52} = 15.649$, $p < 0.001$) and males ($F_{1,54} = 18.308$, $p < 0.001$). For adolescent females, follow-up t-tests determined that this was due to significantly greater active responding at 3 μg/kg (t (18) = -3.440, $p < 0.005$) and 10 μg/kg (t (5.246) = -3.082, $p < 0.05$) + VS presentations, compared to these doses paired with CS presentations. Adolescent males also made significantly more active responses when 3 and 10 μg/kg nicotine (3μg: t (7.044) = -3.119, $p < 0.05$; 10 μg: t (8.132) = -3.905, $p < 0.005$) was paired with VS presentations. Additionally they made more active responses when 30 μg/kg nicotine (t

(23) = -2.769, $p < 0.05$) was paired with VS presentations. These results suggest there may be a difference in the dose-response curves for reinforcement-enhancement (VS) and the primary reinforcement (CS) of nicotine for adolescent males and females.

Figure 2. Mean active and inactive nosepoke responses made by adolescent females

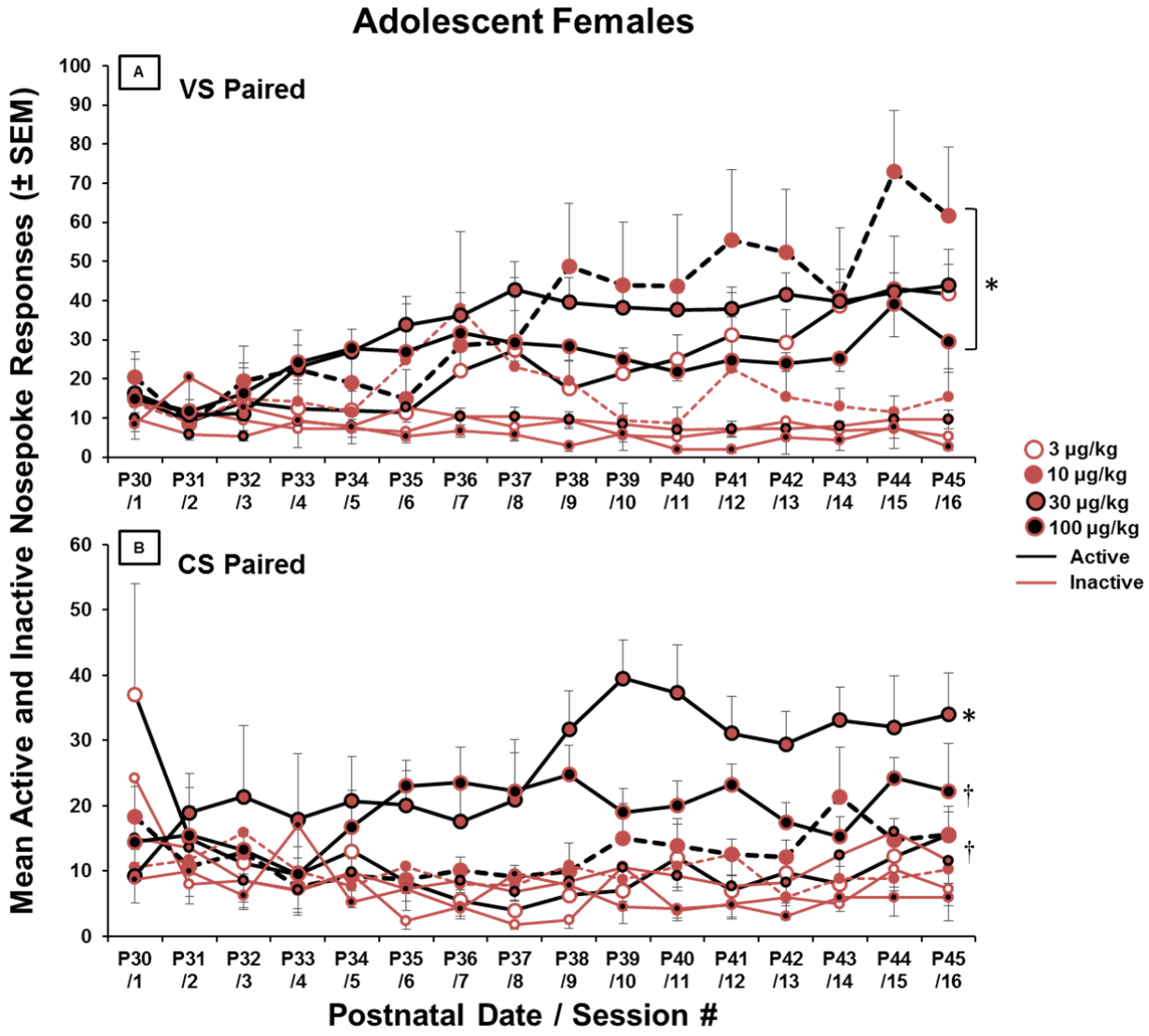

Figure 2. Mean active and inactive nosepoke responses made by adolescent females across sessions on an FR2 schedule of reinforcement, when nicotine infusions were paired with VS presentations (A), or CS presentations (B). Significant difference between active and inactive responses is represented by *. The bracket signifies all doses are different from their inactive responses. Trend for a difference between active and inactive responses is represented by †.

Figure 3. Mean active and inactive nosepoke responses made by adolescent males

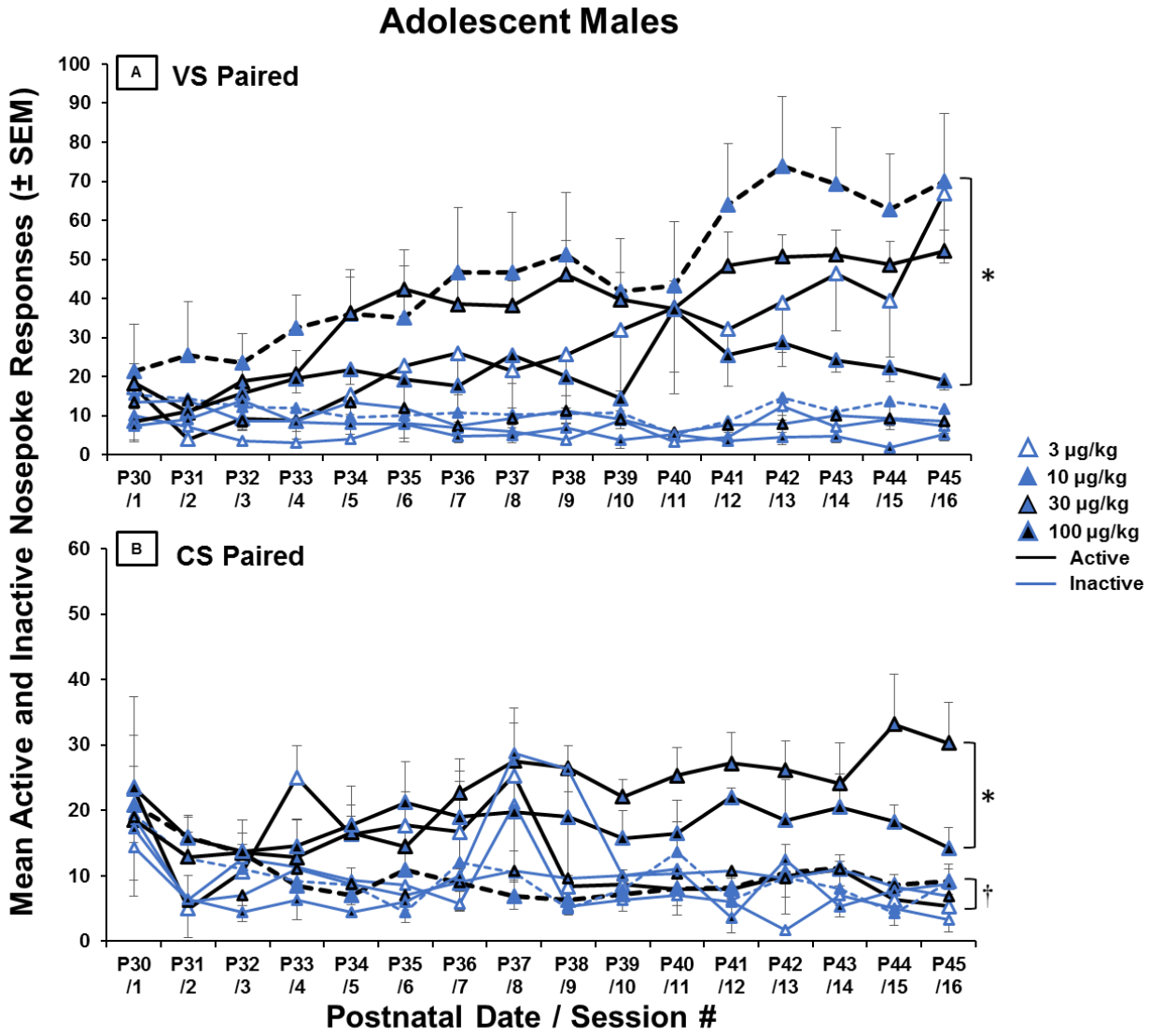

Figure 3. Mean active and inactive nosepoke responses made by adolescent males across sessions on an FR2 schedule of reinforcement, when nicotine infusions were paired with VS presentations (A), or CS presentations (B). Significant difference between active and inactive responses is represented by *. Trend for a difference between active and inactive responses is represented by †. Brackets signify when multiple dose groups in a panel are reflected by the statistical symbol.

Sex by Developmental Stage

To determine the effect of sex and developmental stage on self-administered infusions of nicotine, average infusions earned were compared between groups within each dose and cue condition. Figure 4 describes the average infusions earned across doses for each sex and age group when paired with VS (Figure 4A) or CS (Figure 4B) presentations. When nicotine is paired with VS presentations, all sex and age groups acquire self-administration at all doses tested, with all groups exhibiting a peak at 10 μg/kg. In the VS condition, there was no effect of Sex among adolescents for 3 or 100 μg/kg nicotine. Peak responding by both adolescent groups was reached in the 10 μg/kg + VS condition. A median split was applied to adolescent and adult males responding for 10 μg/kg nicotine + VS. An independent samples t-test was conducted on the animals above the group medians in each age group (n = 4/group), and revealed a significant difference for infusions earned between adolescent and adult males (t (6) = 4.480, $p < 0.005$), with adolescent males earning significantly more infusions than adult males.

There was a significant main effect of developmental stage on infusions earned when paired with VS presentations in the 30 μg/kg nicotine condition ($F_{1,37} = 4.503$, $p < 0.05$), with adolescent males earning significantly more infusions than all other groups. This effect was again primarily due to a difference between male adolescents and adults, as revealed by follow-up t-tests (t (16.647) = 2.577, $p < 0.05$). The inset bar graphs in figure 4A and B show the group average earned infusions across the last three days for 10 and 30 μg/kg nicotine, with the same significant group differences noted. These experiments demonstrate that adolescent and adult male and female rats will reliably self-administer doses of nicotine between 3 and 100 μg/kg when paired with a moderately reinforcing nonpharmacological stimulus.

In the CS paired cue condition, similar to the VS condition, there was no effect of sex among adolescents for 3 or 100 μg/kg nicotine. There was a main effect of age on infusions earned in the 10 μg/kg + CS pairing condition ($F_{1,35}$ = 20.241, $p < 0.001$), with adults earning significantly more infusions than adolescents, as revealed by follow-up t-tests (males: t (7.723) = -3.218, $p < 0.05$; females: t (20) = -2.783, $p < 0.05$). There was no main effect of sex, nor an age X sex interaction. Furthermore, there was no significant effect of age, sex, or an age X sex interaction on responding for 30 μg/kg nicotine ($p > 0.05$). These results demonstrate that there may be a shift in the primary reinforcement of nicotine between adolescents and adults, as adolescents did not acquire self-administration at a dose that supported behavior in adults. However, at a dose that does support acquisition of self-administration for adolescents, peak responding is similar across sexes and both age groups.

Figure 4. Average earned infusions for all groups across nicotine doses and cue conditions

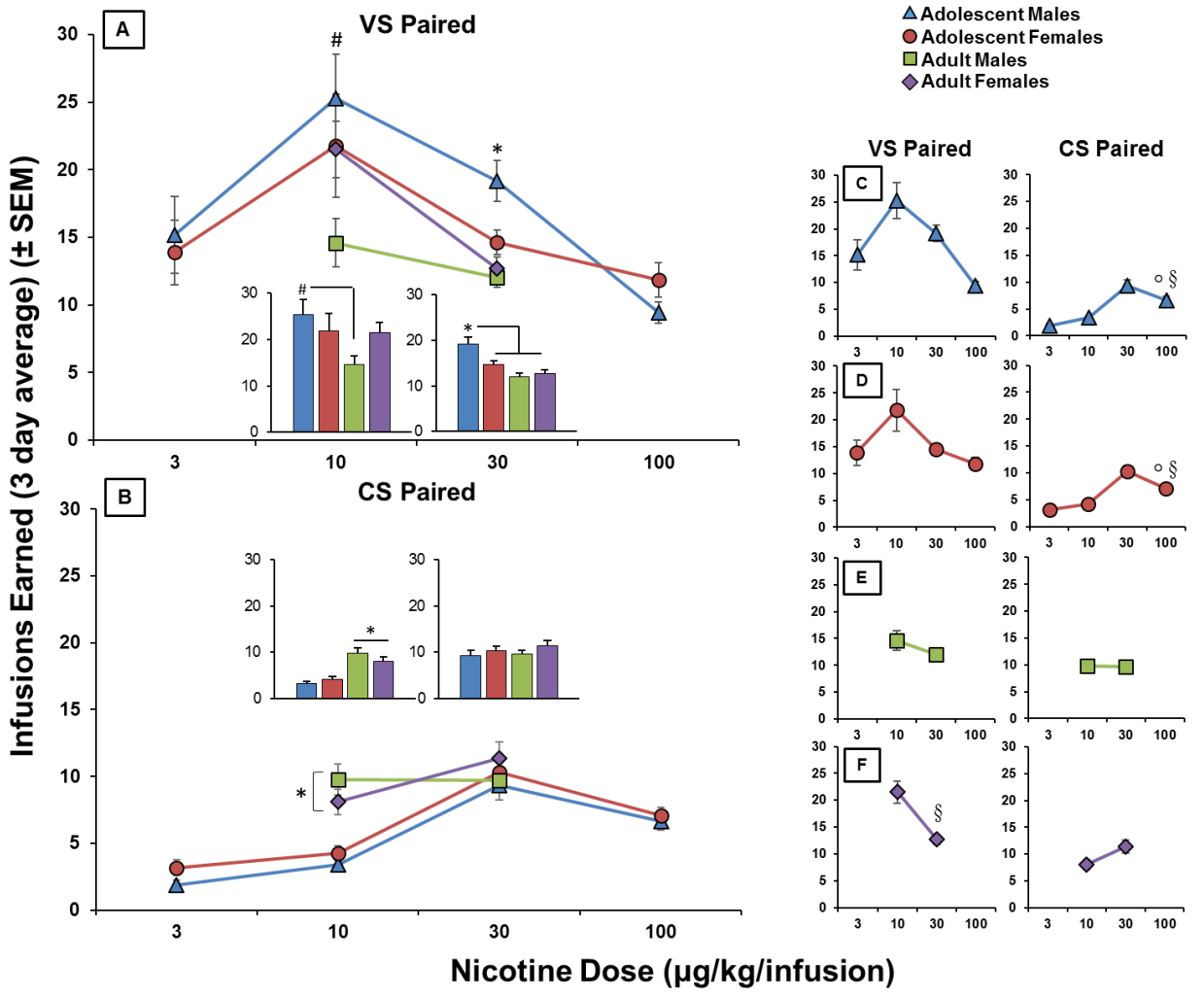

Figure 4. All data points are three day averages of infusions earned across the last three sessions for each sex and age group at each dose, indicated on the x-axis (x-axis is same for all panels). Inset bar graphs (A) and (B) depict average infusions for 10 and 30 µg/kg nicotine, and include the same data shown in each corresponding line graph. Error bars represent standard errors. Significant effect of age is represented by *. Significant difference between age groups among males is represented by #. Data points in panels (A) and (B) are separated by group for adolescent males (C), adolescent females (D), adult males (E), and adult females (F). Within a cue condition, significant difference from 3 µg/kg is represented by ◦. Significant difference from 10 µg/kg is represented by §.

Dose

To address the effect of dose on infusions earned within each sex and age group (four groups total), the same average earned infusions data were analyzed separately for each group across doses, for each of the cue conditions separately. In this case, the key comparisons were across doses within the VS cue condition or the CS condition, for each individual sex and age group. Figure 4 also shows the average infusions earned by each group in VS and CS paired conditions individually by age and sex group (Figure 4C-F). There was no significant effect of dose on infusions earned by adolescent males or females in the VS paired condition (Figure 4C and D; $p > 0.05$). A one-way ANOVA revealed a significant effect of dose for both adolescent males ($F_{3,23} = 5.334$, $p < 0.01$) and females ($F_{3,28} = 5.777$, $p < 0.005$) when nicotine infusions were paired with CS presentations. Post hoc Bonferroni analyses revealed that both adolescent males and females earned significantly more infusions at 30 μg/kg nicotine than at 3 and 10 μg/kg ($p < 0.05$) paired with CS presentations.

In contrast to adolescents, there were no effects of dose on infusions earned by either male or female adults when nicotine was paired with CS presentations (Figure 4E and F; $p > 0.05$), suggesting the threshold for primary reinforcement by nicotine is lower in adults than adolescents. Moreover, adult males did not show an effect of dose when nicotine was paired with VS presentations ($p > 0.05$). In contrast to their age-matched counterparts, an independent samples t-test revealed a significant effect of dose when infusions were paired with VS for adult females (t (8.669) = 2.377, $p < 0.05$), earning significantly more infusions at 10 μg/kg than at 30 μg/kg nicotine.

Comparison of VS to CS

To determine the effect of cue condition pairing with nicotine on infusions earned, average earned infusion data were compared within each age group, across doses and cue conditions. In this case, the key comparison is between VS and CS within a dose. Figure 5 shows a comparison of the average infusions earned across cue conditions and doses for each sex and age group (Figure 5A, adolescents; 5B, adults). A three-way between-groups ANOVA was used to examine the main effects of sex, dose, and cue condition as they relate to infusions earned by either adolescents or adults. In adolescents, there was a significant two-way interaction of dose X cue condition ($F_{3,106} = 4.735$, $p < 0.005$). There was no main effect of sex ($p > 0.05$), as both males and females earned a similar number of infusions in each condition. Planned comparisons between cue conditions for each dose revealed a significant effect of cue condition for all 4 doses tested, with both male and female adolescents earning significantly more infusions when nicotine was paired with the moderately reinforcing VS than when paired with CS presentations (3 μg/kg: $F_{1,17} = 5.775$, $p < 0.05$; 10 μg/kg: $F_{1,34} = 30.841$, $p < 0.001$; 30 μg/kg: $F_{1,43} = 11.914$, $p < 0.005$; 100 μg/kg: $F_{1,12} = 6.158$, $p < 0.05$).

In a similar three-way ANOVA comparing adult groups, there was a significant main effect of cue condition ($F_{1,59} = 12.902$, $p < 0.005$) and a significant cue X dose interaction ($F_{1,59} = 5.881$, $p < 0.05$). Planned comparisons showed a significant main effect of cue at 10 μg/kg ($F_{1,28} = 12.796$, $p < 0.005$), with adult males and females earning significantly more infusions of 10 μg/kg nicotine paired with VS presentations than when paired with CS presentations. There was no effect of cue condition at 30 μg/kg nicotine ($p > 0.05$), as both adult males and females earned a similar number of infusions regardless if nicotine was paired with VS or CS presentations.

Figure 5. Comparison of infusions earned across VS and CS cue conditions for all doses and groups

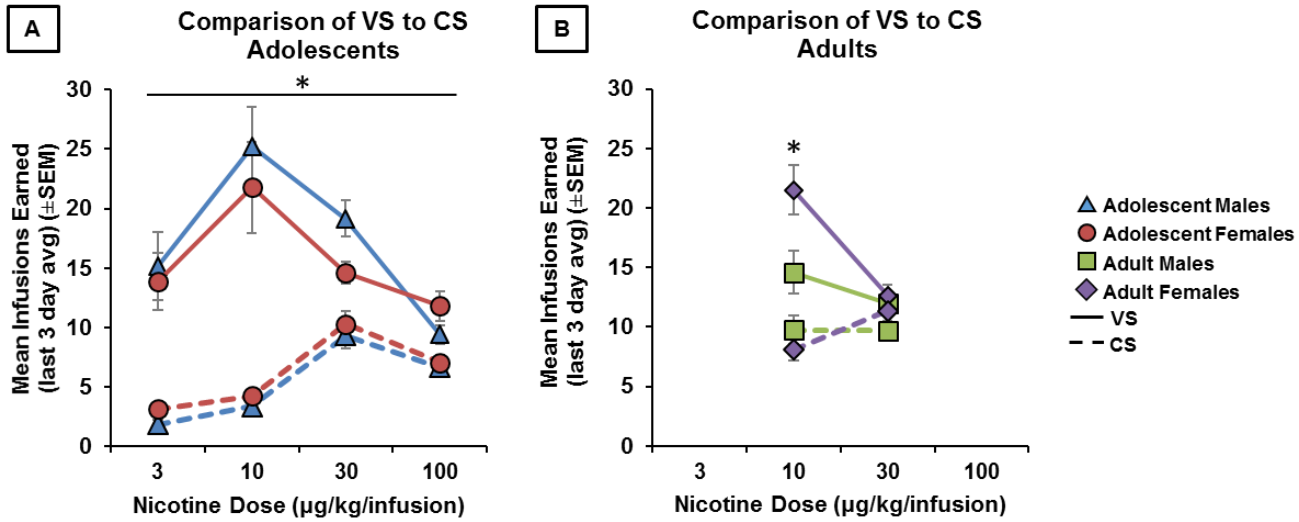

Figure 5. Data points are three day averages of infusions earned during the last three sessions across cue conditions for all doses for adolescents (A) and adults (B). Data is the same as shown in figure 2, collapsed into one panel per age group for direct comparison between sexes. Error bars represent standard errors. Significant effect of cue condition, with increased responding when infusions of nicotine are paired with VS presentations is represented by * (no main effect of sex; significance refers to both males and females within each age group).

Rate and Proportion to Acquire

Figure 6 shows the number of days to fulfill acquisition criterion when nicotine was paired with VS presentations (Figure 6A) or CS presentations (Figure 6B). In the VS paired condition, there was a trend for a two-way interaction of sex X age ($F_{1,75}$ = 3.531, p = 0.064), with adult females and adolescent males acquiring more quickly than adult males and adolescent females. Follow-up comparisons within each age group revealed that this difference may be driven by a significant difference in rate to acquire between adult males and females ($F_{1,26}$ = 5.988, $p < 0.05$), with adult females acquiring faster than adult males at both doses tested (10 and

30 μg/kg). An analysis of the 30 μg/kg + VS condition revealed a non-significant trend for an effect of sex ($F_{1,36}$ = 3.264, p = 0.079), with females acquiring faster than males. Again, this difference was strongest within adults, with a trend towards a significant main effect of sex ($F_{1,13}$ = 3.685, p = 0.077). For all other doses, there was no main effect of sex or age on rate to acquire ($p > 0.05$) when nicotine was paired with VS presentations.

Analysis of the rate to acquire by all groups in the VS condition also revealed a non-significant trend for an effect of dose ($F_{3,75}$ = 2.419, p = 0.079), with higher doses of nicotine supporting more rapid acquisition. Post hoc Bonferroni analyses determined a significant difference between 3 and 100 μg/kg (both doses only tested for adolescents), following the pattern that the high dose of 100 μg/kg nicotine supports more rapid acquisition than the low dose of 3 μg/kg. This significant effect of dose was true for adolescent males only ($F_{3,29}$ = 3.091, $p < 0.05$), due to a significant difference in rate to acquire between 3 and 100 μg/kg ($p < 0.05$). There were no effects of dose on rate to acquire for any other group ($p > 0.05$), all of which exhibit relatively flat acquisition dose-response curves. Moreover, nearly all animals in all doses paired with the moderately reinforcing VS reached acquisition by the end of the experimental period, with a dose-dependent increase in the percentage of rats fulfilling the acquisition criterion.

An analysis of CS paired nicotine infusions was conducted, and no significant main effects or interactions on rate to acquire were found ($p > 0.05$). Neither adolescent males nor females met acquisition criterion for 3 and 10 μg/kg nicotine paired with CS presentations. Because only adults acquired at 10 μg/kg + CS presentations, it appears that there is an effect of age, however there was no effect of sex among adults ($p > 0.05$). Furthermore, there was no effect of age or sex on rate to acquire self-administration of 30 μg/kg paired with CS

presentations ($p > 0.05$). Independent samples t-tests were conducted for each sex and age group to determine the effect of Dose on the rate to acquire self-administration, for the doses at which each age group reached at least 33% acquisition. Adolescent males showed a trend towards an effect of dose (t (11) = 2.075, $p = 0.062$), exhibiting a similar pattern to VS pairing in that higher doses engendered faster acquisition. No other groups showed an effect of dose ($p > 0.05$), again exhibiting relatively flat acquisition dose-response curves. In general, in dose and cue conditions that support acquisition for a group, groups acquire at relatively the same rate regardless of those conditions.

Comparison of VS to CS Pairing

Figure 7 displays the percentage of rats meeting criterion for groups that had at least 33% of subjects reach acquisition. Rats receiving nicotine paired with VS presentations were more likely to meet the acquisition criterion than those receiving infusions paired with CS presentations, particularly at low doses of nicotine (3 and 10 μg/kg). In VS paired conditions, percentage of groups acquiring were all greater than 80%, except for adolescent females self-administering 3 μg/kg (67%). A Fishers Exact Test of the proportion of animals acquiring in each of the dose and cue conditions in which all sex and age groups were tested (10 and 30 μg/kg, CS and VS) showed an effect of age in the 10 μg/kg + CS presentations condition ($p < 0.05$). This is unsurprising as no adolescents acquired in this condition. Furthermore, this condition supported the lowest percentage of acquisition for both adult groups (37.5% of adult females; 62.5% of adult males). While there is a difference between proportions of groups acquiring in different cue conditions, a comparison of the rate to acquire at each dose across cue conditions for each sex and age group showed that in general there was no difference in the rate to acquire, when animals did acquire ($p > 0.05$).

Figure 6. Days to reach acquisition criterion within each group

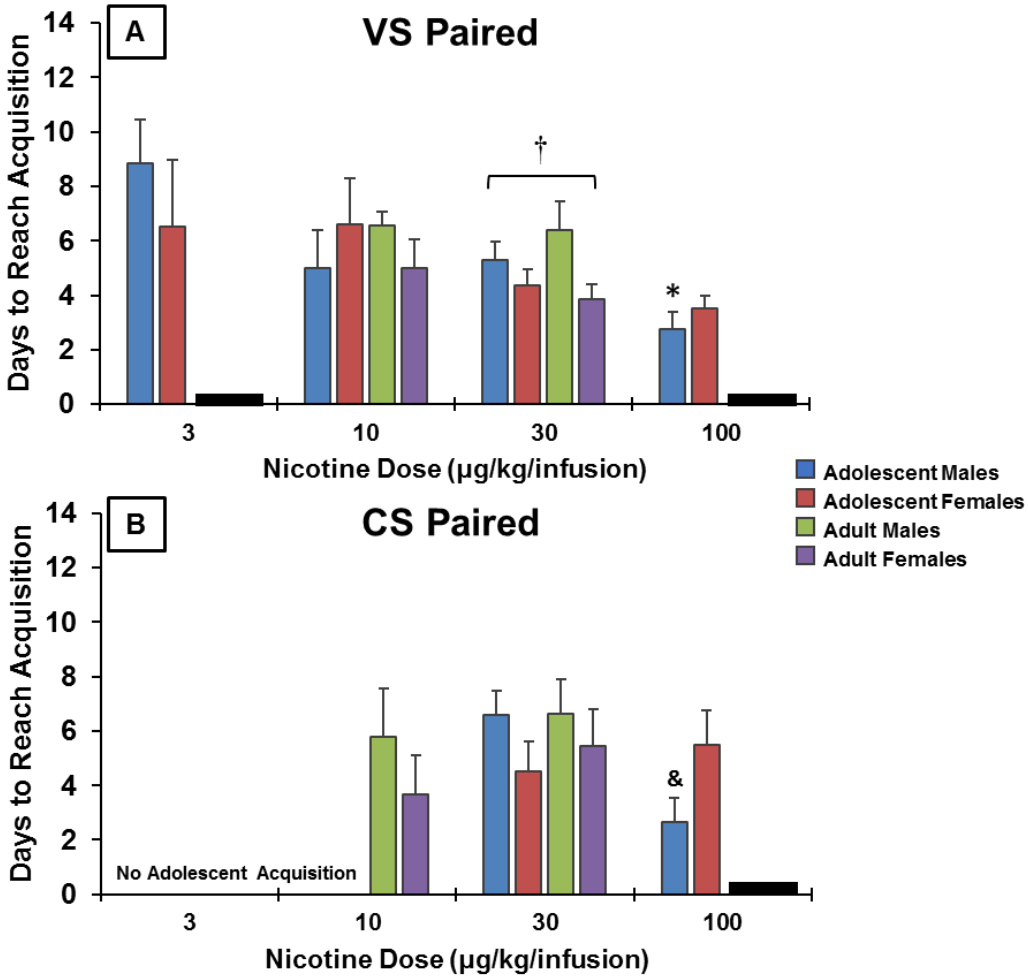

Figure 6. Bars are averages of the first of three consecutive days fulfilling acquisition criterion for each sex and age group at each nicotine dose, paired with VS presentations (A) and CS presentations (B), if at least 33% of the group acquired. Adults were not tested at 3 or 100 µg/kg, symbolized by the black bar covering the x-axis at these doses. Adolescents did not acquire in either 3 or 10 µg/kg paired with CS presentations (only adolescents tested at 3 µg/kg). Error bars represent standard errors. Trend for an effect of sex is represented by †. Significant effect of dose for adolescent males is represented by *; and trend for an effect of dose among adolescent males is represented by &.

Figure 7. Percentage of rats fulfilling acquisition criterion within each group

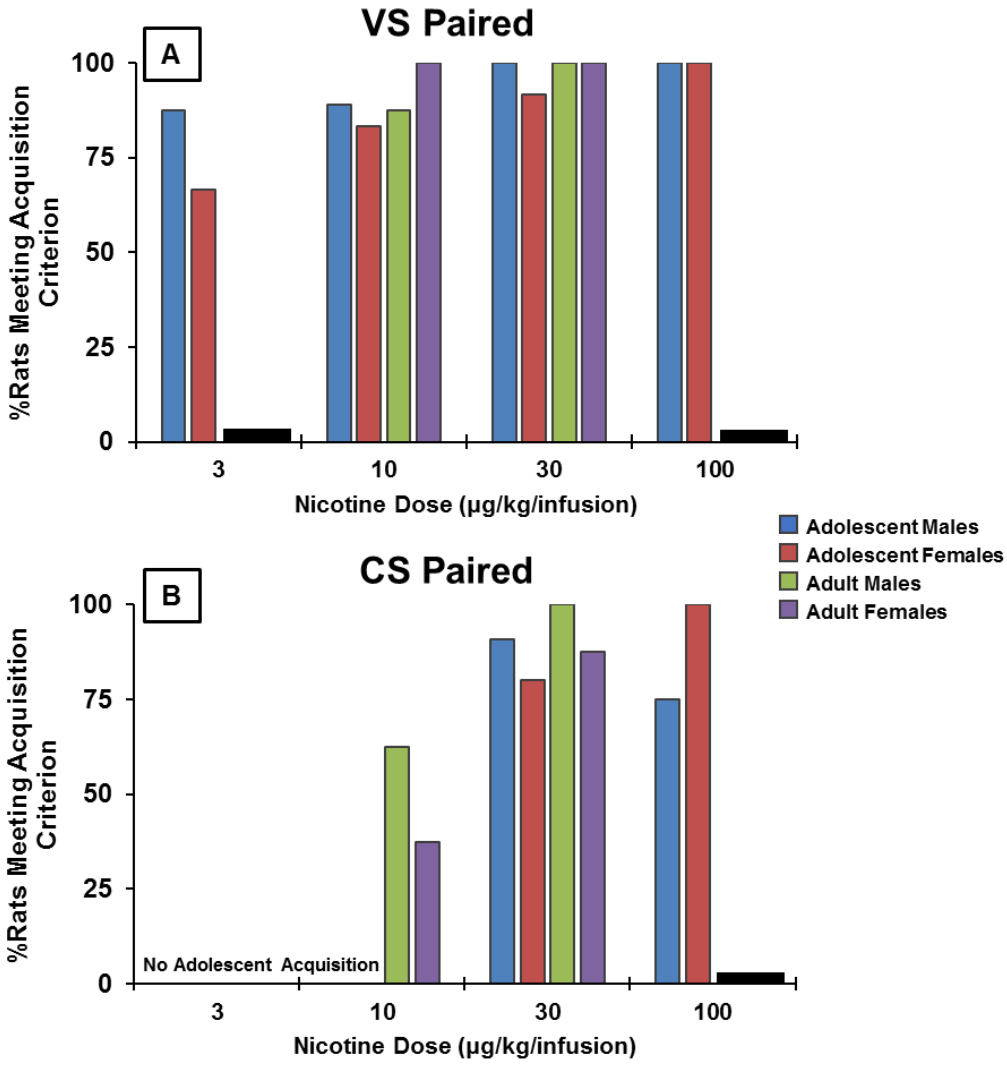

Figure 7. Bars are percentages of rats in each group fulfilling acquisition criterion at each nicotine dose paired with VS presentations (A) or CS presentations (B), if at least 33% of the group acquired. Adults were not tested at 3 or 100 µg/kg, symbolized by the black bar covering the x-axis at these doses. Adolescents did not acquire in either 3 or 10 µg/kg paired with CS presentations (only adolescents tested at 3 µg/kg).

4.0 DISCUSSION

As human smokers predominantly pick up the habit during adolescence, the goal of this study was to explore how adolescent nicotine self-administration behavior differs from adults, and if the same factors (i.e., dose, cue condition) that impact adults also affect adolescent behavior. The present study demonstrates that both male and female adolescents acquired nicotine self-administration, responding robustly for infusions of a range of low and high doses of nicotine paired with VS presentations. Furthermore, nearly all rats (male or female, adolescent or adult) given access to 3, 10, 30, or 100 μg/kg nicotine paired with VS presentations acquired nicotine self-administration. There was little difference in the rate to acquire nicotine self-administration across dose/cue conditions that supported acquisition of behavior for all groups.

The acquisition of nicotine self-administration and ultimate level of behavior reached was essentially equal for adolescent males and females. At low doses of nicotine paired with CS presentations, the lack of a sex effect extended to both groups responding at a similarly low level, and both failing to reach the criterion set to define when acquisition had been reached. CS presentations paired with 10 μg/kg nicotine revealed a reliable developmental-difference. In contrast to both male and female adolescents failing to acquire self-administration of 10 μg/kg nicotine + CS, both male and female adults fulfilled the acquisition criterion and responded fairly robustly, self-administering the same amount as in the 30 μg/kg nicotine + CS condition. Once adolescents acquired at 30 μg/kg + CS, their level of responding was not different from adults.

The differences at 10 μg/kg suggest that there is a developmental shift in the dose-response curve for the primary reinforcement of nicotine. The direction of this difference is in opposition to the developmental-difference observed in the VS-paired condition.

Primary Reward of Nicotine and Reinforcement-Enhancement

In the presence of the CS, adolescent animals responded relatively robustly for nicotine only at or above 30 μg/kg. Adult animals responded to a similar degree as adolescents at 30 μg/kg, and maintained that level of responding at 10 μg/kg, a dose of nicotine considered to be near the threshold for primary reinforcement for adult animals (Smith et al., 2013). This dose fell below the threshold for reinforcement for adolescents. This developmental-difference was reversed at 30 μg/kg nicotine paired with VS presentations, with adolescents earning significantly more infusions than adults. This shift in the dose-response curves as a function of cue condition between adolescents and adults may be due to developmental changes occurring in the brain (Spear, 2000), including maturation of mesocortical dopamine reward pathways (Chambers, Taylor, & Potenza, 2003), and shifts in the level of nicotinic acetylcholine receptor (nAChRs) expression, as well as changes in the specific subunits expressed across adolescence (Doura, Gold, Keller, & Perry, 2008; Leslie et al., 2004).

Differences in adolescent dopaminergic reward circuitry may impact nicotine self-administration initiation and the transition to maintenance use and dependence. Adolescents exhibit disproportionately high risk-taking and novelty-seeking behavior, often involving experimentation with drugs as well as high reactivity to stress (Chambers et al., 2003; Spear, 2000). Adolescence is also marked by reduced formal reasoning and decision-making, corresponding with the delayed development of the prefrontal cortex (Spear, 2000). Alterations to dopaminergic reward pathways may contribute to reduced reward thresholds, and greater

dopamine release in response to rewards, which engender faster acquisition of drug seeking and taking. Variation in the thresholds of use for adolescents versus adult users may pose an issue to national regulations of the nicotine content of commercially available cigarettes.

The truncated CS acquisition dose-response curve we saw in adolescents may additionally be extended to a comparison of the adolescent dose-response curve for nicotine paired with the moderately reinforcing VS stimulus. Adolescents robustly and rapidly acquired self-administration of low doses of nicotine that did not produce self-administration when paired with CS presentations, suggesting that the dose-response curves for reinforcement-enhancement and primary reinforcement of nicotine differ for adolescents. One hypothesis for the mechanism underlying these differing dose-response curves is that adolescents are responding for VS presentations and not specifically for infusions of nicotine. A previous study from our lab (Weaver et al., 2012) treated adolescent male rats (P28-42) with injections of saline or nicotine (0.32 mg/kg, s.c.) prior to a 1-h session in which they could make nosepoke responses on an FR2 schedule for VS presentations. Rats receiving saline earned fewer VS presentations than adolescents in the present study (VS combined with nicotine infusions). The level of responding for VS presentations in the absence of nicotine neared the level of responding for the lowest dose of nicotine tested here, 3 μg/kg, suggesting that perhaps motivation to deliver the VS initially drives responding, which exposes adolescents to nicotine, which in turn enhances the reinforcing value of the VS and further increases behavior.

Another possible explanation for the differing adolescent VS and CS dose-response curves is differential expression of nAChRs during adolescence. Considering recent work from our lab examining self-administration of the cessation aid Varenicline (Levin et al., 2012) in light of its affinity to bind different nAChRs (Coe et al., 2005; Foulds, 2006; Rollema et al., 2007),

suggests that the primary reinforcing and reinforcement-enhancing effects of nicotine may be dissociated and result from actions on different nAChRs subunits. Perhaps the receptor expression profile in adolescents favors receptor subtypes associated with reinforcement-enhancement, rather than primary reinforcement, resulting in acquisition of self-administration of low doses of nicotine only when a nonpharmacological reinforcer is available to be enhanced.

These results provide evidence that nonpharmacological stimuli and the reinforcement-enhancing effects of nicotine impact the acquisition of nicotine self-administration for both male and female adolescents, as has previously been shown for adults. Furthermore, the reinforcement-enhancing effects of nicotine may be particularly important during adolescence, as it may engender acquisition and support use at doses of nicotine that would otherwise likely fail to act as a primary reinforcer. It may be the case that adolescent males and females both are similar to adult females, in that nonnicotine stimuli influence behavior as much or more than the reinforcement of nicotine alone. This is important to consider for understanding what is driving tobacco product use to begin with, and how best to promote cessation.

Importance of Studying Adolescent Nicotine Self-Administration

Human smokers predominantly begin smoking during adolescence; however, most studies utilizing an animal model of nicotine self-administration use adult, male animals. Understanding nicotine reinforcement between adolescents and adults, as well as males and females, is important not only to treat nicotine dependence, but will also be important to anticipate how rates of initiation by new smokers may shift if the nicotine content of cigarettes is altered under the Family Smoking Prevention and Tobacco Control Act. Relatively few published studies have examined adolescent nicotine self-administration, and most studies differ in the specific conditions employed. This variation makes it difficult to fully interpret nicotine-

taking behavior, the contribution of primary nicotine reinforcement vs. reinforcement-enhancement to self-administration, and understanding how the results may reflect human adolescent behavior. Based on human epidemiological data showing robust use by adolescent females (Carroll et al., 2004; Lynch, 2009; Lynch et al., 2002), rodent self-administration studies using females (Chen, Matta, & Sharp, 2007; Levin et al., 2003), and data showing the rapid development of dependence in both adolescent and adult females (Anker & Carroll, 2011; Carroll & Anker, 2010), we hypothesized adolescent females would acquire self-administration faster than any other sex and age group. Our current data demonstrate that both male and female adolescents acquired nicotine self-administration in different cue conditions, however analysis of the impact of sex and age on infusions earned and rate to acquire did not support our hypothesis that adolescents, particularly adolescent females, would respond more for nicotine than adults.

Prototypical developmental changes associated with adolescence, including hormonal changes, occur during P28-42 in rodents (Spear, 2000). This range is approximate and relatively conservative as it certainly differs slightly between males and females: beginning earlier in females, and likely extending later in males. Based on this age range proposed by Spear (2000), as well as other studies examining adolescent self-administration (Lynch, 2009; Shram, Funk, Li, & Le, 2008; Shram, Li, & Le, 2008), we selected initiation ages to ensure that the period during which rats had access to nicotine fell completely during adolescence (P30-45) or adulthood (P90-105). In our adolescent condition, the lack of the anticipated sex and age differences, as seen in previous studies (Chen et al., 2007; Lynch, 2009), suggest that subtle differences between studies may lead to different conclusions. One such methodological difference between studies includes the period within adolescence that animals have access to nicotine. Our adolescents initiated access to nicotine on P30, whereas Chen et al. (2007) began rats during mid-adolescence at P43-

45. The sub-period (i.e., early vs. late adolescence) at which rats gain access to nicotine may ultimately impact rate to acquire and peak level of nicotine intake achieved, as well as contribution of varying levels of hormones (Lynch, 2006, 2009). Our data support this notion; no significant differences between dose groups within cue conditions emerged until later in the adolescent period (closer to P40). Further studies will investigate the consequence of acquisition during sub-periods of adolescence.

Technical Considerations

Beyond consideration of the age at initiation, and the concomitant practical concerns related to animal size, there are a multitude of other issues related to the brevity of adolescence in rodents and the goal of studying the acquisition of self-administration behavior. As has been discussed in detail above, studies utilizing adolescent rats as a model of new smokers have been designed primarily to determine what factors impact the acquisition of nicotine self-administration, in a model that more closely represents human acquisition of smoking. In order to study acquisition of nicotine self-administration, it is important to avoid pre-training with sucrose or food, common in other adolescent studies of nicotine self-administration (Levin et al., 2003; Li et al., 2012; Shram, Funk, Li, & Lê, 2008), as well as priming injections of nicotine at the start of the session (Lynch, 2009). Pre-training with alternative reinforcers complicates interpretation of the rate of responding and acquisition of the same behavior now directed at delivering infusions of nicotine. Rats are making the same behavioral responses, but expectations of reinforcer delivery are discordant. To complicate interpretations further, nicotine has previously been shown to enhance motivation to obtain food (Popke, Mayorga, Fogle, & Paule, 2000) and sucrose (Schassburger et al., 2013). Moreover, prior food training cuts into the brief period of adolescence.

One final detail of our model warranting comment is that all animals, regardless of age, had free access to food throughout the experimental period. Nicotine self-administration studies frequently restrict animals to 80-85% of their free-feeding weight to precipitate acquisition of self-administration and support robust responding (Donny et al., 1998). To avoid detrimental effects on health and growth, as well as compensatory developmental changes and delayed puberty resulting from restricting access to food in adolescent animals (Delemarre-van de Waal, van Coeverden, & Engelbregt, 2002), all animals were treated in a similar fashion and given free access to food. Despite free access, both adult groups acquired self-administration of the dose and cue conditions tested, within the brief experimental period. This condition may make it difficult to compare adult behavior across other studies, but this model is ideal in that it more closely models human behavior and allows for direct comparison between adolescents and adults.

Summary

In conclusion, the present study demonstrates that adolescent male and female Sprague-Dawley rats acquire nicotine self-administration across several doses, when nicotine is paired with a moderately reinforcing nonpharmacological stimulus or an initially neutral stimulus. There was no apparent sex difference between adolescents in self-administration. However, adolescents exhibited a dose-response curve for nicotine paired with CS presentations shifted to the right compared to adults, suggesting a developmental-difference in the primary reward of nicotine. Our finding that a low dose of nicotine may enhance responding for VS presentations in adolescent animals, but not in the absence of the VS, suggests that the reinforcement-enhancing effects of nicotine may be particularly important during adolescence and may sustain use at doses of nicotine that would likely fail to act as primary reinforcers. These results are, on the one

hand, encouraging in that there may be a dose of nicotine at which new initiates will not acquire, suggesting reduction of nicotine in content cigarettes will have a similar effect in adolescents as adults. However, the role of cues and other rewards in sustaining nicotine use in adolescents will be important to investigate in the future, as they did support robust behavior at low doses of nicotine.

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