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Green apple e-cigarette flavorant farnesene triggers reward-related behavior by promoting high-sensitivity nAChRs in the ventral tegmental area

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3. Authors: Skylar Y. Cooper[1], Austin T. Akers[1], and Brandon J. Henderson[1*]

[1]Department of Biomedical Sciences, Marshall University, Joan C Edwards School of Medicine, Huntington, WV, 25703, USA.

4. Author Contributions: SYC and BJH designed research, performed research, analyzed data, and wrote the paper. ATA performed research.

5. Corresponding Author:

Brandon J. Henderson, PhD

Department of Biomedical Sciences

Joan C Edwards School of Medicine at Marshall University

1700 3rd Ave., 410 BBSC, Huntington, WV 25703

Email: Hendersonbr@marshall.edu Phone: 304-696-7316

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ABSTRACT

While combustible cigarette smoking has declined, the use of electronic nicotine delivery systems (ENDS) has increased. ENDS are popular among adolescents, and chemical flavorants are an increasing concern due to the growing use of zero-nicotine flavored e-liquids. Despite this, little is known regarding the effects of ENDS flavorants on vaping-related behavior. Following previous studies demonstrating the green apple flavorant, farnesol, enhances nicotine reward and exhibits rewarding properties without nicotine, this work focuses on the green apple flavorant, farnesene, for its impact on vaping-related behaviors. Using adult C57BL/6J mice, genetically modified to contain fluorescent nicotine acetylcholine receptors (nAChRs), and farnesene doses of 0.1, 1.0, and 10 mg/kg, we observed farnesene-alone produces reward-related behavior in both male and female mice. We then performed whole-cell patch-clamp electrophysiology and observed farnesene-induced inward currents in ventral tegmental area (VTA) putative dopamine neurons that were blocked by the nAChR antagonist, DhβE. While the amplitudes of farnesene-induced currents are ~30% of nicotine's efficacy, this indicates the potential for some ENDS flavorants to stimulate nAChR function. Additionally, farnesene enhances nicotine's potency for activating nAChRs on VTA dopamine neurons. This may be due to changes in nAChR stoichiometry as our data suggests a shift toward high-sensitivity α4β2 nAChRs. Consequently, these data show that the green apple flavorant, farnesene, causes reward-related behavior without nicotine through changes in nAChR stoichiometry that results in an enhanced effect of nicotine on VTA dopamine neurons. These results demonstrate the importance of future investigations into ENDS flavorants and their effects on vaping-related behaviors.

SIGNIFICANCE STATEMENT

Although combustible cigarette use has decreased by ~11% in America over the past two decades, the use of electronic nicotine delivery systems (ENDS) has increased by 135% and 218% among high school and middle school students, respectively, in the last two years alone (Cullen et al., 2018; 2019). Due to the fact that most ENDS users vape flavored nicotine products and the growing use of zero-nicotine flavored e-liquids, it raises the questions of how chemical flavorants alter nicotine addiction and if they increase abuse liability themselves. We show that one chemical flavorant and odorant of green apple, farnesene, causes reward-related behavior on its own. These results increase our understanding on how flavorants promote neurological changes and affect nicotine addiction.

INTRODUCTION

Tobacco use remains the leading preventable cause of disease and death in America and results in nearly half a million deaths per year. Although there has been a decline in the use of combustible cigarettes, the use of electronic nicotine delivery systems (ENDS) has increased tremendously with over three million users between the ages of 14 and 18 (Cullen et al., 2018). The former U.S. Food and Drug Administration (FDA) Commissioner, Scott Gottlieb, stated that he believes the ENDS companies are creating a new demographic market among the youth, rather than simply helping adult smokers quit; which was the original objective for the production of ENDS (FDA, 2018). Since their inception, ENDS have become more of a concern among the adolescent population due to the dramatic increase in use among their age demographic and because of the numerous flavor options available (Cullen et al., 2018; FDA, 2018; Mead et al., 2018; Schneller et al., 2018). Unlike combustible cigarettes which are limited to menthol flavor, there is no restriction on flavored ENDS and currently over 15,000 unique flavors are on the market (Hsu et al., 2018). Additionally, >90% of adolescent and ~70% of adult ENDS users prefer flavored products (Mead et al., 2018; Schneller et al., 2018).

Menthol is the most prominent flavor for tobacco products and was considered to be an inert flavor additive, yet, studies have shown that menthol enhances nicotine reward-related and reinforcement-related behaviors in rodents (Wang et al., 2014; Biswas et al., 2016; Henderson et al., 2017). The menthol-induced enhancement is due to a combination of effects on nicotinic acetylcholine receptor (nAChR) upregulation (Brody et al., 2013; Henderson et al., 2016; Henderson et al., 2017), dopamine neuron excitability (Henderson et al., 2018), dopamine release (Zhang et al., 2018), and TrpM8-dependent mechanisms (Fan et al., 2016). In addition to menthol, similar investigations into other popular ENDS flavors are being conducted. A recent report determined that green apple and other fruit flavors are nearly the most popular among all flavorant options available (Espino-Diaz et al., 2016; Omaiye et al., 2019). An investigation into one chemical flavorant of green apple, farnesol, reported that farnesol produced reward-related behavior in mice in the absence of nicotine (Avelar et al., 2019). Furthermore, this effect was sex-dependent and was found to be caused by changes in nAChR upregulation and ventral tegmental area (VTA) dopamine neuron firing in males only at the doses examined.

Based on these previous investigations, we have examined another chemical flavorant of green apple, farnesene, to determine its effect on vaping-related behaviors. We used conditioned place preference assays to observe farnesene's effect on reward-related behavior, confocal microscopy to observe nAChR changes on midbrain dopamine and GABA neurons associated with the reward pathway, and whole-cell patch-clamp electrophysiology to study changes in midbrain neuron function. These experiments were performed with mice genetically modified to express fluorescently labeled nAChRs and were used in microscopy or electrophysiology following behavioral assays. Following these experiments, we have demonstrated that farnesene enhances nicotine reward-related behavior and prompts reward-related behavior in the absence of nicotine, and thus, may explain the prominence of green apple flavors among ENDS users. Additionally, we identified farnesene acts as a partial agonist for nAChRs and stimulates inward currents on VTA dopamine neurons. Finally, we observed that while farnesene does not exert a pronounced effect on VTA nAChR upregulation, long-term treatment with farnesene alters nAChR stoichiometry to promote the assembly of high-sensitivity nAChRs. Overall, these data identify the significance in studying ENDS flavors and demonstrate the potential underlying mechanisms that may promote the initiation and maintenance of ENDS use among the youth.

MATERIAL AND METHODS

Reagents and Dose Selection – (–)-Nicotine hydrogen tartrate (product number – 1463304) and Farnesene (product number – W383902-100G-K, lot number – MKCB6021) were obtained from Sigma-Aldrich. We determined the relevant dose of farnesene in regard to mouse behavioral assay doses. According to Tierney et al. (2016), flavorants range from 1-20-fold the amount of nicotine in the traditional cigarette as well as ENDS, with an average flavor concentration of ~12 mg/mL (Omaiye et al., 2019). It has been confirmed that 0.5 mg/kg nicotine is rewarding for mice in conditioned place preference assays (Tapper et al., 2004; Henderson et al., 2016; Henderson et al., 2017). Given this dose of nicotine, we determined the clinically relevant dose of farnesene is 0.5 - 10 mg/kg and used a dosing range of 0.1, 1.0, and 10 mg/kg for this study.

Mice – All experiments were conducted in accordance with the guidelines for care and use of animals provided by the National Institutes of Health. Protocols were approved by the Institutional Animal Care and Use Committee at Marshall University. Mice were group housed on a standard 12/12 hr light/dark cycle at 22^{o}C and given food and water *ad libitum*. For all assays, we used α4-mCherryα6-GFP mice (see below), originated from a C57BL/6J strain that are genetically modified to contain α4* and α6* nAChR fluorescent tags. These mice have been shown to exhibit similar levels of nAChRs to wild-type mice and behave similar to wild-type mice in conditioned place preference assays (Mackey et al., 2012; Srinivasan et al., 2016; Henderson et al., 2017; Avelar et al., 2019). Following CPP assays, mouse brains that were homozygous for α4-mCherry and transgenic for α6-GFP were used in confocal microscopy assays (20 mice, see Figure 3). For a portion of the behavioral experiments, wildtype littermate mice and Jackson Laboratory C57BL/6J mice were used alongside α4-mCherryα6-GFP mice. For all experiments, we used adult (3 – 6 months old) mice. Both male and female mice were used and numbers of each are detailed below in the methods for specific experiments and given in detail in corresponding figures.

Our genetically modified α4-mCherryα6-GFP mice were the result of crossing α4-mCherry homozygous knock-in mice (Srinivasan et al., 2016) with α6-GFP bacterial artificial chromosome (BAC) transgenic mice (Mackey et al., 2012). α4-mCherry knock-in mice are backcrossed to C57BL/6J mice every 10 generations while α6-GFP mice are continuously backcrossed to C57BL/6J mice (from Jackson Laboratory; https://www.jax.org/strain/000664).

Genotyping **–** On postnatal day 21, mice were weaned and housed with same-sex littermates. Concomitantly, tail biopsies were taken for genotyping analysis by PCR (Transnetyx, Cordova, TN). Only mice that were transgenic for α6-GFP and homozygous for α4-mCherry were used in confocal assays (see below), with the exception of α6-GFP and α4-mCherry mice used for NFRET controls.

Locomotor Assays **–** Adult male and female non-transgenic α4-mCherryα6-GFP mice (3 - 5 months old) were habituated to the experimental room for ~1 h prior to the experiment (n = 6-7 mice/sex). Mice were placed in an open field (40 x 40 x 36 cm) immediately after an intraperitoneal injection of saline or farnesene (0.1 mg/kg). Distance traveled over a period of 20 minutes was recorded using motion tracking software (SMART 3.0). Number of mice are listed in Figure 1B.

Conditioned Place Preference (CPP) Assays **–** CPP assays were conducted in a three-chamber spatial place-preference apparatus (Harvard Apparatus, PanLab) over a 10-day period, using male and female mice. Time spent in chambers was recorded by motion tracking software (SMART 3.0). The test consisted of three stages: pre-test, injections, post-test. An unbiased protocol was used where 'drugs' (saline, nicotine (0.5 mg/kg), farnesene alone (0.1, 1.0, or 10 mg/kg), or 0.1 mg/kg farnesene plus 0.5 mg/kg nicotine) were given immediately before confinement in the right white/grey chamber on 'drug' days and saline was given immediately before confinement in the left white/black chamber on saline days. Each conditioning period lasted 20 minutes. Prior to the use of experimental mice during each stage, a scrap mouse of the same sex was used to deposit odors for 20 minutes. In the pre-test stage, mice were placed in the central chamber and given 20 minutes of free access to all chambers. Drug-naïve mice that spent >65% of their time in one of the two conditioning chambers were removed from the study and the remaining mice were counter-balanced, similar to previously published methods (Neugebauer et al., 2011; Einstein et al., 2013; Lee et al., 2020). Eight males and five females were excluded. During stage 2, intraperitoneal injections were given in the white/grey chamber (saline control, farnesene, nicotine, or farnesene plus nicotine, dissolved in saline) or white/black chamber (saline). The mice received their designated drug injections on days 2, 4, 6, and 8, and received saline injections on days 3, 5, 7, and 9. In the post-test stage, mice were again placed in the central chamber and given 20 minutes of free access to all chambers. Adult male and female mice, 3-6 months old, were used in CPP assays (60 males and 57 females total). Previous reports have shown that the use of α4-mCherryα6-

GFP mice have displayed no differences in nicotine reward-related behavior as tested by conditioned place preference in comparison to C57BL/6J mice (Henderson et al., 2017; Avelar et al., 2019). Sex differences are further discussed in *Results*. Data are expressed as a change in baseline preference between the post- and pre-test. Drug treatments were blind to experimenters until all data analysis was completed.

Confocal Imaging of Mouse Brain Slices – α4-mCherryα6-GFP mice used in CPP assays were also used in microscopy assays. Following the completion of CPP assays, mice were euthanized with CO_2 and subjected to a swift cardiac perfusion with 10 mL ice-cold saline to reduce autofluorescence in the mCherry emission range. Brains were then swiftly removed, flash frozen with acetone and dry ice, and then stored at -80°C. Brains were coronally sectioned (20 µm) using a cryostat, mounted with Vectashield (Vector labs, H-1000), and coverslipped. We targeted bregma -3.1 mm (anterior-posterior limits of -2.9 to -3.3 mm) because this region gave the most consistent sections that contained a large portion of the VTA, substantia nigra pars reticulata (SNr) and substantia nigra pars compacta (SNc) in a single slice.

A Leica SP5 TCSII confocal microscope was used to excite α6-GFP and α4-mCherry at 488 and 561 nm, respectively. 20X images with a 10X digital zoom were collected for the quantitative measurements of α4-mCherry and α6-GFP neuron raw integrated density (RID). Normalized Förster Resonance Energy Transfer (NFRET) was calculated using the PixFRET ImageJ plug-in to identify α4α6* nAChRs.

All experimenters were blind to drug treatment until all data analysis was completed. Approximately 30-60 VTA dopamine neurons and ≥30 VTA and SNr GABA neurons were imaged. Data from these images were averaged to provide RID values for each mouse. A total of 20 mice were used in confocal assays, aged 3–5 months (n provided in Figure 3).

Neuro-2a Cell Culture and Transient Transfections – Mouse neuroblastoma 2a (neuro-2a) cells were cultured in the following medium: MEM with 5% fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Cells were plated by adding 90,000 or 50,000 cells (microscopy and electrophysiology, respectively) to poly-ɒ-lysine-coated 35mm glass-bottom imaging dishes (MatTek Corporation) and cultured in a humidified incubator (37°C, 95% air, 5% CO_2). Cells were transfected with 500 ng of each nAChR subunit cDNA plasmid (α4-mCherry, α4-GFP, and β2wt or α4-mCherry, α6-GFP, and β2wt depending on intended subtype target and FRET pairing). Following plating procedures, plasmid DNA was mixed with 250 µl of Opti-

MEM and Lipofectamine-3000 was separately added to the Opti-MEM. After 5 min at 24°C, the two solutions were combined and incubated at 24°C for 25 min. Plated neuro-2a cells then received the mixed solution and were incubated for 24h. The next day, Opti-MEM was removed, and the cells received growth medium. 500 nM filter-sterilized farnesene (or sham treatment) was added after replacing the Opti-MEM with standard culture medium. 24 h after drug/sham addition, neuro-2a cells were imaged on a confocal microscope (discussed above) or examined using electrophysiological methods (discussed below).

Whole-Cell Patch-Clamp Electrophysiology – Using brain slices from 3-5-month-old male and female α6-GFP mice, we identified putative dopamine (pDA) neurons of the VTA for recordings. Dopamine neurons of the lateral VTA selectively express α6* nAChRs making our α6-GFP mice suitable for identifying pDA neurons in electrophysiological assays. After recent work detailing the presence of α6* nAChRs on medial VTA glutamate neurons (Yan et al., 2018), we restricted our recordings to the lateral VTA to increase our chance of accurately identifying pDA neurons. Following behavioral assays, mice were anesthetized with CO_2 and then cardiac perfusion was performed using ice-cold NMDG-based artificial cerebral spinal fluid (NMDG-ACSF) saturated with 95% O_2 / 5% CO_2 (carbogen) containing (in mM): 93 NMDG, 2.5 KCl, 1.2 NaH_2PO_4, 10 $MgSO_4$, 0.4 $CaCl_2$, 30 $NaHCO_3$, 5 Na-ascorbate, 3 Na-pyruvate, 2 thiourea, and 25 glucose. Brains were removed and kept in agarose for slicing with a Compresstome® VF-300-OZ (Precisionary Instruments). Coronal brain sections (250 µm) were cut into cold carbogenated NMDG-ACSF to obtain slices containing the VTA (target bregma -3.1 mm; anterior-posterior limits of -2.7 to -3.5 mm) and were then allowed to recover at 32°C in carbogenated NMDG-ACSF for 12-15 min. Following this, slices were transferred to standard ACSF containing (mM): 125 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 26 $NaHCO_3$, and 11 glucose for one hour at 32°C. One hour later, slices were transferred to the recording chamber and continuously perfused with carbogenated ACSF (1.5 - 2.0 ml/min) at 32°C.

Neurons were visualized with an upright microscope (Axio Examiner A1, Zeiss) equipped with an Axiocam 702 mono using DIC near infrared illumination. Blue illumination was used to visualize α6-GFP presence in putative dopamine neurons. Whole-cell patch-clamp techniques were used to record electrophysiological signals with an Integrated Patch-Clamp Amplifier (Sutter). Patch electrodes had resistances of 4 – 10 MΩ when filled with intrapipette solution (in mM): 135 K gluconate, 5 KCl, 5 EGTA, 0.5

$CaCl_2$, 10 HEPES, 2 Mg-ATP, and 0.1 GTP. Recordings were sampled at ≥10 KHz. The junction potential between patch pipette and bath solutions was nulled just before gigaseal formation. Series resistance was monitored without compensation throughout experiments using SutterPatch software. The recording sessions for neurons were terminated if the series resistance changed by more than 20%. Nicotine and farnesene (dissolved in ACSF at pH 7.4) applications were applied using pressure microinjection (Picospritzer III, Parker) at 5 psi. Drug concentrations and duration of applications are given in *Results*. While microinjections of nicotine mitigates many of the complications of usage in brain slices (diffusion in and out of tissue and cells), we used a maximum of 3 nicotine puffs per brain slice.

For the recordings of spontaneous excitatory post-synaptic currents, bath perfusion of ACSF was switched to an ACSF solution containing 100 µM picrotoxin (Sigma-Aldrich, catalog number 124-87-8) to block $GABA_A$ receptors. After 5 minutes, pDA neurons in the VTA were voltage clamped at -65 mV to record sEPSCs.

For cultured cells, 50,000 neuro-2a cells were plated onto sterilized 12 mm circular glass coverslips, placed in 35-mm culture dishes and cultured in a humidified incubator (37°C, 95% air, 5% CO_2). Cells were transfected as described above. For patching of neuro-2a cells we used extracellular solution containing (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose (320 mOsm, pH set to 7.3 with Tris-base). For voltage-clamp experiments, neuro-2a cells were voltage clamped at a holding potential of -55 mV. To avoid nAChR desensitization by repetitive nicotine application we applied drug puffs at ~3 min intervals and continually perfused the recording chamber with extracellular solution.

Statistical Analysis – All results are presented as mean ± SEM and all statistical analyses were performed using Graphpad Prism. CPP data (Figures 1A and 2) were analyzed with either a one-way (drug factor) or two-way ANOVA (sex x drug x interaction) with a *post-hoc* Tukey for means comparison. Unpaired data (figures 1B, 3, 4, 5, 7, and $8E_{1-2}$) were analyzed by t-test. Figure 8B was analyzed with a one-way ANOVA (drug factor) with a *post-hoc* Tukey for means comparison. Complete statistical tests and results are displayed in the statistical table provided (Table 1). Power analyses (G*Power software, www.gpower.hhu.de) were used to determine efficient sample sizes (Tables 2 – 6).

RESULTS

Farnesene-alone prompts reward-related behavior

Here, we examine a chemical flavorant of green apple, farnesene, for its ability to alter reward-related behavior. Using male and female α4-mCherryα6-GFP mice (C57BL/6J background) and farnesene doses of 0.1, 1.0, and 10 mg/kg, we conducted CPP assays to measure reward-related behavior. The previous report that examined another green apple flavorant, farnesol, observed a sex-dependent effect on mice in CPP assays (Avelar et al., 2019). Accordingly, we hypothesized that farnesene may produce a sex-dependent effect given the fact that its chemical structure is very similar to farnesol. We observed a sex-dependent and drug-dependent effect in our CPP assays with the above farnesene doses, as well as a significant interaction between sex and drug factors (two-way ANOVA (sex x drug dose x interaction); sex factor: $F_{(1, 88)} = 10.55$, $p = 0.002$; drug factor: $F_{(5, 88)} = 12.21$, $p < 0.0001$; and interaction factor: $F_{(5, 88)} = 3.045$, $p = 0.014$). Following the significant sex-dependent effect, we examined the effect of farnesene on reward-related behavior separately for male and female mice.

We noted a significant effect of farnesene in male and female mice (males: one-way ANOVA, $F_{(3, 30)} = 5.98$, $p = 0.003$, Figure $1A_1$; females: one-way ANOVA, $F_{(3, 25)} = 9.81$, $p = 0.0002$, Figure $1A_2$). In males, farnesene at a dose of 0.1 mg/kg prompted a significant rewarding effect compared to saline- ($p = 0.0065$), 1.0 mg/kg farnesene- ($p = 0.020$), and 10 mg/kg farnesene-treated mice ($p = 0.005$). Higher doses of farnesene in male mice did not exhibit a significant change from baseline compared to saline, which may mimic nicotine's inverted-U dose response in CPP assays. On the other hand, female mice exhibited a significant change from baseline with all farnesene doses when compared to saline (1.0 mg/kg, $p = 0.013$; 10 mg/kg, $p = 0.040$; Figure $1A_2$), with the greatest change from baseline at 0.1 mg/kg ($p < 0.0001$, Figure $1A_2$). We note, farnesene doses tested may need to be higher in order for the females to observe an inverted-U response similar to the males. Similarly, we may need to test lower doses in both males and females to determine the threshold dose for reward-related behavior. Overall, these data support the idea that farnesene at 0.1 mg/kg is rewarding on its own in both male and female mice. Following these observations, follow-up assays were performed with 0.1 mg/kg dosing in male and female mice.

Additionally, we conducted locomotor assays to determine the effects of acute farnesene treatment on locomotor behavior in male and female mice. It is well understood that nicotine increases locomotor activity in mice (Wall et al., 2017), and was recently determined that green apple flavorant, farnesol, also increased this behavior (Avelar et al., 2019). Here, male and female mice were administered intraperitoneal injections of saline or 0.1 mg/kg farnesene, and locomotor activity was assessed in an open field test (Figure $1B_{1\text{-}2}$). Unlike the previous reports, farnesene did not significantly alter the ambulatory behavior of either male or female mice compared to saline treatment (males, $p < 0.111$, Figure $1B_1$; females, $p < 0.801$, Figure $1B_2$).

Farnesene enhances nicotine reward

Due to the fact that many ENDS users prefer flavored nicotine-containing products, and the findings that farnesene-alone is rewarding (Figure 1A), it is important we study the combining effect of farnesene and nicotine on reward-related behavior. To do so, we performed additional CPP assays using saline, nicotine, and nicotine combined with the peak farnesene dose (0.1 mg/kg). Many previous reports have used 0.5 mg/kg nicotine (intraperitoneal) to produce a rewarding effect in C57 mice (Tapper et al., 2004). Accordingly, we selected this dose for these CPP assays. Only male mice exhibited significant nicotine reward-related behavior (one-way ANOVA, $p = 0.017$ and $p = 0.292$ for males and females, respectively; Figure 2A-B), however in both sexes we noted a significant nicotine + farnesene effect compared to saline (one-way ANOVA; males, $p = 0.002$, Figure 2A; females, $p = 0.0001$, Figure 2B). Additionally, both sexes exhibited enhanced rewarding effects in the nicotine + farnesene group compared to nicotine alone, but only females exhibited a significant enhancement ($p = 0.004$).

Despite effects on reward-related behavior, farnesene does not upregulate nAChRs on VTA dopamine neurons

Following behavioral assays, we performed confocal microscopy to observe potential changes in nicotinic acetylcholine receptor (nAChR) levels based on the long-standing knowledge that chronic nicotine exposure upregulates nAChRs (Nashmi et al., 2003; Nashmi et al., 2007; Henderson et al., 2014; Srinivasan et al., 2016) and that green apple flavorant, farnesol, worked in a similar manner (Avelar et al., 2019). Following

the well-characterized effects of nAChR upregulation in midbrain neurons, we focused on dopamine and GABA neurons in the VTA and GABAergic neurons in the substantia nigra pars reticulata (SNr) (Figure 3A). Using α4-mCherryα6-GFP mice, we examined α4β2* (* denotes other subunits may be present), α6β2*, and α4α6β2* nAChR density of putative dopamine and GABA neurons in the VTA and SNr in response to saline or farnesene treatment. Here, we used the same mice that were employed in conditioned place preference assays to maintain dosing relevant to reward-related behavior. To aid in the identification of putative dopamine neurons in the VTA, we used α6-GFP fluorescence as a marker, as α6-GFP nAChR subunits are highly expressed in dopamine neurons in the lateral VTA (Mackey et al., 2012) (described further in methods for electrophysiology). Given that GFP and mCherry are FRET pairings, we used pixel-based FRET methods to identify nAChRs that contain both α4 and α6 nAChR subunits (Figure 3B). Changes in nAChR number was determined by quantifying a change in raw integrated density (RID) of α4-mCherry or α6-GFP fluorescence. Unlike the previous study by Avelar et al. demonstrating farnesol's ability to upregulate nAChRs (2019), farnesene treatment produced no significant changes in nAChR number within putative dopamine or GABA neurons in the VTA or SNr (Figure 3C-D).

Despite the absence of upregulation, farnesene alters stoichiometry of nAChRs

In addition to examining the upregulation of nAChRs in mouse brain slices, we also investigated farnesene-induced changes in nAChR stoichiometry. Nicotine has long been known to selectively upregulate high-sensitivity nAChRs, including α4* and α6* nAChRs (Kuryatov et al., 2005; Srinivasan et al., 2011), while menthol (in the absence of nicotine) and cytisine have been shown to upregulate low-sensitivity nAChRs (Srinivasan et al., 2012; Henderson et al., 2016). α4β2 nAChRs exist in two stoichiometries – high-sensitivity ($\alpha4_{(2)}\beta2_{(3)}$) and low-sensitivity ($\alpha4_{(3)}\beta2_{(2)}$) (Nelson et al., 2003). α6* nAChR stoichiometries are characterized as α6β2, α6β2β3, or α4α6β2β3 (Lindstrom et al., 1987; Xiao et al., 2011; Henderson et al., 2014).

To determine farnesene's effects on nAChR stoichiometry, we used a pixel-based, normalized Förster Resonance Energy Transfer (NFRET) method (Srinivasan et al., 2012). Aside from quantifying the RID of α4*, α6*, and α4α6* nAChRs, we additionally examined the effect of farnesene treatment on NFRET intensity and pixel count as both are informative measurements regarding the change in number of nAChR pentamers that

contain both an α4 and α6 nAChR subunit (Figure 4). In both males and females we saw no significant changes in the mean NFRET intensity between saline and farnesene treatment groups (Figure $4A_1$ and $4B_1$). Yet, in females-only we observed a significant decrease in the mean pixel count following farnesene treatment (p = 0.048; Figure $4B_{2\text{-}3}$). This indicates that farnesene reduces the number of α4α6* nAChRs on VTA dopamine neurons in female mice. Given that females demonstrated the greatest range in farnesene-induced reward-related behavior, this finding may explain why we only see an effect on pixel count in female mice.

Given that our mouse model only allows analysis of changes in α4α6* nAChR stoichiometry, we used *in vitro* methods to examine both α4α6β2 and α4β2 nAChR stoichiometry following farnesene exposure. Accordingly, we studied nAChR stoichiometry changes using neuroblastoma-2a (neuro-2a) cells that were transiently transfected with α4-mCherry, α6-GFP, and β2wt or α4-mCherry, α4-GFP, and β2wt nAChR subunits to examine changes in α4α6β2 and α4β2 nAChR stoichiometry (Figure 5A and 5C, respectively). Cells transiently transfected to contain α4-mCherryα6-GFPβ2 nAChRs (Figure 5A) were treated with control media or 0.5 μM farnesene for 24 hrs. We selected 0.5 μM farnesene to match previous studies with the similarly structured terpene, menthol (Henderson et al., 2016) and farnesol (Avelar et al., 2019). In farnesene-treated cells we noted a significant decrease in the mean pixel count (p < 0.0001, Figure $5B_1$) and the mean NFRET percentage (p < 0.0001, Figure $5B_2$) which indicates a significant decrease in the number of α4-α6 nAChR subunit pairings. Thus, the data obtained with *in vitro* methods matches those obtained using mouse brain slices. Cells transiently transfected to contain α4-mCherryα4-GFPβ2 nAChRs (Figure 5C) were also treated with control media or 0.5 μM farnesene and we also observed a significant decrease in mean pixel count (p = 0.0149, Figure $5D_1$) and mean NFRET percentage (p < 0.0001, Figure $5D_2$). Accordingly, this indicates a decrease in the number of low-sensitivity $\alpha4_{(3)}\beta2_{(2)}$ nAChRs. These results are further summarized in Figure 6. Here we show that long-term farnesene treatment promotes the inclusion of high-sensitivity nAChRs by transitioning the number of low-sensitivity $\alpha4_{(3)}\beta2_{(2)}$ (Figure 6A) and α4α6β2 (Figure 6B) nAChRs to a majority of high-sensitivity $\alpha4_{(2)}\beta2_{(3)}$ nAChRs.

Farnesene alters VTA dopamine neuron function

Although we found no significant changes in nAChR up- or down-regulation on VTA neurons, the changes in stoichiometry observed in VTA dopamine neurons and neuro-2a cells suggests farnesene alters stoichiometry, potentially toward high-sensitivity α4β2 nAChRs. To examine this in an *in vivo* model we used brain slice whole-cell electrophysiology. Given that high- and low-sensitivity α4β2* nAChRs can be functionally distinguished through concentration-response relationships (Nelson et al., 2003; Xiao et al., 2009), we utilized similar methods to determine if farnesene treatment altered nAChR stoichiometry on putative VTA dopamine neurons. Mackey et al. has previously demonstrated α6 nAChRs exhibit complete overlap with tyrosine hydroxylase in VTA dopamine neurons (2012). Thus, several previous investigations have used α6-GFP fluorescence, in addition to other electrophysiological markers, as a method to identify dopamine neurons within the VTA and SNc (Mackey et al., 2012; Henderson et al., 2016; Henderson et al., 2017; Avelar et al., 2019). However, more recent investigations have shown that α6-GFP nAChRs are present on glutamate neurons in the medial portions of the VTA (Yan et al., 2018). To increase our chances of identifying putative dopamine neurons, we targeted α6-GFP positive cells (Figure 7A$_{1-2}$) in the lateral VTA that exhibited classical electrophysiological markers of dopamine neurons (Margolis et al., 2006; Margolis et al., 2008): hyperpolarizing sag (I_h), firing frequency, and action potential widths >2ms. Together, these features have been proven to provide a robust method of identifying putative dopamine neurons (Mackey et al., 2012; Henderson et al., 2017). We used coronal brain slices obtained from saline or 0.1 mg/kg farnesene treated mice using a dosing paradigm that matches the above CPP protocol and targeted bregma -3.1 to match confocal microscopy assays. Putative VTA dopamine neurons were voltage-clamped at -65 mV and 0.5, 5, 10, and 100 μM concentrations of nicotine were applied using localized pressure injection with a micropipette (5 psi, 10 s applications) (Figure 7B$_{1-2}$, 7C). In the farnesene-treated condition we noted a leftward shift in the concentration-response of nicotine, indicative of a shift toward more high-sensitivity nAChRs (Figure 7C). In addition to recording nicotine-stimulated inward currents, we also recorded spontaneous excitatory post-synaptic currents (sEPSCs) in putative dopamine neurons (Figure 7D). Here, we observed that chronic farnesene increased the baseline frequency (p = 0.0163) and amplitude (p < 0.0001) of sEPSCs on putative VTA dopamine neurons compared to saline treatment (Figure 7E$_{1-2}$).

Farnesene acts as a partial agonist of nAChRs

The previous report on farnesol, another green apple flavorant, showed that farnesol did not stimulate nAChR-mediated currents on its own but likely functions as a noncompetitive antagonist (Avelar et al., 2019). To examine farnesene's pharmacological actions, we first started with acute applications on putative VTA dopamine neurons (Figure 8A-B). We observed that 5 and 500 μM farnesene stimulates inward currents in putative VTA dopamine neurons (mean peak current amplitudes of 27.5 ± 3.3 and 58.5 ± 9.6 pA, respectively, Figure 8B). We note that 0.5 μM farnesene (consistent with *in vitro* NFRET assays) did not produce any notable inward currents (data not shown). Using ACSF containing DhβE (0.3 μM), we observed that farnesene-induced (500 μM) inward currents are dependent on β2* nAChRs (Figure 8A). Finally, we used 100 μM nicotine to obtain a comparative efficacy for farnesene and determined that farnesene possessed ≤ 44.0 ± 7.3% efficacy compared to nicotine (at 100 μM) on putative VTA dopamine neurons. 5 and 500 μM farnesene had a significantly lower peak current amplitude compared to 100 μM nicotine (p = <0.0001 and 0.0031, respectively). In examining peak current amplitudes of nAChRs on dopamine neurons it is difficult to isolate exactly which subtypes are activated. To address this, we used neuro-2a cells transiently transfected to contain either α4-GFPβ2 or α6-GFPβ2β3 nAChRs (Figure 8C). These two nAChR subtypes exhibit distinct sensitivities to nicotine and can be maximally stimulated by 3 μM nicotine (α6-GFPβ2β3 nAChRs) or 100 – 300 μM nicotine (α4-GFPβ2 nAChRs) (Henderson et al., 2014). For neuro-2a cells containing nAChRs, we applied 500 μM farnesene (300 ms applications) and observed mean peak current amplitudes of 21.2 ± 3.0 and 23.8 ± 6.1 pA for α6-GFPβ2β3 and α4-GFPβ2 nAChRs, respectively (Figure $8E_{1\text{-}2}$). Using 3 μM and 100 μM nicotine, we observed mean peak current amplitudes of 109.7 ± 14.3 (p < 0.0001) and 1190.4 ± 192.3 (p < 0.0001) pA for α6-GFPβ2β3 and α4-GFPβ2 nAChRs, respectively (Figure $8E_{1\text{-}2}$). Thus, the comparative efficacy of farnesene to nicotine is 19.3 ± 2.7 and 2.1 ± 0.6% for α6-GFPβ2β3 and α4-GFPβ2 nAChRs, respectively.

Table 1. Statistical Tests and Results

Figure	Type of Test	Interaction/ Main Effect	Statistical Data
1, 2	two-way ANOVA	Sex x Drug	$F_{(5, 88)} = 3.045$, $p = 0.0140$
1, 2	two-way ANOVA	Sex	$F_{(1, 88)} = 10.55$, $p = 0.0016$
1, 2	two-way ANOVA	Drug	$F_{(5, 88)} = 12.21$, $p < 0.0001$
$1A_1$	one-way ANOVA		$F_{(3, 30)} = 5.98$, $p = 0.0025$
$1A_1$	*post-hoc* Tukey		saline vs 0.1 mg/kg; $p = 0.0065$
$1A_1$	*post-hoc* Tukey		saline vs 1.0 mg/kg; $p = 0.9997$
$1A_1$	*post-hoc* Tukey		saline vs 10 mg/kg; $p = 0.9770$
$1A_1$	*post-hoc* Tukey		0.1 mg/kg vs 1.0 mg/kg; $p = 0.0203$
$1A_1$	*post-hoc* Tukey		0.1 mg/kg vs 10 mg/kg; $p = 0.0047$
$1A_1$	*post-hoc* Tukey		1.0 mg/kg vs 10 mg/kg; $p = 0.9680$
$1A_2$	one-way ANOVA		$F_{(3, 25)} = 9.81$, $p = 0.0002$
$1A_2$	*post-hoc* Tukey		saline vs 0.1 mg/kg; $p < 0.0001$
$1A_2$	*post-hoc* Tukey		saline vs 1.0 mg/kg; $p = 0.0126$
$1A_2$	*post-hoc* Tukey		saline vs 10 mg/kg; $p = 0.0402$
$1A_2$	*post-hoc* Tukey		0.1 mg/kg vs 1.0 mg/kg; $p = 0.4631$
$1A_2$	*post-hoc* Tukey		0.1 mg/kg vs 10 mg/kg; $p = 0.2245$
$1A_2$	*post-hoc* Tukey		1.0 mg/kg vs 10 mg/kg; $p = 0.9669$
$1B_1$	Unpaired t-test		saline vs. farnesene (males), $p = 0.111$
$1B_2$	Unpaired t-test		saline vs. farnesene (females), $p = 0.801$
2A	one-way ANOVA		$F_{(2, 23)} = 8.506$, $p = 0.0017$
2A	*post-hoc* Tukey		saline vs nicotine; $p = 0.0173$
2A	*post-hoc* Tukey		saline vs nicotine + farnesene; $p = 0.0022$
2A	*post-hoc* Tukey		nicotine vs nicotine + farnesene; $p = 0.4280$

2B	one-way ANOVA		$F_{(2, 25)} = 13.04$, $p = 0.0001$
2B	*post-hoc* Tukey		saline vs nicotine; $p = 0.2916$
2B	*post-hoc* Tukey		saline vs nicotine + farnesene; $p = 0.0001$
2B	*post-hoc* Tukey		nicotine vs nicotine + farnesene; $p = 0.0041$
$3C_1$	Unpaired t-test		[α4, $p = 0.2239$], [α6, $p = 0.9065$], [α4α6, $p = 0.6259$]
$3C_2$	Unpaired t-test		$p = 0.1268$
$3C_3$	Unpaired t-test		$p = 0.1227$
$3D_1$	Unpaired t-test		[α4, $p = 0.0515$], [α6, $p = 0.4842$], [α4α6, $p = 0.0653$]
$3D_2$	Unpaired t-test		$p = 0.5572$
$3D_3$	Unpaired t-test		$p = 0.6156$
$4A_1$	Unpaired t-test		$p = 0.6343$
$4A_2$	Unpaired t-test		$p = 0.7225$
$4B_1$	Unpaired t-test		$p = 0.5285$
$4B_2$	Unpaired t-test		$p = 0.0480$
$5B_1$	Unpaired t-test		$p < 0.0001$
$5B_2$	Unpaired t-test		$p < 0.0001$
$5D_1$	Unpaired t-test		$p = 0.0149$
$5D_2$	Unpaired t-test		$p < 0.0001$
$7E_1$	Unpaired t-test		$p = 0.0002$
$7E_2$	Unpaired t-test		$p < 0.0001$
8B	one-way ANOVA		$F_{(2, 12)} = 23.05$, $p < 0.0001$
8B	*post-hoc* Tukey		5 μM Farnesene vs 500 μM Farnesene; $p = 0.1823$
8B	*post-hoc* Tukey		5 μM Farnesene vs 100 μM Nicotine; $p < 0.0001$
8B	*post-hoc* Tukey		500 μM Farnesene vs 100 μM Nicotine, $p = 0.0031$
$8E_1$	Unpaired t-test		$p < 0.0001$
$8E_2$	Unpaired t-test		$p < 0.0001$

Table 2. G*Power Statistics – Conditioned Place Preference (Fig. 1 and 2)			
Input Parameters		**Output Parameters**	
Test Family	F Tests	**Noncentrality Parameter**	16.94
Test Type	ANOVA: one-way	**Critical F**	2.21
Type of Analysis	A priori	**Numerator df**	7
Effect size	0.55	**Denominator df**	48
α err prob	0.05	**Total Sample Size**	56
Power	0.8	**Sample size/group**	7
Number of groups	8	**Actual Power**	0.811
Sample size indicates number of mice needed per treatment group.			

Table 3. G*Power Statistics – Locomotor Behavior (Fig. $1B_{1-2}$)			
Input Parameters		**Output Parameters**	
Test Family	T tests (two tails)	**Noncentrality Parameter**	3.21
Test Type	Biserial model	**Critical t**	2.23
Type of Analysis	A priori	**Df**	10
Effect size	0.68	**Total Sample Size**	12
α err prob	0.05	**Sample size/group**	6
Power	0.8	**Actual Power**	0.825
Number of groups	2		
Sample size indicates the number of neurons/cells needed for sufficient power.			

Table 4. G*Power Statistics – Fluorescence Microscopy (Fig. 3 and 4)			
Input Parameters		**Output Parameters**	
Test Family	T tests (two tails)	**Noncentrality Parameter**	3.53
Test Type	Biserial model	**Critical t**	2.57
Type of Analysis	A priori	**Df**	5
Effect size	0.8	**Total Sample Size**	7
α err prob	0.05	**Sample size/group**	≥3
Power	0.8	**Actual Power**	0.803
Number of groups	2		
Sample size indicates number of mice needed per treatment group.			

Table 5. G*Power Statistics – Brain Slice Electrophysiology (sEPSCs, Fig. 7)			
Input Parameters		**Output Parameters**	
Test Family	T tests (two tails)	**Noncentrality Parameter**	3.25
Test Type	Biserial model	**Critical t**	2.26
Type of Analysis	A priori	**Df**	9
Effect size	0.7	**Total Sample Size**	11
α err prob	0.05	**Sample size/group**	6 – 7
Power	0.8	**Actual Power**	0.823
Number of groups	2		
Sample size indicates the number of neurons needed for sufficient power.			

Table 6. G*Power Statistics – Electrophysiology (inward currents, Fig. 8)			
Input Parameters		**Output Parameters**	
Test Family	T tests (two tails)	**Noncentrality Parameter**	3.40
Test Type	Biserial model	**Critical t**	2.36
Type of Analysis	A priori	**Df**	7

Effect size	0.75	**Total Sample Size**	9
α err prob	0.05	**Sample size/group**	≥5
Power	0.8	**Actual Power**	0.830
Number of groups	2		
Sample size indicates the number of neurons/cells needed for sufficient power.			

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DISCUSSION

With the growing popularity of ENDS products among all ages and the large preference for flavored e-liquids (Huang et al., 2018; Mead et al., 2018; Schneller et al., 2018), the goal of our study was to determine how one green apple flavorant, farnesene, may affect vaping-related behaviors using behavioral, neurobiological, and neurophysiological assays. Menthol has long been the most studied flavorant for its large prevalence in combustible cigarettes and is known to enhance nicotine reward (Henderson et al., 2016; Henderson et al., 2017) and nicotine reinforcement (Wang et al., 2014; Biswas et al., 2016). Yet, with the increase in flavorant production with more than 15,000 ENDS flavorant options available to the vaping community (Hsu et al., 2018), there is an urgent need to understand how flavorants alter neurobiology and neurophysiology. A recent investigation revealed farnesol (a green apple flavorant similar in structure to farnesene) behaves similarly to menthol by enhancing nicotine reward; but differs in the fact that it causes reward-related behavior on its own (Henderson et al., 2017; Avelar et al., 2019). Here we noted similar findings: farnesene, a green apple flavorant, causes reward-related behavior on its own. However, one key difference from the previous report is that farnesene produces reward in male and female mice while farnesol only produced an effect with males (Avelar et al., 2019). Additionally, farnesol caused significant upregulation of α6-containing nAChRs (Avelar et al., 2019), but farnesene did not upregulate nAChRs. Instead, farnesene was observed to alter nAChR stoichiometry to promote the expression of more high-sensitivity nAChRs as evidenced by electrophysiology and NFRET assays in mouse brain slices and cultured cells.

This is a very significant finding considering that >90% of adolescents ENDS users and >70% of adult ENDS users prefer flavored products (Mead et al., 2018; Schneller et al., 2018). Given the absence of restrictions on most flavored ENDS, it's important we document the effects that flavorants have on altering the addictive nature of nicotine and determining potential addictive properties in flavorants alone. Although we observed reward-related behavior with farnesene in both sexes, we did note sex differences in our conditioned place preference assays. First, females exhibited reward-related behavior at all doses of farnesene examined (0.1, 1, and 10 mg/kg) while males exhibited reward to only 0.1 mg/kg farnesene. This may be due to the fact that females metabolize farnesene faster than males, similar to nicotine (Matta et al., 2007). Additionally, females did not exhibit significant nicotine reward with 0.5 mg/kg nicotine. To date, nicotine dose-response

data has primarily been in male mice, and those in females are only given via subcutaneous injection (Kota et al., 2008). Due to the lack of nicotine dose-response curve data using intraperitoneal injections in females, this may explain the lack of significant nicotine reward at the given dose. Although there is a significant change from baseline for nicotine + farnesene in both males and females, the change from baseline is less than farnesene-alone in both sexes. While others have reported significant conditioned place preference with 0.5 mg/kg nicotine in CPP assays using both sexes, this could highlight the need to re-examine nicotine dose-responses with both sexes in this assay. Additionally, we would like to note that acute farnesene did not significantly alter locomotor behavior compared to saline treatment in male or female mice. Although nicotine and farnesol were observed to increase locomotor activity in mice, previous studies have demonstrated these changes are likely mediated through α6β2* and α4α6β2* nAChRs (Drenan et al., 2010; Cohen et al., 2012). Given that the farnesene-induced behavioral effects shown here are likely mediated through high-sensitivity α4β2 nAChRs, this may be the reason we did not see any significant changes in locomotor behavior between farnesene- or saline-treated mice.

Although farnesol and farnesene exhibit similar behavioral results, they differ in the cellular aspects that drive reward-related behavior. Based on previously published findings that chronic nicotine exposure promotes the upregulation of high-sensitivity nAChRs on VTA dopamine, VTA/SNr GABA, and habenular neurons and the recent evidence that farnesol acted in a similar manner, our initial investigations focused on the number of α4*, α6*, and α4α6* nAChRs present on dopamine and GABA neurons as a consequence of farnesene exposure. We noted no significant changes between farnesene and saline treatment groups. The reward-related behaviors we noted may not be attributed to nAChR upregulation, but instead by changes in nAChR stoichiometry. While many are well-informed regarding the various subtypes of nAChRs, there also exist distinct stoichiometries that confer different sensitivities to nicotine (Nelson et al., 2003; Tapia et al., 2007). Both our microscopy measurements (NFRET) and brain slice electrophysiology data support the fact that farnesene treatment, consistent with conditioned place preference dosing, produces more high-sensitivity nAChRs on VTA dopamine neurons. This increase in high-sensitivity (likely α4β2) nAChRs results in not only increased sensitivity to nicotine, but also to the endogenous neurotransmitter acetylcholine (ACh).

In the case of changes in stoichiometry, we note there are limitations to our methods. Currently, we possess only the capability to measure α4α6* nAChR stoichiometry *in vivo*. Thus, we required the use of heterologous cells and transfection methods in an *in vitro* system to conduct fluorescence microscopy assays to study changes in α4β2 nAChR stoichiometry. We acknowledge that this may not truly represent *in vivo* systems and we note a distinct difference between the *in vivo* and *in vitro* findings with α4α6* nAChRs (compare Figure 4 to Figure $5B_{1\text{-}2}$). These two systems also differ in the time-dependency of nAChR upregulation and stoichiometry changes. Nicotine or flavorant induced nAChR upregulation and changes in stoichiometry occur over the course of 10-12 days *in vivo* (Henderson et al., 2016; Henderson et al., 2017; Nashmi et al., 2007), whereas the *in vitro* system findings occur in 24 hours (Srinivasan et al., 2011). This difference in time-dependency for *in vitro* preparations is accompanied by the lacking complexity of a cell's environment *in vivo* (Fu et al., 2019). Stoichiometry changes within *in vivo* systems are not only brain region-specific (Fu et al., 2019), but they also rely on unique cellular machinery that is absent in our *in vitro* model. Despite this, our transfection methods follow validated protocols that have matched several *in vivo* mouse and human studies (Srinivasan et al., 2011; Henderson et al., 2017), and we are able to detect a change in nAChR stoichiometry that is consistent with our findings using brain slice electrophysiology as both suggest the presence of more high-affinity nAChRs.

Farnesene treatment significantly increased the frequency and amplitude of sEPSCs. The excitatory inputs to the VTA dopamine neurons we studied are local and distal glutamate neurons (medial VTA, prefrontal cortex) and distal cholinergic neurons (peduncular pontine tegmentum and laterodorsal tegmentum). The inhibitory inputs are GABA neurons in the VTA, rostromedial tegmentum, and interpeduncular nucleus. Thus, the changes in sEPSC frequency and amplitude are likely due to changes in glutamatergic or GABAergic local inputs given that we used a coronal brain slice preparation. While many of the cholinergic inputs are distal and are not present in a coronal slice preparation, some cholinergic signaling is maintained and this is likely a source of elevated sEPSC frequency and amplitude. Regardless, the enhanced frequency and amplitude of sEPSCs in VTA dopamine neurons suggests that farnesene triggers enhanced activity that may drive dopamine release through the mesolimbic pathway and thus contribute to reward-related behavior.

In addition to this, we observed that farnesene itself acts as a partial agonist, likely through α6-containing nAChRs, as it exhibited very low efficacy on α4β2 nAChRs transiently transfected into neuro-2a cells. Here, we must acknowledge that a dose of 500 μM is likely not vaping-relevant but it was necessary to determine the comparable efficacy to nicotine. Despite this, 5 μM farnesene still produced detectable inward currents in VTA dopamine neurons and this suggests that farnesene may also weakly stimulate nAChRs in the VTA. Here, further investigations would need to be conducted to determine the human vaping-relevant concentrations of farnesene that would be present in the brain and how this specific concentration alters neurophysiology. Even with these caveats, this finding is significant, especially in the rising popularity of zero-nicotine e-liquids. Green apple flavors (depending on the specific flavorants present) may directly stimulate nAChRs and this could be the rationale for their popularity.

The data reported in this study suggest that 1) farnesene enhances nicotine reward and displays reward-related behavior through the increase in high-sensitivity nAChRs on VTA dopaminergic neurons; 2) that farnesene changes nAChR stoichiometry and results in an enhanced affinity and potency of nicotine and ACh; and 3) that this ultimately leads to enhanced excitatory postsynaptic currents on VTA dopamine neurons and thus greater likelihood of action potential generation. Overall, this may explain why flavored ENDS are growing in popularity and may contribute to low cessation rates. The finding of enhanced nicotine reward when flavorants are present and the fact that flavorants-alone may be rewarding, further indicates the importance of studying flavorants for their potential to alter vaping-related behaviors. However, there are components of this local VTA circuitry that still need to be elucidated: 1) how are VTA glutamatergic inputs and their nAChRs altered by farnesene; 2) how are α4β2 nAChRs on VTA GABA neurons altered functionally; and 3) what is the net effect of farnesene on dopamine release. Additionally, there are several green apple flavorants that are still unknowns in the scope of how they alter vaping-related behavior (methylbutyl acetate, hexyl acetate, and ethyl acetate). Based on these findings, it is of importance we continue to investigate ENDS flavors for their role in nicotine addiction. With the continuous rise in ENDS use, especially among the adolescent population, it is critical we depict that ENDS flavors are not a simple additive to the devices, instead they are an enhancer to the addictive properties of nicotine and a potential threat to zero-nicotine flavored ENDS users as well.

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Figure Legends

Figure 1. Farnesene-alone produces reward-related behavior in male and female mice. (A_{1-2}) Male and female mice were administered saline or farnesene at doses of 0.1, 1.0, or 10 mg/kg in a CPP assay. (B_{1-2}) Male and female mice were administered saline or 0.1 mg/kg farnesene in an open field locomotor assay. All data are mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$; one-way ANOVA with *post hoc* Tukey (A) or unpaired t-test (B). Exact *p* values are given in the Results section. Number of mice for each treatment group in CPP assays is indicated in parenthesis. Dots within bars represent the CPP scores or locomotor activity from individual mice within the designated treatment groups.

Figure 2. Farnesene (0.1 mg/kg) enhances nicotine reward-related behavior in both sexes. (A-B) Male and female mice were administered saline, nicotine (0.5 mg/kg), or nicotine (0.5 mg/kg) plus farnesene (0.1 mg/kg) in a CPP assay. All data are mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; one-way ANOVA with *post hoc* Tukey. Exact *p* values are given in the Results section. Number of mice for each treatment group is indicated in parenthesis and dots within bars represent the CPP scores from individual mice within the designated treatment group.

Figure 3. Farnesene treatment has no effect on nAChR number in the midbrain. (A_1) Schematic of target mouse brain region (bregma -3.1 mm). (A_2) Sample 10X image of a mouse coronal brain section at approximately bregma -3.1 mm. Scale bar, 250 µm. (B) Sample images of saline and farnesene treated VTA dopamine neurons. Scale bar, 15 µm. (C-D) Raw integrated density (RID) of α4*, α6*, and α4α6* nAChRs of VTA dopamine neurons (C_1 and D_1), α4* nAChRs of VTA GABA neurons (C_2 and D_2), and α4* nAChRs of SNr GABA neurons (C_3 and D_3) in saline and farnesene (0.1 mg/kg) treated male (C) and female (D) mice. All data are mean ± SEM. Unpaired t-test. Dots indicate the RID values from individual mice.

Figure 4. Farnesene alters the stoichiometry of α4α6β2* nAChRs in VTA DA neurons. (A_1 and B_1) Mean NFRET percentage, (A_2 and B_2) mean NFRET pixel count, and (A_3 and B_3) mean pixels / neuron histograms for saline- and farnesene-treated (0.1 mg/kg) VTA dopamine neurons in male (A) and female (B) mice. All data

667 are mean ± SEM. *, $p < 0.05$; unpaired t-test. Exact p values are given in the Results section. Dots within bars
668 represent the values from individual mice within the designated treatment group; n = > 40 neurons per mouse
669 per treatment group.
670
671 **Figure 5. Farnesene favors high-sensitivity nAChRs in neuro-2a cells.** Representative neuro-2a cells
672 transfected with α4-mCherry, α4-GFP or α6-GFP, and β2wt nAChR subunits to produce (A) α4-mCherryα6-
673 GFPβ2 nAChRs or (C) α4-mCherryα4-GFPβ2 nAChRs. Scale bar, 10 μm. Mean NFRET pixel count (B_1 and
674 D_1) and NFRET percentage (B_2 and D_2) treated as control or with 0.5 μM farnesene for (A) α4-mCherryα6-
675 GFPβ2 nAChRs or (C) α4-mCherryα4-GFPβ2 nAChRs. All data are mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; ****,
676 $p < 0.001$; unpaired t-test. Exact p values are given in the Results section. Dots within bars represent the
677 values from individual cells within the designated treatment group; n > 30 cells per condition.
678
679 **Figure 6. Farnesene favors high-sensitivity nAChRs in neuro-2a cells.** (A) α4β2 nAChRs assemble in two
680 stoichiometries and we observed that farnesene treatment shifts a mixed population of HS and LS α4β2
681 nAChRs to a majority of HS α4β2 nAChRs. (B) In examining α4α6β2 nAChRs, under control treatments ~65%
682 of the population are α4α6β2 nAChRs while the remainder is likely α4β2 nAChRs. Following treatment with
683 farnesene, <14% of the nAChRs are α4α6β2 nAChRs.
684
685 **Figure 7. Farnesene enhances the affinity and potency of nicotine.** Representative images of VTA putative
686 dopamine (pDA) neurons in a brain slice (bregma -3.1) were identified through the presence of α6-GFP
687 nAChRs in IR-DIC (A_1) and GFP fluorescence (A_2) imaging modes. Scale bars, 20 μm. (B) Representative
688 inward currents from VTA pDA neurons (α6-GFP positive) with 10 s applications of 500 nM (B_1) or 10 μM (B_2)
689 nicotine in voltage-clamp mode. Arrows indicate start of nicotine puff application and dotted red lines indicate
690 baseline prior to puff and the duration of nicotine application. (C) Average nicotine concentration response of
691 peak-current amplitude of VTA pDA neurons (n = 7 neurons / 4 mice and 5 neurons / 3 mice per nicotine
692 concentration for saline and farnesene-treated mice, respectively). (D) Representative waveforms of sEPSCs
693 from VTA pDA neurons recorded from saline-treated or farnesene-treated mice in the presence of 30 μM

picrotoxin. (E) Mean sEPSC frequency (E_1) and amplitude (E_2) in saline-treated and farnesene-treated mouse brain slices (n = 9 neurons / 4 mice and 9 neurons / 3 mice for saline- and farnesene-treated mice, respectively). For all assays, drug treatments were consistent with the CPP assay paradigm using 0.1 mg/kg farnesene. (C and $E_{1\text{-}2}$) Data are mean ± SEM. ***, $p < 0.005$; ****, $p < 0.0001$; unpaired t-test. Exact p values are given in the Results section. Dots within bars represent the values from individual neurons within the designated treatment group.

Figure 8. Farnesene acts as a partial agonist on nAChRs. (A and B) Voltage-clamp recordings from putative VTA dopamine neurons. (A) 5 and 500 μM farnesene and 100 μM nicotine were applied to putative VTA dopamine neurons. The β2* nAChR antagonist, DhβE (0.3 μM) blocked inward currents stimulated by 500μM farnesene. (B) Mean peak current amplitude for farnesene and nicotine applications on putative dopamine neurons in the VTA. (C, D, and E) Voltage-clamp recordings from neuro-2a cells transiently transfected to contain α4-GFPβ2 and α6-GFPβ2β3 nAChRs. (C) Representative images of neuro-2a cells that contain α4-GFPβ2 or α6-GFPβ2β3 nAChRs. (D) Representative inward currents stimulated by 300 ms applications of 500 μM farnesene on neuro-2a cells containing α4-GFPβ2 or α6-GFPβ2β3 nAChRs. ($E_{1\text{-}2}$) Mean peak current amplitude of 500 μM farnesene and nicotine applications (3 μM and 100 μM Nicotine for α6-GFPβ2β3 and α4-GFPβ2 nAChRs, respectively) on neuro-2a cells containing nAChRs. (B and $E_{1\text{-}2}$) Data are mean ± SEM. **, $p < 0.01$; ****, $p < 0.0001$; one-way ANOVA with *post hoc* Tukey (B) or unpaired t-test (E). Dots represent data from individual neurons or cells. Exact p values are given in the Results section.