UNIVERSITY OF CALIFORNIA, IRVINE

Novel insights into nicotine dependence: The hope for new cessation therapeutics

DISSERTATION

Submitted in partial satisfaction of the requirement for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

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The journey of graduate school is full of excitements, but also mental struggles. I cannot overcome these difficulties without the support and company of many people. My principal investigator, Dr. Christie Fowler, is the beacon that guides me on this journey of graduate school. She puts in so much efforts instructing me to become a better researcher. Even with a busy schedule almost all year around, she is always available for questions and assistance. She provides me the resources to build my own projects and inspires me to actively learning new skills and design new experiments. Her guidance is deeply appreciated, and I cannot be what I am today without her.

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ABSTRACT OF THE DISSERTATION

Novel insights into nicotine dependence: The hope for new cessation therapeutics

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Doctor of Philosophy in Biological Sciences

University of California, Irvine 2022

Associate Professor Christie D Fowler, Chair

Tobacco smoking is considered to be one of the leading causes of preventable death worldwide, and nicotine is the primary reinforcer of tobacco dependence. Current therapeutics for tobacco cessation focus on alleviating the rewarding effects nicotine, but they are only modestly efficacious. Therefore, alternative therapeutics with greater efficacy is required. In this dissertation, the mechanism of nicotine dependence was investigated with different approaches to provide a foundation for the development of novel nicotine cessation treatments. As nicotine metabolism is involved in regulating the reinforcing effects of nicotine, we have demonstrated that inhibiting CYP2A6, the major enzyme responsible for metabolizing nicotine, decreases nicotine self-administration in mice. To address the dramatic increased use of e-cigarettes, we have established a vapor self-administration model in rodents to allow us study how nicotine vapor impact brain circuitry. As the aversive symptoms during nicotine withdrawal are also major factors that prevent people from quitting smoking, the exact neurobiological mechanisms of nicotine withdrawal remain to be elucidated. While previous studies have emphasized cholinergic signaling in the medial habenula in nicotine withdrawal

(Gorlich et al., 2013; Dao et al., 2014; Shih et al., 2014), we have shown that purinergic signaling plays an important role in controlling medial habenula neuronal activity. Together, our studies provide insight into the biological mechanisms underlying nicotine dependence and a foundation to further advance novel nicotine cessation treatments.

INTRODUCTION

A. Substance abuse and nicotine addiction

Substance abuse is defined as compulsive use of psychoactive drugs and substances, including alcohol, cocaine, opioid, and nicotine (WHO, 2020). The use and abuse of these psychoactive drugs often leads to dependence (WHO, 2020). Drug dependence not only exposes people to mental health hazards but also imposes serious problems for the society as a whole (National Institute on Drug Abuse, 2017; Substance Abuse and Mental Health Services Administration, 2018). It has been demonstrated that substance use has high comorbidity with anxiety disorder, panic disorder (Magidson et al., 2012), depression (Torrens et al., 2011), bipolar disorder (Compton et al., 2007), attention-deficit hyperactivity disorder (ADHD) (De Alwis et al., 2014) and psychotic illness (Hartz et al., 2014). In 2017, 19.7 million American adults suffered from a substance use disorder, and substance abuse has cost the American society more than 740 billion dollars annually in loss of workplace productivity, healthcare, and crime-related expenses (National Institute on Drug Abuse, 2017; Substance Abuse and Mental Health Services Administration, 2018). Due to the serious impact of substance abuse on individuals and the society, there is a crucial need for us to better understand the mechanisms of how substance of abuse affects the brain. This will allow us to develop therapeutics to aid people who are suffering from substance abuse.

Tobacco is commonly abused. There are 48 thousand deaths annually due to tobacco smoking in the U.S. (U.S. Department of Health and Human Services, 2014). It has been demonstrated that tobacco smoking increases the risk of many disorders, such as

cardiovascular diseases, diabetes, reproductive disorders and cancer (U.S. Department of Health and Human Services, 2014). Nicotine is one of the many toxins in tobacco which acts as the major psychoactive component and is the primary reinforcer for dependence (Stolerman and Jarvis, 1995).

A decline in cigarette use has been associated with the increasing awareness of the harmful effects of tobacco. However, in recent years, e-cigarette use has become prevalent with technological development (CDC, 2019). Of concern, there has been approximately a 1700% increase in e-cigarette use among adolescents has been observed from 2011 to 2019 (CDC, 2019). The use of e-cigarette can lead to nicotine dependence, resulting a long-lasting impact on learning, memory, attention, and impulse control in the adolescents' developing brain (Yuan et al., 2015). Furthermore, e-cigarette use elevates the risk of developing mental disorders, such as post trauma stress disorder, anxiety, and other substance abuse for other drugs in the future (U.S. Department of Health and Human Services, 2016; Grant et al., 2019). For adults, the e-cigarette use is correlated with the increase of self-reported stress and depression, suggesting the elevated risk of mental health impact from vaping (Xie et al., 2020). Thus, we must understand mechanisms underlying nicotine addiction in order to develop therapeutics to help people with nicotine dependence.

B. Nicotine metabolism and CYP2A6 polymorphism

a. Nicotine metabolism

When nicotine is absorbed through the lungs via inhalation, it enters the circulation within seconds and is metabolized in the liver (Benowitz, 1990). The majority of nicotine is converted to cotinine by the cytochrome P450 enzyme CYP2A6 and the minor nicotine N-oxide by the flavin-containing monooxygenase 3 (Benowitz and Jacob, 1994; Kubota et al., 2006; Ray et al., 2009). Around 10-15% of cotinine remains unchanged, and it is excreted in urine (Benowitz, 1994). Cotinine is further converted into trans-3′hydroxycotinine (3-HC), 5′-hydroxycotinine, cotinine *N*-oxide, cotinine methonium ion, cotinine glucuronide, and norcotinine (Neurath,1994; Benowitz, 2008; Ray et al., 2009).

b. Polymorphism of CYP2A6

In humans, many CYP2A6 variants, ranging from deleted alleles to fully functional alleles, have been identified (Kubota et al., 2006; Mwenifumbo and Tyndale, 2007; Mwenifumbo et al., 2008; Ray et al., 2009; Akrodou, 2015; Benowitz et al., 2016; López-Flores et al., 2017). CYP2A6*1A is the wild-type allele that has normal functionality in metabolizing nicotine (Kutobta et al., 2006; Mwenifumbo et al., 2008; Ray et al., 2009). Other CYP2A6 alleles with altered functionality that have been widely studied are CYP2A6*9 (48T>G in TATA promoter region, decreased enzyme activity) and CYP2A6*12 (10 amino acid substitution, deceased enzyme activity) (Mwenifumbo et al., 2008; Ray et al., 2009; Akrodou, 2015). As for the CYP2A6 alleles that lack enzyme activity, CYP2A6*2 (L160H amino acid substitution) and CYP2A6*4 (full deletion of the allele) are the alleles that have been extensively studied (Yamano et al., 1990; Kitagawa et al., 1999; Ray et al., 2009; Akrodou, 2015). CYP2A6*1A can be found in all ethnic groups and CYP2A6 alleles with null and reduced activity are more prevalent in

people Asian background, including Chinese, Korean and Japanese (Rao et al., 2000; Schoedel et al., 2004; Malaiyandi et al., 2005; Nakajima et al., 2006). Thus, genetic factors contribute to nicotine metabolism, which is thought to underly susceptibility to addiction.

The measurement of 3-HC/cotinine ratio is the validated biomarker for quantifying the activity of CYP2A6 in metabolizing nicotine (Mwenifumbo and Tyndale, 2007; Ray et al., 2009; Benowitz et al., 2009; Benowitz et al., 2016). The conversion from cotinine to 3-HC is selective for CYP2A6, and the 3-HC/cotinine ratio can be sampled from saliva and plasma (Dempsey et al., 2004; Lea et al., 2006; Mwenifumbo and Tyndale, 2007). The CYP2A6 alleles (*2, *4, *9, *12) with reduced or null activity of the enzyme is correlated with lower 3-HC/cotinine ratio (Dempsey et al.,2004; Johnstone et al., 2006; Mwenifumbo and Tyndale, 2007). The CYP2A6 activity is also reflected on the rate of clearance of nicotine to cotinine ($CL_{NIC\text{-}COT}$) (Mwenifumbo and Tyndale, 2007). As the mean rate of $CL_{NIC\text{-}COT}$ in individuals with normal CYP2A6 activity defined as 100%, the mean rate of $CL_{NIC\text{-}COT}$ found in people with reduced activity (*9, *12) is ~80% and the ones with null activity (*2, *4) of CYP2A6 is only ~49% (Mwenifumbo and Tyndale, 2007).

c. Association between CYP2A6 polymorphism and smoking behavior

Differences in CYP2A6 activity has been reported to influence smoking behavior, which includes smoking status and level of dependence (Kutobta et al., 2006; Ray et al., 2009; Akrodou, 2015; López-Flores et al., 2017). It has been reported that decreased or null activity of CYP2A6 is more prevalent in non-smokers (Pianezza et al., 1998; Iwahashi et

al., 2004; Malaiyandi et al., 2005). In contrast, individuals who have CYP2A6*1A alleles are more susceptible to develop nicotine dependence, and exhibit a higher cigarette consumption (Tyndale et al., 1999; Rao et al., 2000; WHO, 2002; Schoedel et al., 2004; Malaiyandi et al., 2005; Minematsu et al., 2006; Mwenifumbo et al., 2007; Ray et al., 2009). Interestingly, a previous study indicates that smokers with normal CYP2A6 activity smoke their first cigarette earlier in the day than people with reduced activity of CYP2A6 (Kobuta et al., 2006). In conclusion, the activity of CYP2A6 can correlate with the status of nicotine dependence in smokers.

It has also been demonstrated that the CYP2A6 polymorphism is correlated with the severity of nicotine withdrawal symptoms and the response to cessation therapeutics (Kobuta et al., 2006; Ray et al., 2009; Akrodou, 2015). Smokers with normal activity of CYP2A6 self-reported higher severity of withdrawal symptoms than smokers with null or reduced activity of CYP2A6 (Kobuta et al., 2006). The difference in the severity of withdrawal symptoms also affects the success rate of nicotine cessation (Ray et al., 2009; Akrodou, 2015). People with lower activity CYP2A6 alleles are almost twice as likely to quit smoking than people with CYP2A6 *1A alleles (Gu et al., 2000). As for the response to nicotine cessation treatments, researchers have investigated bupropion, a dopamine and norepinephrine reuptake inhibitor and noncompetitive antagonist of nAChRs, and nicotine replacement therapy (NRT), a treatment providing low levels of nicotine through transdermal patch and gum (Slemmer et al., 2000). People with reduced CYP2A6 function have higher success rates compared to people with normal CYP2A6 function (32% to 11%) after 6 months of bupropion/nicotine replacement therapy (Yamanaka et al., 2004). The transdermal nicotine patch is also more effective

in people with reduced CYP2A6 function (Patterson et al., 2008). In sum, difference in the rate of nicotine metabolism is one of the key factors that may influence nicotine dependence and the success rate with cessation treatment. Therefore, inhibiting the CYP2A6 activity is an alternative and feasible approach for a cessation therapeutics and the rate of nicotine metabolism in smokers should be taken into consideration to design better treatments for each individual.

C. Nicotinic acetylcholine receptors (nAChRs) and the brain regions involved in the rewarding effects of nicotine

Nicotine is an agonist of nicotinic acetylcholine receptors (nAChRs). It has been demonstrated that nAChRs express in the central and peripheral nervous systems, skeletal muscle, and many other peripheral tissues (Hogg et al., 2003). A total of 12 neuronal nAChRs subunits have been identified, which includes α2-α10 and β2-β4 (Hogg et al., 2003). These nAChR subunits form a heteromeric pentamer, which typically contains at least a α type and β type subunit (Hogg et al., 2003). The α7, α8, and α9 nAChR subunits are known to form a homomeric pentamer but only α7 nAChR is widely distributed in the CNS (Dani and De Biasi, 2001).

Both the binding of nicotine and the endogenous agonist, acetylcholine, with the nAChRs will change the nAChRs to an open conformation for 2-3 milliseconds (Dani and Heinemann, 1996), allowing for the influx of Na^+ and Ca^{2+} into the cell (Fowler et al., 2008). After opening, nAChRs will become desensitized (Dani and Heinemann, 1996). During desensitization, nAChRs will remain closed and the binding of agonists will not open the channel (Dani and Heinemann, 1996). The duration of desensitization varies

depending on what nAChR subunits are present in the channel. It can range from millisecond (e.g. α7 nAChRs) to seconds (e.g. α4β2 subunit-containing nAChRs) (Giniatullin et al., 2005). Thereafter, the receptors take a closed state is accessible for agonist-induced activation.

The expression of nAChRs in the brain is widely distributed. They have been found in the prefrontal cortex (PFC), ventral tegmental area (VTA), hippocampus, amygdala, medial habenula (MHb), interpeduncular nucleus (IPN), and many other brain regions (Tuesta et al., 2011; Dani and Subramaniyan, 2015). The VTA is considered to be the main region that mediates the rewarding effect of nicotine (Dani and De Biasi, 2001; Wu et al., 2013; Rose et al., 2016). Nicotine directly excites VTA dopaminergic neurons which express α4β2 containing nAChRs (Wu et al., 2013; Leslie et al., 2013). These dopaminergic neurons projects to the nucleus accumbens (NAc) and the PFC (Hogg et al., 2003; Wu et al., 2013; Leslie et al., 2013; Subramaniyan and Dani, 2015). The firing of VTA dopaminergic neurons directly elevates dopamine levels in the NAc and PFC (Wu et al., 2013; Subramaniyan and Dani, 2015). The VTA is highly innervated by cholinergic projections originating from the pedunculopontine tegmentum and laterodorsal pontine tegmentum (Pidoplichko et al., 2004). The cholinergic inputs into the VTA mainly project to GABAergic neurons, and only 5% of the dopaminergic neurons in the VTA are innervated by these cholinergic projections (Garzon et al., 1999). Long term nicotine exposure-induced desensitization of α4β2 containing nAChRs mainly blocked the tonic cholinergic excitation into VTA GABAergic neurons (Pidoplichko et al., 2004). Therefore, VTA dopaminergic neurons are disinhibited and dopamine release in the NAc and PFC subsequently increases (Mansvelder et al.,

2002; Pidoplichko et al., 2004). Nicotine also directly acts on α6-containing nAChR in the VTA axon terminals in NAc to regulate dopamine release (Exley et al., 2008). It has also been demonstrated that nicotine directly acts on β2 containing nAChRs in the PFC to increase attention and working memory function (Guillem et al., 2011). Furthermore, it has been proposed that nicotine disrupts the cognitive inhibitory control from the PFC to VTA; therefore, promoting the rewarding seeking behavior (Wu et al., 2013). In conclusion, the rewarding effects of nicotine are mostly mediated by effects on dopaminergic circuits in the brain.

D. Medial Habenula (MHb)- Interpeduncular nucleus (IPN) pathway

The rewarding effect of nicotine is not the sole factor that modulates nicotine dependence. Nicotine aversion and nicotine withdrawal are also key factors regulating nicotine intake. The MHb-IPN pathway has been considered to be highly involved in regulating nicotine aversion and withdrawal based on the results from molecular, behavior, and electrophysiology studies (Fowler and Kenny, 2014; Antolin-Fontes et al., 2015). The following paragraphs will review the anatomical connections, and the studies demonstrating the MHb-IPN pathway participates in the regulation of nicotine aversion and withdrawal.

a. Afferent and efferent projections of the MHb

The MHb is a subregion of the habenula, a bilateral structure that belongs to a part of the epithalamus (Kim and Chang, 2005). The origins of major inputs to the MHb are the triangular septal nucleus (TS) and the septofimbrial nucleus (Quin and Luo, 2009). The

projection from the TS to the MHb releases glutamate and ATP (Sperlagh et al., 1995; Sperlagh et al., 1998; Qin and Luo, 2009). ATP and glutamate are released from different synapses instead of co-releasing from the same synapse (Edwards and Robertson, 1998). Monoaminergic projections to the MHb have also been discovered, including the dopaminergic projection from the VTA, and the noradrenergic inputs from mesencephalic raphe and locus coeruleus (Herkenham and Nauta, 1977; Gottesfeld, 1983).

The MHb also receives GABAergic projections from the medium septum (MS) and nucleus of diagonal band (NDB) (Quin and Luo, 2009). The MHb has a high expression of $GABA_B$ receptors (Charles et al., 2001). Regarding $GABA_A$ receptors expression and function in the MHb, studies demonstrate conflicting results. One study, applying the gramicidin- perforated patch recording technique, has demonstrated that $GABA_A$ receptors mediate fast excitation in MHb neurons since the MHb lacks the expression of the K^+/Cl^- co-transporter 2 (KCC2), which is responsible for extruding the Cl^- out of the neuron (Kim and Chung, 2007). The absence of KCC2 expression on MHb neurons results in high intracellular Cl^- concentration. As the reversal potential of $GABA_A$ receptor is more positive than firing threshold due to high intracellular Cl^- concentration, the activation of $GABA_A$ receptors will elicit a fast excitation of the neuron (Kim and Chung, 2007; Choi et al., 2016). However, another study indicates that no $GABA_A$ receptor-mediated currents could be detected (Wang et al., 2006). Furthermore, the expression of $GABA_A$ receptors has not been found in MHb neurons (Wang et al., 2006). Despite the contradictory results regarding the expression of $GABA_A$ receptors, it has been demonstrated that there is no expression of KCC2 in MHb neurons in multiple studies

(Wang et al., 2006; Kim and Chung, 2007; Markkanen et la., 2014; Choi et al., 2016). The exact mechanism for how MHb neurons regulate intracellular Cl^- concentration without the presence of KCC2 requires further investigation.

The major efferent projections of the MHb are consist of axons to the IPN through the fasciculus retroflexus tract (Herkeham and Nauta, 1979; Qin and Luo, 2009; Antolin-Fontes et al., 2015). The projections from the dorsal MHb to lateral subnuclei of the IPN are glutamatergic and substance-Pergic, whereas the projections from the ventral MHb to central and intermediate nuclei of the IPN are acetylcholine and glutamate co-expressing (Contestabile et al., 1987; Aizawa et al., 2012). It should also be noted that the projections from the MHb to the IPN form a unique circuitry structure. The axons from the MHb zigzag through the central IPN region, forming *en passant* synapses before ending ipsilaterally in the dorsal region of the IPN (Herkeham and Nauta, 1979). In the intermediate region of the IPN, MHb axon terminals from crest synapse with IPN neurons (Lenn et al., 1983). In other words, IPN dendrites form a disc shape and receive projections from both the right and left MHb (Lenn et al., 1983). The crest synapse may serve as a coincidence detector since a similar structure has been proposed to be the coincidence detector in the auditory system (Carr and Konishi, 1990). However, the exact role of the unique structures in the MHb-IPN pathway regulating nicotine addiction remain to be further defined.

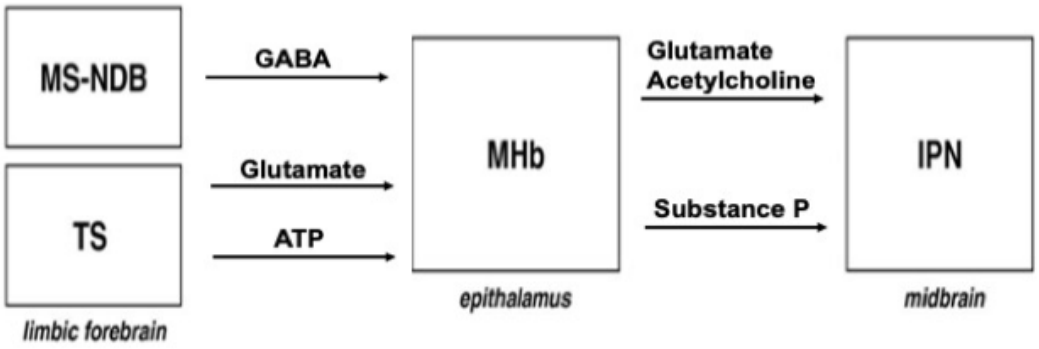

Figure Intro 1. An illustration of the efferent and afferent projections of the MHb, Adapted from Quin and Luo, 2009

b. MHb-IPN pathway and nicotine aversion

The MHb-IPN pathway expresses a high density of α5, α3, and β4 nAChR subunits. Allelic variation of the gene cluster, *CHRNA5-CHRNA3-CHRNAB4*, encoding α5/α3/ β4 subunits of nAChRs is related to increased risk of tobacco addiction (Fowler et al., 2011; Fowler and Kenny, 2014; Antolin-Fontes et al., 2015; Mclaughlin et al., 2015). A single nucleotide polymorphism (SNP) in *CHRNA3*, rs1051730, is strongly associated with higher chances of developing tobacco dependence (Thorgeirsson et al., 2008). The SNP in *CHRNA5,* which results in substituting aspartic acid to asparagine in amino acid residue 398 (D398N), is related to heavy smoking (Bierut et al., 2008), early onset of smoking (Weiss et al., 2008; Berrettini et al., 2008) and self-reported pleasure buzz in human smokers (Sherva et al., 2008). The α5, α3, and β4 subunit nAChR-mediated signaling in the MHb-IPN tract are considered to be highly involved in regulating nicotine aversion (Fowler and Kenny, 2014; Antolin-Fontes et al., 2015, Elayouby et al., 2021). Specifically, it has been demonstrated that mice with genetically knockout of the α5 subunit nAChRs self-administer a higher amount of nicotine compared to wild type mice

(Fowler et al., 2011). Furthermore, α5 subunit nAChRs knockout mice will consume nicotine at a dose that is considered to be aversive to wild type mice, but viral re-expression of α5 subunit nAChRs specifically in the MHb rescue the nicotine intake behavioral phenotype of α5 subunit nAChRs knockout mice (Fowler et al., 2011). Nicotine-induced activation of MHb glutamatergic projections into IPN limits nicotine intake (Fowler et al., 2011) and absence of the α5 subunit nAChR signaling in MHb attenuate the nicotine-induced activation of the glutamatergic input to the IPN, which therefore results in a decreased aversion to nicotine at a higher dose (Fowler et al., 2011). As for β4 subunit nAChR, it has been shown that overexpression of β4 subunit nAChR enhances aversion to nicotine and interestingly, virally expressing α5 subunit nAChR D398N variant in MHb reverses the elevated nicotine aversion phenotype in mice with β4 subunit overexpression (Frahm et al., 2011). It has been proposed that nicotine aversion is modulated by the balanced activity of the β4 subunit and α5 subunit nAChR signaling in the MHb (Frahm et al., 2011). The role of the α3 subunit nAChR-mediated signaling in the MHb-IPN pathway is more challenging to assess since the α3 null mice would die soon after birth (Xu et al., 1999). However, the β4 subunit nAChR is almost always co- localized with the α3 subunit nAChR and the auxiliary α5 subunit nAChR assembles with α3β4 composition to form a functional nAChR (Frahm et al., 2011) and the MHb-IPN tract has a high expression of the α3β4 composition of nAChRs (Zoli et al., 1995). Furthermore, a recent study demonstrated that the knockout down of α3 subunit in the MHb or IPN increases nicotine self-administration (Elayouby et al., 2021). Therefore, α3 subunit nAChR mediated signaling in the MHb-IPN pathway is involved in regulating nicotine aversion.

c. The MHb-IPN pathway and nicotine withdrawal

Abrupt cessation in tobacco smoking will elicit aversive nicotine withdrawal symptoms (Changeux, 2010). These aversive symptoms are one of the major reasons why quitting tobacco smoking is hard to achieve (Changeux, 2010). In humans, nicotine withdrawal elicits a collection of both mental and physical aspects of symptoms, including anxiety, irritability, difficulty concentrating, bradycardia, gastrointestinal discomfort, and weight gain (Shiffman and Jarvik, 1976; Antolin-Fontes et al., 2015). Rodents also demonstrate nicotine withdrawal symptoms when nicotine intake is suddenly ceased, or a nAChR antagonist is administered after chronic nicotine consumption. The physical signs, also called as somatic signs, in rodents include head nods, body shakes, hyperalgesia, and decrease of locomotor activity (Hildebrand et al., 1999; Damaj et al., 2003; Grabus et al., 2005). Affective nicotine withdrawal symptoms in rodents include anxiety-like behavior (Damaj et al., 2003), impaired contextual fear conditioning (Davis et al., 2005), and elevation of the brain reward threshold (Kenny and Markou, 2001).

Besides their role in nicotine aversion, studies have shown that α5 and β4 nAChR subunit-mediated signaling in the MHb-IPN pathway are also involved in regulating nicotine withdrawal (Salas et al., 2009; Antolin-Fontes et al., 2015). It has been demonstrated that α5 null mice demonstrate less somatic signs of withdrawal and withdrawal-induced hyperalgesia after chronic nicotine administration (Jackson et al., 2008; Salas et al., 2009). The administration of the nicotinic receptor antagonist, mecamylamine, specifically into the MHb or IPN, can elicit nicotine withdrawal (Salas et al., 2009). Since the MHb and IPN have high expression of the α5 subunit nAChR, it is

likely that this α5 subunit nAChR- mediated signaling is involved in regulating nicotine withdrawal (Salas et al., 2009; Mclaughlin et al., 2015). As for the β4 nAChR subunit, it has been shown that β4 knockout mice demonstrate less somatic signs after chronic nicotine exposure (Salas et al., 2004b). Long-term nicotine exposure upregulates β4 subunit nAChR expression in the MHb and also in somatostatin positive (SSt+) neurons in the IPN (Zhao-Shea et al., 2013; Meyers et al., 2015). The administration of nicotinic antagonists in the IPN or abrupt cessation in nicotine consumption releases the inhibition from SSt^{+} neurons to MHb glutamatergic axon terminals in the IPN; therefore, the GABAergic neurons in the IPN are activated to elicit somatic signs during nicotine withdrawal (Zhao-Shea et al., 2013).

It should also be noted that other subunits of nAChRs are involved in nicotine withdrawal regulation in the MHb-IPN pathway. There is a strong expression of α2 subunit nAChR expression in the IPN (Ishii et al., 2005). The α2 null mice demonstrate less somatic signs during nicotine withdrawal in a familiar environment (Salas et al., 2009). However, α2 null mice exhibit more somatic signs and also a sex-dependent deficit in cue-induced fear conditioning during nicotine withdrawal in a novel environment (Lotfipour et al., 2013). In conclusion, the results from the studies mentioned above indicate that that α2 subunit- containing nAChRs may be involved in regulating affective signs of nicotine withdrawal.

The unique pace-making firing characteristic of MHb is also considered to regulate nicotine withdrawal (Gorlich et al., 2013; Dao et al., 2014). Almost all MHb neurons generate a spontaneous tonic firing at baseline at a frequency from 2 to 10Hz,

independent of the synaptic input into the MHb (Gorlich et al., 2013). This pace-making electrophysiological property is regulated by hyperpolarization-activated cyclic nucleotide-gated (HCN) channels (Gorlich et al., 2013; Antolin-Fontes et al., 2015). The infusion of HCN channel blocker in the MHb not only inhibits the spontaneous tonic firing but also elicits somatic signs and affective signs on nicotine naive mice (Gorlich et al., 2013). Acute nicotine administration will increase the baseline firing frequency through α3β4 and α5 containing nAChRs (Gorlich et al., 2013; Dao et al., 2014). Chronic nicotine exposure doesn't elevate the firing frequency of the baseline firing in response to nicotine application; however, re-exposure of nicotine during nicotine withdrawal will increase the firing frequency to reach 20Hz or above (Gorlich et al., 2013). Interestingly, it has been shown that acetylcholine levels increase at MHb axon terminals in the IPN when stimulation frequency is 20Hz or above (Ren et al., 2011; Gorlich et al., 2013), although this finding may be confounded by the experimental approach with supraphysiological manipulations. In conclusion, tonic neuronal firing in the MHb mediates aspects of nicotine withdrawal.

E. Purinergic signaling

Adenosine 5'-triphosphate (ATP) mediated fast synaptic currents in the CNS were first discovered in the MHb (Edwards et al, 1992; Burnstock, 2015), identifying that ATP acts as a neurotransmitter. The source of the ATP release and several subtypes of purinergic receptors have been identified in the MHb; however, limited further studies have investigated the role of purinergic transmission in the function of the MHb. The

following paragraphs provide a concise review of purinergic signaling and supporting studies in the MHb.

a. Discovery of purinergic signaling

ATP has been long considered solely as an energy source for the cell. Even with the first study about purinergic signaling describing the effect of adenosine on cardiac muscle in 1929 (Drury and Szent-Gyorgyi, 1929), scientists were still reluctant to accept the concept that ATP can act as a neurotransmitter. It was hypothesized that the presence of extracellular ATP was merely the result of cellular apoptosis. It was not until 1972 that the term “purinergic transmission” was introduced (Burnstock, 1972). Later in the 1990s, the purinergic receptors and the subtypes of these receptors were identified and cloned (Ralevec and Burnstock, 1998). With the discovery of ATP-mediated currents and its receptor, the concept that ATP acts not only as an energy source of the cell but also as a neurotransmitter began to be widely accepted.

b. Purinergic receptors and their subtype

Once ATP is released into the synaptic cleft, ATP will be hydrolyzed into adenosine 5’-diphosphate (ADP) and adenosine 5’-monophosphate (AMP). Later, AMP will be metabolized into adenosine by 5’-nucleotidase (Burnstock, 2006). Different subtypes of purinergic receptors are activated by different metabolites of ATP or ATP itself (Burnstock, 2006).

The purinergic receptors can be divided into two categories: P1 and P2 receptors. Adenosine activates P1 receptors (Burnstock, 2018). There are four subtypes of P1 receptors: A_1, A_2, A_{2B}, A_3 (Fredholm et al., 2001; Burnstock, 2018). All P1 receptors are coupled to adenylate cyclase (Reshkin et al., 2000). A_1 and A_2 receptors activate adenylate cyclase once adenosine binds; whereas A_{2B} and A_3 receptors inhibit adenylate cyclase when activated (Reshkin et al., 2000).

For P2 receptors, there are two main subtypes: P2X and P2Y receptors (Burnstock, 2015). P2X are ionotropic receptors, and they are activated by ATP (Burnstock, 2015). Seven different subunits, $P2X_1$-X_7, for the P2X receptors have been identified (Burnstock, 2015). Three P2X subunits assemble to form a heterotrimer or a homotrimer as a functional receptor (North, 2002). The $P2X_6$ subunit only forms a heterotrimer with other subunits and the $P2X_7$ subunit only forms homotrimer (North, 2002). Homotrimer and heterotrimer have different receptor kinetics and sensitivity to agonists and antagonists. For instance, the $P2X_{1/5}$ heteromer has a higher sensitivity to ATP, comparing to both $P2X_1$ homomer and $P2X_5$ homomer (North, 2002). However, once activated by ATP, all subtypes of the P2X receptor increase the permeability to cations like Na^+ and Ca^{2+} (North, 2002; Burnstock, 2018). For $P2X_5$ homotrimer, it also increases permeability to Cl^- (North, 2002). When being exposed to ATP for extended periods of time in millimolar levels, the $P2X_7$ receptor will convert into a non-selective cytolytic pore conformation, allowing large molecules with 900 Da to permeate into the cytoplasm (Virginio et al., 1999). This increased permeability to large molecules elicits the signal cascade to cell death (Virginio et al., 1999; North 2002; Burnstock, 2007; Savio et al., 2018).

P2Y receptors are G-protein coupled receptors. There is a total of 8 subtypes of P2Y receptors that have been identified: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ (Guzman and Gerevich, 2016). The missing numbers of the P2Y receptors are either being assigned to other non-mammalian species with P2Y homologs or the function as a receptor to extracellular nucleotide has not been fully identified (von Kugelgen and Hoffmann, 2015). In general, P2Y receptors are activated by ATP or ADP and different subtypes exhibit different sensitivity to ATP and ADP (Abbracchio et al., 2005). However, some P2Y subtypes only respond to other nucleotides (Guzman and Gerevich, 2016; Burnstock, 2018). For instance, the $P2Y_1$ receptor can be activated by both ATP and ADP, but ADP is a more potent than ATP (Abbracchio et al., 2006; Guzman and Gerevich, 2016). However, the $P2Y_{11}$ receptor does not respond to ADP, but it will be activated by ATP (Burnstock, 2018). $P2Y_{14}$ and $P2Y_6$ receptors are examples of P2Y receptor subtypes that only respond to nucleotides other than ATP and its metabolites since they activate when uridine 5'-diphosphate (UDP) is present, but not ATP nor ADP (Guzman and Gerevich, 2016). As for the functionality of P2Y receptors, $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors are coupled to G_q protein to release Ca^{2+} from intracellular storage upon receptor activation (Abbracchio et al., 2006). $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors are coupled to G_i protein and inhibit the activity of adenylate cyclase (Abbracchio et al., 2006). Interestingly, the $P2Y_{11}$ receptor is not only coupled to G_q protein to elevate intracellular Ca^{2+} release but also coupled to G_s protein to activate adenylate cyclase (Communi et al., 1999).

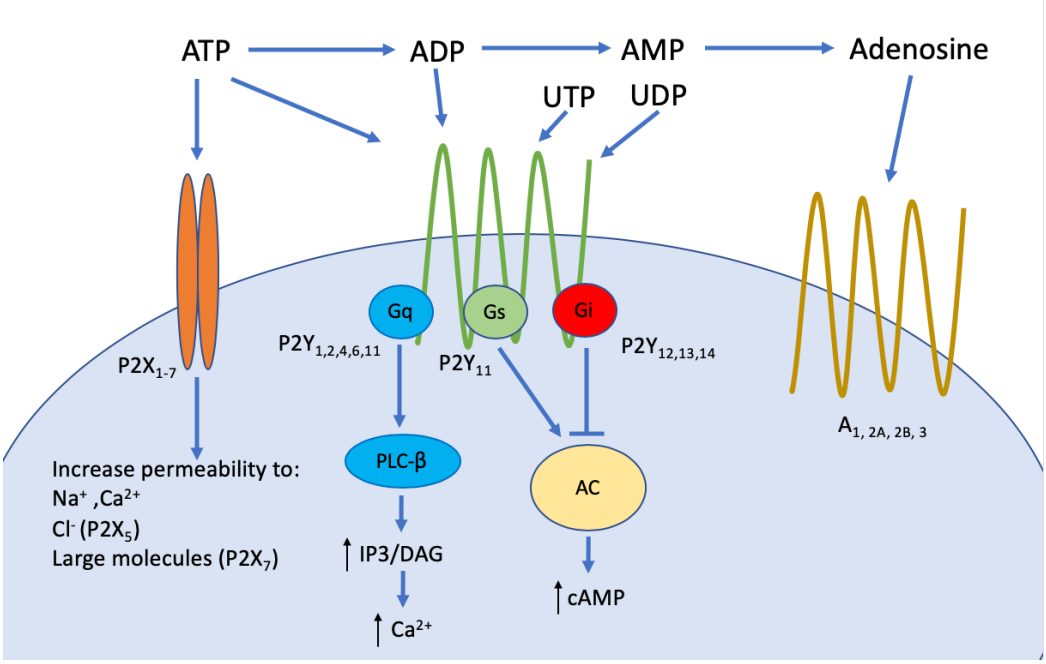

Figure Intro 2. Illustration of ATP receptors, Adapted from Yang and Liang, 2012

c. The expression of purinergic receptors

Purinergic receptors are widely distributed in the body. The expression of purinergic receptors can be found not just in the brain but also in the peripheral nervous system and non-neuronal tissue. One of the many examples is that the $P2Y_1$ receptor is expressed in platelets, and the ADP dependent aggregation of platelets is mediated by $P2Y_1$ receptors (Cattaneo et al., 2004). This section will mainly discuss the expression of purinergic receptors in the brain and what subtypes of purinergic receptors are found in the MHb.

P1 receptors are expressed on neurons in different regions of the brain. The A_1 receptors are highly expressed in the cortex, hippocampus, and cerebellum (Dixon et al., 1996; Bynoe at el., 2015). The A_{2A} receptors have high expression in the NAc and

the striatum (Dixon et al., 1996). The A_{2B} and A_3 receptors have a weaker expression in the brain compared to the expression in peripheral organs (Dixon et al., 1996). P1 receptors are also thought to be expressed on glia and astrocyte to regulate blood-brain barrier permeability (Bynoe et al., 2015). Regarding the expression of the P1 receptors in the MHb, a study applying the whole-cell recording technique has demonstrated that activation of presynaptic A_1 receptor on TS axon terminals inhibits ATP and glutamate release in the MHb (Edwards and Robertson, 1998).

The expression of P2 receptors in the brain has been identified (Burnstock, 2015). For P2X receptors, $P2X_4$, $P2X_5$, and $P2X_6$ are the subunits that can be found in multiple brain regions (Guo et al., 2008; Burnstock, 2015). As for P2Y receptors, $P2Y_1$ receptor is the P2Y receptor subtype that is highly expressed in many brain regions, including the cortex, hippocampus, caudate nucleus, putamen, and globus pallidus (Moore et al., 2000). There are specific regions that have high expression of both P2X and P2Y receptors, such as the hippocampus, basal ganglia, and hypothalamus (Abbracchio et al., 2006; Burnstock, 2015). This expression profile suggests that P2 receptor-mediated signaling may be highly involved in modulating learning and memory, sleep, and locomotion (Abbracchio et al., 2006; Burnstock, 2015).

Astrocytes express P2 receptors and release ATP to neurons. The expression profile of P2 receptors varies depending on the brain region. Astrocytes in the cortex express almost all types of P2X subunits and P2Y subtypes except the $P2X_6$ subunit (Abbracchio and Ceruti, 2006). As for the astrocyte in the NAc, they express $P2Y_1$, $P2Y_4$, and $P2X_{1\text{-}4}$ (Franke et al., 2001; Franke et al., 2004). $CD11b^+$Microglia also

express P2 receptors, and the expression of P2 receptors alters after exposure to the bacterial endotoxin lipopolysaccharide (Bianco et al., 2005; Crain et al., 2009).

The expression of the P2 receptor in the MHb has been confirmed. The results from the immunohistochemistry assays indicate that the MHb expresses $P2X_1$, $P2X_2$, $P2X_5$, $P2Y_4$, and $P2Y_1$ (Kanjhan et al., 1999; Moran-Jimenez and Matute, 2000; Florenzano et al., 2008; Guo et al., 2008; Song et al., 2011). As for the function of P2 receptors, it has been demonstrated that activation of presynaptic $P2Y_4$ receptors increases glutamate release in the MHb (Price et al., 2003). However, more studies are required to further understand the role of other subtypes of the P2 receptor in the MHb.

d. Purinergic receptor studies related to drug addiction

Recent studies have started to reveal that purinergic signaling may play an important role in drug addiction. The A_{2A} receptor is the purinergic receptor that has been most studied m and many findings suggest that the A_{2A} receptor may be a target to treat addiction to stimulants. Elevation of cocaine-induced extracellular dopamine in the NAc does not sustain in A_{2A} receptor knockout mice and the cocaine-mediated hyperlocomotion also decreases, compared to wildtype mice with cocaine administration at the same dose (Wells et al., 2012). This indicates that A_{2A} receptors regulate the acute effect of cocaine (Wells et al., 2012). Self-administration of methamphetamine decreased A_{2A} receptor expression in the NAc (Kavanagh et al., 2015). The antagonism of the A_{2A} receptor in the dorsomedial striatum restores the decision-making deficit caused by methamphetamine (Furlong et al., 2015). The A_{2A} receptor-mediated signaling is also considered to be involved in regulating alcohol

addiction (Micioni Di Bonaventura et al., 2012; Houchi et al., 2013). It has been demonstrated that the blocking A_{2A} receptor reduces voluntary ethanol intake in rats, and an A_{2A} receptor antagonist may be a potential treatment for alcohol abuse (Micioni Di Bonaventura et al., 2012; Houchi et al., 2013).

As for P2 receptors, it has been shown that the expression of $P2X_4$ and $P2X_7$ in the prefrontal cortex and the striatum is decreased during morphine withdrawal (Metryka et al., 2019). Intrathecal infusion of P2X antagonist that specifically blocks $P2X_1$, $P2X_3$ and heteromeric $P2X_{2/3}$ receptors diminishes opioid tolerance in mice (Tai et al., 2010). The $P2X_4$ receptor is considered to be highly involved in regulating alcohol addiction. The difference in the $P2X_4$ receptor expression profile, specifically in the brain regions involving in reward circuitry, may be an indicator of innate alcohol preference. It has been shown that the $P2X_4$ receptor expression in the VTA is reduced in the rat lines selectively bred for high ethanol consumption, compared to the rat lines bred for low ethanol consumption (McBride et al., 2012). Specific knockdown of $P2X_4$ receptor expression in the NAc results in increased ethanol consumption in mice (Khoja et al., 2018). An antiparasitic drug, Ivermectin, which also activates $P2X_4$ receptors, has been proposed to be a novel treatment fighting against alcohol addiction (Franklin et al., 2014).

In conclusion, purinergic signaling may be highly involved in regulating drug-seeking behavior, and it may be a novel target to treat drug dependence. However, more studies are required, especially in regard to nicotine addiction. Since P2 receptors are also

expressed in the MHb, purinergic signaling may also play a role in modulating nicotine intake and withdrawal.

e. Purinergic signaling in synaptic transmission

The activation of P2 signaling may have an inhibitory effect on glutamatergic signaling. It has been demonstrated that activation of the P2X receptors inhibits NMDA receptor-mediated excitatory postsynaptic current (EPSC) (Pankratov et al., 2002). Applying a P2 general blocker can facilitate the formation of long-term potentiation (LTP) in the CA1 region of the hippocampus (Pankratov et al., 2002). The mechanism of how P2X receptor activation inhibits postsynaptic NMDA receptors may be a calcium-dependent NMDA inactivation/desensitization (Legendre et al., 1993). P2X receptors have a high permeability to Ca^{2+} and intracellular Ca^{2+} concentration quickly elevates once P2X receptors are open. Ca^{2+} binds to calmodulin and this Ca^{2+}-calmodulin complex interacts with the NR1 subunit of the NMDA receptor, causing the NMDA receptor inactivation (Zhang et al., 1998). The P2X receptor-mediated signaling may serve as a filter to prevent spontaneous LTP in postsynaptic neurons since even basal synaptic activity can elicit LTP when the P2 blocker suramin is present (Wieraszko, 1995; Guzman and Gerevich, 2016). Regarding the role of P2 receptors in GABAergic transmission, it has been demonstrated that activation of the $P2X_3$ subunit-containing P2X receptors may facilitate presynaptic GABA release, possibly through a Ca^{2+} dependent mechanism (Haugstad et al., 1992; Gomez-Villafuertes et al., 2001).

However, more research is required to understand the exact mechanism of how P2X receptors facilitate GABA release.

The activation of the P2X receptor may have different actions on glutamatergic transmission depending on its location. While activation of postsynaptic P2X receptors inhibits glutamatergic transmission through Ca^{2+} inactivation (Legendre et al., 1993), presynaptic P2X receptors facilitate glutamate release (Sperlagh et al., 2002; Khakh et al., 2003; Rodrigues et al., 2005). It has been demonstrated that presynaptic $P2X_1$ and $P2X_2$ subunit-containing P2X receptors and $P2X_7$ receptors promote glutamate release in the hippocampus (Sperlagh et al., 2002; Khakh et al., 2003; Rodrigues et al., 2005).

P2Y receptors may inhibit glutamatergic transmission as well (Gerevich et al., 2004). $P2Y_1$ receptors inhibit presynaptic glutamate release in rat dorsal ganglion neurons (Gerevich et al., 2004). The activation of the $P2Y_1$ receptor activates the G-protein and the Gβγ subunit is disassociate from the G-protein complex (Guzman and Gerevich, 2016). The Gβγ subunit will bind to voltage-activated calcium channels (VACC) and cause the channel to close; therefore, the activation of the $P2Y_1$ receptor inhibits the activity of the neuron, leading to decrease glutamate release (Gerevich et al., 2004). The activation of presynaptic $P2Y_1$ and $P2Y_2$ receptors also inhibits presynaptic glutamate release in the hippocampus (Rodrigues et al., 2005). As for postsynaptic P2Y receptors, it has been demonstrated that activation of postsynaptic $P2Y_1$ receptors dampens the postsynaptic neurons' activity by directly inhibiting the NMDA receptor and activating outward rectifying potassium channels (Moran- Jimenez and Matute, 2000; Luthardt et al., 2003; Guzman and Gerevich, 2016). P2Y receptors also appear to be

involved in modulating GABAergic signaling (Saitow et al., 2005). A study has shown that applying agonist of $P2Y_1$ receptors will induce a long-lasting increase of stimulation invoked inhibitory postsynaptic current in the cerebellum (Saitow et al., 2005). Presynaptic $P2Y_1$ receptors facilitated GABA release may be calcium dependent since intracellular Ca^{2+} concentration increases while applying $P2Y_1$ receptor agonist (Saitow et al., 2005). Furthermore, the facilitation is blocked while applying Ca^{2+} chelating agent (Saitow et al., 2005). While the activation of presynaptic $P2Y_1$ inhibits glutamate release the activation of presynaptic $P2Y_4$ receptors increases glutamate release, even though both receptors are coupled to G_q protein (Price et al., 2003). Besides $P2Y_1$ and $P2Y_4$ are activated by different agonists, the reason why the activation of $P2Y_1$ and $P2Y_4$ would have different actions on neurotransmission awaits to be further investigated.

F. Different models of nicotine administration in animals

A variety of methods of nicotine administration in animals have been introduced to investigate the factors that contribute to nicotine dependence. Daily subcutaneous or intraperitoneal nicotine injections (Pandey et al., 2011; Lefever et al., 2017; Pushkin et al., 2019) and oral consumption of nicotine-containing solution (Rowell et al., 1983; Robinson et al, 1996; Glick et al., 1998; Gorlich et al., 2013) have been widely applied in previous studies. Both injections and oral intake of nicotine can be easily achieved without costly equipment and complicated techniques. However, repeated injections may induce stress and fear response in animals (Brynildsen et al., 2016). The bitter taste of nicotine may affect the motivation of consuming nicotine-containing solution, even though saccharin is often added to the solution to mask the bitterness (Collins et al., 2012). Oral intake of nicotine solution also results in slower absorption rate of

nicotine and lower levels of plasma concentration (Adriani et al., 2002). Subcutaneous osmotic pumps have been implanted to continuously administer nicotine while avoiding possible stress response from daily injections (Besheer et al., 2003; Dao et al., 2014; Brynildsen et al., 2016). Instead of continuous delivery of nicotine, an intermittent nicotine delivery protocol has been established to better simulate the actual pattern of cigarette use in human smokers (Brynildsen et al., 2016). It has been demonstrated that intermittent delivery of nicotine produced more robust nicotine withdrawal behaviors than continuous delivery (Brynildsen et al., 2016). However, the doses delivered by osmotic-pump results in higher nicotine levels than that found in smokers (Russell et al., 1980; Gorlich et al., 2013)

Injections and osmotic pump delivery of nicotine are methods that have been widely utilized to induce nicotine dependence; however, the motivation of the animals to seek nicotine cannot be measured since they are passively receiving the drug instead of self-administering. Intravenous nicotine self-administration is a reliable model to allow animals to voluntarily intake nicotine (Fowler et al., 2011). The animals are placed in an operant chamber to freely press on the active lever to receive intravenous nicotine infusions or the inactive lever that does not associate with any consequences (Fowler et al., 2011). The motivation of drug seeking can be easily quantified by recording the number of lever presses, and the amount of nicotine that the animals self-administer can be precisely measured (Fowler et al., 2011). The intravenous nicotine self-administration procedure has been viewed as the gold standard model to access the dose-dependent effect of nicotine in rodent, non-human primates and humans

(Goodwin et al., 2015; Jensen et al., 2016; Lallai et al., 2021). However, the procedures of implanting the catheter, especially in mice, may be challenging. Also, the diameter of the vein in adolescence mice is too small to make it technically feasible to insert the tube for nicotine infusion. Therefore, the intravenous nicotine self-administration can only be accessed in adult mice.

With the recent uprising of e-cigarette use in the adolescents, the potential effect of e-cigarette in altering the brain circuitry activities has become a major concern (CDC, 2019). Previous studies have investigated the impact of nicotine to the brain by having the animals passively inhale nicotine vape (George et al., 2010; Alhaddad et al., 2020; Re et al., 2021). Furthermore, nicotine vapor self-administration in rodent has been recently established to further assess the motivation of the animal for nicotine vapor intake (Lallai et al., 2021, see chapter 2). The nicotine vapor exposure model, either passive exposure or self-administration, is the model that best mimics human smoking. No surgery is required and nicotine exposure in adolescent mice is feasible. However, the amount of nicotine that is being inhaled into the animal cannot be directly quantified. Since nicotine's major metabolite cotinine is a biomarker for nicotine intake, measuring the blood cotinine levels after nicotine vapor exposure can evaluate how much nicotine was inhaled (Lallai et al., 2021).

G. Summary

In this dissertation, the mechanisms underlying nicotine dependence have been investigated with different approaches. In chapter 1, we developed a novel inhibitor of

CYP2A5/6, DLCI-1, to decrease nicotine consumption in mice and demonstrated that DLCI-1 has the potential to be a future therapeutic of nicotine cessation. In chapter 2, we established the vapor self-administration protocol for rats and then applied and modified this technique for mice. Finally, we discuss how purinergic signaling alters MHb activity in chapter 3. Taken together, these data have advanced the field by providing a more complete understanding of the factors mitigating the reinforcing aspects of nicotine and the mechanism of nicotine withdrawal, which will be applied to develop more efficacious treatments for tobacco and nicotine cessation.

CHAPTER 1: The novel CYP2A6 inhibitor, DLCI-1 decreases nicotine self-administration in mice

Rationale:

Tobacco addiction remains a leading preventable cause of disease and death worldwide, despite a high percentage of individuals expressing a desire to quit (CDC, 2015). Nicotine, the main psychoactive component in tobacco, exerts its reinforcing effects by acting on neuronal nicotinic acetylcholine receptors (nAChRs) in the brain (Albuquerque et al., 1995; Lena and Changeux, 1998; Tuesta et al., 2011). Although tobacco cigarette use has declined recently, this has unfortunately been contrasted with a dramatic increase in the use of e-cigarettes, which deliver vaporized nicotine (GBD, 2017). While e-cigarettes have been promoted as tobacco cessation aids, product consumption by never-smokers has raised significant concerns for addiction liability, especially among youth (Etter and Bullen, 2014; Gornall, 2015; Primack et al., 2015).

In humans, the majority of nicotine is metabolized to nicotine-$\Delta^{1'-5'}$-iminium ion by the hepatic enzyme, cytochrome P450 2A6 (CYP2A6), followed by the conversion to cotinine by either CYP2A6 itself, or through the subsequent action of aldehyde oxidase or other enzymes (Ray et al., 2009; Raunio and Rahnasto-Rilla, 2012; von Weymarn et al., 2012). Allelic variation in the gene encoding for CYP2A6 in humans has been associated with altered nicotine metabolism, resulting in a differential vulnerability to nicotine dependence, related to the rate of metabolism (Ray et al., 2009; Thorgeirsson et al., 2010). The *CYP2A6* gene is highly polymorphic with allelic variants resulting in

either lower or increased metabolic function (Mwenifumbo et al., 2008; Thorgeirsson et al., 2010; Raunio and Rahnasto-Rilla, 2012). Importantly, individuals with a slow metabolizer CYP2A6 genotype are less vulnerable to develop tobacco dependence than normal metabolizers (Audrain-McGovern et al., 2007; Thorgeirsson et al., 2010; Bloom et al., 2011). It is predicted that a slower metabolism would result in more prolonged actions of nicotine following drug consumption. Therefore, with lower levels of nicotine intake, the amount of drug remaining in the system would accumulate over time, and thus, less drug would be required to achieve sustained activation of nAChRs in mesolimbic brain pathways mediating reinforcement (Tuesta et al., 2011; Fowler and Kenny, 2014). However, under conditions of increasing nicotine intake, activation of the pathways regulating the aversive properties of the drug, such as the medial habenulo-interpeduncular pathway, would be more readily engaged, resulting in a downward behavioral titration of drug consumption (Fowler et al., 2011; Fowler and Kenny, 2014). Supporting this contention, slow metabolizers have been found to consume less nicotine per day, experience less withdrawal symptoms, and are more successful in quitting smoking (Strasser et al., 2007; Patterson et al., 2008; Rodriguez et al., 2011; Liakoni et al., 2019). Conversely, individuals with a higher rate of metabolism exhibit greater levels of nicotine intake, withdrawal symptoms and drug craving (Strasser et al., 2011; Chen et al., 2018a; Liakoni et al., 2019), which may thereby increase the likelihood of relapse during cessation. In a study with the cessation therapeutic bupropion, it was found that individuals with a slow metabolism are similarly successful in achieving abstinence under placebo or bupropion conditions, whereas fast metabolizers show greater benefit from bupropion treatment (Patterson et al., 2008).

Given these findings in humans and the consideration that individuals with allelic variation appear to function normally despite having a reduced CYP2A6 metabolism, drug development efforts to limit nicotine metabolism represents an exciting opportunity for therapeutic modulation. Indeed, initial drug validation efforts along these lines have proven to be effective in smokers. Methoxsalen was shown to inhibit CYP2A6, and in smokers, methoxsalen decreased nicotine metabolism and reduced cigarette consumption (Sellers et al., 2000). Conceptually, this approach could lead to a significant reduction in tobacco product use, thereby limiting exposure to harmful constituents mitigating adverse health effects, and/or provide the means for an individual to gradually reduce intake over time to eventually achieve full smoking cessation, potentially in combination with other therapeutics.

Recently, we developed a potent and selective CYP2A6 inhibitor, DLCI-1, which was shown to exhibit favorable metabolic stability and half-life in human liver microsomes and adheres to Lipinski's rule of five for drug likeness (Denton et al., 2018). Here, we examined whether DLCI-1 would be effective in mediating nicotine consumption in both males and females in a mouse model. The intravenous nicotine self-administration procedure was used as it allows for the most direct and reliable measure of nicotine reinforcement in mice (Fowler and Kenny, 2011). We also assessed the effects of the approved smoking cessation therapeutic, bupropion, on nicotine self-administration, as a means of comparing DLCI-1's relative effectiveness in reducing nicotine intake. As a secondary behavioral measure, mice were examined for nicotine-mediated changes in locomotion. Finally, to ensure that DLCI-1 was selective for decreasing nicotine intake,

as opposed to a general attenuation of lever pressing behavior, the effect of DLCI-1 was assessed during food self-administration.

Material and Methods:

Animals

Male and female wildtype C57BL/6J mice were derived from breeders in our laboratory animal facilities or were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were maintained in an environmentally controlled vivarium on a 12 h reversed light/dark cycle. Food and water were provided *ad libitum* until behavioral training commenced. During food training, subjects were mildly food restricted to 85–90% of their free-feeding bodyweight, and water was provided *ad libitum*. All experiments were conducted in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of California, Irvine.

Drugs

(-)-Nicotine hydrogen tartrate salt (MP Biomedicals, Santa Ana, CA, USA; 0215355491) was dissolved in 0.9% sterile saline and adjusted to pH 7.4. The CYP2A6 inhibitor, (5-(4-ethylpyridin-3-yl)thiophe-2-yl)methanamine (DLCI-1), was prepared as the hydrochloride salt, as previously described (Denton et al., 2018), and then dissolved in 0.9% sterile saline, adjusted to pH 7.4, and administered at a dose of 25 or 50 mg/kg. Doses were derived by combining the generally accepted dose of 5 mL/kg and the IC_{50} value of the compound against CYP2A6 (Denton et al., 2018). Both DLCI-1 and vehicle

were administered p.o. via oral gavage at a volume of 0.1 mL. Bupropion (Sigma Aldrich, St. Louis, MO, USA; B102) was dissolved in 0.9% sterile saline and administered at a dose of 1 or 75 mg/kg, which was injected intraperitoneally at a volume of 10 ml/kg. For liquid chromatography - mass spectrometry analysis of blood samples, nicotine-methyl-D3 and cotinine-methyl-D3, trans-3′-hydroxycotinine-methyl-D3, and standards (nicotine, cotinine and trans-3′- hydroxycotinine) were obtained from Toronto Research Chemicals (North York, Canada).

Intravenous nicotine self-administration

Subjects were food trained and then surgically catheterized as previously described (Fowler et al., 2011; Chen et al., 2018b). Briefly, mice were anesthetized with an isoflurane (1-3%)/oxygen vapor mixture and prepared with intravenous catheters. Catheters consisted of a 6 cm length of silastic tubing fitted to a guide cannula (Plastics One, Roanoke, VA) bent at a curved right angle and encased in dental acrylic. The catheter tubing was passed subcutaneously from the animal's back to the right jugular vein, and a 1 cm length of the catheter tip was inserted into the vein and tied with surgical silk suture. Following the surgical procedure, animals were allowed ≥48 h to recover from surgery, then provided access to respond for food reward until criteria were achieved. Mice were then permitted to acquire intravenous nicotine self-administration during 1 h daily sessions, 6-7 days per week, at the standard training dose of nicotine (0.03 mg/kg/infusion) for eight days and were then transitioned onto the moderate 0.1 mg/kg/infusion dose of nicotine. This dose was chosen since mice exhibit the highest level of responding for this dose across the dose response-curve, and it also

results in a similar level of intake as that found at higher doses with behavioral titration via self-administration (Fowler and Kenny, 2011). Nicotine was delivered through tubing into the intravenous catheter by a Razel syringe pump (Med Associates). Each session was performed using 2 retractable levers (1 active, 1 inactive). Completion of the response criteria on the active lever resulted in the delivery of an intravenous nicotine infusion (0.03 mL infusion volume; FR5TO20 sec schedule). Responses on the inactive lever were recorded but had no scheduled consequences.

After acquisition and achieving stable responding on the 0.1 mg/kg/infusion dose, subjects were examined for the effects of DLCI-1 or bupropion on nicotine intake. For Cohort 1 (n=5 males), mice were orally administered DLCI-1, or vehicle control, 30 minutes prior to each session utilizing a within subject Latin-square design. Following these sessions, mice were placed into the open field for locomotor assessments (see below). In between each DLCI-1 or vehicle administration session, subjects were provided baseline days to ensure stable, reinstated responding prior to the next dose. For Cohort 2 (n=7 males, n=6 females), mice were treated as above, but blood was drawn immediately following each session to examine the levels of nicotine metabolites. After re-establishing baseline responding, mice were then injected with bupropion, or vehicle control, 20 minutes prior to each session utilizing a within subject Latin-square design. In between each bupropion or vehicle administration session, subjects were provided baseline days to ensure stable, reinstated responding prior to the next dose. Catheters were flushed daily with physiological sterile saline solution (0.9% w/v) containing heparin (100 units/mL). Catheter integrity was verified with the ultra short-acting barbiturate anesthetic Brevital (2%, methohexital sodium, Eli Lilly, Indianapolis,

IN) at the end of the study. Behavioral responses were automatically recorded by MedAssociates software.

In summary, the experimental timeline was as follows: Food self-administration (5+ days) → intravenous surgery and recovery (3+ days) → nicotine self-administration: acquisition at the 0.03 mg/kg/infusion dose (8 days) → self-administration of the moderate 0.1 mg/kg/infusion nicotine dose (6 days) → 1st DLCI-1 dose session (vehicle, 25 or 50 mg/kg according to Latin-square design, prior to the self-administration session), immediately followed by either a locomotor test (cohort 1) or blood draw (cohort 2) → baseline self-administration (2+ days) → 2nd DLCI-1 dose session, immediately followed by either a locomotor test (cohort 1) or blood draw (cohort 2) → baseline self-administration (2+ days) → 3rd DLCI-1 dose session, immediately followed by either a locomotor test (cohort 1) or blood draw (cohort 2) → Cohort 1 end. Cohort 2 mice then proceeded to: baseline self-administration (2+days) → 1st bupropion dose session (vehicle, 1 or 75 mg/kg according to Latin-square design, prior to the self-administration session) → baseline self- administration (2+days) → 2nd bupropion dose session → baseline self-administration (2+ days) → 3rd bupropion dose session. One female developed a leaky catheter during the bupropion dosing, so she was removed and two additional females were assessed only with bupropion to achieve sufficient subject numbers.

Ultra-pressure liquid chromatography (UPLC)-mass spectrometry (MS)

Immediately following the self-administration session, blood samples were collected from the mice via the facial vein, placed into a microcentrifuge tube with 20 µl of EDTA

(500mM, pH 8.0) on ice, and centrifuged at 3,000 x g for 20 minutes at 4°C. Plasm serum was collected and stored at -80°C. All blood samples were analyzed at the same time with UPLC-MS. A 6 µl aliquot of each plasma sample was spiked with 6 µl of a mixture of deuterium-labeled internal standards, which included nicotine-methyl-D_3, cotinine-methyl-D_3 and trans-3′- hydroxycotinine-methyl-D_3, each at a concentration of 1 ppm. Fifty µl of 100 % LC-MS grade methanol was added to precipitate out proteins. After vortexing and subsequent centrifugation at 16,000 g for 15 min at 4°C, 35 µl of supernatant was transferred to a sample vial for analysis by ultra-pressure liquid chromatography (UPLC)-mass spectrometry (MS) analysis. *UPLC-MS/MS Conditions:* Plasma samples prepared as described above were analyzed using an ABSciex 6500 LC-MS/MS system (Sciex, Framingham, MA), consisting of an LC-30AD UPLC pump, a SIL30 AC auto sampler, an ACQUITY UPLC BEH HILIC (2.1 mm × 100 mm, 1.7 µm particle size; Waters Corp.) column at 40°C, and a tandem mass spectrometer (QTrap 6500). Ultra-pressure liquid chromatography (UPLC) was performed at a flow rate of 0.4 ml/min using the following conditions: 1.5 min in 15% solvent A, a linear gradient for 1 min to 50% solvent A, 3 min in 50% solvent A, followed by a return to 15% solvent A for 2.5 min; where solvent A is 5 mM NH_4AC (pH 6.7) and 50% acetonitrile (v/v), and solvent B is 5 mM NH_4AC (pH 6.7) and 90% acetonitrile (v/v). The injection volume of each plasma sample was 2 µL. The QTrap 6500 tandem mass spectrometer was equipped with an electrospray ionization probe operated in the positive-ion mode. The mass spectrometer was operated in the multiple reaction monitoring mode and the concentrations of all analytes were determined simultaneously under the following conditions: cotinine *m/z* 177.1>98, cotinine-methyl-D_3 *m/z* 180.1>101, trans-3′-

hydroxycotinine (3HC) *m/z* 193.1>80, and 3HC-methyl-D_3 *m/z* 196.1>80. A collision energy of 27 V was used for cotinine and cotinine-methyl-D_3, and 32 V for 3HC and 3HC-methyl-D_3. A dwell time of 100 msec was used for all analytes, as well as an ion-spray voltage of 5500 V, an ion source gas flow of 50, DP of 30 V, EP of 10 V, CXP of 10 V and source temperature of 450°C. Standard curves were constructed by plotting the ratio of analyte peak area to peak area of the corresponding internal standard (described above) versus analyte concentration for eight analyte concentrations. Standards at concentrations ranging from 0.3 pg/μL to 325.5 pg/μL were used to establish standard curves. Plasma analyte concentrations were determined by measuring the peak area ratios of analyte to internal standard and then calculating analyte concentration from the appropriate standard curve using MultiQuant 2.1.1 software. The quantification limits (signal/noise>10) for each compound were 0.3 pg/μL for cotinine and 0.6 pg/μL for 3HC.

Open field locomotor test

The open field chamber was composed of Plexiglas (35 cm L × 35 cm W × 31 cm H) as described previously (Chen et al., 2018b). Following testing in the intravenous nicotine self- administration session, mice (Cohort 1, n=5) were immediately transferred to the open field chamber. After a 5-minute habituation period, subjects were scored in the open field apparatus for a 15-minute session to assess locomotor activity. Activity was recorded with a video camera and distance travelled, time mobile and center time were scored by two experimenters blinded to the group condition with ANY-Maze Software (Stoelting Co., Wood Dale, IL, USA).

Operant food training

Subjects (n=11 males) were mildly food restricted to 85-90% of free feeding weight and trained to press a lever in an operant chamber (Med Associates, Fairfax, VT, USA) for food pellets (20 mg; TestDiet) up to a fixed-ratio 5, time out 20 s (FR5TO20s) schedule of reinforcement, as previously described (Fowler and Kenny, 2011). Each session was performed using 2 retractable levers (1 active, 1 inactive). Completion of the response criteria on the active lever resulted in the delivery of a food pellet. Responses on the inactive lever were recorded but had no scheduled consequences. Once stable responding was achieved (criteria >30 pellets per session across 3 consecutive sessions), subjects were administered DLCI-1, or vehicle control, 30 minutes prior to the session utilizing a within subject, cross-over design. Subjects were provided at least one baseline day in between test sessions to ensure stable responding prior to the next injection in the series. Behavioral responses were automatically recorded by MedAssociates software.

Statistical analyses

Data were analyzed by a t-test, one-way or two-way ANOVA, as appropriate, with Prism software (GraphPad, La Jolla, CA, USA). Significant main or interaction effects were followed by Bonferroni post-hoc comparison with correction for multiple comparisons. Statistical reporting for ANOVAs are presented as a F-statistic value: $F_{(\text{degrees of freedom for group/treatment number, degrees of freedom for subject number})}$ = test value. The criterion for significance was set at $\alpha=0.05$.

Results

Decreased nicotine self-administration with administration of DLCI-1

Mice were examined for the effects of DLCI-1 (**Fig 1A**) or vehicle on intravenous nicotine self-administration. Since nicotine is mainly metabolized by CYP2A6 in the liver, DLCI-1 or vehicle was administered via oral gavage to allow for first pass absorption and thus more directed targeting to this organ. Administration of the 25 and 50 mg/kg doses of DLCI-1 effectively reduced the number of nicotine infusions earned compared to vehicle control in both males (**Fig 1-1B**) (*repeated measures one-way ANOVA*, $F_{(2,18)} = 25.31$, $p < 0.0001$) and females (**Fig 1-1D**) (*repeated measures one-way ANOVA,* $F_{(2,10)} = 9.88$, $p = 0.0043$). In the post-hoc tests, both the 25 mg/kg and 50 mg/kg doses resulted in significantly lower rates of responding than vehicle control for males ($p < 0.0001$) and females ($p < 0.05$ vehicle vs. 25 mg/kg, $p < 0.01$ vehicle vs. 50 mg/kg). To assess the level of lever pressing behavior, the number of active and inactive lever presses were also examined, and statistically significant differences were established for the active lever across conditions in both males (**Fig 1-1C**) (*repeated measures two-way ANOVA, dose* $F_{(2,18)}= 18.36$, $p < 0.0001$; *lever* $F_{(1,9)} = 39.76$, $p = 0.0001$; *interaction* $F_{(2,18)} = 22.33$, $p < 0.0001$) and females (**Fig 1-1E**) (*repeated measures two- way ANOVA, dose* $F_{(2,10)}= 6.20$, $p = 0.0177$; *lever* $F_{(1,5)} = 13.90$, $p = 0.0136$; *interaction* $F_{(2,10)} = 13.83$, $p = 0.0013$). Specifically, the vehicle injection resulted in a significantly greater number of active lever responses than both the 25 mg/kg and 50 mg/kg doses for both males (*post hoc*, Active 0 vs 25 $p<0.0001$, Active 0 vs 50 $p<0.0001$) and females (*post hoc*, Active 0 vs 25 $p<0.001$, Active 0 vs 50 $p<0.001$).

Significant differences were not found in the rate of responding on the inactive levers. Together, these findings demonstrate that DLCI-1 effectively reduced nicotine intake, without affecting general non-specific inactive lever pressing behavior.

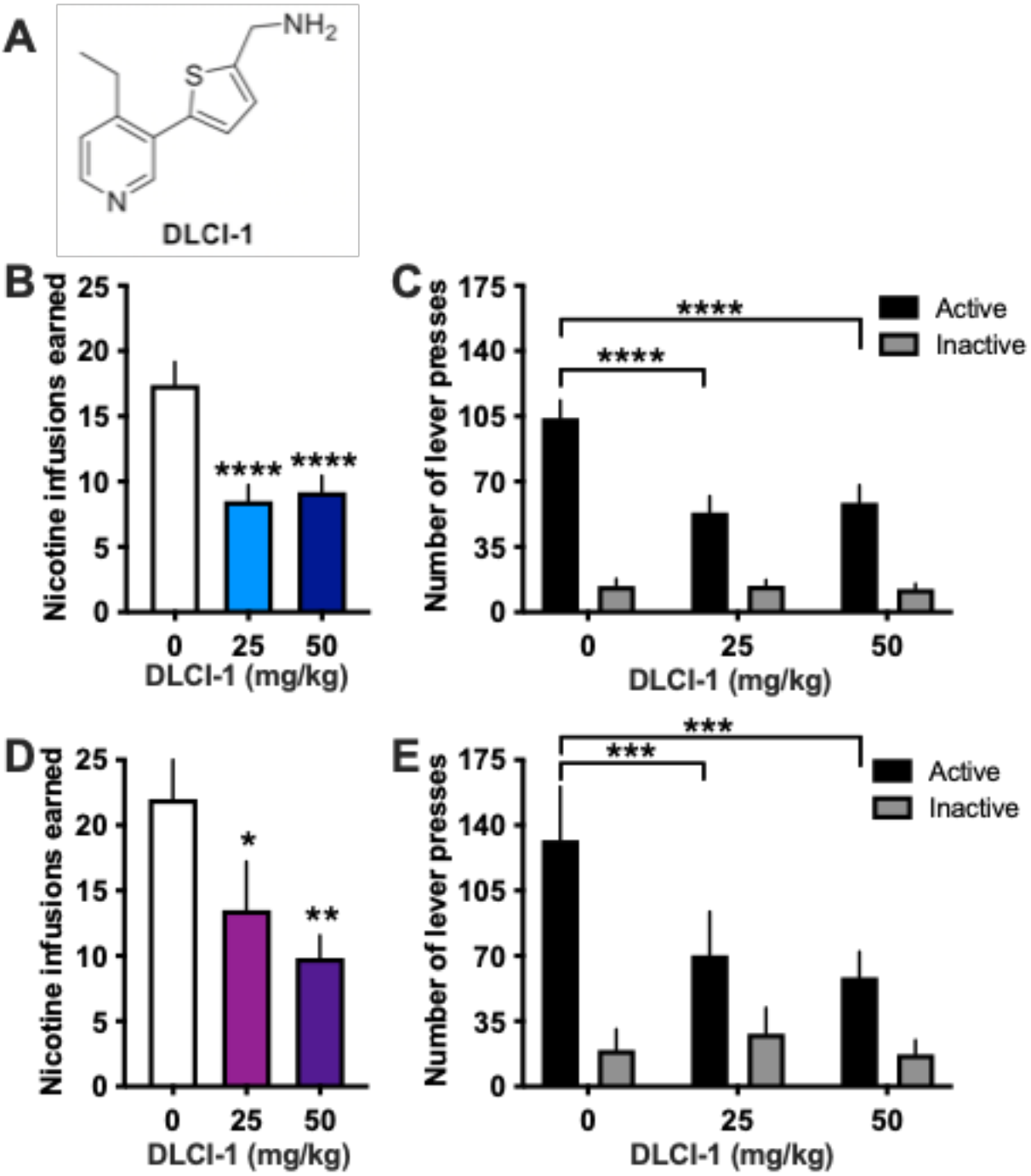

Figure 1-1. The CYP2A6 inhibitor DLCI-1 decreases nicotine self-administration in both male and female mice. Mice were examined for their responding to obtain intravenous nicotine infusions following administration of DLCI-1 or vehicle control. **(A)** Chemical structure of DLCI-1. **(B)** In males (n=10), both doses of DLCI-1 (25 and 50 mg/kg, po) significantly decreased the number of nicotine infusions earned compared to vehicle control. **(C)** When the number of lever presses were examined for the nicotine sessions, male mice exhibited a significant decrease in the number of active lever presses with DLCI-1 pretreatment. In contrast, no significant differences were found in the number of inactive lever presses. **(D)** In females (n=6), DLCI-1 decreased the number of nicotine infusions earned at both doses (25 and 50 mg/kg, po) compared to vehicle control. **(E)** Female mice also exhibited a significant decrease in the number of active lever presses with DLCI-1 pretreatment, but no in the number of inactive lever presses. *p<0.05, **p<0.01, ***p<0.001, as compared to 0 mg/kg vehicle control. Data are presented as mean ± SEM.

Blood samples were collected after nicotine self-administration with DLCI-1 treatment to examine the levels of nicotine's main metabolite, cotinine, which is then metabolized into 3HC. If mice titrate their nicotine levels to maintain a similar level of activation of neural nAChRs across treatments, one would predict that the level of the metabolites would be relatively lower with inhibitor treatment across groups, despite differences in nicotine intake. This supposition considers the fact that each intravenous self-administration session varies in the pattern of nicotine infusions across time and amount of nicotine administered, dependent on the animal's volitional behavior, which may also introduce the possibility of allowing for accumulation of nicotine with DLCI-1 treatment and thus similar levels of metabolites at the session end. Considerations must also be incorporated for differences in the rate of elimination of metabolites throughout the hour session duration via excretion from the body, with earlier infusions during the session likely resulting in greater excretion of metabolites prior to blood collection. Given this, we also analyzed the pattern of nicotine intake across treatment conditions to evaluate the time course of nicotine exposure for each treatment. In males (**Fig 1-2A**), cotinine

levels did not differ between the vehicle and 25 mg/kg treatment (*paired t-test*, $t_{(6)}$ = 1.506, p = 0.0913) or 50 mg/kg treatment (*paired t-test*, $t_{(5)}$ = 0.566, p = 0.2979). In contrast, 3HC levels were higher with vehicle compared to the 25 mg/kg treatment (*paired t-test*, $t_{(6)}$ = 2.816, p = 0.0153), but no differences were found between the vehicle and 50 mg/kg treatment (*paired t-test*, $t_{(5)}$ = 1.608, p = 0.0844). When examining nicotine intake across the 15 min intervals of the session, the vehicle condition maintained a consistently higher level of nicotine intake across the session, which was approximately twice that of the DLCI-1 treatments (**Fig 1-2B**). In females (**Fig 1-2C**), cotinine levels were statistically lower with both the 25 mg/kg treatment (*paired t-test*, $t_{(5)}$ = 2.465, p = 0.0284) and 50 mg/kg treatment (*paired t-test*, $t_{(5)}$ = 3.101, p = 0.0134), as compared to the vehicle. For 3HC, differences were not found between the vehicle and 25 mg/kg treatment (*paired t-test*, $t_{(5)}$ = 1.768, p = 0.0687), but the 50 mg/kg treatment led to lower 3HC levels than the vehicle condition (*paired t-test*, $t_{(5)}$ = 4.670, p = 0.0027). When examining nicotine intake within the hour session in females, all conditions displayed a slight downward trajectory in nicotine consumption (**Fig 1-2D**). Taken together, these findings are indicative of a dynamic process of intake, metabolism and elimination for nicotine and its metabolites throughout the self-administration session.

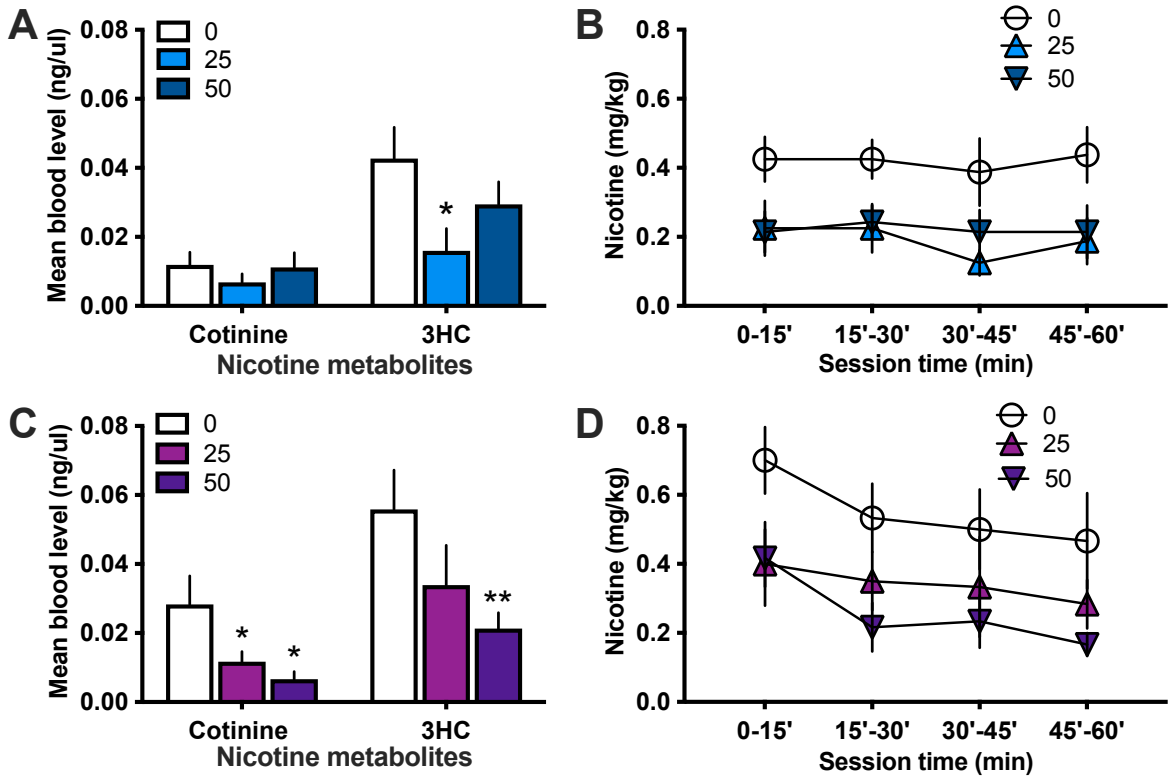

Figure 1-2. Nicotine metabolites in the blood following DLCI-1 treatment and nicotine self-administration. Blood samples were analyzed following the one-hour nicotine self- administration session to examine for differences in metabolite levels. **(A)** In males (n=7), despite a higher level of nicotine intake in the vehicle condition, the levels of cotinine did not differ among treatments immediately after the session. However, the 25 mg/kg DLCI-1 treatment did lead to decreased 3HC levels compared to the vehicle treatment. **(B)** Analysis of the pattern of nicotine self-administered (mg/kg) throughout the session duration reveals that the males maintained a consistent level of responding over each 15 min interval, with consistently lower intake found with DLCI-1 treatment. **(C)** In females (n=6), the 25 and 50 mg/kg DLCI-1 treatment resulted in a lower level of cotinine, and 50 mg/kg DLCI-1 also led to a significant decrease in 3HC, as compared to the vehicle treatment. **(D)** Analysis of the pattern of nicotine self-administered (mg/kg) throughout the session duration in females reveals that the vehicle and DLCI-1 treatments led to consistent levels of responding at higher and lower levels, respectively, and there was an apparent downward shift in intake over the 15 min intervals. *p<0.05, **p<0.01 compared to the 0 mg/kg vehicle control.

Bupropion-induced decrease in nicotine self-administration

To further support the potential of DLCI-1 as a tobacco cessation therapeutic, we assessed the effects of bupropion on nicotine intake for comparison purposes. Interestingly, the effects of bupropion on nicotine self-administration in mice has not been previously investigated. Thus, we examined the impact of a moderate (1 mg/kg) and high (75 mg/kg) bupropion dose; in the rodent literature, the doses of bupropion range from 0.1 to 78 mg/kg (Rauhut et al., 2003; Rauhut et al., 2008; Damaj et al., 2010; Grabus et al., 2012; Hall et al., 2015). The 1mg/kg dose was selected based on prior findings that this dose abolishes nicotine reward in the conditioned place preference paradigm and reverses nicotine- induced tolerance in mice (Damaj et al., 2010; Grabus et al., 2012). The high 75 mg/kg dose was selected since this dose range has been shown to attenuate nicotine self-administration in rats in several prior studies (Rauhut et al., 2003; Rauhut et al., 2008; Hall et al., 2015). In males, we found a significant difference between treatments (**Fig 1-3A**) (*repeated measures one-way ANOVA,* $F_{(2,8)} = 8.249$, $p = 0.0114$). Post-hoc analyses revealed that the high dose (75 mg/kg), but not the moderate (1 mg/kg) dose, induced a significant decrease in nicotine intake compared to the vehicle ($p < 0.01$). However, it is important to note that during testing, the mice exhibited an adverse behavioral state following the high dose bupropion injection, with behaviors representative of withdrawal - shaking, hunched posture, gnawing, lethargy, and piloerection. As a quantitative measure, we examined the latency for the mice to receive their first nicotine infusion during the session, which provides evidence of the ability of the mice to ambulate around the testing chamber and press the lever. It is expected that subjects should attenuate their responding for nicotine with a therapeutic treatment *after* experiencing the interactive effects of the

therapeutic with the first nicotine infusion; thus, subjects would not be expected to differ in the initial latency to first reward. However, we found a significant increase in the latency with the 75 mg/kg high dose bupropion treatment, but not for any other treatment condition with either DLCI-1 or bupropion, thereby providing evidence of the negative behavioral state of the male mice following the high bupropion dose (**Fig 1-3B**) (*One-way ANOVA,* $F_{(5,42)} = 21.05$, $p < 0.0001$; post-hoc $p < 0.0001$ for all conditions compared to 75 mg/kg bupropion). In females, bupropion at both doses resulted in a statistically significant decrease in nicotine intake relative to the vehicle (**Fig 1-3C**) (*repeated measures one-way ANOVA,* $F_{(2,12)} = 20.02$, $p = 0.0002$; post-hoc: vehicle vs. 1 mg/kg, $p < 0.05$; vehicle vs. 75 mg/kg: $p < 0.0001$). At the high 75 mg/kg bupropion dose, similar adverse behavioral effects were noted in the females, which was further confirmed with analysis of the latency to first nicotine infusion among DLCI-1 and bupropion treatments (**Fig 1-3D**) (*One-way ANOVA,* $F_{(5,33)} = 5.863$, $p = 0.0006$; post-hoc $p < 0.05$ for all conditions compared to 75 mg/kg bupropion).

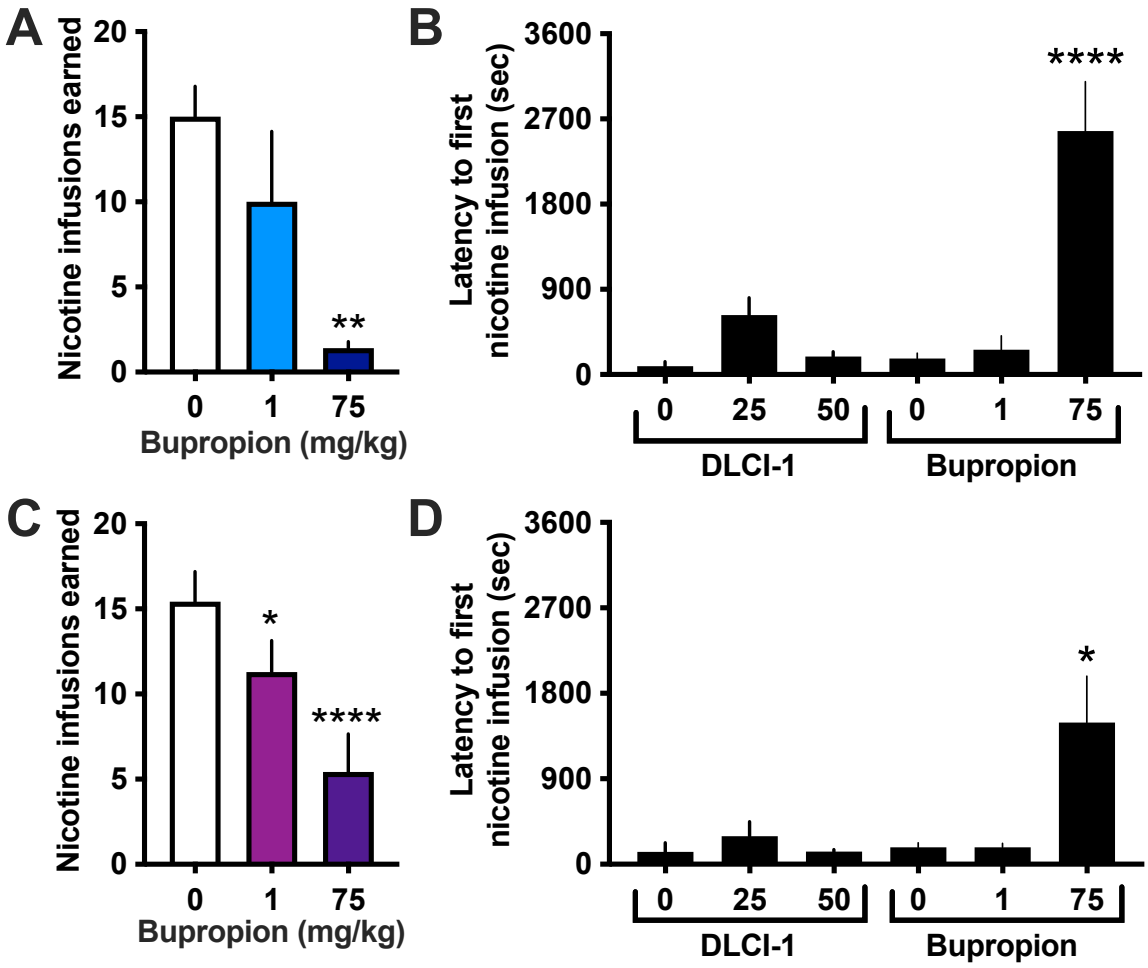

Figure 1-3. Effects of bupropion treatment on nicotine self-administration. Mice were examined to assess whether bupropion would exert a similar reduction in nicotine self- administration as that found with DLCI-1. **(A)** In males (n=5), a significant decrease in nicotine infusions earned was found following the high (75 mg/kg), but not moderate (1 mg/kg), bupropion treatment as compared to vehicle. **p<0.01 vs. 0 mg/kg vehicle. **(B)** Males treated with the high dose of bupropion (75 mg/kg) exhibited significantly delayed latency to respond on the active lever for the first infusion of the session compared to all other treatment conditions, indicating behavioral suppression. ****p<0.0001 vs. all other treatment conditions. **(C)** In females (n=7), both the moderate (1 mg/kg) and high (75 mg/kg) dose of bupropion significantly attenuated the number of nicotine infusions earned. ****p<0.0001 vs. 0 mg/kg vehicle. **(D)** Females administered the high dose of bupropion (75 mg/kg) had a significantly delayed latency to respond on the active lever for the first infusion of the session compared to all other treatments, indicating behavioral suppression. *p<0.05 vs. all other treatment conditions.

No differences in food responding with administration of DLCI-1

To further verify that the differences found with nicotine self-administration were selective to nicotine consumption, we examined the effects of DLCI-1 on operant responding for food pellets under the same fixed ratio 5, time out 20 sec schedule of reinforcement. Separate cohorts of male mice were examined for the 25 mg/kg and 50 mg/kg doses. At the 25 mg/kg dose, subjects did not differ in the amount of food pellets earned compared to vehicle control (**Fig 1-4A**) (*paired t-test*, $t_{(4)} = 0.794$, $p = 0.4716$), nor did they differ in the number of active or inactive lever presses between injection conditions (**Fig 1-4B**) (*repeated measures two-way ANOVA, dose* $F_{(1,4)} = 0.549$, $p = 0.4996$; *lever* $F_{(1,4)} = 53.06$, $p = 0.0019$; *interaction* $F_{(1,4)} = 0.662$, $p = 0.4616$). Similarly, subjects did not differ at the 50 mg/kg dose in either food responding (**Fig 1-4C**) (*paired t-test*, $t_{(5)} = 1.416$, $p = 0.2159$) or in the number of active or inactive lever presses (**Fig 1-4D**) (*repeated measures two-way ANOVA, dose* $F_{(1,5)} = 2.525$, $p = 0.1729$; *lever* $F_{(1,5)} = 339.6$, $p < 0.0001$; *interaction* $F_{(1,5)} = 2.093$, $p = 0.2076$). These data provide evidence that DLCI-1 does not affect lever pressing behavior for food reward or general non-specific inactive lever pressing behavior.

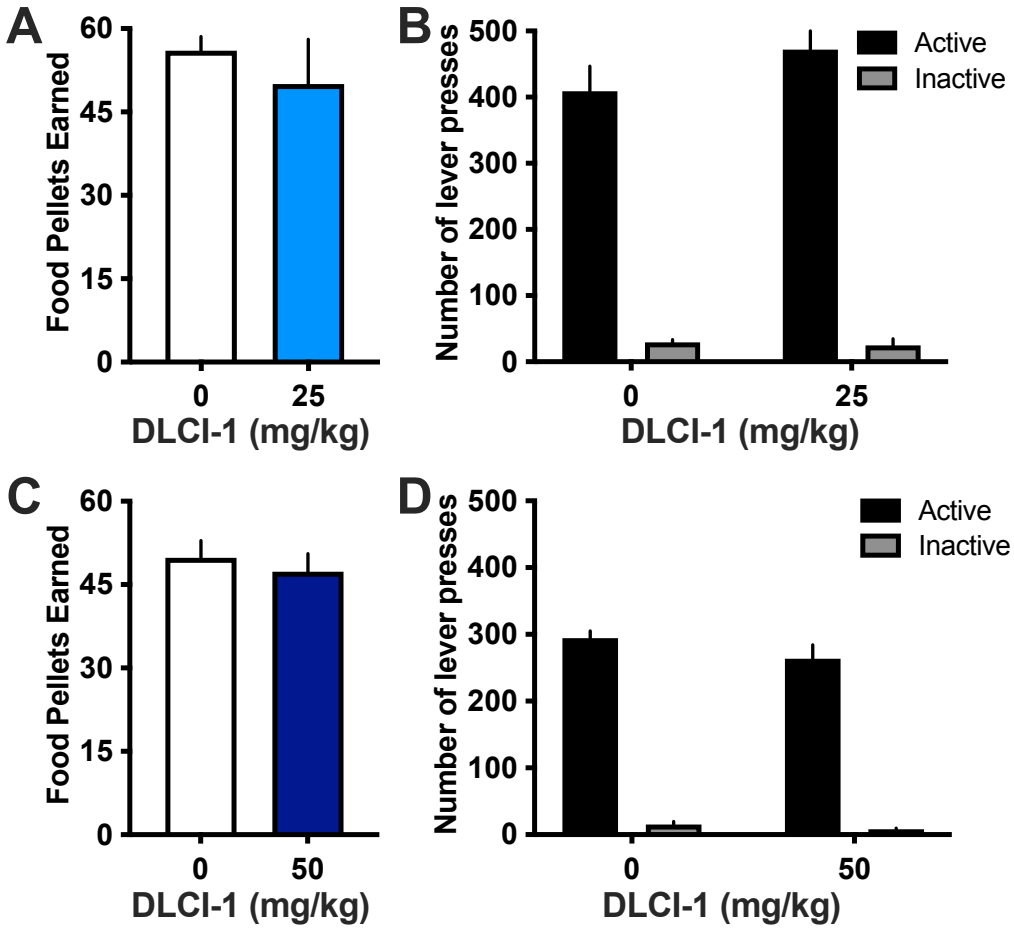

Figure 1-4. No differences in food self-administration following administration of the CYP2A6 inhibitor DLCI-1. Mice were trained to self-administer food pellets and examined for the effects of DLCI-1 pretreatment on behavior (n=5-6/group). **(A-B)** When pretreated with the lower dose of DLCI-1 (25 mg/kg) or vehicle control, mice did not differ in the number of food pellets earned **(A)** or in the number of active and inactive lever presses **(B)**. **(C-D)** Pretreatment with the higher dose of DLCI-1 (50 mg/kg) also did not induce any differences in food self-administration behavior for the number of pellets earned **(C),** or in the number of active and inactive lever presses **(D)**. Data are presented as mean ± SEM.

Effects of DLCI-1 induced lowering of nicotine intake on nicotine-mediated locomotion

Nicotine has been demonstrated to exert hyperlocomotive or hypolocomotive effects at low or high doses, respectively. Thus, following the nicotine session with the higher 50

mg/kg dose or vehicle control, mice were immediately transferred to open field chambers to examine for differences in locomotion. This assessment serves as a secondary measure of DLCI-1's nicotine-mediated effects with the decreased self-administered nicotine level. The mice consumed significantly less total nicotine (mg per kg) within each self-administration session **(Fig 1-5A)** (*paired t-test*, $t_{(4)} = 3.774$, $p = 0.0098$). However, they did not differ in the total distance travelled within the open field **(Fig 1-5B)** (*paired t-test*, $t_{(4)} = 0.969$, $p = 0.3874$) or in the total time mobile **(Fig 1-5C)** (*paired t-test*, $t_{(4)} = 0.433$, $p = 0.6871$). Further, since decreased time in the center of an open field has been proposed to be a correlate of increased anxiety and nicotine has been shown to mediate anxiolytic behavioral effects, the duration of center time was assessed to provide an initial examination of an anxiety-associated response. However, the mice did not differ in the amount of time spent in the center of the open field **(Fig 1-5D)** (*paired t-test*, $t_{(4)} = 0.185$, $p = 0.8622$). These findings indicate that DLCI- 1 decreases nicotine intake, and in doing so, results in similar nicotine-mediated behavioral actions with locomotion. Together, this provides evidence of inhibition of nicotine metabolism with similar *in vivo* actions of nicotine between conditions. Moreover, the food training and locomotor data also support the contention that DLCI-1 does not induce any adverse behavioral effects at the doses administered.

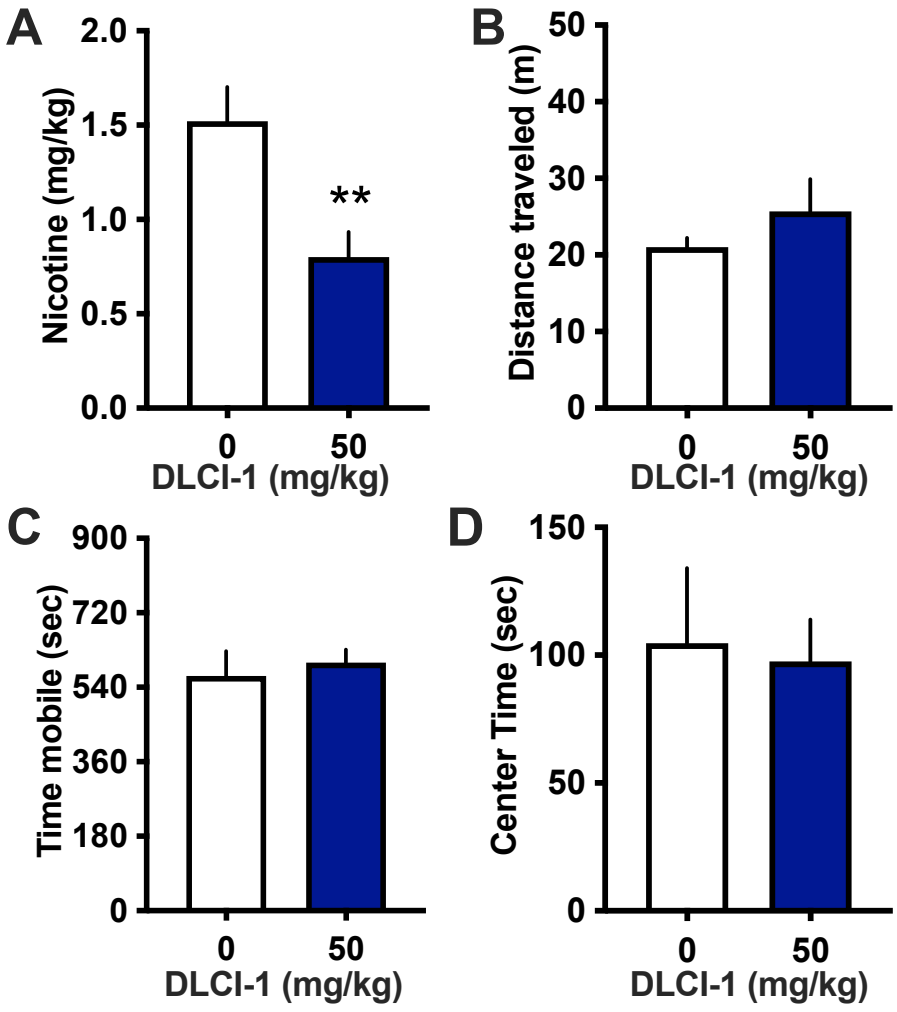

Figure 1-5. Similar locomotor behaviors with DLCI-1 pretreatment despite decreased nicotine exposure. Following the intravenous nicotine self-administration session with vehicle or 50 mg/kg DLCI-1 treatment, male mice (n=5) were immediately examined for their locomotor behaviors in an open field. **(A)** During the nicotine session, DLCI-1 treatment resulted in approximately half of the amount of nicotine intake as that found in the control condition. **p<0.01 **(B-D)** No significant differences were found in the total distance traveled **(B)**, time mobile **(C),** or in the time spent in the center of the open field **(D)**. Data are presented as mean ± SEM.

Discussion

In these studies, we found that oral administration of DLCI-1, a potent, selective and metabolically stable CYP2A6 inhibitor, results in decreased intravenous nicotine self-administration in both male and female mice. Throughout the self-administration session, the mice appeared to consistently titrate their intake across the 15 min session intervals. In males, this resulted in the detection of similar levels of nicotine's metabolites, cotinine and 3HC, in the blood, with the exception of less 3HC found following the 25 mg/kg dose of DLCI- 1. In contrast, females tended to adjust their responding downward throughout the session, and this resulted in significantly less cotinine and 3HC after the self-administration session with the DLCI-1 treatments compared to vehicle. Interestingly, DLCI-1 induced an approximate 50% decrease on nicotine intake in both males and females, which was much greater than one of the first-line therapeutics for tobacco cessation, bupropion, at a moderate dose. Further, the decrease in nicotine intake in the presence of DLCI-1 led to a similar locomotor effect as that found with higher levels of nicotine intake under vehicle conditions, supporting the contention that similar levels of nAChR activation in the brain occurred despite decreased levels of nicotine intake with DLCI-1 treatment. Of further note, these effects were specific to lever pressing for nicotine reward, as differences in inactive lever pressing behavior or food self-administration were not found. Taken together, these data support the conclusion that the novel CYP2A6 enzyme inhibitor, DLCI-1, has therapeutic potential to selectively reduce tobacco and nicotine consumption and thereby promote smoking cessation.

The foundation for these studies is based on findings in humans with CYP2A6 allelic variation and prior reports with methoxsalen, in which lower levels of CYP2A6 nicotine metabolism has been associated with decreased nicotine intake and dependence (Sellers et al., 2000; Strasser et al., 2007; Patterson et al., 2008; Rodriguez et al., 2011; Liakoni et al., 2019). Methoxsalen is classified as a mechanism-based inhibitor; the drug itself becomes metabolically activated by CYP2A6, leading to an active metabolite that irreversibly binds to CYP2A6 and inhibits its activity (Blobaum, 2006). In addition, several studies in mouse models have provided further support for methoxsalen's potential in reducing nicotine dependence associated behaviors (Alsharari et al., 2014; Bagdas et al., 2014). However, besides its actions on CYP2A6, methoxsalen also has carcinogen effects when combined with UV longwave radiation, and this has thereby limited its potential for further development as a smoking cessation therapeutic. In contrast, DLCI-1 was developed as a structural analogue of nicotine, as such, DLCI-1 ascended from a lead directed, medicinal chemical synthesis program with the highest CYP2A6 potency (0.017 μM) of any inhibitor tested, to date, with high selectivity ratios ($IC_{50(CYPXXX)}/IC_{50(CYP2A6)}$) toward all other human drug metabolizing CYPs ranging from >760 for CYP3A4 to >5 for CYP2B6, and good human liver microsomal half-life (40 min). Further, the compound falls within the boundaries of Lipinski's rule of five: calculated octanol-water partition coefficient (cLogP) = 2.108, MW = 218.32, topological polar surface area (tPSA) = 38.38, number of hydrogen bond donors = 1, number of hydrogen bond acceptors = 2 (Denton et al., 2018). Although the compound was designed to selectively inhibit CYP2A6, the possibility of DLCI-1 to enter the brain by

diffusing across the blood brain barrier may occur, which will need to be addressed in future studies.

It is of further note that the current studies were conducted in a mouse model system. Since rats metabolize nicotine primarily with CYP2B1, limitations are imposed when attempting to translate findings from rats to human hepatic function (Matta et al., 2007). In contrast, the mouse isoform CYP2A5 is homologous to human CYP2A6, allowing for a more relatable foundation for future clinical development. Further, it should also be noted that DLCI-1 was dosed orally based on two considerations. First, given that nicotine is mainly metabolized in the periphery by the liver, this allowed us to take advantage of the first pass effect found with gastrointestinal absorption, in which drug uptake occurs via the portal vein into hepatic circulation. Second, this route of administration provides translational relevance to future studies since oral administration is preferred for human dosing.

Interestingly, males and females demonstrated a similar effectiveness with DLCI-1's ability to reduce nicotine self-administration, as both sexes exhibited a decrease in the number of nicotine infusions and reduction of active lever presses with treatment. Thus, this effect was notably robust in consideration of the increased variability in responding typically found in female mice with intravenous nicotine self-administration. When assessing the amount of nicotine self-administered relative to nicotine's metabolites, differences with the DLCI-1 treatment were not consistent between sexes, which may be attributed to several factors. First, one must consider the fast metabolism of nicotine in mice relative to the session duration. Whereas humans exhibit a nicotine half-life ($t_{1/2}$)

of ~2 hours, the plasma nicotine metabolism of mice is estimated to be 6-7 min (Matta et al., 2007). In consideration of this time frame, the measurements in the current study reflect a dynamic process of varying intravenous nicotine infusions (dependent on the mouse's volitional behavior), metabolism and excretion throughout the one hour session, given that blood samples were collected at the session end. Second, the rate of nicotine metabolism has been shown to differ between males and females. Specifically, females have been found to metabolize both nicotine and cotinine faster than males (Matta et al., 2007; Benowitz et al., 2009). In the current study, females exhibited more pronounced changes in nicotine metabolite levels with the treatment-induced differences in nicotine self-administration, which is consistent with the prior findings. Third, chronic cigarette exposure has been shown to lead to decreased nicotine clearance in humans (Benowitz and Jacob, 1994; Benowitz et al., 2009), although it is unknown as to whether this can be attributed to nicotine alone (e.g., as opposed to other constituents in tobacco smoke) and whether mice develop similar changes with chronic exposure. Even so, given that the mice in the current study were chronically self- administering nicotine for at least 13 days prior to the DLCI-1 dosing schedule, our findings support the effectiveness of DLCI-1 as a potential therapeutic consistent with intervention after long-term nicotine exposure.

In an attempt to provide a correlate to the potential effectiveness of DLCI-I compared to other nicotine cessation therapeutics, we examined the effects of bupropion on intravenous nicotine self-administration in mice, which has not been previously investigated in the mouse model. While the 1 mg/kg bupropion dose has been shown to block nicotine's rewarding effects in the conditioned place preference paradigm (Damaj

et al., 2010), this dose was ineffective in altering nicotine self-administration in male mice, while the female mice exhibited a slight decrease in responding at this dose. Thus, comparing the ~50% decrease in nicotine intake found with DLCI-1 to this dose of bupropion, the evidence suggests that DLCI-1 would be more efficacious. However, since studies in rats have used a much higher bupropion dose, we also examined the effects of a 75 mg/kg dose. While both males and females exhibited a substantial decrease in the number of nicotine infusions earned, these effects were likely secondary to general behavioral inhibition and malaise, since the mice receiving the high dose exhibited adverse health-related effects (e.g., shaking, lethargy, gnawing, piloerection, hunched posture). This behavioral inhibition was further confirmed by examining the latency to respond for the first nicotine infusion, in which mice treated with 75 mg/kg bupropion exhibited a substantial delay in pressing the lever for the first infusion compared to all other doses for both DLCI-1 and bupropion. Moreover, the behavioral characteristics evidenced in the mice at this high dose are consistent with that found in humans with bupropion overdose (Stall et al., 2014). Interestingly, doses of bupropion in the intermediate range lead to increased, rather than decreased, nicotine self- administration in rats (Rauhut et al., 2003). Thus, taken together, these findings support the contention that DLCI-1 may serve as an effective therapeutic to promote tobacco and nicotine cessation.

In addition to the effectiveness on addiction-related processes, CYP2A6 inhibitors may also be relevant to tobacco- and nicotine-associated disease. For instance, polymorphism in the *CYP2A6* gene rendering an absence of CYP2A6 metabolic function leads to decreased tobacco use and lung cancer risk (Ariyoshi et al., 2002).

Conversely, an interaction between allelic variation in the *CHRNA5-CHRNA3-CHRNB4* gene cluster, which encode the a5, a3, and b4 nAChR subunits, respectively, and normal CYP2A6 metabolism was found to increase risk for lung cancer, an effect that was interestingly found with both high and lower (e.g., <20 cigarettes per day) nicotine exposure (Wassenaar et al., 2011). Therefore, the resulting effects of CYP2A6 inhibition may serve to mitigate lung cancer risk in patients based on enzymatic levels and genetic predisposition.

In conclusion, the novel CYP2A6 inhibitor, DLCI-1, is a promising CYP2A6 inhibitor, which was rationally designed to decrease nicotine intake. Our data provide the first evidence that DLCI-1 effectively attenuates intravenous nicotine self-administration in a mouse model. In addition to reducing nicotine consumption, pharmacological CYP2A6 inhibition may also lessen withdrawal symptoms to further promote abstinence and/or may ease the transition to achieve abstinence in combination with nicotine replacement approaches, although this needs to be further investigated. Taken together, these studies provide a foundation for further compound development to derive a highly efficacious therapeutic to promote tobacco, as well as e-cigarette, cessation.

CHAPTER 2: Nicotine e-cigarette vape inhalation and self-administration in rodent model

Experiment 1: Nicotine vapor self-administration in rats

Rationale:

Greater than 1.2 billion people use nicotine/tobacco products worldwide, resulting in more than 5 million deaths per year (Jha et al, 2006). The positive reinforcing properties associated with nicotine intake underlie the addictive nature of the drug (Henningfield et al., 1985). Humans consume nicotine through various routes, including tobacco cigarettes, electronic cigarettes (a.k.a., e-cigarettes or vapes), and chewing tobacco. The resulting bioavailability of nicotine is different based on the method of administration in consideration of the pharmacokinetics associated with absorption and distribution. Prior studies have found that nicotine is readily self-administered intravenously by animal models and humans (Johnson,1942; Goldberg et al., 1981; Henningfield et al.,1983; Rose et al., 2003; Fowler et al., 2011), supporting the reinforcing effects of this method of administration. Thus, intravenous nicotine self-administration has been traditionally accepted as having the highest translational validity to human drug use. However, with the recent emergence of e-cigarette products, it should be recognized that vaporized nicotine and constituents found in the vehicle solution may result in different neurochemical, physiological and behavioral alterations related to dependence, which could be different from those observed with other methods of administration (Ponzoni et al., 2015).

Cigarettes typically contain 10-14 mg of nicotine (Kozlowski et al., 1998). With tobacco smoking in humans, ~1-1.5 mg is absorbed systemically via the lungs for one cigarette, and with each inhalation, nicotine reaches the brain within 10-20 s, where the drug binds directly to nicotinic acetylcholine receptors (nAChRs) (Armitage et al., 1975; Benowitz and Jacob, 1984; Benowitz et al.,1988). The nAChRs are ionotropic receptors that permit influx of Na^+ and Ca^{2+}, leading to membrane depolarization, Ca^{2+}-dependent second messenger signaling, and/or presynaptic modulation of neurotransmitter release in many regions of the nervous system. Thus, variability in pharmacokinetics of vaporized nicotine would likely differentially affect the activation of these receptors, leading to important implications for processes mediating dependence. Nicotine is quickly metabolized by CYP450 enzymes in the liver, resulting in a relatively short half-life (~2 - 6 hrs in humans and ~45 min in rats) and subsequent formation of the main metabolite, cotinine, which exhibits a longer half-life (~16 hrs in humans and ~6 hrs in rats) (Matta et al., 2007; Li et al.,2015; Chen et al., 2020). Thus, nicotine exposure levels with self-administration methods can be evaluated by examining cotinine levels in blood plasma (Chen at al., 2020).

E-cigarettes vaporize a solution typically composed of nicotine, propylene glycol, vegetable glycerin, and flavoring agents (Grana et al., 2014). The spread of the e-cigarette market has been rapid, despite many unanswered questions about their safety, efficacy in reducing dependence, and overall impact on public health. At present, individuals across a wide range of ages utilize e-cigarettes, including individuals without a history of smoking tobacco (Pearson et al., 2012; Adkison et al., 2013; Grana et al.,

2014). Remarkably, e-cigarettes have become the most common drug product used by middle- and high-school students (Arrazola et al., 2014; Alsharari et al., 2015; Litt et al., 2016). Compared to tobacco cigarettes, it is thought that e-cigarette consumption results in similar effects of nicotine concentration in the brain and nAChR activity, as well as greater compulsive behavior during intake (Ponzoni et al., 2015). Since e-cigarettes can be consumed more readily in various settings as they may be inhaled inconspicuously and typically do not emit a strong odor, the patterns of use may increase over time for the user. Given this, understanding the differing effects of vaporized nicotine on the brain and relevance to drug addiction is critical ("Clearing the smokes" 2014; Correa et al., 2017; Glasser et al., 2017). Indeed, recent studies have begun to examine nicotine vapor administration in rodent models (George et al., 2010; Ponzoni et al.,2015; Smith et al., 2015; Lefever et al., 2017; Javadi-Paydar et al., 2019), but many questions still remain, including whether the level of intake is titrated similar to intravenous self-administration and whether sex-specific effects are present in the exposure and behavioral levels, factors which are addressed in the current studies.

Here, we first describe our developed model of a robust and reliable procedure for vaporized e-cigarette nicotine self-administration in rats. This protocol is an advance over other current methods, as we find selectivity in the behavioral responses for nicotine vapor on the active lever. To further validate this model, we then examined the effects of modifying the nicotine dose using passive vapor exposure to provide precise dosing across subjects. Next, we determined whether vaporized e-cigarette nicotine exposure results in a similar level of metabolized blood nicotine, as compared to volitional intake

during intravenous nicotine self-administration. Subjects were also examined for nicotine-mediated change in locomotion as a measure of nicotine's actions on the physiological measure. Finally, since women consume more tobacco products than men and studies suggest that women and female rodents are more sensitive to the rewarding effects of nicotine (Perkins et al., 1999; Perkins, 2001; Kota et al., 2008; O'Dell and Torres., 2014), male and female subjects were examined in a within-sex manner. Together, the findings derived from these investigations establish a new procedural model for nicotine vapor exposure, thereby providing an important foundation for future studies.

Material and Methods:

Animals

Adult male and female Wistar rats were purchased from Charles River. Subjects were maintained in an environmentally controlled vivarium on a 12h:12h reversed light:dark cycle. Food and water were provided *ad libitum* until behavioral training commenced. All testing was conducted in the dark phase of the light cycle, when rats are most active. During drug administration procedures, subjects were food restricted to 85-90% of their free-feeding body weight, and water was provided *ad libitum*. All procedures were conducted in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

Drugs

(-)-Nicotine hydrogen tartrate (Cat #0215355491, MP Biomedicals) was dissolved in 0.9% sterile saline (intravenous self-administration) or 50:50 propylene glycol (PG) and vegetable glycerin (VG) solution (e-cigarette vapor) (free base, pH 7.4). Concentrations for intravenous solution are expressed as mg/kg and for aerosol solution as mg/ml.

Experimental Design

Food and vapor nicotine self-administration

Based on prior intravenous nicotine self-administration protocols, initial food training was performed to operantly train the rats to press a lever to receive a reward under an effortful fixed ratio 5 schedule (FR5, in which 5 active lever presses elicits a reward) (Kenny and Markou, 2006; Fowler et al., 2011; Fowler and Kenny, 2011). In the first set, male (n=8) and female (n=8) rats were initially trained to press the active lever in a sealed vapor chamber (340mm x 237mm x 198mm, LJARI) with regulated airflow (1 L/min) for liquid food infusion (50 µl/sec/infusion). Since it is essential that a constant airflow is maintained inside the vapor boxes, the design of the equipment did not allow for a food pellet hopper dispenser, and thus, food reward was provided in a liquid form to ensure consistent control of pressure and airflow. Rewards were earned under the FR5TO20 sec schedule, in which 5 lever presses elicits food delivery and activation of a cue light above the active lever, followed by a 20 sec timeout period. Based on our preliminary studies, we found that the animals would highly respond for a solution of vegetable broth containing 5% sucrose. Once stable responding was achieved (>50 liquid rewards per session across 3 subsequent sessions), subjects were then transitioned to acquire e-cigarette vaporized nicotine during 1 h daily sessions for 7 days on each dose in ascending order (nicotine

concentration: 2.5 mg/ml, 5 mg/ml or 7.5 mg/ml in VG:PG), and the mean of the last 3 days on each dose was used for data analysis. Of note, we did try to employ the higher nicotine dose of 10 mg/ml; however, the solution was cloudy and appeared to precipitate out of solution over time in the 50:50 PG/VG vehicle (pH 7.4), and thus, we did not employ this higher dose in these studies. It is recognized that altering the vehicle and/or pH may permit higher solubility for nicotine. Blood was drawn from the lateral tail vein to assess cotinine levels after final dose sessions. Each session was performed using 2 levers (1 active, 1 inactive). Completion of the response criteria on the active lever resulted in the delivery of a vapor nicotine puff in accordance with the FR5TO20 sec schedule. Responses on the inactive lever were recorded but had no scheduled consequences. Behavioral responses were automatically recorded by Med Associates software. The Med Associates custom computer interface allows for the delivery of vapor generated by a commercial e-cigarette vaporizer under specified controlled conditions (temperature 400°F; 5 sec programmed puff, total vapor time in chamber per puff ~100 sec) (La Jolla Alcohol Research, LJARI). The positive-pressure chamber air flow was vacuum controlled to maintain air delivery through the intake valve at 1 L/min. To ensure that the rats were specifically responding for nicotine, a separate control group (total n=13; 8 males, 5 females) was examined for their responding to vehicle-only vapor across 14 days following initial food training, under identical experimental conditions as above (but no nicotine). Given that we previously found initial exposure to a lower nicotine dose is necessary to establish reliable and consistent responding with intravenous nicotine self-administration (Fowler and Kenny, 2011), a final self-administration group (total n=8; 4

males, 4 females) was examined for their initial responding on the moderate 5 mg/ml dose, without prior access to the lower dose of 2.5 mg/ml.

Passive vapor nicotine administration

Subjects (n=16 males, n=6 females) were exposed to vaporized e-cigarette nicotine during 1 h daily sessions for 7 days (nicotine concentration: 5mg/ml) in the sealed LJARI chambers (340mm x 237mm x 198mm) described above. As an additional control, females were examined for differences in blood cotinine with the same 1 h session under single or dual nicotine vapor exposure conditions (n=4). For each daily session, animals were exposed to nicotine or PG:VG vehicle at a rate of one puff every 5 min (consistent with above: 5 sec programmed puff allowing for ~100 s of vapor presence in chamber); this resulted in 12 total puffs per 1 hr session. The rationale for the number of administrations per 1 hr session was based on the average number of infusions at the 0.03 mg/kg/infusion dose intravenously self-administered by rats in prior studies (Fowler e al., 2011; Tuesta et al., 2017).

Food and intravenous nicotine self-administration

A separate group of subjects (n=8 male, n=6 female) were trained to press a lever in an operant chamber (Med Associates) for food pellets (45 mg; TestDiet) under a fixed-ratio 5, timeout 20 sec (FR5TO20 sec) schedule of reinforcement, as described previously (Fowler et al., 2011; Lallai et al., 2019). For the intravenous self-administration rats, the food was provided in pellet form given the design of the chambers with food hoppers. Once stable responding was achieved (>75 pellets per session across 3 subsequent sessions), subjects were surgically catheterized (Fowler et al., 2011; Lallai et al., 2019). Briefly, rats were anesthetized with an isoflurane (1-3%)/oxygen vapor mixture and

prepared with intravenous catheters. Catheters consisted of a 6 cm length of silastic tubing fitted to guide cannula (Plastics One) bent at a curved right angle and encased in dental acrylic. The catheter tubing was passed subcutaneously from the animal's back to the right jugular vein, and a 2 cm length of the catheter tip was inserted into the vein and tied with surgical silk suture. Following intravenous surgery, animals were allowed ≥48 h to recover from surgery, then permitted again to respond for food reward. Subjects were then transitioned to acquire intravenous nicotine self-administration during 1 h daily sessions for 7 days. Nicotine was delivered through the tubing into the intravenous catheter by a Razel syringe pump (Med Associates). Each session was performed using 2 retractable levers (1 active, 1 inactive). Completion of the response criteria on the active lever resulted in the delivery of an intravenous nicotine infusion (0.03 ml infusion volume; FR5TO20 sec schedule). Responses on the inactive lever were recorded but had no scheduled consequences. Catheters were flushed daily with physiological sterile saline solution containing heparin. Catheter integrity was tested with the ultra-short-acting barbiturate anesthetic Brevital (methohexital sodium, Eli Lilly), and all subjects had catheter patency at the end of the experiment. Behavioral responses were automatically recorded by Med Associates software.

Locomotor assessment

Subjects were examined for nicotine-mediated locomotor effects in an open field chamber prior to any drug exposure (baseline) and immediately after the nicotine exposure sessions on Day 1 and Day 7. This assessment was included in a subset of the rats tested in later cohorts, and thus, the group number included: males (n=6 passive nicotine vapor, n=6 intravenous nicotine self-administration) and females (n=6 passive nicotine vapor,

n=5 intravenous nicotine self-administration). The chamber was composed of plexiglass (35 cm L x 35 cm W x 31 cm H) as described previously (Chen et al., 2018), with a shielded white light lamp ~90 cm above the apparatus for consistent lighting. Prior to testing, animals were habituated by handling for at least 5 mins per day for 2 days. On the first testing day (baseline), they were individually placed into the open field and recorded for a 15 min test. On Day 1 and Day 7, rats were individually placed into the chamber for the 15 min period and then returned to their home cage at the end of each session. Activity was recorded with a video camera and scored with ANYmaze software that divided the field into center and outer edge zones.

Blood cotinine analysis

Collection of blood samples for cotinine measurement was performed from the tail vein. For the first set with vapor self-administration, blood was collected 30 min after the final session on each dose. To limit the potential stress-related impact of repeated blood draws, each subject had blood drawn ≤2 times, with at least 6 days in between each blood collection. For the intravenous nicotine self-administration and passive vapor exposure, blood collection was scheduled as follows: (1) 30 min after chamber-only exposure on Day 0 (baseline), (2) 30 min after the first vapor session (Day 1), and (3) 30 min after the final vapor session (Day 7). On Day 1, a second sample was collected at the post-session 6 hr time point. Serum was separated by centrifugation at 3000 × g for 20 min at 4°C and then stored at −80 °C. The concentration of cotinine was determined with the cotinine ELISA kit (OriGene Technologies, Inc) according to the manufacturer's instructions.

Approach for unbiased data collection

For the studies, each subject was provided with a subject ID number that did not denote group assignment. This information was retained in a secure database. The animals were randomly assigned into experimental groups for testing. Blood samples were coded during analysis to provided blinded conditions, and group identification was revealed after the analysis was completed. To provide further confidence in the findings, behavior was scored by two different experimenters who were blinded to the experimental conditions.

Statistical Analyses

All data were analyzed by a t-test, or one-way or two-way analysis of variance (ANOVA) using Graphpad Prism software (La Jolla, CA), as appropriate. Significant main or interaction effects were followed by Bonferroni post-hoc comparison with correction for multiple comparisons. The criterion for significance was set at $\alpha=0.05$.

RESULTS

Male and female rats self-administer nicotine vapor across doses

Subjects were first examined for their self-administration behavior to earn nicotine vapor puffs according to the fixed ratio 5, timeout 20 schedule of reinforcement. Compared to vehicle vapor, we found that males exhibited a significant increase in their level of responding for nicotine vapor at the 5.0 and 7.5 mg/ml doses (One-way ANOVA, $F_{(3,28)}=4.692$, $p=0.0089$, $R^2=0.3345$; post-hoc, 0 vs. 5 mg/ml dose $p=0.0106$, 0 vs. 7.5 mg/ml dose $p=0.0432$) (**Figure 2-1A**). In contrast, females exhibited a statistically significant increase from control levels only at the 5.0 mg/ml dose (One-way ANOVA, $F_{(3,25)}=3.072$, $p=0.0461$, $R^2=0.2693$; post-hoc 0 vs. 5 mg/ml dose $p=0.0410$) (**Figure 2-**

1B). To verify that these responses were nicotine vapor-directed, the number of lever presses was examined between the active and inactive levers. We found a statistically significant preference for responding on the active lever across all nicotine doses in both males (Two-way ANOVA, *Lever* $F_{(1,56)}$=129.2, p<0.0001, *Dose* $F_{(3,56)}$=4.156, p=0.0100, *Interaction* $F_{(3,56)}$=5.887, p=0.0014; post-hoc, active vs. inactive lever at 2.5 mg/ml p<0.0001, 5 mg/ml p<0.0001, 7.5 mg/ml p<0.0001) (**Figure 2-1C**) and females (Two-way ANOVA, *Lever* $F_{(1,50)}$=152.6, p<0.0001, *Dose* $F_{(3,50)}$=7.691, p=0.0003, *Interaction* $F_{(3,50)}$=4.821, p=0.0050; post-hoc, active vs. inactive lever at 2.5 mg/ml p<0.0001, 5 mg/ml p<0.0001, 7.5 mg/ml p<0.0001) (**Figure 2-1D**). Importantly, no significant differences in lever pressing on the active and inactive lever were found in subjects only exposed to vehicle vapor (e.g., 0 mg/kg dose). This demonstrates a selective responding for nicotine vapor in both males and females across a range of doses, with preference in both sexes at the 5.0 mg/ml dose.

To further confirm the inhalation of nicotine vapor and blood bioavailability, subjects were examined for blood cotinine levels 30 min after each dose. Nicotine's metabolite, cotinine, was detected across all doses of vapor nicotine self-administration. In males, a high level of blood cotinine was detected at the 2.5, 5.0 and 7.5 mg/ml doses (One-way ANOVA, $F_{(3,18)}$=13.93, p<0.0001, R^2 =0.6990; post-hoc, 0 vs. dose 2.5 mg/ml p=0.0437, 5.0 mg/ml p=0.0009, 7.5 mg/ml p<0.0001) (**Figure 2-1E**). In females, a high level of detectable cotinine was found at both the 5.0 and 7.5 mg/ml doses (One-way ANOVA, $F_{(3,14)}$=7.516, p=0.0031, R^2=0.6169; post-hoc, 0 vs. dose 5.0 mg/ml p=0.0011, 7.5 mg/ml p=0.0457) (**Figure 2-1F**). Since significant differences were not found in the level of cotinine with

post-hoc comparisons among the different nicotine doses, the animals appear to have titrated their intake via modified breathing patterns with exposure and by adjusting their rate of lever pressing behavior to receive nicotine vapor.

Next, to further demonstrate that the rats were responding for nicotine vapor specifically, subjects were examined for self-administration of vehicle vapor alone across 14 sessions following food training. We found that both males and females extinguished their responding to low lever-pressing behavior levels. Given the similar behavioral response for males and females in this control condition, their data are combined into one graph (**Figure 2-1G**). This supports the notion that the higher levels of self-administration with nicotine vapor were due to the presence of nicotine. Finally, since prior protocols for intravenous nicotine self-administration found that access to a lower dose is necessary to support self-administration behavior prior to access at a moderate preferred dose, likely due to nicotine's initial aversive effects (Fowler and Kenny, 2011; Fowler and Kenny, 2014), we examined whether this would also occur for nicotine vapor inhalation. Therefore, after food training, subjects were given immediate access to the moderate 5 mg/ml dose of nicotine vapor (e.g., preferred dose in findings above). Under these conditions, the lever pressing behavior for vapor reward was more variable and extinguished across time to low levels (**Figure 2-1H**). These findings provide evidence of nicotine's initial aversive effects at this moderate vapor dose and the need to provide a lower dose for initial acquisition, prior to access to a moderate/high dose, to subsequently allow for sustained self-administration behavior.

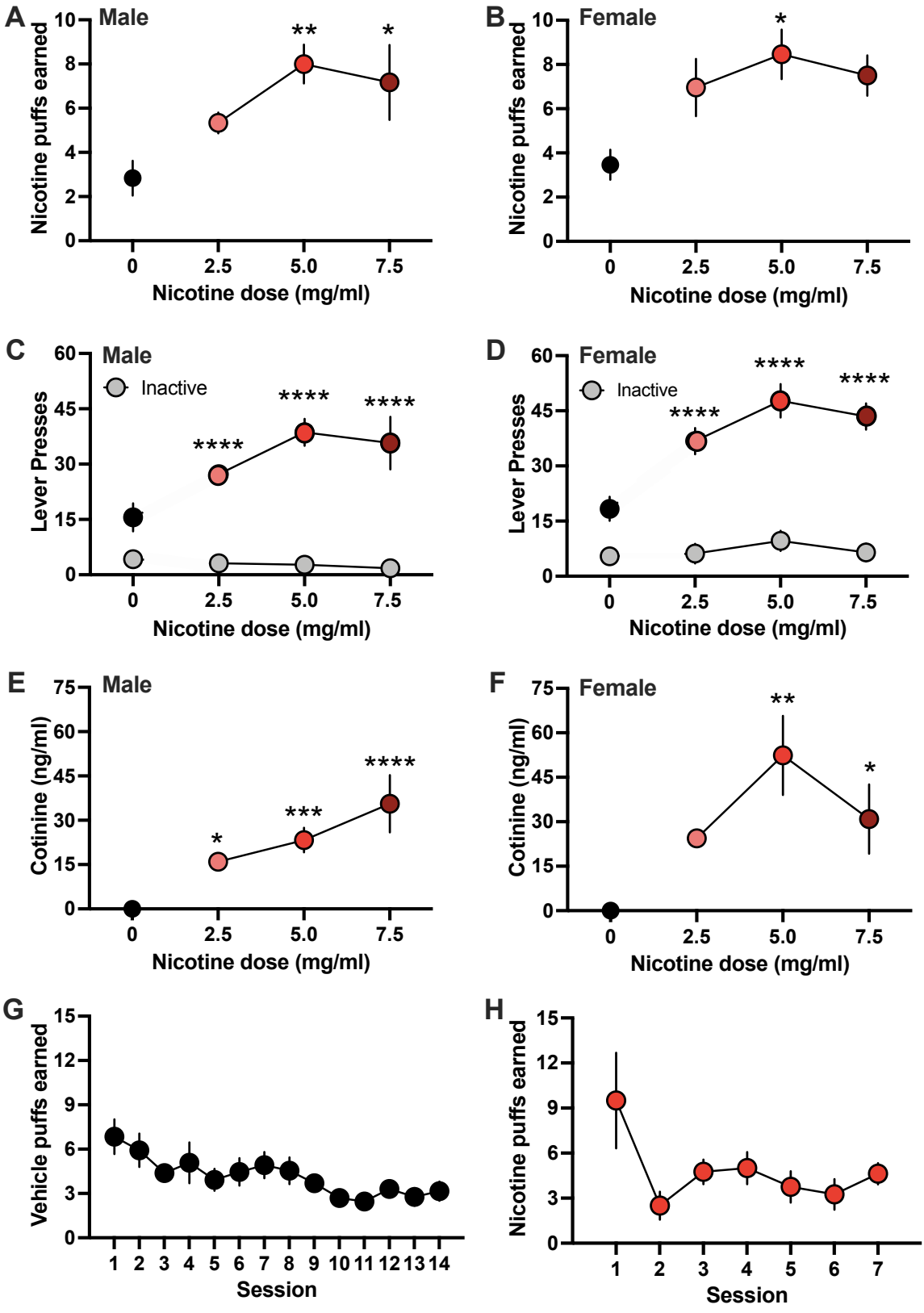
A
Male
Nicotine puffs earned
Nicotine dose (mg/ml)
B
Female
Nicotine puffs earned
Nicotine dose (mg/ml)
C
Male
Inactive
Lever Presses
Nicotine dose (mg/ml)
D
Female
Inactive
Lever Presses
Nicotine dose (mg/ml)
E
Male
Cotinine (ng/ml)
Nicotine dose (mg/ml)
F
Female
Cotinine (ng/ml)
Nicotine dose (mg/ml)
G
Vehicle puffs earned
Session
H
Nicotine puffs earned
Session

Figure 2-1. Nicotine vapor self-administration in male and female rats. Subjects were examined for self-administration behavior at baseline (0 mg/ml, vehicle) and across a range of nicotine doses, which progressively increased from 2.5 to 5.0 to 7.5 mg/ml. **(A)** Male rats (n=8/group) self-administered a significantly increased number of vapor puffs at the 5 and 7.5 mg/ml doses compared to vehicle vapor. *p<0.05, **p<0.01 vs. vehicle control. **(B)** Female rats (n=5-8/group) exhibited increased responding for the delivery of nicotine vapor puffs at the 5.0 mg/kg dose compared to vehicle control. *p<0.05 vs. vehicle control. **(C-D)** When the number of presses on the active and inactive levers were examined, both males **(C)** and females **(D)** exhibited a clear dissociation between the active (colored circles) and inactive (grey circles) levers. This preference in responding on the active lever was present for all nicotine doses examined, but not present for the vehicle control. ****p<0.0001 active vs. inactive lever. **(E-F)** Blood samples were examined for cotinine levels 30 min after the final session of vapor treatment at each dose. **(E)** Cotinine levels progressively increased in male rats as the unit dose increased. *p<0.05, ***p<0.001, ****p<0.0001 vs. vehicle control. **(F)** In females, significant levels of cotinine were found in rats self-administering at the 5 and 7.5 mg/ml concentrations of nicotine. *p<0.05, **p<0.01 vs. vehicle control. **(G)** When rats (n=13 males/females combined) were only provided access to vehicle vapor, their behavioral lever pressing decreased ~3 puffs per hour session, indicating a low baseline level of responding. **(H)** When initially provided access to the higher 5 mg/ml dose, subjects (n=8 males/females combined) decreased their responding across sessions to near baseline levels, indicative of an aversive behavioral response when presented with this higher dose initially. Data are presented as mean ± SEM.

Comparison of nicotine metabolite level with different exposure routes

To ascertain a better understanding of the level of nicotine intake with vapor administration, we next examined passive vapor exposure to provide a controlled dosing schedule across the 1 hr session. This paradigm was compared to intravenous nicotine self-administration, the common technique in the field that provides an exact measure of nicotine bioavailability with volitional intake. After the 1 hr session in males, we found a significant increase in cotinine levels at the 30 min post-session time-point and 5.5 hrs thereafter (Repeated-measures, two-way ANOVA, *Nicotine method* $F_{(1,22)}$=3.57, p=0.072; *Time* $F_{(3,66)}$=56.78, p<0.0001; *Interaction* $F_{(3,66)}$=2.24, p=0.092) (**Figure 2-2A**). Since the immediate effects on Day 1 could be due to stress-related interaction effects with the novel experimental conditions and/or since nicotine metabolism may change with

repeated nicotine exposure across time, we further examined the same subjects after seven consecutive daily nicotine sessions. At the 30 min post-session time-point on Day 7, passive vapor subjects did not differ in the level of cotinine from those that self-administered intravenous nicotine, and further, these values did not significantly differ from Day 1 in male subjects (**Figure 2-2A**). In an initial cohort of males, we also examined whether differences would be present with passive vapor exposure if subjects were placed in the chambers individually or in pairs with a conspecific partner. We found no differences between conditions in the level of cotinine at the various time points, indicating that similar inhalation rates were maintained under the single- vs. dual-exposure conditions (Repeated-measures, two-way ANOVA, Nicotine method $F_{(1,14)}$=0.0721, p=0.7922; *Time* $F_{(2,28)}$=1.138, p=0.3349; *Interaction* $F_{(2,28)}$=1.128, p=0.3379) (**Figure 2-2B**). Thus, these data were compiled with additional cohorts for the passive exposure group shown in Figure 2A, and subsequent exposure sessions in females were performed with two subjects per chamber. As a further analysis of the intravenous nicotine self-administration data, we examined whether the rats differed in the amount of nicotine self-administered on Day 1 or Day 7. There was an overall difference in the amount (mg/kg) of nicotine self-administered (Paired t-test, $t_{(7)}$=2.366, p=0.0499, R^2=0.4444) (**Figure 2-2C**). Therefore, we also examined the amount of nicotine self-administered across the 15-min increments of the session, and we found a significant increase only in the first 15 min epoch on Day 1 compared to Day 7 (Repeated measures, two-way ANOVA, Session Day $F_{(1,56)}$=8.811, p=0.0044; *Time* $F_{(3,56)}$=1.098, p=0.3575; *Interaction* $F_{(3,56)}$=2.477, p=0.0707; post-hoc, Day 1 vs. Day 7 at 0-15' p=0.0177) (**Figure 2-2D**).

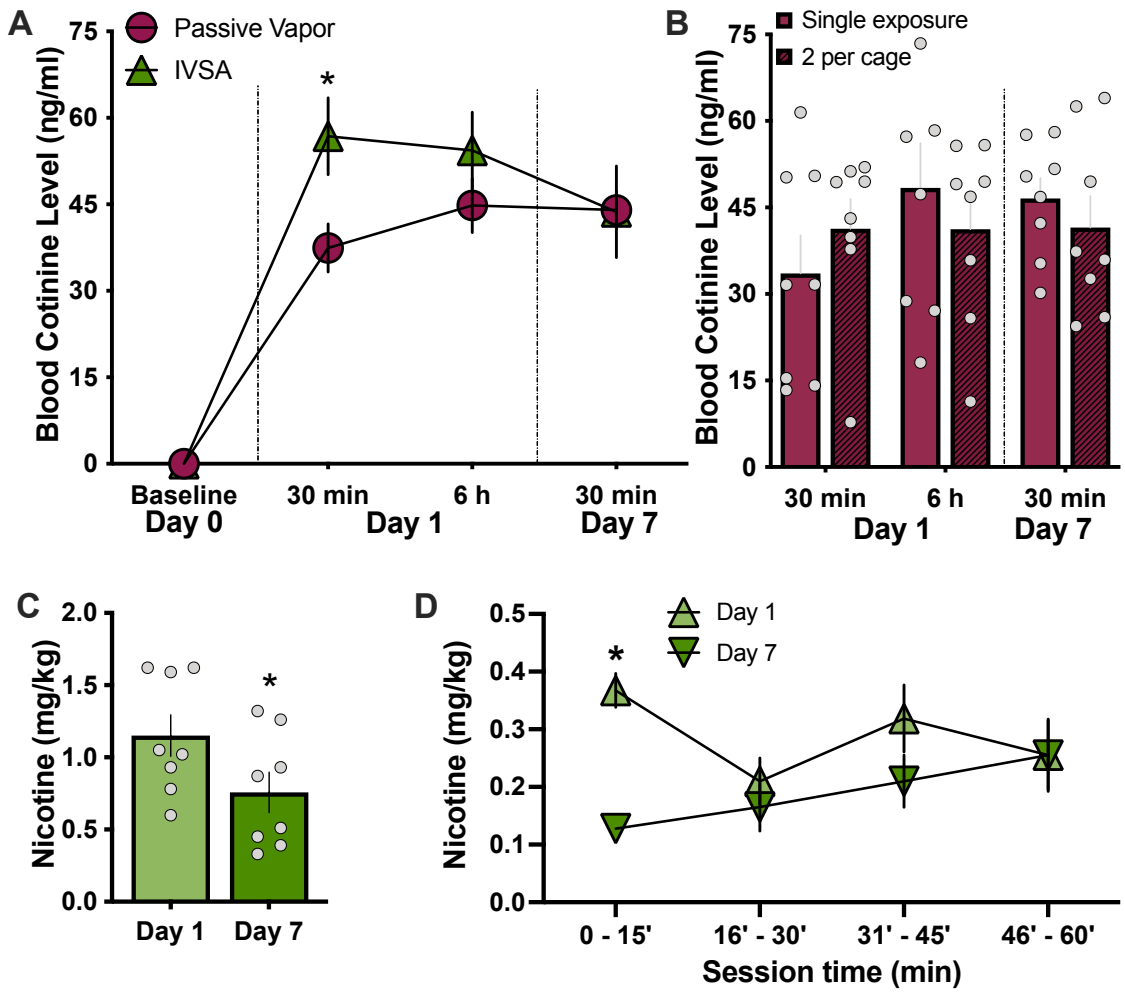

Figure 2-2. Comparison of nicotine metabolite levels following passive vapor or intravenous nicotine self-administration (IVSA) in male rats. Blood samples were collected at baseline (pre-nicotine) or following 1 hr sessions of either nicotine vapor (5 mg/ml) or IVSA (0.03 mg/ml/infusion) from male rats. **(A)** Cotinine levels (n=8-16/group) were decreased with passive vapor exposure 30 min after the first session, as compared to IVSA subjects. However, these differences were not maintained at the later Day 1 time-point of 6 hr, or on Day 7 at the 30 min post-session time-point. *p<0.05 passive vapor vs. IVSA. **(B)** Male rats (n=8/group) exposed to nicotine vapor did not differ in their cotinine levels if placed in the chamber individually (single exposure) or with a cagemate (dual exposure) at all time-points assessed. **(C)** During the nicotine IVSA session (n=8), the rats self-administered a greater net amount of nicotine on Day 1 than on Day 7, which is consistent with the differences found in blood cotinine levels. *p<0.05 Day 1 vs. Day 7. **(D)** When IVSA nicotine intake was examined in 15-min intervals across the 1 hr session, it was found that the males exhibited a significant increase in responding only during the first 15 min interval, and thereafter, they maintained a consistent level of responding. *p<0.05 Day 1 vs. Day 7. Data are presented as mean ± SEM.

For the females, cotinine levels were also assessed following passive nicotine vapor or intravenous nicotine self-administration. Surprisingly, females demonstrated a dramatically different effect based on nicotine exposure route. Across all time points examined in females, passive nicotine vapor exposure resulted in a significantly lower level of cotinine than that found with intravenous nicotine self-administration (Repeated-measures, two-way ANOVA, *Nicotine method* $F_{(1,10)}$=51.87, p<0.0001; *Time* $F_{(3,30)}$=13.89, p<0.0001; *Interaction* $F_{(3,30)}$=3.568, p=0.0255; post-hoc, passive vs. self-administration at time Day 1 - 30 min p=0.0002, 6 hr p=0.0488, Day 7 - 30 min p=0.0017) (**Figure 2-3A**). For intravenous nicotine self-administration, the females self-administered nicotine at a consistent rate, with no differences in nicotine intake between Day 1 and Day 7 (Repeated-measures, two-way ANOVA, *Day session* $F_{(1,40)}$=3.843, p=0.0570; *Time* $F_{(3,40)}$=0.5799, p=0.6317; *Interaction* $F_{(3,40)}$=0.3305, p=0.8033) (**Figure 2-3B**), which corresponded to the similar cotinine levels at the varying blood sampling time-points. Given these unexpected differences between the two administration routes in females, we conducted an additional control study to exclude the possibility that the low levels of cotinine found in females was potentially due to the dual exposure condition. Following single or dual exposure in the chambers, blood was collected at the 30 min post-session time point. Females exposed to passive nicotine vapor alone in the chamber exhibited a similar level of blood cotinine as that found when two females were in the chamber together (Single exposure (n=4) 21.72 $\pm$ 5.923 (mean $\pm$ SEM); Dual exposure (n=4) 23.30 $\pm$ 3.071 (mean $\pm$ SEM); $t_{(6)}$ = 0.2372, p = 0.8204). Thus, these findings further support the striking differences in cotinine levels found between the intravenous nicotine self-administration and nicotine vapor exposure conditions for females.

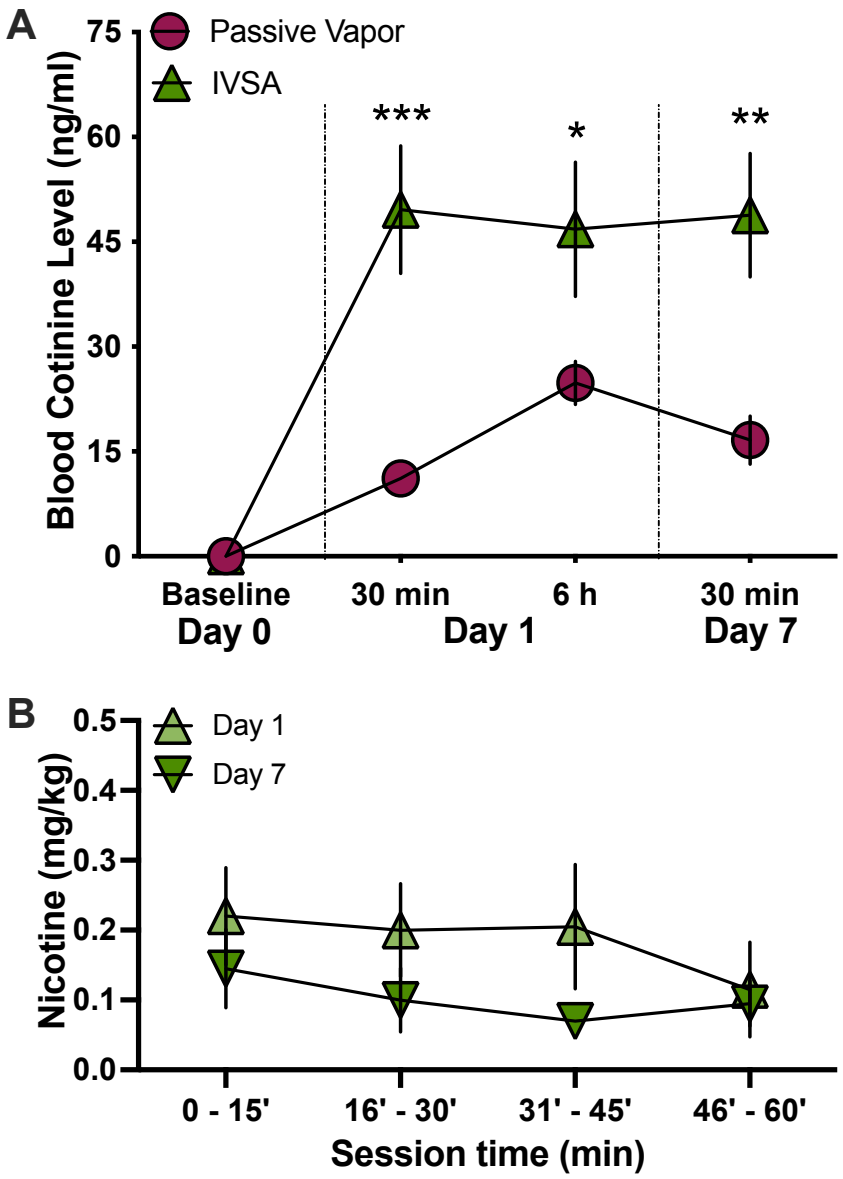

Figure 2-3. Comparison of nicotine metabolite levels following passive vapor or intravenous nicotine self-administration (IVSA) in female rats. Blood samples were collected at baseline (pre-nicotine) or following 1 hr sessions of either nicotine vapor (5 mg/ml) or IVSA (0.03 mg/ml/infusion) from female rats (n=6/group). **(A)** Female cotinine levels were decreased following passive nicotine vapor exposure for all time-points, as compared to IVSA nicotine. *p<0.05, **p<0.01, ***p<0.001 passive vapor vs. IVSA. **(B)** Females did not differ in the amount of nicotine self-administered between Day 1 and Day 7. Data are presented as mean ± SEM.

Effect of nicotine administration route on behavioral locomotion

Peripheral experimenter-administered injections of nicotine at high doses are known to induce hypolocomotion, whereas conversely, lower doses result in hyperlocomotion. However, it has not been previously demonstrated what effect self-administered nicotine has on locomotor behavior in rats. Thus, the goal of this study was two-fold: (1) to examine whether intravenous nicotine self-administration alters locomotor behavior, consistent with prior results from experimenter-administered dosing and (2) to determine whether vapor and intravenous nicotine result in comparable locomotor effects given the similar cotinine levels noted in the above studies. Therefore, we examined whether the male and female rats would differ in their locomotor behavior immediately following the nicotine sessions on Day 1 and Day 7. In males, passive vapor exposure resulted in a significant increase in total distance travelled on the last day of nicotine exposure (Day 7), whereas the intravenous nicotine self-administration resulted in increased locomotion at both the acute and chronic session time points (Day 1 and Day 7) (Repeated measures, two-way ANOVA, *Nicotine method* $F_{(1,10)}$=10.37, p=0.0092; *Time* $F_{(2,20)}$=12.64, p=0.0003; *Interaction* $F_{(2,20)}$=12.69, p=0.0003; post-hoc, passive - Day 0 vs. Day 7 p=0.0028, D1 vs. D7 p=0.0055, self-administration - Day 0 vs. Day 1 p<0.0001, Day 1 vs. Day 7 p=0.0148) (**Figure 2-4A**). To further understand these behavioral differences, we compared the locomotor behavior between groups across 5-min epochs during each 15-min locomotor test. Prior to drug exposure, the subjects did not differ in their distance travelled (Repeated measures two-way ANOVA, *Nicotine method* $F_{(1,10)=}$3.930, p=0.0756; *Time* $F_{(2,20)=}$9.011; p=0.0016; *Interaction* $F_{(2,20)=}$0.0812, p=0.9224) (**Figure 2-4B**), which demonstrates no difference in the baseline level of responding for subjects between

groups. On Day 1, the intravenous nicotine self-administration group displayed a significant increase in distance traveled at all time points assessed (Two-way ANOVA, *Nicotine method* $F_{(1,10)}$=17.55, p=0.0019; *Time* $F_{(2,20)=}$14.21; p=0.0001; *Interaction* $F_{(2,20)}$=1.636, p=0.2197; post-hoc, passive vs. self-administration 0-5' p=0.0008, 5-10' p=0.0040) (**Figure 2-4C**). These findings in males indicate that self-administration of reinforcing doses of nicotine results in hyperlocomotion, which is greater than that found with passive vapor exposure at this timepoint. On Day 7, the groups displayed no difference in their increased locomotor effect across the 15-min session (Two-way ANOVA *Nicotine method* $F_{(1,10)}$=1.823, p=0.2067; *Time* $F_{(2,20)}$=11.78, p=0.0004; *Interaction* $F_{(2,20)}$=0.1741, p=0.8415) (**Figure 2-4D**). Together, these findings suggest that a stress-related effect may have been imposed on Day 1 of the passive vapor exposure, and subsequently, habituation with chronic nicotine vapor sessions allowed for the unmasking of nicotine's behavioral locomotor effect.

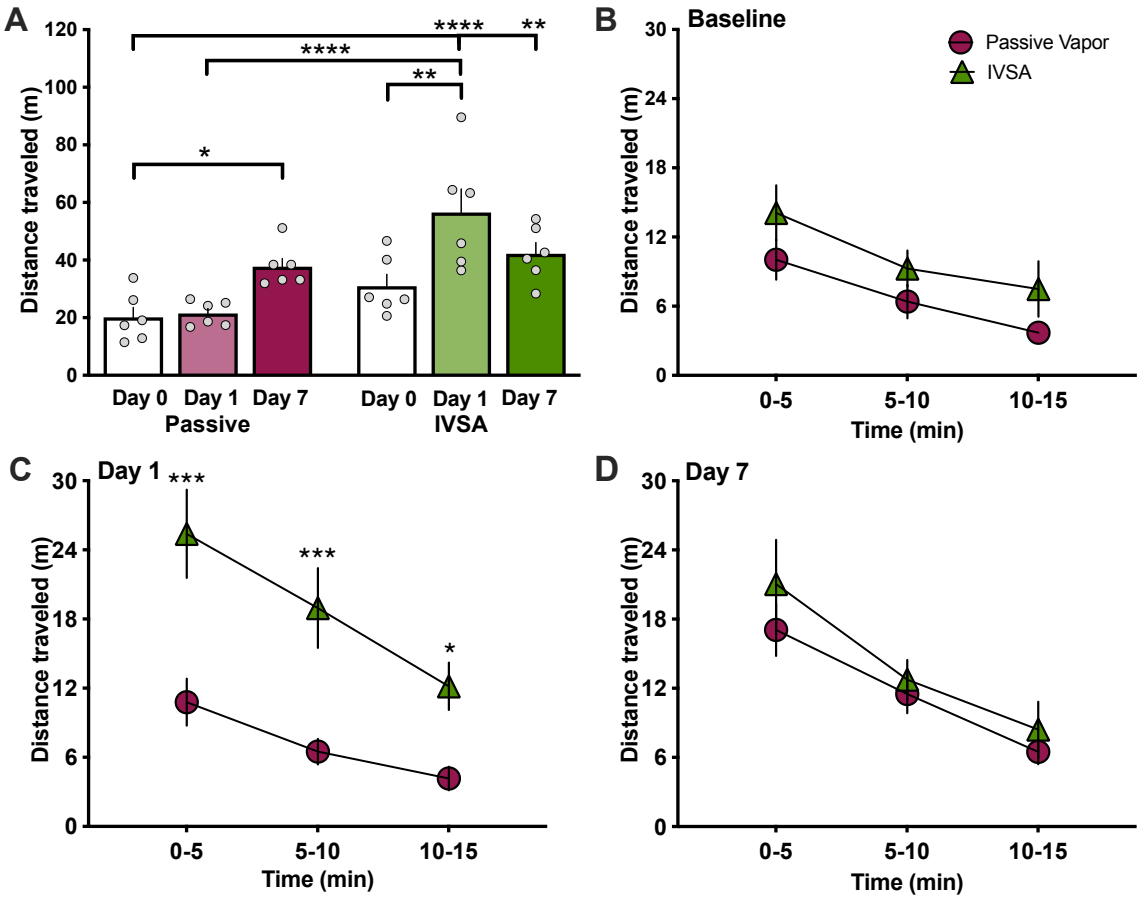

Figure 2-4. In male rats, nicotine vapor exposure and intravenous self-administration (IVSA) both induce a hyperlocomotor effect in male rats. Male rats (n=6/group) were tested for nicotine-mediated locomotor effects in an open field chamber at baseline (pre-nicotine) and immediately after each nicotine sessions. **(A)** Passive nicotine vapor induced a significant increase in locomotor activity on Day 7 of exposure, whereas nicotine IVSA induced a significant increase on Day 1 of exposure, as compared to baseline levels. *p<0.05, **p<0.01, ****p<0.0001. **(B-D)** Locomotor behavior was analyzed in 5 min intervals for each session to compare the level of responding between nicotine passive vapor and IVSA. **(B)** At baseline, there were no differences between groups in distance traveled. **(C)** On Day 1, the IVSA nicotine group exhibited an increase in their distance traveled across the first two 5 min epochs. **p<0.01, ***p<0.001. **(D)** No significant differences were found between groups during the last day of treatment (Day 7). Data are presented as mean ± SEM.

Interestingly, while females exhibited a similar hyperlocomotor effect with intravenous nicotine self-administration, passive vapor exposure did not result in a significant change in behavior (Repeated measures, two-way ANOVA, *Nicotine method* $F_{(1,9)}$=7.695, p=0.0216; *Time* $F_{(2,18)}$=9.663, p=0.0014; *Interaction* $F_{(2,18)}$=4.277, p=0.0302; post-hoc, self-administration Day 0 vs. Day 1 p=0.0017, Day 0 vs. Day 7 p=0.0012) (**Figure 2-5A**). When comparing the groups across the 5-min session epochs, the subjects did not differ at baseline levels (Repeated measures, two-way ANOVA, *Nicotine method* $F_{(1,9)}$=0.0056, p=0.9421; *Time* $F_{(2,18)}$=9.751, p=0.0014; *Interaction* $F_{(2,18)}$=0.9168, p=0.4177) (**Figure 2-5B**). Differences were found between groups on Day 1 with increased locomotion for self-administration compared to passive nicotine vapor (Repeated measures, two-way ANOVA, *Nicotine method* $F_{(1,9)}$=9.896, p=0.0118; *Time* $F_{(2,18)}$=27.92, p<0.0001; *Interaction* $F_{(2,18)}$=0.6084, p=0.5550; post-hoc, passive vs. IVSA 0-5' p=0.0093, 5-10' p=0.0311) (**Figure 2-5C**). However, on Day 7, no differences were found between the groups (Repeated measures, two-way ANOVA, *Nicotine method* $F_{(1,9)}$=4.489, p=0.0551; *Time* $F_{(2,18)}$=0.7410, p=0.4906; *Interaction* $F_{(2,18)}$=1.313, p=0.2935) (**Figure 2-5D**).

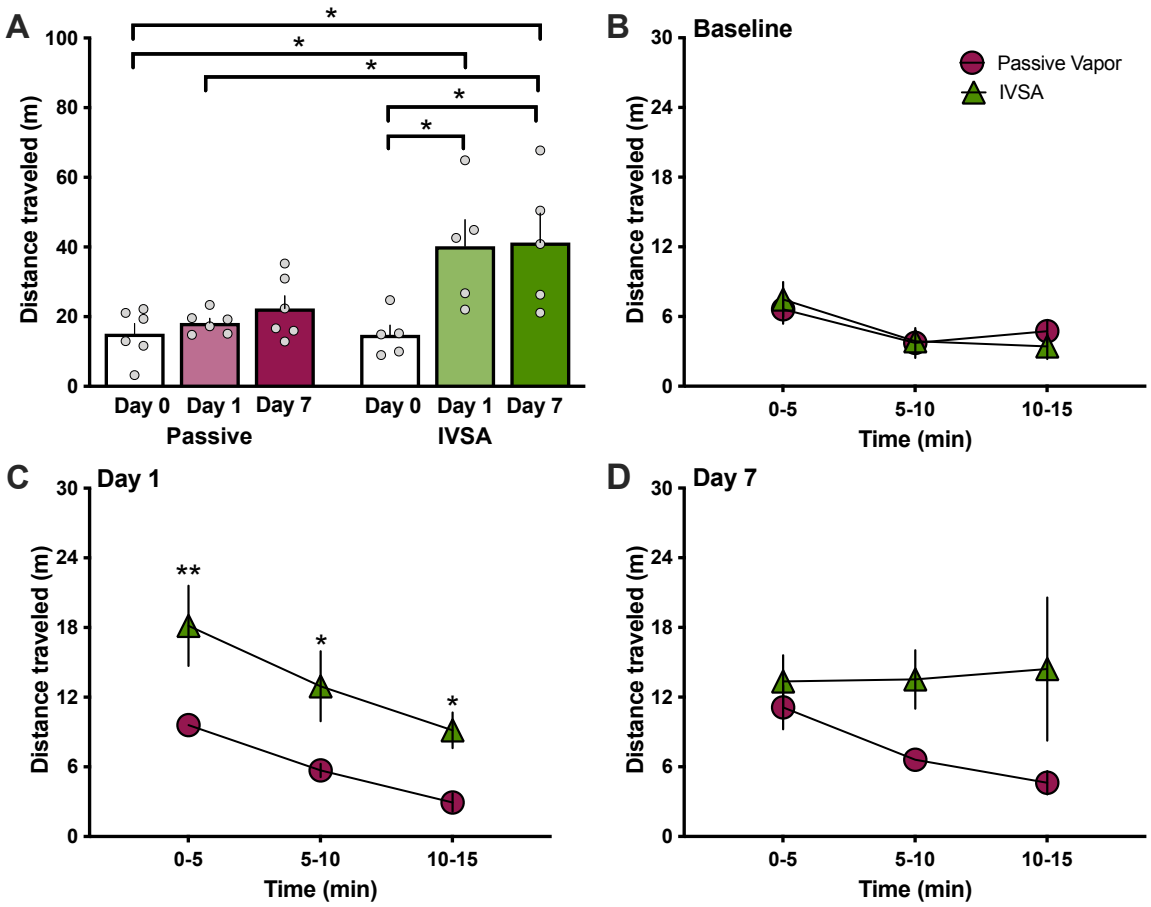

Figure 2-5. In female rats, intravenous nicotine self-administration (IVSA), but not passive vapor exposure, induces a hyperlocomotor effect. Female rats (n=5-6/group) were tested for nicotine-mediated locomotor effects in an open field chamber at baseline (pre-nicotine) and immediately after the nicotine sessions. **(A)** Passive nicotine vapor did not significantly alter locomotion, whereas nicotine IVSA induced a significant increase on Day 1 and Day 7 of exposure, as compared to baseline levels. **p<0.01. **(B-D)** Locomotor behavior was analyzed in 5 min intervals for each session to compare the level of responding between nicotine passive vapor and IVSA. **(B)** At baseline, there were no differences between groups in distance traveled. **(C)** On Day 1, the IVSA nicotine group exhibited an increase in their distance traveled across the first two 5 min epochs. *p<0.05, **p<0.01. **(D)** No significant differences were found between groups during the last day of treatment (Day 7). Data are presented as mean ± SEM.

Effect of nicotine on anxiety-related behaviors in the open field

As additional measures of behavior in the open field, we analyzed two parameters that have been associated with anxiety-related effects in rodents: the time spent in the center

of the arena and freezing time. We found no differences in the time spent in the center of the open field between passive nicotine vapor and intravenous nicotine self-administration for males (Repeated measures, two-way ANOVA, *Nicotine method* $F_{(1,10)}$=1.080, p=0.3231; *Time* $F_{(2,20)}$=0.5051, p=0.6109; *Interaction* $F_{(2,20)}$=0.0609, p=0.9411) (**Figure 2-6A**) and females (Repeated measures, two-way ANOVA, *Nicotine method* $F_{(1,9)}$=1.978, p=0.1932; *Time* $F_{(2,18)}$=0.1878, p=0.8304; *Interaction* $F_{(2,18)}$=0.6601, p=0.5289) (**Figure 2-6B**). Similarly, no differences were found between the groups in freezing behavior for males (Repeated measures, two-way ANOVA, *Nicotine method* $F_{(1,10)}$=0.1805, p=0.6799; *Time* $F_{(2,20)}$=0.3168, p=0.7321, *Interaction* $F_{(2,20)}$=0.4652, p=0.6347) (**Figure 2-6C**) and females (Repeated measures, two-way ANOVA, *Nicotine method* $F_{(1,9)}$=0.8220, p=0.3882; *Time* $F_{(2,18)}$=1.041, p=0.3733, *Interaction* $F_{(2,18)}$=0.6236, p=0.5472) (**Figure 2-6D**).

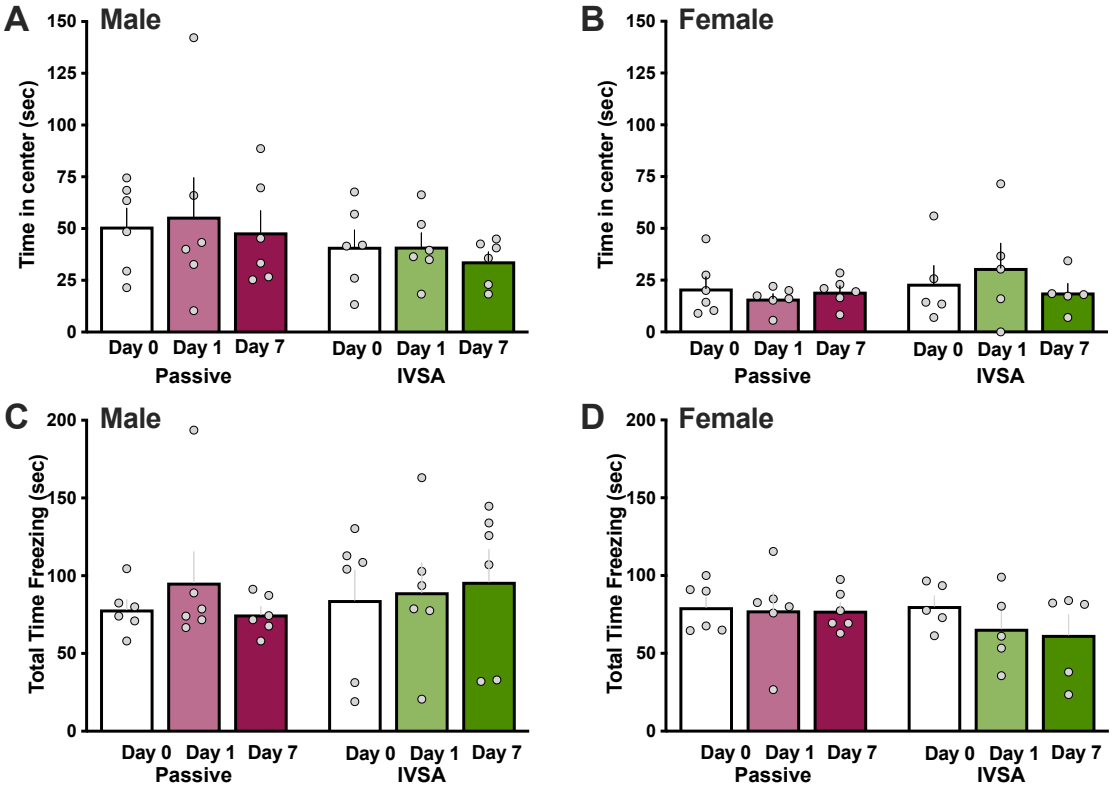

Figure 2-6. No effect of nicotine on anxiety-related behaviors in the open field. Male (n=6/group) and female (n=5-6/group) subjects were further examined for indicators of behaviors associated with anxiety on Day 0 (baseline, no nicotine), and immediately after nicotine sessions on Day 1 and Day 7. **(A-B)** Time in the center of the open field did not differ across all exposures or time-points for both males **(A)** and females **(B)**. **(C-D)** Freezing time was examined but no differences were found across all exposures or time-points for both males **(C)** and females **(D)**. Data are presented as mean ± SEM.

Discussion

In these studies, we developed and validated a reliable procedure for vapor self-administration of nicotine in rats. In establishing this method, we compared to the 'gold-standard' procedure of voluntary drug intake in animal models - intravenous nicotine self-administration. Both males and females exhibited robust and selective responding for vaporized nicotine at the dose of 5 mg/ml, a dose which also elicited a high level of nicotine's metabolite cotinine in blood plasma within both sexes. These effects were specific to the presence of nicotine, as behavioral responding was not maintained when subjects were provided access to vehicle puffs alone. Similar levels of cotinine were observed following passive vapor or intravenous drug administration in males, indicating vapor exposure can elicit comparable levels of nicotine as that found with intravenous self-administration. Interestingly, lower levels of cotinine were detected in females with passive vapor exposure as compared to intravenous self-administration, suggesting either differential inhalation rates for vapor or altered nicotine metabolism with lung inhalation.

Importantly, the current studies provide an advance over prior investigations in the field. For instance, a recent study from Smith *et al.* (2020) employed a vapor self-administration

method in rats. However, in their studies, they were not able to observe dissociative responding between the active and inactive lever, suggesting that the rats did not discriminate the drug-delivering lever. In self-administration protocols, a significant difference between the active and inactive levers supports the contention of directed drug-seeking behavior, consistent with what we found in the current studies. The reasons for such a difference may be either (1) different chamber environments, or (2) effort required to elicit drug delivery. While Smith and colleagues utilized a low-effort fixed-ratio 1 (FR1) schedule of reinforcement, our studies employed a more effortful fixed ratio 5 (FR5) schedule (Smith et al., 2020). Thus, by imposing a higher effort to receive vaporized nicotine delivery, the rats may have been able to more clearly dissociate the contributions of pressing each individual lever. This is similar to our prior findings with intravenous nicotine self-administration in mice and a recent study with self-administration of vaporized cannabis extracts under increasing schedules of reinforcement in rats (Fowler et al., 2011; Freels et al., 2020).

The presence of nicotine following systemic administration is commonly assessed by measuring its main metabolite, cotinine (Hukkanen et al., 2005; Chen et al., 2020). After inhalation, nicotine is absorbed rapidly into the lungs, resulting in a high blood nicotine concentration that quickly declines due to absorption into tissues, including the brain. An average cigarette delivers roughly 10–30 μg/kg, typically resulting in 10–50 ng/ml peak nicotine plasma levels (Benowitz et al., 1984; Matta et al., 2007). In our study, cotinine concentrations in the plasma after vapor and intravenous administration were in the range of ~10-60 ng/ml for both males and females, similar also to that found in prior passive

vapor exposure studies (Lefever et al., 2017; Javadi-Paydar et al., 2019). Approximately 70– 80% of nicotine is converted to cotinine (Benowitz et al., 1994; Benowitz et al., 2009), which supports the level of vapor nicotine inhalation occurring with the current protocol conditions. Interestingly, pulmonary tissue appears to function as a short-term depot for nicotine binding, which may in part explain the sustained levels of cotinine found at the 6 hr time point in our current investigations (Brewer et al., 2004).

The lower levels of cotinine found at the 30-min post-session time point in males with passive vapor and at all time points in females may be further explained by differences in inhalation and/or stress-related behavioral effects. First, prior studies have found that nicotine detection in the arterial blood is slower if through the lung tissue, as compared to systemic injection (Rose et al., 1999). Second, the pH of smoke particulate may modulate pulmonary absorption; although the nicotine solution pH was adjusted to 7.4 in these studies, heating of the solution for vapor delivery may alter the pH upon smoke delivery (Pankow, 2001). Third, male and female rats have been shown to exhibit differences in nicotine metabolism (Kyerematen et al., 1988), and compared to men, women exhibit a shorter half-life and a quicker elimination of nicotine to yield a significantly higher elimination rate value (Prather et al., 1993; Benowitz, 1999). Thus, nicotine metabolism differences may have partially contributed to the effects across doses. Finally, stress-related behavioral effects may alter breathing patterns, thereby modulating the net amount of nicotine inhaled through the lungs. In particular, this effect may explain the differences found between day 1 and day 7 of exposure, as the animals became habituated to the passive vapor puffs across 7 consecutive sessions and thus were likely

experiencing lower stress levels. Interestingly, a recent study has demonstrated that females exhibit an increase in α5 nicotinic receptor subunit expression in the interpeduncular nucleus following chronically administered nicotine, which was also correlated with anxiety-like behavior (Correa et al., 2019). Thus, differential effects related to an interaction between nicotine modulation of brain function and anxiety-related behaviors may underlie the effects found in females.

We also found increased locomotion in males with both passive inhalation and intravenous nicotine self-administration. These findings are similar to that previously found with passive vapor exposure in male rats (Javadi-Paydar et al., 2019). In contrast, females only exhibited increased locomotion following intravenous nicotine self-administration. Importantly, these behavioral findings are supported by the similar levels of blood cotinine found in males with both methods of nicotine exposure. Nicotine administration has been shown to increase plasma adrenocorticotropic hormone (ACTH) and corticosterone, leading to an increase in locomotion through dopamine release in the nucleus accumbens (Matta et al., 1987; Caggiula et al., 1994; Rouge-Pont et al., 1998; Matt et al., 2007). In contrast, females exhibited lower levels of cotinine with passive exposure, which is reflective of the lack of locomotor effect. Given that experimenter-administered nicotine may induce hypolocomotion at high doses, or hyperlocomotion at lower doses, these findings support the notion that lower doses in the hyperlocomotion range are more reinforcing. It should also be noted that with experimenter administered nicotine, hyperlocomotor effects are more likely to occur with repeated exposure, which

is also consistent with our findings in males for passive vapor exposure and further supports the notion of potential stress-related effects in the first vapor session (Ksir, 1994). Female and male rats have been previously shown to differ in nicotine-mediated reward and reinforcement. For these studies, we sought to establish and validate a vapor self-administration protocol, but given that females and males have been shown to differ in various assessments and with nicotine metabolism, we sought to examine each sex independently. However, it is still worth considering potential sex differences as a foundation for future studies. For instance, compared to male rats, nicotine's rewarding effects are enhanced in females in the conditioned place preference paradigm, females acquire intravenous nicotine self-administration quicker at a low nicotine dose, females demonstrate higher elasticity in demand for nicotine in a behavioral economics assessment, and females exhibit higher motivation to obtain nicotine infusions with the progressive ratio schedule or under extended access conditions (23 hr) (Torres et al., 2009; Donny et al., 2000; Flores et al., 2019; Chellian et al;, 2020). Age-related effects have also been found in the reinforcing effects of nicotine on brain reward thresholds, with adolescent females exhibiting a higher sensitivity to nicotine compared to males (Xue et al., 2020). Interestingly, with specific relevance to nicotine vapor inhalation, a recent study in humans has found that an e-cigarette puff results in a higher concentration of brain nicotine in women compared to men, as demonstrated with ^{11}C-nicotine PET (Solingapuram et al., 2020). In our study, although the males and females exhibited a similar rate of nicotine vapor self-administration across the dose response function, it is therefore possible that differences were present in brain nicotine concentration between

sexes. Thus, it will be interesting in future studies to directly compare males and females under varying experimental conditions.

In addiction-related processes, stress is known to be a key factor mediating drug intake and relapse-related behaviors, and among these factors, social isolation represents a well-established model of chronic stress, which has been shown to interfere with the mechanisms underlying dependence for different drugs, such as amphetamine, cocaine, ethanol and nicotine (Matta et al., 1993; Howes et al., 2000; Whitaker et al., 2013; Koob et al., 2014; Lallai et al., 2016). Here, we found similar levels of blood cotinine with one or two rats present during vapor delivery in the chamber. This is an important finding since it establishes the validity of group housing for vapor drug exposure, thereby overcoming the issue of single administration necessary with intravenous self-administration paradigms due to the presence of the tubing attachment within the operant chamber. Future studies will be of great interest that focus on the impact of social environment on drug-related effects with this vapor exposure method.

In conclusion, we have developed a robust and reliable method of vapor nicotine self-administration that has high translational relevance to human e-cigarette and tobacco intake. The protocol for vapor exposure was shown to result in comparable blood levels of nicotine's metabolite cotinine, compared to intravenous nicotine self-administration. We have also found intriguing differences within each sex based on the route of nicotine intake, and such differences will be important to investigate in future studies to ascertain a better understanding of the underlying mechanisms for lung and/or brain function.

Further, it will be important in future studies to employ the vapor protocol under long-term exposure conditions and with solid vape cartridges that contain a higher dose of vapor nicotine to better elucidate behavioral and metabolic changes occurring with vaporized nicotine inhalation. Since vapor exposure requires no surgical manipulations, this delivery method will also be optimal to examine a range of ages (e.g., young, adolescent, young adult, adult, aged) and interactive effects with disease models (e.g., Alzheimer's disease, COVID-19 viral infection, etc.). Through these efforts, the scientific findings ascertained may better inform tobacco/nicotine regulatory science for effective control polices and may also lead to more efficacious therapeutic interventions for nicotine dependence (Jensen et al., 2016).

Experiment 2: Nicotine vapor self-administration in mice

Rationale:

With nicotine vapor self- administration model in rat established, we also seek to have mice reliably self-administer nicotine vapor. While more transgenic models available in mice, it allows us to explore more the role of specific brain circuits or neuron population in nicotine dependence. Furthermore, the smaller size of the mice can be an advantage for certain techniques such as optogenetics (Ellenbroek and Youn et al., 2016). The smaller size of the mice brain makes the light easier to penetrate and manipulate the activity of targeted neurons (Ellenbroek and Youn et al., 2016). In this study, we have provided evidence to show that mice can voluntarily intake nicotine vapor. Our preliminary data demonstrated that mice can reliably self-administer nicotine vapor. We have validated that mice intake nicotine by measuring their blood cotinine level after

vapor exposure. Furthermore, we have injected mecamylamine, a general acetylcholine receptor antagonist, to examine whether nicotine is the primary reinforcer for lever pressing behavior. The vehicle vapor self-administration in mice is also applied to examine whether the vehicle itself is a reinforcer. In sum, we have demonstrated that nicotine vapor self-administration model in mice is feasible, and it will be a great model to allow us to further study the mechanism of nicotine dependence.

Material and Methods:

Animals

C57BL/6J wildtype male and female mice (8-15 weeks old) were bred in our laboratory animal facilities. Mice were maintained in the vivarium with a 12hr reversed light /dark schedule. Food and water were provided *ad libitum* until food training. During food training and vapor self-administration, subjects were mildly food restricted to 85–90% of their free-feeding bodyweight. All experiments were performed in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of California, Irvine.

Drug

(-)-Nicotine hydrogen tartrate (Cat #0215355491, MP Biomedicals) was dissolved in 0.9% sterile saline (intravenous self-administration) or 50:50 propylene glycol (PG) and vegetable glycerin (VG) solution (e-cigarette vapor) (free base, pH 7.4). Concentrations for aerosol solution as mg/ml. Mecamylamine (Cat #2843, Tocris) was dissolved in 0.9% sterile saline.

Food training and vapor self-administration

The food training and vapor self-administration protocol were modified from the established protocol from food training and vapor-self administration in rats. The mice (n =2) were initially trained to press the active lever in a sealed vapor chamber (19 cm × 12 cm × 18 cm, LJARI) with regulated airflow (1 L/min) for liquid food infusion (50 µl/s/infusion). Because it is essential that a constant airflow is maintained inside the vapor boxes, the design of the equipment did not allow for a food pellet hopper dispenser, and thus, food reward was provided in a liquid form to ensure consistent control of pressure and airflow. Rewards were earned under the FR5TO20 sec schedule, in which five lever presses elicits food delivery and activation of a cue light above the active lever, followed by a 20-s timeout period. Based on our preliminary studies, we found that the animals would highly respond for a solution of vegetable broth containing 5% sucrose. Once stable responding was achieved (>50 liquid rewards per session across three subsequent sessions), subjects were then transitioned to acquire e-cigarette vaporized nicotine during 1-h daily sessions. The mice were placed on nicotine with a concentration of 2.5 mg/ml (vapor duration: 2 secs) for three days and later transitioned to 5mg/ml for 7 days. After 7 days in 5mg/ml (vapor duration: 2 secs) of nicotine, the mice were transitioned to nicotine at a concentration of 7.5 mg/ml (vapor duration: 2 secs). For vehicle self-administration, a separate cohort of mice (n= 4) has undergone food training with the same protocol and later transitioned to vaporized vehicle for 7 days.

Mecamylamine injection before nicotine vapor self-administration

The mouse has undergone food training with the same protocol and later transferred to nicotine vapor self-administration at a dose 2.5 mg/ml. After 7 days of vapor self-administration, the mouse is injected with mecamylamine (2 mg/kg) 30 minutes before the session for 4 days to examine if antagonizing the nAChRs alters the lever pressing behavior.

Cotinine analysis

The blood is collected from the facial vein immediately after the seventh session of nicotine vapor exposure at 5mg/ml and 7.5mg/ml. Serum was separated by centrifugation at 3000× g for 20 min at 4 °C and then stored at −80°C. The concentration of cotinine was determined with the cotinine ELISA kit (OriGene Technologies, Inc) according to the manufacturer's instructions.

Statistical analysis

All data were analyzed by a t test, or one-way or two-way analysis of variance (ANOVA) using Graphpad Prism software (La Jolla, CA), as appropriate. Significant main or interaction effects were followed by Bonferroni post hoc comparison with correction for multiple comparisons. The criterion for significance was set at $\alpha = .05$.

Results:

Mice self-administer nicotine vapor across 5mg/ml and 7.5mg/ml

The mice demonstrated stable intake of nicotine vapor at 5mg/ml after transitioned from 2.5 mg/ml nicotine vapor self-administration. They continued to reliably consume nicotine at 7.5 mg/ml **(Figure 2-7A, C).** The differentiation of the number of active and inactive lever presses at 5 mg/ml and 7.5 mg/ml of nicotine further supports that the mice were deliberately presses on the lever for nicotine intake **(Figure 2-7B, D).**

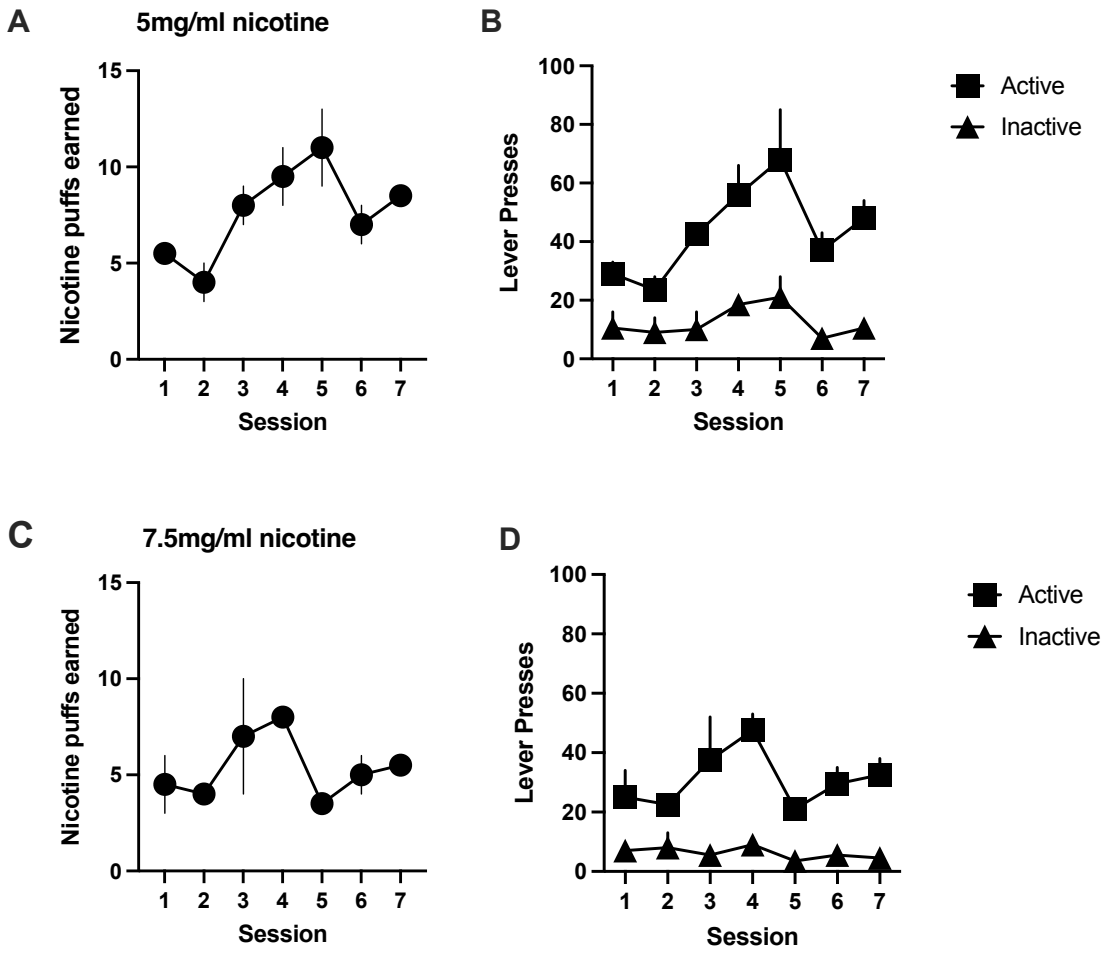

Figure 2-7. Nicotine vapor self-administration at 5 mg/ml and 7.5 mg/ml. (A) The mice demonstrated a stable intake at nicotine with a concentration of 5 mg/ml across 7 sessions **(B)** The number of active lever and inactive lever presses at concentration of 5mg/ml nicotine **(C)** The mice demonstrated a stable intake at nicotine with a concentration 7.5 mg/ml across 7 sessions **(D)** The number of active lever and inactive

lever presses at a concentration of 7.5 mg/ml nicotine. Data are presented as mean ± SEM.

The blood cotinine analysis validates the nicotine vapor intake in mice

To validate that mice were inhaling the nicotine vapor during the session, the blood was collected for cotinine analysis after the seventh session of 5 mg/ml and 7.5 mg/ml nicotine vapor self-administration. The blood cotinine level after 5 mg/ml and 7.5 mg/ml nicotine vapor self-administration are similar **(Figure 2-8).**

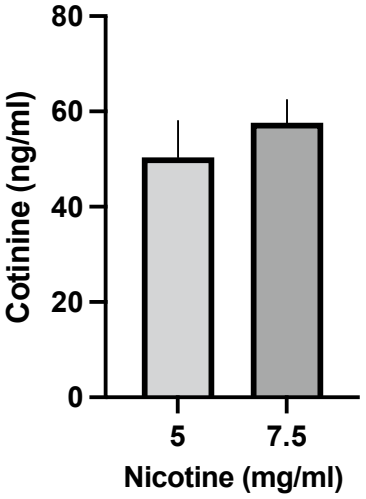

Figure 2-8. Blood cotinine level after 5 mg/ml and 7.5 mg/ml nicotine vapor self-administration. Data are presented as mean ± SEM.

Mecamylamine injection decreases the lever pressing behavior during nicotine vapor self-administration.

To confirm that nicotine is the primary reinforcer for lever pressing behavior, the general nAChRs antagonist, mecamylamine, was injected before nicotine vapor self-administration. If the mice were pressing the lever with the intention to receive nicotine, administering mecamylamine should attenuate this behavior since nicotine wouldn't be able to act on the nicotinic receptors to sustain vapor self-administration. The injection of mecamylamine, with a concentration of 2 mg/kg, resulted a significant decrease of the nicotine vapor earned in mice (*unpaired t-test*, $t_{(8)} = 2.374$, $p = 0.0450$, $R^2=0.4133$). Therefore, it indicates the nicotine is the primary reinforcer during vapor self-administration **(Figure 2-9).**

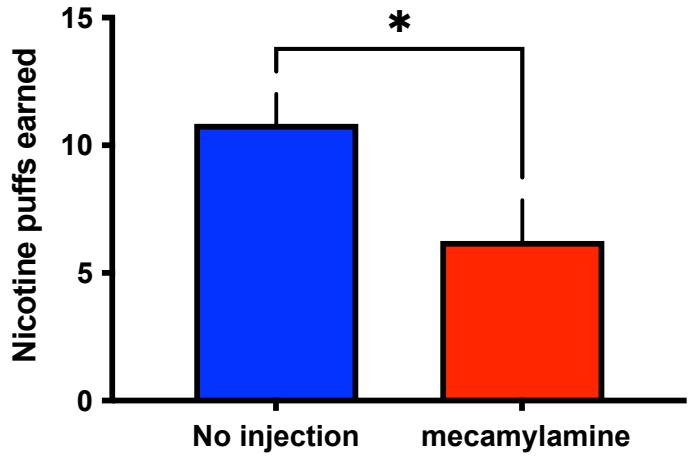

Figure 2-9. The injection of mecamylamine attenuated lever pressing behavior in mice during nicotine vapor self- administration. *p<0.05 mecamylamine vs. baseline, Data are presented as mean ± SEM.

The extinction of vehicle vapor self-administration indicates vehicle itself is not a reinforcer for lever pressing behavior

To ensure that vehicle itself does not act as a reinforcer during vapor self-administration, vehicle self-administration was conducted after food training. The mice quickly decreased their lever pressing behavior at the second session of vehicle self-administration **(Figure 2-10A)**. The extinction of vehicle self- administration is also reflected on the numbers of active lever and inactive lever pressing. The number active lever quickly dropped at the second session and remained to be similar as the number of inactive lever pressing throughout session 2 to 7 **(Figure 2-10B)**.

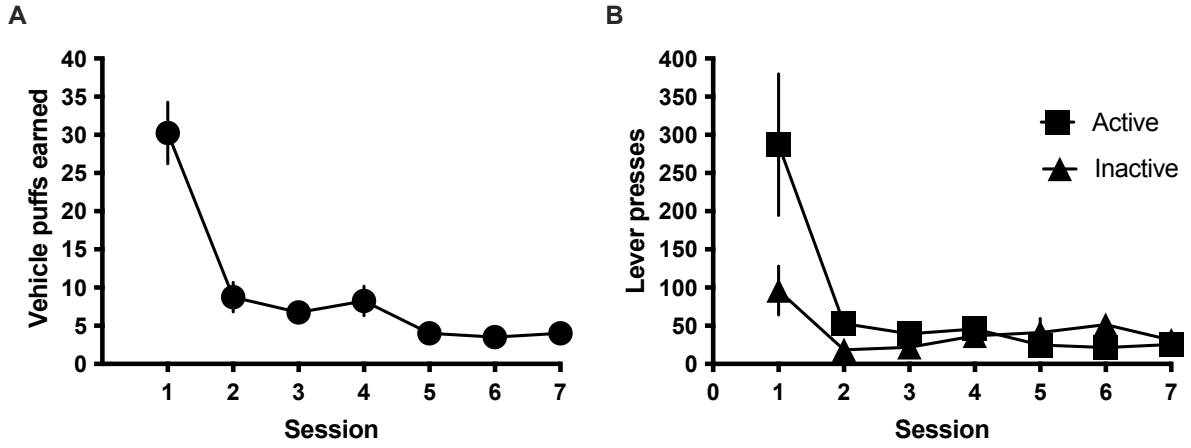

Figure 2-10. Vehicle vapor self-administration. (A) The number of vehicle vapor earned. **(B)** The active and inactive lever presses during vehicle vapor self-administration. Data are presented as mean ± SEM.

Discussion

In this study, we have demonstrated promising results that mice can self-administer nicotine vapor at a dose of 5 mg/ml and 7.5 mg/ml. The measurement of blood cotinine level confirmed the consumption of nicotine vapor. The injection of general acetylcholine

receptor antagonist, mecamylamine, significantly decreased the number of nicotine vapor earned. Therefore, it indicates that nicotine acts as the primary reinforcer for lever pressing behavior. Furthermore, the extinction of lever pressing behavior during vehicle self-administration supports the idea that vehicle itself does not act as a reinforcer.

The effect of e-cigarette has been investigated in mice with passive vapor exposure (George et al., 2010; Alhaddad et al., 2020; Re et al., 2021). Previous study has shown that mice can self-administer nicotine vapor on FR1 schedule (Henderson et al., 2020). While this study provides significant contribution to our understanding of nicotine vapor self-administration and the reinforcing property in the flavor additives in commercial e-cigarette liquid, there are limitations in their experimental design (Henderson et al., 2020). The results observed during nicotine vapor self-administration under FR1 schedule may be a consequence of accidental nosepokes on the active side. In a sperate cohort, the mice showed higher preference on the active side for nosepokes for vapor self-administration that contains both nicotine and menthol. It is likely that the mice were seeking for menthol instead of nicotine since menthol itself can act as a reinforcer and increase the amount of nicotine intake (Garrett and Ahijevych, 2010; Lester et al., 2017). Our preliminary data provides a foundation to support that mice can self-administer nicotine vape on a FR5 schedule with a distinction between the number of active lever pressing and the number of inactive lever pressing. Furthermore, the blood cotinine level after 5 mg/ml and 7.5 mg/ml nicotine exposure suggest that the mice were self-titrating their nicotine intake. The self-titrating behavior of nicotine consumption has been observed in different models of self-administration in mice

including oral intake and intravenous infusion (Fowler et al., 2011; Calarco et al., 2017). Human smokers also unconsciously self-titrate their nicotine consumption by more frequent inhalation of lower strength cigarettes (Ashton et al., 1979; Woodard and Tunstall-Pedoe, 1993). While more blood cotinine measurements should be conducted after different concentrations of nicotine exposure to fully validate the self-titrating behavior of nicotine vapor consumption in mice, the vapor self-administration in mice is an optimal model to mimic human smoking.

Once the model of nicotine vapor-self administration in mice is established, both transgenic mouse lines and optogenetics can be incorporated into the study to further investigate the brain circuit activity change in nicotine vapor intake. Furthermore, the growing popularity of vaping devices have contributed to the increasing use of both cannabis and nicotine (Johnston et al., 2020; Nicksic et al., 2020). Administration of both nicotine and cannabis may lead to higher dependence of both substances since tobacco use may lead to higher numbers of cannabis withdrawal symptoms, and vice versa (Ream et al., 2008; Okoli et al., 2008; Rabin et al., 2015). However, the current treatments are tackling either solely nicotine or cannabis dependence instead of co-use of both and the efficacy of the treatments is low (Rabin et al., 2015). This model will be a great addition as a research model to advance our understanding of nicotine dependence and also the impact the co-exposure to cannabis and nicotine to develop better cessation therapeutics.

CHAPTER 3. ATP neurotransmission modulates habenular activity during nicotine withdrawal

Rationale:

Nicotine is the primary reinforcer for tobacco smoking, which is one of the leading causes of preventable death. With the awareness of the harmful effects of tobacco, there has been a decline in tobacco smoking; however, this has been accompanied by an increase in e-cigarette use, especially among adolescents (CDC, 2019). Nicotine has been shown to alter neurodevelopment (Luna et al., 2010; Siqueira et al., 2017; Ren and Lotfipour, 2019; Mahajan et al., 2021), leading to persistent changes in later behavior into adulthood (Pushkin et al., 2019). Thus, it is critical to understand how nicotine alters brain circuit activity during adolescence.

The rewarding effects of nicotine, nicotine aversion, and nicotine withdrawal are all major factors regulating nicotine dependence (Fowler and Kenny, 2014; Antolin-Fontes et al., 2015). It has been shown that the medial habenula (MHb)- interpeduncular nucleus (IPN) pathway is highly involved in modulating nicotine aversion and withdrawal (Salas et al., 2004a; Salas et al., 2004b; Salas et al., 2009; Fowler et al., 2011; Gorlich et al., 2013; Dao et al., 2014; Shih et al., 2014; Fowler and Kenny, 2014; Antolin-Fontes et al., 2015). The MHb neurons exhibit a characteristic of unique spontaneous firing with a frequency of 2-10Hz, which has been proposed to regulate nicotine withdrawal symptoms (Gorlich et al., 2013; Dao et al., 2014). Acute nicotine elevates neuronal firing frequency in the MHb (Gorlich et al., 2013). Interestingly, following chronic nicotine

exposure, the baseline firing rate is unaltered, but re-exposure to nicotine significantly increases firing frequency greater than that found with acute nicotine exposure (Gorlich et al., 2013). This finding suggests that MHb neuronal activity becomes altered with chronic nicotine and may thus thereby underlie withdrawal.

Nicotine acts in the brain on the neuronal nicotinic acetylcholine receptors (nAChRs). Of note, the ventral MHb has been shown to express a high density of nAChRs, especially those containing the α5, α3 and β4 subunits, in cholinergic neurons (Fowler et al., 2011; Gorlich et al., 2013; Shih et al., 2014; Dao et al., 2014). Acute nicotine exposure elevates neuronal activity, for instance via activation of postsynaptic α3β4 nAChRs (Shih et al., 2014). Further, injection of general nAChR antagonist, mecamylamine, in the MHb elicits somatic withdrawal symptoms in mice chronically exposed to nicotine (Salas et al., 2009), supporting the role of these MHb receptors in withdrawal behaviors. Besides cholinergic signaling, neurokinin receptors and HCN channels have been shown regulate nicotine withdrawal (Gorlich et al., 2013; Dao et al., 2014). Specifically, in nicotine naive mice, injection of a HCN antagonist into the MHb elicits somatic withdrawal symptoms (Gorlich et al., 2013). Presynaptic α5 subunit-containing nAChRs facilitate the activation of neurokinin signaling, resulting in increased MHb neuronal firing, and in mice chronically exposed to nicotine, MHb injection of a neurokinin 1 or neurokinin 3 antagonist induces somatic withdrawal symptoms (Dao et al., 2014). While these findings demonstrate the functional importance of MHb neurons in withdrawal symptomology, other signaling mechanisms discretely expressed within these circuits may have additional modulatory roles on endogenous function. Of importance, ATP was first shown to act as a neurotransmitter in the MHb (Edwards et al.,1992). ATP acts on

P2X and $P2Y_1$ receptors, both of which are expressed in the MHb (Kanjhan et al., 1999; Moran-Jimenez and Matute, 2000; Florenzano et al., 2008; Guo et al., 2008; Song et al., 2011). Activation of P2X receptors elicits Na^+ and Ca^{2+} influx (North, 2002; Burnstock, 2018), and activation of $P2Y_1$ receptors leads to G-protein coupled signaling, thereby increasing intracellular Ca^{2+} concentrations (Abbracchio et al., 2006; Guzman and Gerevich, 2016). *In vitro*, mutual inhibition has been shown between P2X receptors and $\alpha 3\beta 4$-containing nAChRs (Decker and Galligan 2009; Gorlich et al.,2013; Eggan and McCallum, 2017). Furthermore, the activation of $P2Y_1$ receptors enhances the inward current of the HCN channel (Huang et al., 2010), suggesting that activation of the $P2Y_1$ receptor may counteract withdrawal-related effects. Thus, these prior findings support the hypothesis that purinergic signaling modulates the MHb neuronal activity that underlies the withdrawal state.

In these studies, we sought to determine whether nicotine acts on circuit function to alter ATP signaling and whether ATP neurotransmission regulates habenular firing during the naïve and nicotine withdrawal state. First, we utilized G-protein coupled receptor activation-based ATP sensor (GRAB$_{ATP}$) imaging to monitor ATP release activity in the MHb. The GRAB$_{ATP}$ sensor is a modified human $P2Y_1$ receptor that elicits robust florescence signal in response to extracellular ATP (Wu et al., 2021). Given that nicotine increased habenular ATP release and blocking purinergic receptors attenuated nicotine-mediated effects on neuronal firing, we next employed patch clamp electrophysiology to examine the effects of activation of P2X and $P2Y_1$ receptors on habenular neuronal activity, and these receptors' further interaction with nicotine, under both acute and chronic nicotine states. Both males and females were investigated to

probe for sex-specific effects. Together, our studies reveal the functional relevance of extracellular ATP in the habenula and further demonstrate that purinergic signaling plays an important role in habenular neuronal activity during nicotine withdrawal.

Materials and Methods

Animals

C57BL/6J wildtype male (n=39) and female (n= 44) mice were bred in our laboratory animal facilities. Subjects were investigated during adolescence, PND21-PND50. Mice were maintained in the vivarium with a 12hr reversed light /dark schedule. Food and water were provided *ad libitum* until sacrificed for patch clamp recording or GRABATP imaging. All experiments were performed in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of California, Irvine.

Drug

The P2X receptor general agonist, αβ-methylene adenosine 5'-triphosphate trisodium (ATP) salt (Tocris Bioscience, 3209), $P2Y_1$ specific agonist, [(1*R*,2*R*,3*S*,4*R*,5*S*)-4-[6-Amino-2-(methylthio)-9*H*-purin-9-yl]-2,3-dihydroxybicyclo[3.1.0]hex-1-yl]methyl] diphosphoric acid mono ester trisodium salt (MRS2365) (Tocris Bioscience, 2157), general P2 antagonist suramin (Sigma-Aldrich, S2671) and (-)-Nicotine hydrogen tartrate salt (MP biomedicals, Santa Ana, CA, USA; 0215355491) were dissolved in 0.9% sterile saline and adjusted to pH 7.4. Based on prior studies, the following concentrations were used: 100 μM of αβ-methylene adenosine 5'-triphosphate trisodium salt solution and 30 μM of suramin solution (Edwards et al.,1992; Pankratov et

al.,2002). Based on previous electrophysiology studies in the MHb (Dao et al.,2014; Morten et al.,2018), 1 μM nicotine was used to validate that the recordings are retrieved from cholinergic neurons. To avoid a possible ceiling effect of neuronal firing, 0.5 μM nicotine was chosen to study the interaction between cholinergic and purinergic signaling mechanisms.

Nicotine exposure paradigm

To investigate the role of purinergic signaling in the MHb during a state of nicotine withdrawal, mice were subject to subcutaneous nicotine injection at a concentration of 1.5 mg/kg for 10 days. The electrophysiology recordings or GRABATP imaging were conducted 24 hrs after the last injection.

Acute brain slicing

Mice were fully anesthetized with isoflurane, followed by transcardiac perfusion of 10ml of 4°C NMDG-HEPES artificial cerebrospinal fluid (aCSF) containing (in mM): 92 NMDG, 2.5 KCl, 1.25 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 2 thiourea, 5 Na-ascorbate, 3 Na-pyruvate, 0.5 $CaCl_2 \cdot 2H_2O$, and 10 $MgSO_4 \cdot 7H_2O$, saturated with carbogen (95% O_2/5% CO_2) and pH value adjusted to the range of 7.3-7.4. The transcardiac perfusion of the pre-chilled NMDF-HEPES aCSF solution is used to rapidly slow the metabolism of the brain to decrease neuronal death rate during slicing (Ting et al., 2018). The brain was quickly extracted and 250μm coronal slices of the MHb were sectioned using a MicroSlicer Zero 1N (Ted Pella). After sectioning, slices were transferred into the beaker filled with 150 ml, 32°C NMDG-HEPES aCSF and the Na^+

spike-in procedure is performed by adding Na^+ spike-in solution (2M NaCl in NMDG-HEPES aCSF) every 5mins. The Na^+ spike-in procedure allows the brain slices to gradually accommodate to higher extracellular Na^+ concentration (Ting et al., 2018). This procedure can both increase neuronal survival rate and reliability of the gigaseal formation during patch clamp (Ting et al., 2018). After adding the Na^+ spike-in solution 5 times, the brain slices were transferred to HEPES holding solution containing (in mM): 92 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 2 thiourea, 5 Na-ascorbate, 3 Na-pyruvate, 2 $CaCl_2 \cdot 2H_2O$, and 2 $MgSO_4 \cdot 7H_2O$, saturated with carbogen (95% O_2/5% CO_2) and pH value adjusted to the range of 7.3-7.4. The brain slices were incubated at room temperature at least 1hr before recording.

Patch clamp and drug application

Brain slices were transferred to the recording chamber on Scientific SliceScope (Scientifica) and submerged under 32°C recording solution containing (in mM): 124 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 24 $NaHCO_3$, 5 HEPES, 12.5 glucose, 2 $CaCl_2 \cdot 2H_2O$, and 2 $MgSO_4 \cdot 7H_2O$, saturated with carbogen (95% O_2/5% CO_2) and pH value adjusted to the range of 7.3-7.4. The rate of recording solution perfusing into the recording chamber was 5 ml/min. Ventral MHb neurons were visualized by infrared different interference contrast microscopy (Olympus). Whole-cell recordings were obtained using borosilicate glass pipette electrodes (Sutter Instrument) with resistance 3-5 MΩ. The electrodes were filled with a cesium methanesulfonate based internal solution containing (in mM): 135 KMeSO3, 10 HEPES, 4 $MgCl_2$, 4 MgCl2, 4 Na2ATP, 0.4 NaGTP, 10 Sodium creatine phosphate with a pH value adjusted to 7.3 using KOH.

Data were acquired by a Multiclamp 700B amplifier (Molecular Devices) and a Digidata 1550B digitizer (Molecular Devices) and recorded with pCLAMP 10 (Molecular Devices). All signals were filtered at 5 kHz and sampled at 20 kHz. The series resistance was monitored by applying a hyperpolarizing 10 mV voltage step in voltage clamp mode to calculate the resistance using Ohm's law. If the series resistance varied above 20% across the experiment, the data were excluded due to concerns with the giga-seal state.

Ventral MHb neurons were maintained at -50 mV under current clamp to provide standardized conditions. The baseline was recorded for 5 mins before drug application. Drugs were applied across 0.5 secs with a picosprizer (Parker Hannifin). Drug action was quantified during the 5 secs immediately after picospritzer application. The borosilicate glass pipettes (3-5 MΩ) containing αβ-methylene ATP or MRS2365 were placed ~20 μm away from recording neurons and the pressure meter on the picosprizer was set to 10-12 PSI. To assess the interaction between cholinergic and purinergic signaling, the baseline was recorded for 2 mins followed by bath application of 0.5 μM nicotine for 10 mins. With the picospritzer, αβ-methylene ATP or MRS2365 was applied onto the recording neuron for 0.5 secs while nicotine bath application was maintained for an additional 10 mins. To examine cell activity during nicotine withdrawal, brain slices were obtained from mice injected with nicotine daily for 10 prior days, with the same tissue processing and patch clamp procedures described above.

To examine endogenous purinergic signaling in the presence of an antagonist, neurons were held at -50 mV in current clamp. The aCSF was applied for 5 mins, followed by

bath application of: (1) 5 mins suramin (30 μM), (2) 5 mins of suramin (30 μM) and nicotine (0.5 μM), and (3) 10 mins nicotine (0.5 μM), in sequential order. Neuronal activity was recorded and firing frequency was calculated during each drug bath application. To ensure a return to baseline levels following suramin application, nicotine was bath applied for 10 mins, and the neuronal activity during the last 5 mins was analyzed.

Stereotaxic surgery

The AAV-GRABATP sensor was microinjected directly into the MHb using a stereotaxic device. Mice were anesthetized with 1-3% of isoflurane/ oxygen mixture. The head was fixed and adjusted to flat-skull position on the Kopf stereotaxic frame. Microinjection of 0.5 µl AAV9-hSyn-ATP1.0 (titer: 5*10^13 v.g./ml, WZ Biosceinces, Catlog #YL006001-AV9) was administered bilaterally in the MHb (AP: -1.2 mm, Midline: ±1.06, DV: -2.47mm, injectors at a 20° angle towards midline) at a rate of 0.25µl/min. The injectors remained in place for additional 3 mins before moving 0.2 mm up the dorsal axis, and another 0.5µl of AAV9-hSyn-ATP1.0 was administered at the same infusion rate across 2 min. The injectors remained in the injection site for 3mins before removed from the brain.

Fluorescence imaging of GRABATP in brain slices

After 2.5 weeks from the AAV9-hSyn-ATP1.0 injection, acute brain slices at a 250 μm thickness were obtained from the mice, similar to that described above. Images were acquired on the electrophysiology recording microscope with SciCam Pro Camera

(Scientifica). The frame rate of the image was set to 2 frames per second, and the binning mode at 2 x 2 on the Ocular software (Scientifica). MHb neurons expressing GRABATP were visualized using 488nm LED light. The baseline activity of ATP release in MHb was recorded for 2.5 mins before bath applying 1 μM of nicotine. After 5 mins nicotine exposure (to allow the recording chamber to be fully submerged with nicotine solution), fluorescence activity indicative of ATP release was recorded for 8+ mins.

Statistical Analysis

The number of subjects for each experiment was based on the results of power analysis performed in G*Power 3.1 software (effect size= 0.4, $p<0.05$, power= 0.95). T-tests or analysis of variance (ANOVAs) was performed to fit the experimental design. Following significant main and interaction effects, a Tukey *post-hoc* test was used, with correction for multiple comparisons. Data analysis was performed in GraphPad Prism 7.0 software. The criterion of significance was a=0.05.

Results

Nicotine induces ATP release in the MHb

The expression of purinergic receptors in the MHb have been documented; however, ATP release activity in this brain region remained to be confirmed (Sperlagh et al., 1995; Sperlagh et al., 1998; Kanjhan et al., 1999; Moran-Jimenez and Matute, 2000; Price, G. D., et al., 2003; Florenzano et al., 2008; Guo et al., 2008; Song et al., 2011). Thus, we employed GRABATP sensor imaging to directly monitor real-time ATP release,

which is reflected on the change of fluorescence signal (**Figure 3-1A-D**). In the MHb, we did not visualize changes in GRABATP sensor fluorescence under endogenous conditions, suggesting minimal, if any, ATP baseline release (**Figure 3-1A**). Thus, we next sought to determine whether acute nicotine would alter ATP extracellular activity. Perfusion of 1μM nicotine induced an increase in fluorescence in discrete regions of the brain section, indicative of nicotine-mediated ATP release (**Figure 3-1B, C**). Given that the MHb is highly involved in regulating nicotine withdrawal (Gorlich et al., 2013; Dao et al., 2014; Shih et al., 2014; Fowler and Kenny, 2014; Antolin-Fontes et al., 2015), we next hypothesized that signaling dynamics may be altered following chronic nicotine exposure. In brain sections from mice exposed to nicotine across 10 days, we observed limited ATP release activity in the MHb at baseline, similar to that found in the naïve tissue **(Figure 3-2A)**. Interestingly, re-exposure to nicotine during nicotine withdrawal increased florescence across the duration of recording **(Figure 3-2B, C)**. While these observations suggest that nicotine potentiates ATP signaling during the withdrawal state, quantitative analysis of neuronal activity is warranted to support this conclusion.

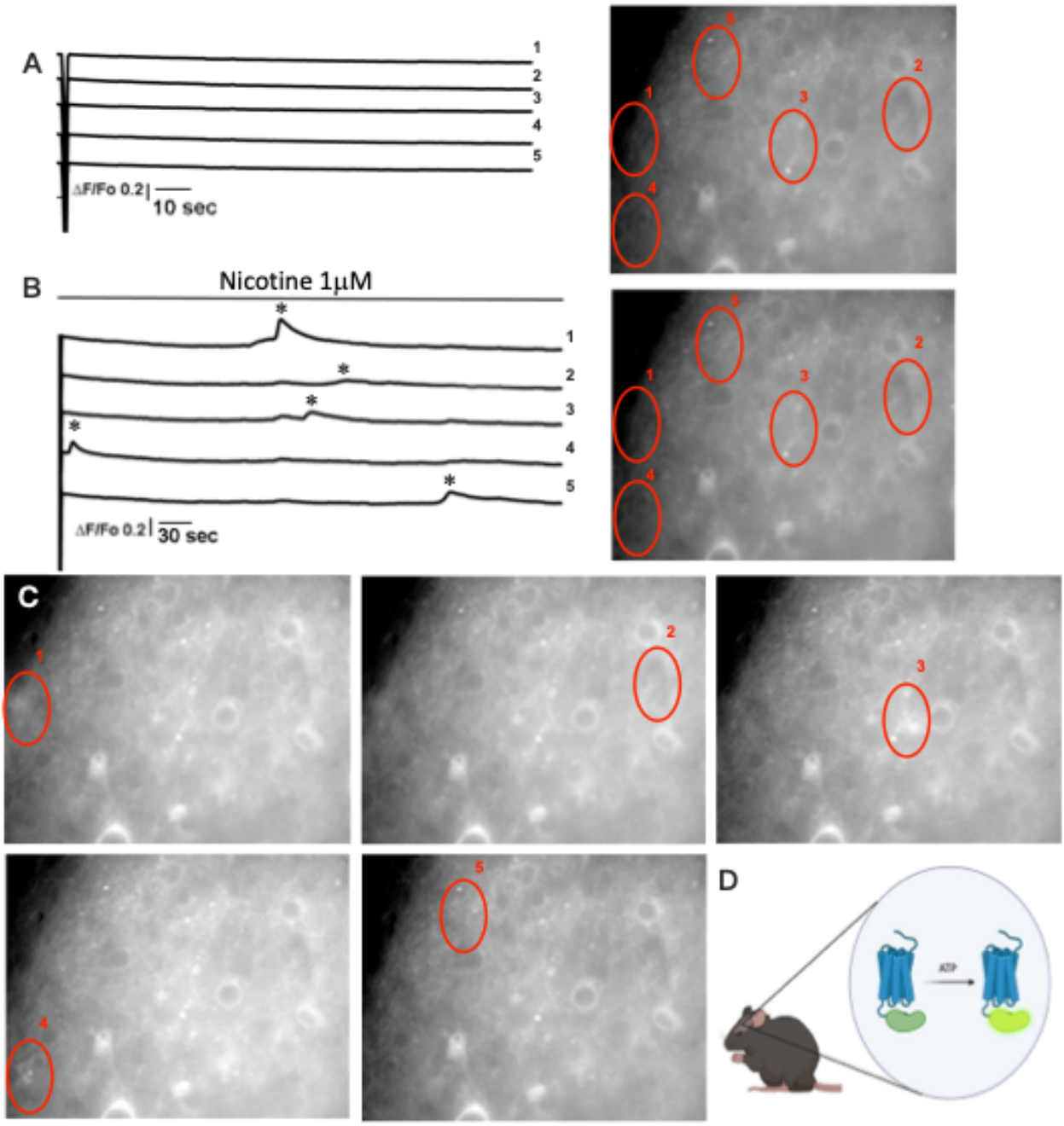

Figure 3-1. Acute nicotine exposure induces GRABATP fluorescence signal in the MHb. MHb sections expressing the GRABATP sensor were imaged to monitor ATP release activity. **(A)** At baseline with aCSF perfusion, little to no changes in fluorescence signal was detected as show with the traces (left) and representative microscopy image (right). In the images, the numbers and red ovals correspond to each respective numbered trace. **(B)** During 1μM nicotine aCSF perfusion, changes in fluorescence signal were observed in the MHb, as shown with the traces (left). The right image shows the baseline level of signal. **(C)** Representative images show the peak of each ΔF/F0

signal trace during 1μM nicotine aCSF perfusion (denoted with ∗ on each numbered trace). **(D)** Illustration of GRAB$_{ATP}$ sensor expressed in the MHb of the brain, in which the presence of extracellular ATP induces a change in fluorescence signal.

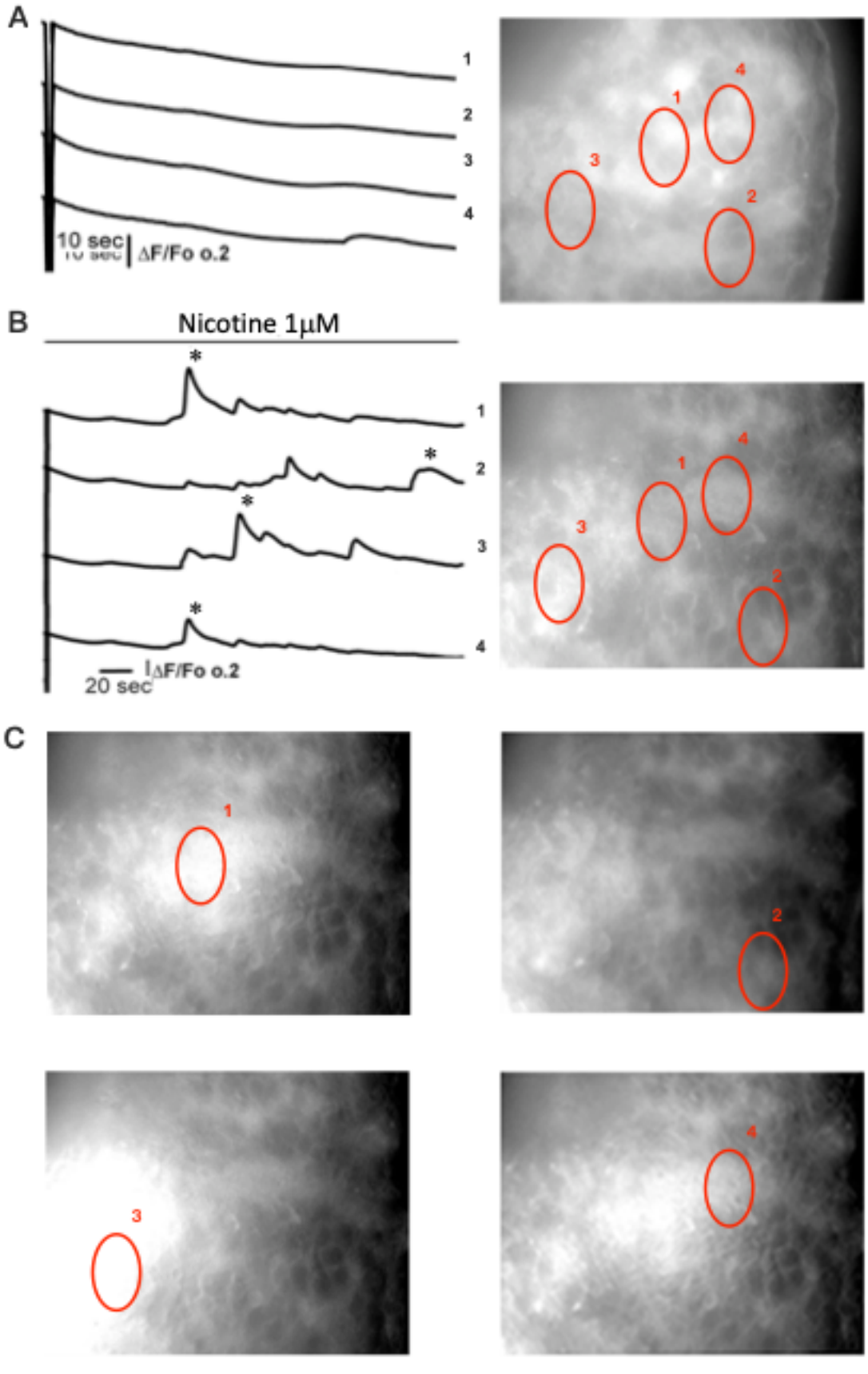
A
1
2
3
4
10 sec
ΔF/Fo o.2
B
Nicotine 1μM
1
2
3
4
20 sec
ΔF/Fo o.2
C

Figure 3-2. During nicotine withdrawal, re-exposure to nicotine induces a robust GRABATP fluorescence signal in the MHb. MHb sections expressing the GRABATP sensor were imaged to monitor ATP release activity. **(A)** Mice were treated with 1.5 mg/kg nicotine for 10 days, and 24 hrs later, brain sections were examined for GRABATP fluorescence. During withdrawal with aCSF perfusion, little to no changes in fluorescence signal was detected, as show with the traces (left) and representative microscopy image (right). In the images, the numbers and red ovals correspond to each respective numbered trace. **(B)** During 1μM nicotine aCSF perfusion, nicotine re-exposure induced changes in fluorescence signal in the MHb, as shown with the traces (left). The right image shows the baseline level of signal. **(C)** Representative images show the peak of each $\Delta F/F_0$ signal trace during 1μM nicotine aCSF re-exposure (denoted with ∗ on each numbered trace).

Blockage of purinergic signaling demonstrates interaction with nicotine

Next, we sought to examine whether endogenous ATP signaling alters neuronal firing in the MHb at baseline. Brain sections were perfused with aCSF containing (1) the P2 general receptor antagonist, suramin, to block endogenous purinergic signaling, (2) nicotine alone, or (3) co-perfusion of nicotine and suramin. Data analysis revealed significant differences among the groups (Repeated measure, one way ANOVA $F_{(3,18)} = 8.601$, $p = 0.0009$, $R^2 = 0.5891$) (**Figure 3-3**). The post-hoc indicated no significant difference in the firing frequency between baseline and suramin perfusion ($p= 0.9998$). This finding is consistent with the observation for GRABATP sensor imaging, in which limited ATP release was observed without the presence of nicotine. Therefore, given that nicotine exposure appeared to elevate ATP signaling with the GRABATP sensor, we then investigated the effect of blocking the action of nicotine-mediated ATP release on habenular neuronal activity by co-perfusing suramin and nicotine. Nicotine induced a significant increase in neuronal firing from baseline levels ($p = 0.002$), and comparing between suramin alone and nicotine alone ($p = 0.0024$). Interestingly, suramin prevented the nicotine-mediated elevation in firing frequency, which was significantly

different from the level of firing found during nicotine alone (p= 0.0079) (**Figure 3-3B, C**). Since both males and females were used, we further examined whether a sex difference was present, but no significant differences were found between males and females in the effect of the suramin on the nicotine-mediated firing frequency increase **(Table 3-1)**. Moreover, one prior study suggested that higher concentrations of suramin may act on NMDA and AMPA receptors (Suzuki et al., 2004), but no statistically significant effects were found at 30 μM in their study, which was the dose used herein. Nevertheless, we conducted a control experiment to assess suramin alone, and no differences were observed in neuronal firing (Paired t-test, $t_{(4)}$=0.5663, p=0.6015, R^2=0.07421; Baseline, 2.682±0.6243 (Mean±SEM), Suramin, 2.840±0.7833). Therefore, these findings suggest a putative interaction between purinergic signaling and cholinergic signaling in MHb cholinergic neurons.

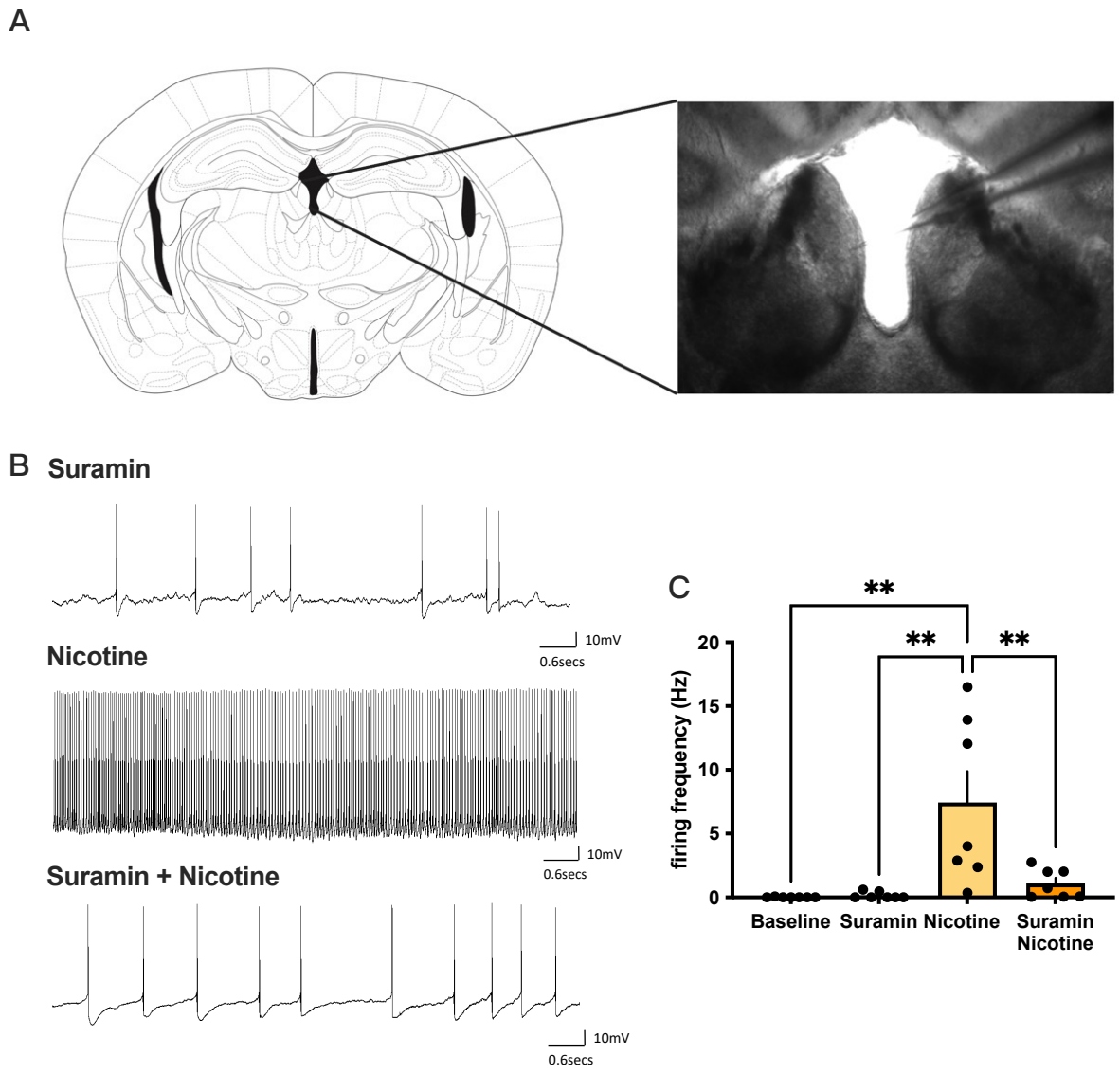

Figure 3-3. Perfusion of the P2 general antagonist prevented the nicotine-mediated firing frequency increase in MHb neurons. (A) Recordings were obtained from neurons located in the ventral part of the MHb. The general P2 antagonist suramin was perfused to examine the effects of endogenous ATP signaling on neuronal firing in the MHb (n=7). **(B)** Representative traces during suramin, nicotine, and co-perfusion. **(C)** Suramin alone did not elicit any significant effect on altering the firing frequency, whereas nicotine increased firing frequency. When suramin and nicotine were co-applied, blockade of the P2 receptors prevented nicotine's ability to increase firing frequency in the MHb neurons. **p<0.01, ***p<0.001, as compared to other conditions. Data are presented as mean ± SEM.

ATP facilitates neuronal firing via P2X and P2Y$_1$ receptors

To first determine whether ATP alone can alter neuronal activity, we examined the effects of the P2X and $P2Y_1$ receptor agonists on firing frequency. It was previously demonstrated that activation of P2X receptors leads to influx of Na^+ and Ca^{2+} (Pankratov et la., 2009; Yang et al., 2012; Burnstock 2015). Therefore, activating the P2X receptors would be expected to induce an excitatory effect on neuronal activity in the MHb. As for $P2Y_1$ receptors, it has been shown that activation of these membrane receptors increases intracellular Ca^{2+} through G-protein coupled signaling (Abbracchio et al., 2006; Guzman and Gerevich, 2016). Furthermore, in mesencephalic trigeminal neurons, ATP was demonstrated to act on $P2Y_1$ receptors to facilitate HCN channel currents (Huang et al., 2010). In the MHb, it has been demonstrated than applying an HCN antagonist abolishes neuronal firing (Gorlich et al., 2013), and thus, activating $P2Y_1$ receptors may promote neuronal activity in this region. In sum, the current evidence from the field leads to the hypothesis that activation of both P2X and $P2Y_1$ receptors would result in elevation of habenular neuronal firing frequency. As a first control measure to validate the approach, we applied vehicle (0.9% saline) with picospritzer application, and no significant change was found in the firing frequency (Paired t-test, $t_{(4)} = 1.730$, $p = 0.1587$, $R^2 = 0.4280$) (**Figure 3-4A, B**). No differences were observed between males and females in this assessment (**Table 3-1**). Next, to investigate the effects of the P2X receptor, we applied the general agonist, $\alpha\beta$-methylene ATP, onto habenular cholinergic neurons, and we found a significant increase in firing frequency with the P2X agonist (Paired t-test, $t_{(13)} = 3.886$, $p = 0.0019$, $R^2 = 0.5374$) (**Figure 3- 4C, D**). Thereafter, we examined the function of the $P2Y_1$ receptor with the specific agonist, MRS2365, and observed a significant increase in

firing frequency in the cholinergic neurons (Paired t-test, $t_{(12)} = 5.545$, $p = 0.0001$, $R^2 = 0.7193$) (**Figure 3-4E, F**). These data indicate that both the P2X and $P2Y_1$ receptors can induce an increase in MHb activity independent of the presence of nicotine.

To further investigate whether ATP differentially affects neuronal activity during nicotine withdrawal, we then examined the effects of the agonists in mice treated with nicotine across 10 consecutive days prior. Thus, brain sections were examined at the 24 hr withdrawal time point, with no nicotine present. Application of the P2X receptor agonist (Paired t-test, $t_{(11)} = 4.377$, $p = 0.0011$, $R^2 = 0.6352$) (**Figure 3-5A, B**) or the $P2Y_1$ receptor agonist (Paired t-test, $t_{(6)} = 3.679$, $p = 0.0103$, $R^2 = 0.6929$) (**Figure 3-5C, D**) significantly increased firing frequencies in cholinergic neurons during nicotine withdrawal. Of note, no difference was found in these studies between males and females for both the effects of P2X or $P2Y_1$ agonists under baseline or withdrawal conditions **(Table 3-1).** To further investigate whether the effects were greater with P2X or $P2Y_1$ agonist application, we then derived the change in neuronal firing from baseline and examined across data sets, but no statistically significant differences were found (Nicotine naïve state, P2X agonist: 2.586±0.6656 (Mean±SEM), $P2Y_1$ agonist: 3.888±0.7603; Nicotine withdrawal state, P2X agonist: 2.835±0.6477, $P2Y_1$ agonist: 3.644±0.9903; One way ANOVA $F_{(3,43)} = 0.7546$, $p = 0.5258$, $R^2 = 0.05001$).

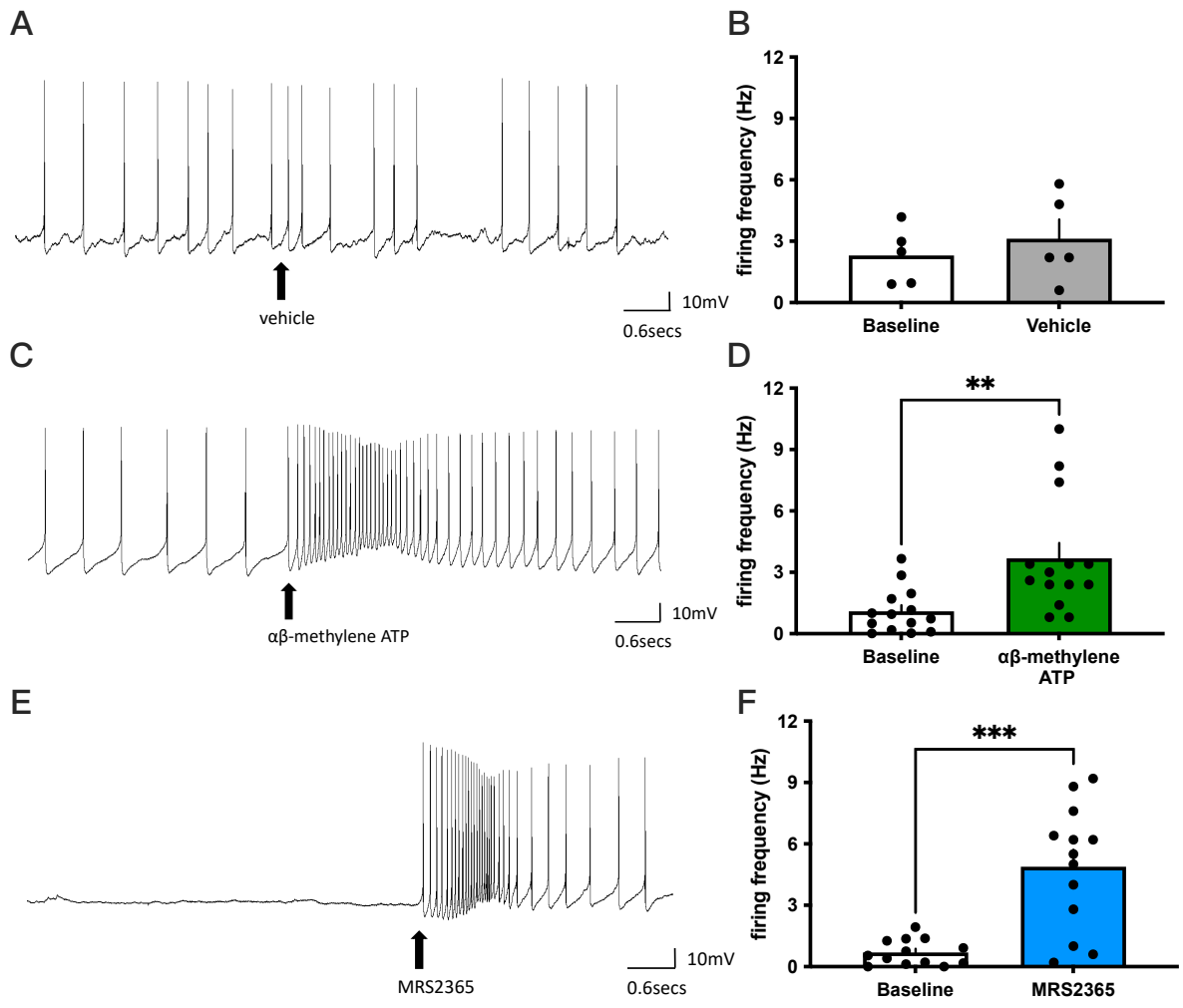

Figure 3-4. The P2X and P2Y$_1$ receptor agonists increase firing frequency in cholinergic neurons. MHb neuron activity was recorded, and then the agonists for the P2X receptor and P2Y$_1$ receptor were applied to examine changes in neuronal firing. **(A)** Representative trace of neuronal activity during vehicle application. The arrow indicates the time point of vehicle application. **(B)** Vehicle application did not alter firing frequency across time (n=5). **(C)** Representative trace of the firing frequency change P2X agonist application. The arrow indicates the time point of αβ-methylene ATP application. **(D)** The P2X receptor agonist significantly increased firing frequency in neurons (n=14). **(E)** Representative trace of the firing frequency change during P2Y$_1$ agonist application. The arrow indicates the time point of MRS2365 application. **(F)** The activation of P2Y$_1$ receptors significantly increased firing frequency (n=13). **p<0.01, ***p<0.001, as compared to baseline. Data are presented as mean ± SEM.

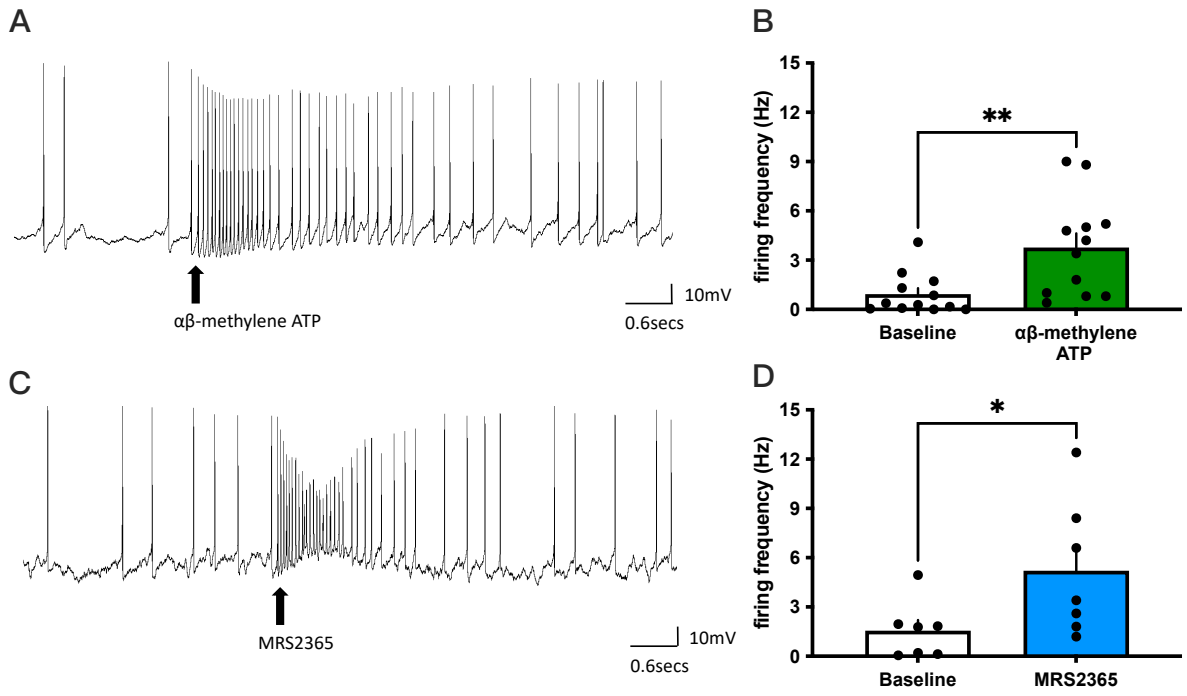

Figure 3-5. P2X and $P2Y_1$ receptor agonists application during nicotine withdrawal increases firing frequency. Twenty-four hrs after nicotine, MHb neuronal activity was recorded, and the agonists of the P2X or $P2Y_1$ receptors were applied. **(A)** Representative trace of the firing frequency change during P2X agonist application. The arrow indicates the time point of αβ-methylene ATP application. **(B)** The P2X receptor agonist significantly increased firing frequency during nicotine withdrawal (n=12). **(C)** Representative trace of the firing frequency change during $P2Y_1$ agonist application. The arrow indicates the time point of MRS2365 application. **(D)** The $P2Y_1$ receptor agonist significantly increased firing frequency during nicotine withdrawal (n=7). *p<0.05, **p<0.01, as compared to baseline. Data are presented as mean ± SEM.

Interaction between nicotinic and ATP signaling during acute nicotine exposure

A prior study reported a potential mutual inhibition between P2X receptors and cholinergic signaling in HEK-293 cells and cultured myenteric neurons (Decker and Galligan, 2009). Given this and our findings above, we hypothesized that the mutual inhibition between purinergic P2X and cholinergic singling would limit the effects of the P2 agonist on neuronal activity. Consistent with this expectation, application of αβ-methylene ATP did not induce a significant change in firing frequency during nicotine

perfusion (Repeated measure, one way ANOVA $F_{(2,24)} = 14.29$, $p < 0.0001$, $R^2 = 0.3191$) (**Figure 3-6A, B**). The post-hoc results indicate that while nicotine perfusion increased firing frequency above baseline levels (baseline vs. nicotine p = 0.0091, baseline vs. nicotine with αβ-methylene ATP, p < 0.0001), no difference was found between nicotine and co-exposure to nicotine and αβ-methylene ATP (p= 0.4550). Furthermore, no significance differences were found between males and females during nicotine perfusion with the P2X agonist (**Table 3-1**). We next examined whether a similar effect is found between cholinergic and $P2Y_1$ receptor mediated signaling and significant differences were found among the treatment groups (Repeated measure, one way ANOVA $F_{(2,20)} = 21.14$, $p < 0.0001$, $R^2 = 0.6789$) (**Figure 3-6C, D**). The post-hoc analysis revealed a significant increase in firing frequency during nicotine exposure (p = 0.0446), and a further statistically significant increase with application of the $P2Y_1$ agonist MRS2365 during nicotine (p= 0.0026). The co-treatment group also differed from baseline firing frequency levels (p < 0.0001). No sex differences were found with co-administration of the $P2Y_1$ receptor and nicotine (**Table 3-1**). These data support the notion of mutual inhibition between the P2X receptor and nicotine, but not the $P2Y_1$ receptor.

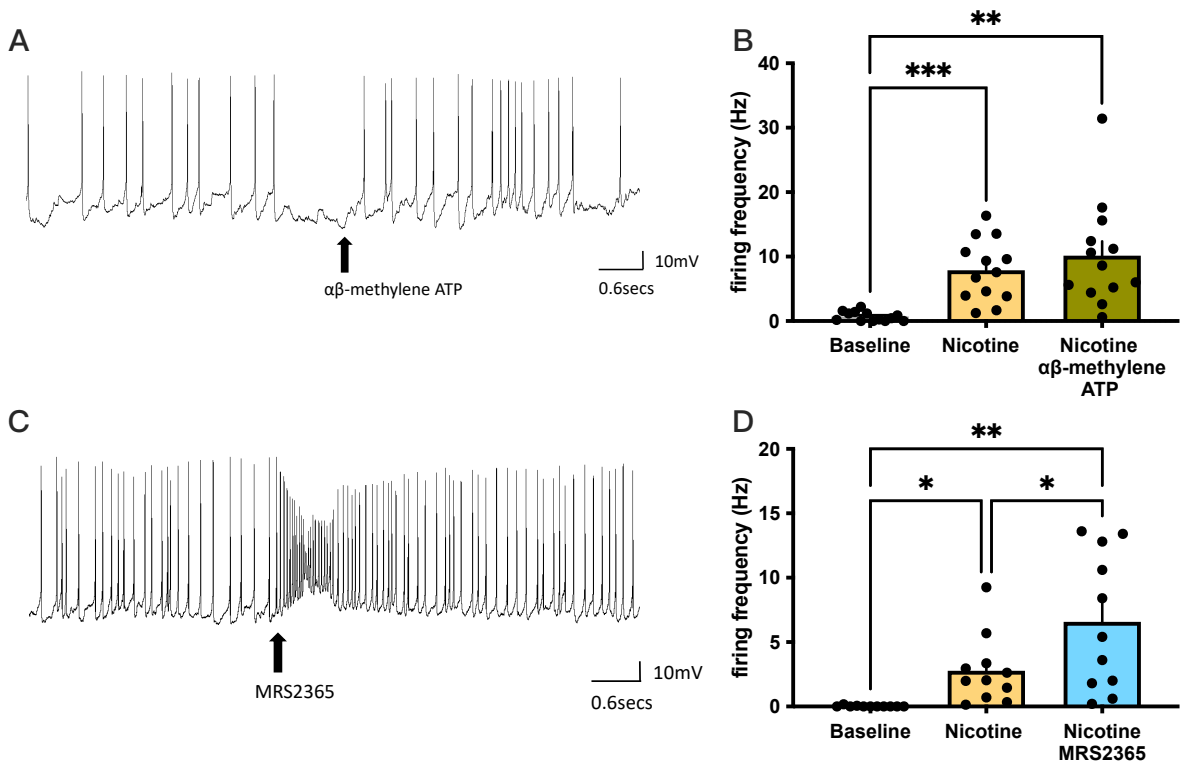

Figure 3-6. Co-exposure to acute nicotine and the $P2Y_1$ receptor agonist potentiates neuronal firing in cholinergic MHb neurons. The interaction between purinergic signaling and nicotinic signaling was examined by applying the purinergic receptor agonists during nicotine perfusion to MHb cholinergic neurons. **(A)** Representative trace of the neuronal firing when the P2X agonist was applied during nicotine perfusion. The arrow indicates the time point of αβ-methylene ATP application. **(B)** The P2X receptor agonist did not further elevate the firing frequency during nicotine perfusion (n=13). **(C)** Representative trace of the neuronal firing change when the $P2Y_1$ agonist was applied during nicotine perfusion. The arrow indicates the time point of MRS2365 application. **(D)** $P2Y_1$ receptor agonist application further elevated the firing frequency during nicotine perfusion (n=11). *p<0.05, **p<0.01, ***p<0.001, as compared to other conditions. Data are presented as mean ± SEM.

Nicotine withdrawal alters the interactive effects of purinergic signaling and nicotine

Given the importance of this neuronal population in mediating nicotine withdrawal and previously documented changes in cellular activity following chronic nicotine exposure (Gorlich et al., 2014; Dao et al., 2014), we sought to determine whether the interaction

between purinergic and cholinergic signaling mediates the altered state of circuit function. Following 10 days of nicotine exposure, the effects of P2X agonist were examined with nicotine re-exposure and with the addition of αβ-methylene ATP (Repeated measure, one way ANOVA $F_{(2,28)} = 39.11$, $p = 0.0151$, $R^2 = 0.2555$) (**Figure 3-S1**). Nicotine increased the firing frequency from baseline ($p = 0.0020$), and this effect was further potentiated in the presence of αβ-methylene ATP and nicotine ($p < 0.0001$). Co-exposure of nicotine and αβ-methylene ATP was also significantly different from baseline ($p < 0.0001$). However, when males and females were separately analyzed, a significant difference was found based on sex. Specifically, the potentiated firing frequency in the presence of nicotine and αβ-methylene ATP was readily observed in females (Repeated measure, one way ANOVA $F_{(2,14)} = 34.03$, $p < 0.0001$, $R^2 = 0.8302$; post-hoc, baseline vs. nicotine $p = 0.0494$, baseline vs. nicotine and αβ-methylene ATP, $p < 0.0001$, nicotine vs. nicotine and αβ-methylene ATP, $p = 0.0002$) (**Figure 3-7A, B**). However, a significant potentiated effect was not found in males (Repeated measure, one way ANOVA $F_{(2,12)} = 13.04$, $p = 0.0001$, $R^2 = 0.6848$; post-hoc, baseline vs. nicotine, $p= 0.0232$, baseline vs. nicotine + αβ-methylene ATP, $p = 0.0008$, nicotine vs. nicotine + αβ-methylene ATP, $p=0.1636$) (**Figure 3-7C, D**). Thus, we next compared the change in firing frequency by subtracting the values for nicotine and αβ-methylene ATP from the values for nicotine alone. Interestingly, this change in firing frequency was significantly higher in females compared to males (Unpaired t-test, $t_{(13)} = 3.422$, $p = 0.0045$, $R^2 = 0.4739$) (**Figure 3-7E**), thereby demonstrating a notable sex difference in cholinergic neuron response. Finally, the effects of the $P2Y_1$ receptor agonist was also examined with nicotine re-exposure during withdrawal (Repeated measure, one way

ANOVA $F_{(2,24)} = 9.927$, $p = 0.0007$, $R^2 = 0.4527$) (**Figure 3-7F, G)**. The post-hoc analysis revealed a significant increase in neuronal firing frequency with nicotine ($p = 0.0054$) and co-exposure of nicotine and MRS2365 ($p = 0.0010$). However, there was no difference in the response to nicotine, either with or without MRS2365 ($p= 0.7789$). Further, no significant difference was found between males and females during withdrawal with the $P2Y_1$ receptor agonist **(Table 3-1).**

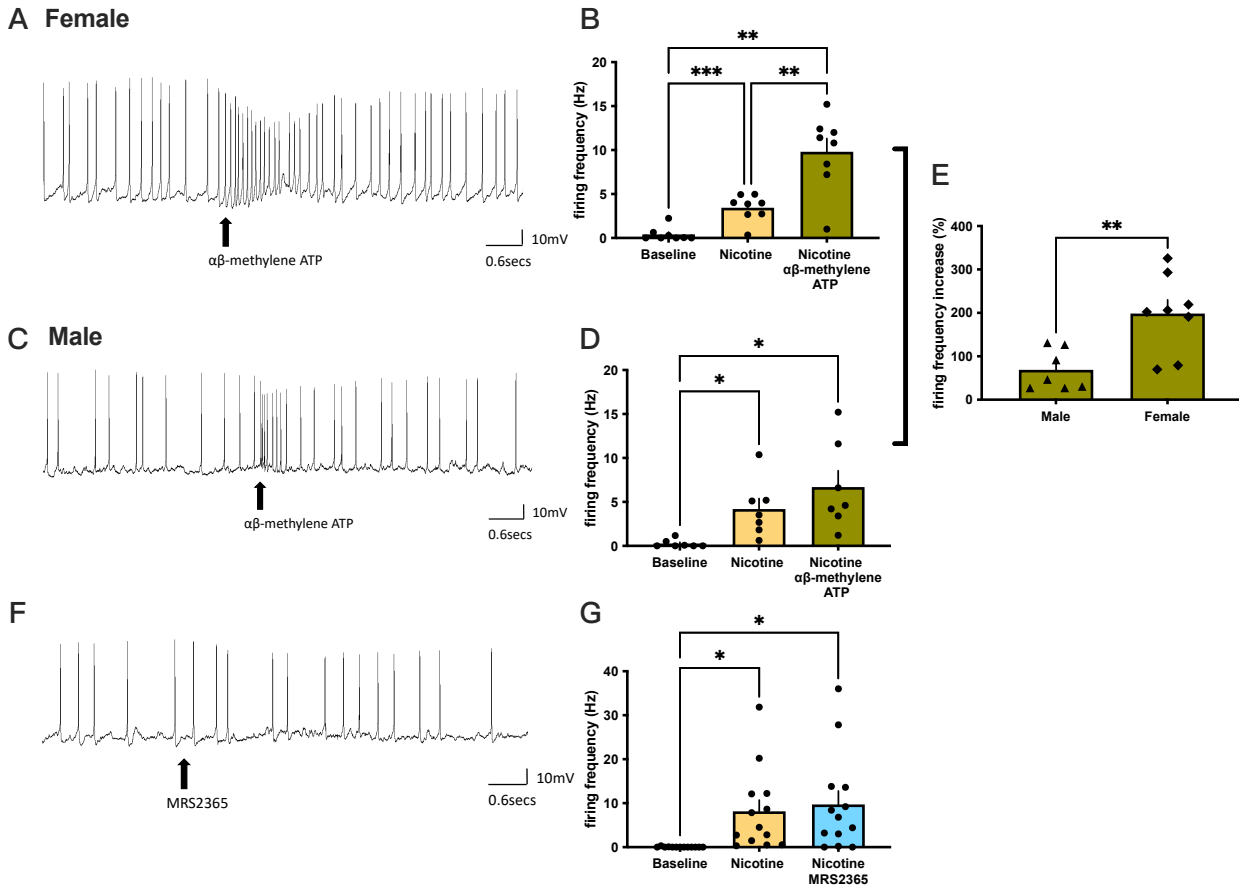

Figure 3-7. During nicotine withdrawal, co-exposure to nicotine and the P2X receptor agonist potentiates neuronal firing in female, but not male, cholinergic MHb neurons. The interaction between purinergic signaling and cholinergic signaling during nicotine withdrawal (24-hr post nicotine) was examined by applying purinergic receptor agonists during nicotine perfusion. **(A)** Representative trace of neuronal firing change in females when the P2X agonist was applied during nicotine perfusion. The arrow indicates the time point of αβ-methylene ATP application. **(B)** In females, the P2X receptor agonist significantly potentiated the firing frequency during nicotine perfusion (n=8). **(C)** Representative trace of neuronal firing in males when the P2X agonist was

applied during nicotine perfusion. The arrow indicates the time point of αβ-methylene ATP application. **(D)** In males, application of the P2X receptor agonist did not significantly elevate the firing frequency during nicotine perfusion (n=7). **(E)** When comparing the P2X agonist effects with nicotine between withdrawal male and female mice, the females exhibited a significantly higher firing frequency elevation than that found in males. **(F)** Representative trace of the neuronal firing when the $P2Y_1$ receptor agonist was applied during nicotine perfusion. The arrow indicates the time point of MRS2365. **(G)** Application of $P2Y_1$ receptors did not elevate the firing frequency during nicotine perfusion in males and females (n=13). *p<0.05, **p<0.01, ***p<0.001, as compared to other conditions. Data are presented as mean ± SEM.

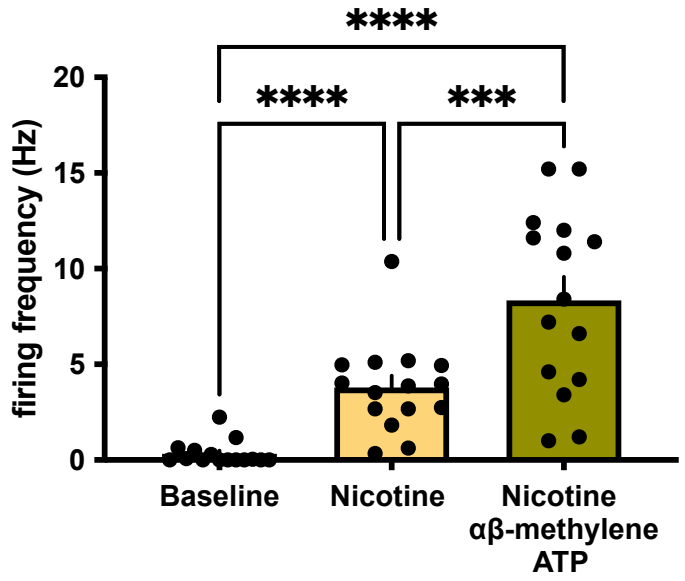

Figure 3-S1 Combined data set of male and female neuronal recordings demonstrating P2X receptor agonist potentiates firing frequency in the MHb when re-exposing to nicotine during withdrawal. The interaction between P2X receptor-mediated signaling and cholinergic signaling was examined during nicotine withdrawal. Application of αβ-methylene ATP significantly increased firing frequency when nicotine was perfused when both males and females were included in the analysis (n=15). ***p<0.001, ****p<0.0001. Data are presented as mean ± SEM.

Figure	Statistical Test	P-value
Figure 3-3C, Suramin	Unpaired t-test $t_{(5)} = 1.909$, $R^2 = 0.4216$	0.1145
Figure 3-4B, Vehicle	Unpaired t-test, $t_{(3)} = 1.44$, $R^2 = 0.4087$	0.2455

Figure 3-4D, αβ-methylene ATP Nicotine naive	Unpaired t-test, $t_{(12)} = 1.674$, $R^2 = 0.1893$	0.1200
Figure 3-4F, MRS2365 Nicotine naive	Unpaired t-test, $t_{(11)} = 0.9556$, $R^2 = 0.07665$	0.3598
Figure 3-5B, αβ-methylene ATP Nicotine withdrawal (no nicotine applied)	Unpaired t-test, $t_{(11)} = 0.1073$, $R^2 = 0.00104$	0.9165
Figure 3-5D, MRS2365 Nicotine withdrawal (no nicotine applied)	Unpaired t-test, $t_{(5)} = 0.2358$, $R^2 = 0.01100$	0.8229
Figure 3-6B, Nicotine + αβ-methylene ATP Acute Nicotine	Unpaired t-test, $t_{(11)} = 1.126$, $R^2 = 0.1033$	0.2843
Figure 3-6D, Nicotine + MRS2365 Acute Nicotine	Unpaired t-test, $t_{(9)} = 0.7889$, $R^2 = 0.0646$	0.4505
Figure 3-7G, Nicotine + MRS2365 Withdrawal and nicotine re-exposure	Unpaired t-test, $t_{(11)} = 0.8077$, $R^2 = 0.05599$	0.4364

Table 3-1. Statistical analyses of the change from baseline values for each treatment, comparing between males and females.

Discussion

In these studies, we reveal that purinergic signaling modulates neuronal activity in the MHb and that these actions become dysregulated with nicotine exposure. Our findings provide evidence that nicotine induces the release of ATP, as observed with the GRAB$_{ATP}$ sensor, and nicotine's effects are interactive with purinergic signaling on the post-synaptic cholinergic neuron, as evidenced by a decrease in nicotine-mediated firing with the P2 antagonist. Both the P2X and $P2Y_1$ receptors independently increase cholinergic neuron firing during a nicotine naïve or withdrawal state. However, in the presence of nicotine, a dramatically different effect becomes evident, which was based on sex and history of drug exposure. Specifically, for the P2X receptor, the agonist had

no observable effect on nicotine-mediated activity, but during nicotine withdrawal, exposure to nicotine and the P2X receptor agonist induced a potentiative effect, which was surprisingly only observed in females. In contrast, for the $P2Y_1$ receptor, a potentiative effect of the agonist and nicotine was found in nicotine-naïve subjects, but during nicotine withdrawal, there was no effect of the agonist on nicotine-mediated firing. These findings suggest that mutual inhibition between purinergic and nicotinic signaling may occur during the first exposure to nicotine with the P2X receptor and following chronic nicotine exposure with the $P2Y_1$ receptor. Taken together, our data provide a complex understanding of the interactions between purinergic and nicotinic signaling on habenular cholinergic neurons and further reveal the impact of nicotine on novel mechanisms leading to altered circuit function.

Independent effects of P2X and $P2Y_1$ receptors in the MHb

Our studies indicate that activation of P2X receptors induce an excitatory effect on neuronal activity in the MHb in naïve subjects. While our studies are the first to reveal the role of purinergic signaling in the MHb on neuronal activity, previous studies have investigated P2X receptors in synaptic transmission in other regions of the brain. For instance, in the CA1 region of the hippocampus, application of the P2X receptor agonist inhibits NMDA receptor-mediated excitatory postsynaptic currents, whereas the P2 general antagonist facilitates the formation of long-term potentiation (Pankratov et al., 2002). It has been proposed that the influx of Ca^{2+} through the P2X channel leads to calcium-dependent NMDA inactivation/desensitization (Legendre et al., 1993; Zhang et al., 1998). While initial findings suggested that NMDA receptors were not expressed in

the MHb (Robertson et al.,1999), more recent cell-specific investigations with transgenic mice have identified functional NDMA receptors in cellular populations of the MHb (Otsu et al., 2018). Thus, these findings indicate that NMDA receptor modulation may be a downstream mediator of P2X function. However, in our studies, we examined the immediate effects of P2X receptor activation on cellular firing, which would have occurred in a more discrete time frame than that required for NMDA receptor modulation through downstream intracellular calcium signaling. Furthermore, we observed an increase in neuronal activity with the agonist, not a decrease as found in the prior NMDA receptor study (Pankratov et al., 2002).

Interestingly, another possibility is that activation of presynaptic P2X receptors may facilitate glutamate release, as demonstrated in the hippocampus (Sperlagh et al., 2002; Khakh et al., 2003; Rodrigues et al., 2005), which would thereby increase AMPA and/or NMDA receptor activation. However, it has also been shown that activation of presynaptic $P2Y_1$ receptors dampen the glutamatergic transmission via inhibition of presynaptic glutamate release in the hippocampus (Rodrigues et al., 2005), and activation of postsynaptic $P2Y_1$ receptors inhibits NMDA receptor-mediated currents in prefrontal and parietal cortex (Luthardt et al., 2003). Given these opposing effects of P2X and $P2Y_1$, one would expect that each agonist would have opposing effects on habenular activity. However, we found increased neuronal activity with either the P2X or $P2Y_1$ agonist, suggesting that different mechanisms predominate in the MHb. As noted above, ATP has been shown to act on $P2Y_1$ receptors to facilitate HCN channel currents in mesencephalic trigeminal neurons (Huang et al., 2010). Given the importance of HCN channels in maintaining spontaneous MHb neuronal activity (Gorlich

et al., 2013), the increased neuronal firing evidenced with activation of the $P2Y_1$ receptor likely involves interaction with HCN channel signaling via downstream calcium-mediated mechanisms.

Interaction between purinergic signaling and acute nicotine

While the P2X receptor agonist and nicotine independently increased neuronal firing, we did not observe an additive effect on firing frequency during co-exposure. This finding may be due to either a ceiling-related effect with cellular activity, or mutual inhibition. Given that higher firing frequencies have been observed in habenular neurons with other experiments (e.g., during nicotine withdrawal and (Gorlich et al., 2013)), a putative ceiling-related effect can be excluded. Interestingly, mutual inhibition between P2X and cholinergic signaling has been suggested by other reports (Decker and Galligan, 2009). Specifically, co-application of ATP and acetylcholine led to a significantly lower current than the predictive summation when ATP or acetylcholine were individually applied, as demonstrated in HEK-293 cells expressing α3β4 nAChRs and P2X receptors (Decker and Galligan, 2009). Another study in acutely dissociated sympathetic neurons also demonstrated mutual inhibition between P2X receptors and nAChR mediated signaling (Searl et al., 1998). However, it should be noted that such inhibitory effects have not been documented in all cell types that express both purinergic and nicotinic receptors, including visceral sensory ganglion (Reyes et al., 2006) and cultured sympathetic neurons (Rogers et al., 1997). Such discrepancies are likely due to the different expression profile of P2X receptor and nAChR subtypes across cellular populations. Since α3β4 nAChRs and $P2X_2$ receptors are both

expressed in the MHb (Zoli et al., 1995; Kanjhan et al., 1999), consistent with the cellular populations demonstrating mutual inhibition (Decker and Galligan, 2009), these findings support the conclusion of mutual inhibition for the effects observed in our study.

Interaction between purinergic signaling and nicotine re-exposure during withdrawal

The P2X agonist induced a potentiated effect on neuronal firing during re-exposure to nicotine in female subjects undergoing withdrawal. One of the possible mechanisms for this finding is the interaction between purinergic and neurokinin signaling. Neurokinin can modulate nicotine-induced firing frequency in MHb neurons and may play a role in nicotine withdrawal (Dao et al., 2014). Further, neurokinin has been shown to potentiate ATP-mediated currents in dorsal root ganglion neurons (Wang et al., 2001). Thus, an interaction between neurokinin and P2X may have resulted in the effects observed. However, while this prior study included both males and females (Dao et al., 2014)., potential sex differences were not examined, and as such, this would not explain why we only observed the differences in females. Moreover, it should be noted that neurokinin would be expected to be present during the acute nicotine application, for which we did not find a potentiated effect. Thus, it remains to be determined whether chronic nicotine induces changes in neurokinin signaling that could explain the differences found during the withdrawal state, or whether such nicotine exposure induces sex-dependent effects on the expression of ATP, neurokinin and/or nicotinic signaling mechanisms. For instance, it has been demonstrated that, in the IPN during nicotine withdrawal, females exhibit an increase in expression of the α5 nAChR subunit,

while males have an increase in the expression of the α2 and α3 nAChR subunits (Correa et al., 2019). Thus, these changes may lead to differences in circuit function, thereby altering symptoms underlying nicotine dependence. Indeed, in humans, women suffer more from adverse symptoms during nicotine withdrawal and are more likely to relapse during quit attempts (Swan et al., 1993; Bjornson et al., 1995; Rojas et al.,1998). Thus, the current findings may provide evidence for a sensitized state in the female brain during nicotine withdrawal, in which nicotine re-exposure leads to altered neuronal activity that promotes further relapse.

Interestingly, with regard to $P2Y_1$ receptor-mediated signaling, we did not observe any potentiation in neuronal activity with nicotine and the agonist, contrary to that observed during acute nicotine exposure. This provides evidence that changes in cellular mechanisms occurred during chronic nicotine exposure, leading to an effect that is reminiscent of the mutual inhibition observed with the P2X receptor in naïve subjects. However, given that the P2X is an ionotropic receptor, and $P2Y_1$ is a metabotropic receptor, the molecular interactions resulting in the effect on neuronal firing likely differ. Thus, it will be of interest in future studies to examine whether such nicotine exposure leads to altered expression of purinergic receptors or downstream intracellular signaling mechanisms.

Limitations

First, for the antagonist study, the general P2 receptor antagonist was employed given that specific antagonists are not available for all of the P2 receptor subtypes. Thus, in the future, it would be interesting to examine the more specific effects of each P2

receptor subtype by manipulating expression at either the pre- or post-synaptic membrane. Secondly, astrocytes appear to express P2X and P2Y receptors, which may contribute to regulating circuit function (Franke and Illes, 2006; Burnstock, 2007; Butt, 2011; Franke et al., 2012). For instance, the $P2X_3$ receptors on astrocytes located in the trigeminal caudal nucleus have been implicated in neuropathic pain (Mah et al., 2017). Further, hippocampal astrocytes expressing $P2Y_1$ receptors appear to enhance neuroprotection through interleukin signaling, but these receptors have also been implicated in astrocyte hyperactivity in an Alzheimer's disease mouse model (Fujita et al., 2009; Delekate et al., 2014). Thus, further studies focused on astrocyte signaling in the MHb during nicotine exposure may reveal additional insight.

Conclusions

By examining the actions of the nAChR agonist, nicotine, on circuit function these data have multiple implications. For instance, the endogenous neurotransmitter, acetylcholine, acts on nicotinic receptors in the MHb, and as such, these findings provide insight into endogenous circuit function. Secondly, humans consume nicotine recreationally, and as such, our findings provide further evidence for the effects of nicotine with tobacco or e-cigarette use. We focused our study in mice during adolescence since increasing use of e-cigarette use in teenagers has become a serious societal and individual health concern. Adolescents are more prone to develop nicotine dependence, and the impact of nicotine on the adolescent developing brain may lead to long-term changes in neural function and mental health (Luna et al., 2010; U.S. Department of Health and Human Services, 2016; Siqueira et al., 2017; Grant et al.,

2019; Ren and Lotfipour, 2019; Mahajan et al., 2021). Finally, these findings reveal an alternative mechanism mediating nicotine withdrawal that can be potential targets for future therapeutic development for nicotine cessation.

DISCUSSION

In this dissertation, different factors that regulate nicotine intake and withdrawal have been discussed. We have shown that inhibiting CYP2A6, the major enzyme that is responsible for metabolizing nicotine, decreases nicotine consumption. This indicates that the metabolic rate of nicotine is an important factor mediating nicotine reinforcement. Since the uprising of e-cigarette use has become a growing concern, a research model is required to allows us to investigate the impact of e-cigarette to brain circuitry. Therefore, we established the nicotine vapor self-administration model in rats and showed promising data that mice may also voluntarily intake nicotine vapor. As most of the studies focus on nicotinic signaling for deciphering the mechanism of nicotine withdrawal, other signaling pathways are also significant in regulating nicotine withdrawal. We have revealed that purinergic signaling modulates MHb activity, and the interaction between purinergic signaling and nicotinic signaling becomes differentially altered during nicotine withdrawal. In sum, our studies have advanced our understanding of nicotine dependence and provided a foundation to develop alternative and better nicotine cessation treatments.

Advancing cessation therapeutics

As the efficacy of current FDA-approved treatments for nicotine cessation are low, inhibiting CYP2A6 activity is a feasible approach for new therapeutics. The success rate of staying abstinence after one year was only 20.5% with varenicline and 18.6% with bupropion (Benli et al., 2017). Although the combination of varenicline and nicotine

replacement therapy (NRT) may increase the success rate of nicotine cessation by 15%, it can also cause higher incidence of adverse effect such as nausea, sleep disturbance, constipation, skin reactions to nicotine patch and depression (Koegelenberg et al., 2014). While more studies correlate the nicotine metabolic rates with the severity of nicotine dependence and smoking behavior, a tailored cessation treatment based of CYP2A6 genotype may be more efficient than standard therapeutic options (Kutobta et al., 2006; Heitjan et al., 2008; Ray et al., 2009; Akrodou, 2015; López-Flores et al., 2017). A combination of CYP2A6 inhibitor with standard treatments can result a higher efficacy in treating nicotine dependence.

While it is widely recognized that CYP2A6 is mainly expressed in liver, it should be noted that it is also found in the brain (Higashi et al., 2007a). Difference in CYP2A6 activity in the brain may influence the duration of nicotine activation in nAChRs and therefore, altering smoking behavior. For instance, high activity of CYP2A6 in MHb may alter the duration of nAChRs activation on cholinergic neurons and therefore affecting the aversive response to nicotine. Furthermore, it has been shown that CYP2A6 activity is induced by estrogen via activation of estrogen receptors (Higashi et al., 2007b). The activity of CYP2A6 may be enhance by estrogen if CYP2A6 and estrogen receptors are co-expressed in the same region of the brain (Higashi et al., 2007b; Zarate et al., 2017). Although CYP2A6 inhibitor, DLCI-1 induced similar effect in inhibiting nicotine intake between male and female mice (Chen et al., 2020), the interaction between CYP2A6 and estrogen may be the potential mechanism for higher relapse rate in females (Swan et al., 1993; Bjornson et al., 1995; Rojas et al.,1998; Allen et al., 2016). Future studies

should address the action of CYP2A6 in the brain and its interaction with estrogen to better understand the factors mediating nicotine dependence and to design personalized cessation treatments.

CYP2B6, another enzyme which also metabolizes nicotine, may participate in nicotine reinforcement (Ray et al., 2009; Garcia et al., 2015). Although CYP2B6 plays a minor role in hepatic metabolism of nicotine, it is expressed in the brain (Ray et al., 2009; Garcia et al., 2015). Thus, the activity of CYP2B6 can influence the nicotine level in smoker's brain. Furthermore, bupropion is metabolized by CYP2B6 and the activity of CYP2B6 can affect the efficacy of the treatment (Lee et al., 2007). It has been shown that smokers with reduced activity in CYP2B6 responded better to bupropion as cessation treatment (Lee et al., 2007; Akrodou, 2016). Interestingly, the expression of CYP2B6 gene is regulated by estrogen via binding with estrogen receptors in human breast cancer cell (Lo et al., 2010). Although it remains to be investigated whether the CYP2B6 gene expression is also regulated by estrogen in the brain, the interaction between CYP2B6 and estrogen can be another potential factor that contributes to sex difference in nicotine dependence (Allen et al., 2016). Studying the relationship between the enzyme activity of CYP2B6 in brain and nicotine dependence may contribute to the development of alternative treatments with higher efficacy.

Novel approaches to investigate nicotine dependence during adolescence

Adolescent smoking has always been a concerning issue as adolescent brains are more susceptible to develop nicotine dependence (Karpinski et al., 2010; Doubeni et al.,

2010; Strong et al., 2016; Siqueira et al., 2017; Ren and Lotfipour, 2019; Mahajan et al., 2021). Even monthly smoking can elevate the risk of developing nicotine dependence in adolescents by 10-fold as compared to adult smokers (Doubeni et al., 2010). While the brain during adolescence is in a critical stage of developing cognitive control and executive function, it is vulnerable to the influence of nicotine (Luna et al., 2010; Siqueira et al., 2017; Ren and Lotfipour, 2019; Mahajan et al., 2021). The use of nicotine during adolescence elevated the risk of developing mental illness, like anxiety and depression, and other drug abuse (U.S. Department of Health and Human Services, 2016; Grant et al., 2019; Ren and Lotfipour, 2019). With appealing additives and flavors have been manufactured into e-cigarettes to mask the harshness of nicotine, adolescents are more attracted to initiate vaping (Kreslake et al., 2008; Tierney et al., 2016; Siqueira et al., 2017). While there is a recent uprising use of e-cigarette, there is a lack of treatments for nicotine cessation specific for adolescents, as most of the therapeutics are developed based on adult smoker studies (CDC, 2019; Gray et al., 2019). Varenicline did not improve the end-of-treatment abstinence rate after a 12-week treatment in participants with the age ranging from 14-21. (Gray et al., 2019). Six weeks of 300 mg bupropion per day in adolescents (age from 14-17) resulted only 14.5% of abstinence at the end of the treatment (Muramoto et al., 2007). Furthermore, the use of bupropion increases the risk of suicidal thoughts and behavior in adolescents (Karpinski et al., 2010). The NRT, including gums and transdermal patches, have been used to help adolescent to quit smoking (Karpinski et al., 2010). However, as we demonstrated, chronic nicotine exposure leads to alteration of brain circuit activity. NRT may increase the risk of developing nicotine dependence and the likelihood to administer other drugs

(Karpinski et al., 2010). Therefore, a tailored nicotine cessation treatment for adolescents is required. The vapor self-administration model is the optimal model that simulate human vaping and we can utilize this model to develop therapeutic interventions to prevent adolescents to develop nicotine dependence. Furthermore, it should be acknowledged that adolescents may experience different withdrawal symptoms than adults. Adult rats demonstrated significant higher conditional placed aversion and somatic signs compared to adolescent rats during mecamylamine-precipitated withdrawal (Shram et al., 2008). In humans, adult smokers reported to experience higher rates of restless during nicotine withdrawal while there is a higher rate of withdrawal related nervousness reported in adolescent smokers (Pergadia et al., 2010). Thus, the differences of nicotine withdrawal between adolescents and adult should be further investigated and taken into consideration while designing specific treatments for adolescent smokers.

To understand nicotine withdrawal during adolescence, we have demonstrated that purinergic signaling modulates MHb activity during nicotine withdrawal. Purinergic signaling may be a potential therapeutic target to develop cessation treatment for people during adolescence. We have shown that exposure to P2X agonist potentiates neuronal firing in the MHb while re-exposure to nicotine during withdrawal in females. If increasing of MHb neuronal firing frequency is correlated with elevated aversion responses to nicotine, treating women with P2X agonist during relapse can elevate the aversion reaction to nicotine; therefore, nicotine consumption will decrease. As women have higher rate of relapse (Swan et al., 1993; Bjornson et al., 1995; Rojas et al.,1998),

P2X agonist may be utilized to help women during adolescence to quit smoking more effectively than traditional therapeutics. However, it remains to be further investigated of how MHb activity is involved in nicotine withdrawal. Furthermore, it will be interesting to study whether the interaction between purinergic signaling and nicotinic signaling alters during nicotine withdrawal in adulthood.

Not just the purinergic signaling itself, the interaction of different signals and purinergic signals and how this interaction modulate neuronal firing in the MHb should be studied to fully understand the role of MHb activity in nicotine withdrawal. Previous studies indicate that there may be interactions between purinergic signaling with HCN channel mediated and neurokinin signaling (Wang et al., 2001; Huang et al., 2010; Gorlich et al., 2013; Dao et al., 2014). In addition, opioid signaling pathway may interact with purinergic signaling in the MHb. It has been demonstrated that the infusion of naloxone potentiates P2X receptor mediated signaling in cardiac sympathetic sensory nerves (Fu et al., 2019). In addition, blocking $P2X_4$ or $P2X_7$ receptor in medial prefrontal cortex enhances the analgesia effect in morphine tolerant mice (Tai et al., 2010). As for MHb, the expression of μ- opioid receptor has been identified (Gardon et al., 2014; Boulos et al., 2019). Mice with conditional knockout of μ- opioid receptor on β4 nAChR subunit expressing neurons showed less morphine withdrawal symptoms when injected with naxolone or mecamylamine (Boulos et al., 2019). This finding indicates that there is a possible crosstalk between cholinergic signaling and opioid signaling (Boulos et al., 2019). It is likely that purinergic signaling may interact with opioid signaling in the MHb. Furthermore, this interaction may participate in modulating nicotine intake behavior. The

effect of opioid signaling to MHb activity should be first identified before investigating the interaction between purinergic signaling and opioid signaling in the MHb.

The role of purinergic signaling in neurodegenerative diseases and pain have been intensely studied; however, the role of purinergic signaling in nicotine addiction remains to be further explored (Burnstock, 2017). Not just in the MHb, P2 receptors are also expressed in brain regions, such as VTA and prefrontal cortex, that are important in regulating nicotine intake (Xiao et al., 2008; Zeng et al., 2021). In VTA, P2 receptor mediated signaling is involved in regulating the activity of GABAergic neuron terminals that innervate dopaminergic neurons (Xiao et al., 2008). Interestingly, activation of P2X receptors and $P2Y_1$ receptors in GABAergic terminals result in different actions on dopaminergic neurons in VTA (Xiao et al., 2008). Activation of P2X receptors in GABAergic terminals elevates the frequency of spontaneous inhibitory postsynaptic currents (sIPSCs), whereas activation of $P2Y_1$ receptors in GABAergic terminals decreases the frequency of sIPSCs (Xiao et al., 2008). As we proposed that P2X agonist can be a potential cessation therapeutic, it can be applied to increase the activity of GABAergic terminals acting on dopaminergic neurons in the VTA. The inhibition of dopaminergic neurons in VTA may lead to alleviation of the rewarding effect of nicotine. It remains to be further investigated how purinergic signaling modulates dopaminergic neurons activity in VTA specifically during nicotine withdrawal. In conclusion, besides cholinergic signaling, purinergic signaling may also play a significant role in nicotine dependence and it can be an alternative mechanism for future cessation therapeutic target.

Conclusion

While the current treatments for nicotine dependence are only modestly effective, we should consider all factors that regulates nicotine dependence, not just the reinforcing effect of nicotine, to develop treatments that are more efficacious. More studies are required to understand how metabolism of nicotine and nicotine withdrawal regulates nicotine dependence to provide a foundation for designing a tailored and effective treatment. Furthermore, different approaches should be implanted to consider age as an important factor in regulating nicotine dependence to better assist adolescents in nicotine cessation.

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