Adult consequences of repeated nicotine vapor inhalation in adolescent rats

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Abstract

Introduction: There has been a recent resurgence in nicotine inhalation in adolescents due to the popularity and availability of Electronic Nicotine Delivery Systems (ENDS). Data show almost five times as many US high-school seniors inhale nicotine vapor daily compared with those who smoke tobacco. This study was conducted to determine the impact of repeated adolescent vapor inhalation of nicotine on behavior in adulthood in rats.

Methods: Male and female Sprague-Dawley rats were exposed to 30-minute sessions of ENDS vapor inhalation, twice daily, from Post-Natal Day (PND) 31 to PND 40. Conditions included vapor from the propylene glycol (PG) vehicle or nicotine (30 mg/mL in the PG). Animals were assessed for effects of nicotine on open field (PND 74-105) and wheel activity (PND 126-180) and for the self-administration of nicotine vapor (PND 285-395). Plasma levels of nicotine and cotinine were assessed in separate groups of male and female Wistar and Sprague-Dawley rats after a single nicotine inhalation session.

Results: Group mean plasma nicotine ranged from 39 to 59 ng/mL post-session with minimal strain or sex differences detected. Adolescent nicotine exposure modestly enhanced sensitivity to the locomotor stimulating effects of nicotine (0.1-0.8 mg/kg, s.c.) in an open field in female rats, but didn't change effects of nicotine on wheel activity. Female rats exposed to nicotine (30 mg/mL) vapor as adolescents responded more vigorously than PG exposed females for nicotine vapor in a FR5 challenge.

Conclusions: The data suggest repeated adolescent nicotine vapor inhalation leads to enhanced liability for nicotine self-administration in adulthood in female rats, but minimal change in spontaneous locomotor behavior.

Introduction

Inhalation of nicotine has experienced a recent resurgence in adolescents due to the popularity and widespread availability of Electronic Nicotine Delivery Systems (ENDS), most commonly referred to as e-cigarettes. Monitoring the Future survey data in the United States showed that 11.7% of high-school seniors, and 6.9% of tenth graders, vaped nicotine daily in 2019, while rates of daily tobacco smoking had reached a decades' low of 2.4% and 1.3% in these populations (Johnston et al., 2021); approximately 25% of high-school

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seniors vaped nicotine in the past month. The 2022 National Youth Tobacco Survey in the United States found that 3.3% of middle school students and 14.1% of high school students reported current ENDS use, while only 1.0% of middle school and 2.0% of high school students smoked cigarettes (Park-Lee et al., 2022). There is an obvious concern that reductions in the harm associated with cigarette smoking will be at least partially replaced by similar and possibly novel harms associated with vaping for nicotine content. About 20-21% of adults ages 18-22 vaped nicotine at least monthly, compared with 10-13% of those in their later teens in 2020 (Schulenberg et al., 2021). This is consistent with vaping habits that started to increase in prevalence in adolescent populations during the past half-decade continuing on with use patterns in some adult individuals. Concerningly, pre-teen rates of nicotine use increased during the initial months of COVID-19 related stay-at-home orders, while alcohol use declined (Pelham et al., 2021).

A recent review points out the need for scientific research on the health impact of ENDS-based exposure to nicotine and furthermore identifies several domains in which adolescents have previously been found to be at increased risk (Fowler et al., 2018). Some of this research can be greatly enhanced by the use of traditional pre-clinical laboratory models in, e.g., rats, to isolate risks due to ENDS from social and environmental factors which may be associated with human adolescent behavioral patterns. The goal of this study was therefore to determine if there are any lasting consequences of repeated vapor inhalation of nicotine in adolescent rats. For this, we used an ENDS-based system that has been previously shown effective in delivering active nicotine doses to adult rats of either sex (Javadi-Paydar et al., 2019b; Lallai et al., 2021; Montanari et al., 2020), to pregnant rats (Breit et al., 2022) and to mice (Cooper et al., 2021; Echeveste Sanchez et al., 2022; Henderson and Cooper, 2021). ENDS-based systems have shown efficacy for nicotine self-administration in rats (Smith et al., 2020) and mice (Cooper et al., 2021; Henderson and Cooper, 2021) and repeated daily exposure to nicotine via ENDS vapor inhalation leads to withdrawal following discontinuation in adult rats (Montanari et al., 2020).

There is evidence that nicotine injection during adolescence alters responses in rats during adulthood. For example, Renda and colleagues found that male rats treated with nicotine (1.0 mg/kg, s.c.) once daily from Post-Natal Day (PND 28-41) did not intravenously self-administer more nicotine as adults under initial Fixed Ratio (FR1) or a Progressive Ratio contingency, but did self-administer slightly more under a Fixed-Interval 1 minute procedure (Renda et al., 2020). Cardenas and Lotfipour (Cardenas and Lotfipour, 2022) reported increased self-administration of methamphetamine in male rats treated with nicotine by i.v. injection from PND 28-31 and tested for self-administration from PND 32-36. Daily nicotine injection (0.6 mg/kg, s.c.) from PND 28-42 enhanced pain response and reduced efficacy of morphine in the formalin test when rats were assessed as young adults circa PND 72 (Khalouzadeh et al., 2022). Given that inhalation of drugs can produce a similar subjective high at a lower plasma concentration even compared with intravenous delivery in humans (Cook et al., 1993), it is critical to determine whether inhalation produces lasting consequences. Recent work from Breit and colleagues has provided evidence that this is may be the case; prenatal exposure to nicotine via ENDS vapor was shown to impair adolescent motor coordination in rats (Hussain et al., 2022).

The vapor inhalation model for delivery of nicotine to rat subjects is still relatively new with relatively few data available regarding plasma levels of nicotine, or the metabolite cotinine, across rat age, sex and strain. In one recent report, Espinoza and colleagues (Espinoza et al., 2022) found that Wistar rats exposed to 14 days of nicotine vapor (generated from 12 mg/mL in a vegetable glycerin / propylene glycol vehicle) for 90 minutes each day during the adolescent interval (PND 33-46) exhibited more nosepokes in the nicotine vapor delivery port during this passive drug delivery than did air-exposed control groups. Serum cotinine levels of ~190-220 ng/mL were reported in all three groups other than female adolescents which were significantly higher (~380 ng/mL). These results compare with ~165 ng/mL in *adult* male Wistar rats exposed to 10 daily 60-minute sessions of nicotine vapor (generated from a 80 mg/mL concentration in a PG/VG vehicle)

reported by Montanari and colleagues (Montanari et al., 2020).

To the end of directly comparing nicotine distribution and cotinine generation across sex and two common rat strains, within a single lab model, we first conducted a study of plasma concentrations of nicotine and cotinine after vapor exposure and after subcutaneous injection. This is particularly critical since methods related to vapor puffing protocols, session duration, and rat strain have varied across this emerging literature (Espinoza et al., 2022; Javadi-Paydar et al., 2019b; Montanari et al., 2020). Similarly, the behavioral and physiological effects of vapor inhalation of nicotine have not yet been reported for adolescent rats, so we next determined if effects on locomotor activity and thermoregulation previously reported in adult animals (Javadi-Paydar et al., 2019b) would generalize to adolescents of each sex. Prior evidence suggests similar effects since nicotine injection (0.2-0.4 mg/kg, s.c.) similarly increased activity in a photo cell chamber in male and female Sprague Dawley rats of adolescent (PND 40) and adult (PND 90) ages (Bryson et al., 1981). The main study then exposed groups of adolescent rats to repeated (30-minute sessions, twice daily for 10 days) inhalation of nicotine vapor or that of the PG vehicle, to determine lasting effects in adulthood. Animals were assessed during adulthood for responses to nicotine in spontaneous locomotor behavior in open field and activity wheels, and for nicotine vapor self-administration.

Methods

Subjects

Male and (N=22) and female (N=54) Sprague-Dawley (Envigo/Harlan) and male (N=6) and female (N=6) Wistar (Charles River) rats were used for this study. The vivarium was kept on a 12:12 hour reversed light-dark cycle, and behavior studies were conducted during the vivarium dark period. Food and water were provided ad libitum in the home cage. Animal body weights were recorded weekly, beginning at 6 weeks of age (PND 36) and continuing through the end of the study. Experimental procedures were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committees of The Scripps Research Institute and of the University of California, San Diego and were consistent with recommendations in the NIH Guide (Garber et al., 2011).

Drugs

Nicotine bitartrate (Sigma-Aldrich, St. Louis, MO) was dissolved in propylene glycol (PG; Fisher Scientific) at a concentration of 30 and 60 mg/mL for vapor inhalation studies and dissolved in physiological saline for subcutaneous injection studies. PG was used as the vehicle for consistency and comparability with our prior reports on the impact of nicotine vapor inhalation (Javadi-Paydar et al., 2019a; Javadi-Paydar et al., 2019b).

Plasma Nicotine and Cotinine Analysis

Plasma nicotine and cotinine concentrations were quantified using liquid chromatography/mass spectrometry (LCMS) as previously reported (Javadi-Paydar et al., 2019b). Briefly, 50 ul of plasma were mixed with 50 ul of deuterated internal standard (100 ng/ml cotinine-d3 and nicotine-d4; Cerilliant). Nicotine and cotinine (and the internal standards) were extracted into 900 ul of acetonitrile and then dried. Samples were reconstituted in 100 uL of an acetonitrile/water (9:1) mixture. Separation was performed on an Agilent LC1200 with an Agilent Poroshell 120 HILIC column (2.1mm x 100mm; 2.7 um) using an isocratic mobile phase composition of acetonitrile/water (90:10) with 0.2% formic acid at a flow rate of 325 uL/min. Nicotine and cotinine were quantified using an Agilent MSD6130 single quadrupole interfaced with electrospray ionization and selected ion monitoring [nicotine (m/z=163.1), nicotine-d4 (m/z=167.1), cotinine (m/z=177.1) and cotinine-d3 (m/z=180.1)]. Calibration curves were generated daily using a concentration range of 0-200 ng/mL with observed correlation coefficients of 0.999.

Apparatus

Vapor Inhalation: An e-cigarette based vapor inhalation system (La Jolla Alcohol Research, Inc) which has previously been shown to deliver active doses of heroin, cannabidiol, Δ9-tetrahydrocannabinol (THC), oxycodone, methamphetamine and nicotine (Gutierrez et al., 2021; Javadi-Paydar et al., 2019a; Javadi-Paydar et al., 2019b; Nguyen et al., 2016; Nguyen et al., 2019) was used for these studies. Vapor was delivered into sealed vapor exposure chambers (209 mm W X 234 mm H X 259 mm L, passive vapor exposure; 152 mm W X 178 mm H X 330 mm L, vapor self-administration; La Jolla Alcohol Research, Inc, La Jolla, CA, USA) through the use of e-vape controllers (Model SSV-3 or SVS-200; 58 watts, 0.24-0.26 ohms, 3.95-4.3 volts, ~214 °F; La Jolla Alcohol Research, Inc, La Jolla, CA, USA) to trigger SMOK Baby Beast Brother TFV8 sub-ohm tanks. Tanks were equipped with V8 X-Baby M2 0.25 ohm coils. MedPC IV software was used to schedule and trigger vapor delivery (Med Associates, St. Albans, VT USA). For passive vapor exposure, 6 second vapor puffs were triggered at 5-minute intervals. The apparatus and settings were the same for all drug conditions. The chamber air was vacuum-controlled by a chamber exhaust valve (i.e., a "pull" system) to flow room ambient air through an intake valve at ~1 L per minute. This also functioned to ensure that vapor entered the chamber on each device triggering event. The vapor stream was integrated with the ambient air stream once triggered. Airflow was initiated 30 seconds prior to, and discontinued 10 seconds after, each puff. For vapor self-administration, airflow was continuous, and 1 second vapor puffs were triggered for each reinforcer delivery.

Open Field: Testing was conducted in plastic chambers with dimensions of 80 cm (L) X 44 cm (W) X 33 cm (H). Locomotor activity was recorded videographically using webcams (Logitech Model C270) mounted approximately 1 meter above the arena. Analysis of behavior was conducted off-line using ANY-maze behavior tracking software (Stoelting) with analysis configured for the Center (43 cm X 11 cm) versus the Peripheral Zones. The experiment was run under white light illumination to facilitate the video tracking.

Activity Wheels: Experimental sessions were conducted in white illuminated procedure rooms with activity wheels that attached to a typical housing chamber with a door cut to provide access to the wheel (Med Associates; Model ENV-046), using approaches previously described (Gilpin et al., 2011; Miller et al., 2013; Taffe et al., 2021). The lights were turned on for maximum comparison with the open field experiment. Rats were given access to the wheel in acute 30-minute sessions during which wheel rotation (quarter-rotation resolution) activity was recorded at 10-minute intervals. One 30-minute habituation session was conducted for each animal prior to initiating the experimental sessions.

Experiments

Experiment 1: Plasma nicotine and cotinine after vapor inhalation or subcutaneous injection

Groups (N=6) of male and female Sprague-Dawley and Wistar rats were implanted with indwelling jugular catheters as previously described (Nguyen et al., 2020; Nguyen et al., 2018). The goal was to determine if any major substantive sex or strain differences in nicotine exposure resulted from identical inhalation parameters and whether any differences were also observed following parenteral injection of nicotine. Following recovery, experiments were conducted in which blood samples were collected after a 30-minute nicotine (30 mg/mL) vapor inhalation session (35, 50, 120, 240 minutes after vapor initiation) or after a 1 mg/kg, s.c. injection (15, 30, 60, 120 minutes post-injection). If a sample could not be obtained for a given timepoint, the available samples were included, resulting in the sample size as described in the relevant figures, below. The blood draw studies were conducted consistent with published recommendations for good practices (Diehl et al., 2001).

Experiment 2: Nicotine inhalation in adolescent rats

Separate studies were conducted in groups of male (N=8) and female (N=8) peri-adolescent Sprague-Dawley rats to verify efficacy of the nicotine vapor inhalation procedure in younger animals since our prior studies (Javadi-Paydar et al., 2019a; Javadi-Paydar et al., 2019b) had been conducted in adults. Rats arrived in the laboratory on Post-Natal Day (PND) 22 and were implanted with radio-telemetry transmitters (Data Sciences International, St. Paul, MN, USA; TA11TA-F20) on PND 31 using aseptic technique, inhalation anesthesia and post-operative recovery as previously described (Nguyen et al., 2020; Taffe et al., 2015). One animal of each sex did not survive through surgical recovery, thus N=7 per group for the studies. Vapor experiments were initiated on PND 43 with a baseline assessment, the first set of nicotine vapor experiments were conducted on PND 49 and PND 52, THC vapor inhalation experiments not herein reported on PND 57 and PND 60, and then a repetition of nicotine on PND 63 and PND 66. The goal was to determine if body temperature responses to nicotine vapor in late adolescence are similar to those reported for the adult age range, using identical vapor inhalation conditions (Javadi-Paydar et al., 2019b). The nicotine experiments determined the effects of PG inhalation versus nicotine (30 mg/mL in the PG) inhalation, in a counterbalanced order within groups, on spontaneous activity and body temperature responses. Three males had a significantly aberrant drop in body temperature following nicotine vapor on the same experimental day for the first experiment, and it was noted that room temperature was ~0.2° C colder than for any other study day. For this reason the nicotine experiment was repeated a second time. Comparison of the temperature and activity means with those three animals excluded with the full sample results from the repetition study confirmed qualitative similarity of results for both male and female rats. Thus, these pre-planned inhalation conditions (nicotine 30 mg/mL for 30 minutes) were used for Experiment 2. Subsequent experiments conducted in this group of animals have been previously reported (Gutierrez et al., 2021).

Experiment 3: Effect of repeated adolescent nicotine inhalation on open field locomotion in adult rats

Groups of male (N=8 per treatment) and female (N=8 per treatment) Sprague-Dawley rats arrived in the laboratory at PND 25 and were exposed to either PG or nicotine (30 mg/mL in the PG) vapor in 30-minute sessions twice per day (6 h interval; 0800/1400) for 10 days from PND 31 to PND 40. Adolescent inhalation was done in the vivarium dark cycle but under white light illumination. Locomotor activity was assessed from PND 74 to PND 105 in open field using video tracking with analysis by ANYMaze; assessment was conducted during the vivarium dark cycle but under white light illumination to facilitate the tracking. One 30-minute baseline recording session was conducted to habituate the animals to the procedure and apparatus. Thereafter, dose conditions (0.0, 0.2, 0.8 mg/kg nicotine, s.c.) were assessed in a counter balanced order and then (0.0, 0.1, 0.4 mg/kg nicotine, s.c.) were assessed in a counter-balanced order. Doses were assessed 3-4 days apart.

Experiment 4: Effect of repeated adolescent nicotine inhalation on wheel activity in adult rats

The groups from Experiment 3 were assessed for wheel activity in adulthood, using chamber-adjacent activity wheels (Med Associate Model #ENV-046) with quarter rotation counts recorded every 15 minutes during 1 h sessions; these were conducted under white light illumination for greater comparison with the open field study. Animals were first exposed to two baseline sessions (5-7 days apart, starting PND 126) without any pre-treatment to habituate them to the procedure. Thereafter (PND 165- PND180), sessions were run immediately after pre-injection with nicotine (0.0, 0.2, 0.4, 0.8 mg/kg, s.c.) with doses administered in a counter-balanced order, with a minimum 3-4 day interval between doses.

Experiment 5: Effect of repeated adolescent nicotine inhalation on nicotine self-administration in adult rats

From PND 285 onward the groups of rats from Experiment 3-4 were assessed for the self-administration of nicotine (30 mg/mL) vapor in

30-minute sessions. Four inhalation chambers with nose-poke manipulanda and two chambers with lever manipulanda were used, with the assignment of the boxes counterbalanced across groups. A response on the drug-associated hole/lever (FR-1 response requirement) resulted in illumination of the cue light and delivery of a 1 second puff of vapor. This was followed by a 20 second timeout during which the cue light remained illuminated and hole/lever responses were recorded but led to no consequences. Schedule: The intervals between successive sessions were [2, 3, 2, 15, 6, 5, 2, 4 days] for the initial 9 acquisition sessions. Animals were next evaluated with a range of concentrations (0, 10, 30 or 60 mg/mL nicotine in the PG) under an FR1 response contingency evaluated in a counterbalanced order across sequential test sessions. The schedule was 2-4 days between sessions for each individual.

After the concentration-substitution procedure rats were returned to FR1 / Nic (30 mg/mL) for one session, after which the response contingency was increased to FR5. The rationale is that increased responding on the drug-associated manipulandum is interpreted as evidence of drug-seeking, as in (Chaudhri et al., 2005). An unexpected facilities flooding emergency disrupted the behavioral testing rooms after the 3rd FR5 session. Animals were idled for three weeks and then re-started under the same FR5 contingency before returning to FR1 for four additional sessions. This latter study was based on a prior report that female rats initiated at 6 weeks of age increase their intake more than female rats initiated at 8 weeks of age in an intravenous self-administration procedure when the contingency is returned to FR1 from FR8 (Levin et al., 2011). Analysis thus first focused on the first three FR5 sessions compared with the immediate prior FR1 session. The second analysis compared the post-interruption FR5 sessions and the subsequent FR1 sessions with the FR1 session conducted prior to the FR5 manipulation.

Data Analysis

Plasma concentrations of nicotine and cotinine were analyzed by mixed-effect models to account for missing values. A within-subject factor of Time after vapor initiation / injection and a factor for Group were included. Telemetry data were analyzed by 5-minute bin, including three pre-inhalation baseline values and data from 40-120 minutes after the start of inhalation. A within-subject factor of Time after vapor initiation and a factor for Group were included in the ANOVA.

In all analyses, a criterion of $P<0.05$ was used to infer that a significant difference existed. Any significant main effects were followed with post-hoc analysis using Tukey (multi-level factors), Sidak (two-level factors) or Dunnett (treatments versus control within group) correction. All analysis used Prism for Windows (v. 9.4.0; GraphPad Software, Inc, San Diego CA).

Results

Experiment 1: Plasma nicotine and cotinine

Plasma nicotine and cotinine after vapor inhalation

Concentrations of nicotine and cotinine in plasma were first analyzed across the four groups for each analyte (**Figure 1**), and the mixed-effects analyses confirmed significant effects of Time, of Group and/or the interaction on plasma *nicotine* [Group: n.s.; Time: $F(1.291, 17.21) = 172.4$, $P<0.0001$; Interaction: $F(9, 40) = 2.39$, $P<0.05$], and plasma *cotinine* [Group: $F(3, 17) = 9.03$, $P<0.001$; Time: $F(1.219, 16.25) = 240.4$, $P<0.0001$; Interaction: $F(9, 40) = 2.55$, $P<0.05$], concentrations. To further parse the interaction for nicotine, follow-up analysis was conducted collapsing first across rat strain [Sex: $F(1, 19) = 4.51$, $P<0.05$; Time: $F(1.394, 21.37) = 180.7$, $P<0.0001$; Interaction: $F(3, 46) = 5.97$, $P<0.005$] and next across rat sex [Strain: n.s.; Time: $F(1.247, 19.12) = 141.2$, $P<0.0001$; Interaction: n.s.]. The post-hoc test failed to confirm significant differences between male and female groups at any specific time-point. Post-hoc analysis of the Time factor, collapsed across group, confirmed that plasma concentrations of nicotine at each time-point were significantly different from the concentrations at every other timepoint.

Follow-up analysis of the cotinine

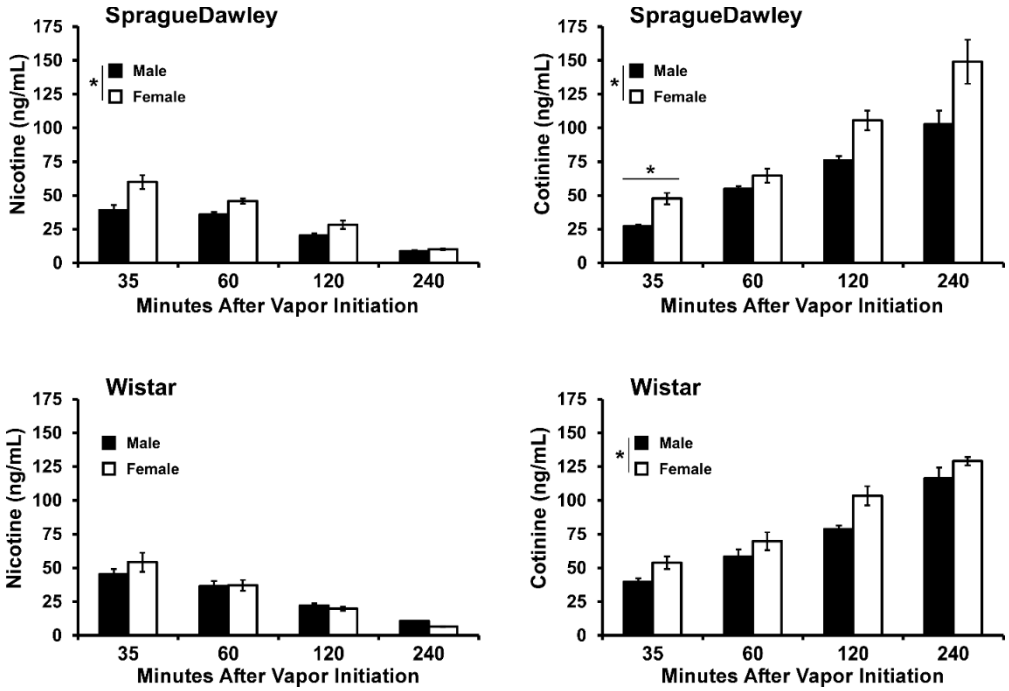

Figure 1: *Mean (±SEM) plasma concentrations of nicotine and cotinine for Male (N=6) and Female (N=4 35 min; N=3 60-240 min) Sprague-Dawley and Male WI (N=6 35 min; N=5 60-120; N=3 240) and Female (N=5 35 min; N=4 60-240 min) Wistar rats after vapor exposure to nicotine for 30 minutes. A significant difference between sexes, within strain, is indicated with *.*

concentrations were also conducted first across rat strain to determine any effect of sex [Sex: F (1, 19) = 24.46, P<0.0001; Time: F (1.259, 19.31) = 218.2, P<0.0001; Interaction: F (3, 46) = 3.472, P<0.05] and next across rat sex to determine any effect of rat strain [Strain: n.s.; Time: F (1.307, 20.04) = 178.9, P<0.0001; Interaction: n.s.]. The post-hoc analysis confirmed significantly higher cotinine concentrations in the female rats, across strain, 35, 120 and 240 minutes after vapor initiation. Higher cotinine levels in female versus male Sprague-Dawley rats were also confirmed 35 minutes after vapor initiation. Post-hoc analysis of the time factor confirmed that plasma concentrations of cotinine at each time-point were significantly different from the concentrations at every other timepoint for both male and female groups.

Plasma nicotine and cotinine after subcutaneous injection

The female rats with patent catheters were too few for the subcutaneous injection study, thus it was opted to collect samples 15 and 120 minutes after injection by acute venipuncture under inhalation anesthesia (with recovery in-between sampling points). Statistical comparisons were therefore limited to the 15- and 120-minute samples, however the intervening time points for the males are also presented in **Figure 2** for reference. The mixed-effects analysis of all four groups confirmed a significant effect of Time post-injection [F (1, 18) = 35.78, P<0.0001] but not of group for nicotine concentrations. This was also the outcome when the analysis was limited to each strain, to each sex, collapsed across strain or collapsed across sex. The mixed-effects analysis of all four groups confirmed a significant effect of Time post-injection [F (1, 18) = 35.78, P<0.0001] and of Group [F (3, 19) = 4.58; P<0.05] for cotinine concentrations. The post-hoc test confirmed that there was a significant difference in cotinine concentrations between strains within both male and female groups at the 120-minute time point. There was also a difference between Sprague-Dawley males and Wistar females at this time-point.

Experiment 2: Nicotine vapor in adolescent rats

There was a modest and short-lived reduction in body temperature observed in both sexes of

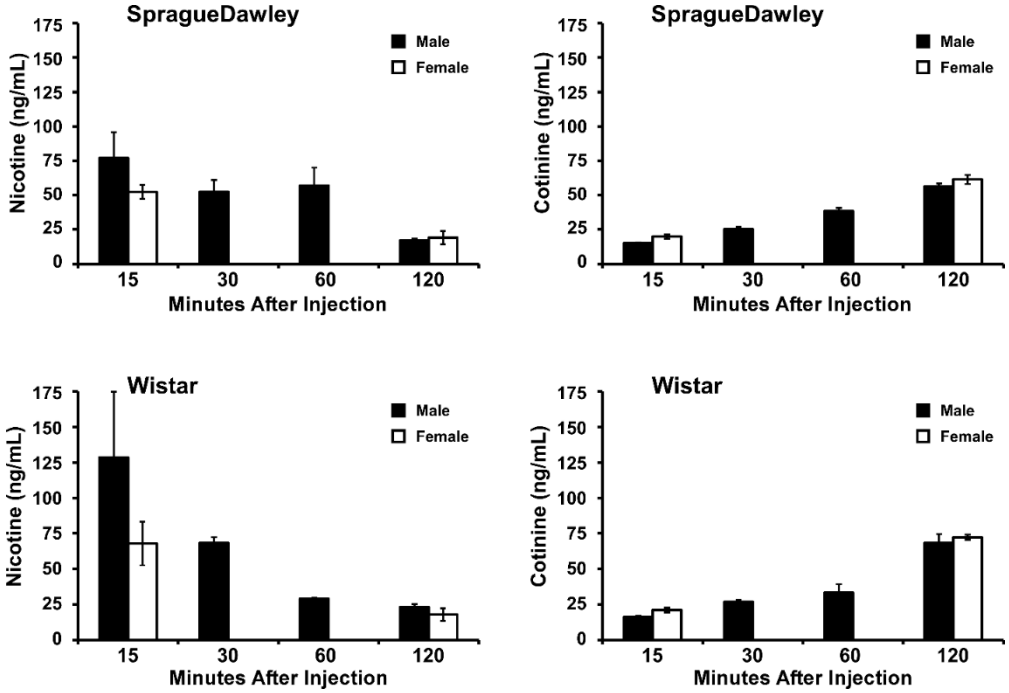

Figure 2: *Mean (±SEM) plasma nicotine and cotinine concentrations observed in male (N=6) and female (N=6) Sprague-Dawley and in male (N=4-5 per time point out of N=5 total rats) and female (N=6) Wistar rats, after 1 mg/kg, nicotine s.c..*

peri-adolescent rats after nicotine inhalation (**Figure 3**), similar to what we reported previously for adult male Sprague-Dawley rats after a single 30 min nicotine vapor exposure (Javadi-Paydar et al., 2019b), thus confirming active doses are delivered by this technique in adolescent animals. No significant effects on activity rate were observed. The two-factor analysis confirmed significant effects of Group [F (3, 24) = 9.31; P<0.0005] and the interaction of Group with Time after vapor initiation [F (57, 456) = 5.40; P<0.0001] on body temperature. The post-hoc analysis further confirmed that body temperature was lower after nicotine inhalation compared with PG inhalation in female (40-55 minutes after the start of inhalation) and male (40-50 minutes after the start of inhalation) rats. The post-hoc test likewise confirmed that male rat temperature was lower than female rat temperature after PG (all three baseline samples, 40, 50-65 minutes after vapor initiation) or nicotine (50-60 minutes after vapor initiation) inhalation.

The two-factor analysis of activity confirmed significant effects of Group [F (3, 24) = 22.42; P<0.0001] and of Time [F (19, 456) = 6.96; P<0.0001], but not the interaction of factors, on activity rates. The post-hoc test of the marginal means for Group confirmed that female activity was higher in each treatment condition compared with male activity in each treatment condition, however there was no significant difference in activity associated with treatment condition within either sex.

Experiment 3: Effect of repeated adolescent nicotine inhalation on open field locomotion in adult rats

Growth: There were no group differences in body weight associated with the adolescent treatment across the first 36 weeks of the study interval; males were ~75% heavier than females by the end of this interval.

Baseline locomotion: The initial three factor ANOVA confirmed that baseline locomotion in the open field was affected significantly by Sex [F (1, 28) = 8.67; P=0.01], by Time [F (11, 308) = 102.3; P<0.0001] within the session and by the interaction of Time with Sex [F (11, 308) = 3.64; P<0.0001], but not by adolescent treatment nor any interaction of adolescent treatment with the other factors (**Figure 4**). The follow up two-way ANOVA collapsed across adolescent treatment confirmed that there were significant effects of

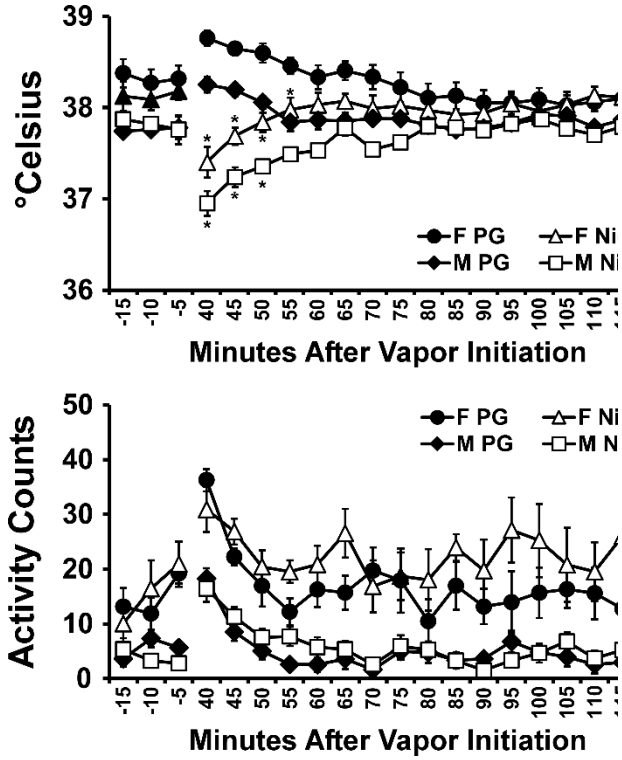

***Figure 3**: Mean (±SEM) temperature and activity rates for male (N=7) and female (N=7) rats assessed after inhalation of vapor from the propylene glycol (PG) vehicle or nicotine (30 mg/mL in the PG) on PND 63 and 66. A significant difference between inhalation conditions, within sex, is indicated with *.*

Sex [F (1, 30) = 9.21; P<0.005], of Time [F (11, 330) = 102.6; P<0.0001] and of the interaction [F (11, 330) = 3.65; P<0.0001] on locomotor activity. The post-hoc test confirmed a sex difference in the 5-20 minute bins. Similarly, reduced activity compared with the first time bin was confirmed for all subsequent bins for all (female and male) rats. Within the female group, activity was significantly lower compared with the 15 minute (lower in the 30-60 minute bins) and 20 minute (lower in the 30, 40-60 minute bins) timepoints. In the male group, however, activity was only significantly lower than the 15-minute observation in the final time bin. In the follow-up ANOVA collapsed across sex there was a significant effect of Time [F (11, 330) = 94.61; P<0.0001] on activity but not of adolescent treatment or the interaction of factors. The post-hoc test confirmed activity was significantly lower compared with the first time bin for all subsequent bins for both treatment groups.

<u>*Nicotine injection:*</u> Distance traveled in the entire session was analyzed by injection dose of nicotine (0, 0.1, 0.4, 0.8 mg/kg, s.c.) and by original adolescent treatment group (**Figure 5**). The two-factor ANOVA confirmed significant effects of Dose [F (3.214, 89.98) = 11.39; P<0.0001], Group [F (3, 28) = 8.58; P<0.0005] and the interaction of Dose with Group [F (12, 112) = 3.54; P<0.0005] on distance traveled. The post-hoc test confirmed increased activity compared with saline injection in the female/Nicotine (0.4, 0.8 mg/kg) and male/Nicotine (0.8 mg/kg) groups. Activity was also higher in the female/Nicotine group after 0.4 mg/kg injection compared with either of the 0.2 or 0.8 mg/kg doses and higher in the male/Nicotine group after 0.8 mg/kg compared with the 0.1 mg/kg dose. In the female/PG group, activity was significantly reduced after the 0.2 mg/kg dose compared with either of the 0.1 or

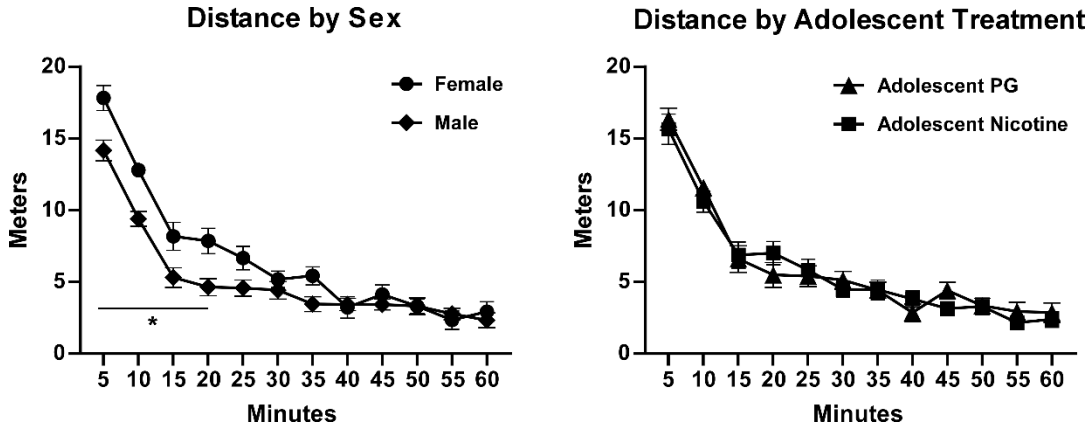

***Figure 4:** Mean (N=16 per group; ±SEM) distance traveled in the open field arena by 5 minute time bin segments. The data are presented collapsed across adolescent treatment (left panel) and collapsed across sex (right panel) to reflect the statistical analysis (see text).*

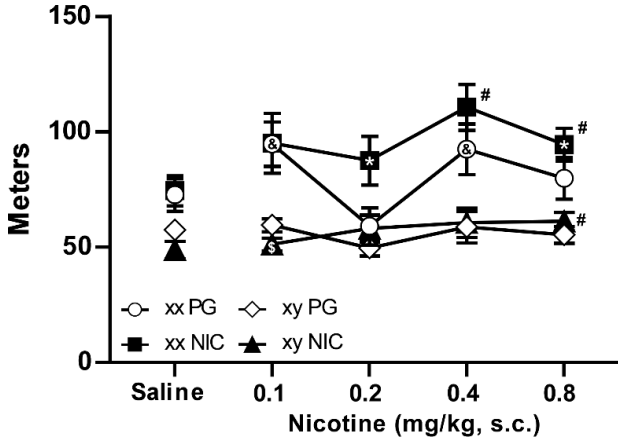

Figure 5: *Mean (N=8 per group; ±SEM) distance traveled in the open field after injection with saline or nicotine for male (xy) and female (xx) rats treated in adolescence with inhalation of vapor from the PG vehicle or NICotine (30 mg/mL). A significant difference from saline is indicated with #, from the 0.2 dose with &, from the 0.4 dose with * and from the 0.8 dose with $.*

0.4 mg/kg doses. Activity by 5-minute bin is depicted in **Supplementary Materials Figure S1**.

Experiment 4: Effect of repeated adolescent nicotine inhalation on wheel activity in adult rats

The injection of nicotine suppressed wheel activity in both male and female rats (**Figure 6**). The total session wheel counts were first analyzed by Sex, Adolescent Treatment and nicotine Dose (0.0-0.8 mg/kg, s.c.). The three-way ANOVA confirmed that total session wheel activity was significantly affected by Dose [F (2.682, 75.10) = 21.03; P<0.0001], by Sex [F (1, 28) = 46.10; P<0.0001] and by the interaction of Dose with Sex [F (3, 84) = 6.0; P<0.001]. There was no significant effect of Adolescent Treatment nor of the interaction with any other factors. Collapsed across adolescent treatment there were again significant effects of Dose [F (3, 90) = 22.17; P<0.0001], of Sex [F (1, 30) = 48.15; P<0.0001] and of the interaction [F (3, 90) = 6.32; P<0.001]. The post hoc test of the marginal mean for Dose confirmed wheel activity was significantly lower after 0.8 mg/kg compared with all other conditions, and lower after 0.4 mg/kg compared with saline injection. This same pattern was also confirmed within the female group, but no specific differences within the male group were confirmed in this analysis. A follow-up ANOVA of the male groups' activity confirmed a significant effect of Dose [F (3, 42) = 9.64; P<0.0001], but not of Adolescent Treatment, and the post hoc test confirmed significantly lower wheel activity after 0.8 mg/kg compared with saline and the 0.2 mg/kg condition, as well as after 0.4 mg/kg compared with saline injection. Activity in the two baseline sessions is depicted in **Supplementary Materials Figure S2**. Activity in the nicotine session is depicted by 15-minute time segment in **Supplementary Materials Figure S3**.

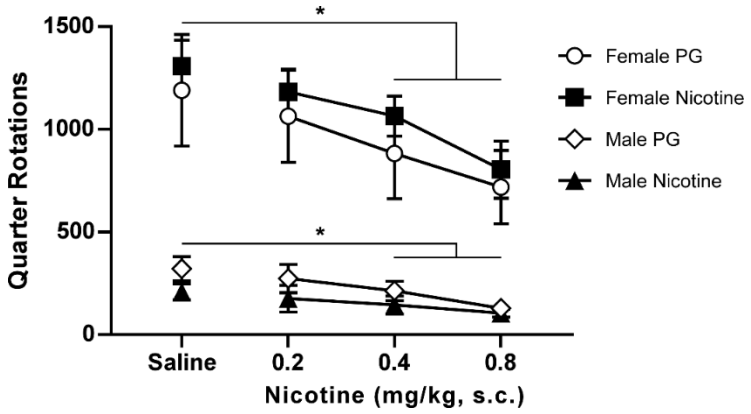

Figure 6: *Mean (±SEM) wheel activity after subcutaneous injection with saline or nicotine for male (N=8 per group) and female (N=8 per group) rats treated in adolescence with repeated inhalation of vapor from the propylene glycol (PG) vehicle or Nicotine (30 mg/mL in the PG). A significant difference from Saline, within sex and collapsed across adolescent treatment, is indicated with *.*

Experiment 5: Effect of repeated adolescent nicotine inhalation on nicotine vapor self-administration in adult rats

Male and female rats responded for different numbers of nicotine vapor (30 mg/mL) puffs in the 30-minute self-administration sessions. The first nine acquisitions sessions and a tenth training dose session conducted after four dose-substitution sessions (see **Supplementary Materials Figure S4**) are presented in **Figure 7**. The number of vape deliveries was significantly altered by rat Sex [F (1, 28) = 30.11; P<0.0001] and by the Session [F (9, 252) = 3.18; P<0.005] in the 3-way ANOVA (**Figure 7A**).

There were no significant effects of Adolescent treatment alone or in interaction with other factors on vapor deliveries. Collapsed across all groups, fewer deliveries were obtained in sessions 4, 7-10 relative to the first session as confirmed by the Dunnett post-hoc test. Each of the female groups obtained significantly morevapor deliveries than each of the male groups, but the treatment groups within each sex did not differ significantly from each other. The percentage of drug-associated responses was significantly altered by the session [F (9, 252) = 3.98; P<0.0001] in the 3-way ANOVA (**Figure 7B**), but there were no significant effects of any other factor. Collapsed across group, rats expressed a higher percentage of drug-associated responses in sessions 5-10 relative to the first session, confirmed by the Dunnett post-hoc test.

FR increment: An unexpected facilities emergency disrupted behavioral testing after the 3rd FR5 session. Animals were idled for three weeks and then re-started. Analysis thus first focused on the first three FR5 sessions compared with the immediate prior FR1 session (i.e., Session 10 in Figure 7) as a baseline. A follow-up analysis was conducted to determine the effect of altering the response contingency from FR5 to FR1 when testing resumed after the disruption.

In the initial part of the experiment, there was a decrease in the deliveries of nicotine vapor following an increase of the response contingency to FR5 (**Figure 8A**). The three-factor analysis confirmed that there was a significant effect of Session [F (3, 84) = 30.80; P<0.0001], of Sex [F (1, 28) = 29.54; P<0.0001], Adolescent treatment [F (1, 28) = 5.41; P<0.05], and of the interaction of Session with Sex [F (3, 84) = 6.66; P<0.0005] on vape deliveries. The post-hoc analysis further confirmed a difference between adolescent treatment conditions for the female rats on the FR1 and the third FR5 sessions. Vape deliveries were significantly lower in all of the FR5 sessions, compared with the FR1 session for both female groups and in the first FR5 session for the male nicotine-exposed group. In addition, significantly more

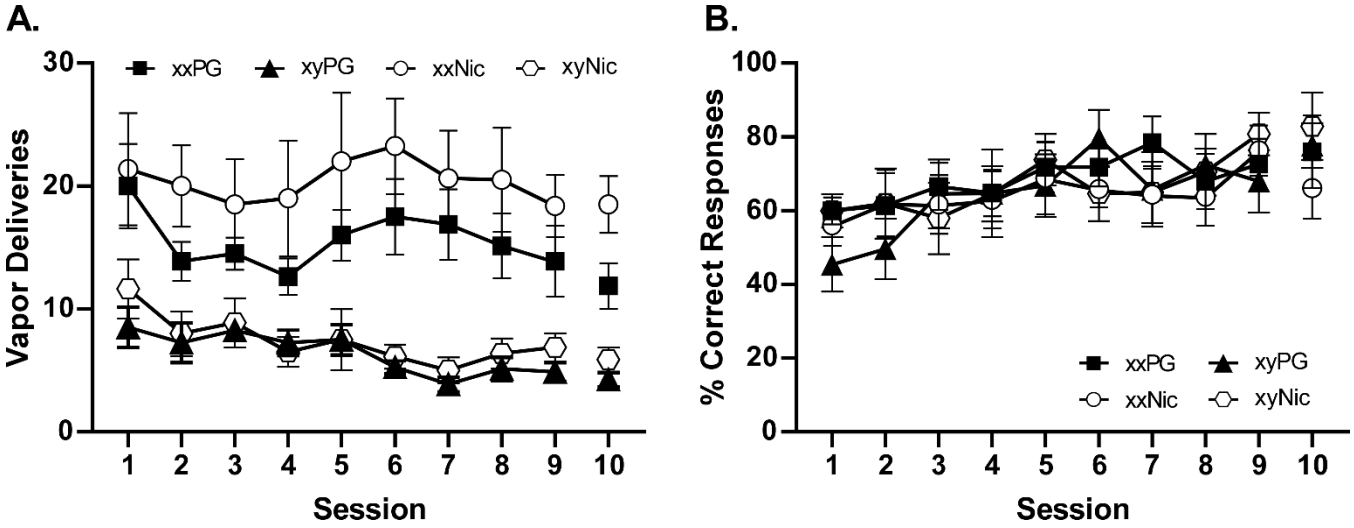

Figure 7: *Mean (N=8 per group; ±SEM) A) nicotine (30 mg/mL) vapor deliveries and B) Percent correct responses (on the drug-associated manipulandum) in the initial nine 30-minute sessions and a 10th session conducted after a 4-session dose-substitution. Intervals between sessions ranged from 2-5 days except for a 15-day interval between Session 4 and Session 5.*

vape deliveries were obtained by the nicotine-exposed female group in the third FR5 session compared with the first FR5 session. Responses on the drug-associated manipulandum (**Figure 8B**) were significantly affected by Session [F (3, 84) = 23.28; P<0.0001], of Sex [F (1, 28) = 23.34; P<0.0001], Adolescent treatment [F (1, 28) = 4.54; P<0.05], and the interaction of Session with Sex [F (3, 84) = 5.88; P<0.005], as confirmed in the 3-way ANOVA. The post-hoc test further confirmed that more correct responses were made by the PG-treated females (FR5 sessions 2 and 3) and the nicotine-treated females (all three FR5 sessions) compared with the FR1 session. Responses differed between the two female groups in the final FR5 session, and the nicotine-exposed group emitted significantly more responses inthe third FR5 session compared with the first FR5 session. There were no differences in percent of responses directed at the drug-associated manipulandum confirmed (**Figure 8C**).

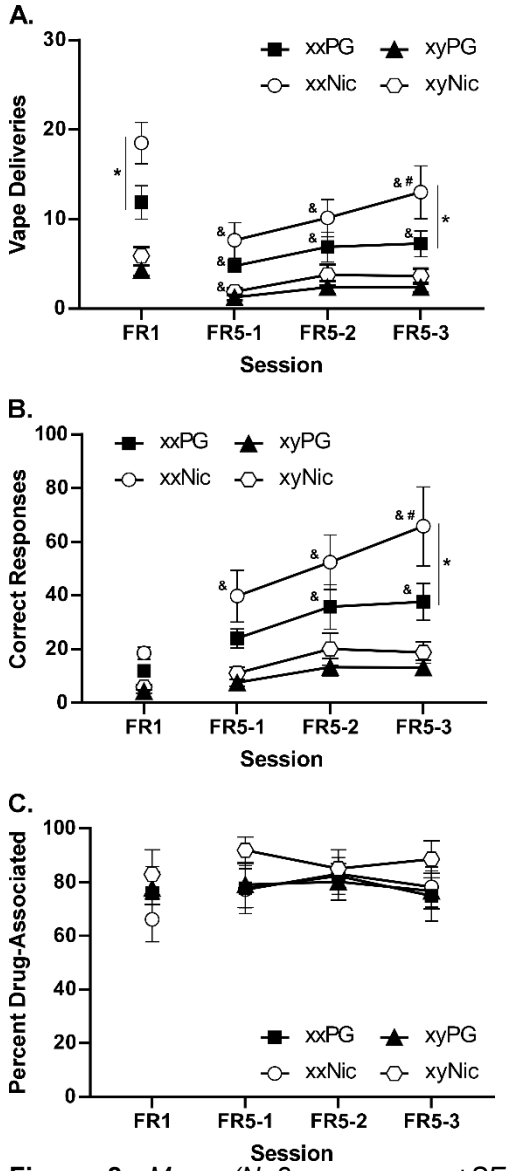

Figure 8: *Mean (N=8 per group; ±SEM) A) nicotine (30 mg/mL) vapor deliveries, B) responses on the drug-associated manipulandum and C) percent responses on the drug-associated manipulandum obtained under Fixed Ratio (FR) 1 and then under FR 5 conditions for three sessions for 40 week old adult male (xy) and female (xx) rats exposed repeatedly during adolescence to vapor from the PG vehicle or Nicotine (30 mg/mL). A significant difference between treatment groups for a given session is indicated with *, a significant difference from the FR1 session within group with &, and a significant difference from the first FR5 session within group by #.*

The analysis of the second part of the experiment confirmed differences in vapor deliveries associated with of Sex [F (1, 27) = 43.94; P<0.0001], Session [F (7, 189) = 29.47; P<0.0001], and the interaction of Session with Sex [F (7, 189) = 10.21; P<0.0001], as confirmed in the 3-way ANOVA. (**Figure 9A**). There were no significant effects of Adolescent treatment alone or in interaction with other factors. Likewise, responses on the drug-associated manipulandum (**Figure 9B**) were significantly affected by Sex [F (1, 27) = 32.68; P<0.0001], Session [F (7, 189) = 18.68; P<0.0001], and the interaction of Session with Sex [F (7, 189) = 4.32; P<0.0005], as confirmed in the 3-way ANOVA. There were no significant effects of Adolescent treatment alone or in interaction with other factors on responses. There were no differences in percent of responses directed at the drug-associated manipulandum confirmed in the analysis (**Figure 9C**).

Discussion

The study provides further evidence that Electronic Nicotine Delivery Systems (ENDS) vapor inhalation of nicotine can be used in rats to model the inhalation route of administration, which is being used increasingly by humans. The data show that ENDS inhalation results in plasma nicotine and cotinine levels that are consistent with those observed in human smokers, as well as in rat models using a

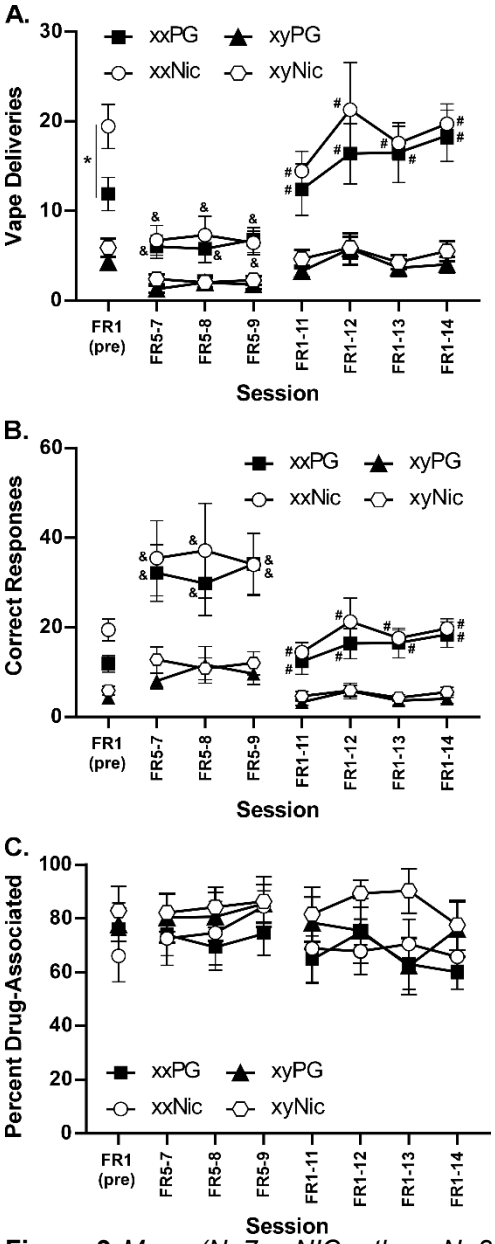

Figure 9 *Mean (N=7 xxNIC, others N=8 per group; ±SEM) A) nicotine (30 mg/mL) vapor deliveries, B) responses on the drug-associated manipulandum and C) percent responses on the drug-associated manipulandum obtained under Fixed Ratio (FR) 1, FR 5 and then FR1 conditions adult male (xy) and female (xx) rats exposed repeatedly during adolescence to vapor from the PG vehicle or Nicotine (30 mg/mL). A significant difference between treatment groups for a given session is indicated with *, a significant difference from the FR1 session within group with &, and a significant difference from the first FR5 session within group by #.*

parenteral injection route of administration. It was further demonstrated that there are physiological effects of nicotine vapor inhalation in adolescent rats that are similar to those produced in adult rats after vapor inhalation. Finally, it was shown that repeated adolescent exposure to nicotine vapor inhalation increases the locomotor stimulant effects of nicotine (s.c.) and increases the vapor self-administration of nicotine, at least in female rats. Effects of repeated adolescent nicotine vapor inhalation were limited, since no effects of this treatment on baseline locomotor activity in the open field or on wheel activity were observed.

The ENDS based inhalation model was shown to be as effective in adolescent rats as it is in adult rats. The vapor inhalation of nicotinereduced adolescent rats' body temperature in a manner similar to effects that we have reported in prior studies for adult rats, using similar exposure parameters (30 mg/mL nicotine, 30-minutes). These effects lasted about 20-30 minutes after the cessation of nicotine inhalation, as with adult rats (Javadi-Paydar et al., 2019b). Thus, physiologically relevant conditions were used in the repeated dosing approach. Although we previously reported *increased* locomotor activity, as measured with the radio-telemetry system after nicotine vapor inhalation (Javadi-Paydar et al., 2019a; Javadi-Paydar et al., 2019b), this was not observed in the adolescents. In those reports, however, the activity was measured within the vapor inhalation chambers without any animal transfer to a different recording chamber because some prior studies (unpublished) had found that a boost in activity associated with transfer to another recording chamber obscured any drug-related changes in activity.

The nicotine and cotinine levels observed after a 30-minute inhalation with a 30 mg/mL nicotine concentration were consistent with those reported by us for Sprague-Dawley male rats using the same exposure parameters (Javadi-Paydar et al., 2019b), but extend those data to show the time-course for conversion of nicotine to cotinine for up to four hours after the start of inhalation. In addition, we contrasted nicotine and cotinine in both male and female rats of Wistar and Sprague-Dawley strains. Although some group differences in nicotine and cotinine were confirmed, there were only minor

sex and strain differences in terms of mean concentrations of either cotinine or nicotine. The nicotine and cotinine levels were also similar to levels reported for male Wistar rats exposed to vapor from a 40 mg/mL nicotine concentration over 60 minutes using slightly different puffing parameters (Montanari et al., 2020) but were higher than those reported for pregnant rats exposed to nicotine (36 mg/mL) vapor using different puffing parameters for 30 minutes (Breit et al., 2022). The cotinine levels were also similar to those reported in a mouse model of vapor inhalation (Cooper et al., 2021). The available data to date, including herein, therefore shows that the vapor inhalation approach is repeatable and generalizable to produced target levels of nicotine in rodent subjects. The inhalation approach used here and in our prior study have generated plasma nicotine levels comparable to a 0.8-1.0 mg/kg, s.c. injection of nicotine, a commonly used range for behavioral studies in rats, and was a range which produced locomotor suppressive effects in this study.

Sex differences in activity were observed in the telemetry, open field and wheel assays, with females more active in all cases. This is highly consistent with most reports of activity in rats as measured with open field video or beam-break technology, radio-telemetry and activity wheels (Krentzel et al., 2020; Lee et al., 2019; Verlangieri, 1979). Injection of nicotine significantly increased open field activity in the male (0.8 mg/kg, s.c.) and female (0.4, 0.8 mg/kg, s.c.) groups treated as adolescents with nicotine vapor, but not the PG-exposed groups. Female and male rats were equivalently sensitive to the wheel activity suppressing effects of nicotine, expressing a similar degree of suppression in the first 15 min after 0.2-0.8 mg/kg, s.c., doses. The effect of nicotine, delivered by s.c. injection or vapor inhalation, on wheel activity is similar to a suppressive effect of 0.4 mg/kg nicotine, s.c., injection on wheel activity (Bryson et al., 1981). Another report found ~0.25 mg/kg, s.c. to be the peak of an inverted-U dose response for increased wheel activity in female Sprague-Dawley rats (Gillman et al., 2010), again showing that nicotine can either increase or decrease rodent activity depending on dose, assay and other factors.

Vapor *self-administration* is a relatively novel procedure, although some recent evidence has been provided for the self-administration of nicotine (Lallai et al., 2021; Smith et al., 2020), heroin (Gutierrez et al., 2020), fentanyl (McConnell et al., 2021), sufentanil (Vendruscolo et al., 2018) and cannabis extracts (Freels et al., 2020). The present results show that male and female rats will self-administer nicotine by vapor inhalation without any prior operant training (as in Lallai et al. 2021). Across the entire study within group, rats self-administered similar numbers of vapor deliveries under a FR1 contingency in sessions of only 30 min duration. Introduction of a higher response contingency (FR5) led to increased numbers of responses on the drug-associated manipulandum, which was associated with group mean behavioral discrimination of over 75% responses on the drug-associated manipulandum. Male and female animals exhibited similar levels of behavioral discrimination, but females responded consistently for more vapor deliveries. Since there is considerable evidence that female rats will self-administer many drugs at a higher rate than male rats in intravenous procedures (Anker and Carroll, 2011), it is possible to view this outcome as a positive control that is also consistent with self-administration. The impact of adolescent nicotine vapor exposure on self-administration was limited to the initial stages of the study for female rats. A somewhat variable mean difference during acquisition led to a significant difference in the initial FR5 investigation where the nicotine-exposed animals self-administered more vapor deliveries in the baseline FR1 session and recovered more quickly from the introduction of FR5, leading to a mean difference on the third session. After the unavoidable 3-week delay, these differences were no longer present.

Sex differences were observed throughout the study, in both PG- and nicotine-exposed groups. Prior evidence for sex-differences in nicotine self-administration is mixed with some studies finding no sex difference and some reporting a difference. The reported differences may depend on any of several methodological variables, the influences of which are poorly understood. A recent report found that female Sprague-Dawley rats intravenously self-administer fewer nicotine infusions than do male Sprague-Dawley rats, while no sex-difference in

Long-Evans rats was observed (Leyrer-Jackson et al., 2021). A study of heterogeneous stock adolescent rats derived from eight founder strains reported increased oral nicotine intake in female rats (Wang et al., 2018). There is also varied evidence of female Sprague-Dawley rats self-administering / acquiring more / faster (Sanchez et al., 2014), less/slower (Levin et al., 2011) or the same (Chaudhri et al., 2005; Donny et al., 2000; Pittenger et al., 2016) as male rats of the same strain. Similarly, female Wistar rats have been reported as self-administering / acquiring more / faster (Chellian et al., 2021; Uribe et al., 2020), less/slower (Swalve et al., 2016) or the same as male rats of the same strain and a similar diversity of sex-associated outcome has been reported for Long Evans rats (Leyrer-Jackson et al., 2021; Li et al., 2014). Another study found that female rats intravenously self-administer higher amounts of nicotine only under FR5, but not FR1, and as in the present study, responses only partially compensated (i.e.12-19 responses under FR1, 30-70 under FR5) for the increased response requirement (Chaudhri et al., 2005). Male and female Sprague-Dawley rats intravenously self-administer identical numbers of infusions of nicotine when self-administration is started in adolescence (PND 34) after food-reinforced operant training (Carreno and Lotfipour, 2022) and adult female and male Wistar rats self-administered similar amounts of *nicotine vapor*, also following food-reinforced operant training (Lallai et al., 2021). Finally, a meta-analytic review of twenty studies concluded evidence for female rats self-administering nicotine at higher rates, with an estimate of the magnitude of effect of 0.18 standard deviations (Flores et al., 2019). Where investigated, only minimal effects or no effects of estrous stage have been reported (Donny et al., 2000; Levin et al., 2011).

Overall, these data do not illustrate significant lasting consequences of repeated adolescent nicotine exposure by vapor inhalation on activity patterns in open field or on an exercise wheel. These data do, however, point to a potential liability for moderately enhanced nicotine self-administration in adulthood, in female rats.

Declaration of Interests: The authors report no financial conflicts of interest that would influence the outcomes reported in this manuscript.

Acknowledgements: The authors are grateful to Eric L. Harvey, Ph.D., for running plasma analysis studies. These studies were supported by the Tobacco Related Disease Research Program (T31IP1832, MAT), a UCSD Chancellor's Post-doctoral Fellowship (AG), UCSD IRACDA (AG) and the NIH (R01 DA042211, MAT). None of the funding bodies had any influence on the study design, data interpretation, manuscript creation or in the decision of when and what to publish from the studies conducted.

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