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Effects of repeated nicotine vapor exposure and withdrawal on somatic signs, anxiety-like behavior, and brain reward thresholds in male Sprague Dawley rats

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EFFECTS OF REPEATED NICOTINE VAPOR EXPOSURE AND WITHDRAWAL ON SOMATIC SIGNS, ANXIETY-LIKE BEHAVIOR, AND BRAIN REWARD THRESHOLDS IN MALE SPRAGUE DAWLEY RATS

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Dedication

Lovingly dedicated to my parents Miguel Martínez, and Gregoria Gonzalez de Martínez, for promoting higher education in our household, and for their sacrifices to provide my siblings and I, a better quality of life by immigrating to the United States. Also dedicated to my siblings, Miguel Martínez Jr., and Alexa Martínez for their unconditional support and never-ending love.

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Chapter 1: Introduction

1.1 Relevance of e-cigarettes

Electronic nicotine delivery systems, also known as e-cigarettes, have been developed to allow nicotine vapor consumption and assist individuals with smoking cessation from conventional tobacco cigarettes (Caponnetto, Russo et al. 2013, Cai and Wang 2017). However, recent rises in recreational e-cigarette use have been attributed to individuals who have not previously engaged in traditional cigarette use due to their well-known negative health effects (Javadi-Paydar, Kerr et al. 2019). According to the CDC, increased marketing and versatile flavorings of e-cigarettes has also led to increased recreational usage of these systems, especially among the adolescent and young adult population (Singh, Arrazola et al. 2016, Cai and Wang 2017, Cullen, Liu et al. 2019, Bandi, Cahn et al. 2021). Recent reports on e-cigarette use have shown that while adult use in the U.S. has increased modestly from 3.0% in 2017 to 4.5% in 2019 (Wang, Asman et al. 2018, Cornelius, Wang et al. 2020), a dramatic increase in adolescent use has been observed, with use in this population increasing from 11.7% in 2017 to 27.5% in 2019 (Bold, Kong et al. 2021). This increase in the recreational use of e-cigarettes is alarming considering the well-known addictive properties of nicotine and highlight the need for research on this novel nicotine delivery system (Cai and Wang 2017).

1.2 Nicotine abuse liability

Addiction to drugs of abuse, such as nicotine, is a complex disease that is characterized by many components. Koob and Le Moal (2001) describe an addiction cycle that is comprised of three major components: preoccupation-anticipation, binge-intoxication, and withdrawal-negative affective states. Importantly, the authors note that different drugs and patterns of use can result in differences in the intensity of each component of the addiction cycle. One recent study

investigated the abuse liability (i.e., tendency to use the drug excessively) of e-cigarettes against Food and Drug Administration approved nicotine inhalers and users' own brand of traditional cigarettes. This study found that e-cigarette abuse liability was higher than that seen with nicotine inhalers, but lower than that observed with traditional cigarettes (Maloney, Breland et al. 2019). Additionally, comparable plasma nicotine levels following exposure to the highest nicotine concentration against traditional cigarettes suggests the potential for nicotine dependence with repeated e-cigarette use.

1.3 NICOTINE WITHDRAWAL

Increases in drug intake and withdrawal, following repeated nicotine exposure via intravenous self-administration and osmotic mini pumps has been well documented in pre-clinical models, particularly rodents (O'Dell, Chen et al. 2007, Flores, Cruz et al. 2020). Research with rats has also shown that repeated nicotine exposure may also result in neuroadaptations that underlie long-term increases in drug intake and withdrawal (Berke and Hyman 2000). A recently published study reports important initial steps for characterizing the effects of nicotine vapor on drug intake and withdrawal. In this study researchers demonstrated that previous exposure to nicotine vapor during adolescence results in an increased propensity to self-administer nicotine later in adulthood through intravenous self-administration (Kallupi, de Guglielmo et al. 2019). The authors also go on to demonstrate that while spontaneous withdrawal from nicotine vapor did not increase anxiety levels as indicated by the elevated plus maze, increases in somatic withdrawal signs were observed (Kallupi, de Guglielmo et al. 2019). Together, these findings demonstrate that nicotine vapor exposure in rodents, results in addictive behaviors similar to those seen following more traditional routes of exposure and calls for further characterization of the effect of nicotine vapor on the brain and behavior.

1.4 BRAIN STIMULATION REINFORCEMENT

While there are a number of drug-induced neuroadaptations that are believed to drive changes in drug intake and withdrawal, aberrant dopamine signaling in the brain reward system is perhaps the most well defined (Koob and Le Moal 2005, 2008). Drug effects on the brain reward system can be studied in rodents by using Intracranial Self-Stimulation (ICSS) of the mesolimbic dopamine reward pathway that projects from the ventral tegmental area to the nucleus accumbens. ICSS allows animals to deliver electrical stimulations to brain reward structures through a response manipulandum. Stimulating electrodes are aimed at specific structures of the brain reward pathway, including the medial forebrain bundle (MFB), a relatively larger section of the mesolimbic pathway (Carlezon and Chartoff 2007). Electrical stimulations to the MFB are often measured in amplitudes and amplitudes that produce high rates of responding are presumed to reflect general brain reward thresholds. Electrical stimulation of the brain reward pathway with ICSS does indeed reinforces response behavior and this technique is often used to investigate the effects of drug exposure on the brain reward system.

Stimulation threshold baselines can be established for individual subjects and changes in these thresholds after drug administration have been assessed to determine increases or decreases in brain reward sensitivity (Markou and Koob 1992). Specifically, Koob and his colleagues suggest that elevations in ICSS reward thresholds relative to baseline reflect decreases in reward sensitivity observed during negative psychological states, such as those seen with withdrawal. Conversely, decreases in ICSS thresholds relative to baseline may suggest increases in brain reward sensitivity, such as that seen immediately following drug intake (see Figure 1). Studies have indeed demonstrated decreases in ICSS thresholds immediately following nicotine injections and increases in ICSS threshold during withdrawal from repeated nicotine injections (Johnson,

Hollander et al. 2008, Fowler, Tuesta et al. 2013). Identifying brain reward threshold changes during and after nicotine vapor exposure will be an important step towards characterizing the biological and behavioral effects of this new and highly popular nicotine delivery system.

1.5 OBJECTIVES

Experiment 1: This experiment investigated the effects of repeated nicotine vapor exposure on somatic withdrawal signs and anxiety-like behavior in rats. Withdrawal in human users of traditional nicotine products is well described, therefore examining withdrawal behaviors following vapor exposure is a necessary first step for nicotine vapor research.

Experiment 2: This experiment investigated the effects of acute and repeated nicotine vapor exposure on brain reward function. More specifically, this experiment assessed the effects of nicotine vapor exposure and withdrawal on ICSS brain reward thresholds in rats. Findings will provide valuable insight on how nicotine vapor can affect the brain reward system.

Chapter 2: Methods

2.1 SUBJECTS

Adult male Sprague-Dawley rats (n=56) and male Wistar rats (n=16) were obtained from an outbred stock of animals (Envigo Inc., Indianapolis, IN) for Experiment 1 and Experiment 2, respectively. Animals in both experiments were housed in a humidity and temperature-controlled vivarium (22°C) with a 12hr light/dark cycle and *ad libitum* access to food and water, except as noted below. Animals in Experiment 1 will be pair-housed, while animals in Experiment 2 will remain individually housed to minimize electrode implant displacement. All experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas of El Paso.

2.2 APPARATUS

Nicotine vapor system: A Four Chamber Benchtop Passive E-vape Inhalation System (La Jolla Alcohol Research Inc., San Diego, CA) was used to deliver nicotine vapor to animals in both experiments. The system is composed of four chambers large enough to house two rats per chamber (interior dimension of 14.5" L x 10.5" W x 9.0" H). Each chamber has two valve ports that are located on opposite walls and allow connection to vapor tubing. One valve port was connected via PVC tubing to a small vacuum that provided airflow in the chamber at 0.6 L per minute. The vacuum outlet was connected to a Whatman HEPA filter (Millipore Sigma, Darmstadt, Germany), then onto a building exhaust system that safely removed the nicotine vapor from the chambers. The other valve port was connected via PVC tubing to a TFV4 mini-tank (4.9-volt, 65.0W; Smok Inc, Shenzhen, China), where the nicotine e-liquid was heated by a 0.42 Ω atomizer coil. Each nicotine concentration and vehicle control (24 mg/mL, 12 mg/mL, and 0

mg/mL) is assigned its own tubing and exposure chambers are thoroughly cleaned between exposures to avoid cross-contamination between experimental groups.

Operant chambers: Operant chamber (12" L x 9.5" W x 11.5" H, Med-Associates, Inc., Fairfax, VT) used in Experiment 2 were Plexiglas boxes with grid flooring, houselights, and a fixed wheel manipulandum attached to a wall of the Plexiglas box. The wheel manipulandum is used to deliver electrical brain reward self-stimulations. A stimulator provides electrical stimulations that are sent through a commutator (2-channel, 305-plugs) located in each operant chamber. The commutator is attached to a commutator balance arm (0-20.3 cm, above chamber). A spring leash (5-100 cm, 2-channel; PTechnologies, Roanoke, VA) is screwed onto the commutator on one end and an electrode pedestal implanted into the animal on the other. A modified top permits the animal to move freely during training.

Elevated plus maze: The elevated plus maze (EPM) was used in Experiment 1 to assess anxiety-like behavior, as a measure of withdrawal. The EPM consists of two closed arms and two open arms (10 cm W x 50 cm L). The closed arms had walls that were 20 cm in height and the maze was elevated 50 cm from the ground. All EPM testing was conducted under a red light, as differences in light exposure may influence behavior when using the elevated plus maze.

2.3 DRUGS

The present study used flavorless nicotine e-liquids containing nicotine in its freebase form in a 50/50 vegetable glycerin/propylene glycol vehicle. All nicotine e-liquids were purchased from the commercial vendor Vapor Chef (VC Tobacco #13; Bristol, PA). Nicotine e-liquid concentrations of 24 mg/mL and 12 mg/mL, as well as a 0 mg/mL nicotine vehicle control were used for Experiment 1, while 0 mg/mL or 24 mg/mL were used for Experiment 2. For Experiment 1 injections of nicotine ditartrate salt (0.8 mg/kg, s.c., expressed as a base) and the non-selective

nicotinic receptor antagonist mecamylamine (3.0 mg/kg, s.c., salt) were used (National Institute on Drug Abuse; Bethesda, MD). Both of these drugs were dissolved in 0.9% sterile saline and the pH of nicotine ditartrate salt was adjusted to 7.4 using a pH meter with chloride and hydroxide titration.

2.4 PROCEDURES FOR EXPERIMENT 1

Nicotine vapor exposure: Animals were allowed to acclimate to the vivarium for at least 1 week prior to nicotine vapor exposures. Once acclimated, animals began 9 consecutive days of experimental procedures (see Figure 2 for a schematic of Experiment 1 timeline). Experiment 1 had a total of seven experimental groups (n=8/group) divided as: home cage (HC), vapor chamber (VC), 0 mg/mL vehicle control, 12 mg/mL nicotine vapor, 24 mg/mL nicotine vapor, 0.8 mg/kg nicotine injection, and saline injection. The HC group remained in their home cages for the entirety of the experiment, while the vapor chamber group was placed in vapor chambers without any vapor exposure.

For daily vapor exposures, animals with vapor exposure were placed in chambers with their respective cage mate. Pseudo-randomized assignment to vapor exposure groups was used due to the paired housing and exposure of the animals. The total time of nicotine vapor exposure sessions for Experiment 1 and 2 was 89 minutes for each nicotine concentration. Each session consisted of four cycles that lasts eighteen minutes and thirty seconds with an additional five-minute inter-exposure interval between each cycle. A 3-second nicotine puff is delivered into each chamber at the beginning of a cycle followed by a two-minute inter-puff interval, for a total of ten 3-second puffs every cycle. To model average daily nicotine vapor consumption in human e-cigarette users, a total forty puffs were delivered to the rats per daily nicotine vapor exposure sessions (Qasim,

Karim et al. 2018). To help minimize nicotine contamination, lower nicotine concentration groups underwent vapor exposure before higher concentration groups during daily exposure sessions.

Tail blood collection: To confirm systemic absorption of nicotine using our nicotine vapor inhalation system, tail blood (.5 mL) was collected for each nicotine concentration on exposure day 6. This ten-minute procedure included brief anesthetization of each animal using 2.5-3% isoflurane gas in oxygen. Blood was collected using Eppendorf vials and centrifuged at (6900 r. p. m. x 4°C), before blood serum extractions were collected in separate vials and stored in a collection box placed in a -80°C freezer. Post-surgical topical analgesics were applied to the tail after sample collection. Cotinine detection was assessed utilizing an ELISA kit (Cal Biotech Inc, El Cajon, CA), allowing for duplicate of the same sample. All standards, reagents, and substrate amounts were determined based on instructions provided in the ELISA kit.

Withdrawal sign measures: On Day 7 of nicotine vapor exposure somatic signs were measured in all animals. Animals were placed in a Plexiglas box (30cm x 29 cm) for assessment of somatic signs. Following acclimation to the box, rats were injected with mecamylamine (3.0 mg/kg, s.c.) and an additional ten-minute waiting period was required before precipitated withdrawal sign assessment. Number of somatic signs were recorded for ten minutes based on the following list of known indicators of withdrawal: blinks, yawns, teeth chatters, gasps, writhes, body shakes, headshakes, ptosis, and grooming (Harris, Pentel et al. 2011, Flores, Cruz et al. 2020).

Elevated plus maze (EPM): On Day 9 of vapor exposure, anxiety-like behavior, reflected by entries in the EPM, was assessed in all animals. Animals acclimated to the testing room in their home cage for ten minutes, followed by an injection of mecamylamine (3.0 mg/kg, s.c.; Harris, Pentel et al. 2011). As with somatic sign assessment, a ten-minute waiting period following

mecamylamine administration was required before placing animals in the middle of the EPM, facing an open arm. Assessment of behavior during precipitated withdrawal was based on closed versus open arm entries, across five minutes.

2.5 PROCEDURES FOR EXPERIMENT 2

Electrode implantation: Animals were anesthetized using isoflurane 2.5-5% gas in oxygen. Once anesthetized, rats were administered an intraperitoneal injection of saline (3.0 ml), a subcutaneous injection of flunixin (0.2 ml), and a subcutaneous injection of lidocaine on the scalp (0.1 ml). Animals were then positioned and secured in a stereotactic frame, with a flat skull position established by matching dorsal-ventral coordinate measures. Following screw placements, a 5mm incisor bar elevation was applied, and a unilateral untwisted electrode was implanted into one hemisphere (left and right hemisphere, counterbalanced) with the tip of the electrode targeting the medial forebrain bundle (MFB) at the level of the lateral hypothalamus (AP: -0.5 mm from bregma; ML: +- 1.7 mm; DV: -9.3 mm from the skull surface; Paxinos & Watson, 2007). After electrode placement, 2-3 screws were secured with acrylic cement glue to create a "cap" around the surgical site. Postsurgical analgesics (i.e. Neosporin) were administered according to institutional regulations. Rats recovered from surgery for 5-7 days before any behavioral training was initiated.

ICSS training: Following recovery from surgery, animals began training in the ICSS task (see Figure 3 for a schematic of Experiment 2 timeline). In phase I of training, animals were tested individually in ICSS operant chambers. All animals started training with a default stimulation frequency of 100Hz and 120 microamperes (μA), which were delivered freely following ¼ rotations of the response wheel. If low wheel responding was observed in an animal after approximately 5 minutes, 10-20 μA adjustments were made to the ICSS stimulation within

sessions. Animals were trained in phase I until the criteria of 100 spins of the response wheel was achieved within 5 minutes.

For phase II training, trials begin with a noncontingent stimulation, followed by a variable post-stimulation response window (7.5 s) during which delivery of a second stimulus was contingent upon a ¼ turn of the response wheel. Animals were required to spin the response wheel for 30 contingent electrical stimulations, at 5 different inter-trial intervals (ITI). ITIs were presented in ascending order (1, 3, 5, 10, and 15 sec), with one ITI assigned per day. Non-contingent and contingent stimulations were matched and daily sessions began with μA-stimulations that produced successful responding on the previous training day. If low wheel responding was observed in an animal during a session, 10-20 μA adjustments were made to the stimulations for the following training sessions.

In phase III we attempt to identify μA thresholds that produced maximal responding to non-contingent stimulations in individual rats. To determine these thresholds, μA thresholds "blocks", consisting of 3 trials per block, were automatically adjusted within daily session by the MedPC program based on each animal's responding to the non-contingent stimulus. When no response was detected for 2 out the 3 trials at a given μA-block, the stimulation increased by 5μA. When responding was detected for 2 out the 3 trials at a given μA-block the stimulation decreased 5μA. In each daily session, 2 descending and 2 ascending μA-block series were presented, starting with a descending session and progressing in an alternating manner. The threshold for each of the 4 series was defined as the midpoint between the two μA-blocks immediately preceding a shift in series order. For phase III training day 1, the starting μA-setting was based on the last μA-setting successfully presented on the last day of phase II. On all subsequent phase III training days, the starting μA-setting was based on the average of the 4 identified series thresholds, plus 30 μA. If

an animal maintains responding for 4 to 5 days on phase III they were moved to phase IV. If responding is not maintained across 4 to 5 days on phase III, they were returned to phase II training.

Phase IV followed the same procedures used in phase III training. During phase IV, the average of the four alternating μA-block series is identified as the rat's threshold for that day. This phase ended when all thresholds were stable, meaning that there was less than 10% variability of amplitude increases and decreases across 3 training days. Response latencies were also identified and defined as the point between the start of the non-contingent stimulus and a ¼ turn response on the wheel (Chellian, Wilks et al. 2021).

Nicotine vapor exposure: Once rats achieved stability criteria for phase IV, they were pseudo-randomly assigned into one of two groups 0 mg/mL vehicle control or a 24 mg/mL nicotine vapor concentration (n=8/group) and underwent 14 consecutive days of vapor exposure immediately prior to ICSS testing, using daily vapor exposure parameters described in the Procedures section for Experiment 1. On day 1 of exposure, acclimation to vapor chambers was achieved by placing the rats in the chambers (without vapor) for 30 minutes before vapor exposure began.

ICSS threshold testing: Animals were tested in ICSS using procedures described for Phase IV training. Testing occurred immediately following each day of the 14 days of nicotine vapor exposure and for an additional 14 days following nicotine vapor exposure cessation. This approach allowed us to assess the effects of acute and repeated nicotine vapor exposure, and spontaneous withdrawal from repeated nicotine vapor exposure, on ICSS brain reward thresholds.

Histology: Whole brains were collected following decapitation on withdrawal day 14, following ICSS testing, for electrode placement verification. Brains were stored for 3-4 days in 4% PFA diluted using 1X PBS, then placed in a 30% sucrose/70% PFA solution for 6 days. Brains

were sliced at 40 μm using a cryostat and tissues were mounted on gelatin-coated slides. Tissues were dehydrated using distilled water, followed by increasing concentrations of ethanol (i.e., 50, 70, 95, and 100%, 3 min each) for compatibility with xylene (Thermo Fisher Scientific, Waltham, MA). Tissues were then gradually rehydrated through descending concentrations of ethanol (i.e.,100, 95, 70, and 50%, 3 min each) before staining by dipping tissues in thionine (Thermo Fisher Scientific, Waltham, MA). Tissues were again dehydrated through ascending ethanol concentrations and xylene for compatibility with mounting medium, and left to dry. A line of DPX was placed across slides, which were then sealed with a coverslip and left to dry overnight. TIFF-formatted images were taken using a bright-field microscope with an x5 magnification (Carl Zeiss Corporation, Thornwood, NY) and an EXi monochrome camera (TeledyneQimaging, Inc., Surrey, British Columbia) with Volocity Software (Ver. 6.1.1; Quorum Technologies, Inc.,Puslinch, Ontario).

2.6 STATISTICS

Statistical analyses used in Experiment 1 included a one-way ANOVA with LSD post-hoc tests for cotinine, somatic withdrawal signs, and entries. Statistical analysis for Experiment 2 included mixed-model ANOVAs with t-tests post-hoc analysis for response latencies, threshold μA-settings, and threshold percent changes from baseline (average of last 3 days of Phase IV training thresholds for nicotine vapor exposure and average of last 3 days of nicotine vapor exposure thresholds for nicotine vapor withdrawal). Bonferroni post-hoc tests were also conducted for all analyses and significant LSD and t-tests that were also found to be significant with Bonferroni are denoted with an asterisk (*).

Chapter 3: Results

3.1 EXPERIMENT 1

3.1.1 Blood plasma cotinine levels

Figure 4 illustrates blood plasma cotinine levels from tail vein blood that was extracted from all groups following 6 days of nicotine vapor exposure. One-way ANOVA revealed a main effect of group [$F_{(6,49)}$=157.58; $p \leq 0.001$]. Post-hoc analyses show that rats in the 12 mg/mL and 24 mg/mL nicotine vapor groups expressed higher cotinine levels than HC, VC, 0 mg/mL, and saline injection groups ($p \leq 0.001$*). Similarly, post-hoc tests revealed significantly higher cotinine levels in the 0.8 mg/kg nicotine injection group when compared to the saline injection group ($p \leq 0.001$*). When comparing groups exposed to nicotine vapor exposure to group exposed to nicotine injections, post-hoc analyses show that the 12 mg/mL group had significantly lower cotinine levels ($p \leq 0.001$*), while the 24 mg/mL group had higher cotinine levels, than the 0.8 mg/kg nicotine injection group ($p \leq 0.001$*). Finally, a comparison of cotinine levels between the 12 mg/mL nicotine vapor group and 24 mg/mL nicotine vapor group showed that the 24 mg/mL group had significantly higher cotinine levels than the 12 mg/mL group ($p \leq 0.001$*), following 6 days of vapor exposure. In summary, higher nicotine concentration exposure resulted in increased levels of cotinine when compared to animals in control groups.

3.1.2 Somatic signs of withdrawal

Figure 5 illustrates somatic signs that were assessed in all groups on day 7 of nicotine vapor exposure, following mecamylamine administration. A one-way ANOVA revealed a main effect of exposure group [$F_{(6,49)}$=3.84; $p \leq 0.003$]. Post-hoc analyses revealed that the 12 mg/mL and 24 mg/mL nicotine vapor group displayed more somatic signs when compared to the HC group ($p \leq 0.005$, $p \leq 0.001$*, respectively). The 24 mg/mL nicotine vapor group was also found to display

more somatic signs than saline injection, VC, and 0 mg/mL vehicle control groups ($p \leq 0.05$). These findings demonstrate that increases in somatic withdrawal signs were displayed in animals exposed to higher nicotine concentrations when compared to controls.

3.1.3 Elevated plus maze open arm entries

Figure 6 illustrates anxiety-like behavior that was assessed in all groups on day 9 of nicotine vapor exposure, following mecamylamine administration. A one-way ANOVA revealed a main effect of treatment group [$F_{(6,49)}=2.38$; $p \leq 0.05$]. Post-hoc analysis revealed that the 24 mg/mL nicotine vapor group had fewer open arm entries than the HC group ($p \leq 0.05$) and that the 0.8 mg/kg nicotine injection group had fewer open arm entries when compared to the saline injection group ($p \leq 0.05$). Results in the EPM test show that exposure to higher nicotine concentrations resulted in fewer open arm entries in the EPM when compared to the control group.

3.2 EXPERIMENT 2

3.2.1 ICSS Thresholds

Figure 7 illustrates ICSS threshold measures as μA values during the 14 days of nicotine vapor exposure, as well as the 14 days immediately following cessation of nicotine vapor exposure. Two rats from each treatment group were removed from the study, due to a failure to complete ICSS training or electrode dislodgement during the training, resulting in a final total of 6 rats per group. Mixed-model ANOVA comparing nicotine vapor exposure groups across the 14 days of treatment revealed a main effect of time [$F_{(13,126)}=1.84$; $p \leq 0.05$], such that all rats exhibited a slight increase in threshold across the 14 days of treatment (Figure 7A). No main effect or interaction was observed for treatment group during nicotine vapor exposure days. Mixed-model ANOVA comparing nicotine vapor exposure groups across the 14 days immediately following cessation of nicotine vapor exposure revealed no main effect or interaction for either time or treatment group

(Figure 7B). Overall, thresholds between vehicle control and 24mg/mL nicotine concentration, were comparable immediately following daily exposure to vapor. During withdrawal days thresholds between 0 mg/mL vehicle control and 24 mg/mL again remained similar.

3.2.2 ICSS Thresholds Percent Change

Figure 8 illustrates ICSS thresholds measures as percent change from baseline during the 14 days of nicotine vapor exposure, as well as the 14 days immediately following cessation of nicotine vapor exposure. Mixed-model ANOVA comparing nicotine vapor exposure groups across the 14 days of treatment revealed no main effect or interaction for time or treatment group (Figure 8A). Mixed-model ANOVA comparing nicotine vapor exposure groups across the 14 days immediately following cessation of nicotine vapor exposure revealed a main effect of group [$F_{(1,10)}$=11.01; $p \leq 0.01$]. No main effect or interaction was observed for time during days immediately following cessation of nicotine vapor exposure. Post-hoc analysis comparing the nicotine vapor treatment groups on each day immediately following cessation of nicotine vapor exposure revealed higher percent changes in the 24 mg/mL nicotine vapor exposure group, when compared to the 0 mg/mL vehicle control group, on days 1, 3, 8, 9, and 13 ($p \leq 0.05$, Figure 8B). In summary, thresholds expressed as percent change were comparable in the 0 mg/mL vehicle control and 24 mg/mL nicotine concentration groups immediately following daily vapor exposure. Interestingly, thresholds during withdrawal days were significantly different between 0 mg/mL vehicle control and 24 mg/mL nicotine concentration groups, with the 24 mg/mL nicotine concentration group displaying higher threshold percent changes when compared to the 0 mg/mL vehicle control group.

3.2.3 Response latencies

Figure 9 illustrates ICSS response latencies during the 14 days of nicotine vapor exposure, as well as the 14 days immediately following cessation of nicotine vapor exposure. Mixed-model ANOVA comparing nicotine vapor exposure groups across the 14 days of nicotine vapor exposure revealed no main effect or interaction for time or treatment group (Figure 9A). Similarly, mixed-model ANOVA comparing nicotine vapor exposure groups across the 14 days immediately following cessation of nicotine vapor exposure revealed no main effect or interaction for time or treatment group (Figure 9B). These findings suggest that exposure to 24 mg/mL nicotine does not significantly increase ICSS response latencies when compared to the 0 mg/mL vehicle controls.

3.2.4 Electrode placement verification

Figure 10 illustrates ICSS electrode placement in the MFB for all rats, as plotted onto figures of a rat brain atlas (Paxinos & Watson, 2007). Gross observations of electrode placement suggest that of the 12 rats completing ICSS training and test, 7 rats had placement in the MFB, with 3 rats having electrode placements just outside of the MFB (2 from the 0 mg/mL vehicle control group and 1 from the 24 mg/mL vapor group) and 2 rats did not have electrode placement confirmed (1 from the 0 mg/mL vehicle control group and 1 from the 24 mg/mL vapor group).

Chapter 4: Discussion

The findings of my thesis demonstrate that our novel nicotine vapor system creates significant withdrawal-like states in male rats following cessation of repeated nicotine vapor exposure. Our model produced somatic withdrawal signs and anxiety-like behavior, similar to those observed with more well-established routes of nicotine administration in rodents. Effects of nicotine vapor exposure on the brain reward system was also assessed and findings demonstrate brain reward changes through relative increases in brain reward stimulation thresholds. Specifically, increases in ICSS brain reward thresholds' percent change was observed following cessation of repeated nicotine vapor exposure (i.e., nicotine withdrawal).

Increased cotinine biomarkers observed following nicotine vapor exposure: A major nicotine metabolite, cotinine, has a longer half-life (15-19 hours) compared to nicotine (2-3 hours), and other plasma nicotine biomarkers (Benowitz 1996, Buccafusco and Terry 2003, Avila-Tang, Al-Delaimy et al. 2013). This characteristic makes cotinine detection reliable for assessment of nicotine vapor exposure. Findings from Experiment 1 show that exposure to nicotine vapor (12 and 24 mg/mL) did indeed increase cotinine levels relative to negative controls on day 6 of exposure. When comparing animals in the 24 mg/mL group to animals in the 12 mg/mL group, we found that the group with the higher concentration of nicotine also had higher levels of cotinine, as expected. Additionally, we found that when compared to the group receiving 0.8 mg/mL nicotine injections across treatment days, the 24 mg/mL vapor group had slightly higher cotinine levels, and the 12 mg/mL group had slightly lower cotinine levels. The 0.8 mg/kg dose was selected as a positive control as it is considered a moderate injection dose of nicotine that has been shown to produce cotinine levels in rats similar to those seen in in traditional cigarette smokers (Vieira-Brock, Andrenyak et al. 2013, Allen, Wasserman et al. 2019). While the nicotine e-liquid

concentrations used in our experiment produce cotinine levels that were slightly outside of the range produced by the 0.8 mg/kg injection, they did produce cotinine levels comparable to those observed when using other traditional doses and routes of administration (Shoaib and Stolerman 1999, Torres, Gentil et al. 2013, Craig, Zhao et al. 2014), as well as cotinine levels observed in human e-cigarette users (Flouris, Chorti et al. 2013, Marsot and Simon 2016). Others have demonstrated that the use of passive nicotine vapor exposure in rodents also effectively models pharmacokinetics related to behavioral and metabolic changes in human e-cigarette users (Shao, Lopez et al. 2019). However, one limitation to consider with rodent vapor exposure, is the limited control of total nicotine administered to the subjects. Systemic delivery of nicotine in animals via vapor inhalation can indeed fluctuate based on subject size and inhalation rate, as well as location within the vapor exposure chamber. Overall, the translational framework of the described rodent vapor inhalation system can uniquely contribute to the literature on nicotine addiction by providing a reliable and valid animal model of human e-cigarette consumption (Matta, Balfour et al. 2007).

Increased severity of withdrawal behavior observed following nicotine vapor exposure: Increases in physical withdrawal and anxiety-like behavior following cessation of nicotine exposure have been observed across animal species. Somatic behavioral signs of withdrawal have been thoroughly characterized in rodents (Malin et al., 1992), while decreases of open arm entries in an elevated plus maze are commonly assessed as a measure of anxiety-like behavior (Walf and Frye 2007). In our studies, Experiment 1 assessed both somatic signs and open arm entries in an elevated plus maze during precipitated withdrawal from repeated nicotine vapor exposure. The results revealed that rats exposed to 24 mg/mL nicotine vapor yielded more somatic withdrawal signs (e.g., blinks, yawns, writhes etc.) relative to those in all control groups, while rats exposed to 12 mg/mL nicotine vapor yielded more somatic withdrawal signs relative to only those in the

home cage control group. Similar evidence suggests that repeated exposure to nicotine using more traditional routes of administration also leads to increases in somatic withdrawal signs (Skjei and Markou 2003).

Regarding entries to the elevated plus maze open arms, analyses showed that animals receiving 24 mg/mL nicotine vapor had significantly fewer open arm entries than animals that were left in their home cages throughout the experiment. Decreases in open arm entries on an elevated plus maze have previously been observed in mice exposed to other concentration of e-cigarette vapor or to cigarette smoke (Ponzoni, Moretti et al. 2015). Interestingly, a more recent study also showed decreases in open arm entries following nicotine vapor exposure in male and female rats, despite a 3-week abstinence period between last day of vapor exposure and testing (Smith, Kallupi et al. 2020), suggesting that nicotine induced anxiety-like behavior may be a long-lasting characteristic observed during withdrawal. To our surprise, our model produced only modest decreases in open arm entries when tested immediately following day 9 of nicotine vapor exposure. Studies have shown that females display increases in anxiety-like behavior compared to male rats on the EPM and these differences have been suggested to be mediated by ovarian hormones and stress responses (Torres, Pipkin et al. 2015, Flores, Cruz et al. 2020). Similar results have also been observed in female mice following chronic nicotine exposure (Caldarone, King et al. 2008). Therefore, a limiting factor in the current study, possibly explaining the modest effect of anxiety-like behavior observed, was that we did not include female rats in our investigation. However, the EPM has shown to have face validity in its ability to measure anxiety in rodents, reflected by avoidance into open vs closed arms of the maze. In the EPM the animals must choose to enter into dark protected areas or into a lit open vulnerable space. Since rats are nocturnal animals they naturally prefer the darker protected areas. Other behaviors displayed in open arms

such as freezing, immobility and defecation are anxiety-related and increased in open arms compared to closed arms (Pellow et al., 1985; Walf and Frye, 2007).

Increased brain reward thresholds observed following nicotine vapor exposure: Previous research investigating the effects of nicotine exposure and withdrawal on brain reward function using ICSS have administered nicotine using injections, minipumps, infusions, and smoke exposure. In the current study we examine, for the first time, the effects of nicotine vapor exposure and withdrawal on ICSS thresholds. Our analysis identified no significant changes in threshold μAs or threshold percent change from baseline, during repeated nicotine vapor exposure. The findings of the current study however, are not entirely surprising as previous literature examining ICSS thresholds during nicotine exposure have produced mixed findings (Huston-Lyons and Kornetsky 1992, Epping-Jordan, Watkins et al. 1998, Harrison, Gasparini et al. 2002, Chellian, Wilks et al. 2021). The notion of variability in ICSS reward thresholds during nicotine exposure may be due to differences in sample size per group, differences in methodology used between experiments, and the resulting variation in pharmacokinetics. For example, differences in the effects of nicotine delivery via osmotic minipumps on ICSS have been attributed to differences in minipump infusion rates (Epping-Jordan, Watkins et al. 1998, Xue, Behnood-Rod et al. 2020). For our experiment, a larger sample size per group could have helped reduce variability in this task.

Unlike previous work examining ICSS thresholds during nicotine exposure, previous studies using ICSS has consistently shown elevations in reward thresholds in rats during nicotine withdrawal, regardless of administration method (Watkins, Stinus et al. 2000, Kenny, Gasparini et al. 2003, Muelken, Schmidt et al. 2015, Tan, Xue et al. 2019). Such effects have also been displayed following cessation of other drugs of abuse classes that include, but are not limited to, depressants, opiates and stimulants, (Schulteis, Markou et al. 1995, Chartoff, Sawyer et al. 2012,

Holtz, Radke et al. 2015). As with previous reports on the effects of nicotine withdrawal on ICSS, the current study revealed significant changes in thresholds during withdrawal from nicotine vapor exposure. Furthermore, threshold elevations corroborate findings from Experiment 1, where our nicotine vapor model was able to produce somatic signs and anxiety-like behavior during precipitated nicotine vapor withdrawal.

No significant changes in response latencies were observed during or after repeated nicotine vapor exposure in Experiment 2, suggesting that repeated nicotine vapor exposure does not produce immediate or long-term increases in behavioral response time. The effects of nicotine on response latencies in ICSS have produced mixed findings in previous work. For example, one study found that increases in nicotine injection doses (0.125, 0.25, and 0.5mg/kg) produced no effect in response latencies ICSS (Harrison, Gasparini et al. 2002). Alternatively, another more recent study found that injection of nicotine significantly decreased response latencies (Xue, Behnood-Rod et al. 2020). While increases in response latencies should be expected due to nicotine's well defined psychomotor stimulant effect, significant increases in response latencies could represent performance effects resulting from nicotine's stimulant effects, rather than effects of nicotine on brain reward processes. Therefore, another potential limitation of the present study is that locomotor activity was not directly assessed during ICSS training or testing sessions. Assessment of activity during inter-trial intervals can help identify locomotor effects of nicotine that are outside of contingent reward responding (Schaefer and Michael 1986). It is important to note, however, that mixed results on locomotor activity do exist and are likely due to the differences in rat strains, nicotine concentrations, nicotine's biphasic locomotor effect, and route and timeline of administration (Schaefer and Michael 1986, Samaha, Yau et al. 2005, Rauhut, Zentner et al. 2008). For example, repeated intravenous infusions of 30μg/kg nicotine have been

found to produce locomotor sensitization, while repeated peripheral injections of this same dose resulted in tolerance to nicotine's locomotor stimulant effect, suggesting that route of administration plays a role in nicotine's effects on activity (Lenoir, Tang et al. 2013).

Chapter 5: Conclusion

Future directions and conclusions: While the current study showed differences in behavioral measures of withdrawal, it also highlights the need for further investigation of nicotine vapor systems on both the brain and behavior. Future studies should investigate effects of e-cigarette vapor in vulnerable populations, such as females, which display enhanced anxiety-like behavior during nicotine withdrawal (Flores, Cruz et al. 2020). Additionally, a recent study suggests that differences in intake patters of nicotine vapor between males and females may underlie observed differences in cotinine levels (Lallai, Chen et al. 2021). Future work should also include an assessment of the effects of nicotine vapor on adolescents, due to the dramatic increase in e-cigarette use in this population and differences in nicotine withdrawal observed between adolescent and adults (O'Dell, Bruijnzeel et al. 2006, Xue, Behnood-Rod et al. 2020). In addition to investigating the effects of nicotine vapor exposure in specific populations, future studies should continue to investigate the unique pharmacology of nicotine e-liquid and vaping behaviors, including how different concentrations of nicotine, intake patterns, chemical additives (e.g., salt), and chemical reaction products (e.g., nicotyrine) contribute to its effects on the brain and behavior (Sleiman, Logue et al. 2016, Gholap, Kosmider et al. 2020).

The recent increase in e-cigarette use observed in adolescents and young adults is driven by a shift in risk perception related to the negative health effects of e-cigarettes (Gorukanti, Delucchi et al. 2017, Bandi, Cahn et al. 2021). The dramatic increase of e-cigarette use in these populations highlights the immediate need for research on the effects nicotine vapor has on the brain and behavior. This data is needed for the development of important educational campaigns and regulation policies (Collins, Glasser et al. 2019). Additionally, while many traditional cigarette smokers seek smoking cessation through e-cigarettes, studies have not provided clear evidence on

whether these systems are effective for smoking cessation compared to other nicotine replacement therapies (Pokhrel and Herzog 2015, Villanti, Feirman et al. 2018). A more thorough investigation of the marketing, perceptions, and health effects of these novel nicotine delivery systems will be required to better understand their long-term impact on society, and particularly, in vulnerable populations.

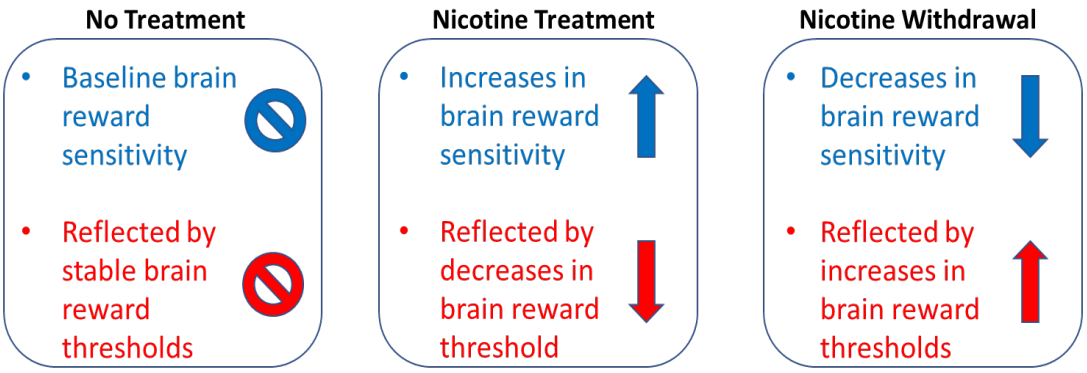

Figure 1. ICSS brain reward threshold changes during nicotine treatment and withdrawal. Text in blue suggest differences in the presumed brain reward sensitivity, while text in red suggest quantifiable changes in thresholds (commonly measured in μA or percent change in μA), following intracranial self-stimulation behavior.

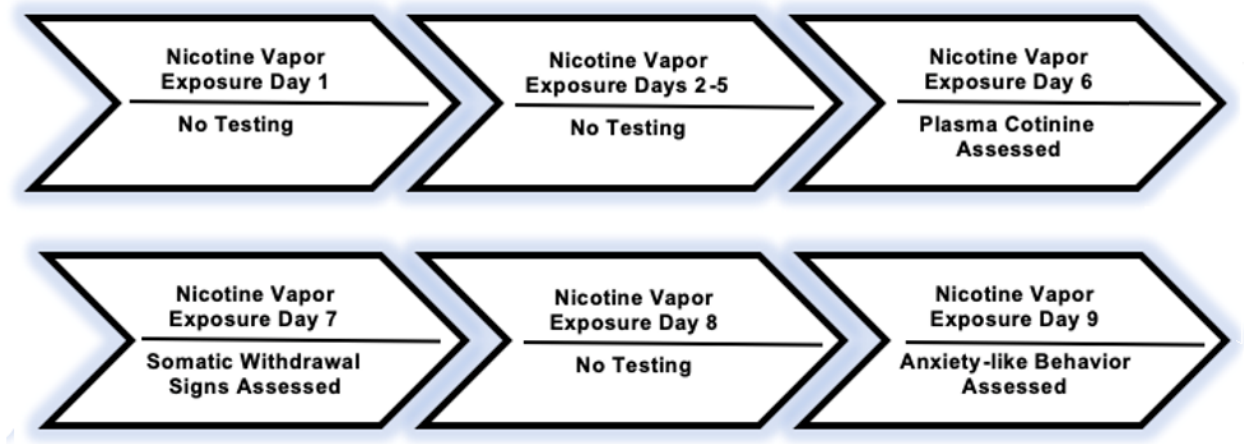

Figure 2. Timeline for Experiment 1

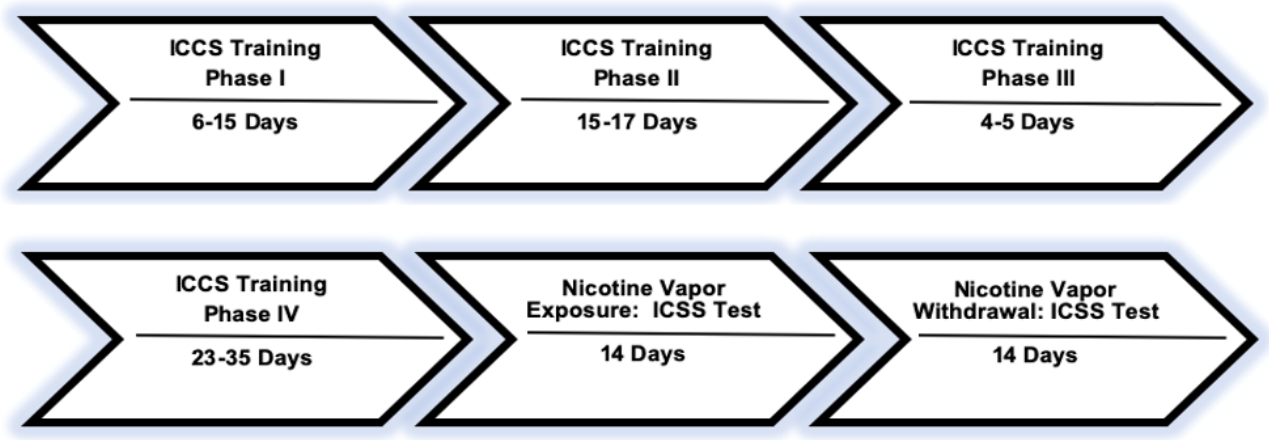

Figure 3. Timeline for Experiment 2

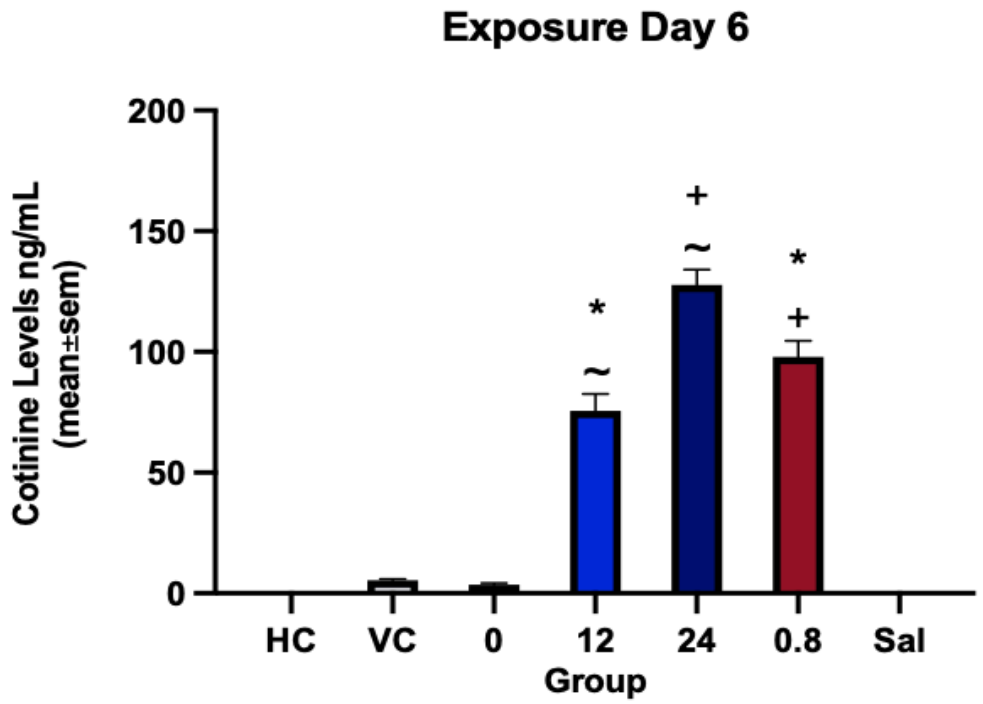

Figure 4. Blood plasma cotinine levels following 6 days of nicotine vapor exposure.

Animals exposed to 12 and 24 mg/mL nicotine vapor, as well as 0.8 mg/kg nicotine injection displayed significantly higher cotinine levels than home cage (HC), vapor chamber (VC), 0 mg/mL (0), and saline injection (Sal) control groups. Asterisks (*) indicate significant difference from all control groups and 24 mg/mL group, plus sign (+) indicates significant difference from all control groups and 12 mg/mL group, and tilde (~) indicates significant difference from all control groups and 0.8 mg/kg nicotine injection group. Critical p-value is 0.05.

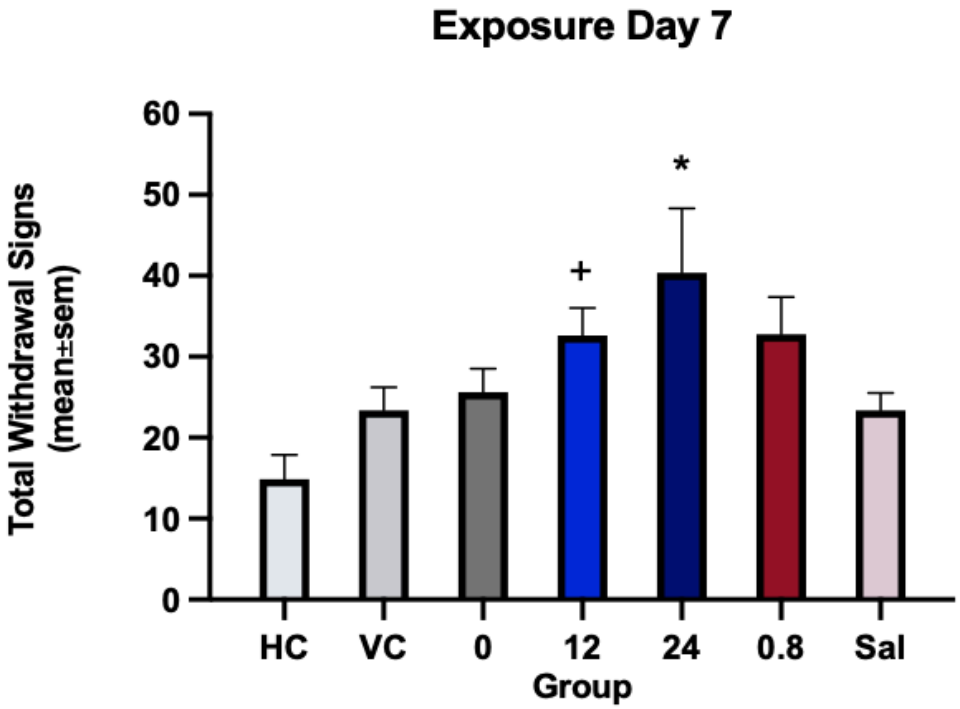

Figure 5. Total withdrawal signs following 7 days of nicotine vapor exposure.

Animals exposed to 24 mg/mL nicotine vapor displayed significantly more somatic signs than home cage (HC), vapor chamber (VC), 0 mg/mL (0), and saline injection (Sal) control groups. Animals exposed to 12 mg/mL nicotine vapor displayed significantly more somatic signs than home cage (HC) control group. Asterisks (*) indicate significant difference from HC, VC, 0, and Sal control group, while plus sign (+) indicates significant difference from HC control group. Critical p-value is 0.05.

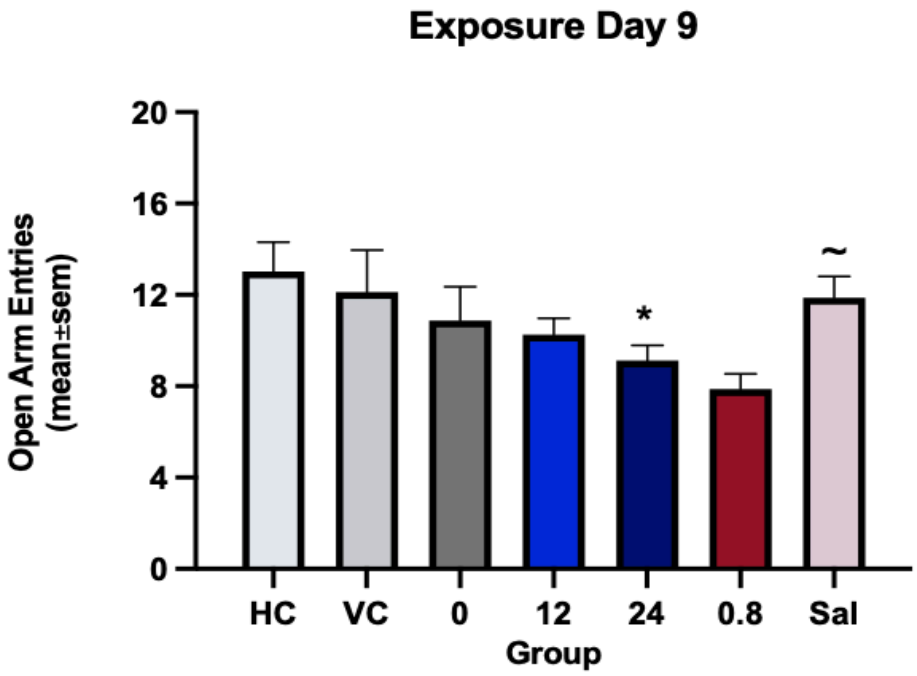

Figure 6. Open arm entries in elevated plus maze following 9 days of nicotine vapor exposure.

Animals exposed to 24 mg/mL nicotine vapor displayed significantly less open arm entries than the home cage (HC) control group. Animals exposed to 0.8 mg/kg nicotine injections displayed significantly more open arm entries than the saline injection (Sal) control group. Asterisks (*) indicate significant difference from HC control group and tilde (~) indicates significant difference from Sal control group. Critical p-value is 0.05.

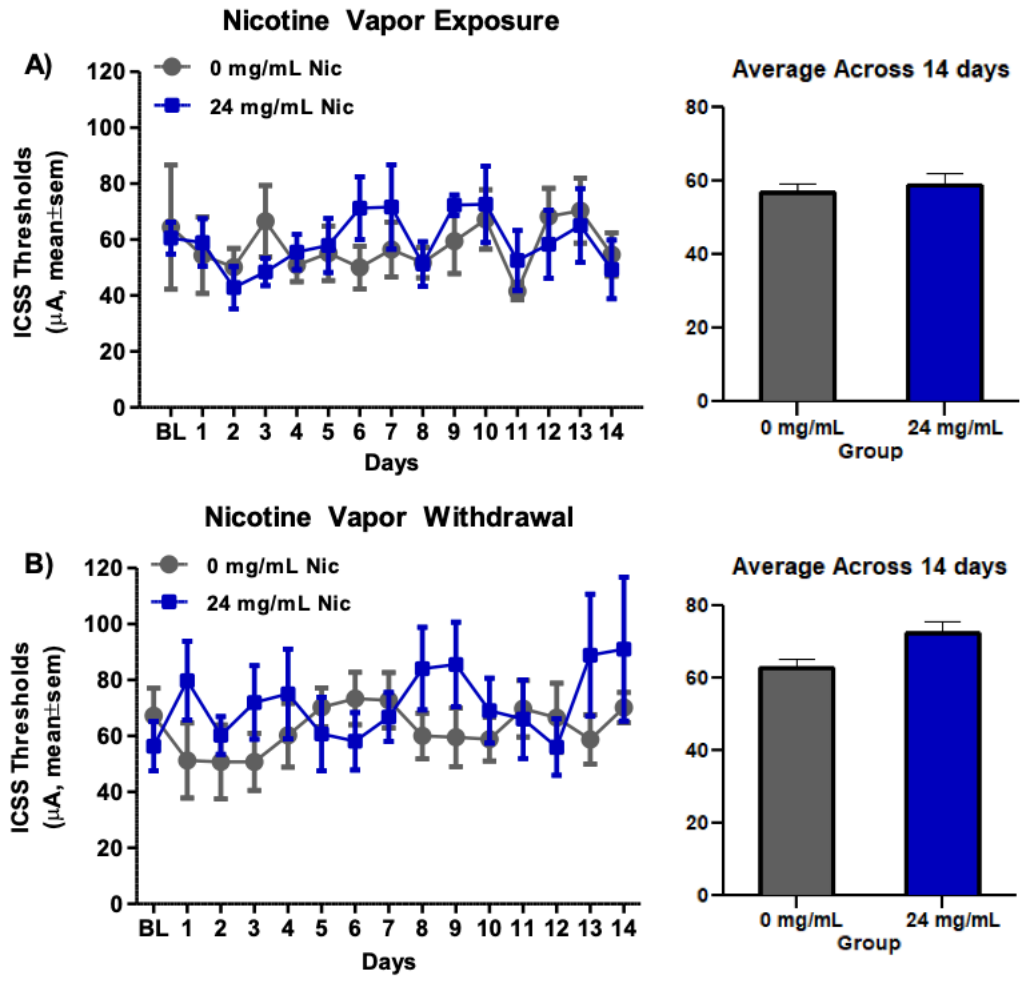

Figure 7. ICSS thresholds in microamperes during nicotine vapor exposure and withdrawal.

No significant differences in reward threshold microamperes were seen across 14 days of vapor exposure (A) or across 14 days of withdrawal (B) when comparing animals exposed to 24 mg/mL nicotine vapor (blue squares) to animals exposed to 0 mg/mL vehicle control (grey circles). Critical p-value is 0.05.

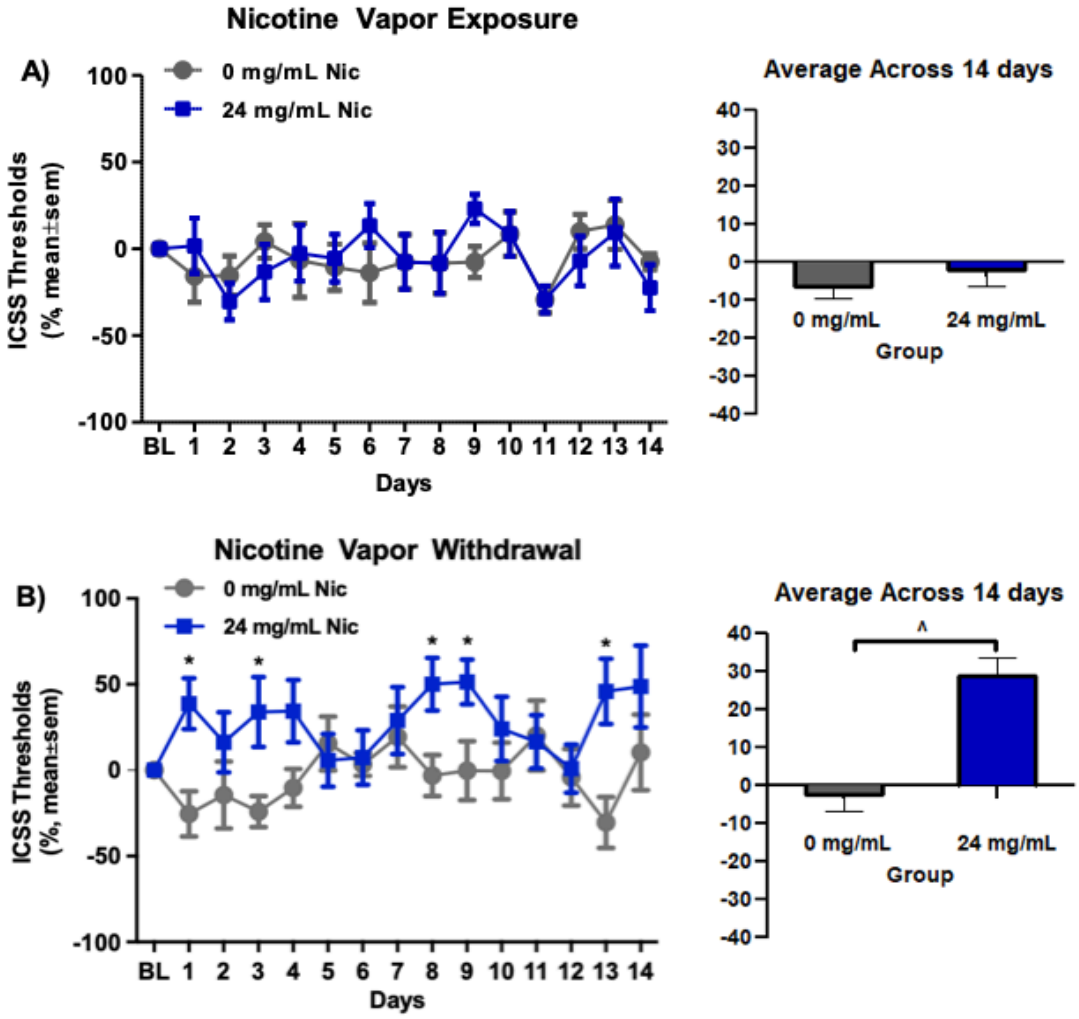

Figure 8. ICSS thresholds as percent change from baseline during nicotine vapor exposure and withdrawal.

A) No significant differences were seen across 14 days of vapor exposure. B) Across 14 days of withdrawal the 24 mg/mL nicotine vapor group (blue squares) displayed a significantly higher threshold percent change from baseline than the 0 mg/mL vehicle control (grey circles). Up arrow (^) indicates significant main effect of treatment group across 14 days nicotine vapor withdrawal and asterisks (*) indicate significant difference between treatment groups on given day. Critical p-value is 0.05.

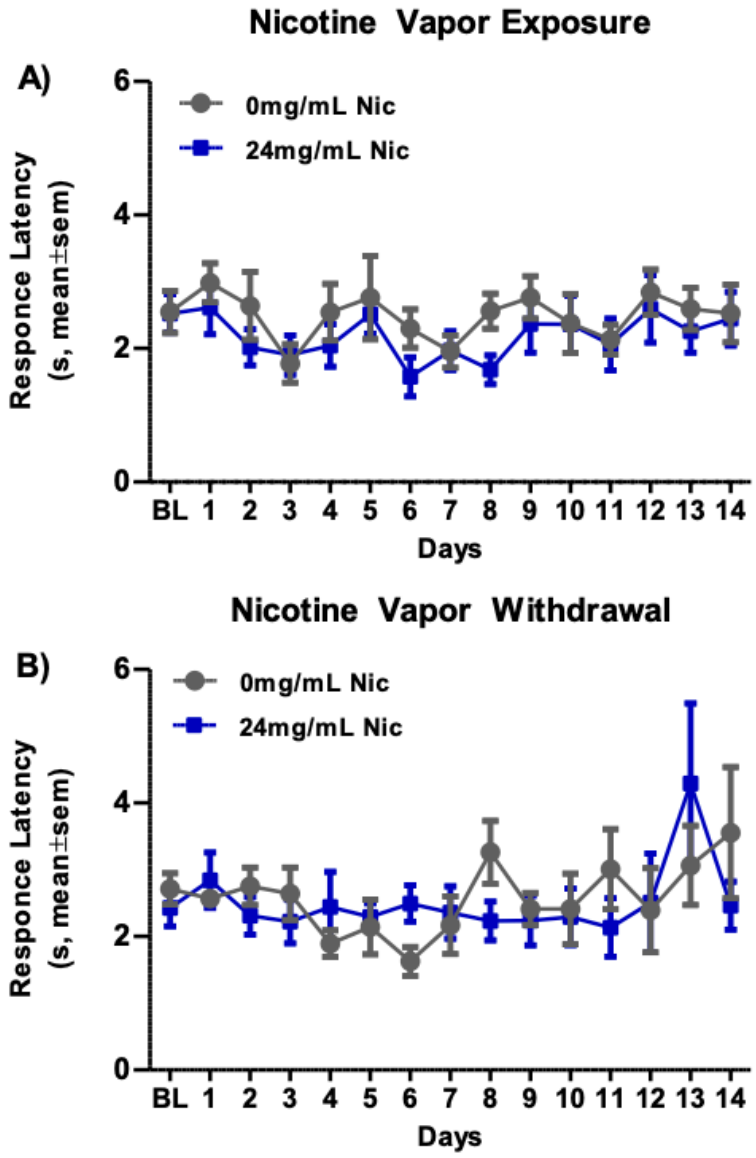

Figure 9. ICSS response latencies in seconds during nicotine vapor exposure and withdrawal.

No significant differences in response latencies were seen across 14 days of vapor exposure (A) or across 14 days withdrawal (B) when comparing animals exposed to 24 mg/mL nicotine vapor (blue squares) to animals exposed to 0 mg/mL vehicle control (grey circles). Critical p-value is 0.05.

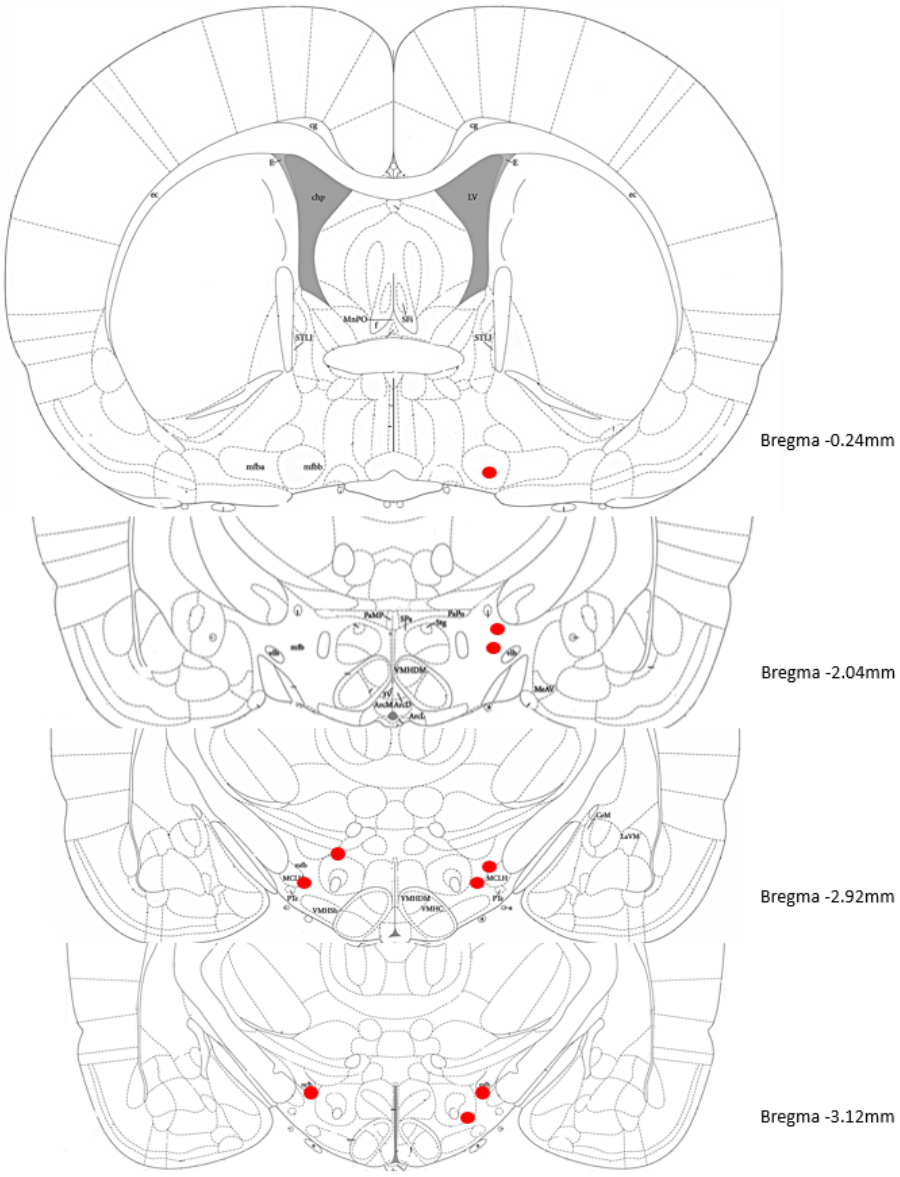

Figure 10. ICSS electrode placement in the MFB.

Gross observations of electrode placement suggest that 7 rats had placement in the MFB, with 3 rats having electrode placements just outside of the MFB (2 from the 0 mg/mL vehicle control and 1 from the 24 mg/mL vapor group).

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Curriculum Vitae

Michelle Martínez was born and raised in El Paso, Texas for the majority of her life. She lived in several cities before the age of 5, including Lexington, Missouri and Las Vegas, Nevada, as her parents sought better employment opportunities following their move to the United States. Michelle attended Mission Early College High School (MECHS), a high school that promoted higher education for lower income families, where she was able to obtain both an Associate of arts degree in psychology and a high school diploma in 2013. Thereafter, she attended the University of Texas at El Paso following graduation and continued to pursue a B.S. in psychology with a minor in biology and graduated in 2016. As an undergraduate, Michelle joined the bilingual cognition lab under the mentorship of Dr. Wendy Francis, which she later continued her research as a graduate student in the Social, Cognitive, and Neuroscience doctoral program in fall 2017. She presented her contributions to dissertations and first-year project at regional conferences such as ARMADILLO and presented her work at Vancouver, British Columbia part of Psychonomics, an international conference. In Spring 2019, she decided to pursue a behavioral neuroscience doctoral degree under the supervision of Dr. Ian Mendez, where she developed her thesis work around the behavioral and neurobiological mechanisms that drive and may contribute to withdrawal in addiction. She has presented her research at several conferences including Biology, Behavior and Chemistry, National Hispanic Science Network and Society for Neuroscience. She also has a publication in *Journal of Experimental Psychology: Learning, Memory, and Cognition* and has an additional manuscript under review.