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Nicotine-free vapor inhalation produces behavioral disruptions and anxiety-like behaviors in mice: Effects of puff duration, session length, sex, and flavor

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Abstract

Electronic-cigarette's (ECIGs) popularity has grown over the last decade and changed the way individuals administer nicotine. Preclinical research is imperative for understanding the addictive properties and health-risks associated with ECIG use; however, there is not a standard dosing regimen used across research laboratories. The main objective was to determine how vapor puff durations, administration session length, and flavored e-liquid alters general and mood-disorder related behaviors while providing a foundation of vapor administration parameters. Adult male and female C57BL/6 mice were exposed to several nicotine-free unflavored vapor puff durations (1, 3, 6, or 10 sec) and vapor administration session lengths (10 and 30 mins) then measured on the following assays: locomotor activity (LMA), tail suspension test (TST), and light-dark test. The effects of mecamylamine and the time-course of vapor-induced depression of LMA also were assessed. Additionally, mice were exposed to flavored (strawberry and adventurers tobacco blend) vapor inhalation and measured on locomotor activity, tail suspension test, and light-dark test. Following both 10 and 30 min vapor administration session, there was a puff duration-dependent decrease in distance traveled, time in center, and rearing. The vapor-induced depression of LMA was not mediated by nicotine or nicotinic acetylcholine receptor (nAChR) activation and lasted 60-90 mins. The 10 sec puff duration produced an anxiogenic-like effect in the light-dark test by decreasing the time spent in the light side. Vapor inhalation did not significantly alter TST behavior. No significant effects of sex or flavor were found. The anxiogenic-like effects of nicotine-free vapor inhalation are concerning as many adolescents vape nicotine-free flavored e-liquid, and there is an association between ECIGs and mood disorders. Additionally, these studies demonstrate that vapor puff duration, but not vapor administration session length, is an important variable to consider during research design as it can become a confounding variable and alter baseline behaviors.

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1. Introduction

Over the last decade, electronic-cigarettes (ECIGs) have changed nicotine drug-taking behaviors in adolescents and young adults (Mirbolouk et al., 2018; Uddin et al., 2020; Wang et al., 2020). One of the main reasons for the increased use of ECIGs is the customizability of the ECIG tank (e.g. vape pens, mods, etc.) and JUUL devices. The ECIG tank devices allow the user to manipulate several variables including, but not limited to, nicotine concentration (nicotine freebase), e-liquid flavor, tank, atomizer, and wattage (Cullen et al., 2018). The JUUL device is discreet as it is shaped like a flash drive, but has several limitations as compared to the ECIG tank devices. For example, the JUUL device uses “pods” that come prefilled with specified nicotine concentrations (3% = 35 mg/ml, 5% = 59 mg/ml, nicotine salt base) and are only available in menthol or tobacco flavor (due to FDA regulations; https://www.fda.gov/media/133880/download). There are reports of people vaping nicotine, marijuana/cannabis, and alcohol (Fuster et al., 2020; MacLean et al., 2017; Pokhrel et al., 2020; Uddin et al., 2020). ECIGs were initially marketed as a safe alternative to smoking; however, the safety profile of ECIGs remains unclear. Preclinical research has become imperative for evaluating the short- and long-term risks associated with ECIG use.

In 2020, over 25% of American teens reported using ECIGs (19.6% high school students; 4.7% middle school students) (Wang et al., 2020). Several reports have found that 22-45% of adolescent ECIG users self-report using a nicotine-free e-liquid (Ambrose et al., 2015; Audrain-McGovern et al., 2019; Cullen et al., 2018; Morean et al., 2018; Pepper et al., 2018; U.S. Department of Health and Human Services, 2016). Because research has shown that a significant proportion of adolescents use ECIGs for the flavors, not nicotine, the main objective of this paper is to gain a better understanding of the impact that nicotine-free e-liquid vapor has on general locomotor and mood-disorder related behaviors. Here we evaluated the effects of nicotine-free, unflavored and flavored, e-liquid on general behavior (locomotor activity and rearing), anxiety-like behaviors (open field time in center and light-dark test), and depressive-like behavior (tail suspension test) in mice. These findings provide insight into how nicotine-free e-liquid may affect mental health in the clinical population.

The second objective of this paper was to determine how vapor puff durations, administration session length, flavored e-liquid, and sex alter behavior to provide a foundation of administration parameters that will allow for vapor drug administration with minimal effects on baseline behavior. The preclinical vapor inhalation field is still in its infancy as the majority of studies were not published until 2014 or later (except for George et al., 2010). Many of these early studies used lab-built or modified anesthesia equipment since commercial manufacturing of vape equipment was not an option at the time (George et al., 2010; Lefever et al., 2017a, b; McGrath-Morrow et al., 2015; Ponzoni et al., 2015). Commercial-built vape machines are now readily available and provide more control over experimental settings such as the puff duration, number of puffs administered per session,

session length, wattage settings, etc. Unlike pharmacological and neuroscience research that uses mg/kg to administer drugs, there is not a standard dosing regimen (i.e. puff duration, session length, e-liquid ratio, wattage, etc.) for the nicotine vapor inhalation field (details can be found in table 1). For example, puff durations for vapor inhalation studies vary from 1 sec to continuous exposure for hours, and the vapor administration session lengths also vary drastically between studies from 5 mins to 14 hours. The present study found that nicotine-free vapor inhalation altered some, but not all behaviors, and these behavioral changes could mask or enhance drug effects, limiting our ability to translate these results to the clinical population. The objectives of this paper directly align with the needs identified by the National Academies of Sciences, Engineering, and Medicine, and National Institutes of Health on ECIGs research (National Academies of Sciences, Engineering, and Medicine, 2018; Walton et al., 2015).

2. Methods

2.1. Subjects and Ethics Statement

Experiments were conducted in 334 C57BL/6 mice (162 males, 172 females). Mice were either purchased from Charles River Laboratories (Raleigh-Durham, NC) or bred at Weber State University with breeder pairs purchased from Charles River Laboratories. No differences were observed between purchased and bred mice. Animals were at least 8 weeks of age at the beginning of testing. All mice were group housed (3-4 per cage) in standard Plexiglass cages (18.5cm x 29.5cm x 12.5cm) in a temperature-controlled vivarium (20-22 °C). Mice had ad libitum access to food and water, except during the experimental procedures. All experiments were conducted during the light-on phase with lights on at 6:00 am (12-hour light/dark cycles). All procedures were approved by the Institutional Animal Care and Use Committee at Weber State University and complied with federal guidelines (Institute of Laboratory and Animal Resources, 2011).

2.2. Drugs

For vapor administration, unflavored, strawberry flavored (10%), or adventurer blend tobacco flavored (10%) nicotine-free e-liquids were used which consisted of a 50:50 oil blend of propylene glycol (PG) and vegetable glycerin (VG) (vaporvapes.com, Sand City, CA, USA). The nonselective nicotinic antagonist, mecamylamine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in a 0.9% saline solution and administered subcutaneously at a volume of 10 ml/kg. Treatment of 1.0 mg/kg mecamylamine was administered 30 mins before vapor inhalation (Honeycutt et al., 2020; Lefever et al. 2017; Walters et al., 2006). All drug and vapor treatments were randomized across mice using a Latin-Square design, and test sessions were separated by at least 48 hrs.

Vapor inhalation administration methods were adapted from published literature (Cooper et al., 2020; Honeycutt et al., 2020; Lefever et al., 2017; Lefever et al., 2019; Montanari et al., 2020). One E-Vape™ delivery system was used to provide vapor administration (Model SVS200, La Jolla Alcohol Research, Inc.). This E-Vape™ system is comprised of a digital interface, a vapor generator, Plexiglass chamber, and an air pump (Fig. 1B). The digital interface controlled the puff duration, inter-puff interval, and session length. The vapor

generator was equipped with a Cloud Beast tank (TFV8) and 0.15Ω atomizer (SMOK, Nanshan District, Shenzhen, China). Battery wattage output was set to 125W for optimal performance designated by SMOK specifications. The hose that was connected to the top of the Cloud Beast tank delivered vapor into a transparent Plexiglass chamber (29 cm × 20 cm × 15 cm) with a 1L/min airflow. Vapor puffs were delivered every 2 mins for either 10 mins (6 puffs total) or 30 mins (16 puffs) based on the experiment. Puff duration (1, 3, 6, or 10 sec) was an independent variable in most studies and varied based on the experiment. Mice were removed from the chamber after the last puff completely cleared the chamber (See Figure 1 for vapor clearance times and E-Vape™ set up).

2.3. Experiments

2.3.1. Vapor chamber clearance times based on puff duration—One trained observer recorded the vapor clearance times for each puff duration (1, 3, 6, and 10 sec) with the observer starting the timer once the puff was complete. Clearance times for each puff duration were collected in quadruplicates.

2.3.2. Comparison of puff duration and session length on LMA—The first experiment was to determine the behavioral effects of puff durations with a 10 min session. This study used a repeated measures design and consisted of 6 sessions that were conducted in 9 male and 9 female mice. Locomotor activity (LMA; distance traveled), time in the center of the open field (time in center; 20cm x 20 cm starting ~4cm from the sides), rearing (vertical counts) variables were tracked using three open field Plexiglass arenas (28cm × 28cm × 20cm) fitted with three 16-beam IR arrays (version 7; Med Associates, Inc., St. Albans, VT). Open field arenas were each enclosed in a sound-attenuating cabinet equipped with a house light and a small fan. Mice received a 60 min habituation session before the start of vapor inhalation studies. For the test sessions, animals were placed in the E-Vape™ chamber and exposed to nicotine-free unflavored vapor for 10 mins at various puff durations (1, 3, 6, or 10 sec), with puffs occurring every 2 mins for a total of 6 puffs. Directly following vapor administration, animals were placed into the open field arenas for 30 mins. Additionally, mice completed a baseline session in which mice were placed into the locomotor activity chambers for 30 mins without being exposed to vapor inhalation.

In the next experiment, we used a new group of mice (8 males and 9 females) following the methods described above; however, we increased the vapor inhalation session time to 30 mins. Mice were exposed to nicotine-free unflavored vapor with 1, 3, and 6 sec puff durations occurring every 2 mins for a total of 16 puffs. The 10 sec puff was excluded from this study due to harmful effects found in 3 mice following a 30 min vapor inhalation session, which included difficulty breathing, lethargy, and a white haze over the eyes of the mice.

2.3.3 Effects of mecamylamine on vapor-induced depression of LMA—This study was conducted in 9 male and 9 female mice with identical methods to the first study that used a 10 min vapor administration session. The following conditions were tested: baseline, saline + 10 sec puff (nicotine-free e-liquid), 1.0 mg/kg mecamylamine + no puff (placed in vape chamber, but no vapor exposure), and 1.0 mg/kg mecamylamine + 10 sec

puff session (nicotine-free e-liquid). Mice were pretreated with 1.0 mg/kg mecamylamine, or saline, 30 mins before vapor administration and placed back into their home cage. After vapor inhalation, mice were placed into the locomotor activity chambers for 30 mins.

2.3.4. Time course of vapor-induced depression of LMA—A time-course experiment was conducted to determine the duration of the vapor-induced depression of LMA (n= 6 males and 6 females). Methods were consistent with previous studies that used a 10 min vapor administration session. Following vapor administration, mice were returned to home cages for designated durations (0, 10, 30, 60, or 100 mins) before being placed into the open field arenas.

2.3.5. Effects of puff duration on anxiety-like behavior using the light-dark test—The light-dark test is a behavioral assay that can be used to measure the anxiolytic- and anxiogenic-like effects in rodents. This behavioral assay evaluates the tendency of mice to explore a novel environment while avoiding aversive stimuli such as a bright light over an open field (Crawley and Goodwin, 1980). Increased time spent in the dark side is interpreted as an anxiogenic-like effect. Dark box inserts (28cm x 15cm x 21cm with a 5.1cm x 5.4cm arched opening in the center; Med-Associates) were placed on the right side of the open-field arena. An LED array, consisting of forty-eight 5050 SMD LEDs, was mounted 36.2 cm above the left side of the open field arena producing 650 lux of illuminance (Govee, Shenzhen, Nanshan District, China). Each 5 min test session started by placing a mouse into the outer front corner of the dark side (Saavedra et al., 2020). Duration in the light side, latency to first light zone entry, and entries into the light side were collected using Activity Monitor software (version 7: Med-Associates). This light-dark study evaluated a puff duration effect curve consistent with the first LMA study using a 10 min vapor administration session. For this study, a between-subjects design was used to limit habituation to the testing arena and bright light. Mice in the control group were placed in the E-Vape™ chamber for 10 mins but did not receive vapor administration (n = 8-11 per group).

2.3.6. Effects of puff duration on depressive-like behaviors using the tail suspension test (TST)—TST methods were consistent with those previously described (Saavedra et al., 2020). In brief, mice were suspended by their tails, with paper adhesive tape, from a metal bar attached to a retort stand at approximately 35 cm above the countertop, for a test session duration of 6 min. Each test session was videotaped. Scoring of immobility time was completed by two trained observers blinded to the treatment. The scores were averaged between the two observers. A mouse hanging motionless without showing escape-related behaviors was defined as immobile. Escape-related behaviors were defined as attempting to climb their tail, a running motion using all limbs, or strong shaking of the body. Subtle movements involving only the forelimbs were not counted as escape-related behaviors. The first TST study evaluated a puff duration effect curve consistent with the light-dark study. Mice in the control group were placed in the E-Vape™ chamber for 10 mins and did not receive vapor administration (n = 6-11 per group).

2.3.7. Effects of flavored e-liquid on LMA, light-dark test, and TST—Three flavor experiments were conducted to determine how flavor additives might alter the

behavioral effects of vapor inhalation. A positive/natural flavor (Strawberry), a negative/non-natural flavor (Adventurer Blend Tobacco), and nonflavored e-liquid were used. For the LMA study, methods were consistent with previous studies that used a 10 min vapor administration session and varied puff duration (n = 8-9 female mice per group). Only female mice were used because no major sex differences were observed in the first set of LMA studies. The female data from experiment 1 (Fig. 2A-C) were used to compare the effects of flavors on LMA. The light-dark test and TST were used to determine if the flavor additives would enhance, or attenuate, the anxiogenic-like effects of vapor inhalation or produce a depressive-like behavior, respectively (n = 10-11 per group, mixed-sex). Based on the results from the first light-dark test and TST, mice received a 10 sec puff every 2 mins for a 10 min administration session. Control groups were placed into the E-Vape™ chamber for 10 mins and did not receive vapor administration.

2.4. Data Analysis

For the sex comparison and flavor LMA studies, a two-way mixed factor analysis of variance (ANOVA) was used, with “puff duration” or “treatment condition” as within-subjects factors and “sex” or “flavor” as between-subjects factors. For the light-dark test and TST sex comparison studies, two-way between-subjects ANOVAs were used, with “sex” and “puff duration” as between-subjects factors. One-way between-subjects ANOVAs were used for the chamber clearance, time course, and light-dark test and TST flavor studies. All significant ANOVAs were followed by a Tukey post hoc test (significance set at $p<0.05$). Data were analyzed in GraphPad Prism version 8.0.2 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Vapor chamber clearance times based on puff duration

Vapor chamber clearance time is rarely reported in preclinical studies; however, this variable is important as it determines how long an animal is exposed to the vapor and/or drug. Puff duration significantly increased the time needed for the vapor to completely clear the passive vapor chamber, with the 10 sec puff taking an additional 90 sec to clear the vapor as compared to the 1 sec puff (Fig. 1A; $F(3, 12) = 315.20$, $p < 0.001$).

3.2. Effects of puff duration and session length on LMA

Figure 2 shows the effects of 10 and 30 min vapor administration sessions on LMA. Following the 10 min vapor administration session (Fig 2, top panels), all puff durations significantly decreased distance traveled for female mice ($p < 0.01$); whereas only the 10 sec puff decreased distance traveled for the male mice ($p < 0.05$) (main effect of puff duration, $F(4, 64) = 15.40$, $p < 0.001$; no main effect of sex, $F(1, 16) = 1.28$, $p = 0.28$; interaction, $F(4, 64) = 2.85$, $p < 0.05$; Fig. 2A). Female mice had a puff duration-dependent decrease in time in center as compared to vehicle ($p < 0.05$). The male mice had a significant increase in time in center after the 1 sec puff ($p < 0.01$), however, male mice had a significant decrease in time in center at all other puff durations as compared to the 1 sec puff ($p < 0.05$) (main effect of puff duration, $F(4, 64) = 10.24$, $p < 0.001$; no main effect of sex, $F(1, 16) = 0.57$, $p = 0.46$; interaction, $F(4, 64) = 4.50$, $p < 0.01$; Fig. 2B). The 6 and 10 sec puffs significantly

decreased rearing in female mice as compared to baseline rearing ($p < 0.05$). The 1 sec puff increased rearing in male mice; however, the 3, 6, and 10 sec puffs all decreased rearing as compared to the 1 sec puff ($p < 0.05$) (main effect of puff duration, $F(4, 64) = 9.21$, $p < 0.001$; no main effect of sex, $F(1, 16) = 2.84$, $p = 0.11$; interaction, $F(4, 64) = 3.54$, $p = 0.01$; Fig. 2C).

Following the 30 min vapor administration session (Fig 2, middle panels), the 6 sec puff significantly decreased distance traveled regardless of sex (main effect of puff duration, $F(3, 45) = 4.41$, $p < 0.01$; no main effect of sex, $F(1, 15) = 0.84$, $p = 0.37$; no interaction, $F(3, 45) = 1.92$, $p = 0.14$; Fig. 2D). All puff durations significantly decreased time in center (main effect of puff duration, $F(3, 45) = 14.83$, $p < 0.001$; no main effect of sex, $F(1, 15) = 0.01$, $p = 0.91$; no interaction, $F(3, 45) = 0.46$, $p = 0.71$; Fig. 2E). Mice had a significant decrease in rearing after the 3 and 6 sec puff durations (main effect of puff duration, $F(3, 45) = 9.01$, $p < 0.001$; no main effect of sex, $F(1, 15) = 0.31$, $p = 0.59$; no interaction, $F(3, 45) = 0.67$, $p = 0.58$; Fig. 2F).

3.3 Effects of mecamylamine on vapor-induced depression of LMA

Previous studies have found that high doses of nicotine (24-30 mg/ml) vapor inhalation decreased LMA and this decrease in LMA is reversed by the nicotine antagonist mecamylamine (Lefever et al., 2017). A mecamylamine antagonist study was conducted to determine if inadvertent nicotine (i.e. contamination from vendor) was contributing to the vapor-induced depression of LMA. Under control conditions (i.e. saline pretreatment) the 10 sec puff significantly decreased distance traveled, time in center, and rearing in male and female mice (Fig 2, bottom panels). Pretreatment with 1.0 mg/kg mecamylamine did not alter the significant effects of the 10 sec puff, indicating that nicotine was not contributing to the effects of vapor inhalation on LMA (Distance Traveled [Fig. 2G]: main effect of treatment, $F(3, 48) = 26.29$, $p < 0.001$; main effect of sex, $F(1, 16) = 13.63$, $p < 0.01$; no interaction, $F(3, 48) = 2.09$, $p = 0.118$; Time in Center [Fig. 2H]: main effect of treatment, $F(3, 48) = 24.86$, $p < 0.001$; main effect of sex, $F(1, 16) = 6.97$, $p = 0.01$; interaction, $F(3, 48) = 5.05$, $p < 0.01$; Rearing [Fig. 2I]: main effect of treatment, $F(3, 48) = 15.25$, $p < 0.001$; main effect of sex, $F(1, 16) = 0.17$, $p = 0.68$; interaction, $F(3, 48) = 1.34$, $p = 0.27$).

3.4. Time course of vapor-induced depression of LMA

Figure 3 shows the time course of effects for the 10 sec puff on distance traveled, time in center, and rearing. No sex differences were found; therefore, the data for the male and female mice were combined for all analyses. The vapor inhalation depressed of distance traveled at 0, 10 and 30 mins after the completion of the vapor administration session ($F(5, 55) = 9.83$, $p < 0.001$; Fig. 3A), while the vapor inhalation depressed rearing at 0, 10, 30 and 60 mins after administration ($F(5, 55) = 8.73$, $p < 0.001$; Fig. 3C). Although there was a trend toward significance, vapor inhalation failed to significantly alter time in center ($F(5, 55) = 2.22$, $p = 0.065$; Fig. 3B).

3.5. Effects of puff duration on anxiety-like behavior

We used the light-dark test to further evaluate the effects of vapor inhalation on anxiety-like behaviors. The light-dark test is a behavioral assay that can be used to measure the

anxiolytic- and anxiogenic-like effects in rodents. This assay evaluates the tendency of mice to explore a novel environment while avoiding aversive stimuli such as a bright light over an open field with increased time spent in the dark side is interpreted as an anxiogenic-like effect. Figure 4 shows the effects of vapor inhalation on anxiety- and depressive-like behaviors in male and female mice. The light-dark test (top three panels) was used to further evaluate the anxiogenic-like effect of nicotine-free e-liquid found in the open field test (i.e. decreased time in center). The 10 sec puff significantly decreased the time spent in the light side ($p < 0.05$) and the latency to first entry into the dark side ($p < 0.001$) (Additionally, treatment with the 6 and 10 sec puff decreased the overall number of entries into the light side ($p < 0.01$) (Duration in Light Side [Fig. 4A]: main effect of puff duration, $F(4, 80) = 4.47$, $p < 0.01$; no main effect of sex, $F(1, 80) = 2.59$, $p = 0.11$; no interaction, $F(4, 80) = 0.17$, $p = 0.95$; Latency to First Entry into Light Side [Fig 4B]: main effect of puff duration, $F(4, 80) = 8.23$, $p < 0.001$; no main effect of sex, $F(1, 80) = 1.76$, $p = 0.18$; no interaction, $F(4, 80) = 0.46$, $p = 0.77$; Entries into the Light Side [Fig. 4C]: main effect of puff duration, $F(4, 80) = 8.60$, $p < 0.001$; no main effect of sex, $F(1, 80) = 1.55$, $p = 0.21$; no interaction, $F(4, 80) = 2.13$, $p = 0.08$).

3.6. Effects of puff duration on depressive-like behaviors

TST was used to further evaluate the effects of puff duration on mood disorder-related behaviors (i.e. depressive behaviors), and to determine if puff duration produced a global depression of behaviors or if it was exclusive to specific types of behaviors (Figure 4, bottom panel). All puff durations tested failed to alter immobility time in TST (no main effect of puff duration, $F(4, 69) = 0.72$, $p = 0.58$; no main effect of sex, $F(1, 69) = 1.08$, $p = 0.30$; no interaction, $F(4, 69) = 0.29$, $p = 0.89$; Fig. 4D).

3.7. Effects of flavored e-liquid on LMA, light-dark test, and TST

We conducted flavored e-liquid experiments to determine how flavor additives might alter the behavioral effects found in LMA, light-dark test, and TST. Figure 5 shows the effects of flavored e-liquid on LMA, anxiety-like and depressive-like behaviors. The effects of puff duration (unflavored and flavored) were consistent with the behavioral effects found in the sex comparison experiments such that higher puff durations depressed LMA, produced an anxiogenic-like effect in the light-dark test, and did not alter immobility time in TST. The strawberry flavor produced significantly smaller reductions of distance traveled (Fig. 5A), time in center (Fig. 5B), and rearing behavior (Fig. 5C) as compared to the unflavored and tobacco flavored e-liquid (Distance Traveled [Fig. 5A]: main effect of puff duration, $F(4, 92) = 36.46$, $p < 0.001$; main effect of flavor, $F(2, 23) = 3.40$, $p = 0.05$; no interaction, $F(8, 92) = 1.05$, $p = 0.41$; Time in Center [Fig. 5B]: main effect of puff duration, $F(4, 92) = 10.95$, $p < 0.001$; main effect of flavor, $F(2, 23) = 5.49$, $p < 0.05$; no interaction, $F(8, 92) = 1.04$, $p = 0.41$; Rearing [Fig. 5C]: main effect of puff duration, $F(4, 92) = 21.46$, $p < 0.001$; main effect of flavor, $F(2, 23) = 8.54$, $p < 0.01$; no interaction, $F(8, 92) = 1.28$, $p = 0.27$).

The 10 sec puff of flavored and unflavored e-liquid vapor inhalation produced similar significant decreases in the time spent in the light side ($F(3, 36) = 5.35$, $p < 0.01$; Fig 5D), did not significantly alter the latency to first light side entry ($F(3, 36) = 2.52$, $p = 0.07$; Fig. 5E) or number of entries ($F(3, 36) = 2.84$, $p = 0.05$; Fig. 5F). The 10 sec puff of flavored and

unflavored e-liquid vapor inhalation did not significantly alter immobility time in TST ($F(3, 39) = 0.36$, $p = 0.78$; Fig. 5G).

4. Discussion

Although ECIGs have been available in the United States for approximately 15 years, the preclinical vapor inhalation field is relatively new and continues to evolve as new technology is introduced in the field. There is a need to understand the basic effects of vapor inhalation as well as the effects of drug administration via vapor inhalation. This is the first study to systematically evaluate the effects of nicotine-free vapor puff duration, administration session length, sex, and e-liquid flavors on rodent behaviors. There were three main findings. First, nicotine-free vapor inhalation produced anxiety-like effects (i.e. anxiogenic effect) in two behavioral assays (light-dark test and reduced time in center in LMA). There has been a long-standing positive association between combustible cigarette use and mood disorders (Fluharty et al., 2017), and this association is also present among ECIG users (Bianco, 2019; Grant et al., 2019; Hefner et al., 2019). This finding is a concern as 22-45% of adolescents use nicotine-free e-liquid in their ECIGs and vapor inhalation might exacerbate feelings of anxiety in individuals with a mood disorder (Ambrose et al., 2015; Audrain-McGovern et al., 2019; Morean et al., 2018; Pepper et al., 2018; U.S. Department of Health and Human Services, 2016). It remains unclear how the addition of nicotine or repeated administration would alter the anxiogenic-like effects produced by nicotine-free vapor inhalation. Future studies need to determine how e-liquids containing low and high nicotine concentrations alter the anxiogenic-like and depression of LMA produced by vapor inhalation as nicotine generally produces an inverted U-shaped curve in behavioral assays due to its steep dose response curve. For example, systemically administered nicotine at low, but not high, doses have been shown to produce anxiolytic-like effects in mice and may attenuate the anxiogenic-like effects of vapor inhalation (Anderson and Brunzell, 2012; Anderson and Brunzell, 2015).

Second, we found that longer puff durations disrupt some, but not all, behaviors. The behavioral effects of puff duration appear to be behavior-specific and not an overall depression of motor control. For example, vapor inhalation decreased LMA and rearing but failed to influence immobility time in TST. Moreover, the time course of vapor-induced depression of LMA and rearing differed significantly as LMA was restored after 30 min, while rearing did not fully recover until 100 mins. We hypothesized that longer administration session lengths would exacerbate the vapor-induced depression of LMA because the mice received three times more vapor puffs (16 vs 6), however, this was not the case, indicating that the depression of LMA has reached a floor effect. The 30 min vapor administration session may have produced a longer time course for the vapor-induced depression of LMA; however, that was not measured in this study. The mechanism responsible for the decrease in LMA and anxiogenic-like effects is not clear. We have shown that nicotinic acetylcholine receptor (nAChR) activation is not responsible for these behavioral effects as mecamylamine failed to block the decrease in LMA following the 10 sec puff of vapor (Fig. 2A-C). Mecamylamine reverses nicotine (24 and 30 mg/ml) vapor induced decreases in LMA indicating that the nicotine-free vapor induced depression of LMA in the present study is produced through a novel mechanism (Lefever et al., 2017).

Third, the strawberry and tobacco flavored e-liquids produced depression of LMA and anxiety-like effects similar to unflavored e-liquid. Although there was a main effect of flavor in the LMA studies, these effects were likely a result of differences in baseline behavior for the strawberry flavor groups as the shapes of the puff duration curves are similar. Moreover, the addition of flavor in the e-liquids did not significantly enhance or attenuate the behavioral disruption produced unflavored e-liquid in any of the behavioral studies. This is in direct contrast to a report by Cooper et al., 2020, that found a green apple and menthol flavor significantly increased nicotine vapor self-administration as compared to unflavored nicotine vapor, and nicotine-free green apple flavor was able to maintain vapor self-administration responding. Clinical studies also report that flavors increase the reinforcing properties of ECIGs as well as the amount of nicotine consumed (St.Helen et al., 2017; Goldenson et al., 2016). Furthermore, injected green apple (farnesol) and menthol flavors enhance nicotine's reinforcing properties via upregulation of nAChRs and dopaminergic neuron excitability (Avelar et al., 2019; Henderson et al., 2017). Taken together, flavor effects are behavior-dependent such that they are likely to alter behaviors that assess the rewarding properties or drug-seeking behaviors (i.e. conditioned place preference and self-administration) over other behaviors (i.e. locomotor activity, anxiety-like, depressive-like, etc.).

As the preclinical vapor inhalation field moves forward, there is a need for standardization of dosing regimens across research groups to allow for replication between laboratories. This can be difficult as research groups are using lab-built or modified anesthesia equipment (plans for lab-build vape equipment can be found via Frie et al., 2020; Hilpert et al., 2019) or different commercial vendors (machines are available from La Jolla Alcohol Research Inc, Scireq [inExpose], e~Aerosols, and Teague enterprises). Unfortunately, the lab-built machines are sometimes limited in their ability to control dosing parameters and the commercial machines vary widely in the method of delivery (nose only vs whole-body), chamber size, and ability to adjust parameters. Table 1 provides information on puff duration, administration session length, dosing regimen, type of equipment for published vapor inhalation studies, and main outcome for all nicotine vapor inhalation studies available at the time this paper was submitted. The puff duration for vapor inhalation studies varies from 1 sec to 10 sec with the most common puff duration being 10 secs; however, several studies did not provide information on puff duration details or used continuous exposure for hours. Vapor administration session lengths also vary drastically between studies (5 mins to 14 hrs). One of the most important variables in vapor inhalation studies is the total vapor exposure time, or vapor clearance time after each puff, as this determines how long rodents are exposed to the drug. Unfortunately, total vapor exposure time and clearance time are rarely reported. Standardizing total vapor exposure or vapor clearance times would provide consistency across laboratories and standardize drug dosing for animals. Puff duration, wattage, and chamber size all contribute to total vapor exposure and/or vapor clearance time, and these parameters can be adjusted within each laboratory to meet a standard once established. Drug exposure studies would be required for each drug class to determine the total vapor exposure required to produce brain and plasma concentrations consistent with systemic injections in rodents and/or clinical populations. Although this would be time-consuming and difficult, it is important for moving the vapor inhalation field forward.

Currently, the variability in vapor administration parameters makes it difficult to compare results between laboratories and will likely result in replication issues.

Conclusion

Although many perceive ECIGs are a safer alternative to combustible cigarettes, we demonstrate that ECIGs (vapor inhalation) are not harmless, as nicotine-free vapor inhalation produced anxiety-like behaviors, as well as disrupted general locomotor behaviors (Amrock et al., 2016; Cooper et al., 2018; Pericot-Valverde et al., 2017). As many consumers of nicotine-free ECIGs are adolescents, long-term effects of ECIGs on their mental health remains unclear. Additionally, the present study determined that puff duration is an important factor in vapor inhalation (vaping) studies. Specifically, puff duration is directly related to the time it takes for the vapor to be cleared from the chamber (Figure 1A), therefore, puff duration is a key determinant in overall vapor exposure of subjects. Moreover, appropriate control groups (i.e. no vapor exposure and/or nicotine-free vapor exposure) are necessary for determining drug effects, as unadulterated vapor inhalation can be sufficient to alter measured behaviors. Methodological inconsistencies can lead to uncertainty in interpreting results and identifying critical variables, such as total vapor exposure or puff duration. Increasing standardization of methodology and measurement of important variables will increase generalizability and translational value of preclinical vapor inhalation (vape) research throughout the field.

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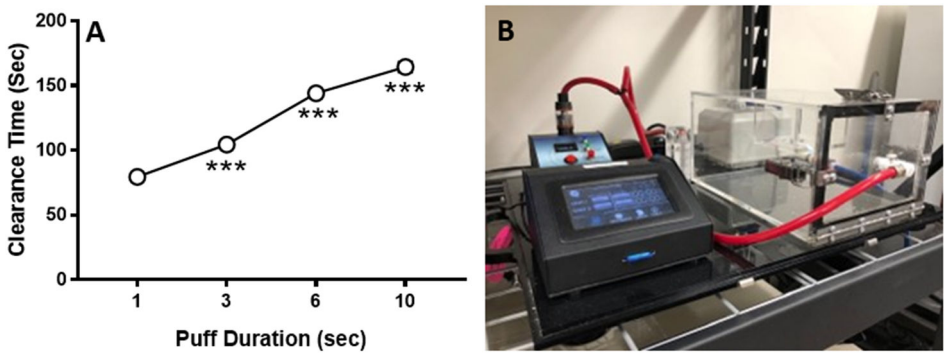

Figure 1.

Vapor chamber clearance times and E-Vape™ delivery system set up. (A) Vapor chamber clearance times based on puff duration. (B) The E-Vape™ system is comprised of a digital interface, a vapor generator, Plexiglass animal chamber, and an air pump. The digital interface controlled the puff duration, inter-puff interval, and session length. The vapor generator was equipped with a Cloud Beast tank (TFV8) and 0.15Ω atomizer. A significant ANOVA was followed by Tukey posthoc test. Filled points represent significant effect versus 1 sec puff ($p < 0.001$). All data show mean ± SEM ($n = 4$ per puff duration)

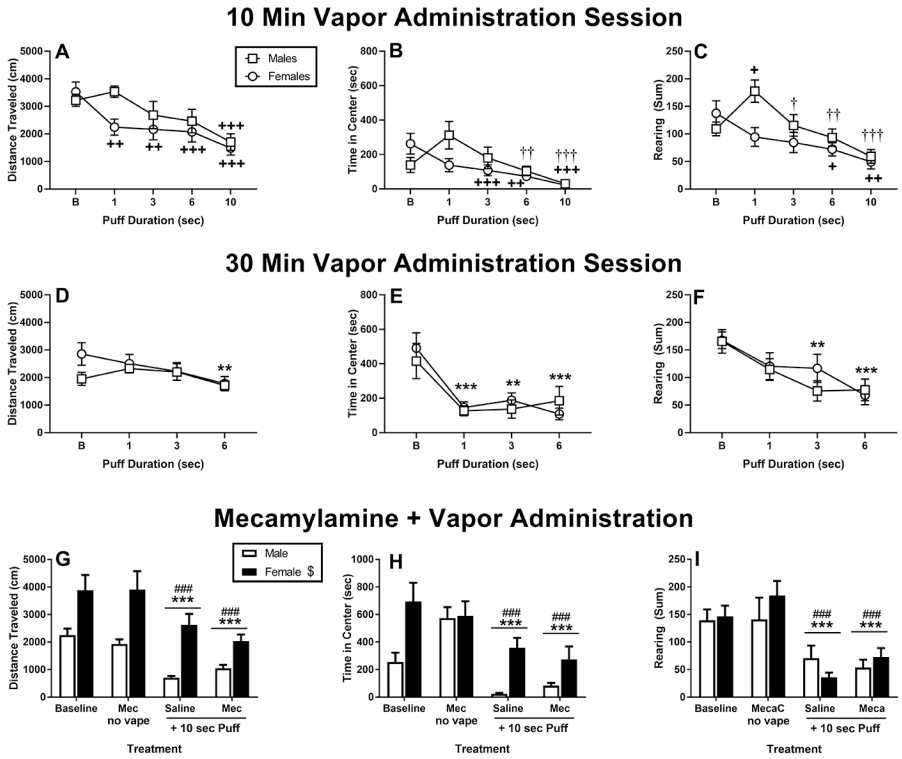

Figure 2.

Effect of vapor puff duration on locomotor activity based on vapor administration session length. Top panels show the effects of sex and puff duration on distance traveled (A), time in center (B) and rearing behavior (C) following a 10 min vapor administration session (6 puffs total). Middle panels show the effects of sex and puff duration on distance traveled (D), time in center (E) and rearing behavior (F) following a 30 min vapor administration session (16 puffs total). Bottom panels show the effects of sex and mecamylamine on vapor-induced depression of LMA following a 10 min vapor administration session (6 puffs total). All significant ANOVAs were followed by a Tukey post hoc test. +$p < 0.05$, ++$p < 0.01$, +++$p < 0.001$ represent significant interaction effect from baseline. †$p < 0.05$, ††$p < 0.01$, †††$p < 0.001$ represent significant interaction effect from 1 sec. **$p < 0.01$, ***$p < 0.001$ represent significant main effect of puff duration versus baseline. ###$p < 0.001$ represent significant main effect of treatment versus 1.0 mg/kg mecamylamine + no vapor inhalation. \$$p < 0.05$ represent significant main of sex (G, H). All data show mean ± SEM for 8 to 9 mice per group.

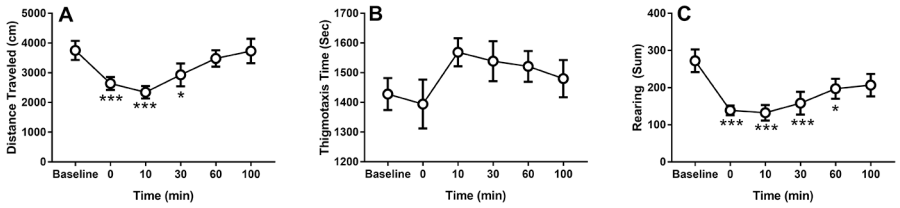

Figure 3.

Time course of effects produced by 10 sec vapor puff on locomotor activity. The 10 sec puff decreased distance traveled (A), time in center (B) and rearing (C). Time (min) represents the time after the 10 min vapor administration session until mice were placed in the open field arena. All significant ANOVAs were followed by a Tukey post hoc test. **$p < 0.01$, ***$p < 0.001$ represent significant main effect of puff duration versus baseline. All data show mean ± SEM for 12 mice (male = 6; female = 6).

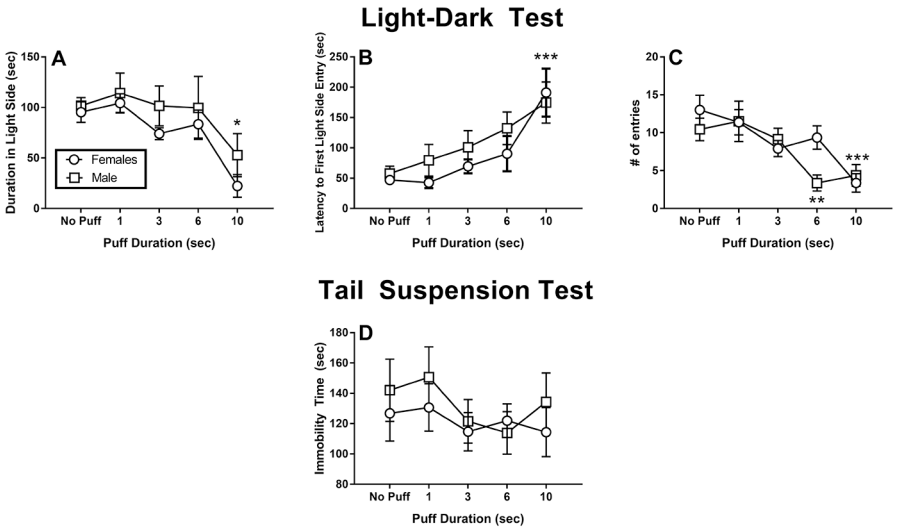

Figure 4.

Effects of vapor puff duration on light-dark test and tail suspension test. Top panels show the effects of sex and puff duration on duration in the light side (A), latency to first enter the light side (B), and number of entries into the light side (C). Bottom panel shows the effects of sex and puff duration on immobility time from TST (D). All significant ANOVAs were followed by a Tukey post hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ represent significant main effect of puff duration versus no vapor puff control. All data show mean ± SEM for 6-11 mice.

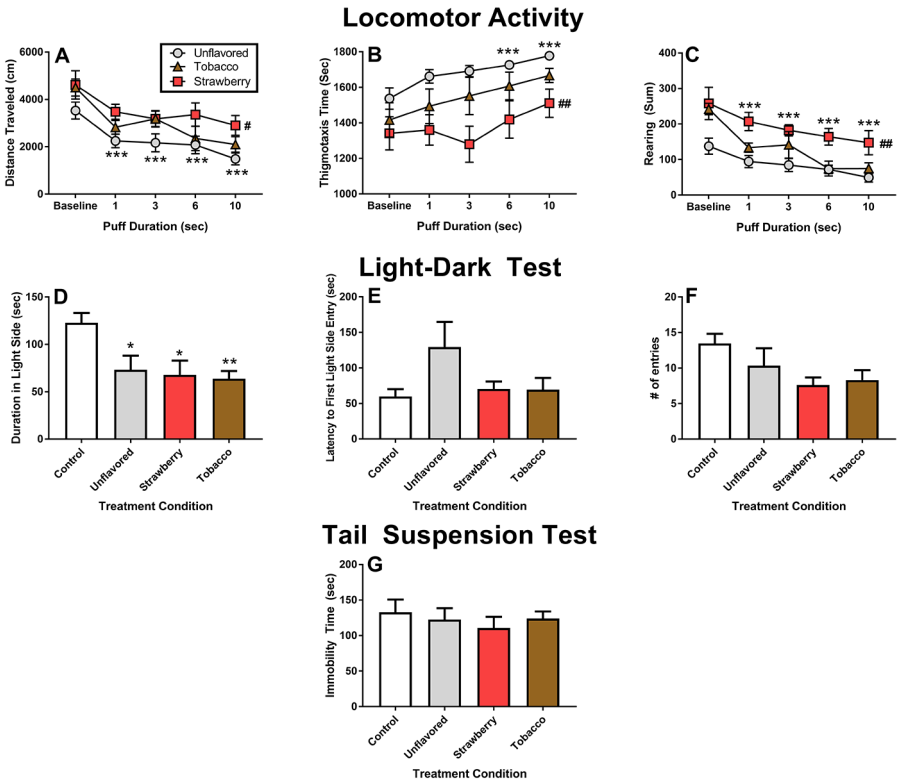

Figure 5.

Effects of flavored e-liquid on LMA, light-dark test, and tail suspension test. Top panels show the effects of flavored vapor and puff duration on distance traveled (A), time in center (B), and rearing behavior (C). Middle panels show the effects of flavored vapor on duration in the light side (D), latency to first enter the light side (E), and number of entries into the light side (F). Bottom panel shows the effects of flavored vapor on immobility time from TST (G). All significant ANOVAs were followed by a Tukey post hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ represent significant main effect versus no vapor puff control. #$p < 0.05$, ##$p < 0.01$ represent significant main effect of flavored vapor versus unflavored and tobacco flavored vapor. All data show mean ± SEM for 8-11 mice.

Table 1.

Dosing parameters for nicotine vapor inhalation studies

Puff Duration	Administration Session Length	Dosing Regimen	Vehicle (ratio)	Equipment	Main outcome Measured	Citation
N/A (continuous)	2, 8, or 14 hr	Continuous during session for 1 or 7 days	N/A	Commercially purchased (La Jolla Alcohol Research, Inc.)	Behavior	George et al., 2010
N/A (continuous)	12 hr	Continuous for session for 12 days	N/A	Commercially purchased (La Jolla Alcohol Research, Inc.)	Behavior	Gilpin et al., 2014
8 ml *	30 min	Three sessions per day for seven weeks	Not provided	Lab built; modified rodent ventilator Ugo Basile, (model 7025)	Behavior	Ponzoni et al., 2015
6 sec	20 min	6-sec puffs every 15 sec	PG (100%)	Lab built; modified Masterflex peristaltic pump (model 7523-80)	Behavior	Smith et al., 2015
10 sec	13 min	Multiple 5 min exposures to 10 sec puff	PG:VG (50:50)	Lab built; modified iStick30W (ELeaf) and EZ-177 Sure-Seal 1L induction anesthesia chamber	Behavior	Lefever et al., 2017
10 sec	15 or 30 min	Four 10-sec puffs at 2-sec intervals every 5 min	PG (100%)	Commercially purchased (La Jolla Alcohol Research, Inc.)	Behavior	Javadi-Paydara et al., 2019
10 sec	5 min	5 min exposures to 10 sec puff	PG:VG (50:50)	Lab built; modified iStick30W (ELeaf) and EZ-177 Sure-Seal 1L induction anesthesia chamber	Behavior	Lefever et al., 2019
3 sec	3 hr	Self-administration: Fixed ratio 1 or 3	PG:VG (30:70)	Commercially purchased (La Jolla Alcohol Research, Inc.)	Behavior	Cooper et al., 2020
3 sec	10 min	3-sec puff was delivered every 2 mins, exposed daily for 5 days	PG:VG (50:50)	Commercially purchased (La Jolla Alcohol Research, Inc.)	Behavior	Honeycutt et al., 2020
3 sec	1 hr	3-sec nicotine puff delivered every 2 min for 11 days	PG:VG (50:50)	Commercially purchased (La Jolla Alcohol Research, Inc.)	Behavior	Montanari et al., 2020
6 sec	20 min	Pups were exposed to 6-sec puffs every 15 sec for postnatal days 1 and 2, and 2 times per day for postnatal days 3-9	PG (100%)	Lab built; modified Masterflex peristaltic pump (model 7523-80)	Health Risk Related	McGrath-Morrow et al., 2015
2 sec	1.5 hr	2-sec puff every 10 sec, 2 times per day for 2 weeks	PG:VG (NJOY bold)	Commercially purchased smoke machine (modified Jaeger-Baumgartner CSM 2080. CH Technologies)	Health Risk Related	Sussan et al., 2015
35 mL *	1 hr	Exposed daily, 5 days per week from postnatal weeks 4 to 10; exposed 2 times per day, 5 days per week from postnatal weeks 4 to 10	PG (100%) or VG (100%)	Lab-built; modified Innokin iTaste MVP2.0 aerosolizer	Health Risk Related	Larcombe et al., 2017

Puff Duration	Administration Session Length	Dosing Regimen	Vehicle (ratio)	Equipment	Main outcome Measured	Citation
6 sec	20 min	6-sec puff delivered every 15 sec, exposed daily for 1 week or 3 weeks	PG (100%)	Lab built; modified Masterflex peristaltic pump (model 7523-80)	Health Risk Related	Laube et al., 2017
4 sec	3 hr	4-sec puff delivered every 30 sec, exposed daily, 5 days per week, for 12 weeks	PG:VG (50:50)	Commercially purchased (e~Aerosols)	Health Risk Related	Lee et al., 2018
5 sec	15 min	Female breeders were exposed to four 5-sec puffs delivered every 20 sec 2 times daily, 5 min between sessions, for 6 weeks prior to gestation until up to postnatal day 20 of pups	PG:VG (50:50)	Lab built; modified KangerTech NEBOX	Health Risk Related	Chen et al., 2018
20 mL *	7 min	16 puffs delivered every 2-min, 4 times per day with 30 min between sessions for 3 days or 4 weeks	PG:VG (50:50)	Lab built; modified eRoll devices (Joye Technology)	Health Risk Related	Glynos et al., 2018
N/A	2 hr	48 mg of nicotine per day for 5 weeks	PG:VG (ratio not provided)	Commercially purchased (Teague Enterprises)	Health Risk Related	Reinikovaite et al., 2018
4 sec	3 hr	4-sec puffs delivered every 30 sec, exposed daily for 12 weeks	PG:VG (50:50)	Commercially purchased (e~Aerosols)	Health Risk Related	Orimoloye et al., 2019
10 sec	1 hr	Exposed 3 times daily, 10 min between sessions, for 14 days	PG:VG (50:50)	Commercially purchased (InExpose, SciReq)	Health Risk Related	Shi et al., 2019
4 sec	4 hr	Exposed daily 5 days per week for 54 weeks	isopolypropylene glycol:VG (50:50)	Commercially purchased (e~Aerosols)	Health Risk Related	Tang et al., 2019
5 sec	30 min	Exposed 2 times daily for 6 weeks	PG:VG (50:50)	Lab built; modified KangerTech NEBOX	Health Risk Related	Chen et al., 2020

PG:VG = propylene glycol (PG):vegetable glycerin (VG) ratio; N/A = not applicable

* indicates the use of mL instead of puff duration