CELLULAR AND BEHAVIORAL EFFECTS OF NICOTINE VAPOR EXPOSURE ON MOUSE MESOLIMBIC REWARD CIRCUITRY

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ABSTRACT

ManHua Zhu: Cellular and behavioral effects of
nicotine vapor exposure on mouse mesolimbic reward circuitry
(Under the Direction of Melissa Herman)

Nicotine is a highly addictive compound in tobacco products such as combustible cigarettes and electronic cigarettes. Although the trends for smoking combustible cigarettes in the United States have decreased over the last couple decades, the emergence of electronic nicotine delivery systems (ENDS) products have grown in popularity and use in adults and adolescents. These products are often marketed as a safer alternative to combustible cigarettes, however the effects of electronically delivered nicotine vapor exposure on the brain remain unclear. In the first set of experiments, we validated and utilized a preclinical electronic nicotine vapor exposure paradigm to examine the behavioral and cellular effects of vaporized nicotine. We found that a single session versus repeated sessions of intermittent nicotine vapor exposure differentially altered body temperature, locomotion, and neuronal activity in a stress-relevant nucleus, the central amygdala (CeA). In the second set of experiments, we examined the effects of different nicotine concentrations and formulations (freebase versus salt) delivered via vapor exposure. We found sex differences by concentration and formulation in serum nicotine levels, neuronal activity in the CeA and ventral tegmental area (VTA), and anxiety-like and motivated behavior. In the last set of experiments, we investigated the neuronal activity and inhibitory control of corticotropin-

releasing factor receptor 1 (CRF1) neurons in the VTA following acute and chronic nicotine vapor exposure. VTA CRF1 neurons, which are mainly dopaminergic and project to the nucleus accumbens, showed sex-specific basal differences in phasic inhibition. Following acute nicotine vapor exposure, tonic inhibition was enhanced in females but reduced in males, and overall neuronal activity was increased in both sexes. These effects were no longer observed following chronic nicotine vapor exposure suggestive of neuroadaptations that occur with repeated nicotine vapor exposure. Overall, these findings demonstrated that exposure to electronic nicotine vapor induces sex-, exposure timing-, nicotine dose-, and formulation-specific changes on reward and stress related neural populations and circuits, which may have significant implications for understanding how ENDS use impacts human populations.

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LIST OF ABBREVIATIONS

CeA	Central amygdala
CPA	Conditioned place aversion
CPP	Conditioned place preference
CRF or CRH	Corticotropin-releasing factor or Corticotropin-releasing hormone
CRF1	Corticotropin-releasing factor receptor 1
CTA	Conditioned taste aversion
E-cig	Electronic cigarettes
ENDS	Electronic nicotine delivery system
FDA	US Food and Drug Administration
GFP	Green fluorescent protein
HPA	Hypothalamic-pituitary-adrenal
IHC	Immunohistochemistry
IP	Intraperitoneal
LDT	Laterodorsal tegmentum
LHb	Lateral habenula
NAc	Nucleus accumbens
nAChR	Nicotinic acetylcholine receptors
PG/VG	Propylene glycol/ Vegetable glycerol
PVN	Paraventricular nucleus of the hypothalamus
SC	Subcutaneous
VTA	Ventral tegmental area
WHO	World Health Organization

CHAPTER 1: INTRODUCTION

Nicotine is a highly addictive substance found in tobacco products and its continued use can lead to nicotine dependence or addiction. The most current Diagnostic and Statistical Manual of Mental Disorders (DSM-5) from the American Psychiatric Association defines tobacco use disorder as a “problematic pattern of tobacco use leading to clinically significant impairment or distress.” Tobacco use disorder is characterized by tobacco craving and seeking, continued use despite negative consequences, development of tolerance, and expression of withdrawal symptoms following abrupt cessation of tobacco use (DSM-5). Smoking and the use of tobacco products have many deleterious effects on the body that contribute to heart/cardiovascular diseases, lung/respiratory diseases, cancer, diabetes, and stroke, as well as others (CDC, 2014). Tobacco use is frequently comorbid with other psychiatric conditions such as anxiety, depression, schizophrenia, and attention deficit disorders. Additionally, tobacco use is often concomitant with use of other drugs of abuse leading to polysubstance use. With increased societal awareness of the known adverse effects of tobacco use, many people who smoke turn to smoking cessation treatments to curb craving and withdrawal symptoms. Currently, there are seven FDA approved medications, many falling into the nicotine replacement therapy category, such as over-the-counter nicotine patch or nicotine gum. Other smoking cessation medications such as varenicline and bupropion require a prescription. The most

effective cessation treatments often combine medication with behavioral or counseling therapy (García-Gómez et al., 2019). The development and availability of these different smoking cessation treatments have helped many to quit smoking and the percentage of adults in the United States that currently smoke has declined from ~21% in 2005 to ~12% in 2020 (Cornelius et al., 2022). However, despite the overall cultural shift away from acceptance of smoking and the decreasing smoking population, smoking remains as the leading cause of preventable death. Tobacco use caused more than 480,000 deaths per year in the United States (CDC, 2014) and more than seven million deaths per year globally (WHO, 2017). Economically, cigarette smoking related health conditions cost the US more than $240 billion in healthcare spending and close to $185 billion in lost productivity (Shrestha et al., 2022).

Combustible cigarettes versus electronic nicotine delivery systems (ENDS)

Many of the known negative health effects from smoking cigarettes result from the exposure to the smoke created during the combustion of tobacco in traditional cigarettes. Electronic nicotine delivery systems (ENDS) such as electronic cigarettes, often referred to as e-cigarettes or e-cigs, were first developed as an alternative method of delivering nicotine without burning tobacco and exposure to tobacco smoke. These devices come in a variety of sizes and shapes but all function similarly by using a battery to heat up a heating element that vaporizes a liquid solution into an aerosol that is inhaled by the user. The liquid solutions (sometimes referred to as e-liquids) often contain nicotine and/or other additives including flavorants. These devices have been marketed as a ‘safer’ alternative to combustible tobacco cigarette smoking and have

become a culturally accepted and practiced form of nicotine replacement therapy, similar to nicotine gum and nicotine patches. However, electronic cigarettes are currently not approved by the US Food and Drug Administration (FDA) as a form of smoking cessation aid. While combustible tobacco cigarette use has continued to decline over the years, the prevalence of e-cigarette use has increased over the last couple decades. The percentage of adults in the US who currently use e-cigarettes increased from 2.8% in 2017 to 3.7% in 2020 (Cornelius et al., 2022). Among current adult e-cigarette users, ~40% formerly smoked cigarettes, ~37% currently still smoke cigarettes, and ~23% have never smoked cigarettes (CDC, 2021). These statistics show that while e-cigarettes were able to help some former smokers with smoking cessation, the emergence of e-cigarette use has also increased the population of dual use (both combustible and electronic cigarettes) and promoted nicotine use in a population that never smoked combustible cigarettes before. ENDS use is especially prevalent in the adolescent and young adult demographic with a report showing that ~25% of high school seniors have used ENDS products in the past month and ~12% who use ENDS daily (NIDA, 2019). The health effects, especially long-term effects, of electronically delivered nicotine are still not well understood and remain an area of further scientific investigation to help better inform policies regarding and regulating ENDS use in different age groups. Additionally, because ENDS and other vapor products often contain the addictive compound nicotine, the rewarding and abuse liability of these products must be further examined and evaluated.

Nicotine formulation and concentration

In addition to the wide range in the variety of ENDS products, the composition of the heated solution (e-liquids) commercially available is also highly variable. The base components of e-liquids consist of propylene glycol (PG) and vegetable glycerol (VG) however, the ratio of the two solutions can vary. Additionally, additives such as nicotine and many different flavorants, such as apple, menthol, tobacco, or spearmint, are added to the PG/VG base solution to cater to individual preferences and differences in use. To complicate the plethora of combinations even more, the formulation and concentration of nicotine found in e-liquid can also be quite variable. Nicotine can be in its unprotonated form, referred to as freebase nicotine. The addition of organic salts protonates the nicotine and results in nicotine salt. Most earlier versions of ENDS products included nicotine freebase, however, newer models such as Juul devices have switched to nicotine salts. Previous surveys have found that users report increased smoothness and reduced bitterness with nicotine salts as compared to freebase nicotine (Leventhal et al., 2021; O'Connell et al., 2019). The concentration of nicotine found in commercialized e-liquids can range from 0 to 90 mg/ml however, labeling is not always consistent across different e-liquid products and studies have found that the concentration on the label is often not accurate or consistent with actual nicotine content (National Academies of Sciences et al., 2018). With the rapid emergence of these ENDS products and the multitude of different customizable components, pre-clinical and clinical studies are still required to fully understand the effects of each of these components.

Nicotine reward seeking behavior

Many behavioral phenotypes of nicotine's rewarding and reinforcing effects have been demonstrated across multiple forms of nicotine delivery in preclinical animal models. Rats will lever-press to self-administer intravenously delivered nicotine (Gilpin et al., 2014; O'Dell et al., 2007). The self-administration of nicotine in rodents is also observed with nicotine delivered orally via drinking water (Adriani et al., 2002; Wong et al., 2020) and intracranially directly into the ventral tegmental area (Husson et al., 2020; Ikemoto et al., 2006; Maskos et al., 2005). More recently, studies have shown that this nicotine self-administration behavioral phenotype is also observed with inhalation of nicotine in vapor form in both rats (Lallai et al., 2021) and mice (Henderson & Cooper, 2021). However, nicotine's pleasurable effects follow an inverted U-shaped dose curve with high levels of nicotine leading to aversive effects and thus make it more challenging to elicit escalation of drug-seeking with nicotine as compared to other drugs of abuse such as cocaine, methamphetamine, alcohol, etc. Similarly, reports have shown that humans who smoke are able to self-titrate the level of nicotine within the body to maintain a 'pleasurable' level throughout the day (Ashton et al., 1979), but escalation of tobacco/nicotine in humans can develop over many years (Kim et al., 2009). In addition to the nicotine-seeking behavior demonstrated by nicotine vapor self-administration paradigms, rats have also shown conditioned placed preference to the nicotine vapor-paired chamber of a two-chambered box, indicative of a preference to nicotine vapor over vehicle solution vapor (Frie et al., 2020). Another hallmark of nicotine dependence is the expression of withdrawal symptoms following cessation of nicotine use. In humans, nicotine withdrawal symptoms include, but are not limited to, strong cravings

for nicotine/smoking, irritability and restlessness, trouble concentrating, sleep deficits, weight gain, anxiety, and depression (Hughes et al., 1991). Rodent animal models in nicotine withdrawal display spontaneous somatic signs of withdrawal that include head shakes, paw tremors, jumping, paw/genital licking, blinks, gasps, writhes, and ptosis (Malin et al., 1992). These withdrawal symptoms can also be precipitated with injections of mecamylamine, a non-selective antagonist of nicotinic acetylcholine receptors (nAChR) that nicotine acts on in the brain (Malin et al., 1994). These studies highlight the reinforcing properties of nicotine that drive drug-seeking behaviors in both animal and human models.

Nicotine and the reward circuitry

Nicotine activates the brain's mesolimbic reward circuitry. Canonically, nicotine binds to nicotinic acetylcholine receptors (nAChRs) on dopaminergic neurons in the ventral tegmental area (VTA) and drives the release of dopamine into the nucleus accumbens (NAc). The signaling between the VTA and NAc makes up the mesolimbic reward pathway and underlies the encoding and processing of rewarding stimuli in the brain. In addition to the dopaminergic neurons, the VTA contains a very heterogenous cell population including neurons that are glutamatergic, GABAergic, and even neurons co-expressing more than one neurotransmitter (Morales & Margolis, 2017). Many researchers have investigated the effects of nicotine in the brain and reward circuitry through the lens of understanding how different nAChRs subunit composition impact nicotine binding and receptor activation. Nicotinic acetylcholine receptors are pentameric ligand-gated ion channels found in muscle and in the brain. The five

subunits are arranged across the cell membrane to form a pore that is permeable to monovalent (Na^+ and K^+) and divalent (Ca^{2+}) cations. These channels have extracellular binding sites for endogenous compounds such as acetylcholine as well as exogenous compounds such as nicotine that can both stabilize the channel in its open conformation and allow the flow of cations through the center pore. The two most common subtypes of nAChRs in the brain are the heteromeric α4β2 and homomeric α7 subunit combinations, though many different combinations of nAChR subunits exist, each with distinct kinetic and desensitization properties (Picciotto et al., 2012). In VTA dopamine neurons, the β2 nAChR subunit directly increases dopamine firing and drives release of dopamine in the nucleus accumbens (Picciotto et al., 1998). Dopamine neuron excitability is modulated by both glutamatergic and GABAergic neurons in the VTA. Presynaptic GABAergic neurons express α4 and β2 nAChR subunits that transiently increase inhibitory transmission onto dopamine neurons, but these receptors quickly desensitize and thus show prolonged reduction in inhibitory transmission. Presynaptic glutamatergic neurons express α7 nAChR subunits that increase excitatory transmission onto dopamine neurons, and these receptors show lower desensitization (Mansvelder et al., 2002; Wooltorton et al., 2003). Thus, the combination of both inhibitory GABAergic signaling and excitatory glutamatergic signaling results in a net shift toward excitation. Following repeated or chronic nicotine use, the ability of nicotine to activate this mesolimbic reward pathway and encode reward is altered. Specifically, nAChR expression in the VTA is upregulated resulting in decreased tonic firing of VTA dopaminergic neurons (Grieder et al., 2012). Concurrently, there is a reduction in dopamine neurotransmitter levels in the downstream NAc (Fung et al., 1996; Hildebrand

et al., 1998). Two smoking cessation medications specifically target these brain mechanisms; varenicline binds to nAChRs to block nicotine's ability to bind and bupropion blocks dopamine reuptake to counteract the downregulation of dopamine transmission (García-Gómez et al., 2019).

Nicotine in stress and anxiety disorders

Many surveys around the world have found that smoking is positively correlated with a variety of anxiety disorders such as generalized anxiety disorder, panic disorder, and obsessive-compulsive disorder (Lawrence 2009). Additionally, smokers often cite relieving stress or anxiety as a reason to smoke and continue smoking (Doherty et al., 1995; Parrott, 1993), and elevated anxiety levels increase the risk of smoking relapse in female smokers (Piper 2010). Immediately after smoking a cigarette, smokers do indeed report decreased subjective stress levels, however, between cigarettes when nicotine levels are low, smokers report increased stress levels (Parrott, 1994). Additionally, longitudinal studies found that adults who smoked heavily during their adolescence are at a higher risk of developing agoraphobia, generalized anxiety disorder, and panic disorder (Johnson 2000). Similarly, preclinical studies have found that nicotine plays an important role in regulating stress and anxiety behaviors, and vice versa, stress can drive relapse to nicotine seeking (Bruijnzeel et al., 2009). One of the main brain regions involved in stress regulation is the central amygdala (CeA), as it is involved with integrating emotionally-relevant sensory information to coordinate appropriate behavioral responses. The CeA is a mainly GABAergic nuclei that receives glutamatergic inputs from the lateral and basolateral amygdala and GABAergic inputs

from the intercalated cells and local GABA neurons. The CeA's main projection targets include the bed nucleus of the stria terminalis and locus coeruleus for stress response, the periaqueductal gray for pain processing, and the VTA for reward-related behavior (Gilpin et al., 2015). Acute administration of nicotine has been shown to increase neuronal activation, as measured by cFos expression, in the CeA and bed nucleus of the stria terminalis in male rats (Cao et al., 2007), but also attenuate the increase in restraint stress-induced increase in cFos (Hsu et al., 2007). Additionally, activation of α2-adrenergic receptors in the CeA has been found to attenuate the stress-induced reinstatement of nicotine seeking in male rats (Yamada & Bruijnzeel, 2011). Although nicotine has historically been shown to increase VTA dopamine firing, recent studies have found that a primarily medial subpopulation of VTA dopamine neurons decrease firing in response to nicotine (Eddine et al., 2015). A subsequent study from the same group further discovered that the subpopulation of dopamine neurons that are inhibited by nicotine are specifically an amygdala-projecting population that is involved in modulating nicotine-induced anxiety-like behaviors (Nguyen et al., 2021).

Nicotine and the corticotropin-releasing factor (CRF) system

Although stress and anxiety are complex conditions that are mediated through multiple central and peripheral aspects of the nervous system, one area of significance is the corticotropin-releasing factor (CRF) system. CRF, or also referred to as corticotropin releasing hormone (CRH), is released by the hypothalamus as a part of the hypothalamic-pituitary-adrenal (HPA) axis. Activation of the HPA axis leads to the release of the primary stress hormone, cortisol, that underlies many of the peripheral

responses to stress, such as increased sweating, increased heart rate, pupil dilation, and reduced digestion. Upstream to the cortisol release, the CRF neuropeptide can exert central effects through the binding of CRF receptors. There are two main subtypes of CRF receptors: CRF receptor 1 (CRF1) and CRF receptor 2 (CRF2). Both receptor subtypes are expressed throughout the brain but CRF binds to CRF1 with higher affinity (Dedic et al., 2018). The CRF system has also been implicated in the actions of many drugs of abuse such as cocaine (Blacktop et al., 2011; Vranjkovic et al., 2018), alcohol (Agoglia et al., 2020; Herman et al., 2016), and nicotine (Grieder et al., 2014; Uribe et al., 2020). Acute nicotine exposure can activate the HPA axis and chronic exposure can alter basal HPA axis activity and blunt the HPA axis response to stress (Rohleder & Kirschbaum, 2006). In the CeA, nicotine and CRF systems are involved in modulating pain and anxiety-like behavior. Rats in nicotine withdrawal show increased CRF and CRF1 mRNA in the CeA, and application of a CRF1 antagonist within the CeA reduced the nicotine dependence-induced hyperalgesia phenotype (Baiamonte et al., 2014). Rats with long access to nicotine self-administration developed an abstinence-induced increase in anxiety-like behavior that was prevented by infusion of a CRF1 antagonist into the CeA (Cohen et al., 2015). CRF has also been implicated in mediating the negative affective states and dysphoria associated with nicotine withdrawal (Bruijnzeel et al., 2012; Bruijnzeel et al., 2009; Marcinkiewcz et al., 2009). In the VTA, studies have also shown that CRF increases VTA dopamine firing through activation of CRF1 receptors (Wanat et al., 2008; Zalachoras et al., 2022). VTA CRF1 neurons mainly project to the nucleus accumbens core, and the activation of these neurons helps coordinate reward reinforcement behaviors and enhance dopamine release (Heymann

et al., 2020). Knockout of CRF1 in midbrain dopamine neurons results in increased anxiety-like behavior and reduced dopamine release in the prefrontal cortex (Refojo et al., 2011). Additionally, chronic nicotine exposure has been shown to increase expression of CRF mRNA in the VTA (Grieder et al., 2014), and activation of the CRF-CRF1 system coordinates withdrawal-induced increases in nicotine self-administration (George et al., 2007). Although there are clear links between nicotine and the CRF1 system, the specific cellular effects of nicotine vapor exposure on VTA CRF1 neurons remain understudied.

Given the significant rise and prevalence of ENDS use, the overall goal of this dissertation was to better understand the cellular and behavioral impacts of electronic nicotine vapor exposure on reward and stress systems using mouse animal models. In chapter two, we aimed to validate a preclinical model of electronic nicotine vapor exposure in male mice and investigate its effects centrally on CeA neuronal activity and peripherally on body temperature and locomotor activity. In chapter three, we examined how different nicotine concentrations and formulations (salt vs freebase) impact CeA and VTA neuronal activity and anxiety-like behavior in female and male mice. In chapter four, we focused on the CRF1-expressing neuronal population in the VTA and examined how acute and chronic nicotine vapor exposure impacts neuronal activity and inhibitory signaling. Finally, in the last chapter, we will discuss the overall findings presented in this dissertation and their implications in the field of electronic nicotine vapor research. We will also discuss some interesting future lines of investigation and questions that arise from these findings. We hope that the studies in this dissertation will

shed light on the overall understanding of the impact of ENDS on reward and stress systems to improve our understanding of the neurobiology of nicotine vapor exposure and to better inform policy governing ENDS use in humans and advance the development of potential ENDS use cessation therapeutics.

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CHAPTER 2: ELECTRONIC NICOTINE VAPOR EXPOSURE PRODUCES DIFFERENTIAL CHANGES IN CENTRAL AMYGDALA NEURONAL ACTIVITY, THERMOREGULATION, AND LOCOMOTOR BEHAVIOR IN MALE MICE[1]

Introduction

Nicotine, as a component of tobacco smoke or other nicotine delivery devices, is a highly addictive drug. Nicotine addiction is characterized by repeated cycles of intake culminating in the need for regular consumption and withdrawal symptoms during periods of abstinence (Markou, 2008). These behaviors appear to be mediated by central adaptations at the cellular level that can lead to long-lasting changes in structure and function of neurons and neuronal networks following repeated drug exposure and withdrawal (Lüthi & Lüscher, 2014). Repeated exposure to drugs of abuse has been shown to produce diminished effects over time, which can contribute to the development of tolerance and promote increases or maintenance of drug-seeking behavior. Although previous work has identified important effects of nicotine on central and peripheral function (Picciotto & Kenny, 2013; Picciotto & Mineur, 2014), studies integrating the effects of nicotine exposure via vapor inhalation are lacking, as are studies comparing how the effects of nicotine vapor exposure change over time. As the route of administration has been shown to have differential effects on the metabolism

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and pharmacokinetics of nicotine (Benowitz et al., 2009), it is imperative to consider the impact of route of administration in preclinical studies and studies examining the effects of nicotine via a nicotine vapor model are warranted.

Historically, the most common method for nicotine delivery in humans was the smoking of tobacco products like cigarettes. However, the number of current adult smokers has been steadily declining (Wang et al., 2018) and the use of electronic vapor (or e-vape) systems is increasing in prevalence and popularity (Chaffee et al., 2017; Palazzolo, 2013), particularly among younger populations, with an estimated 27.5% of high school students and 10.5% of middle schools students reporting current use of e-cigarettes (Cullen et al., 2019). Vaping is commonly thought of as having less associated health risks compared to tobacco smoking and has been suggested as a replacement method for smoking cessation (Palazzolo, 2013). However, recent studies have shown that vaping can produce cytotoxic effects on airway tissue (Ghosh et al., 2018; Herman & Tarran, 2020; Lerner et al., 2015), and the effects of vaping on neuronal function and addictive behaviors remain unclear. Recent studies using nicotine vapor models similar to the one used here have demonstrated that nicotine vapor exposure alters temperature regulation and locomotor function in rats (Javadi-Paydar et al., 2019; Lallai et al., 2021), produces nicotine-induced conditioned place preference (Frie et al., 2020), as well as spontaneous and mecamylamine-precipitated somatic signs of withdrawal (Montanari et al., 2020). Additionally, self-administration of nicotine vapor has recently been demonstrated in rodents (Cooper et al., 2021; Lallai et al., 2021; Smith et al., 2020) and it has been shown that self-administration of nicotine vapor can be enhanced with the addition of e-liquid flavors such as green apple and

menthol (Cooper et al., 2021). Preclinical models utilizing electronic nicotine vapor are an important tool to investigate cellular and brain region-specific mechanisms involved in the stages of electronic nicotine vapor exposure and the development of nicotine dependence. Whereas other models of nicotine exposure offer significant advantages like of the ability to deliver more precisely-timed systemic or intravenous nicotine dosages or voluntary oral consumption, electronic nicotine vapor exposure offers better translational relevance with regards to its real-world nicotine consumption by inhalation. However, the field at large suffers from an incomplete understanding of the basic parameters of electronic nicotine vapor exposure (i.e. nicotine concentration, exposure frequency/length, etc) and the relevant cellular and behavioral consequences of different exposure paradigms.

Mechanistically, nicotine asserts its effects through the binding of nicotinic acetylcholine receptors, which are expressed throughout the nervous system (Picciotto & Mineur, 2014). A number of brain regions have been identified as targets of nicotine-induced plasticity, including the mesolimbic reward pathway and the amygdala (Adinoff, 2004). The central amygdala (CeA) is a central component of the limbic system and confers emotional relevance to internal and external sensory input to coordinate appropriate behavioral responses. In this context, the CeA has been implicated in numerous adaptive behaviors [feeding, fear learning, stress (Ciocchi et al., 2010; Douglass et al., 2017; Gilpin et al., 2015)] and maladaptive conditions [anxiety, depression, chronic stress, addiction (Bolton et al., 2018; Gilpin et al., 2015; Kenny et al., 2009; Koob et al., 1998)]. Nicotine has been shown to produce variable effects on CeA activity and plasticity dependent on dose, timing, and route of administration

(Brunzell et al., 2003; Valjent et al., 2004). Additionally, specific ensembles of neurons in the CeA have also been shown to contribute to the incubation of nicotine craving as evidenced by increased nicotine seeking following chronic intravenous nicotine self-administration and withdrawal (Funk et al., 2016). The CeA has been implicated in the central effects of nicotine, however the effects of acute and repeated nicotine vapor exposure on CeA electrophysiological activity and synaptic transmission remains understudied.

One of the primary goals of nicotine research is to understand how nicotine exposure impairs or dysregulates cellular functions to produce long-lasting maladaptive changes to brain circuitry and neuroplasticity. The present study utilizes a preclinical model of electronic nicotine vapor (e-vape) exposure to study the cellular and physiological consequences of acute and repeated exposure on CeA neuronal activity, thermoregulation, and locomotion.

Materials and Methods

Animals

For all experiments, adult male C57BL/6J mice (total N=104, The Jackson Laboratory) were used. All mice were group housed in a temperature- and humidity-controlled 12 hr light/dark (7am lights on, 7pm lights off) facility with ad libitum access to food and water and access to environmental enrichment. All experimental procedures were approved by the Institutional Animal Care and Use Committee.

Drugs

(-)-nicotine free base and propylene glycol (PG) were purchased from Sigma. Vegetable glycerol (VG) was purchased from Fisher. DNQX (10 mM), AP-5 (50 mM), and CGP55845A (1 mM) were purchased from Tocris Bioscience.

Electronic Nicotine Vapor Delivery System

Mice were placed in chambers for vaporized delivery of 120 mg/ml (-)-nicotine free base (Sigma N3876) in a 30/70 (v/v) propylene glycol (PG, Sigma P4347)/vegetable glycerol (VG, Fisher G33-500) solution or PG/VG control solution. Either 120 mg/ml Nic or PG/VG solution was filled into e-vape tanks (Baby Beast Brother, Smok) that were then screwed into the vapor generator (95Watt, Model SVS200, La Jolla Alcohol Research, Inc) that triggers the heating of the vape solution into vapor. The vape generator was connected to the e-vape controller (Model SSV-1, La Jolla Alcohol Research, Inc) that controls duration and frequency of vape delivery. The air-tight chambers are connected to a vacuum system that constantly pulls room air through the chambers at ~1 L per minute and ensures that each triggered vape is pulled into the chamber. Each 3 sec vape puff takes ~1minute to clear the chamber. Vape exposure sessions start between 9:00-10:00AM during the animals' light cycle, but vapor exposure was administered in the dark with lights off in the room. Repeated exposure was performed on consecutive days over the same approximate time period. After vape exposure, mice were returned to their home cage and regular housing facility.

Serum Analysis of Nicotine and Metabolites

Trunk blood from mice were collected immediately following acute or last session of repeated vape exposure. In animals used for slice electrophysiology experiments, trunk blood was collected following rapid decapitation. In animals used for cFos experiments, trunk blood was collected from cardiac puncture following pentobarbital (150mg/kg, ip) injection prior to perfusion. Separate cohorts of animals were used for measuring nicotine and cotinine levels following a single 3-sec 120 mg/ml nicotine vape (N= 6) and time course following acute vape (N= 4/time point). Trunk blood samples were spun down in a centrifuge and the serum layer was then collected and stored at -20°C before it was analyzed for nicotine and cotinine using liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described (Ghosh et al., 2019).

Slice Electrophysiology

Immediately following acute electronic vapor exposure (PG/VG N=6, Nic N=6) or the last session of repeated electronic vapor exposure (PG/VG N=6, Nic N=6), mice were rapidly decapitated, and brains were extracted and placed into an ice-cold sucrose solution containing (in mM): sucrose 206.0; KCl 2.5; CaCl2 0.5; MgCl2 7.0; NaH2PO4 1.2; NaHCO3 26; glucose 5.0; HEPES 5. Coronal slices (300μM thick) containing the central amygdala (CeA) were prepared with a Leica VT1000S (Leica Microsystems) and incubated in oxygenated (95% O2/5% CO2) artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl 120; KCL 2.5; EGTA 5; CaCl2 2.0 MgCl2 1.0; NaH2PO4 1.2; NaHCO3 26; Glucose 1.75; HEPES 5 for 30 min at 37°C, followed by 30 min equilibration at room temperature (20-22°C). For all recordings, patch pipettes (4-7 MΩ;

King Precision Glass Inc.) were filled with internal solution containing (in mM) KCl 145; EGTA 5; MgCl2 5; HEPES 10; Na-ATP 2; Na-GTP 0.2 and slices were superfused with oxygenated aCSF (described above). Cell firing was measured using the juxtacellular (cell-attached) configuration in gap-free voltage-clamp recording mode while membrane properties and spontaneous inhibitory postsynaptic currents (sIPSCs) were measured using whole-cell voltage-clamp (V_{hold} = -60 mV) recording modes in the presence of glutamate and $GABA_B$ receptor antagonists (DNQX, 20 μM; AP5, 50 μM; CGP55845A, 1 μM) to isolate $GABA_A$ receptor currents. All recording data were acquired with Multiclamp 700B amplifier (Molecular Devices), low pass filtered at 2–5 kHz, digitized (Digidata 1440A; Molecular Devices), and stored on a computer using pClamp 10 software (Molecular Devices).

Immunohistochemistry

Immediately following acute electronic vape exposure (PG/VG N=8, Nic N=8) or the last session of repeated electronic vapor exposure (PG/VG N=9, Nic N=9), mice were anesthetized with pentobarbital (150 mg/kg, i.p.) and perfused with 1X phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS. Brains were postfixed in 4% PFA at 4°C overnight then transferred to 30% sucrose in PBS at 4°C until brains sank. Brains were serially sectioned at 40 μM using either a cryostat (Leica CM3050S, Leica Biosystems) or a microtome (HM450, Thermo Fisher) and slices were stored in 0.01% sodium azide in PBS at 4°C.

Four to five sections containing the CeA from each animal were selected for cFos immunoreactivity. Sections were designated as anterior (bregma -0.70 to -0.94), middle (bregma -1.06 to -1.34), or posterior (bregma -1.46 to -1.58) CeA using a mouse brain atlas (Franklin & Paxinos, 2008) as reference. All sections were washed in PBS for 10 min then incubated with 50% methanol in PBS for 30 min, 3% hydrogen peroxide in PBS for 5 min, and blocking solution (0.3% Triton X-100 (Thermo-Fisher), 1% bovine serum albumin (BSA; Sigma) for 1 hr, all at room temperature (RT). Slices were then incubated at 4°C with rabbit anti-cFos primary antibody (1:3000, Millipore Sigma; ABE457) in blocking solution for 24-48 hrs. Sections were washed with Tris, NaCl, Tritonx-100 (TNT) buffer and Tris, NaCl, blocking reagent (Perkin-Elmer, TNB) buffer then incubated in goat anti-rabbit horse radish peroxidase (HRP, 1:200, Abcam ab6721) in TNB buffer for 30 min followed by another round of TNT buffer washes. Fluorescence signal in the CeA were amplified by incubating in tyramide conjugated fluorescein (1:50) in TSA amplification diluent (Akoya Biosciences, NEL741001KT) for 10 min at RT. Slices were washed again with TNT buffer before being mounted onto slides using Vectashield (Vector labs; H1500) and coverslipped. Fluorescent signal in the CeA was detected and imaged on a fluorescent microscope (Nikon Eclipse 6600) under 20x objective.

Body temperature

Core body temperatures were measured in mice (PG/VG N= 10, Nic N=10) immediately upon removal from the vapor chambers using a digital thermometer (Body Temperature Thermometer, 50316, Stoelting Co., Wood Dale, IL), with a mouse rectal

probe (#RET; 3/4” length, 0.028” diameter; Braintree Scientific, Braintree, MA). Repeated measures were taken from the same animals following one 3-Hr vape session (acute), five 3-Hr vape sessions (repeated), and 72-hrs following repeated vape session (withdrawal).

Open field locomotion

This test predicts how animals respond when introduced into a novel open arena and is used to capture spontaneous activity measures. The apparati are square white Plexiglas (50 x 50 cm) open fields illuminated to 360 lux in the center. Following body temperature assessment, each animal was placed in the center of the field and distance traveled and velocity were recorded during a 10-minute observation period and analyzed using Noldus Ethovision XT software.

Statistical Analysis

Membrane characteristics and excitability cell-attached firing data were analyzed with Clampfit 10.6 (Molecular Devices). Frequency, amplitude, and decay of spontaneous IPSCs (sIPSCs) were analyzed and visually confirmed using a semiautomated threshold-based detection software (Mini Analysis). Electrophysiological data are reported as individual cell and averaged by animal and Grubb’s outlier test was used to find and remove outliers in data sets. Quantification of immunohistochemistry was performed in a blinded manner on 2-6 sections spanning anterior-posterior axis of CeA per animal using ImageJ. Statistical analysis of all experimental parameters was carried out using Prism 9.0 (GraphPad). Experimental parameters were analyzed and

compared between groups using unpaired two-tailed t-test, one-way, or two-way ANOVA, with Sidak's repeated measures where appropriate. All data are expressed as mean ± SEM with $p<0.05$ set as the threshold for statistical significance.

Results

Electronic nicotine vapor exposure paradigm

To establish a model of passive electronic nicotine vapor exposure in mice, we employed a commercially available system from La Jolla Alcohol Research, Inc comprised of vacuum-controlled chambers (**Fig. 1.1A**) where time-triggered vapes are delivered (**Fig. 1.1B**), and cleared from the chamber in approximately one minute. To mimic the intermittent pattern of vaping in humans, we set the exposure parameters to deliver 3 sec vapes with 10 min intervals between vapes. To investigate the difference following a single vape exposure versus multiple vape exposures, we placed mice (1-5 per chamber) into the vape chambers (**Fig. 1.1A, B**) and exposed them to one of two vape paradigms, acute or repeated, respectively. In the acute vape exposure, mice were exposed to a 3-sec vape every 10 minutes over a 3-hour session (**Fig. 1.1C left**). In the repeated vape exposure, mice were exposed to the same 3-hr session, but for five consecutive days (**Fig. 1.1C right**). In both acute and repeated exposure paradigms, mice were separated into two groups where one group was exposed to vehicle control propylene glycol/vegetable glycerol (PG/VG) and the other was exposed to 120 mg/ml nicotine in PG/VG.

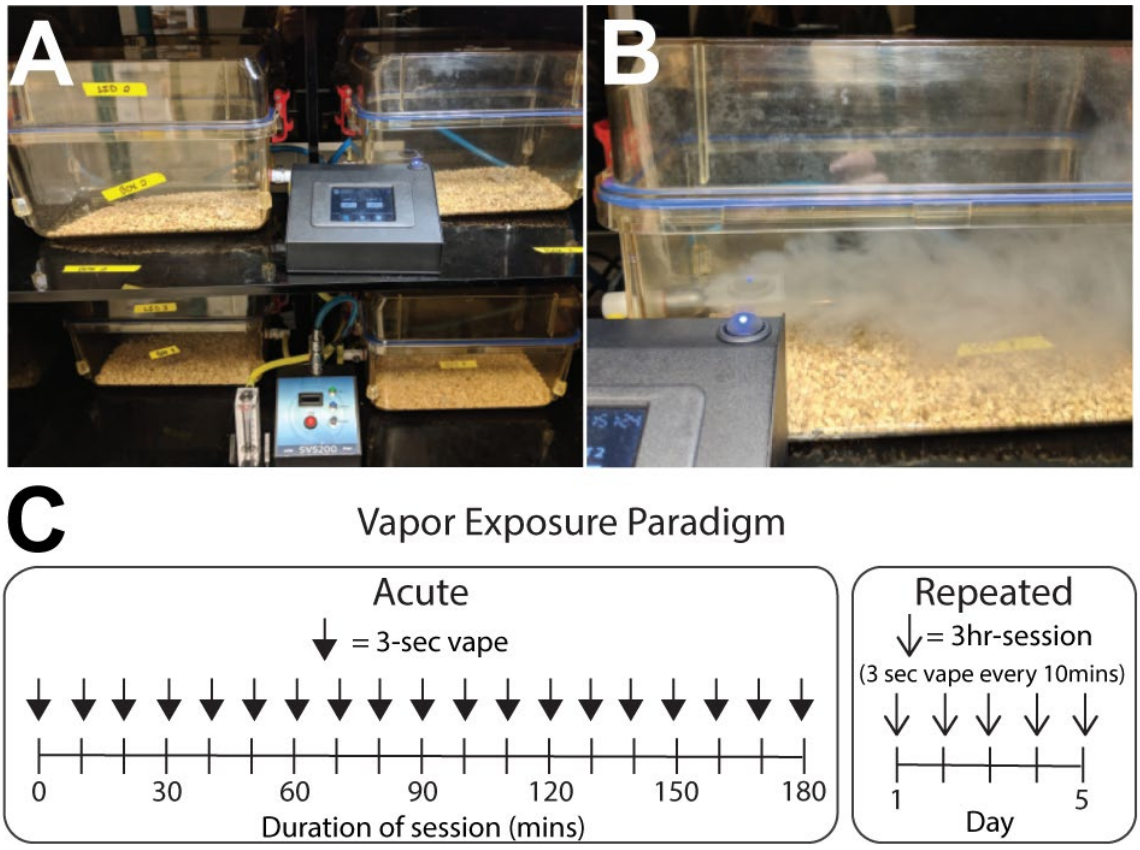

Figure 1.1- Mouse electronic nicotine vapor system, exposure parameters, and exposure paradigm (acute and repeated). (A) Mouse electronic nicotine vapor delivery system including vapor chambers, vapor generator, and e-vape controller. **(B)** Example of a 3-sec vapor delivery inside the chamber. **(C)** Vapor exposure paradigm of acute (left) and repeated (right) exposure.

Body weight and nicotine metabolism following electronic nicotine vapor exposure

To examine the impact of acute and repeated nicotine vapor exposure on body weight, mice exposed to PG/VG control or 120 mg/ml nicotine were weighed daily over the course of the exposure paradigm. Following acute exposure, average body weights of PG/VG and nicotine groups were not significantly different (PG/VG 30.92 ± 1.08 g, N=13; Nic 29.69 ± 0.94 g, N=13; t=0.8625, df=24, p=0.40, unpaired t-test, **Fig. 1.2A**). In

mice with repeated exposure, two-way ANOVA of body weight data showed no interaction of day x vape content ($F(4, 88) = 0.04896$, $p=0.9954$), no main effect of vape content ($F(1, 22) = 0.1302$, $p=0.7217$), but a main effect of day ($F(2.653, 58.36) = 19.47$, ****$p<0.0001$, **Fig. 1.2A**). However, a post hoc Sidak's multiple comparison's test show no significant differences between PG/VG and Nic groups for each day. This suggests that the body weight of mice exposed to 120 mg/ml nicotine was not significantly different from mice exposed to PG/VG control in both acute and repeated exposure paradigms and that electronic nicotine vapor exposure does not negatively impact the maintenance of body weight compared to PG/VG controls.

To examine nicotine metabolism following vapor exposure, we measured levels of serum nicotine and the nicotine metabolite cotinine. In a separate cohort of animals (N=6), we found that following a single 3-sec vape of 120 mg/ml nicotine, average serum nicotine was 20.21 ± 4.37 ng/ml and average serum cotinine was 4.862 ± 0.53 ng/ml (**Fig. 1.2B**). In mice exposed to an acute 3-hr session of vape (PG/VG N=13, Nic N=14), serum nicotine and cotinine were significantly greater in the 120 mg/ml Nic group as compared to PG/VG controls (serum nicotine: PG/VG 4.938 ± 1.679 ng/ml, Nic 319.2 ± 63/93 ng/ml, $t=4.804$, $df=25$, ****$p<0.0001$, unpaired t-test, **Fig. 1.2C left**; serum cotinine: PG/VG 8.045 ± 3.55 ng/ml, Nic 267.2 ± 44.29 ng/ml, $t=5.616$, $df=25$, ****$p<0.0001$, unpaired t-test, **Fig. 1.2C middle**). Serum nicotine and serum cotinine levels from acute 120 mg/ml nicotine vaped animals were positively correlated (slope= 0.3916, intercept= 142.3, $r(12)= 0.5562$, *$p=0.0389$, **Fig. 1.2C right**). In a separate cohort of mice, we measured serum nicotine and cotinine at 0 Hr, 1 Hr, 2 Hr, and 24 Hr, following an acute 3-hr vape session (PG/VG 0 Hr N=4, Nic 0 Hr N=4, Nic 1 Hr N=4, Nic

2 Hr N=4, Nic 24 Hr N=4). In comparison to the 0 Hr PG/VG control group, serum nicotine and cotinine were significantly greater in animals exposed to acute 120 mg/ml Nic at 0 Hr, 1 Hr, and 2 Hr but this difference was eliminated at 24 Hr (serum nicotine: 0 Hr PG/VG 1.280 ± 0.97 ng/ml, 0 Hr Nic 378.6 ± 36.94 ng/ml, 1 Hr Nic 311.9 ± 85.44 ng/ml, 2 Hr Nic 205.6 ± 18.88 ng/ml, 24Hr Nic 3.488 ± 1.102 ng/ml, $F_{(4, 15)}$=16.70, ****p<0.0001, one-way ANOVA, **Fig. 1.2D left**; serum cotinine 0 Hr PG/VG 0.2625 ± 0.08 ng/ml, 0 Hr Nic 377.9 ± 64.58 ng/ml, 1 Hr Nic 334.7 ± 25.08 ng/ml, 2 Hr Nic 409.2 ± 67.00 ng/ml, 24 Hr Nic 2.283 ± 0.57 ng/ml, $F_{(4, 15)}$=22.80, ****p<0.0001, one-way ANOVA, **Fig. 1.2D right**). Similar to acute exposure, mice exposed to repeated vape exposure (PG/VG N=18, Nic N=18) showed significantly greater serum nicotine and cotinine in the 120 mg/ml Nic group as compared to PG/VG controls (serum nicotine: PG/VG 2.06 ± 0.74 ng/ml, Nic 346.0 ± 51.40 ng/ml, t=7.119, df=34, ****p < 0.0001, unpaired t-test, **Fig. 1.2E left**; serum cotinine: PG/VG 0.59 ± 0.15 ng/ml, Nic 502.9 ± 38.63 ng/ml, t=13.00, df=34, ****p<0.0001, **Fig. 1.2E middle**). Serum nicotine and serum cotinine levels from repeated 120 mg/ml nicotine vaped animals had a positive relationship but were not significantly correlated (slope=0.2609, intercept=406.9, r(16)= 0.3472, p=0.1581, **Fig. 1.2E right**). Taken together, these data show that animals exposed to acute and repeated 120 mg/ml nicotine have higher serum nicotine and cotinine levels as compared to its PG/VG control groups.

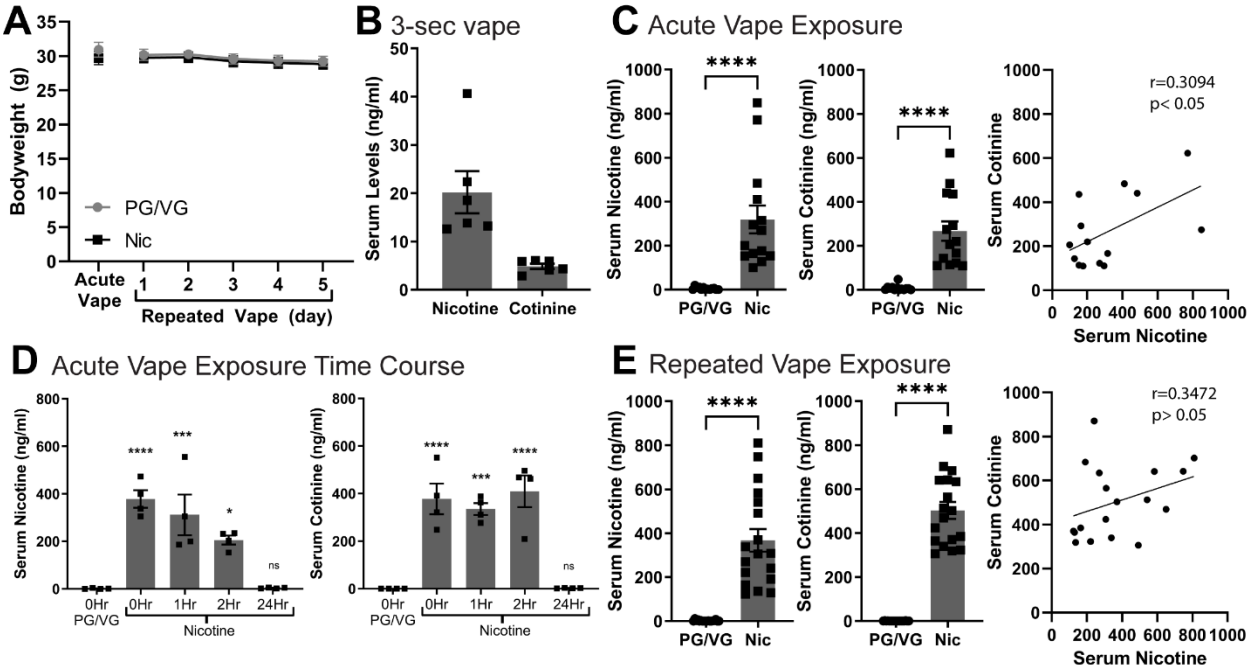

Figure 1.2- Serum nicotine and cotinine levels and bodyweight following acute and repeated electronic nicotine vapor exposure. **(A)** Body weights of animals exposed to acute (PG/VG N = 13, Nic N = 13) and repeated (PG/VG N = 12, Nic N = 12) vape (2-way ANOVA: main effect of day ****p < 0.0001). **(B)**, Serum nicotine and cotinine levels following a single 3-s 120 mg/ml nicotine vape (N = 6). **(C)**, Serum nicotine (left, unpaired t-test ****p < 0.0001) and cotinine (middle, unpaired t-test ****p < 0.0001) following acute PG/VG control (N = 13) or 120 mg/ml nicotine (N = 14) 3-h vape and correlation of serum nicotine and cotinine (right) **(D)** Time course of serum nicotine (left, 1-way ANOVA ****p < 0.0001; Dunnett's compared to 0 Hr PG/VG: 0 Hr Nic ****p < 0.0001, 1 Hr Nic ***p < 0.0005, 2 Hr Nic *p < 0.05, 24 Hr Nic not significant) and cotinine (right, 1-way ANOVA ****p < 0.0001; Dunnett's compared to 0 Hr PG/VG: 0 Hr Nic ****p < 0.0001, 1 Hr Nic ***p < 0.0005, 2 Hr Nic ****p < 0.0001, 24 Hr Nic not significant) following acute PG/VG or 120 mg/ml nicotine vape (PG/VG 0 h N = 4, Nic 0 h

N = 4, Nic 1 h N = 4, Nic 2 h N = 4, Nic 24 h N = 4). **(E)** Serum nicotine (left, unpaired t-test ****p < 0.0001) and cotinine (middle, unpaired t-test ****p < 0.0001) following repeated PG/VG control (N = 18) or 120 mg/ml nicotine (N = 18) vape and correlation of serum nicotine and cotinine (right).

Effects of acute electronic nicotine vapor exposure on central amygdala neuron activity

Once we established an electronic nicotine vape exposure model, we next investigated the impact of acute vape exposure on neuronal activity in the central amygdala (CeA) using both electrophysiological and immunohistochemical techniques. First, we examined the membrane properties of CeA neurons from PG/VG and 120 mg/ml Nic-exposed male mice. Membrane capacitance, membrane resistance, time constant, and membrane potential were not statistically different between the two vape groups by unpaired t-test (**Fig. 1.3A**). We then examined inhibitory synaptic transmission by measuring spontaneous inhibitory postsynaptic currents (sIPSCs) in CeA neurons from PG/VG and 120 mg/ml Nic mice and found no significant difference in sIPSC frequency by individual cells (PG/VG 1.08 ± 0.17 Hz, n=13 cells; Nic 1.45 ± 0.20 Hz, n=16 cells; t=1.397, df=27, p=0.1737, unpaired t-test; **Fig. 1.3C left**) or averaged by animal (PG/VG 1.08 ± 0.05 Hz, N= 4 animals; Nic 1.58 ± 0.24 Hz, N=6 animals; t=1.697, df=8, p=0.1282, unpaired t-test; **Fig. 1.3C right**). Similarly, we found no significant difference in sIPSC amplitude by individual cells (PG/VG 45.81 ± 2.10 pA, n=13 cells, Nic 45.11 ± 2.88 pA, n=16 cells; t=0.1879, df=27, p=0.8524, unpaired t-test; **Fig. 1.3D left**) or averaged by animal (PG/VG 45.65 ± 2.37 pA, N=4 animals; Nic 47.91 ± 4.24 pA, N=6 animals; t=0.4024, df=8, p=0.698, unpaired t-test; **Fig. 1.3D right**).

However, when we examine cell firing, we found a significantly greater baseline firing rate in CeA neurons from the 120 mg/ml Nic group compared to PG/VG controls when analyzed by individual cell (PG/VG 0.94 ± 0.16 Hz, n=15 cells; Nic 1.73 ± 0.23 Hz, n=12 cells; t=2.638, df=25, *p=0.0141, unpaired t-test; **Fig. 1.3F left**) and average by animal (PG/VG 0.98 ± 0.18 Hz, N=6 animals; Nic 1.84 ± 0.32 Hz, N=6 animals; t=2.340, df=10, *p=0.0414, unpaired t-test; **Fig. 1.3F right**). We then examined neuronal activity across the anterior-posterior (AP) span of the CeA by immunohistochemical labeling of cFos, an immediate early gene marker for cell activity. Two-way ANOVA revealed a main effect of AP coordinates (F (2, 96) = 14.57, ****p<0.0001) and vape content (F (1, 96) = 9.942, **p=0.0022; **Fig. 1.3H**). Post hoc Sidak's multiple comparison test found that the middle CeA of mice exposed to 120 mg/ml Nic possessed a greater number of cFos positive cells than that of mice exposed to PG/VG control vape (*p=0.0384; **Fig. 1.3H**). This is consistent with our CeA firing data following acute vape (**Fig. 1.3F**) as a majority of the electrophysiological recordings are from cells in the middle CeA. Taken together, these data indicate that exposure to an acute 3 hr session of 120 mg/ml Nic electronic vapor increases activity of CeA neurons as compared to PG/VG controls.

A Acute Vape	Membrane Capacitance Cm (pF)	Membrane Resistance Rm (MΩ)	Time Constant Tau (ms)	Membrane Potential Vm (mV)
PG/VG	45.3 ± 5.6	571.2 ± 74.5	546.5 ± 86.1	-55.9 ± 2.1
NIC	57.3 ± 6.1	598.5 ± 94.5	716.0 ± 109.4	-52.3 ± 2.0

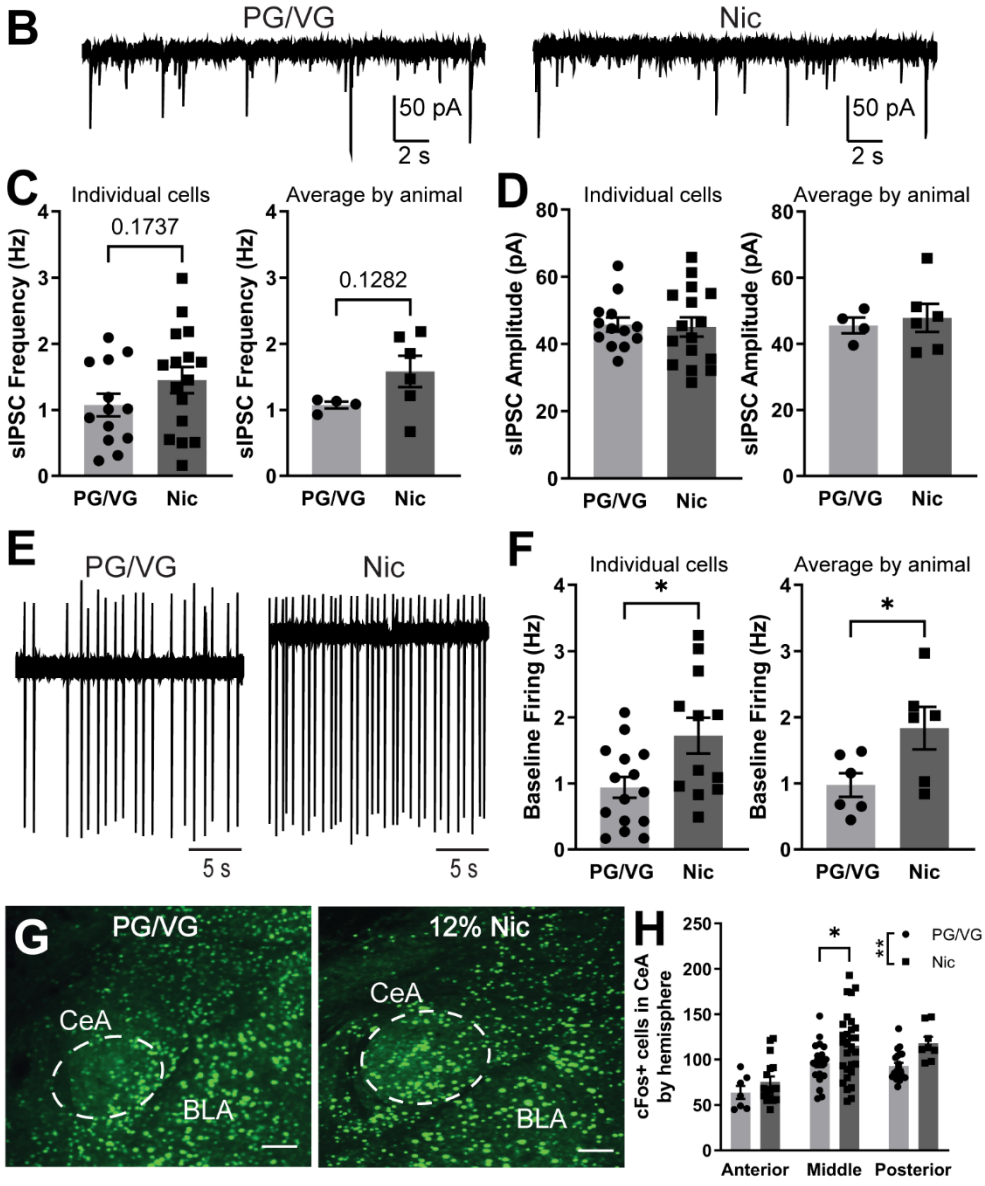

Figure 1.3- Inhibitory transmission and neuronal activity in the central amygdala (CeA) following acute electronic nicotine vapor exposure. (A) Membrane properties of CeA neurons from mice exposed to acute PG/VG control (N = 6 animals, n = 15 cells) or 120 mg/ml nicotine (N = 5 animals, n = 17 cells) vape. **(B)** Representative traces of sIPSCs in CeA neurons from mice exposed to acute

PG/VG control (left) or 120 mg/ml nicotine vape (right). **(C)** Summary of sIPSC frequency by individual cells (left) and averaged by animal (right) in CeA neurons from mice exposed to acute PG/VG (N = 4 animals, n = 13 cells) or 120 mg/ml nicotine (N = 6 animals, n = 16 cells) vape. **(D)** Summary of sIPSC amplitude by individual cells (left) and averaged by animal (right) in CeA neurons from mice exposed to acute PG/VG (N = 4 animals, n = 13 cells) or 120 mg/ml nicotine (N = 6 animals, n = 16 cells) vape. **(E)** Representative traces of cell-attached firing in CeA neurons from mice exposed to acute PG/VG control (left) or 120 mg/ml nicotine vape (right). **(F)** Summary of cell-attached firing frequency by individual cells (left, unpaired t-test *$p < 0.05$) and averaged by animal (right, unpaired t-test *$p < 0.05$) in CeA neurons from mice exposed to acute PG/VG (N = 6 animals, n = 15 cells) or 120 mg/ml nicotine (N = 6 animals, n = 12 cells) vape. **(G)** Representative micrograph of fluorescently labeled cFos in the CeA of mice exposed to acute PG/VG or 120 mg/ml nicotine vape. Scale bar: 100 μm. **(H)** Summary of cFos-positive cells by hemisphere across anterior to posterior CeA of mice exposed to acute PG/VG (n = 48 hemispheres in 8 animals) or 120 mg/ml nicotine (n = 54 hemispheres in 8 animals) vape (2-way ANOVA: main effect of AP ****$p < 0.0001$ and vape content **$p < 0.01$; Sidak's Middle CeA *$p < 0.05$).

Effects of repeated electronic nicotine vapor exposure on CeA neuron activity

Previous research has shown that repeated exposure to nicotine can cause differential changes in synaptic transmission and/or neuronal activity in a number of

brain regions (De Biasi & Dani, 2011; Picciotto & Mineur, 2014). Here, we examined how CeA activity is changed following repeated (5 day) exposure to 120 mg/ml nicotine electronic vapor. We first examined the membrane properties of CeA neurons from PG/VG and 120 mg/ml Nic-exposed mice and found that membrane capacitance, membrane resistance, time constant, and membrane potential were all not significantly different between the two groups (**Fig. 1.4A**). We then examined inhibitory transmission in CeA neurons and found no significant difference in spontaneous IPSC frequency by individual cells (PG/VG 1.07 ± 0.25 Hz, n=12 cells; Nic 0.87 ± 0.21 Hz, n=12 cells; t=0.6145, df=22, p=0.5452, unpaired t-test; **Fig. 1.4C left**) or averaged by animal (PG/VG 1.00 ± 0.20 Hz, N=6 animals; Nic 0.86 ± 0.15 Hz, N=6 animals; t=0.5681, df=10, p=0.5825, unpaired t-test; **Fig. 1.4C right**). Similarly, we found no significant difference in sIPSC amplitude by individual cells (PG/VG 42.24 ± 3.35 pA, n=12 cells, Nic 46.31 ± 3.58 pA, n=12 cells; t=0.8301, df=22, p=0.4154, unpaired t-test; **Fig. 1.4D left**) or averaged by animal (PG/VG 44.38 ± 5.26 pA, N=6 animals; Nic 46.66 ± 3.47 pA, N=6 animals; t=0.3626, df=10, p=0.7244, unpaired t-test; **Fig. 1.4D right**). We also found no significant difference in baseline firing between the PG/VG and 120 mg/ml Nic groups when analyzed by individual cell (PG/VG 0.76 ± 0.11 Hz, n=15 cells; Nic 1.16 ± 0.19 Hz, n=18 cells; t=1.731, df=31, p=0.0934, unpaired t-test; **Fig. 1.4F left**) and average by animal (PG/VG 0.80 ± 0.16 Hz, N=6 animals; Nic 1.22 ± 0.25 Hz, N=6 animals; t=1.457, df=10, p=0.1759, unpaired t-test; **Fig. 1.4F right**). When we examined cFos expression across anterior-posterior CeA, two-way ANOVA analysis revealed a main effect of vape content (F (1, 82) = 7.219, **p=0.0087; 1.**Fig.1 4H**). However, post hoc Sidak's test revealed no significant differences between PG/VG and 120 mg/ml Nic

groups in anterior, middle, or posterior CeA (**Fig. 1.4H**), which is consistent with the electrophysiological data. Together, these data suggest that in contrast to the effects observed with acute exposure, repeated exposure to 120 mg/ml Nic vapor does not result in increased CeA activity as compared to PG/VG controls.

A **Repeated Vape**	Membrane Capacitance Cm (pF)	Membrane Resistance Rm (MΩ)	Time Constant Tau (ms)	Membrane Potential Vm (mV)
PG/VG	50.7 ± 6.1	679.4 ± 135.1	635.8 ± 117.6	-50.0 ± 2.4
NIC	46.3 ± 6.4	524.1 ± 81.2	472.2 ± 88.1	-45.9 ± 1.8

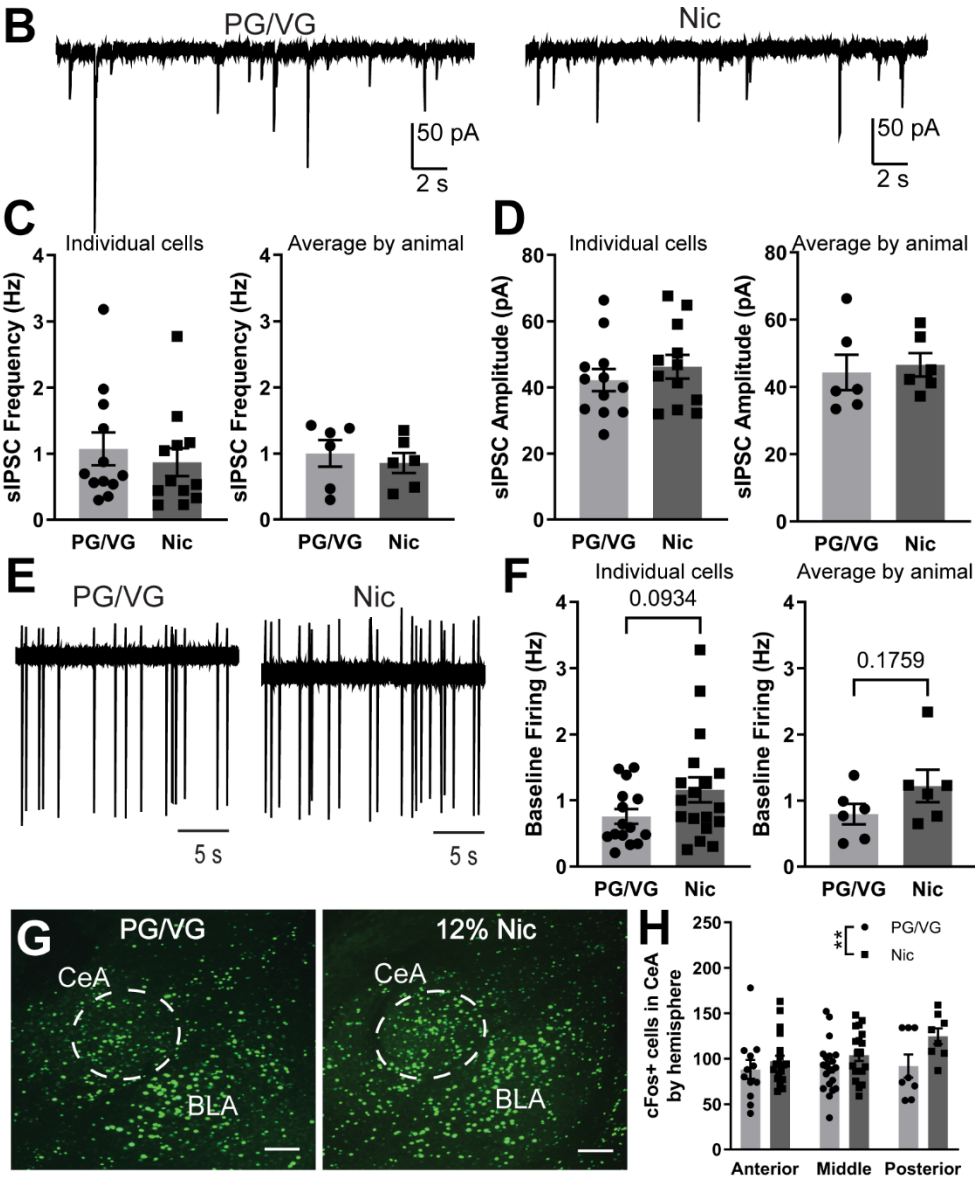

Figure 1.4- Inhibitory transmission and neuronal activity in the CeA following repeated electronic nicotine vapor exposure. **(A)** Membrane properties of CeA neurons from mice exposed to repeated PG/VG control (N = 6 animals, n = 17 cells) or 120 mg/ml nicotine (N = 6 animals, n = 18 cells) vape. **(B)** Representative traces of sIPSCs in CeA neurons from mice exposed to repeated PG/VG control (left) or 120 mg/ml nicotine vape (right). **(C)** Summary of sIPSC frequency by individual cells (left) and averaged by animal (right) in CeA neurons from mice exposed to repeated PG/VG (N = 6 animals, n = 12 cells) or 120 mg/ml nicotine (N = 5 animals, n = 12 cells) vape. **(D)** Summary of sIPSC amplitude by individual cells (left) and averaged by animal (right) in CeA neurons from mice exposed to repeated PG/VG (N = 6 animals, n = 12 cells) or 120 mg/ml nicotine (N = 5 animals, n = 12 cells) vape. **(E)** Representative traces of cell-attached firing in CeA neurons from mice exposed to repeated PG/VG control (left) or 120 mg/ml nicotine vape (right). **(F)** Summary of cell-attached firing frequency by individual cells (left) and averaged by animal (right) in CeA neurons from mice exposed to repeated PG/VG (N = 6 animals, n = 15 cells) or 120 mg/ml nicotine (N = 6 animals, n = 18 cells) vape. **(G)** Representative micrograph of fluorescently labeled cFos in the CeA of mice exposed to repeated PG/VG or 120 mg/ml nicotine vape. Scale bar: 100 μm. **(H)** Summary of cFos-positive cells by hemisphere across anterior to posterior CeA of mice exposed to repeated PG/VG (n = 41 hemispheres in 9 animals) or 120 mg/ml nicotine (n = 47 hemispheres in 9 animals) vape (2-way ANOVA: main effect of vape content **p < 0.01).

Body temperature and locomotion following acute and repeated electronic nicotine vapor exposure

After identifying the metabolic, molecular, and cellular changes following acute and repeated electronic nicotine vapor exposure, we examined the *in vivo* impact of electronic nicotine vapor after a single acute session, after repeated 5 day sessions, or 72 Hr after the final session of the repeated sessions (withdrawal). As nicotine has been reported to have hypothermic effects (Javadi-Paydar et al., 2019), we first measured body temperature of mice immediately following vape exposure. Two-way ANOVA revealed an interaction between exposure schedule x vape content ($F (2, 36) = 16.58$, ****$p<0.0001$) as well as main effects of exposure schedule ($F (2, 36) = 24.90$, ****$p<0.0001$) and vape content ($F (1, 18) = 68.24$, ****$p<0.0001$) alone. Post hoc Sidak's multiple comparisons test revealed significantly lower body temperature in acute Nic (****$p<0.0001$) and repeated Nic (****$p<0.0001$), but not in withdrawal, as compared to PG/VG controls (PG/VG N=10 animals, Nic N=10 animals; **Fig. 1.5A**). We then assessed locomotor activity using the open field test. In both parameters measured (distance and velocity), two-way ANOVA revealed an interaction between exposure schedule x vape content (distance $F (2, 36) = 14.89$, ****$p<0.0001$; velocity $F (2, 36) = 14.83$, ****$p<0.0001$) and a main effect of exposure schedule (distance $F (2, 36) = 13.48$, ****$p<0.0001$; velocity $F (2, 36) = 13.50$, ****$p<0.0001$). Post hoc Sidak's multiple comparisons test revealed significantly less distance travelled (****$p<0.0001$) and at a slower velocity (*$p=0.0175$) in acute Nic as compared to PG/VG controls (**Fig. 1.5B)**. However, the opposite was revealed in repeated Nic with significantly more distance travelled (****$p<0.0001$) and at a faster velocity (*$p=0.0315$) as compared to PG/VG controls (PG/VG N=10 animals, Nic N=10 animals; **Fig. 1.5C**). In both distance and

velocity measures for mice in withdrawal (72 Hr after repeated exposure), 120 mg/ml Nic was not significantly different than PG/VG controls. Collectively, these data demonstrate that acute and repeated exposure to 120 mg/ml nicotine vapor produce consistent hypothermic effects but divergent effects on locomotion, both of which are reversed in withdrawal.

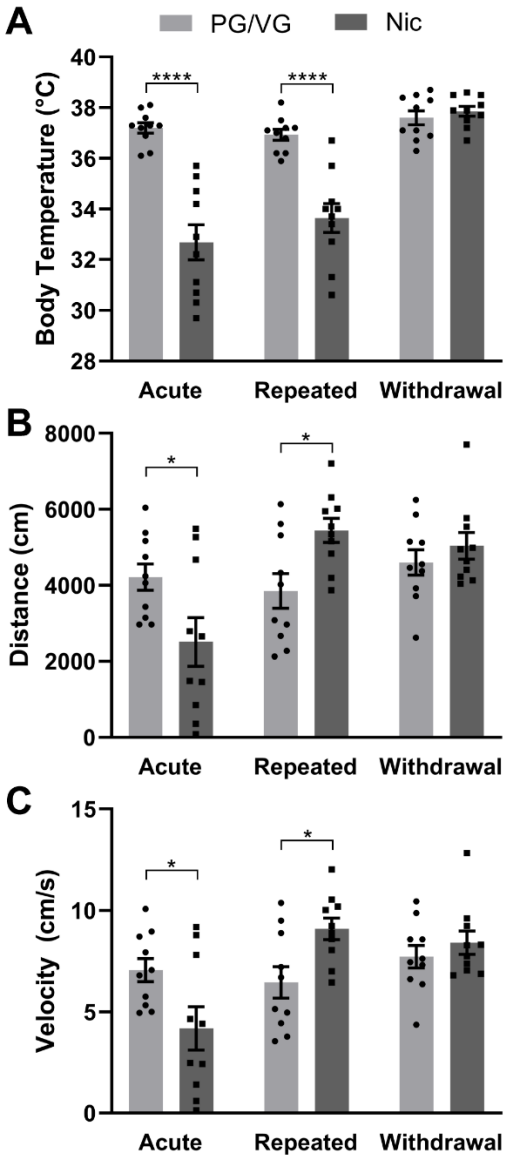

Figure 1.5- Body temperature and locomotion following acute and repeated electronic nicotine vapor exposure. (A) Body temperature of mice following acute, repeated, or withdrawal (2-way ANOVA interaction ****p < 0.0001: main effect of exposure schedule ****p < 0.0001 and vape content ****p < 0.0001; Sidak's acute ****p < 0.0001, repeated ****p < 0.0001) from PG/VG or 120 mg/ml nicotine vapor exposure (PG/VG N = 10, Nic N = 10, repeated measures). **(B)** Distance traveled in open field assay in mice following acute, repeated, or withdrawal (2-way ANOVA interaction ****p < 0.0001: main effect of exposure schedule ****p < 0.0001; Sidak's acute *p < 0.05, repeated *p < 0.05) from PG/VG or 120 mg/ml nicotine vapor exposure (PG/VG N = 10, Nic N = 10, repeated measures). **(C)** Locomotor velocity in open field assay in mice following acute, repeated, or withdrawal (2-way ANOVA interaction ****p < 0.0001: main effect of exposure schedule ****p < 0.0001; Sidak's acute *p < 0.05, repeated *p < 0.05) from PG/VG or 120 mg/ml nicotine vapor exposure (PG/VG N = 10, Nic N = 10, repeated measures).

Discussion

These studies utilized a preclinical model of electronic nicotine vapor exposure to examine the efficiency and reliability of modeling electronic vapor exposure in male mice and to examine the effects of acute (single session) and repeated (five daily sessions) nicotine vape exposure. The data presented here demonstrate that male mice will tolerate electronic nicotine vapor exposure sessions characterized by repeated intermittent vapes and that repeated intermittent vaping results in significant nicotine

levels in the blood and produces exposure paradigm-specific neuronal and behavioral effects. Specifically, acute exposure to electronic nicotine vapor produced significant increases in central amygdala (CeA) activity that were not observed following repeated exposure. Peripherally, significant decreases in core body temperature were observed in male mice exposed to both acute and repeated electronic nicotine vapor session. Decreased locomotion was observed following acute but increased locomotion was observed following repeated electronic nicotine vapor exposure. Collectively, these data provide evidence for the utility of vapor exposure models in mice and demonstrate how both central and peripheral systems are differentially affected by both acute and repeated electronic nicotine vapor exposure.

The electronic nicotine vapor exposure method used in these studies has several notable advantages over previous models, but also some limitations that should be considered. One advantage of the electronic nicotine vapor exposure paradigm is that it mimics the delivery method used by humans, in that the nicotine is delivered in vapor form and the route of delivery is through inhalation. This model also mimics the administration method seen in social situations in which electronic cigarettes are common. Route of administration of nicotine is an important determining factor in the timing and magnitude of the reinforcing effects as well as the behavioral effects of nicotine exposure. In previous studies nicotine has been administered through experimenter-delivered injections (Kasten et al., 2016) or subcutaneous minipumps (LeSage et al., 2002). These models provide the benefit of standardized serum nicotine levels, however these have limitations related to surgery and/or injection induced stress. A voluntary route of nicotine delivery is oral consumption with bottle choice which

showed nicotine-induced hypothermic and locomotor effects (DeBaker et al., 2020; Kasten et al., 2016; O'Rourke et al., 2016). However, this model of nicotine exposure does not replicate human consumption of nicotine and raises questions regarding taste preference when paired with other substances like sucrose. Nicotine administered via inhalation of vapor has become an emerging route of human consumption thus, preclinical studies should aim to reflect this route of administration.

The experimental parameters were chosen to mimic the experience of vaping which is characterized by brief periods of nicotine vapor inhalation (i.e. ‘puffs’) interspersed with periods of regular air inhalation. The 3 s vape period was chosen as it was sufficient to fill the cage with vapor and was consistent with vape duration of a puff in human e-cig users (Dawkins et al., 2016; Hiler et al., 2017). Studies in humans have also found that when given the ad libitum vape over the course of an hour, experienced male vapers have an average number of 48 puffs (Dawkins et al., 2016). In our paradigm, we’ve chosen the longer interval of 10 minutes between vape deliveries to mimic early recreational use with limited time periods. This also allow for clearance of the previous vape and to provide a period of regular air inhalation between vapes. Finally, the 3 h group exposure was chosen to model nicotine use that typically occurs in discrete time periods of exposure.

One important issue to consider in electronic nicotine vapor exposure paradigms is dosage. Commercial e-liquids offer a variety of nicotine concentrations, ranging from 3, 6, 12, 18, or 36 mg/ml. Studies in humans have found that a puff inhaled by an experienced e-cig user, as compared to a native e-cig user, is longer in duration and larger in volume and thus produce higher plasma nicotine levels. This effect was

observed across multiple nicotine concentrations (Hiler et al., 2017) and emphasized how nicotine consumption via inhalation can be impacted by the variability in vapor topography. The 120 mg/ml nicotine concentration was chosen based on a ten-fold increase in what is found in commercial e-liquids (12 mg/ml) to account for the reduction in vapor volume as it is passively inhaled from chamber air as opposed to direct inhalation into the airway as observed with humans. This concentration was further validated by the pharmacologically relevant serum nicotine and cotinine levels (**Fig 2**) and consistent with cotinine levels observed in human heavy smokers (Lawson et al., 1998). Previous preclinical studies have used lower nicotine vapor concentrations (1-80 mg/ml) with lower serum nicotine and cotinine levels (Cooper et al., 2021; Frie et al., 2020; Javadi-Paydar et al., 2019; Lallai et al., 2021; Montanari et al., 2020; Smith et al., 2020), however lower concentrations have produced variable effects. Although 120 mg/ml nicotine concentration was relatively high compared to previous work and human vape products, future studies will compare serum nicotine levels at different nicotine concentrations. Another consideration of the current approach is the use of passive exposure, which allows for precise control of experimental parameters, but does not account for the volitional aspect of nicotine intake. As so little is known about the impact of electronic vapor exposure in mice, passive exposure was preferable for the direct comparison of cellular and behavioral consequences of electronic nicotine vapor exposure without the confounding factor of variable intake or timing of exposure. However, since voluntary administration remains an important aspect of studying volition in drugs of abuse and self-administration of drugs delivered by vapor inhalation in rodents has been demonstrated with nicotine (Cooper et al., 2021; Lallai et al., 2021;

Smith et al., 2020), cannabis (Freels et al., 2020; Glodosky et al., 2020) and heroin (Gutierre et al., 2020), future studies will examine cellular and behavioral consequences of voluntary nicotine vapor self-administration to explore the impact of volition.

One important caveat to the current study is that all experiments were performed only in male mice, precluding our ability to identify any relevant sex differences in electronic nicotine vapor exposure. As females represent a significant proportion of the current vaping population (USDHHS, 2016), studies examining the impact of nicotine vapor exposure in female subjects are important and warranted. Previous studies in rats have demonstrated sex differences in nicotine metabolism (Kyerematen et al., 1988) and the effects of nicotine on anxiety-like behaviors (Torres et al., 2013). A recent study examining nicotine vapor self-administration in male and female rats identified that while both males and female rats will self-administer nicotine vapor in roughly equivalent levels, passive vapor exposure produced significantly lower serum cotinine levels in females as compared to males (Lallai et al., 2021). In addition, male rats displayed an increase in locomotion following repeated passive nicotine vapor exposure, while females did not, suggesting the potential for sex differences in behavioral sensitivity to passive nicotine vapor. Collectively, these data suggest that there may be important sex differences in the effects of nicotine vapor exposure in mice, which will be the subject of future studies.

The CeA has previously been implicated in the central effects of nicotine, however results were variable and largely dependent on dose, model, and timing of exposure. Exposure to a single three-hour session of electronic nicotine vapor exposure resulted in significant increases in central amygdala (CeA) neuronal activity as

measured by electrophysiological assessment of neuronal firing and by immunohistochemical assessment of the activity marker cFos. The CeA is a primarily GABAergic nucleus composed of interneurons and projection neurons (Pitkänen & Amaral, 1994) and inhibitory microcircuits within the CeA have been implicated in fear learning (Haubensak et al., 2010) and in the plasticity observed with acute and chronic ethanol exposure (Herman et al., 2016). However, the inhibitory inputs onto CeA neurons (as measured by sIPSCs) do not appear to be modulated by acute or repeated electronic nicotine vapor exposure, and the increases in neuronal activity following acute exposure were observed independent of changes in inhibitory signaling. In contrast to what was observed with acute exposure, following five days of repeated electronic nicotine vapor sessions, there was no increase in CeA activity observed by either electrophysiological or immunohistochemical evaluation. We used both electrophysiological (firing) and immunohistochemical (cFos) measures of neuronal activity in the CeA to examine single cell- versus population-specific changes in CeA activity following electronic nicotine vapor exposure. Our results from the two measures show similar direction of change in acute exposure and no change in repeated exposure.

These neuronal activity findings are consistent with previous studies where an increase in CeA cFos was found following a single subcutaneous injection of acute nicotine, but no increase in CeA cFos was observed following chronic nicotine exposure using osmotic minipumps. However, they also found increased cFos with a single subcutaneous nicotine injection following chronic osmotic minipump nicotine exposure (Salminen et al., 1999). Increased CeA cFos was also observed after 14 days of

withdrawal from chronic nicotine self-administration (Funk et al., 2016). A single intraperitoneal injection of nicotine increased phosphorylated extracellular regulated kinase (pERK) in the CeA twenty minutes after drug administration (Valjent et al., 2004), however a study of voluntary nicotine drinking found no increase in amygdala pERK after an acute (1.3 hour) drinking session and only saw a significant increase in amygdala pERK after chronic (28-30 day) drinking (Brunzell et al., 2003). Taken together, these studies suggest that the CeA is differentially engaged with different nicotine exposure models and at different timepoints of exposure and withdrawal.

The electronic nicotine vapor exposure system utilized in this study has also been employed in prior work examining the impact of electronically-generated nicotine vapor in rodents. One study in rats reported decreased core body temperature following nicotine vapor exposure (Javadi-Paydar et al., 2019), which is consistent with our findings as well as what has been shown following nicotine intraperitoneal (ip) injection (Javadi-Paydar et al., 2019) and subcutaneous (sc) injection (Akinola et al., 2019; Levin et al., 2003) and suggest that nicotine's effect on body temperature are similar across rodent species and route of nicotine administration. Additionally, the decrease in locomotion we observed with acute nicotine vapor is consistent with studies where nicotine was delivered through ip or sc injections (Akinola et al., 2019; Levin et al., 2003). Interestingly, the same rat study that reported similar decreases in core body temperature found no difference in locomotion between PG/VG and nicotine groups following a single 30 min vapor exposure (Javadi-Paydar et al., 2019). This divergence from our findings may be due to the difference in timing and duration of vape exposure of experiments compared to ours (single 30 min vape vs. 3 hr session of 3 sec vape

every 10mins, respectively) or the location of locomotor activity test (inhalation chamber vs novel open arena, respectively). However, following repeated nicotine vapor, increased locomotion was observed in multiple studies (Javadi-Paydar et al., 2019; Lallai et al., 2021), which is consistent with our findings suggesting another parameter that is consistent across rodent species.

Collectively, these studies provide important evidence for how acute and repeated exposure to electronic nicotine vapor can produce differential effects in the CeA and on specific behaviors. The development and more wide-spread use of preclinical models of electronic nicotine vapor exposure will allow for more detailed studies on the impact of vaping on additional brain regions and behaviors that could lead to an improved understanding of how vaping effects the human brain to promote the development of nicotine dependence specific to the vaping route of delivery. The importance of this work is underscored by the increasing prevalence of nicotine vaping and the prevailing assumption that since vaping represents a safer alternative to tobacco smoking, it is less ‘dangerous’ or harmful of an activity. It will be important for scientific research to continue apace with human user experience so that neurobiological underpinnings of clinically-relevant nicotine vapor exposure models can be used to understand the impacts of vaping on human populations.

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CHAPTER 3: ELECTRONIC VAPORIZATION OF NICOTINE SALT OR FREEBASE PRODUCES DIFFERENTIAL EFFECTS ON METABOLISM, NEURONAL ACTIVITY, AND BEHAVIOR IN MALE AND FEMALE C57BL/6J MICE[2]

Introduction

Electronic Nicotine Delivery Systems (ENDS) represent the most currently used and available form of nicotine system, and their popularity has rapidly grown with ~3.7% of young adults (18-24) using ENDS (9.1 million) (Cornelius et al., 2022). With this growing popularity and the variability in ENDS devices, the availability and use of different nicotine formulations and concentrations has also increased (Ozga et al., 2022). Two types of nicotine formulations are predominant within the market, nicotine freebase and nicotine salt. Nicotine freebase is highly volatile and when vaporized it enters the bloodstream through the mouth/upper respiratory tract. This solution is often described as bitter and harsh. Nicotine salt, on the other hand, is made by adding organic acids into freebase nicotine which results in protonated nicotine salt. When vaporized, this solution travels further down the respiratory tract and is absorbed into the bloodstream by the alveoli, which is more similar to the absorption that occurs with cigarettes. The addition of organic acids into nicotine freebase is reported to increase its smoothness and reduce its bitterness (Leventhal et al., 2021; O'Connell et al., 2019).

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The smoothness of the nicotine salt has made it more popular amongst first time smokers (Leventhal et al., 2021) but there is a relatively equal prevalence of nicotine salt and nicotine freebase amongst ENDS users (Cohen et al., 2022). Humans who have smoked nicotine salt showed higher nicotine serum levels than those who used nicotine freebase. At a higher concentration, nicotine salt was also able to closely resemble nicotine serum levels reached when smoking a cigarette (O'Connell et al., 2019) which suggests other underlying physiological differences might result from consuming nicotine salt and nicotine freebase.

Potential physiological differences between nicotine formulations remain unclear, as there are few studies that investigate the pharmacokinetic effects of nicotine salt and freebase in preclinical models. It has been previously shown in rats that a single subcutaneous injection of nicotine freebase reached and maintained higher serum nicotine levels than a single injection of nicotine salt (Han et al., 2022). Using an e-vape self administration paradigm, Henderson and Cooper et. al (2021), found that mice exposed to nicotine salt sought more nicotine deliveries than their freebase counterparts. In addition, mice exposed to nicotine salt yielded higher plasma cotinine levels than mice exposed to freebase (Henderson & Cooper, 2021). Thus suggesting that nicotine salt is metabolized at a faster rate and therefore more vapor puffs are required to maintain an effect. These serum results were similar to previous clinical studies (O'Connell et al., 2019) and when taken into consideration with the perceived smoothness of nicotine salt vapor, nicotine salts can potentially pose a higher likelihood of developing dependence than nicotine freebase in clinical populations.

Further, potential differences between nicotine salt and nicotine freebase and sex differences can arise in behavioral effects observed after vapor exposure. One preclinical study in rats found sex differences where male rats exposed to nicotine vapor showed higher serum cotinine levels than females, as well as hyperlocomotion following passive vape administration (Lallai et al., 2021). These effects could be due to differences in absorption or metabolism however, the possibility of sex differences in metabolic enzyme levels should also be considered. Previous studies have also shown that nicotine vapor exposure results in changes in thermoregulation and locomotor function (Honeycutt et al., 2020; Javadi-Paydar et al., 2019; Lallai et al., 2021; Zhu et al., 2021). A comprehensive review of preclinical and clinical studies found that male rats and mice showed increased anxiety following prenatal and postnatal nicotine exposure. A number of caveats such as variability in methodology and inconsistency in literature reporting prevented the authors from reaching any conclusions (Sikic et al., 2022). We have also previously shown that acute nicotine vapor exposure increases activity in the central amygdala (CeA), however that study only investigated a single nicotine formulation (freebase) and was only performed in male mice (Zhu et al., 2021). Few studies have examined the effects of formulation on behavior or brain region-specific activity in a preclinical model. The goal of the current study was to investigate the physiological and behavioral effects of different nicotine formulations and concentrations in male and female mice following a single session of vapor, to determine the changes after initial exposure to nicotine vapor. In order to further understand the effects of nicotine formulation on anxiety-like and reward-related behavior we specifically examined activity in the CeA due to its association with anxiety

and substance use disorders (Gilpin et al., 2015). We also targeted the ventral tegmental area (VTA) for its role in reward signaling and nicotine effects (Markou, 2008). Additionally, we examined the impact of formulation and concentration on nicotine metabolism and anxiety-like and motivated behavior.

Materials and Methods

Animals

Female and male adult (10- 12 weeks) C57/BL6J mice were obtained from Jackson Labs and group-housed in a temperature- and humidity-controlled 12hr light/dark (7am lights on) facility with ad libitum food and water access. All experimental procedures were approved by the UNC Institutional Animal Care and Use Committee.

Drugs

(-)- Nicotine free base ($C_{10}H_{14}N_2$; Sigma Aldrich) was diluted to a 3% (30 mg/ml) nicotine concentration in 1:1 propylene glycol (Sigma Aldrich):vegetable glycerol (Fisher, PG/VG). Nicotine ditartrate dihydrate salt, 98% ($C_{10}H_{14}N_2 \cdot 2C_4H_6O_6 \cdot 2H_2O$; Sigma Aldrich) was dissolved in 1:1 PG/VG to make 1% (10 mg/ml) and 3% (30 mg/ml) nicotine concentration, taking into account the difference in molecular weight of the salt to match the nicotine content of the nicotine freebase concentrations.

Electronic Nicotine Vapor Exposure

Mice were exposed to electronic nicotine vapor in airtight vacuum-controlled chambers as previously described (Zhu et al., 2021). Briefly, PG/VG control, 3%

nicotine freebase, 1% nicotine salt, or 3% nicotine salt solutions are added to electronic nicotine vape tanks (Baby Beast Brother, Smok) to be heated and vaporized (95 watt, 0.25Ω, 200℃, SVS200, Scientific Vapor). The vacuum pressure allows the vaporized solution to be circulated through the chambers at ~1 L/min flow rate. Vapor delivery was triggered by e-vape controllers (SSV-1, La Jolla Alcohol Research) that are set to deliver vapor for 3 seconds every 10 minutes for a total of 3 hours (Fig 1, left).

Nicotine Serum Analysis

Immediately following an acute vapor exposure session, trunk blood was collected from cardiac puncture. Nicotine and cotinine levels were analyzed through liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described previously (Ghosh et al., 2019; Zhu et al., 2021).

Immunohistochemistry (IHC)

Immediately following an acute vapor exposure session, mice were anesthetized with isoflurane and perfused with 1x phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS. Brains were left to postfix overnight in 4% PFA before transferring to 30% sucrose in PBS at 4°C. Brains were then sectioned at 40 μm using a microtome (HM450, Thermo Fisher Scientific) and stored at 4°C in 0.01% sodium azide in PBS. Slices containing the CeA and VTA were washed in PBS for 10 mins, incubated in 50% methanol in PBS for 30 mins, washed for 5 mins in 3% hydrogen peroxide and incubated in blocking solution (0.3% Triton X-100; Thermo Fisher), 1% bovine serum albumin (BSA; Sigma) for 1hr. They were then incubated for 48 hrs at 4°C in rabbit anti-

cFos primary antibody (1:3000, Millipore Sigma; ABE457) in blocking solution. Slices were washed with Tris, NaCl, Triton X-100 (TNT) buffer for 10 mins. Followed by a Tris, NaCl, blocking reagent (TNB; PerkinElmer) wash for 30 mins, a 30 min incubation in Horse Radish Peroxide (HRP; 1:200, Abcam ab6721) and a 5 minute washes in TNT. Fluorescence signal was amplified by incubating the slices in Cy3 [1:50] TSA amplification diluent (Akoya Biosciences, NEL744001KT) for 10 min at room temperature. CeA slices were then washed in TNT buffer for 10 minutes and mounted onto slides with Vectashield DAPI with hard-set (Vector labs; H1500-10). In VTA slices, tyrosine hydroxylase expression was labeled by an additional 10 min washes in PBS, and a 60 min incubation in blocking solution (0.3% Triton X-100, 10% Normal Donkey Serum (NDS), and 1% Bovine serum (BSA)). VTA slices were then incubated overnight in primary antibody (Mouse anti-TH [1:1000], Sigma T1299) in blocking solution at 4°C. Finally the slices were washed for 10 mins in PBS and incubated for 2 hrs in secondary antibody (Donkey anti mouse [1:700], Abcam ab150105) in PBS, washed in PBS and mounted onto slides using Vectashield DAPI (Vector labs; H1500). Fluorescence signals were detected and imaged on fluorescent microscopes (Nikon Eclipse 6600 for CeA and Keyence BZ-X800 for VTA).

Novelty Suppressed Feeding (NSF) Test

To assess the effects of nicotine formulation on anxiety-like behavior following a single vapor session, the novelty suppressed feeding test was conducted. All mice received highly palatable food (Froot Loop, Kellogg's) in their home cage 48 hours prior to testing and were food deprived 24 hours prior to testing. Testing was conducted in a

sound-attenuated behavior cabinet with a clean, empty rat cage inside and equipped with a light source set at 200 lux. Following vapor exposure described above, each mouse was placed inside an empty cage with a Froot Loop to record their latency to feed time as a measure of anxiety-like behavior. Once the mouse engaged in feeding or reached the maximum time limit of 10 minutes, the time was recorded and the NSF post-test commenced where the mouse was transferred back to its home cage and allowed 10 minutes to feed on a pre-weighed amount of Froot Loops. The weight of Froot Loops consumed was calculated as a measure of motivation to feed.

Statistical Analysis

Statistical analysis and data visualization were performed using Prism 9.0 (GraphPad). Data were analyzed and compared using one-way or two-way ANOVAs with post hoc Tukey's multiple comparisons as well as pearson's correlation with $p<0.05$ as the criterion for statistical significance. All data sets are expressed as mean ± SEM.

Results

Serum nicotine and cotinine analysis following exposure to nicotine freebase vs nicotine salt

To investigate the effects of nicotine formulation (freebase vs salt) and nicotine concentration (1% vs 3%), we separately exposed female and male mice to vapor composed of propylene glycol/vegetable glycerol (PG/VG, control), 3% nicotine freebase (3% FB), 1% nicotine salt (1% salt), or 3% nicotine salt (3% salt). Mice were exposed to a single vapor session comprised of 3 second vapor deliveries every 10 minutes for a total of 3 hours. Immediately following exposure, serum was collected and

mice were perfused for immunohistochemistry (**Fig 2.1A**). Serum nicotine levels showed a significant sex x vapor content interaction (2-Way ANOVA, $p=0.0058$, $F(3, 31) = 5.039$) and a main effect of vapor content (#$p<0.0001$, $F(3, 31) = 142.0$, **Fig 2.1B**). Post-hoc Tukey's multiple comparisons showed that mice of both sexes that were exposed to 3% FB displayed higher levels of serum nicotine than those exposed to 3% salt. Additionally, females exposed to 3% FB had higher serum nicotine levels than males exposed to 3% FB (**Fig 2.1B**). Levels for serum cotinine, the main metabolite of nicotine, also showed a significant sex x vapor content interaction (2-Way ANOVA, $p=0.0335$, $F(3, 31) = 3.289$) and a main effect of vapor content (#$p<0.0001$, $F(3, 31) = 18.18$), and post-hoc Tukey's showed that females exposed to 3% FB had higher serum cotinine levels than males exposed to 3% FB (**Fig 2.1C**). Overall, these data indicate that nicotine vapor in freebase formulation produced higher serum nicotine levels than the salt formulation of the same concentration and within the freebase formulation group, females had higher serum nicotine and cotinine levels than males.

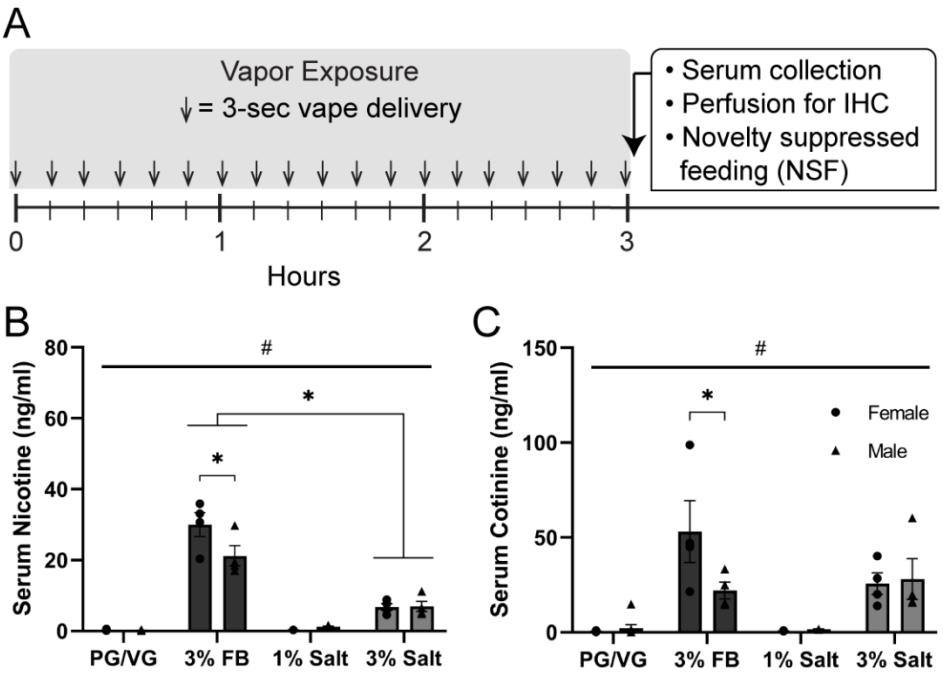

Figure 2.1- Experimental timeline and serum nicotine and cotinine following nicotine vapor exposure. **(A)** Experimental timeline of vapor exposure (3 second vapor every 10 minutes for 3 hours), followed by serum collection and perfusion for immunohistochemistry or NSF behavioral assay. **(B)** Serum nicotine levels in females and males following PG/VG, 3% nicotine freebase (3% FB), 1% nicotine salt (1% salt), or 3% nicotine salt (3% salt) vapor exposure. Significant interaction ($p=0.0058$) and #Main effect of vapor content ($p<0.0001$); *Post-hoc Tukey's: Female 3% FB vs Male 3% FB, Female and Male 3% FB vs 3% salt. **(C)** Serum cotinine levels in females and males following PG/VG, 3% nicotine freebase (FB), 1% nicotine salt, or 3% nicotine salt vapor exposure. Significant interaction ($p=0.0335$) and #Main effect of vapor content ($p<0.0001$); *Post-hoc Tukey's: Female 3% FB vs Male 3% FB.

Neuronal activation in the central amygdala following exposure to nicotine freebase vs nicotine salt

To measure changes in neuronal activity in the central amygdala (CeA, **Fig 2.2A**) we performed immunohistochemistry and labeled for cFos in female (**Fig 2B, top**) and male (**Fig 2.2B, bottom**) mice exposed to PG/VG, 3% FB, 1% salt, and 3% salt. In females, we observed no significant difference in average cFos expression between groups (1-Way ANOVA, p=0.5118, F= 0.8153, **Fig 2.2C**). In males however, we observed a significant increase in average cFos expression in the 3% FB, 1% salt, and 3% salt groups as compared to PG/VG controls (1-Way ANOVA, #p=0.0007, F=10.99, **Fig 2.2D**). We then combined the female and male data to examine sex differences using 2-way ANOVA. We found a significant effect of vapor content (#p=0.0415, F (3, 26) = 3.158, **Fig 2.2E**), however, post hoc multiple comparisons between specific groups did not yield any significant differences. We also correlated CeA cFos expression with nicotine serum levels in females, males, and mice of both sexes and found no significant correlation (Female and Male r= -0.083, p=0.73; Female r=-0.098, p=0.79; Male r= -0.115, p=0.75, **Fig. 2.2F**). Taken together these data suggest that in females, CeA activation is unaffected by vapor exposure, but in males CeA activity is increased following nicotine vapor exposure, regardless of concentration or formulation.

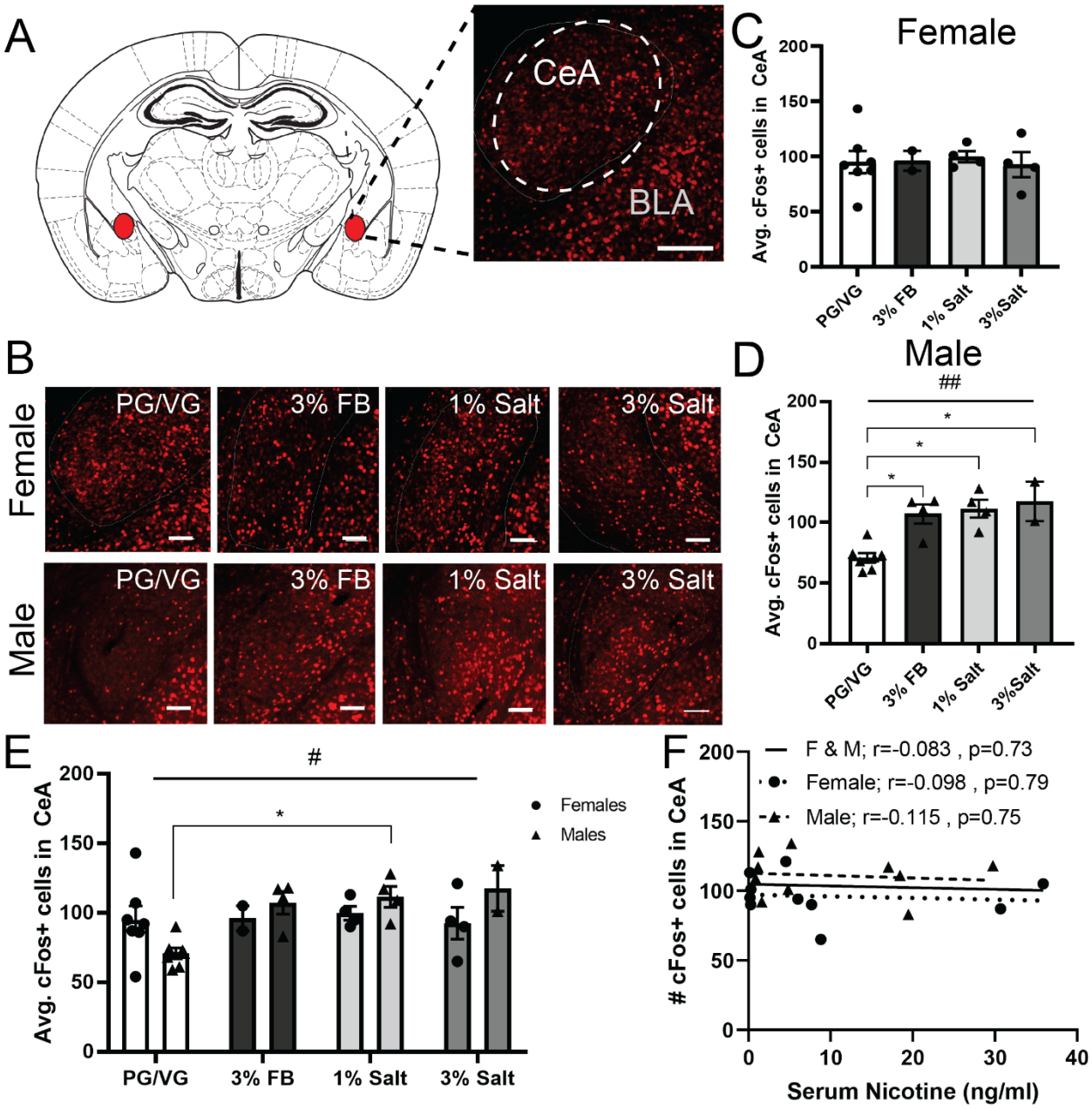

Figure 2.2- Neuronal activation in the central amygdala area following exposure to nicotine freebase vs. nicotine salt (A) Representative image of cFos (red) in the central amygdala. Scale bar = 100 µm **(B)** Representative image of cFos (red) expression in females (top row) and males (bottom row) exposed to PG/VG, 3% nicotine freebase (3% FB), 1% nicotine salt (1% salt), or 3% nicotine salt (3% salt) vapor. Scale bars = 100 µm **(C)** Average cFos expression in females

following exposure to different vapor content. 1-way ANOVA, p= 0.5118. **(D)** Average cFos expression in males following exposure to different vapor content. 1-Way ANOVA, #p=0.0007 *Post-hoc Tukey's Male: PG/VG vs 3% FB, PG/VG vs 1% salt. PG/VG vs 3% salt. **(E)** Average cFos expression in females and males exposed to different vapor content. 2-way ANOVA, #p=0.0415 *Post-hoc Tukey's multiple comparison test Male: PG/VG vs 1% salt **(F)** Correlation of averaged cFos expression in females,males, and both sexes combined with serum nicotine levels.

Neuronal activation in the ventral tegmental area following exposure to nicotine freebase vs nicotine salt

To investigate neuronal activation in the ventral tegmental area (VTA, **Fig 2.3A**) and its colocalization with the dopaminergic population, we performed immunohistochemistry to label for cFos and tyrosine hydroxylase (TH) in female (**Fig 2.3B, top**) and male (**Fig 2.3B, bottom**) animals exposed to PG/VG, 3% FB, 1% salt, or 3% salt. In females, we found a significant difference in the average cFos expression in the VTA between vapor groups (1-Way ANOVA, #p=0.0037, F=7.214, **Fig 2.3C**) and post hoc Tukey's multiple comparisons show that cFos expression was higher in the 3% FB group compared to PG/VG (p=0.0036), 1% salt (p=0.0097), and 3% salt (p=0.0306) groups. In males, there was no significant difference in the average cFos expression in the VTA between vapor groups (1-Way ANOVA, p=0.1057, F=2.402, **Fig 2.3D**). When the data from the two sexes were combined to examine potential sex differences using 2-Way ANOVA, we found a main effect of vapor content (#p=0.0001, F (3, 30) = 9.460) and specifically, that females exposed to 3% FB showed increased cFos expression as

compared to female PG/VG (p=0.0005), 1% salt (p=0.0024), and 3% salt (p=0.0157) vapor groups (post hoc Tukey's, **Fig 2.3E**). We next examined activity in dopaminergic neurons by quantifying colocalization of cFos and TH and again found a main effect of vapor content (2-Way ANOVA, $p<0.0001$, $F(3, 30) = 12.18$). Specifically, females exposed to 3% FB showed increased cFos and TH colocalization as compared to female PG/VG (p=0.0006) and 1% salt (p=0.0013) vapor groups (post hoc Tukey's, **Fig 2.3F**). We correlated VTA cFos expression with nicotine serum levels in females, males, and mice of both sexes and found a significant positive correlation with female and male data combined ($r= 0.63$, $p= 0.0011$) and female only data ($r= 0.69$, $p= 0.013$) but not with male only data (Males $r= 0.46$, $p= 0.13$, **Fig 2.3G**). The significant correlation in both sexes combined is likely driven by the females. We performed a similar correlation analysis to examine the relationship between cFos expression in the TH population of the VTA and serum nicotine and found a positive correlation with female and male data combined ($r= 0.68$, $p= 0.0003$), female only data ($r= 0.69$, $p= 0.014$), and male only data ($r= 0.65$, $p= 0.022$, **Fig 2.3H**). Taken together, these data suggest that females exposed to nicotine vapor in 3% freebase formulation show significantly increased VTA neuronal activity as compared to exposure to either the 1% or 3% salt formulation, the increased activity is observed in both global VTA as well as specifically in the VTA dopaminergic population, and the increased activity is likely driven by the nicotine exposure as activity was correlated with serum nicotine levels.

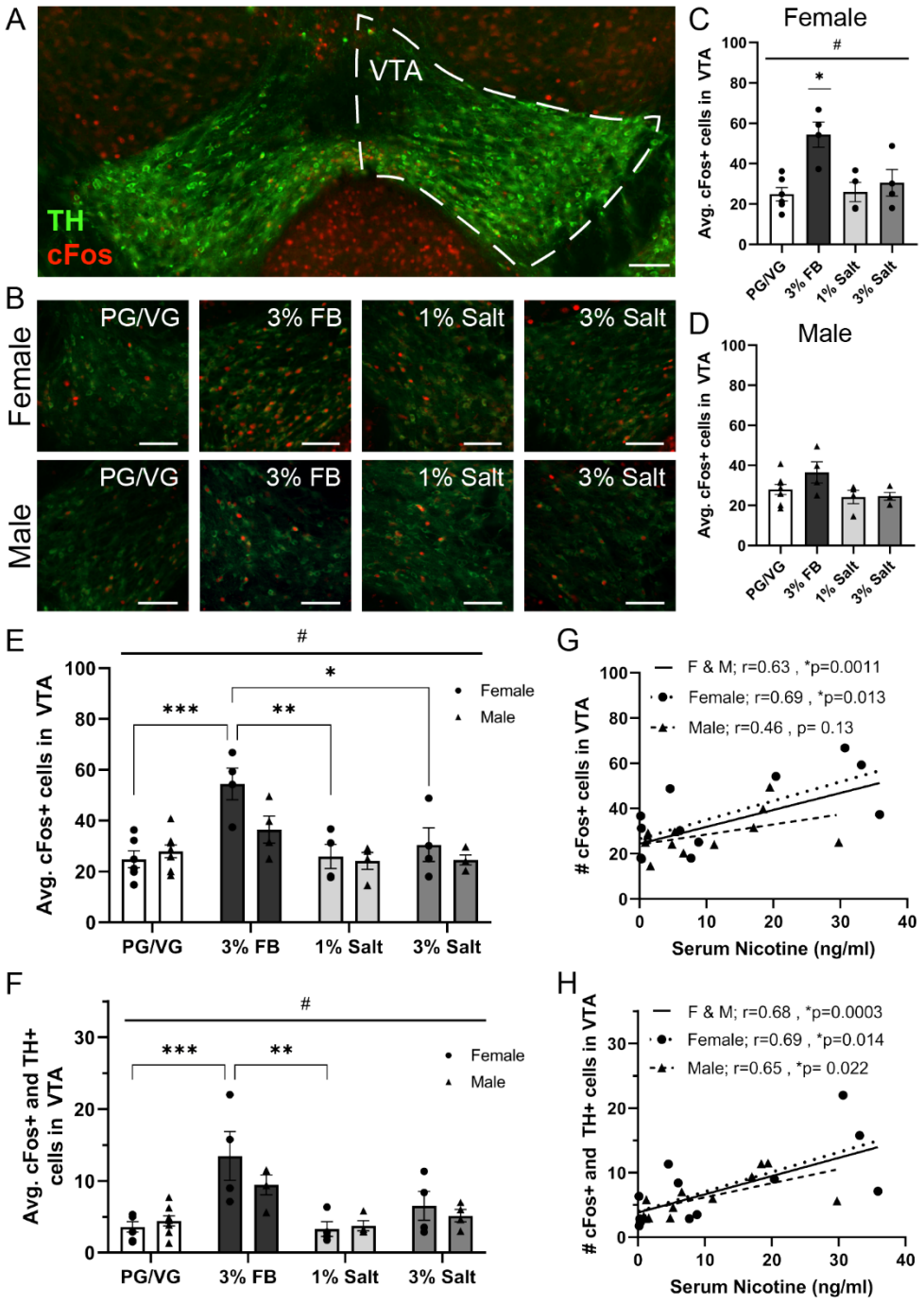

Figure 2.3- Neuronal activation in the ventral tegmental area following exposure to nicotine freebase vs. nicotine salt **(A)** Representative image of the ventral tegmental area (right hemisphere outlined with dotted line) labeled with tyrosine hydroxylase (TH, green) and cFos (red). Scale bar = 100 µm **(B)** Representative

image of double labeling of tyrosine hydroxylase (TH, green) and cFos (red) expression in females (top row) and males (bottom row) exposed to PG/VG, 3% Nicotine Freebase (3% FB), 1% Nicotine Salt (1% Salt), or 3% Nicotine Salt (3% Salt) vapor. Scale bars = 100 μm **(C)** Average cFos expression in females following exposure to different vapor content. #1-way ANOVA, p=0.0037 *Post-hoc Tukey's Female: 3% FB vs PG/VG, 3% FB vs 1% Salt, 3% FB vs 3% Salt. **(D)** Average cFos expression in males following exposure to different vapor content. 1-Way ANOVA, p=0.1057 **(E)** Average cFos expression in females and males exposed to different vapor content. #Main effect of vapor content, p=0.0001 *Post-hoc Tukey's Female: 3% FB vs PG/VG, 3% FB vs 1% Salt, and 3% FB vs 3% Salt. **(F)** Average cFos and TH colocalization in females and males exposed to different vapor content. #Main effect of vapor content, p<0.0001 *Post-hoc Tukey's Female: 3% FB vs PG/VG and 3%FB vs 1% Salt.

Anxiety-like and motivated behavior following exposure to nicotine freebase vs nicotine salt

Following a single vapor session of either PG/VG, 3% FB, 1% salt, or 3% salt, anxiety-like behavior was assessed using the NSF test (**Fig 2.4A**). Females displayed no significant difference in latency to feed time between the PG/VG, 3% FB, 1% salt, and 3% salt groups (1-way ANOVA, p=0.6399, **Fig 2.4B**) suggesting no difference in anxiety-like behavior following a single vapor session. In contrast, there was a main effect of vapor content when comparing male latency to feed times (1-way ANOVA, #p=0.0453, F=3.057, **Fig 2.4C**) with trends of the 3% FB group displaying a longer latency to feed time and more anxiety-like behavior compared to the PG/VG group

(p=0.0680) and the 1% salt group (p=0.0904). During post-test food consumption (**Fig 2.4D**), females displayed no significant difference between the PG/VG, 3% FB, 1% salt, and 3% salt groups (1-way ANOVA, p=0.3928, **Fig 2.4E**) suggesting no group differences in overall motivation to feed. There was a main effect of vapor content in the male NSF post-test (1-way ANOVA, #p=0.0192, F=3.914, **Fig 2.4F**) and post hoc Tukey's multiple comparisons show that the male 3% FB group consumed significantly less food during the post-test compared to the PG/VG group (p=0.0497) and the 3% salt group (p=0.0264) but not the 1% salt group. Collectively, these findings show that vapor exposure did not alter anxiety-like behavior and motivation to feed in females. However, male mice exposed to 3% nicotine freebase vapor displayed lower motivation to feed and had trends toward an increase in anxiety-like behavior.

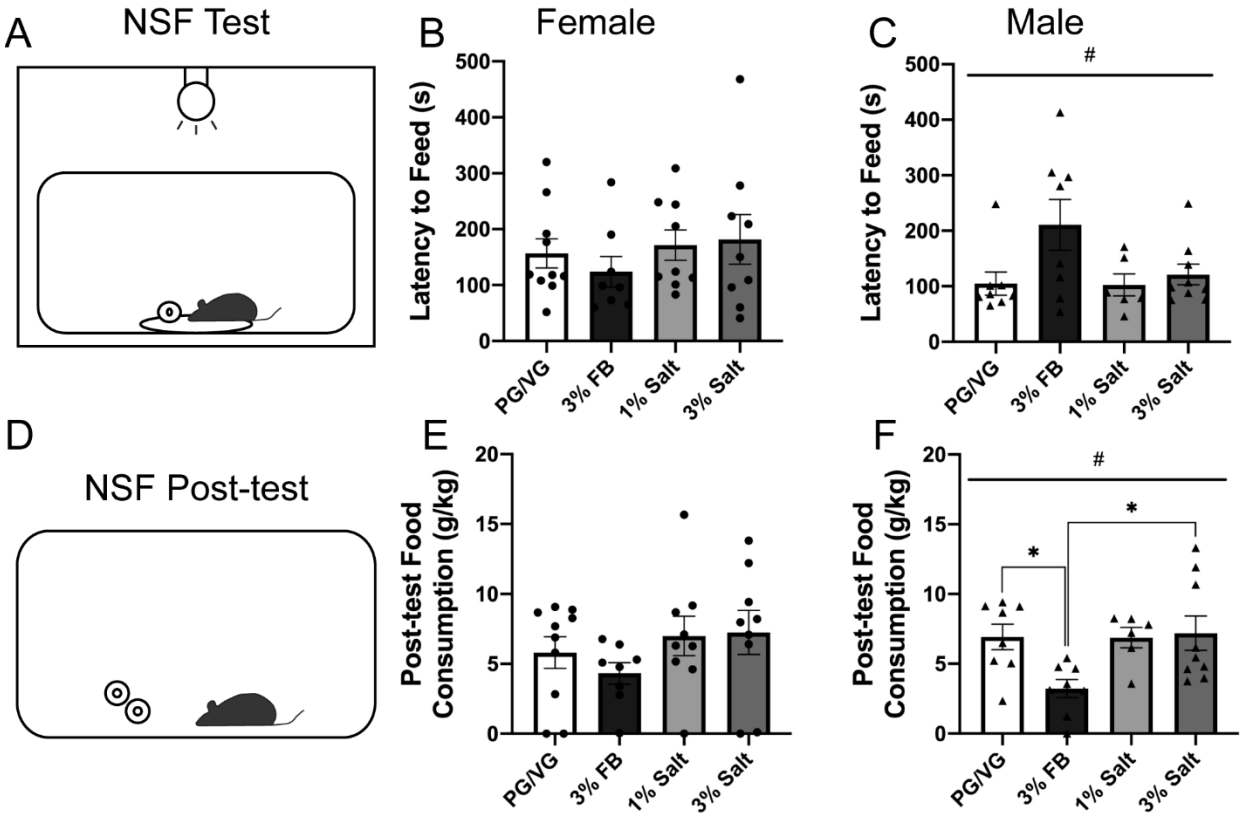

Figure 2.4 - Anxiety-like and motivated behavior following exposure to nicotine freebase vs nicotine salt **(A)** Diagram of the novelty-suppressed feeding (NSF) test. **(B)** Latency to feed time measured in females following exposure to PG/VG, 3% nicotine freebase (3% FB), 1% nicotine salt (1% salt), or 3% nicotine salt (3% salt) vapor. 1-way ANOVA, p=0.6399 **(C)** Latency to feed time measured in males following exposure to PG/VG, 3% FB, 1% salt, or 3% salt vapor. #1-way ANOVA, p=0.0453 **(D)** Diagram of the NSF post-test. **(E)** Post-test food consumption measured in females following exposure to PG/VG, 3% FB, 1% salt, or 3% salt vapor. 1-way ANOVA, p=0.3928 **(F)** Post-test food consumption measured in males following exposure to PG/VG, 3% FB, 1% salt, or 3% salt vapor. #1-way ANOVA, p=0.0192 *Post-hoc Tukey's: PG/VG vs. 3% FB and 3% FB vs. 3% salt

Discussion

The results of this study provide important insight into the role of formulation and concentration in the sex-specific effects of electronically vaporized nicotine on nicotine metabolism, region-specific neuronal activity, and anxiety-like and motivated behavior in female and male C57BL/6J mice. Notably, nicotine vapor composed of 3% nicotine freebase produced significantly higher serum nicotine levels as compared to 1% and 3% nicotine salt formulations in both males and females, and females in the 3% freebase group displayed higher serum nicotine and serum cotinine levels compared to males. Despite these sex-specific differences in serum nicotine levels by formulation and concentration, nicotine vapor exposure of any concentration or formulation

significantly increased activity in the central amygdala (CeA), but only in males. CeA activity in females exposed to nicotine vapor was not significantly different from PG/VG controls, although females displayed higher basal activity. In contrast to the CeA, only the 3% freebase group displayed increased activity in the ventral tegmental area (VTA) and this was only observed in females in both the overall VTA neuronal population and specifically in the dopaminergic VTA population. In anxiety-like and motivated behavior, females were relatively unaffected by nicotine vapor exposure, however anxiety-like behavior in males was impacted by vapor exposure, an effect primarily driven by increased anxiety-like behavior in the 3% freebase group. Males in the 3% freebase group also exhibited parallel reductions in motivated behavior. Collectively this work demonstrates the differential (and sex-specific) impacts of nicotine vapor exposure of different formulations and concentrations. These findings have significant relevance for the metabolic, neuronal and behavioral consequences of vaping in both males and females.

When studying the physiological, metabolic and behavioral effects of drugs of abuse it is imperative to take into consideration sex differences as well as delivery methods (Han et al., 2022; Lallai et al., 2021). Previous preclinical studies have noted a difference in nicotine and cotinine serum levels in male and female mice exposed to nicotine, with males showing higher nicotine serum levels than females (Henderson & Cooper, 2021; Lallai et al., 2021). However, they failed to further investigate the reason behind the nicotine metabolite differences such as enzyme levels. While previous clinical studies included both male and female participants (Leventhal et al., 2021; O'Connell et al., 2019), they did not look at sex differences that might arise from

differences in nicotine metabolism or questionnaire responses. Thus resulting in a limited understanding of the sex-specific effects that nicotine salt and freebase have in a clinical population. One limitation of clinical research is the inability to directly examine the effect nicotine exposure might have on the activation of specific brain regions. Following immunohistochemical analysis in the CeA, we found that males displayed higher neuronal activation following exposure to nicotine vapor regardless of formulation or concentration, while females did not. When quantifying cFos expression in the VTA, which is known for its role in reward signaling, only females showed an effect of vapor content with the 3% freebase group demonstrating higher VTA activation compared to other nicotine vapor-exposed groups. Following the novelty suppressed feeding test, females were unaffected by nicotine exposure while males demonstrated an effect of vapor content with a trend toward increased anxiety-like behavior in the 3% freebase group. These results suggest that sex could potentially influence how nicotine freebase or salt alters reward-seeking and anxiety-like behaviors.

Nicotine metabolite levels are a good indication of the rate at which nicotine is absorbed and then metabolized in the body. Once ingested, nicotine is rapidly metabolized into the more stable metabolite, cotinine, which is often used as a marker of nicotine use. In a human population, vaping nicotine salt resulted in higher serum nicotine levels than vaping nicotine freebase (O'Connell et al., 2019), suggesting that nicotine salt is more effectively absorbed by the body. Clinical studies report that nicotine salt is associated with more positive vaping experiences (Leventhal et al., 2021), which raises the possibility of an altered puff topography (increased puff volume or number of puffs) based on preference that may promote increased absorption. Here,

we observed that nicotine freebase vapor exposure produced higher nicotine serum levels, however this is not likely due to altered respiratory rates as the freebase vapor is generally considered more aversive. Preclinical studies on the effect of formulation on metabolism are contradictory. One study conducted in rats found that following a single subcutaneous dose of nicotine freebase, serum nicotine levels were higher than those in rats injected with nicotine salts (Han et al., 2022), which are consistent with the results of the current study. However, in another study, mice exposed to nicotine salt vapor had significantly higher serum cotinine levels than mice exposed to nicotine freebase. Additionally, serum cotinine levels overall appear higher in males than in females (Henderson & Cooper, 2021). These results differ from the current study where mice exposed to 3% nicotine freebase displayed significantly higher serum nicotine levels than those exposed to 3% nicotine salt, and females in the 3% freebase group displayed higher serum nicotine levels than males. These differences could be due to different concentrations of nicotine (30 mg/ml of nicotine freebase and 30 mg/ml of nicotine salt in our study as opposed to 6mg/ml) (Henderson & Cooper, 2021). Furthermore, the higher serum levels we found in females compared to males could suggest the need for more nicotine in order to maintain reinforcing effects. Clinical studies measuring urine and saliva Nicotine Metabolite Ratios (NMR) in both male and female smokers found that female smokers had higher NMR than males (Jain, 2020). The higher serum nicotine levels observed in females could be due to increased absorption or diminished metabolism. The possibility of sex differences in nicotine metabolizing enzymes should also be considered. Further studies are required to determine the specific metabolic mechanisms driving this effect as well as any

differences that may arise following chronic exposure as this paper focused on a single acute exposure.

The CeA has been implicated in anxiety disorders and substance use such as the central effects of nicotine (Gilpin et al., 2015). However the extent of CeA involvement and specific actions of nicotine in the CeA remain controversial and are potentially dependent on the method, amount, and time-course of nicotine exposure. One study found that acute nicotine exposure of a single intraperitoneal injection increased CeA phosphorylated extracellular regulated kinase (pERK) expression 20 minutes after injection (Valjent et al., 2004), however a study of voluntary nicotine drinking found no increase in amygdala pERK after an acute (1.3 hour) drinking session and only saw a significant increase in amygdala pERK after chronic (28-30 day) drinking (Brunzell et al., 2003). Studies of the immediate early gene cFos are also contradictory. One study found that a single subcutaneous injection of nicotine increased cFos in the CeA, that there was no change in cFos after chronic nicotine exposure, but that cFos was increased with acute nicotine administration after chronic exposure (Salminen et al., 1999). We previously reported increased cFos and neuronal firing in the CeA after a single session of electronic nicotine vapor exposure, but no change in cFos or firing after five days of repeated sessions of nicotine vapor (Zhu et al., 2021). Another report observed increased CeA cFos after withdrawal from chronic nicotine self-administration (Funk et al., 2016). However, exposure to different formulations and/or concentrations of nicotine may differentially impact CeA neuronal activity and subsequent affective behavior in a sex-specific manner. In females, there was no significant difference in CeA activity between groups which could be associated with the lack of nicotine effects

on anxiety-like or motivated behavior observed for these groups in the NSF test. In males, CeA activity was significantly increased in the 3% freebase, 1% salt, and 3% salt groups compared to the PG/VG control group. However, only the male 3% freebase group displayed increased anxiety-like behavior and decreased motivated behavior. The use of the NSF test to examine anxiety-like behavior was first developed in studies using only males; however, this test has since been validated in females. Conditions such as anxiogenic stimuli should be considered when analyzing results from the NSF test and other behavioral assays. A study assessing how various factors (e.g. body weight, social isolation) affected food consumption and anxiety-like behavior in male and female C57Bl/6j mice in the NSF test found that estrous cyclicity was not the main source of variability in stress-induced feeding response but rather sex-specific effects of social isolation duration. All other variables tested resulted in similar anxiety-like responses from males and females (Francois et al., 2022). In our study the group-specific differences in behavior could be associated with the higher levels of serum nicotine observed in the 3% freebase groups. As acute nicotine exposure has been associated with increased anxiety (Biala & Budzynska, 2006), the higher serum nicotine levels in males could contribute to the increased anxiety-like behavior and decreased motivated behavior in this group compared to other nicotine vapor-exposed groups. However, females in the 3% freebase group also had significantly elevated serum nicotine levels, even higher levels than males, but did not display increased anxiety-like behaviors, potentially due to elevated basal CeA activity. In addition, as nicotine has appetite-suppressing properties (Mineur et al., 2011), it is possible that the elevated serum nicotine levels in the male 3% freebase group contributed to the decreased

motivated behavior seen in NSF, however this effect was not observed in females, suggesting that it is not a global suppression. Some limitations of using the NSF test, specifically in our study, include the stimulant effects of nicotine on locomotion and appetite. In future studies, it may be worthwhile to use an additional behavioral assay to assess anxiety-like behavior to support and validate the behavioral responses measured in the NSF test. They should also employ different (or multiple) tests of anxiety-like behavior to further validate the lack of effects of nicotine vapor on anxiety-like behavior in female mice.

The VTA and its dopaminergic activity is thought to underlie reward signaling in the brain and is a major target of drugs of abuse, including nicotine (Picciotto & Kenny, 2021). Nicotine has previously been shown to increase cell firing (Picciotto et al., 1998; Yin & French, 2000) and cFos expression (Baur et al., 2018) in the VTA. Additionally, studies have found that both rats (Lallai et al., 2021) and mice (Henderson & Cooper, 2021) will self-administer nicotine vapor. In our study, we found that expression of the neuronal activity marker cFos in the VTA is significantly increased in females, but not males, exposed to 3% freebase vapor as compared to PG/VG controls and both 1% and 3% nicotine salt vapor. This effect is preserved in the dopaminergic population in the VTA which indicates that nicotine freebase specifically activates the dopaminergic population in the VTA more than nicotine salt, suggestive of increased reward signaling. The increased cFos expression was correlated with the serum nicotine levels indicating that these effects are likely driven by elevated levels of nicotine itself. These results may appear to contradict a previous study which found that in an electronic nicotine vapor self-administration model, female and male mice nosepoke more for nicotine salt vapor

than nicotine freebase vapor (Henderson & Cooper, 2021). However, we found that serum nicotine levels were significantly lower in the 3% nicotine salt groups as compared to the 3% nicotine freebase group which potentially could explain the higher reward-seeking behavior in the nicotine salt group in that study. If nicotine salt produces less serum nicotine than freebase nicotine, then the animals will nosepoke more to reach a level of serum nicotine that is similar to freebase nicotine exposure. Overall, our current findings indicate that nicotine formulation (freebase vs salt) can produce different serum nicotine and cotinine levels as well as differentially activate the dopaminergic VTA population which may underlie differences observed in the nicotine vapor self-administration studies. Additional experiments are required to further understand the mechanistic differences of nicotine freebase vs salt vapor exposure and how chronic exposure affects nicotine metabolism, CeA and VTA neuronal activation, and anxiety-like and motivated behaviors.

As ENDS use increases in prevalence and popularity, it is important that preclinical studies investigate nicotine vapor exposure that models human use in an array of aspects such as frequency and volume of inhalation as well as emerging biochemical and technological adaptations to ENDS devices. The results of the present study highlight important sex differences in the effects of nicotine vapor exposure by formulation and concentration. These differences could have significant consequences in the acute effects of vaping as well as the development of nicotine dependence through ENDS usage in men and women. It is important to note that the changes here only reflect immediate effects following acute exposure, while clinical populations likely reflect a more prolonged chronic use. Future studies will investigate the role of

formulation and concentration on long-term ENDS usage and on withdrawal or cessation of use.

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CHAPTER 4: SEX DIFFERENCES IN THE IMPACT OF ELECTRONIC NICOTINE VAPOR ON CORTICOTROPIN-RELEASING FACTOR RECEPTOR 1 NEURONS IN THE MOUSE VENTRAL TEGMENTAL AREA[3]

Introduction

Canonically, nicotine's addictive properties involve activation of nicotinic acetylcholine receptors on dopaminergic neurons in the ventral tegmental area (VTA) driving dopamine release in the nucleus accumbens (NAc). Nicotine also produces distinct patterns of excitatory glutamatergic and inhibitory GABAergic inputs onto VTA dopamine neurons increasing overall excitability (Mansvelder & McGehee, 2000; Mansvelder et al., 2002). Although nicotine typically increases VTA dopamine neuron activity, nicotine is also inhibitory in amygdala-projecting VTA dopamine neurons and increases anxiety-like behaviors (Nguyen et al., 2021). Rodents will self-administer nicotine intravenously (Gilpin et al., 2014; O'Dell et al., 2007; Picciotto et al., 1998), intracranially into the VTA (Husson et al., 2020; Ikemoto et al., 2006; Maskos et al., 2005), and through inhalation (Cooper et al., 2021; Lallai et al., 2021). However, the impact of nicotine vapor from electronic nicotine delivery systems (ENDS) on specific VTA neuronal populations and mesolimbic reward circuitry remains unclear.

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Nicotine also modulates stress and anxiety behaviors and stress-induced relapse to drug seeking (Doherty et al., 1995; Fidler & West, 2009; Parrott, 1993). The corticotropin-releasing factor (CRF) system regulates stress responding and is implicated in cocaine (Blacktop et al., 2011; Han et al., 2017; Vranjkovic et al., 2018), alcohol (Agoglia et al., 2022; Agoglia et al., 2020; Herman et al., 2016; Newman et al., 2018), and nicotine addiction (Grieder et al., 2014; Uribe et al., 2020). Nicotine activates the hypothalamic-pituitary-adrenal axis driving CRF release in the thalamus (Rohleder & Kirschbaum, 2006). Chronic nicotine exposure alters basal hypothalamic-pituitary-adrenal (HPA) axis activity (Rohleder & Kirschbaum, 2006) and increases CRF mRNA expression in the VTA (Grieder et al., 2014). CRF increases VTA dopamine neuron firing via CRF receptor 1 (CRF1) (Wanat et al., 2008; Zalachoras et al., 2022). VTA CRF1 neurons project to the NAc core and activation of these neurons' cell bodies and terminals coordinates reward reinforcement behavior and enhance dopamine release, respectively (Heymann et al., 2020). Conversely, CRF1 deletion in midbrain dopamine neurons increases anxiety-like behavior (Refojo et al., 2011) and CRF1 antagonism reduces footshock-induced reinstatement of nicotine seeking (Bruijnzeel et al., 2009).

Nicotine's VTA effects and modulation of anxiety and stress have been well-studied, however, less is known about VTA CRF1 neurons and their response to electronic nicotine vapor, especially in females. ENDS products provide an opportunity for pre-clinical studies to examine sex- and population-specific effects of nicotine vapor exposure. We used male and female CRF1-GFP and CRF1-Cre mice to determine how acute and chronic nicotine vapor exposure dysregulates activity and inhibitory control of VTA CRF1 neurons.

Materials and Methods

Animals

Adult CRF1-GFP (Justice et al., 2008) or CRF1-Cre mice were bred in-house and group-housed in temperature- and humidity-controlled 12hr light/dark (7am lights on) facilities with ad libitum food and water access. All experimental procedures were approved by the UNC Institutional Animal Care and Use Committee.

Drugs

Freebase (-)-nicotine (N3876) and propylene glycol (PG, P4347) were purchased from Sigma. Vegetable glycerol (VG, G33-500) was purchased from Fisher. Glutamate receptor antagonists DNQX (0189) and DL-AP5 (3693) as well as GABA receptor antagonists CGP 52432 (1246) and SR 95531 (GBZ, 1262) were all purchased from Tocris Bioscience.

Immunohistochemistry

Mice were perfused and tissue sectioned and processed as previously described (Zhu et al., 2021). Sections were incubated with primary antibodies: mouse anti-TH (1:1000; T1229, Sigma), chicken anti-GFP (1:1000; ab13970, Abcam), rabbit anti-cFos (1:3000; ABE457, Millipore) and secondary antibodies: Alexa 555 goat anti-mouse (1:250; A21424, Invitrogen), Alexa 647 goat anti-mouse (1:200; 115-605-003, JacksonImmuno), Alexa 488 donkey anti-chicken (1:700; 703-545-155, JacksonImmuno) or goat anti-rabbit horse radish peroxidase (1:200; ab6721, Abcam) followed by tyramide-conjugated Cy3 (1:50) diluted in TSA amplification diluents (Akoya

Biosciences, NEL741001KT). Sections were mounted (Vectashield mounting medium, H-1200, Vector Labs), cover-slipped, and imaged (Leica SP8 confocal). Quantification was performed by blinded experimenters using ImageJ (NIH).

Stereotaxic Intracranial Microinjection

Mice were anesthetized with isoflurane (2-4%) for stereotaxic (Kopf Instruments) intracranial infusions (100 nl/min) into target regions. CRF1-cre mice were injected with AAV5-hSyn-DIO-eGFP (500 nL/hemisphere; 50457-AAV5; titer ≥ 7×10^{12} vg/mL, Addgene) into the VTA (ML ±0.60, AP -3.2, DV -4.5). CRF1-GFP mice were injected with red retrograde beads (250 nL/hemisphere; Lumafluor) into the NAc (ML ±0.65, AP +1.48, DV -4.75).

Slice Electrophysiology

Immediately after completion of last vapor exposure, mice were rapidly decapitated and brains extracted into sucrose solution containing (in mM): sucrose 206.0; KCl 2.5; CaCl2 0.5; MgCl2 7.0; NaH2PO4 1.2; NaHCO3 26; glucose 5.0; HEPES 5. Coronal slices (250-300 μM) were incubated in oxygenated (95% O2/5% CO2) artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl 130; KCL 3.5; CaCl2 2; MgSO47H2O 1.5; NaH2PO4H2O 1.25; NaHCO3 24; Glucose 10 at 37□C (30 min) and room temperature (30 min). Recordings were performed 1-8 hours following decapitation with pipettes (4-7 MΩ) filled with internal solution (in mM) KCl 145; EGTA 5; MgCl2 5; HEPES 10; Na-ATP 2; Na-GTP 0.2. Spontaneous action potentials were measured in whole-cell current clamp and action potential half width and threshold to

fire were quantified by Clampfit 11.1 (Molecular Devices). Rheobase was determined using current clamp with cell held at -70 mV and increments of 5 or 10 pA steps. Inhibitory transmission was measured using whole-cell voltage-clamp (Vhold= -60 mV) recording mode with glutamate receptor antagonists (20 μM DNQX and 50 μM AP5) and GABAB receptor antagonist (1 μM CGP 52432). All recordings were obtained at a sampling rate of 10,000 Hz with a Multiclamp 700B amplifier (Molecular Devices), low pass filtered at 2-5 kHz, digitized (Digidata 1440A; Molecular Devices), and stored on a computer (pClamp 10 software; Molecular Devices). Nicotine (1 μM, freebase) and gabazine, a GABAA receptor antagonist (100 μM; SR 955531) were focally applied by a y-tube positioned in close proximity to the recorded cell. Firing frequency was quantified by Clampfit. Spontaneous inhibitory postsynaptic currents (sIPSCs) from stable recording periods with ≥60 events were analyzed using MiniAnalysis (Synaptosoft). Focal nicotine (1 μM)-induced sIPSCs were analyzed for ~2 minutes as previously reported (Mansvelder et al., 2002). Tonic current was determined using a Gaussian fit to an all-points histogram as previously described (Glykys & Mody, 2007).

Electronic Nicotine Vapor Exposure

Mice were placed in airtight vacuum-controlled chambers (~1 L/min air circulation). Vaporizers (95 watt, SVS200, Scientific Vapor) heated (up to 200°C) solutions in e-vape tanks (Baby Beast Brother, Smok) with coils (0.25 Ω resistance, Smok). Mice were exposed to vaporized e-liquid solution of either 12% (v/v, or 120 mg/mL) (-)-nicotine freebase in a 50/50 (v/v) propylene glycol/vegetable glycerol (PG/VG) or PG/VG control solution (Zhu et al., 2021). E-vape controllers (SSV-1,

LJARI) trigger 3 second vapor deliveries every 10 minutes over a 3-hour session (acute) or daily 3 hour sessions for 28 days (chronic).

Nicotine and Cotinine Serum Analysis

Trunk blood was collected immediately following final vapor exposure. Samples were centrifuged and serum was collected and stored at -20°C. Samples were analyzed for nicotine and cotinine, a nicotine metabolite, using liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described (Ghosh et al., 2019).

Statistical Analysis

Data were analyzed (Prism 9.0, GraphPad) and compared using unpaired two-tailed t-tests (with Welch's correction where appropriate), one-sample t-tests (theoretical mean = 100), and two-way ANOVAs with post-hoc Tukey's where appropriate. Variance (F test or Bartlett's test) and normality (Kolmogorov-Smirnov test) were assessed. All data are expressed as mean ± SEM with $p<0.05$ as the criterion for statistical significance.

Results

Characterization of VTA CRF1 neurons

GFP+ (CRF1) neurons were expressed predominantly in the lateral region of the VTA (**Fig 3.1A, top**) and colocalized with tyrosine hydroxylase (TH+), a marker of dopaminergic neurons (**Fig 3.1A, bottom**). ~40% of TH+ VTA neurons co-expressed CRF1 (**Fig 3.1B**) and ~80% of CRF1 neurons co-expressed TH (**Fig 3.1C**). No sex

differences were observed in co-expression of either CRF1 or TH populations (**Fig 3.1B, C**). We injected AAV-hSyn-DIO-eGFP into the VTA (**Fig 3.1D, E left**) of CRF1-cre mice and observed terminal expression in the NAc (**Fig 3.1E, right**), suggesting that CRF1 neurons are part of the mesolimbic VTA-NAc circuitry.

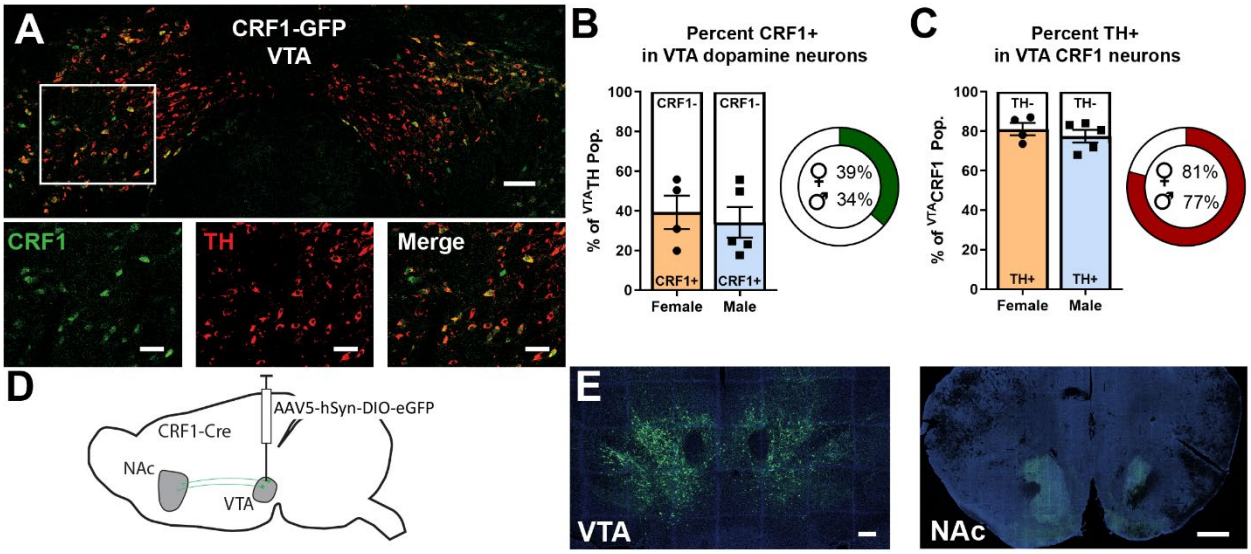
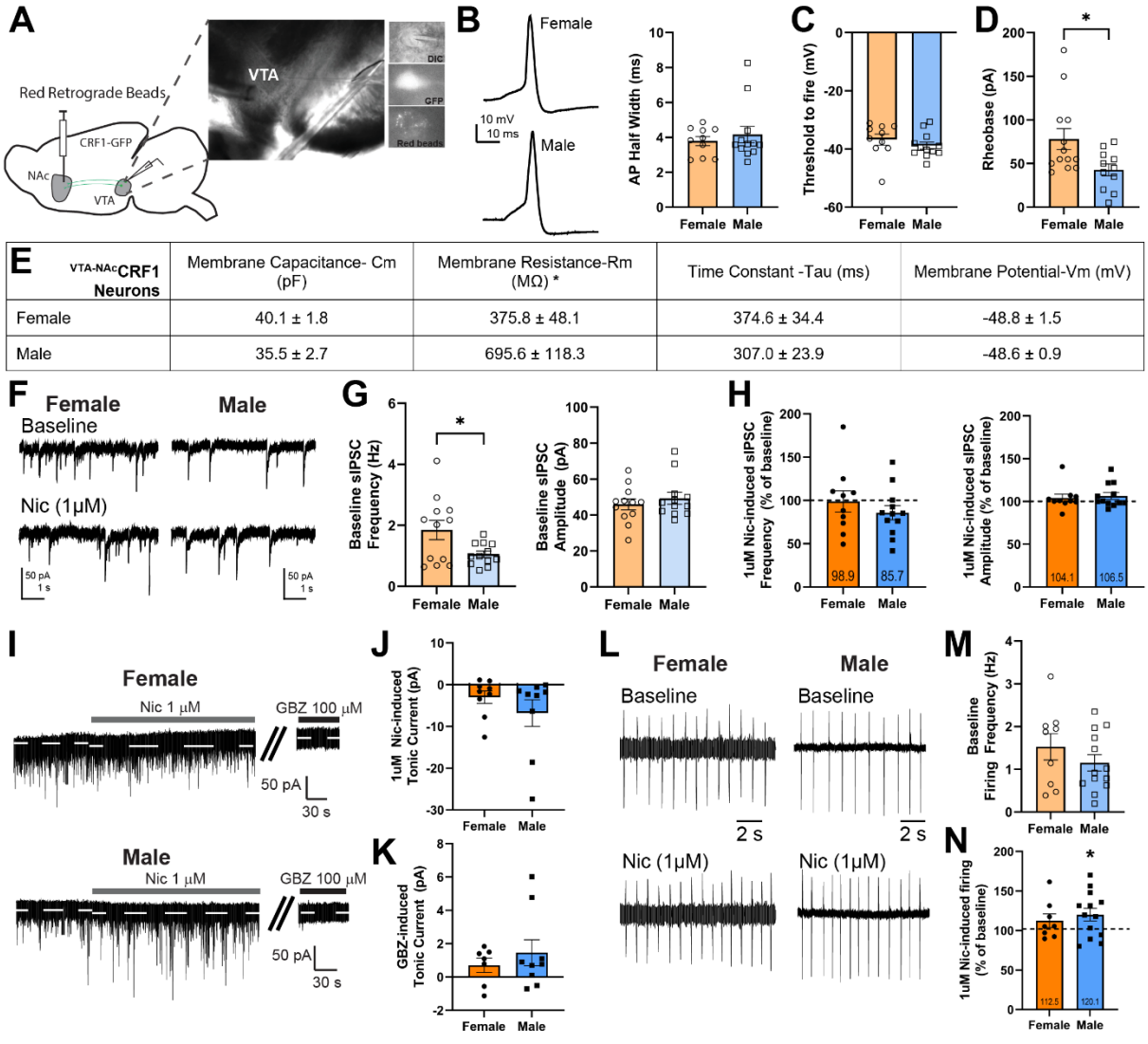


Figure 3.1- Immunohistochemical characterization of VTA CRF1 neurons. **(A)** Top: Representative image of a coronal section of the VTA, scale bar=100 µm. Bottom: Magnified view (white box in top image) of CRF1 (GFP), tyrosine hydroxylase (TH), and merged channels, scale bar=50 µm. **(B)** Percentage of TH neurons in the VTA that are CRF1 positive (colored) and negative (white) in females (N=4) and males (N=5); unpaired t-test, p=0.67, t=0.44, df=7. Inset: averaged percentage of CRF1+ neurons in female and male mice. **(C)** Percentage of CRF1 neurons in the VTA that are TH positive (colored) and negative (white) in females (N=4) and males (N=5); unpaired t-test, p=0.45, t=0.79, df=7. Inset: averaged percentage of TH+ neurons in female and male mice. **(D)** Schematic of viral strategy to probe VTA CRF1 neuron projections.

(E) Left: VTA injection site, scale bar=200 μm. Right: Terminal expression in the NAc, scale bar=500 μm. Data represented as mean ± SEM.

To determine electrophysiological characteristics of VTA CRF1 neurons that project to NAc ($^{VTA\text{-}NAc}$CRF1 neurons), we injected retrograde beads into the NAc of CRF1-GFP mice (**Fig 3.2A**) and performed electrophysiological recordings in the VTA, targeting neurons co-expressing GFP (CRF1) and beads (**Fig 3.2A, inset**). Using whole cell current clamp with no holding current, we measured spontaneous action potential characteristics in both sexes (**Fig 3.2B, left**) and did not observe any differences in action potential shape or spiking properties. Specifically, action potential half width (**Fig 3.2B, right**) and threshold to fire (**Fig 3.2C**) were not significantly different between the two sexes, however rheobase was significantly higher in females as compared to males (unpaired t-test *p=0.024, t=2.428, **Fig 3.2D**). Additionally, we found no significant differences in membrane properties, except increased membrane resistance in males (unpaired t-test *p=0.017, t=2.51, **Fig 3.2E**). Voltage-clamp recordings of spontaneous inhibitory post-synaptic currents (sIPSCs, **Fig 3.2F**) were performed and sIPSC frequency was significantly higher in females (unpaired t-test *p=0.033, t=2.38, **Fig 3.2G, left**) but sIPSC amplitude showed no sex differences (**Fig 3.2G, right**). Focal nicotine (1 μM) did not alter sIPSC frequency or amplitude (**Fig 3.2H**) in either sex when normalized to baseline. Focal nicotine also induced a tonic inhibitory current in $^{VTA\text{-}NAc}$CRF1 neurons of both sexes (**Fig 3.2I, J**) that was marginal in overall magnitude but was consistently observed and was partially reversed by 100 μM gabazine (**Fig 3.2K**). Cell-attached recordings of spontaneous firing (**Fig 3.2L**) found no sex differences in

baseline firing (**Fig 3.2M**) but focal nicotine induced a significant (~20%) increase in normalized firing frequency in males (*p=0.027, t=2.52; **Fig 3.2N**). These data indicate that $^{VTA\text{-}NAc}$CRF1 neurons in females display greater baseline phasic inhibition, but males display greater focal nicotine-induced firing. Focal nicotine induces tonic inhibition in both sexes.



E $^{VTA\text{-}NAc}$CRF1 Neurons	Membrane Capacitance- Cm (pF)	Membrane Resistance-Rm (MΩ) *	Time Constant -Tau (ms)	Membrane Potential-Vm (mV)
Female	40.1 ± 1.8	375.8 ± 48.1	374.6 ± 34.4	-48.8 ± 1.5
Male	35.5 ± 2.7	695.6 ± 118.3	307.0 ± 23.9	-48.6 ± 0.9

Figure 3.2- Electrophysiological properties and effects of focal nicotine in $^{VTA\text{-}NAc}$CRF1 neurons. (A) Schematic of red retrobead injection in the NAc and

electrophysiological recording in the VTA in CRF1-GFP mice. Inset: Coronal section of VTA (4x) with recording electrode (right) and y-tube (left) and example of recorded neuron (60x) with differential interference contrast (DIC, top), GFP/CRF1 expression (middle), and red bead expression (bottom). **(B)** Example traces of spontaneous action potential (AP) waveform in female (top left) and male (bottom left) and averaged action potential half width (right); unpaired t-test, $p>0.05$. **(C)** Averaged threshold to fire; unpaired t-test, $p>0.05$. **(D)** Averaged rheobase; unpaired t-test, $*p=0.024$, $t=2.428$, $df=22$. **(E)** Membrane properties of $^{VTA\text{-}NAc}$CRF1 neurons from female (n=17 cells, N=8 mice) and male (n=28 cells, N=11 mice) mice. Males showed higher membrane resistance than females; unpaired t-test, $*p=0.017$, $t=2.51$, $df=35.1$. **(F)** Example traces of spontaneous inhibitory post-synaptic currents (sIPSCs) in $^{VTA\text{-}NAc}$CRF1 neurons from female (left) and male (right) mice at baseline and after 1 μM focal nicotine application. **(G)** Baseline sIPSC frequency (left) in $^{VTA\text{-}NAc}$CRF1 neurons were higher in female (n=12 cells, N=6 mice) than male (n=12 cells, N=8 mice) mice; unpaired t-test with Welch's correction, $*p=0.033$, $t=2.38$, $df=13.4$. Baseline sIPSC amplitude (right) in $^{VTA\text{-}NAc}$CRF1 neurons from female (n=12 cells, N=6 mice) and male (n=12 cells, N=8 mice) mice; unpaired t-test, $p>0.05$. **(H)** 1 μM nicotine-induced change in sIPSC frequency (left) normalized to baseline in $^{VTA\text{-}NAc}$CRF1 neurons from female (n=10 cells, N=5 mice) and male (n=12 cells, N=8 mice) mice; unpaired t-test and one sample t-test, $p>0.05$. 1 μM nicotine-induced change in sIPSC amplitude normalized to baseline in $^{VTA\text{-}NAc}$CRF1 neurons from female (n=10 cells, N=5 mice) and male (n=12 cells, N=8 mice) mice; unpaired t-

test and one sample t-test, p>0.05. **(I)** Example traces of 1 μM nicotine-induced and gabazine (GBZ, 100 μM) reversed tonic current in $^{VTA\text{-}NAc}$CRF1 neurons of female (top) and male (bottom) mice. **(J)** 1 μM nicotine-induced inhibitory tonic current in $^{VTA\text{-}NAc}$CRF1 neurons of female (n=9 cells, N=4 mice) and male (n=9 cells, N=6 mice) mice; unpaired t-test with Welch's correction, p>0.05. **(K)** 100 μM Gabazine (GBZ)-induced reversal of tonic current in $^{VTA\text{-}NAc}$CRF1 neurons of female (n=7 cells, N=4 mice) and male (n=9 cells, N=6 mice) mice; unpaired t-test, p>0.05. **(L)** Example traces of cell-attached firing in $^{VTA\text{-}NAc}$CRF1 neurons from female (left) and male (right) mice at baseline and after 1 μM focal nicotine application. **(M)** Baseline firing frequency in $^{VTA\text{-}NAc}$CRF1 neurons of female (n=9 cells, N=5 mice) and male (n=13 cells, N=9 mice) mice; unpaired t-test, p>0.05. **(N)** 1 μM nicotine-induced change in firing frequency normalized to baseline in $^{VTA\text{-}NAc}$CRF1 neurons from female (n=8 cells, N=5 mice) and male (n=13 cells, N=9 mice; one sample t-test, *p=0.027, t=2.52, df=12) mice. Data are represented as mean ± SEM.

Serum nicotine metabolite levels following acute and chronic electronic nicotine vapor exposure

Acute vapor exposure consisted of a 3-hr session (3-sec vapor deliveries every 10 minutes, **Fig 3.3A**). Mice exposed to acute 12% nicotine vapor showed significantly elevated serum nicotine (2-way ANOVA, main effect of vapor content ****p<0.0001, F(1,40)=32.75; PG/VG vs Nic: Female ***p=0.0003, Male **p=0.0055; **Fig 3.3B, left**) and serum cotinine, the primary metabolite of nicotine (2-way ANOVA, main effect of vapor content ****p<0.0001, F(1,40)=53.80; PG/VG vs Nic: Female- ****p<0.0001, Male-

****p<0.0001; **Fig 3.3B, right**). Chronic exposure consisted of daily 3-hr sessions for 28 days (**Fig 3.3C**). A similar effect to the acute animals, but at a larger magnitude, was observed following chronic vapor exposure where both female and male mice showed significantly elevated serum nicotine (2-way ANOVA, main effect of vapor content ****p<0.0001, F(1,30)=57.68; PG/VG vs Nic: Female- ****p<0.0001, Male- ***p=0.0002; **Fig 3.3D, left**) and serum cotinine (2-way ANOVA, main effect of vapor content ****p<0.0001, F(1,30)=41.95; PG/VG vs Nic: Female- **p=0.0087, Male- ****p<0.0001; **Fig 3.3D, right**). These data demonstrate that nicotine delivery through electronic vapor is an effective route of administration that produces significant serum nicotine and cotinine levels following both acute and chronic exposure, however serum levels were more elevated following chronic exposure suggesting potential impairment of nicotine metabolism.

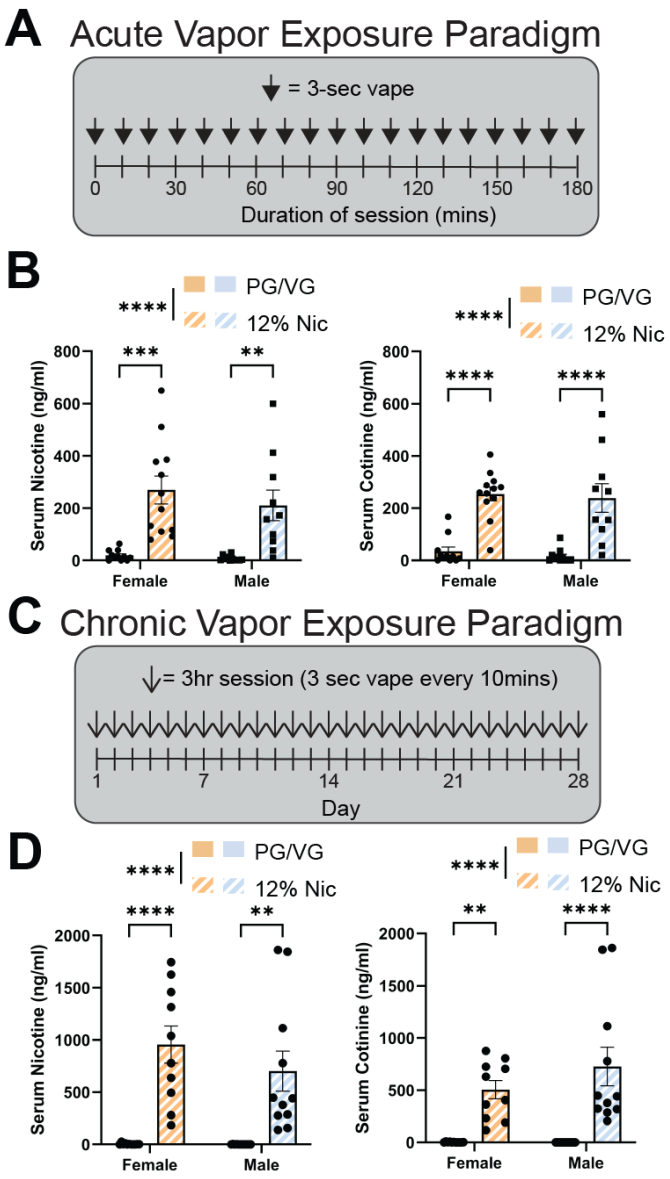


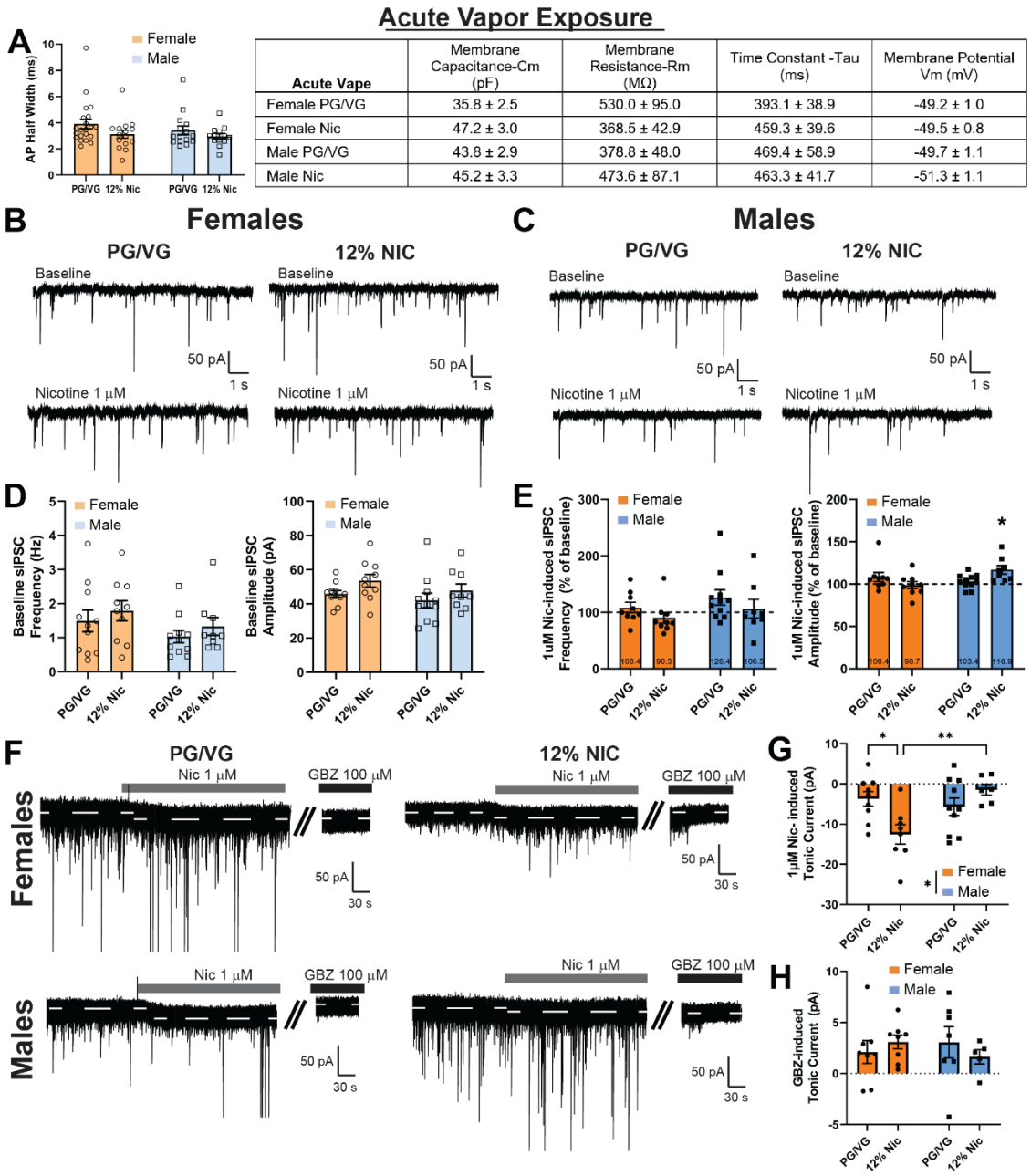
Figure 3.3. Nicotine and cotinine serum levels following acute and chronic electronic nicotine vapor exposure. **(A)** Acute vapor exposure paradigm depicting a 3 hour session of 3 second vape every 10 minutes. **(B)** Serum nicotine (left; 2-way ANOVA, main effect of vapor content ****p<0.0001, F (1, 40) = 32.75, post hoc Tukey's Female PG/VG vs. Nic ***p=0.0003, Male PG/VG vs. Nic **p=0.0055) and serum cotinine (right; 2-way ANOVA, main effect of vapor content ****p<0.0001, F (1, 40) = 53.80, post hoc Tukey's Female PG/VG vs. Nic

****p<0.0001, Male PG/VG vs. Nic ****p<0.0001) levels following acute PG/VG or 12% nicotine vape exposure in female and male mice. **(C)** Chronic vapor exposure paradigm depicting daily 3 hour sessions of 3 second vape every 10 minutes over the course of 28 days. **(D)** Serum nicotine (left; 2-way ANOVA, main effect of vapor content ****p<0.0001, F (1, 40) = 43.42, post hoc Tukey's Female PG/VG vs. Nic ****p<0.0001, Male PG/VG vs. M Nic **p=0.0013) and serum cotinine (right; 2-way ANOVA, main effect of vapor content ****p<0.0001, F (1, 40) = 37.20, post hoc Tukey's Female PG/VG vs. F Nic **p=0.0071, Male PG/VG vs. Nic ****p<0.0001) levels following chronic PG/VG or 12% nicotine vape exposure in female and male mice. ***p<0.005, ***p<0.0005, ****p<0.0001.* Data are represented as mean ± SEM.

The effects of acute vapor exposure on inhibitory signaling in $^{VTA\text{-}NAc}$CRF1 neurons

We exposed CRF1-GFP mice to acute PG/VG or 12% nicotine vapor (**Fig 3.3A**) and performed slice electrophysiology targeting $^{VTA\text{-}NAc}$CRF1 neurons using bead injections as described above (**Fig 3.2A**). We found no significant difference in action potential half width (2-way ANOVA, p>0.05; **Fig 3.4A, left**) or threshold to fire between groups (2-way ANOVA, p>0.05, Female PG/VG -40.2 ± 1.37, Female Nic -38.0 ± 1.28, Male PG/VG -39.6 ± 1.27, Male Nic -39.9 ± 0.73). Rheobase showed an interaction (2-Way ANOVA, *p=0.049, Female PG/VG 35.7 ± 5.57, Female Nic 50.4 ± 7.14, Male PG/VG 59.7 ± 9.54, Male Nic 42.7 ± 7.42) but post hoc Tukey's did not yield any significant comparisons. Additionally, we found no significant differences in membrane properties (**Fig 3.4A, right**), except membrane capacitance (main effect of vapor

content *p=0.035, F(1,77)=4.59, but p>0.05 by Tukey's multiple comparisons test). In females (**Fig 3.4B**) and males (**Fig 3.4C**), there were no significant differences in baseline sIPSC frequency or amplitude (**Fig 3.4D**). In both sexes, focal nicotine (1 μM) produced no change in sIPSC frequency when normalized to baseline (**Fig 3.4E, left**). However, normalized sIPSC amplitude in 12% nicotine-exposed males was significantly increased (*p=0.011, t=3.41; **Fig 3.4E, right**). Focal nicotine (1 μM) induced a tonic current that was comparable in PG/VG groups but enhanced in 12% nicotine-exposed females and reduced in 12% nicotine-exposed males (interaction **p=0.005, F (1, 29) = 9.49; main effect of sex *p=0.039, F (1, 29) = 4.68; Female: PG/VG vs Nic *p=0.024, Female Nic vs. Male Nic **p=0.0091; **Fig 3.4F, G**). Gabazine (GBZ, 100 μM) partially reversed the focal nicotine-induced tonic current (**Fig 3.4H**). These data suggest that phasic inhibition in $^{VTA\text{-}NAc}$CRF1 neurons is largely unaffected but focal nicotine-induced tonic inhibition was bidirectionally dysregulated in a sex-specific manner following acute nicotine vapor exposure.



Acute Vape	Membrane Capacitance-Cm (pF)	Membrane Resistance-Rm (MΩ)	Time Constant -Tau (ms)	Membrane Potential Vm (mV)
Female PG/VG	35.8 ± 2.5	530.0 ± 95.0	393.1 ± 38.9	-49.2 ± 1.0
Female Nic	47.2 ± 3.0	368.5 ± 42.9	459.3 ± 39.6	-49.5 ± 0.8
Male PG/VG	43.8 ± 2.9	378.8 ± 48.0	469.4 ± 58.9	-49.7 ± 1.1
Male Nic	45.2 ± 3.3	473.6 ± 87.1	463.3 ± 41.7	-51.3 ± 1.1

Figure 3.4.- Inhibitory signaling in $^{VTA\text{-}NAc}$CRF1 neurons following acute vapor exposure. **(A)** Averaged action potential half width (left) and membrane properties (right) of $^{VTA\text{-}NAc}$CRF1 neurons from male and female mice exposed to acute PG/VG (Female n=23 cells, N=6 mice; Male n=22 cells, N=5 mice) or 12% nicotine vapor (Female n=15 cells, N=4 mice; Male n=21 cells, N=5 mice) with a

main effect of vapor content on membrane capacitance (2-way ANOVA *p=0.035, F(1,77)=4.59). **(B)** Example of spontaneous inhibitory post-synaptic currents (sIPSCs) in $^{VTA\text{-}NAc}$CRF1 neurons from female mice exposed to PG/VG (left) or 12% nicotine (right) at baseline and after 1 μM nicotine application. **(C)** Example of sIPSCs in $^{VTA\text{-}NAc}$CRF1 neurons from male mice exposed to PG/VG (left) or 12% nicotine (right) at baseline and after 1 μM nicotine application. **(D)** Baseline sIPSC frequency (left) and amplitude (right) in $^{VTA\text{-}NAc}$CRF1 neurons from female and male mice exposed to PG/VG (Female n=11 cells, N=5 mice; Male n= 11 cells, N=4 mice) or 12% nicotine (Female n=10 cells, N=4 mice; Male n= 8 cells, N=5 mice); 2-way ANOVA p>0.05. **(E)** 1 μM nicotine-induced change in sIPSC frequency (left) and amplitude (right) normalized to baseline in $^{VTA\text{-}NAc}$CRF1 neurons from female and male mice exposed to PG/VG (Female n=9 cells, N=5 mice; Male n=11 cells, N=4 mice) or 12% nicotine (Female n=9 cells, N=4 mice; Male n= 8 cells, N=5 mice). Normalized sIPSC amplitude show vapor content x sex interaction (2-way ANOVA *p=0.010, F (1, 33) = 7.45) and specifically, the male 12% Nic group shown significant increase from baseline (one sample t-test *p=0.011, t=3.41, df=7). **(F)** Example traces of 1 μM nicotine-induced and gabazine (GBZ) reversed tonic current in $^{VTA\text{-}NAc}$CRF1 neurons from female (top) and male (bottom) mice exposed to PG/VG (left) or 12% nicotine (right). **(G)** 1 μM nicotine-induced tonic current in $^{VTA\text{-}NAc}$CRF1 neurons from female and male mice exposed to PG/VG (Female n=9 cells, N=5 mice; Male n=10 cells, N=4 mice) or 12% nicotine (Female n=8 cells, N=4 mice; Male n= 6 cells, N=3 mice). Tonic current showed a vapor content x sex interaction

(**p=0.005, F (1, 29) = 9.49), main effect of sex (*p=0.039, F (1, 29) = 4.68) and post hoc Tukey's significance in Female: PG/VG vs Nic (*p=0.024) and Female Nic vs. Male Nic (**p=0.0091) by 2-way ANOVA. **(H)** Gabazine (GBZ, 100 μM) reversal of tonic current in $^{VTA\text{-}NAc}$CRF1 neurons of female and male mice exposed to PG/VG (Female n=8 cells, N=4 mice; Male n=7 cells, N=4 mice) or 12% nicotine (Female n=8 cells, N=4 mice; Male n=6 cells, N=3 mice); 2-way ANOVA p>0.05. Data are represented as mean ± SEM.

The effects of acute vapor exposure on spontaneous firing of $^{VTA\text{-}NAc}$CRF1 neurons

We performed cell-attached recordings in female (**Fig 3.5A**) and male (**Fig 3.5B**) mice exposed to acute PG/VG or 12% nicotine vapor. We observed higher baseline firing frequency in males as compared to females (main effect of sex *p=0.028, F (1, 49) = 5.11; **Fig 3.5C**), however with a marginal overall effect size. Focal nicotine (1 μM)-induced changes in firing normalized to baseline was significantly higher in females exposed to either PG/VG or 12% nicotine as compared to males (main effect of sex *p=0.025, F (1, 37) = 5.44; **Fig 3.5D**). These data suggest that acute exposure does not affect spontaneous firing in $^{VTA\text{-}NAc}$CRF1 neurons. However, females exposed to either PG/VG or 12% nicotine vapor show lower baseline firing and focal nicotine-induced increases in firing.

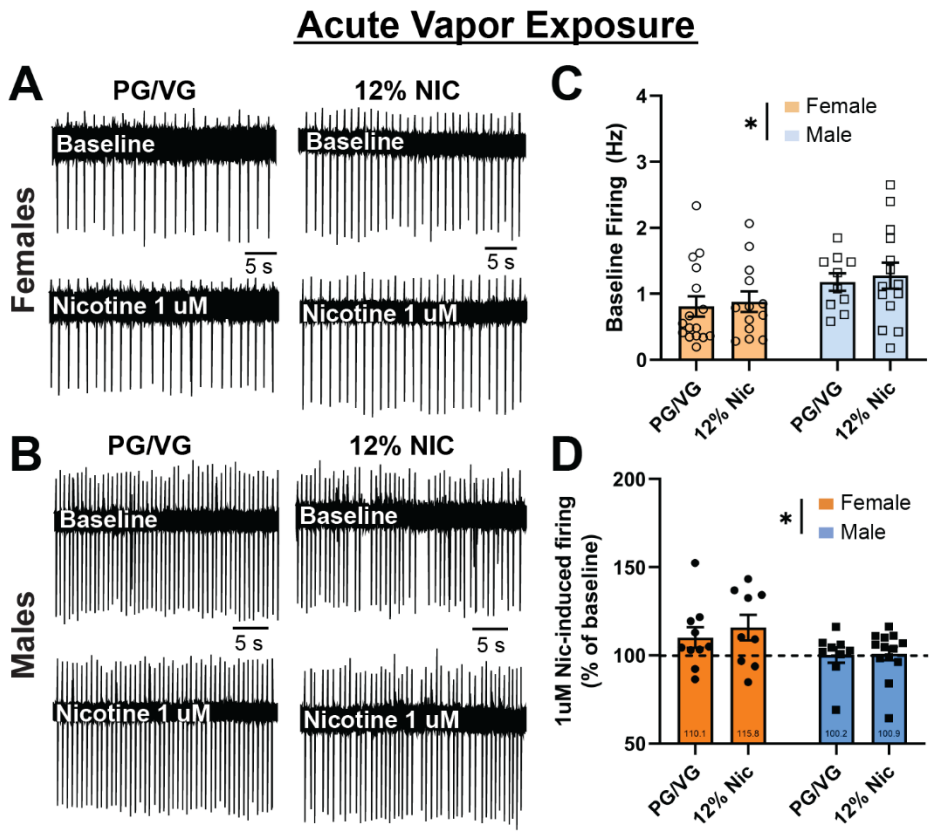


Figure 3.5- Spontaneous firing in $^{VTA\text{-}NAc}$CRF1 neurons following acute vapor exposure. **(A)** Example of spontaneous firing in $^{VTA\text{-}NAc}$CRF1 neurons from female mice exposed to acute PG/VG (left) or 12% nicotine (right) at baseline and after 1 μM focal nicotine application. **(B)** Example of spontaneous firing in $^{VTA\text{-}NAc}$CRF1 neurons from male mice exposed to PG/VG (left) or 12% nicotine (right) at baseline and after 1 μM nicotine application. **(C)** Baseline firing in $^{VTA\text{-}NAc}$CRF1 neurons from females and males exposed to acute PG/VG (Female n=16 cells, N=5 mice; Male n=10 cells, N=4 mice) or 12% nicotine (Female n=13 cells, N=5 mice; Male n=14 cells, N=4 mice); 2-way ANOVA, main effect of sex *p=0.028, F (1, 49) = 5.11. **(D)** 1 μM nicotine-induced change in firing in $^{VTA\text{-}NAc}$CRF1 neurons from female and male mice exposed to acute PG/VG (Female n=10 cells, N=5 mice; Male n=9 cells, N=4 mice) or 12% nicotine (Female n=9

cells, N=5 mice; Male n=13 cells, N=4 mice); 2-way ANOVA, main effect of sex *p=0.025, F (1, 37) = 5.44. Data are represented as mean ± SEM.

The effects of acute vapor exposure on VTA subpopulation activity

To examine overall activity in the VTA we performed immunohistochemistry to label cFos, tyrosine hydroxylase (TH), and CRF1 (**Fig 3.6A**) following acute vapor exposure as described above (**Fig 3.3A**). Animals of both sexes exposed to acute 12% nicotine vapor showed an overall increase in number of cFos+ neuron expression in the VTA (main effect of vapor content *p=0.022, F (1, 13) = 6.80; **Fig 3.6B**). Specifically, cFos expression was increased in the TH+ (dopaminergic) VTA population in both sexes exposed to 12% nicotine compared to PG/VG (main effect of vapor content ***p=0.0002, F (1, 13) = 27.47; post hoc Tukey's: Female PG/VG vs Nic *p=0.014, Male PG/VG vs Nic *p=0.011; **Fig 3.6C**). cFos expression in the CRF1+ VTA population was significantly higher in acute 12% nicotine-exposed mice as compared to PG/VG-exposed mice (main effect of vapor content *p=0.013, F (1, 13) = 8.35), however, post hoc tests did not pull out any significant comparisons (**Fig 3.6D**). cFos expression in TH+ and CRF1+ co-expressing neurons was also increased in 12% nicotine vapor-exposed animals (main effect of vapor content *p=0.013, F (1, 13) = 8.28), but post hoc comparisons were only significant for females (PG/VG vs Nic *p=0.048; **Fig 3.6E**). Overall, these data indicate that acute nicotine vapor exposure increases activity in VTA dopamine and VTA CRF1 neuronal populations of both sexes but with more profound effects in females.

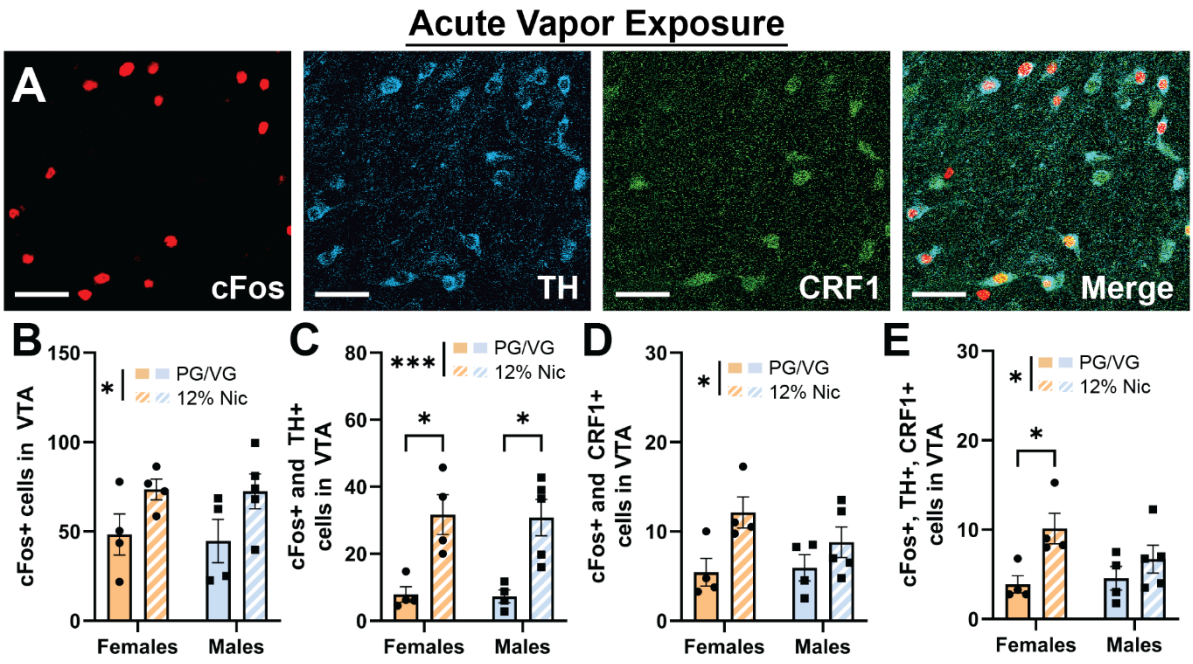


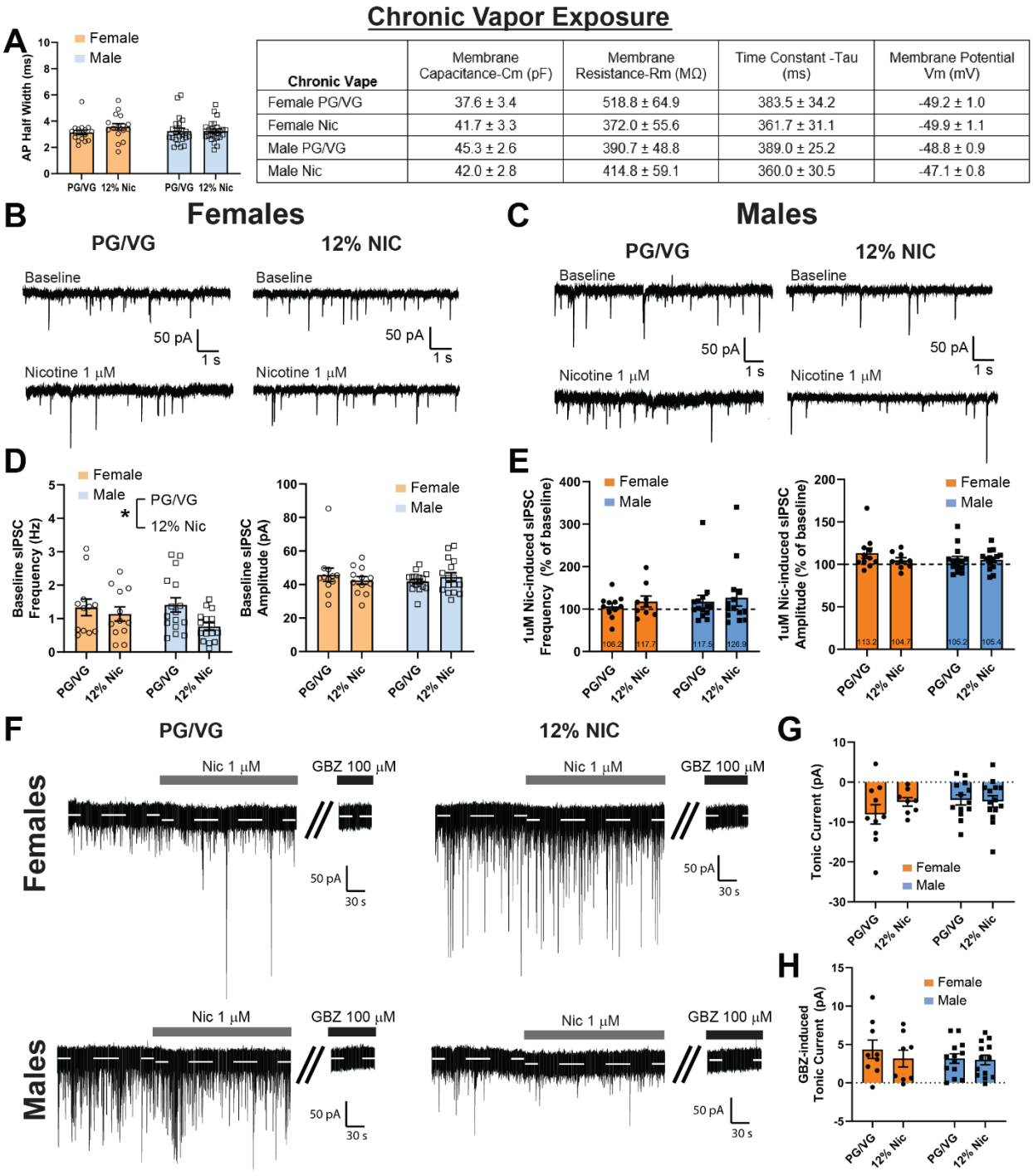
Figure 3.6- Changes in population activity in the VTA following acute vapor exposure. **(A)** Representative images of cFos, tyrosine hydroxylase (TH), CRF1 expression, and merged images of the VTA of a female nicotine vapor exposed mouse. Scale bar = 50 µm. **(B)** Number of cFos+ neurons in the VTA in females and males exposed to acute PG/VG (Female N=4, Male N=4) or acute 12% nicotine (Female N=4, Male N=5); 2-way ANOVA, main effect of vapor content *p=0.022, F (1, 13) = 6.80. **(C)** Number of VTA neurons that express cFos and TH in females and males exposed to acute PG/VG (Female N=4, Male N=4) or acute 12% nicotine (Female N=4, Male N=5); 2-way ANOVA, main effect of vapor content ***p=0.0002, F (1, 13) = 27.47, post hoc Tukey's: Female PG/VG vs Nic *p=0.014, Male PG/VG vs Nic *p=0.011. **(D)** Number of VTA neurons that express cFos and CRF1 in females and males exposed to acute PG/VG (Female N=4, Male N=4) or acute 12% nicotine (Female N=4, Male N=5); 2-way ANOVA, main effect of vapor content *p=0.013, F (1, 13) = 8.35, 2-way ANOVA). **(E)**

Number of VTA neurons that express cFos, TH, CRF1 in females and males exposed to acute PG/VG (Female N=4, Male N=4) or acute 12% nicotine (Female N=4, Male N=5); 2-way ANOVA, main effect of vapor content *p=0.013, F (1, 13) = 8.28, post hoc Tukey's Female PG/VG vs Nic *p=0.048. Data are represented as mean ± SEM.

The effect of chronic vapor exposure on inhibitory signaling in $^{VTA\text{-}NAc}$CRF1 neurons

$^{VTA\text{-}NAc}$CRF1 neurons from CRF1-GFP mice exposed to chronic vapor (as described in **Fig 3.3C**) display no significant differences in action potential half width between groups (2-way ANOVA, p>0.05; **Fig 3.7A, left**) but there was a significant main effect of sex (2-way ANOVA, *p>0.042, post hoc Tukey's p>0.05) in threshold to fire (Female PG/VG -41.6 ± 1.43, Female Nic -40.2.0 ± 1.40, Male PG/VG -39.2 ± 0.90, Male Nic -37.8 ± 0.88). Rheobase (2-Way ANOVA, p>0.05, Female PG/VG 57.4 ± 10.2, Female Nic 50.5 ± 6.04, Male PG/VG 63.0 ± 5.59, Male Nic 54.6 ± 7.59) and other membrane properties (**Fig 3.7A, right**) were also not significantly different across all groups. sIPSCs were recorded from females (**Fig 3.7B**) and males (**Fig 3.7C**) exposed to chronic PG/VG or chronic 12% nicotine vapor. We found that baseline sIPSC frequency was lower in 12% nicotine-exposed mice (main effect of vapor content *p=0.039, F (1, 52) = 4.51), likely driven by males, however post hoc Tukey's test was not significant between groups (**Fig 3.7D, left**). Baseline sIPSC amplitude was not altered in either sex exposed to PG/VG or 12% nicotine (**Fig 3.7D, right**). When 1μM nicotine-induced changes in sIPSC frequency and amplitude were normalized to baseline there were no significant differences between sexes from either PG/VG or 12%

nicotine groups (**Fig 3.7E**). Focal nicotine induced a tonic inhibitory current in $^{VTA\text{-}NAc}$CRF1 neurons of both sexes exposed to either PG/VG or 12% nicotine (**Fig 3.7F, G**) that was partially reversed with 100 μM gabazine (**Fig 3.7H**). These data suggest that chronic nicotine vapor exposure reduced presynaptic phasic inhibition in $^{VTA\text{-}NAc}$CRF1 neurons from males and the sex difference in tonic inhibition observed following acute nicotine vapor exposure was no longer present.



Chronic Vape	Membrane Capacitance-Cm (pF)	Membrane Resistance-Rm (MΩ)	Time Constant -Tau (ms)	Membrane Potential Vm (mV)
Female PG/VG	37.6 ± 3.4	518.8 ± 64.9	383.5 ± 34.2	-49.2 ± 1.0
Female Nic	41.7 ± 3.3	372.0 ± 55.6	361.7 ± 31.1	-49.9 ± 1.1
Male PG/VG	45.3 ± 2.6	390.7 ± 48.8	389.0 ± 25.2	-48.8 ± 0.9
Male Nic	42.0 ± 2.8	414.8 ± 59.1	360.0 ± 30.5	-47.1 ± 0.8

Figure 3.7- Inhibitory signaling in $^{VTA\text{-}NAc}$CRF1 neurons following chronic vapor exposure. **(A)** Averaged action potential half width (left) and membrane properties of $^{VTA\text{-}NAc}$CRF1 neurons from male and female mice exposed to chronic PG/VG (Female n=24 cells, N=4 mice; Male n= 33 cells, N=6 mice) or

12% nicotine vapor (Female n=25 cells, N=5 mice; Male n= 32 cells, N=6 mice). **(B)** Examples of spontaneous inhibitory post-synaptic currents (sIPSCs) in $^{VTA\text{-}NAc}$CRF1 neurons from female mice exposed to PG/VG (left) or 12% nicotine (right) at baseline and after 1 μM nicotine application. **(C)** Examples of spontaneous inhibitory post-synaptic currents (sIPSCs) in $^{VTA\text{-}NAc}$CRF1 neurons from male mice exposed to PG/VG (left) or 12% nicotine (right) at baseline and after 1 μM nicotine application. **(D)** Baseline sIPSC frequency (left; 2-way ANOVA, main effect of vapor content *p=0.039, F (1, 52) = 4.51) and amplitude (right; 2-way ANOVA, p>0.05) from female and male mice exposed to either PG/VG (Female n=13 cells, N=4 mice; Male n=16 cells, N=6 mice) or 12% nicotine (Female n=12 cells, N=5 mice; Male n=16 cells, N=6 mice). **(E)** 1 μM nicotine-induced change in sIPSC frequency (left) and amplitude (right) normalized to baseline in $^{VTA\text{-}NAc}$CRF1 neurons from female and male mice exposed to PG/VG (Female n=11 cells, N=4 mice; Male n=14 cells, N=6 mice) or 12% nicotine (Female n=9 cells, N=5 mice; Male n=14 cells, N=6 mice); 2-way ANOVA and one sample t-test, p>0.05. **(F)** Example traces of 1 μM nicotine-induced and gabazine (GBZ) reversed tonic current in $^{VTA\text{-}NAc}$CRF1 neurons from female (top) and male (bottom) mice exposed to PG/VG (left) or 12% nicotine (right). **(G)** 1 μM nicotine-induced tonic current in $^{VTA\text{-}NAc}$CRF1 neurons from female and male mice exposed to PG/VG (Female n=10 cells, N=3 mice; Male n=13 cells, N=6 mice) or 12% nicotine (Female n=8 cells, N=5 mice; Male n=14 cells, N=6 mice); 2-way ANOVA, p>0.05. **(H)** Gabazine (GBZ, 100 μM) reversal of tonic current in $^{VTA\text{-}NAc}$CRF1 neurons from female and male mice exposed to

PG/VG (Female n=9 cells, N=3 mice; Male n=12 cells, N=6 mice) or 12% nicotine (Female n=8 cells, N=5 mice; Male n=13 cells, N=5 mice); 2-way ANOVA, $p>0.05$. Data are represented as mean ± SEM.

The effect of chronic vapor exposure on spontaneous firing of $^{VTA\text{-}NAc}$CRF1 neurons

We performed cell-attached recordings in female (**Fig 3.8A**) and male (**Fig 3.8B**) mice exposed to chronic PG/VG or 12% nicotine vapor and observed no differences in baseline firing in either vapor condition (**Fig 3.8C**). When normalized to baseline, focal nicotine (1 μM) did not significantly change spontaneous firing in either sex or vapor group (interaction *p=0.045, $F(1, 38) = 4.29$; **Fig 3.8D**). These data suggest that sex differences in baseline firing and effects of focal nicotine on firing in $^{VTA\text{-}NAc}$CRF1 neurons following acute exposure is reversed following chronic exposure to PG/VG or 12% nicotine vapor.

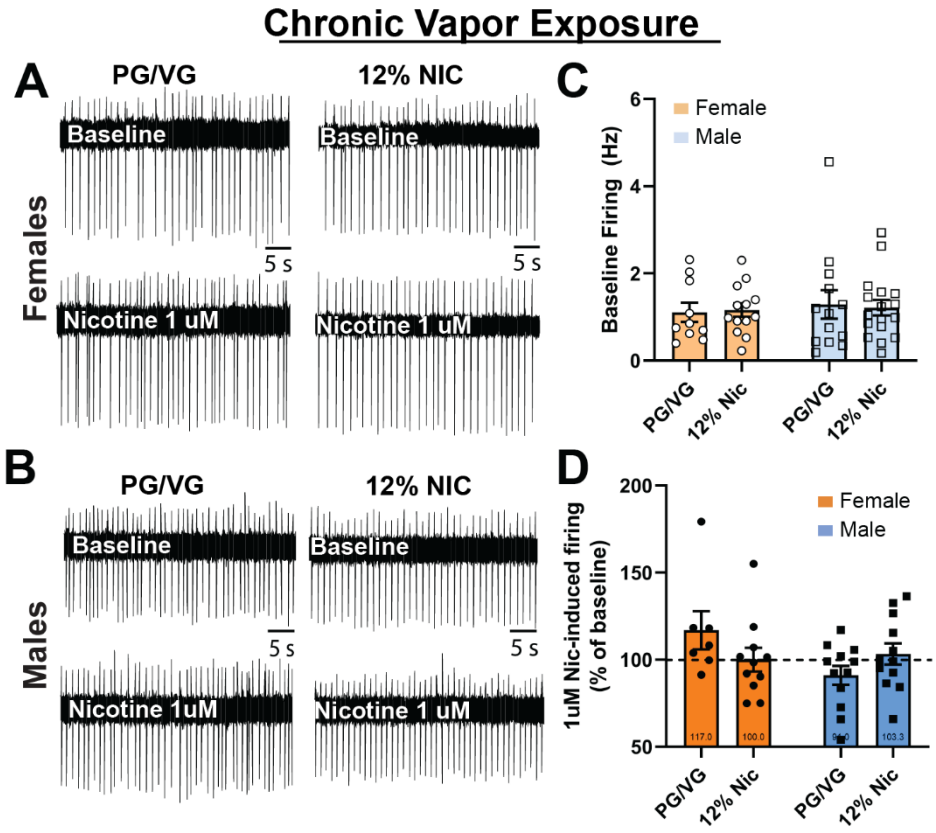


Figure 3.8- Spontaneous firing in $^{VTA\text{-}NAc}$CRF1 neurons following chronic vapor exposure. **(A)** Example of spontaneous firing in $^{VTA\text{-}NAc}$CRF1 neurons from female mice exposed to chronic PG/VG (left) or 12% nicotine (right) at baseline and after 1 μM focal nicotine application. **(B)** Example of spontaneous firing in $^{VTA\text{-}NAc}$CRF1 neurons from male mice exposed to chronic PG/VG (left) or 12% nicotine (right) at baseline and after 1 μM focal nicotine application. **(C)** Baseline firing in $^{VTA\text{-}NAc}$CRF1 neurons from females and males exposed to chronic PG/VG (Female n=10 cells, N=4 mice; Male n=13 cells, N=5 mice) or 12% nicotine (Female n=13 cells, N=5 mice; Male n=17 cells, N=5 mice); 2-way ANOVA, $p>0.05$. **(D)** 1 μM nicotine-induced change in firing in $^{VTA\text{-}NAc}$CRF1 neurons from female and male mice exposed to chronic PG/VG (Female n=7 cells, N=4 mice; Male n=12 cells, N=5 mice) or 12% nicotine (Female n=11 cells, N=5 mice; Male

n=12 cells, N=4 mice); 2-way ANOVA, interaction *p=0.045, F (1, 38) = 4.29. Data are represented as mean ± SEM.

The effect of chronic vapor exposure on VTA subpopulation activity

To examine overall activity in the VTA following long term exposure to vapor, we exposed animals to either PG/VG or 12% nicotine chronically (as described in **Fig 3.3C**) and performed immunohistochemistry to label cFos, tyrosine hydroxylase (TH), and CRF1 (**Fig 3.9A**). We found that animals from both sexes exposed to chronic 12% nicotine vapor show overall increased number of cFos+ neurons (main effect of vapor content *p=0.045, F (1, 15) = 4.80, **Fig 3.9B**). cFos expression in the TH+ VTA population was increased in 12% nicotine-exposed mice compared to PG/VG-exposed mice (main effect of vapor content *p=0.010, F (1, 15) = 8.62) and post hoc tests showed a significant difference between vapor groups in females but not males (Female: PG/VG vs nicotine *p=0.044; **Fig 3.9C**). There was no difference in cFos expression in the CRF1+ or the TH+/CRF1+ VTA population in either sex from both vapor groups (**Fig 3.9D, E**). These data indicate that chronic nicotine vapor exposure maintains elevated activity in VTA dopaminergic neurons, especially in females. However, the increased activity in CRF1+ neurons following acute exposure is reduced to levels similar to PG/VG after chronic exposure.

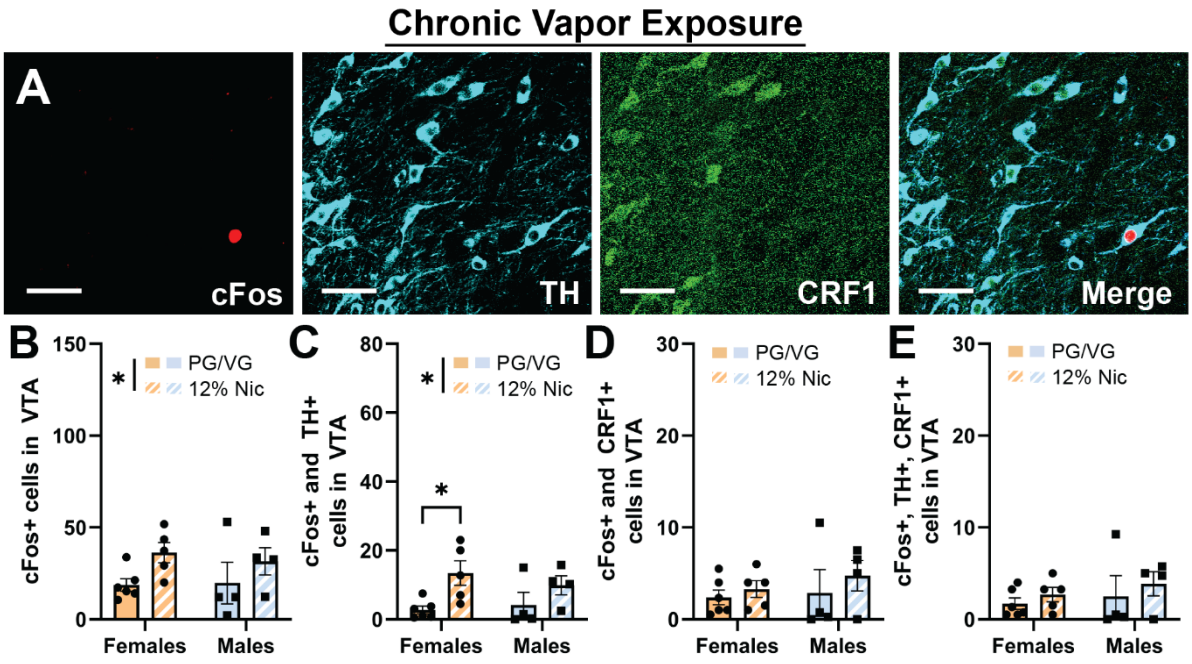


Figure 3.9- Changes in population activity in the VTA following chronic vapor exposure. **(A)** Representative image of cFos, tyrosine hydroxylase (TH), CRF1 expression, and merged images of the VTA of a male nicotine vapor exposed mouse. Scale bar = 50 µm. **(B)** Number of cFos+ neurons in the VTA in females and males exposed to chronic PG/VG (Female N=4, Male N=4) or chronic 12% nicotine (Female N=4, Male N=5); 2-way ANOVA, main effect of vapor content *p=0.045, F (1, 15) = 4.80. **(C)** Number of VTA neurons that express cFos and TH in females and males exposed to chronic PG/VG (Female N=6, Male N=4) or chronic 12% nicotine (Female N=5, Male N=4); 2-way ANOVA, main effect of vapor content *p=0.010, F (1, 15) = 8.62, post hoc Tukey's: Female PG/VG vs nicotine *p=0.044. **(D)** Number of VTA neurons that express cFos and CRF1 in females and males exposed to chronic PG/VG (Female N=6, Male N=4) or chronic 12% nicotine (Female N=5, Male N=4); 2-way ANOVA, p>0.05. **(E)** Number of VTA neurons that express cFos, TH, CRF1 in females and males

exposed to chronic PG/VG (Female N=6, Male N=4) or chronic 12% nicotine (Female N=5, Male N=4); 2-way ANOVA, $p>0.05$. Data are represented as mean ± SEM.

Discussion

VTA CRF1 neurons are predominately dopaminergic and project to the NAc, suggesting involvement in the mesolimbic reward pathway. There are sex differences in phasic inhibition and nicotine-induced firing in $^{VTA\text{-}NAc}$CRF1 neurons, but both sexes displayed focal nicotine-induced tonic inhibition. Following acute nicotine vapor exposure, phasic inhibition and firing in $^{VTA\text{-}NAc}$CRF1 neurons were largely unaffected, but focal nicotine-induced tonic inhibition was enhanced in females and reduced in males. Activity of the CRF1 dopaminergic VTA population increased in both sexes following acute nicotine vapor exposure, but especially in females. Chronic nicotine vapor exposure reduced phasic inhibition in $^{VTA\text{-}NAc}$CRF1 neurons from both sexes and the sex-specific bidirectional changes in tonic inhibition were no longer observed. Additionally, activity of the CRF1 dopaminergic VTA population was no longer elevated following chronic nicotine vapor exposure. Collectively, these findings demonstrate sex-specific differences in inhibitory control and response to focal nicotine in $^{VTA\text{-}NAc}$CRF1 neurons from naïve mice, and sex-specific changes in activity and inhibitory control following acute nicotine vapor exposure that were lost following chronic exposure. These findings identify important sex- and exposure-dependent changes in mesolimbic CRF1 population activity and how electronic nicotine vapor selectively impacts reward and stress circuitry in males and females.

Electronic nicotine delivery systems (ENDS) use has increased dramatically, however, the effects of nicotine vapor on brain reward and stress circuitry remain unclear. Previous studies have used alternative forms of nicotine delivery like subcutaneous minipumps (Grieder et al., 2014; Salas et al., 2009), drinking water (DeBaker et al., 2020; Wong et al., 2020), intraperitoneal ((Akers et al., 2020; Caruso et al., 2018; Pauly et al., 1992), or intravenous (O'Dell et al., 2007; Picciotto et al., 1998) injections. This study used a nicotine vapor inhalation model that allows nicotine to reach the brain on a timescale similar to nicotine from cigarettes (Solingapuram Sai et al., 2020), which is a primary determinant of nicotine reinforcement (Henningfield & Keenan, 1993). Additionally, vapor liquids can contain higher nicotine concentrations than traditional cigarettes, which may cause differential engagement of the reward circuitry. Our previous study found that a single 3-second 12% nicotine vapor produced mouse serum nicotine levels (Zhu et al., 2021) that are comparable to human serum levels in cigarette smokers (Benowitz et al., 1988; Chellian et al., 2021) and electronic cigarette users (St Helen et al., 2016). Additionally, serum cotinine levels in mice following acute nicotine vapor exposure (Fig 3.3B, right) were comparable to those found in humans who smoke cigarettes daily or use e-cigarettes daily (Rapp et al., 2020). However, with repeated nicotine vapor administrations (acute and chronic exposure paradigms), we did observe serum nicotine levels higher than what have been reported in humans. This discrepancy can be due to a variety of potential factors including the species' different nicotine metabolism rates (mice have faster nicotine metabolism than humans), route of inhalation and absorption (mice inhale the vapor mainly through the nose whereas humans inhale vapor through the mouth), time of

sample collection (mouse samples in our study collected immediately following last vape session vs timing of human sample collection can vary depending on the study), and naturalistic mouse behavior (potential ingestion of nicotine deposits on fur when grooming). Future studies will examine nicotine's effects at lower concentrations as well as incorporating self-administration to better model animal's motivation to seek nicotine.

Clinical studies suggest that women, more than men, report stress as a major factor promoting nicotine use (Fidler & West, 2009). Sex differences in nicotine metabolism (Johnstone et al., 2006; Kandel et al., 2007) could also contribute to neurobiological effects, highlighting the gap in knowledge on sex-specific effects and the need for female subjects in pre-clinical research. We examined $^{VTA\text{-}NAc}$CRF1 neurons in both sexes and found that females displayed higher basal phasic inhibition and enhanced tonic inhibition after exposure to acute nicotine vapor compared to males. These data indicate that female VTA CRF1 neurons are under greater inhibitory control which may explain female-specific decreases in dopamine release in the nucleus accumbens with administration of the nicotinic receptor antagonist, mecamylamine (Carcoba et al., 2018). Overexpression of CRF in the nucleus accumbens has also been shown to enhance the reinforcing effects of nicotine in females (Uribe et al., 2020). Future experiments examining the effects of CRF in both female and male $^{VTA\text{-}NAc}$CRF1 neurons will shed light on how stress affects reward processing in the VTA.

We found that approximately 80% of the CRF1 neurons in the VTA are dopaminergic which means that the remaining ~20% are non-dopaminergic and can potentially overlap with other cell types of the VTA. Given that the VTA CRF1 population

is mainly expressed in the lateral VTA it is likely that the remaining 20% are primarily GABAergic however, CRF1 neurons located in the most medial region of the lateral VTA may also be glutamatergic or represent a mixed transmitter population. In naïve males, we observed an increase in firing induced by application of 1uM of nicotine, consistent with previous studies from VTA dopamine neurons in slice recordings (Mansvelder & McGehee, 2000; Picciotto et al., 1998; Pidoplichko et al., 1997; Yin & French, 2000). However, following administration of nicotine intravenously, in vivo single cell extracellular recording from VTA dopamine neurons found that nicotine produces increased firing in some and decreased firing in others (Eddine et al., 2015; Nguyen et al., 2021). This inhibition required D2 receptor activation (Eddine et al., 2015) and was found in the amygdala-projecting population to mediating anxiety-like behavior (Nguyen et al., 2021). These discrepancies in nicotine's effect in the VTA may be due to differences in route of nicotine administration (in vivo vapor vs. in vivo IV vs. in vitro bath application) and cell type population in the VTA (dopamine/GABA/Glut or projection-specific). Following acute nicotine vapor exposure, VTA CRF1 neuronal activity as measured by cFos was increased in both sexes but neither sex showed increases in baseline spontaneous firing. These seemingly contradictory results underscore the importance of the temporal aspect of nicotine's effects. Mice used for both methods of neuronal activity measurement were sacrificed immediately following the last vapor session. However, spontaneous firing was measured following slice preparation and at least an hour-long incubation where neurons were potentially in cellular withdrawal with variable levels of nicotine remaining in the brain. Whereas cFos expression reflects a snapshot of neuronal activity ~30-60 minutes prior to the time of sacrifice (Chaudhuri et

al., 2000) while the animals are actively being exposed to nicotine vapor. Additionally, the increased cFos following acute and dampened cFos expression following chronic nicotine vapor exposure is similar to what was observed previously in a study examining the patterns of activity following systemic nicotine exposure (Baur et al., 2018) and is potentially regulated by desensitization of nAChRs. This is also consistent with the idea of tolerance where the initial effects and responses to a drug diminish with repeated administration of the drug over time as homeostatic changes develop that oppose the effects of the initial response and potentially more drug is required to achieve effects similar to initial exposure. Phasic GABAergic inhibitory inputs are transiently increased but quickly desensitized leading to an overall increase in excitability in VTA dopamine neurons (Mansvelder & McGehee, 2000; Mansvelder et al., 2002). Although we demonstrate that VTA CRF1 neurons are primarily dopaminergic, phasic inhibitory signaling in $^{VTA\text{-}NAc}$CRF1 neurons was not sensitive to either focal or in vivo nicotine, in contrast to previous work in VTA dopamine neurons. This difference may be due to differences in cell types (dopaminergic vs. GABAergic or glutamatergic), species (rat vs mouse), sex (male vs both sexes), and/or age (adolescent vs adult). However, our data suggest that the $^{VTA\text{-}NAc}$CRF1 population displays differential responses to nicotine exposure compared to the VTA dopaminergic population. These differences could underlie potentially distinct roles in stress/anxiety and/or addiction.

VTA dopamine neurons are modulated by both phasic (Grieder et al., 2014; Mansvelder et al., 2002) and tonic (Darnieder et al., 2019; Tossell et al., 2021) inhibition. Canonically, phasic inhibition mediates rapid point-to-point signaling while tonic inhibition regulates network activity by persistent inhibitory conductance

(Semyanov et al., 2004). In GABAergic VTA neurons, inhibitory tonic currents have been found to be mediated through the δ-containing GABAA receptor with higher receptor mRNA expression and tonic current in female mice and act to disinhibit VTA dopamine neurons (Darnieder et al., 2019). In dopaminergic VTA neurons, inhibitory tonic currents are mediated through αβε¬-containing GABAA receptors and directly reduce the excitability of those neurons (Tossell et al., 2021). Although inhibitory tonic current has been observed in the VTA, how nicotine impacts this tonic has previously been understudied. In this study, we observed tonic inhibition induced by focal nicotine in naïve female and male $^{VTA\text{-}NAc}$CRF1 neurons and this tonic current was enhanced in females and reduced in males following acute nicotine vapor exposure. We speculate that the enhanced tonic observed in females following acute nicotine vapor exposure is potentially due to increased expression of (δ- or αβε¬-containing) GABAA receptors at the extrasynaptic membrane. Concurrently, the sex difference may also be mediated via differential expression or desensitization of nicotinic acetylcholine receptors (nAChRs) on VTA dopaminergic neurons or upstream on GABAergic neurons to alter GABA release and/or reuptake. The enhanced tonic inhibition in females could potentially decrease reward signaling by dampening glutamatergic signaling whereas the reduced tonic inhibition in males could enhance glutamatergic signaling and drive reward signaling. This hypothesis may potentially provide the mechanistic basis for sex differences in motivation for nicotine seeking where women are more likely to seek nicotine to relieve stress and men are more likely to seek nicotine for its rewarding properties. Additionally, these findings suggest that nicotine's effect on excitability in the $^{VTA\text{-}NAc}$CRF1 population may be modulated more by tonic inhibition than phasic inhibition

as previously observed in VTA dopamine neurons (Grieder et al., 2014; Mansvelder et al., 2002). Interestingly, the bidirectional sex difference in focal nicotine-induced tonic inhibition was no longer observed following chronic nicotine vapor, suggesting neuroplastic adaptations such as nACh receptor desensitization, internalization, or even decreased gene expression, given the prolonged timeframe, that can diminish the effect of focal nicotine application and dampen the ability of tonic inhibition to regulate $^{VTA\text{-}NAc}$CRF1 activity after chronic exposure. Distinct changes following acute but not repeated nicotine vapor exposure have been observed in our previous work in the central amygdala (Zhu et al., 2021). Additionally, these dampened effects following chronic exposure may be interesting to investigate following withdrawal from nicotine. Overall, these differential effects of nicotine on tonic inhibition, and thus, the excitability of $^{VTA\text{-}NAc}$CRF1 neurons may play a role in the modulation of the reward circuit and the development and maintenance of addiction.

The CRF/CRF1 system is involved in reward processing and has been implicated in many different drugs of abuse. In the context of nicotine, studies report increased CRF mRNA in the VTA following chronic nicotine exposure (Grieder et al., 2014) and CRF1 blockade reduced reinstatement of nicotine seeking (Bruijnzeel et al., 2009). Activation of VTA dopamine CRF1 neurons at their cell bodies has been shown to coordinate reward reinforcement behavior and activation at terminals in the NAc core increase dopamine release (Heymann et al., 2020). Additionally, CRF peptide increases VTA dopamine neuron firing via CRF1 (Wanat et al., 2008; Zalachoras et al., 2022) and knockout of CRF1 in midbrain dopamine neurons has been shown to increase anxiety-like behavior (Refojo et al., 2011). Previous studies have found Crf1 mRNA expression

in GABAergic neurons in the VTA (Zalachoras et al., 2022), however the location of the protein expression (dendrites/cell body vs terminals) and their role in stress/anxiety and nicotine addiction remain unclear. We found that CRF1 VTA neurons were mainly dopaminergic and project to the nucleus accumbens, consistent with previous reports (Heymann et al., 2020), however, $^{VTA\text{-}NAc}$CRF1 neurons did not respond to nicotine in the same way as canonical VTA dopamine neurons. Given the relevance of CRF1 in stress signaling, this suggests that these neurons may be uniquely modulated in the context of stress and anxiety. Future studies examining how nicotine reward processing is affected by stress and anxiety and how anxiety-like behaviors are affected by nicotine exposure are required to understand the intersection of divergent (or convergent) processing in CRF1 VTA neurons in different behavioral conditions.

Collectively, these studies illustrate how CRF1 neurons, which are relevant to stress, are also involved in encoding nicotine reward and how acute and chronic electronic nicotine vapor exposure produce different effects on activity and inhibitory control of VTA CRF1 neurons in females and males. These sex- and exposure-dependent changes in the mesolimbic CRF1 population add to our understanding of the neurobiological underpinnings involved in the development of nicotine addiction. It is imperative that we continue to study how nicotine vapor affects the brain and engages specific reward pathways to better understand nicotine dependence and aid in the development of therapeutics to prevent and mitigate nicotine addiction.

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CHAPTER 5: DISCUSSION

The prevalence of electronic nicotine delivery system (ENDS) use, colloquially referred to as ‘vaping,’ has risen dramatically over the last couple of decades. However, the effects of ENDS use on reward and stress related neural populations and circuits remain understudied, especially in the pre-clinical setting. The overall goal of this dissertation was to investigate the cellular and behavioral effects of electronic nicotine vapor exposure in female and male mice and begin to elucidate the behavioral and neuronal changes that result from ENDS use. In chapter two, we utilized and validated a preclinical model of electronic nicotine vapor exposure system and its ability to reliably deliver nicotine to mice by inhalation. We found that a single session vs repeated sessions of intermittent nicotine vapor exposure differentially altered neuronal activity in the CeA, body temperature, and locomotion in male mice. In chapter three, we investigated how vapor exposure of different nicotine concentrations and formulations (salt versus freebase) affect CeA and VTA neuronal activity and anxiety-like behavior in female and male mice. We found that exposure to nicotine freebase vapor produced higher levels of serum nicotine in both sexes, with greater increases in females. Male mice showed an overall increase in CeA activity independent of formulation and concentration but elevated anxiety-like behavior and decreased motivation to feed were observed primarily in the nicotine freebase-exposed mice. Female mice showed no changes in CeA activity, increased VTA activity (specifically in the dopaminergic

population), but no changes in anxiety-like behavior or motivation to feed. In chapter four, we focused on the CRF1-expressing neurons in the VTA and examined how acute and chronic nicotine vapor exposure impacted neuronal activity and inhibitory signaling. We found that VTA CRF1 neurons are a mainly dopaminergic population that projects primarily to the nucleus accumbens. These neurons show sex differences in basal phasic inhibition but respond differently to nicotine than previous reports in dopamine neurons. Following acute nicotine vapor exposure, tonic inhibition was enhanced in females but reduced in males and overall neuronal activity was increased in VTA dopaminergic CRF1 neurons. Following chronic nicotine vapor exposure, the bidirectional change in tonic inhibition was no longer observed and VTA dopaminergic CRF1 activity was no longer elevated compared to controls, suggesting neuroplastic changes that develop with chronic nicotine exposure. Overall, these findings demonstrate that electronic nicotine vapor exposure has differential cellular and behavioral effects on reward and stress related neural populations. The multiplicity of these effects is largely dependent on different factors such as nicotine vapor exposure timing, dose/concentration, formulation, and sex. Thus, continued research using electronic nicotine vapor models is necessary to further examine its impact on behavior and neural circuits.

Preclinical nicotine vapor exposure model- advantages and disadvantages

The studies presented in this dissertation utilized a nicotine vapor exposure model with the overall goal of better replicating human ENDS use in preclinical research. Decades of nicotine research have employed many different ways to deliver

nicotine to research animal models, including intravenous, subcutaneous, intraperitoneal, and oral routes of administration. However, these delivery methods do not model human nicotine use, which is most commonly through inhalation. One of the main properties that significantly contributes to the abuse liability of cigarette smoking is the fast rate at which nicotine reaches the brain and the learned association of nicotine to the immediate rewarding effects (Henningfield & Keenan, 1993). This is why nicotine replacement therapies such as nicotine patch and nicotine gum are effective smoking cessation methods as the slower nicotine delivery rate allows for more gradual increases in nicotine levels in the blood and brain that can help alleviate the nicotine withdrawal symptoms without the high abuse liability of inhaled nicotine (West et al., 2000). With the exception of intravenous nicotine administration, many of these pre-clinical models do not deliver nicotine to the brain at a rate that is comparable to cigarette smoking in humans. Though the intravenous route is fast, the downside is that it is quite invasive as it requires prior surgical procedures to implant catheters for nicotine delivery. With the development of electronic cigarettes and different ENDS products, these methods can be adapted to deliver nicotine vapor in preclinical research models. In the studies presented in this dissertation, we validated and utilized a preclinical model of an electronic nicotine vapor delivery system to study its effects in mouse animal models. In comparison to many of the previous preclinical models of nicotine delivery, electronic nicotine vapor inhalation is most similar to human use and can deliver nicotine to the brain at fast rates (Solingapuram Sai et al., 2019; St Helen et al., 2016). Additionally, this method of nicotine delivery is relatively non-invasive compared to other models that may require surgical procedures (such as catheter or

osmotic pump implants) or may induce stress with repeated injections (intraperitoneally or subcutaneously). However, there are some limitations to the electronic nicotine vapor model. In the studies presented in this dissertation, electronic nicotine vapor was delivered to mice in a passive/non-contingent manner which may mimic second-hand exposure more so than volitional nicotine use. This passive delivery limits our investigation into motivated nicotine vapor seeking-behavior and the underlying mechanisms that may differ from non-contingent exposure. One of the main challenges that come with nicotine self-administration, not just with nicotine vapor, but across multiple routes of nicotine administration, is achieving escalation in self-administration behavior that is similar to other drugs of abuse such as cocaine or methamphetamine. Nicotine's rewarding effects follow an inverted U-shaped dose response where the rewarding concentration is at the peak of the inverted U-shape and higher concentrations of nicotine can be aversive. This nicotine property makes it challenging to achieve escalation of nicotine self-administration due to self-titration. Although self-administration of nicotine vapor is still quite novel in the field of nicotine research, several research groups have begun to develop different nicotine vapor self-administration protocols for rodent animal models by pre-training with sucrose or food pellet (Lallai et al., 2021) or pairing a flavorant, such as menthol with the nicotine vapor (Cooper et al., 2021; Henderson & Cooper, 2021). However, additional studies using self-administration paradigms to deliver nicotine vapor are still necessary to fully understand the potential differences in the role of reward and stress mechanisms that are involved with voluntary ENDS use.

Nicotine use in adolescence

Both adult and adolescent populations use ENDS products, but the history of previous nicotine use between the two populations are quite different. Prior to current ENDS use, many adults have had some nicotine use, mostly through smoking, whereas most adolescents did not and ENDS use is their first exposure to nicotine. Years of previous research using non-vapor delivery of nicotine have found that aspects of nicotine reward, dependence, aversion, withdrawal, and stress response in adults are vastly different in adolescents. Studies in rodents found that adolescent animals show higher nicotine intake in intravenous self-administration (Levin et al., 2007; Levin et al., 2003) and oral self-administration models (Adriani et al., 2002). Similarly, using a conditioned place preference (CPP) paradigm to examine the conditioned rewarding effects of nicotine, adolescent animals exhibit CPP for a wider range of nicotine doses than adult counterparts (Kota et al., 2008; Kota et al., 2007; Torres et al., 2008). Mechanistically, adolescents showed increased VTA dopamine neuron firing (Jobson et al., 2019) and elevated dopamine release (Corongiu et al., 2020) following subcutaneous nicotine injection. Together, these studies indicate that nicotine's reinforcing properties are enhanced in adolescents. On the other hand, aversive properties of nicotine are diminished in adolescents. In one study, conditioned placed aversion (CPA) that was induced by higher concentrations of nicotine was observed in adults, but not in adolescents (Torres et al., 2008). In another study, adult rats trained to associate nicotine with a flavored solution developed a dose-dependent conditioned taste aversion (CTA), but adolescent rats were resistant to the formation of CTA (Shram et al., 2006). Adolescents also experience less severe spontaneous and

mecamylamine-precipitated withdrawal symptoms following prolonged nicotine use (O'Dell et al., 2006; Shram et al., 2008). Additionally, nicotine use in adolescents produced blunted activation of the HPA axis as indicated by reduced neuronal activity in the paraventricular nucleus (PVN) of the hypothalamus and reduced plasma corticosterone levels (Cao et al., 2007). Interestingly, anxiety-like behavioral tests such as open field, light/dark box, and elevated plus maze showed increased anxiety-like behavior in adolescents following nicotine injection (Adriani et al., 2004) and in adults with adolescent nicotine exposure (Jobson et al., 2019). These anxiety-like behavioral phenotypes may potentially be mediated through downstream effects of CRF and CRF1 receptors within the brain in areas like the CeA and VTA rather than the peripheral effects via classical activation of HPA axis, but further investigation is required to examine this hypothesis.

Although the different aspects of nicotine use in adolescent animal models have been well studied, many of these findings have not yet been examined with nicotine delivered through vapor exposure. The studies presented in this dissertation have all been conducted in adult mouse models and very few pre-clinical studies have examined electronic nicotine vapor exposure in an adolescent population. In one study, rats were trained in a conditioned place preference (CPP) paradigm to associate one side of a chamber to 2, 4, or 8 minutes of mango flavored nicotine vapor exposure and the other side to a vehicle vapor exposure. They found that adult rats show nicotine vapor CPP with the 8-minute exposure, but adolescent rats show nicotine vapor CPP with both 4 minute and 8 minute exposures (Frie et al., 2020). These findings agree with previous CPP studies with nicotine injected subcutaneously (Kota et al., 2007) and suggest that

adolescent rats show enhanced nicotine CPP and find nicotine rewarding at lower doses compared to adult rats. However, the Frie et al. study was only conducted in male rats which limits the potential elucidation of sex-specific differences. We observed several sex-specific effects in our studies, which could have significant implications in adolescent models of nicotine vapor exposure. The nicotine vapor in the Frie et al. study contained a flavorant which can add complexity to the understanding of the rewarding effects of nicotine alone but can also better model adolescent preference for flavored ENDS products in the human population (Zare et al., 2018). Pre-clinical studies on flavors in ENDS products are still limited, however, recent studies from the Henderson group started to examine the reinforcing effects of flavorants on their own and flavorants with nicotine in a vapor self-administration model. They found that certain flavorants alone (i.e. green apple) were able elicit self-administration behavior, while other flavorants (i.e. menthol) were not. However, when either the green apple or menthol flavorant was paired with nicotine, self-administration behavior was enhanced (Cooper et al., 2021). Overall, given the high prevalence of ENDS use in adolescents and the heightened risk of developing nicotine dependence during adolescence, it is vital that future studies examine the effects of nicotine vapor exposure in adolescent animal models with special consideration of sex and flavorants.

The VTA in nicotine reward and aversion

VTA neurons are canonically known for their role in reward encoding and processing, however, recent studies have provided evidence for their involvement in aversion as well. GABAergic neurons in the VTA show increased neuronal firing in

response to an aversive air puff stimulus. These GABAergic neurons, though still responsive to rewarding cues and stimuli, show different temporal activity patterns than that of VTA dopamine neurons (Cohen et al., 2012), suggesting that the VTA GABAergic population may underlie both reward and aversion through different mechanisms. Processing of both reward and aversion is not limited to GABAergic neurons in the VTA but have also been shown in dopaminergic populations in the VTA. Studies from the Lammel group have examined how input- and output- specific VTA dopamine populations underlie encoding of aversive stimuli and coordinating of aversive behavior. Specifically, they found that by using a reward and aversion conditioning task, dopaminergic terminals in the lateral shell of the NAc are excited in response to rewarding stimuli but inhibited during aversive stimuli. However, dopaminergic terminals in the ventral medial shell of the NAc are excited during aversive stimuli and learned aversive cues (de Jong et al., 2019). VTA dopaminergic neurons are also involved in coordinating reward and aversion-associated behaviors dependent on the inputs received. Specifically, GABAergic inputs from laterodorsal tegmentum (LDT) mediate conditioned place preference (CPP) whereas glutamatergic inputs from the lateral habenula (LHb) mediate conditioned place aversion, CPA, (Lammel et al., 2012). As mentioned previously, nicotine's effects follow an inverted U dose curve where high doses of nicotine are aversive. A recent study has shown that intravenous delivery of nicotine at a low dose induces CPP whereas nicotine at a high dose induces CPA. Using fiber photometry to examine bulk calcium activity, high-dose nicotine produced biphasic dopamine activity with an inhibitory early component and an excitatory late component. Additional examination using the dLight dopamine sensor in the

downstream NAc reveal a projection-specific divergence with an early dip in dopamine release in the NAc lateral shell followed by a late surge in dopamine release in the NAc medial shell (Liu et al., 2022). Further circuit mapping and pharmacological experiments suggested that the encoding of the aversive properties of high dose nicotine were mediated by activation of α7 nAChRs in LDT GABA terminals that inhibit VTA dopamine neurons and concurrent desensitization of α4β2 nAChRs on VTA DA neurons resulting in the overall inhibition during the early component. Optogenetic inhibition of the LDT GABAergic terminals in the VTA was able to eliminate the high-dose nicotine-induced CPA phenotype (Liu et al., 2022). In addition to the heterogeneity in cell type populations within the VTA, recent work has suggested heterogeneity in cell type-specific function in regard to the encoding of reward and aversion, especially in the context of nicotine.

In chapter two, we found that vaporized freebase nicotine produces higher serum nicotine levels than nicotine salt of the same concentration. Additionally, within animals exposed to freebase nicotine, females showed higher serum nicotine levels than males. Although nicotine levels taken from blood samples are not synonymous to measures of the nicotine levels found in brain, the two are highly correlated as nicotine is able to easily cross the blood brain barrier (Benowitz et al., 2009). The higher serum nicotine levels from freebase nicotine vapor may suggest that freebase nicotine may more easily trigger an aversive response, especially in females, than nicotine salt. This hypothesis is supported by surveys from vape users that report nicotine salts to be smoother and less bitter than freebase (Leventhal et al., 2021; O'Connell et al., 2019). However, further investigation is required to fully understand if differences in nicotine formulation itself, or

if formulation differences in absorption, rate at which different formulations reach the brain, and/or concentration of nicotine that reaches the brain may differentially engage the underlying brain mechanisms that mediate nicotine-induced aversive behaviors. In chapter three, we examined the impact of nicotine vapor exposure on the CRF1 population in the VTA. We found that although VTA CRF1 neurons are primarily dopaminergic and project to the NAc, they do not respond to nicotine similarly to what has previously been shown in VTA dopamine neurons. It is possible that VTA CRF1 neurons underlie mechanisms of nicotine that intersect with stress and/or aversion but additional experiments are required to uncover how the CRF/CRF1 system in the VTA may be involved in more complex aspects of nicotine exposure and behavior.

Nicotine withdrawal and relevance to stress

In this dissertation, we have examined the behavioral and cellular effects of acute, repeated, and chronic exposure to nicotine vapor and found interesting sex-specific and nicotine formulation-dependent effects in both the CeA and the VTA. However, we have not examined the time point of withdrawal from prolonged nicotine vapor exposure. One of the main components that drives continued nicotine use in nicotine dependent individuals is the experience of physical and psychological withdrawal symptoms that result from acute or short-term withdrawal of nicotine use (Parrott, 1999). Many pre-clinical studies using non-vapor administration of nicotine have examined the expression of spontaneous withdrawal and precipitated withdrawal with the administration of the nAChR blocker, mecamylamine. In addition to the physical somatic signs of nicotine withdrawal, studies have found specific changes in neural

mechanisms that underlie nicotine withdrawal. Specifically, rodents in spontaneous withdrawal following prolonged subcutaneous nicotine exposure showed decreased VTA dopamine tonic firing (Grieder et al., 2012) and in mecamylamine-precipitated withdrawal, dopamine levels in the NAc are reduced (Hildebrand et al., 1998). This suggests an overall dysregulation of the reward-related circuits that were activated with acute nicotine exposure. Very few research groups have examined withdrawal following nicotine vapor exposure. One study has found that rats exposed to non-contingent nicotine vapor exposure indeed display spontaneous and mecamylamine-precipitated withdrawal (Montanari et al., 2020) similar to other modes of nicotine delivery, however, the underlying neural mechanisms mediating this effect remain to be determined. One may presume it is mediated through a similar dysregulation of the reward-related circuits, however the differences in the slow delivery of nicotine subcutaneously versus the fast delivery nicotine vapor and non-contingent versus self-administration delivery may involve different underlying neural mechanisms and different patterns of neural activity.

Nicotine withdrawal has also been linked to stress and anxiety-like behaviors in a bidirectional manner. Rodents in nicotine withdrawal show increased anxiety-like behaviors measured by a variety of behavioral assays such as elevated plus maze (Damaj et al., 2003; Irvine et al., 2001), light dark box (Costall et al., 1989), and acoustic startle (Helton et al., 1993; Jonkman et al., 2005). Rodents trained to self-administer nicotine showed increased drug seeking behavior during withdrawal (George et al., 2007). Additionally, this increase in drug seeking behavior is also observed with presentation of a conditioned cue (Markou et al., 2018) and footshock-stress (Bruijnzeel

et al., 2009; Buczek et al., 1999). The CRF and CRF1 system has been implicated in the link between stress and nicotine withdrawal. Mecamylamine-induced increases in intracranial stimulation threshold, which is thought to indicate deficits in brain reward function and model nicotine withdrawal, was prevented with intracranial administration of CRF peptide directly into CeA and NAc shell (Marcinkiewcz et al., 2009) but also intracranial administration of a CRF1 antagonist into the CeA (Bruijnzeel et al., 2012). CRF1 antagonism has also been shown to reduce stress-induced reinstatement of nicotine seeking (Bruijnzeel et al., 2009). Additionally, animals in spontaneous withdrawal from subcutaneously delivered nicotine show increased CRF mRNA expression in the VTA, and downregulation of the VTA CRF mRNA was able to reduce anxiety-like behavior and prevent the expression of withdrawal-induced CPA (Grieder et al., 2014). A similar effect was observed with mecamylamine-precipitated withdrawal where one study found increased CRF peptide in the CeA as measured by microdialysis and CRF1 antagonism prevented abstinence-induced increases in nicotine intake (George et al., 2007). However, many of the studies implicating the role of CRF and CRF1 in mediating different aspects of nicotine withdrawal have been conducted only in males and predominately in rats. In chapter three we found interesting sex differences in inhibitory control of VTA CRF1 neurons from naïve mice as well as mice exposed to an acute session of electronic nicotine vapor. Although many of the effects from naïve and acute exposed animals were no longer observed in chronic exposed animals, previous work suggests that the VTA CRF1 neuronal population may be involved in mediating the effects of nicotine withdrawal. Additionally, the sex-differences we observed in the VTA CRF1 neuronal population may shed light on the known sex

differences in stress processing and nicotine seeking and relapse in the human population.

Collectively, the studies presented in this dissertation show that electronic nicotine vapor exposure produced differential behavioral and cellular effects that were dependent on sex, vapor exposure timing, nicotine dose, and formulation. We hope that these studies will be the beginning of many to build a more comprehensive understanding of the neurobiological effects of ENDS on reward and stress systems. We have explored a few different future research directions: the impact of nicotine vapor exposure in adolescents, the potential aversive properties of nicotine vapor, and withdrawal from nicotine vapor, however many additional compelling lines of research remain. Further understanding of the impact of ENDS in preclinical models will help inform regulatory policies on ENDS use and potentially advance the development of therapeutics for cessation of ENDS use.

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