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Non-nicotine constituents in e-cigarette aerosol extract attenuate nicotine's aversive effects in adolescent rats

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ABSTRACT

Background: Development of preclinical methodology for evaluating the abuse liability of electronic cigarettes (ECs) in adolescents is urgently needed to inform FDA regulation of these products. We previously reported reduced aversive effects of EC liquids containing nicotine and a range of non-nicotine constituents (e.g., propylene glycol, minor tobacco alkaloids) compared to nicotine alone in adult rats as measured using intracranial self-stimulation. The goal of this study was to compare the aversive effects of nicotine alone and EC *aerosol extracts* in adolescent rats as measured using conditioned taste aversion (CTA), which can be conducted during the brief adolescent period.

Methods and Results: In Experiment 1, nicotine alone (1.0 or 1.5 mg/kg, s.c.) produced significant CTA in adolescent rats in a two-bottle procedure, thereby establishing a model to study the effects of EC extracts. At a nicotine dose of 1.0 mg/kg, CTA to Vuse Menthol EC extract, but not Aroma E-Juice EC extract, was attenuated compared to nicotine alone during repeated two-bottle CTA tests (Experiment 2a). At a nicotine dose of 0.5 mg/kg, CTA to Vuse Menthol EC extract did not differ from nicotine alone during the first two-bottle CTA test but extinguished more rapidly across repeated two-bottle tests (Experiment 2b).

Conclusions: Non-nicotine constituents in Vuse Menthol EC extracts attenuated CTA in a two-bottle procedure in adolescents. This model may be useful for anticipating the abuse liability of ECs in adolescents and for modeling FDA-mandated changes in product standards for nicotine or other constituents in ECs.

1. Introduction

Electronic cigarettes (ECs) are devices that aerosolize a liquid, typically containing nicotine and several other chemicals (e.g., propylene glycol (PG), vegetable glycerin (VG), flavorings), that is inhaled by a user (Brandon et al., 2015; Glasser et al., 2017; National Academy of Sciences, 2018; Walton et al., 2015). EC use has risen greatly over the past decade, especially among youth. For example, past 30-day EC use increased by over 11-fold (from 1.5%–16%) among adolescents in the U.S. between 2011 and 2015, and EC use now surpasses combustible cigarette use in this population (Arrazola et al., 2015; Glasser et al., 2017; National Academy of Sciences, 2018; Singh et al., 2016). More recently, the FDA declared that EC use has reached epidemic levels among adolescents (FDA, 2018). In response to increasing concerns regarding ECs, the FDA Center for Tobacco Products (CTP) issued a deeming rule extending its regulatory authority to ECs (FDA, 2016). Development of appropriate methods and measures for evaluating the abuse liability of ECs could help facilitate the development of effective FDA CTP regulatory policies.

Animal models provide numerous advantages for evaluating tobacco products (see Donny et al., 2012; LeSage et al., 2018), including the ability to examine critical phenomena that are difficult or impossible to study experimentally in humans due to practical or ethical considerations (e.g., the abuse liability of ECs in adolescents). Most animal models of tobacco addiction involve administration of nicotine alone. However, tobacco products including ECs contain various other

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constituents that can mimic or enhance nicotine's effects or produce addiction-related effects themselves (e.g., minor alkaloids, monoamine oxidase (MAO) inhibitors such as the β-carbolines harman and norharman (also called harmane and norharmane), and volatile organic compounds (VOCs) such as acetaldehyde) (e.g., Arnold et al., 2014; Bardo et al., 1999; Belluzzi et al., 2005). Models involving mixtures of constituents derived from tobacco products are needed to determine whether and how these constituents interact to influence tobacco abuse liability. Such interactions may be studied using exposure to cigarette smoke or EC aerosol (Bruijnzeel et al., 2011; Harris et al., 2010; Ponzoni et al., 2015; Small et al., 2010). However, these inhalational models do not allow dissociation of the direct central nervous system (CNS) effects of cigarette smoke or EC aerosol from its sensory effects (e.g., taste, smell), and accurate dosing can be challenging (e.g., Rotenberg and Adir, 1983).

Our laboratory has evaluated the addiction-related effects of EC liquids that contain a combination of nicotine and various non-nicotine constituents (e.g., minor alkaloids, PG) (Harris et al., 2017, 2018b; LeSage et al., 2016b; Smethells et al., 2018). Because EC liquids are administered parenterally, they bypass most, if not all, of the sensory peripheral effects of nicotine and other constituents (e.g., taste, smell), which facilitates study of the direct CNS effects of these constituents. Parenteral administration of extracts also allows for more precise dosing than inhalational models. This approach may therefore be useful for evaluating EC abuse potential, similar to how extracts prepared from cigarette smoke or smokeless tobacco have been useful for studying the abuse liability of those products (e.g., Berman et al., 2018; Brennan et al., 2014; Costello et al., 2014; Harris et al., 2015b; LeSage et al., 2016a).

We previously reported that three different EC liquids produce less acute aversive/anhedonic effects compared to nicotine alone at a high nicotine dose as measured by elevations in intracranial self-stimulation (ICSS) thresholds in adult rats (Harris et al., 2018b; LeSage et al., 2016b). Because nicotine's aversive effects can limit the use of tobacco products (e.g., Hoft et al., 2011; Jensen et al., 2015; Sartor et al., 2010; Shiffman and Terhorst, 2017; Svyryd et al., 2016), an attenuation of these effects could potentially promote EC use. While extending these findings to adolescent rodents is clearly of interest, ICSS requires a prolonged training protocol that is difficult to accomplish during the brief adolescent period in rodents.

Conditioned taste aversion (CTA) is a sensitive, well-established measure of the aversive effects of drugs (Davis and Riley, 2010; Kumar et al., 1983; Verendeev and Riley, 2012) and involves a brief training protocol that is feasible in adolescents (Hurwitz et al., 2013; Shram et al., 2006). In a commonly used CTA procedure, water-deprived animals are allowed access to a novel flavored solution such as saccharin (i.e., the conditioned stimulus) and then injected with a drug such as nicotine (i.e., the unconditioned stimulus, US). This conditioning procedure, sometimes referred to as a one-bottle test but here referred to as a conditioning session, is repeated across several days. During a subsequent two-bottle test, animals are allowed concurrent access to the flavored solution and water, and no injections are administered. Reduced consumption of the flavored solution in drug-injected animals during conditioning or during the two-bottle test compared to saline-injected controls is considered an aversive effect (Davis and Riley, 2010; Kumar et al., 1983; Verendeev and Riley, 2012). The two-bottle test generally provides greater sensitivity to the effects of experimental manipulations (e.g., drug dose, etc.) (Davis and Riley, 2010; Verendeev and Riley, 2012). For example, in adult rodents, nicotine often produces only modest CTA during conditioning but robust CTA in a two-bottle test (Kumar et al., 1983; Pescatore et al., 2005; Shoaib and Stolerman, 1995; Shoaib et al., 2000). The greater sensitivity of a two-bottle CTA test may be particularly important when studying adolescent rodents, which are reported to be less sensitive than adults to nicotine aversion (e.g., Dannenhoffer and Spear, 2016; Shram et al., 2006; Torres et al., 2008). A further advantage of a two-bottle test is that it allows for determination of preference scores (i.e., intake of the flavored solution expressed as a percentage of total liquid (flavored solution + water) intake), which controls for any differences between groups in terms of total liquid consumed (e.g., Merluzzi et al., 2014).

Once a CTA is established, extinction of this behavior can be induced by repeatedly presenting the previously drug-paired flavor in the absence of the drug. Similar to the strength of the conditioned behavior initially acquired, resistance to extinction of CTA and other conditioned behaviors is often related to intensity of the US (see Annau and Kamin, 1961; Dragoin, 1971; Elkins, 1973; James and Mostoway, 1968). However, resistance to extinction of CTA can also be associated with behavioral and neurobiological mechanisms that are distinct from those associated with CTA during acquisition or the first two-bottle test (Durcan et al., 1988; Mickley et al., 2005; Sakata et al., 2013). Resistance to extinction of CTA is therefore a valid measure of the aversive strength of the drug US and may provide unique information to complement findings obtained during conditioning or the first two-bottle CTA test.

The overall goal of this study was to compare the effects of nicotine alone and EC aerosol extracts on CTA in two-bottle tests in adolescent rats. We used EC aerosol extracts rather than EC refill liquids because they more accurately simulate EC use in humans via aerosol exposure and may also contain a unique profile of behaviorally relevant constituents (e.g., acetaldehyde, toluene) compared to EC refill liquid (see Famele et al., 2017; Jo and Kim, 2016; Kim and Kim, 2015). The fact that some of the constituents potentially present in EC aerosol extract (e.g., acetaldehyde, toluene) can themselves induce CTA (Brown et al., 1978; Miyagawa et al., 1984) argues against the possibility that EC extracts would have reduced aversive effects compared to nicotine alone. However, the levels of these constituents in ECs are relatively low (Cheng, 2014; Goniewicz et al., 2014), and drugs that independently produce CTA may nonetheless produce attenuated CTA when administered in combination (Brown et al., 1979; Busse et al., 2005; Kunin et al., 1999). Importantly, the sensitivity and utility of CTA for testing drug interactions (e.g., Loney and Meyer, 2019; Rinker et al., 2008b; Switzman et al., 1981) supports its use for characterizing the aversive effects of EC extracts regardless of the results.

The goal of Experiment 1 was to establish in our laboratory the methodology for reliably inducing CTA to nicotine alone in adolescents in two-bottle tests based on general procedures used by others to study CTA to nicotine or other drugs in adolescents (Cobuzzi et al., 2014; Hurwitz et al., 2013; Shram et al., 2006). This model was subsequently used to compare the effects of nicotine alone and EC aerosol extracts prepared from Aroma E-Juice or Vuse Menthol EC liquid that contained the same nicotine dose (i.e., 1.0 mg/kg, Experiment 2a). Experiment 2b evaluated whether the attenuated CTA to Vuse Menthol EC extract compared to nicotine alone observed in Experiment 2a would generalize to a lower nicotine dose (0.5 mg/kg). We used this dose rather than the 1.5 mg/kg nicotine dose studied in Experiment 1 because the higher dose was not well-tolerated (see Results). The 0.5 mg/kg nicotine dose would also be expected to produce more clinically relevant nicotine serum levels (see Craig et al., 2014). Aroma E-Juice EC extract was not studied in Experiment 2b because it did not produce attenuated CTA compared to nicotine alone in Experiment 2a.

2. Methods

2.1. Animals

Experimentally naïve male and female adolescent Holtzman rats (postnatal day (PND) 22–24 on arrival) were obtained from Harlan/Envigo (Indianapolis, IN). Rats were housed in same-sex pairs in tub cages in a temperature- and humidity-controlled colony room under a 12 h light/dark cycle with free access to food and water. Rats were allowed a 12-day acclimation period prior to onset of behavioral testing, which was conducted during the light (inactive) phase of the

light/dark cycle. All procedures were approved by The Institutional Animal Care and Use Committee (IACUC) of the Hennepin Health Research Institute (formerly Minneapolis Medical Research Foundation) in accordance with the 2011 NIH Guide for the Care and Use of Laboratory Animals and the 2003 National Research Council Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research.

2.2. Drugs

For preparation of nicotine alone, (-)-nicotine (Sigma Chemical Co., St. Louis, MO) was dissolved in sterile saline. EC aerosol extract was prepared using Aroma E-Juice Whole Tobacco Alkaloid (WTA) EC refill liquid (Dark Honey Tobacco flavor) (http://www.aromaejuice.com, Scottsdale, AZ) or Vuse Menthol EC liquid (http://vusevapor.com, Winston-Salem, NC). Aroma E-Juice is advertised as containing higher levels of minor alkaloids than other EC liquids and produced attenuated ICSS threshold-elevating (aversive/anhedonic) effects compared to nicotine alone in our previous study (LeSage et al., 2016b). According to the label, this EC liquid contained 80% VG and 20% PG and had a nicotine concentration of 24 mg/ml. Vuse Menthol was used because it is one of the most popular ECs on the market (Day et al., 2017). Study of a mentholated product was also of interest because menthol can have addiction-related CNS effects and influence nicotine pharmacokinetics (Alsharari et al., 2015; Biswas et al., 2016; Fan et al., 2016; Henderson et al., 2017). According to the manufacturer, Vuse Menthol EC liquid contained 48 mg/ml nicotine and an unspecified amount of menthol flavoring. No other ingredient information was provided for this product.

EC aerosol extracts were prepared as described in the Supplementary Methods. In contrast to the EC *liquids* used in our previous studies (e.g., Harris et al., 2018b; LeSage et al., 2016b), these *extracts* are derived from EC *aerosol* produced by a smoking machine under puffing conditions based on those in EC users. These extracts are therefore expected to contain most, if not all, of the chemicals that result from heating and aerosolizing the EC liquid, thereby providing more clinically relevant exposure conditions than administering non-aerosolized EC liquid. Nicotine concentration was determined, and extracts were diluted to the appropriate nicotine concentration for each study using saline. Thus, the concentration of all constituents in extract decreased in proportion to the nicotine concentration in the extract (i.e., the ratio of nicotine and non-nicotine constituents was constant across nicotine dose). Use of ETOH as a solvent allows for extraction of both volatile and non-volatile constituents that are non-water soluble, and ETOH has been used in numerous preclinical studies evaluating addiction-related effects of cigarette smoke extracts (Brennan et al., 2015, 2013a; Brennan et al., 2013b). EC extracts contained either 6% ETOH (1.0 mg/kg nicotine dose; Experiment 2a) or 3% ETOH (0.5 mg/kg nicotine dose; Experiment 2b) after dilution. Due to the low volumes administered ($\leq$0.2 ml), these concentrations resulted in ETOH doses ($\leq$9.4 or 4.7 mg/kg ETOH for the 1.0 or 0.5 mg/kg nicotine dose, respectively) that are considerably lower than those needed to induce CTA in adolescents ($\geq$2,000–3,500 mg/kg) (Schramm-Sapyta et al., 2010, 2014). Nonetheless, to control for any effects of ETOH itself on CTA, the same concentration of ETOH was added to the nicotine alone and saline control solutions in Experiments 2a and 2b. All formulations were sterile-filtered and their pH adjusted to 7.4 prior to use. All formulations were administered s.c. in a volume of 1 ml/kg, and nicotine doses are expressed as the base.

2.3. Initial EC extract constituent analysis

EC extracts were analyzed for concentrations of nicotine, the solvents PG and VG, minor tobacco alkaloids (e.g., nornicotine), and the β-carbolines harman and norharman as described in the Supplementary Methods.

2.4. Routine nicotine assay

Nicotine concentrations in prepared solutions of nicotine alone and EC extracts were measured by gas chromatography with nitrogen phosphorus detection according to standard protocol in our laboratory (Hieda et al., 1999).

2.5. CTA apparatus

CTA conditioning and testing was conducted in hanging wire-mesh cages (35 cm x 16.5 cm x 17.5 cm). Either one or two 25 ml graduated drinking tubes (product # 900003, Dyets, Inc., Bethlehem, PA) were attached to the inside of each cage with a tube holder (product # 901100, Dyets, Inc.). The spout of each drinking tube was located ≈ 5.5 cm above the floor of the cage.

2.6. Experiment 1: CTA to nicotine alone

2.6.1. Conditioning

The general procedure was based on that previously used to study CTA induced by nicotine or other drugs in adolescent rats (Cobuzzi et al., 2014; Hurwitz et al., 2013; Shram et al., 2006). Following a 24 h period of water deprivation, male and female adolescents (PND = 34–36) were placed in a novel wire-mesh cage containing a 0.1% saccharin solution for 30 min. This saccharin concentration has been used in numerous studies demonstrating CTA to nicotine or other drugs in adolescent or adult rats (e.g., Cobuzzi et al., 2014; Hurwitz et al., 2013; Kumar et al., 1983; Lancellotti et al., 2001; Pescatore et al., 2005; Shram et al., 2006). The saccharin bottle was subsequently removed, and animals remained in the cage for twenty minutes. Rats were then injected s.c. with either saline (Sal; total n = 19, n = 9–10 per sex), 1.0 mg/kg nicotine alone (1.0 Nic, total n = 19, total n = 9–10 per sex), or 1.5 mg/kg nicotine alone (1.5 Nic, total n = 25, n = 12–13 per sex). The twenty-minute interval between saccharin exposure and injections was based on previous studies demonstrating CTA to nicotine or other drugs in adolescents or adult rats (Cobuzzi et al., 2014; Hurwitz et al., 2013; Shram et al., 2006). We used high nicotine doses because nicotine doses up to 0.8 mg/kg fail to induce CTA in adolescent males tested under a similar protocol (Shram et al., 2006). Immediately following injection, rats were returned to their home cages and allowed ad lib access to water for 2 h. to avoid dehydration. Water intake was not measured during this period. This conditioning procedure was repeated each day for a total of 4 days, with rats receiving the same nicotine dose each day.

2.6.2. Two-Bottle Tests

Twenty-one hours after the final conditioning session, a two-bottle CTA test was conducted by allowing animals concurrent access to 0.1% saccharin and water for 30 min. No injections were administered, and position of the water and saccharin bottles (left or right) was counterbalanced across rats to control for side preferences. Beginning immediately following the two-bottle test, rats were allowed 48 h. ad lib access to water in their home cages followed by a 24 -h period of water deprivation. To examine extinction of CTA, the two-bottle test procedure described above was repeated each day for 5 consecutive days. Rats were allowed 2 h. access to water in their home cage following each two-bottle test. Fluid intake was measured by weighing the drinking tubes before and after each one- or two-bottle test. The two-bottle tests (primary outcome) were conducted from PND 38–40 to PND 45–47 (i.e., from mid-adolescence to late adolescence/early adulthood) (Chen et al., 2007; Spear, 2000).

2.7. Experiments 2a and 2b: CTA to nicotine alone and EC extracts

Experiment 2a replicated Experiment 1 except that adolescents received saline (Sal; total n = 15, n = 7–8 per sex), 1.0 mg/kg nicotine

alone (1.0 Nic, total n = 17, n = 8–9 per sex), 1.0 mg/kg Vuse Menthol EC extract (1.0 Vuse, total n = 17, n = 8–9 per sex), or 1.0 mg/kg Aroma E-Juice EC extract (1.0 Aroma E-Juice, total n = 14, n = 6–8 per sex). The same procedures were repeated in Experiment 2b, except that rats received Sal (total n = 12, n = 5–7 per sex), 0.5 mg/kg nicotine alone (0.5 Nic, total n = 14, n = 7 per sex), or 0.5 mg/kg Vuse Menthol EC extract (0.5 Vuse: total n = 11, total n = 5–6 per sex). We used a 0.5 mg/kg nicotine dose to avoid toxicity observed with the 1.5 mg/kg dose used in Experiment 1. Pilot data (not shown) indicated that 0.5 mg/kg nicotine alone produced significant CTA. As described above, the saline and nicotine alone control solutions contained 6% ETOH (Experiment 2a) or 3% ETOH (Experiment 2b) to match the ETOH concentration of the EC extracts.

2.8. Statistical analyses

The primary outcome in each study was saccharin preference (ml saccharin consumed / ml of total liquid (saccharin + water) consumed x 100%) during two-bottle tests. Secondary outcomes included saccharin consumed (in ml) during conditioning sessions and total ml consumed (saccharin + water) during two-bottle tests. Each of these outcomes was analyzed using separate 3-factor ANOVAs with group/formulation and sex as between-subject factors and session as a within-subject factor. Degrees of freedom for all ANOVAs were adjusted using the Greenhouse-Geisser correction to account for possible violations of sphericity. As appropriate, effects of group were compared at each conditioning or test session using independent sample Welch's t-tests with a Bonferroni correction. Welch's t-tests were used to account for unequal variances across groups.

3. Results

3.1. Initial EC extract constituent analysis

Menthol was detected in Vuse Menthol EC extract but not in Aroma E-Juice Dark Honey EC extract (Table 1). Levels of the solvents PG and VG were higher in Aroma E-Juice EC extract compared to Vuse Menthol. Levels of minor alkaloids (expressed as % of nicotine) were generally similar in Vuse Menthol and Aroma E-Juice EC extracts. Levels of the β-carbolines harman and norharman in EC extracts were either very low (Aroma E-Juice) or not detectable (Vuse Menthol) (Table 1).

Table 1
Levels of nicotine, menthol, solvents, minor alkaloids, and β-carbolines (μg/ml) in Vuse Menthol and Aroma E-Juice Dark Honey EC extracts. Data in parentheses indicate relative levels of each minor alkaloid or β-carboline (expressed as % of nicotine) in that solution. Total Minor = combined level of all measured minor alkaloids. ND = Not detected.

	Vuse Menthol EC Extract	Aroma E-Juice EC Extract
Nicotine	15,000	12,000
Menthol	5,990	ND
Solvents		
Propylene glycol	126,000	501,000
Vegetable glycerin	258,000	464,000
Minor Alkaloids		
Nornicotine	4.55 (0.030%)	0.68 (0.006%)
Anabasine	1.03 (0.007%)	0.39 (0.003%)
Anatabine	1.02 (0.007%)	2.31 (0.019%)
Myosmine	4.7 (0.031%)	4.7 (0.039%)
β-nicotyrine	164.0 (1.093%)	210 (1.75%)
Total Minor	*175.3 (1.169%)*	*218.1 (1.82%)*
β-carbolines		
Harman	ND	0.007 (0.00006%)
Norharman	ND	0.07 (0.0006%)

3.2. Body weights

Weights did not differ significantly between groups in any experiment (data not shown). Weights of males (mean g ± SEM, averaged across all experiments) on the first conditioning day, first two-bottle test day, and final two-bottle test day were 131.4 ± 2.3 g, 145.1 ± 2.0 g, and 191.9 ± 2.5 g, respectively. Females weighed 111.7 ± 1.7 g, 122.9 ± 1.9 g, and 152.8 ± 2.1 g, respectively, on these same days.

3.3. Experiment 1: CTA to nicotine alone

3.3.1. Data exclusion

All data for 1 male in the 1.5 Nic group were excluded due to an overdose during conditioning.

3.3.2. Saccharin consumed during conditioning

There were significant main effects of group ($F(2,57) = 5.4$, $p < 0.0001$) and session ($F(2.2,124.1) = 19.4$, $p < 0.0001$) on saccharin consumption as well as a significant group x session interaction ($F(4.4,124.1) = 7.9$, $p < 0.0001$). Because there was no significant main effect of sex or significant interactions between sex and the other variables, data were collapsed across sex for post hoc analyses. Compared to the Sal group, saccharin consumption was significantly lower in the 1.0 Nic group during session 4 ($t(31.8) = 3.1$, $p < 0.05$) (Fig. 1A). Consumption was lower in the 1.5 Nic group than in the Sal group during sessions 3 ($t(25.8) = 3.4$, $p < 0.01$) and 4 ($t(27.8.0) = 5.0–4.6$, $p < 0.01$). No other significant differences were observed between groups during any session (Fig. 1A).

3.3.3. Total liquid consumed during two-bottle tests

Total liquid (saccharin + water) consumption during two-bottle tests was ≈2-3 ml lower in the 1.0 Nic and 1.5 Nic groups compared to the Sal group (see Supplementary Data).

3.3.4. Saccharin preference during two-bottle tests

There were significant main effects of group ($F(2,55) = 6.8$, $p < 0.01$) and session ($F(3.8,207.3) = 39.9$, $p < 0.0001$) on saccharin preference, as well as a significant group x session interaction ($F(7.5, 207.3) = 2.9$, $p < 0.01$). There was no main effect of sex or interactions related to this variable. Analysis of data collapsed across sex indicated that saccharin preference was significantly lower in the 1.0 Nic and 1.5 Nic groups compared to the Sal group during sessions 1–5 ($t(19.5–39.5) = 2.6–4.7$, $p < 0.05$ or 0.01). The 1.0 Nic and 1.5 Nic groups did not differ from the Sal group during session 6, reflecting extinction of CTA (Fig. 1B). The 1.0 Nic and 1.5 Nic groups did not differ from each other during any session (Fig. 1B).

3.4. Experiment 2a: CTA to nicotine alone, Vuse Menthol EC extract, and Aroma E-Juice EC extract (Nicotine dose = 1.0 mg/kg)

3.4.1. Saccharin consumed during conditioning

There was a significant main effect of session ($F(2.7,149.9) = 11.3$, $p < 0.0001$) on saccharin consumption but no effect of group. Although consumption appeared lower in the 1.0 Nic, 1.0 Vuse, and 1.0 Aroma E-Juice groups compared to the Sal group during session 4 (see Fig. 2A), the interaction between group and session was not significant ($F(8.2,149.9) = 1.8$, $p = 0.08$). There was no main effect of sex or interactions between sex and the other variables.

3.4.2. Total liquid consumed during two-bottle tests

Total liquid consumed during two-bottle tests was ≈ 1.0 ml lower in the 1.0 Aroma E-Juice group compared to the Sal group. Total liquid consumption in the other groups did not differ from the Sal group (see Supplementary Data).

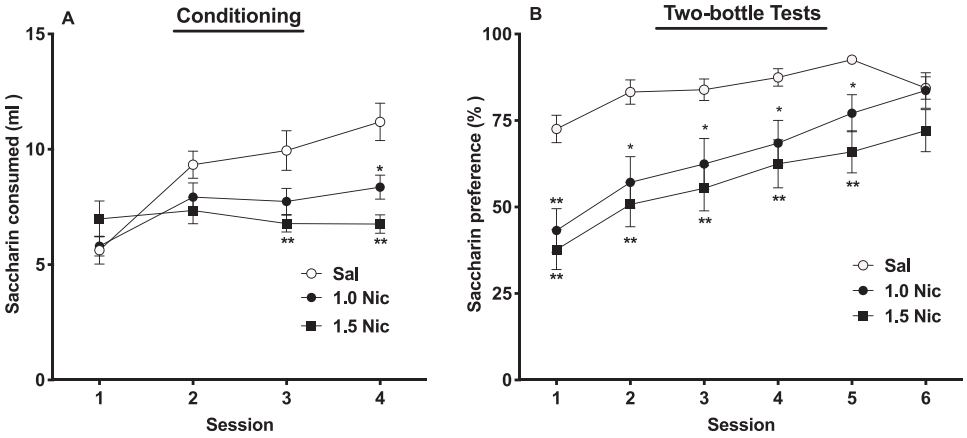

Fig. 1. (A) Saccharin consumed (in ml) during conditioning in male and female adolescent rats receiving saline (Sal), 1.0 mg/kg nicotine alone (1.0 Nic), or 1.5 mg/kg nicotine alone (1.5 Nic) in Experiment 1. (B) Saccharin preference (ml saccharin consumed / ml total liquid consumed x 100%) during two-bottle tests. *,**,** Different from Sal at that session $p < 0.05$ or 0.01.

3.4.3. Saccharin preference during two-bottle tests

There were significant main effects of group ($F(3,55) = 4.2$, $p = 0.01$) and session ($F(3.7,202.2) = 24.4$, $p < 0.0001$) on saccharin preference, but the interaction between group and session was not significant ($F(11.0,202.2) = 1.7$, $p = 0.09$). Because there was no main effect of sex and no interactions related to this variable, data were collapsed across sex. Comparison of marginal means across all 6 sessions indicated that saccharin preference was significantly lower in the 1.0 Nic ($t(157.3) = 6.1$, $p < 0.01$), 1.0 Vuse ($t(166.4) = 3.1$, $p < 0.05$), and 1.0 Aroma E-Juice ($t(129.3) = 7.1$, $p < 0.01$) groups compared to the Sal group (Fig. 2B). Saccharin preference was significantly higher in the 1.0 Vuse group compared to the 1.0 Nic group ($t(199.9) = 2.8$, $p < 0.05$) and 1.0 Aroma E-Juice group ($t(164.0) = 3.8$, $p < 0.01$), reflecting an attenuation of CTA in the 1.0 Vuse group. Despite the lack of an interaction between group and session (see above), magnitude of this attenuation appeared greatest during the first session (Fig. 2B). The 1.0 Nic and 1.0 Aroma E-Juice groups did not differ from each other (Fig. 2B).

3.5. Experiment 2b: CTA to nicotine alone and Vuse Menthol EC extract (Nicotine dose = 0.5 mg/kg)

3.5.1. Saccharin consumed during conditioning

There was no effect of group, but there was a significant effect of session ($F(2.7,84.3) = 16.2$, $p < 0.001$) and a significant group x session interaction ($F(5.4,84.3) = 2.4$, $p < 0.05$) (Fig. 3A). There was also a significant main effect of sex ($F(1,31) = 12.6$, $p < 0.001$), reflecting higher saccharin consumption in males than in females (mean saccharin consumption ± SEM = 8.5 ± 0.3 ml versus 7.1 ± 0.3 ml), but no interactions between sex and the other variables. Subsequent comparison of data collapsed across sex indicated no significant differences between groups during any session (Fig. 3A).

3.5.2. Total liquid consumed during two-bottle tests

Total liquid consumption during two-bottle tests did not differ between groups (Supplementary Data).

3.5.3. Saccharin preference during two-bottle tests

There were significant main effects of group ($F(2,33) = 7.9$, $p < 0.01$) and session ($F(3.2,107.2) = 35.9$, $p < 0.0001$) on saccharin preference and a significant interaction ($F(6.5,107.2) = 4.1$, $p < 0.001$). There was no main effect of sex and no significant interactions related to this variable. Comparison of group data collapsed across sex indicated that saccharin preference was lower in the 0.5 Nic group compared to the Sal group during sessions 1–4 ($t(18.6–23.0) = 2.8–5.0$, $p < 0.05$ or 0.01) (Fig. 3B). In contrast, preference was lower for the 0.5 Vuse group compared to the Sal group during the first session only ($t(18.1) = 3.8$, $p < 0.01$), reflecting more rapid extinction of CTA (Fig. 3B). Saccharin preference in the 0.5 Vuse group was also significantly higher than in the 0.5 Nic group during session 2 ($t(24.2) = 3.2$, $p < 0.05$).

4. Discussion

These studies provide the first characterization of the behavioral effects of an EC aerosol extract containing nicotine and a range of non-nicotine constituents in a preclinical model. Experiment 1 established methodology to reliably elicit significant CTA to nicotine alone in male and female adolescents using a two-bottle procedure. In Experiment 2a, CTA to 1.0 mg/kg Vuse Menthol EC extract, but not Aroma E-Juice EC

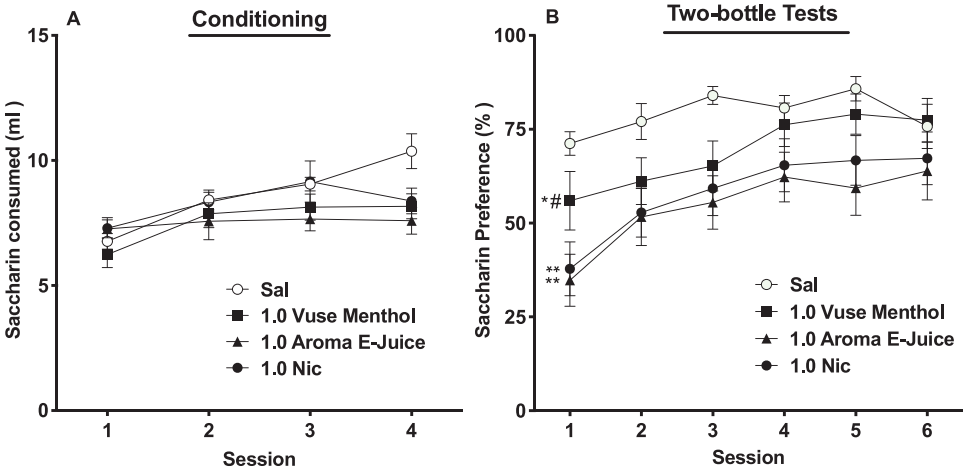

Fig. 2. (A) Saccharin consumed during conditioning in adolescents receiving Sal, 1.0 mg/kg nicotine alone (1.0 Nic), 1.0 mg/kg Vuse Menthol EC extract (1.0 Vuse), or 1.0 mg/kg Aroma E-Juice EC extract (1.0 Aroma E-Juice) in Experiment 2a. (B) Saccharin preference during two-bottle tests in Experiment 2a. *,** Different from Sal (marginal means across sessions), $p < 0.05$ or 0.01. # Different from 1.0 Nic and 1.0 Aroma E-Juice (marginal means across sessions), $p < 0.05$.

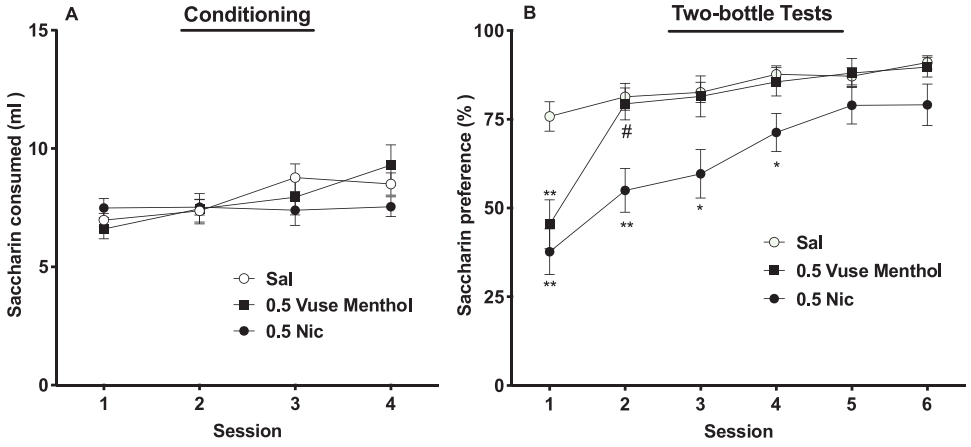

Fig. 3. (A) Saccharin consumed during conditioning in adolescents receiving Sal, 0.5 mg/kg nicotine alone (0.5 Nic), or 0.5 mg/kg Vuse Menthol EC extract (0.5 Vuse) in Experiment 2b. (B) Saccharin preference during two-bottle tests in Experiment 2b. *,*,* Different from Sal at that session, $p < 0.05$ or 0.01. # Different from 0.5 Nic at that session, $p < 0.05$.

extract, was attenuated compared to nicotine alone during repeated two-bottle CTA tests. In Experiment 2b, CTA to 0.5 mg/kg Vuse Menthol EC extract did not differ from nicotine alone during the first two-bottle test but extinguished more rapidly across subsequent tests.

Nicotine's aversive effects can limit the use of tobacco products in both adolescents and adults (e.g., Hoft et al., 2011; Jensen et al., 2015; Sartor et al., 2010; Shiffman and Terhorst, 2017; Svyryd et al., 2016). To the extent that our findings reflect reduced aversive effects of Vuse EC extract, they suggest that non-nicotine constituents in Vuse EC aerosol could increase Vuse EC consumption in adolescents by attenuating nicotine's aversive effects. However, lower CTA to a drug is associated with greater i.v. self-administration of that drug in some studies but not others (Davis and Riley, 2010; Fowler et al., 2011, 2013; Lancellotti et al., 2001; Martin et al., 1999), a discrepancy that may reflect differences in factors including route, dose, and/or contingency across models (for discussion, see Davis and Riley, 2010). Although outside the scope of the current studies, direct comparison of i.v. self-administration of nicotine alone and Vuse Menthol EC extract, particularly under conditions in which nicotine intake is likely limited by its aversive effects (e.g., during a unit dose escalation protocol), is needed to clarify to relevance of our findings to EC consumption. Regardless, our data suggest that non-nicotine constituents in Vuse Menthol ECs can alter the CNS-mediated adverse effects of nicotine exposure. Such an effect, either alone or in combination with behavioral factors that could attenuate nicotine aversion (e.g., conditioned effects of flavors), could potentially contribute to addiction or other health consequences of ECs.

It is unclear which non-nicotine constituent(s) in Vuse Menthol extracts contributed to the modest attenuation of nicotine-induced CTA. Certain minor alkaloids (e.g., nornicotine) can be behaviorally active but only at concentrations much higher than those in the current EC extracts (Caine et al., 2014; Harris et al., 2015a; Marusich et al., 2017). Furthermore, minor alkaloids typically enhance rather than attenuate the behavioral effects of nicotine (Clemens et al., 2009; Desai et al., 2016). Another constituent that may have contributed to the effects of Vuse extract is propylene glycol (PG), which can attenuate nicotine aversion as measured using ICSS (Harris et al., 2018a). However, Aroma E-Juice EC extract contained even higher levels of PG than Vuse Menthol (see Table 1) but did not produce attenuated CTA compared to nicotine alone. Our findings also do not reflect effects of the ß-carbolines harman and norharman, which were not present in Vuse Menthol EC extract.

Menthol was detected in Vuse Menthol and may have contributed to the effects of this EC extract. Parenterally administered menthol can influence nicotine pharmacokinetics and nicotine's addiction-related behavioral and neurobiological effects (Alsharari et al., 2015; Biswas et al., 2016; Fan et al., 2016; Henderson et al., 2017). For example, menthol can inhibit nicotine-induced activation of α4ß2 nicotinic acetylcholine receptors (nAChRs) (Hans et al., 2012), which play a critical role in nicotine aversion (Shoaib et al., 2002, 2000). Such an effect could reduce the ability of nicotine to induce CTA, analogous to the effects of reducing the nicotine dose. However, the relative insensitivity of CTA to nicotine dose in this study and others (e.g., Kumar et al., 1983; Dannenhoffer and Spear, 2016) suggests possible involvement of non-cholinergic mechanisms. In this regard, menthol can influence several other neurotransmitter systems implicated in nicotine aversion including dopamine and GABA (Watt et al., 2008; Zhang et al., 2018).

The role of menthol and/or other non-nicotine constituents in our findings could be addressed by evaluating effects of cocktails containing nicotine and one or more of these constituents at concentrations that are similar to those in EC extracts. Evaluating effects of these non-nicotine constituents in the absence of nicotine would also be of interest. For example, although rarely reported, some drugs can produce a conditioned taste preference rather than CTA under certain conditions (Lett and Grant, 1989; Mucha and Herz, 1985; Parker et al., 1973). The ability of parenterally administered flavorants to activate gustatory receptors (Bradley and Mistretta, 1971; Bures and Buresova, 1989) could also potentially lead to a conditioned taste preference. It is therefore possible that the attenuated CTA for Vuse extract reflects conditioned taste preference to one or more non-nicotine constituents in EC extract superimposed on a CTA for nicotine, rather than an attenuation of nicotine aversion *per se*. Evaluating effects of isolated non-nicotine constituents in this model could address this possibility. However, this account is still consistent with the general conclusion that non-nicotine constituents in Vuse EC aerosol extract attenuated its aversive effects.

The difference in the pattern of results across nicotine doses in Experiments 2a and 2b complements previous reports that different mechanisms can contribute to the acquisition versus extinction of CTA induced by nicotine or other aversive stimuli (e.g., lithium) (Durcan et al., 1988; Mickley et al., 2005; Sakata et al., 2013). For example, extinction of CTA can be selectively mediated by the prefrontal cortex (Akirav et al., 2006; Mickley et al., 2005). It is unclear which of these mechanisms, if any, account for our findings. Future mechanistic studies are needed to address this issue.

The similar CTA for 1.0 mg/kg Aroma E-Juice EC extract and nicotine alone is inconsistent with the attenuated ICSS threshold-elevating (aversive/anhedonic) effects of the same dose of Aroma E-Juice EC liquid in adults (LeSage et al., 2016b). Methodological factors that could account for this discrepancy include age of the animals, dependent measure, dosing regimen, and our current use of an EC aerosol extract rather than an EC liquid. Relevant to the latter point, when expressed as % of nicotine, the Aroma E-Juice EC aerosol extract contained lower levels of minor alkaloids compared to the liquid of this same product (see Table 1 in LeSage et al., 2016a, 2016b). This is consistent with another report of limited aerosolization of minor

alkaloids from EC liquid (Famele et al., 2017). Other behaviorally active constituents that were not measured (e.g., VOCs) may also have differed between the aerosol extract and liquid for this product (see Jo and Kim, 2016; Kim and Kim, 2015). Comparison of the effects of Aroma E-Juice EC extract and liquid on ICSS and CTA, respectively, as well as further chemical evaluation of both formulations, would help clarify the role of formulation (i.e., EC aerosol extract versus liquid) in the different findings across studies.

Some groups injected with nicotine alone or EC extracts had lower total liquid (saccharin + water) consumption during two-bottle tests compared to saline controls. This indicates that conditioned decreases in saccharin consumption were not fully offset by compensatory increases in water consumption in these groups. This effect, which has been reported in several prior CTA studies (e.g., Buresova and Bures, 1984; Cobuzzi et al., 2014; Merluzzi et al., 2014), was generally modest (≈1–3 ml difference in total liquid consumption between groups). Importantly, total consumption in the affected groups (≥8–9 ml) was still sufficient to determine saccharin preference scores. There was also no clear relationship between total liquid intake and saccharin preference scores in either the current study or in prior studies (e.g., Merluzzi et al., 2014), consistent with the notion that these measures are independent. Therefore, while its causes are unclear, the reduced total liquid intake observed in some groups does not appear to confound our conclusions.

Given that nicotine injections can reduce water consumption in adult rats for up to two hours (Clarke and Kumar, 1984; Munster and Battig, 1975), nicotine or EC extracts may have suppressed water consumption during the two-hour period of ad lib water access following each conditioning session. This could have influenced motivation to drink during the conditioning session conducted the next day or during the first two-bottle test. This possibility cannot be directly addressed because water intake during this period was not measured. However, it is unclear whether a hypodypsic effect of nicotine would occur in adolescents, which are less sensitive to several effects of high nicotine doses (e.g., locomotor suppression, aversion) compared to adults (Elliott et al., 2004; Schmidt et al., 2015; Shram et al., 2006). The fact that adolescents were water-deprived in our study may also have reduced the ability of nicotine to suppress drinking compared to the non-water-deprived adults studied in Clarke and Kumar (1984), although nicotine did produce a modest hypodypsic effect in water-deprived adults in Munster and Battig (1975). Importantly, a nicotine-induced suppression of water intake following each conditioning session in the current study would result in greater deprivation and presumably greater liquid consumption during the conditioning or two-bottle test session conducted the next day. However, groups receiving nicotine or EC extracts had either lower or similar saccharin consumption (conditioning sessions) or total liquid consumption (first two-bottle test) compared to saline controls in all experiments. Any differences in water deprivation between groups would also likely have resulted in differences in animal weights (see Armstrong et al., 1980; Collier and Levitsky, 1967), but this was not observed. Even in the unlikely event that groups differed in terms of water intake during conditioning, this would not have influenced our primary outcome (saccharin preference during two-bottle tests), which is independent of total liquid consumed.

Both males and females were included in these studies to model the heterogeneity of participants in human studies and to allow for *exploratory* analyses of sex differences. Sex did not influence the effects of nicotine alone or EC extracts in any experiment. These data are consistent with the lack of sex differences in CTA to nicotine alone in adult rats (Rinker et al., 2008a) and provide the first characterization of the generality of effects of tobacco extracts across sexes. Because these studies were not powered to detect sex differences, we cannot rule out the possibility that we would have observed sex differences in the effects of nicotine alone and/or EC extracts if larger group sizes were used. However, apart from a modest trend for females to have greater CTA than males at the 1.5 mg/kg dose of nicotine alone in Experiment 1 (p-value = 0.20), data appeared consistent across sexes in all of the current studies.

The current studies used adolescent rodents in an attempt to model EC exposure in youth, which is of greatest concern to public health. However, as mentioned above, adolescent and adult rodents can differ in their sensitivity to several of nicotine's behavioral effects, including aversion (e.g., Shram et al., 2006; Torres et al., 2008; Vastola et al., 2002). There are also reports of age differences in sensitivity to the addiction-related effects of non-nicotine constituents (Belluzzi et al., 2005; Sershen et al., 2009). Extending the current approach to adults therefore represents an important area for further study.

We administered EC extracts parenterally in an attempt to bypass their peripheral sensory effects and isolate their direct CNS effects. However, parenteral administration of solutions can produce certain peripheral sensory effects that contribute to CTA (Bradley and Mistretta, 1971; Bures and Buresova, 1989). In addition, while we have measured brain nicotine levels following i.v. infusion of EC liquid (LeSage et al., 2016b), we did not measure levels of nicotine and non-nicotine constituents in the brain following injection of EC extracts in this study to confirm a central site of action. Nonetheless, most behavioral effects of nicotine including CTA are primarily mediated centrally (e.g., Kumar et al., 1983; Reavill et al., 1986; Shoaib et al., 2000). It is therefore most likely that our findings reflect CNS rather than peripheral effects of nicotine and non-nicotine constituents in EC extracts, although other approaches (e.g., measurement of constituent levels in brain, evaluating effects of central administration of extracts, etc.) are needed to confirm this.

While the focus of this study was on the CNS effects of EC extracts, peripheral sensory effects (e.g., taste, "throat hit") of ECs also play an important role in the abuse potential of these products (e.g., Audrain-McGovern et al., 2016; Goldenson et al., 2016; National Academy of Sciences, 2018). Future studies evaluating the effects of oral or inhalational exposure to EC aerosol in this and other models of abuse liability could provide insights into how peripheral and central effects of ECs interact to contribute to their abuse liability.

Grigson (1997) has proposed that CTA may actually be a measure of drug reward rather than aversion. According to the "reward comparison hypothesis", CTA occurs because the flavored solution used during conditioning has low reward value compared to the highly rewarding drug that is subsequently administered. Animals later avoid the flavor because of its low relative reward value (i.e., "anticipatory contrast"), with greater CTA indicating greater drug reward (see Grigson, 1997). According to this view, our findings indicate that Vuse extract may be less rewarding (rather than less aversive) than nicotine alone. This alternate interpretation could be evaluated by comparing Vuse extract and nicotine alone in other assays such as i.v. self-administration or place conditioning.

In summary, our findings suggest that non-nicotine constituents in Vuse Menthol EC extract modestly attenuate its aversive effects in adolescents using CTA. Further evaluation of these and other EC extracts in preclinical models may be useful for anticipating the abuse liability of ECs in adolescents, as well as for modeling FDA-mandated changes in product standards for nicotine or other constituents in ECs.

Contributors

Andrew Harris and Mark LeSage supervised the conduct of the study and were responsible for the conception and design of the study. Peter Muelken and Yayi Swain assisted with developing specific protocols, daily conduct of the experiment, and compiling data. Maciej Goniewicz and Mary Palumbo prepared the EC aerosol extracts and conducted the analyses of propylene glycol, vegetable glycerin, and menthol. Vipin Jain and Irina Stepanov conducted the analyses of the minor alkaloids nornicotine, anabasine, anatabine, and myosmine, and of the ß-carbolines harman and norharman. Andrew Harris wrote the first draft of the

manuscript. All authors contributed to and have approved the final manuscript.

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Declaration of Competing Interest

M. Goniewicz served as a member of a scientific advisory board to Johnson and Johnson. There are no other conflicts to disclose.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.drugalcdep.2019.05.023.

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