THE EFFECTS OF NICOTINE AND OPIOID CO-USE ON DOPAMINERGIC AND GABAERGIC ACTIVITY IN THE VENTRAL TEGMENTAL AREA

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This thesis is dedicated to Troy Periera and to all the other friends, sons, brothers, daughters, sisters, mothers, fathers, etc. who have lost their lives to opioids. Fighting an addiction is an admirable but too often fruitless effort. Here is to honoring that struggle.

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ABSTRACT

THE EFFECTS OF NICOTINE AND OPIOID CO-USE ON DOPAMINERGIC AND GABAERGIC ACTIVITY IN THE VENTRAL TEGMENTAL AREA

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The opioid epidemic has emerged as one of the leading public health concerns of the 21st century. It has been paralleled by a rise in the popularity of e-cigarettes, which has facilitated increased nicotine use among adolescents. Clinical studies have revealed that the co-use of nicotine and opioids is exceedingly common; most individuals with opioid use disorders (OUDs) also report smoking, and smoking is a strong predictor of opioid misuse. Preclinical studies have explored the biological basis for the clinical phenomenon, revealing that co-exposure to nicotine and opioids may produce cross-sensitization to drug reward. Considered the canonical reward pathway, the mesolimbic dopamine system primarily comprises dopaminergic (DAergic) neurons projecting from the ventral tegmental area (VTA) to the nucleus accumbens (NAc). The VTA also contains a substantial population of y-aminobutyric acid (GABA) neurons, which provide local inhibitory control over DA neurons, but also project distally. Though nicotine and opioids have each separately been shown to alter the activity of VTA DA and GABA neurons, little is known about the way that simultaneous use of these drugs may further modify the mesolimbic system. This dissertation establishes a physiologically relevant model for the co-use of nicotine and opioids, with chronic exposure to nicotine vapor beginning in adolescence, followed by a 6-week co-exposure to both nicotine vapor and morphine via continuous two-bottle choice (C2BC), during adulthood. We use this drug administration paradigm in conjunction with calcium imaging (fiber photometry) to compare how the response of VTA DA and GABA neurons to nicotine vapor changes following exposure to chronic adolescent nicotine and then co-exposure to nicotine + morphine. We report that VTA DA neurons are especially sensitive to the effects of adolescent nicotine exposure, whereas VTA GABA neurons are uniquely altered by co-exposure to nicotine and morphine. In this way, we highlight the feasibility and utility of studying polysubstance use in preclinical models and point to VTA GABA neurons as a potential therapeutic target for the co-treatment of concurrent nicotine and opioid dependencies.

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CHAPTER 1: A GENERAL INTRODUCTION

1.1 Opioid Use Disorder in the United States

1.1.1 Definition and Prevalence of Opioid Use Disorder

Opioid Use Disorder (OUD) is broadly defined as a problematic pattern of opioid use that leads to significant problems or distress (American Psychiatric Association, 2013). More specifically, individuals with OUD are likely to experience social, professional, and physical consequences resulting from their drug use. These varied consequences include significant time spent using or seeking drugs, cravings, multiple attempts to stop using, tolerance leading to increased drug usage, drug use in risky situations, withdrawal, and inability to meet responsibilities (American Psychiatric Association, 2013). Worldwide, OUD afflicts nearly 16 million individuals, and 2.1 million Americans report the misuse of opioids (Chang et al., 2018). Of particular concern, OUD has also become much more deadly in recent years. In the last decade, there was a near quadrupling of opioid-related overdose deaths in the United States, from 21,809 in 2010 to 80,411 in 2021 (Spencer et al., 2022). This increase in mortality can be traced to the introduction of potent synthetic opioids like fentanyl, which ultimately led the U.S government to declare the opioid epidemic a public health emergency in 2018 (Jones et al., 2020; U.S. Government Accountability Office, 2018).

1.1.2 The discovery of morphine: a powerful analgesic with high abuse liability

Historical evidence suggests that, as long ago as 3000 BC, the opium poppy, *Papaver somniferum*, was cultivated by ancient Sumerians and ingested for its euphoric and analgesic properties (Salavert et al., 2020). However, it wasn't until 1806 that German pharmacist Friedrich Wilhelm Adam Sertürner first isolated morphine from the opium poppy. He named morphine "Morphium" after the Greek god of sleep and dreams "Morpheus" (Krishnamurti & Rao, 2016). Morphine provided a way for physicians to manage pain with regulated doses and

mitigated the risk of overdose associated with raw poppy juice, which could be variable in potency (Hamilton & Baskett, 2000). Three more active compounds, codeine, thebaine, and papaverine, were subsequently isolated from the poppy plant. Later, scientists would generate hundreds of other opioid derivatives, including semi-synthetic compounds, like heroin, oxycodone and buprenorphine, and synthetic compounds, like methadone and fentanyl. Yet, today, morphine remains the most widely used analgesic and the standard against which all other new opioids for the clinical management of pain are measured (Dowell et al., 2023; Pathan & Williams, 2012).

Although the analgesic properties of morphine and other opioids were revolutionary for the clinical treatment of acute and chronic pain, the abuse liability of these drugs also became quickly apparent. In the United States, morphine addiction became prominent after the Civil War, when, over the course of 4 years, nearly 10 million opium pills were prescribed to soldiers to treat pain associated with battlefield injuries (Brownstein, 1993). At the same time, because of the invention of the hypodermic needle in 1856, it became easier for doctors to use morphine to treat a variety of physical and mental ailments (Paladini et al., 2023). Accordingly, between 1840 and 1890, opioid use in the U.S. rose over 538%. Doctors were especially apt to use morphine to treat issues specific to women, including menstrual cramps, morning sickness, and, of course, general “hysteria” (Presley & Lindsley, 2018). By the turn of the 20th century, hundreds of thousands of Americans, 60% of whom were women, were addicted to morphine and the United States was in the midst of its first opioid epidemic (Golub et al., 2015; Pasternak & Pan, 2013)

1.1.3 A brief history of the 21st century opioid epidemic

Throughout the 20th century, there were continual fluxes in the prevalence of opioid use disorder in the United States, driven by a variety of factors, namely economic turmoil, war and

racism. The roots of the 21st century opioid epidemic trace back to the mid-1990s, when Purdue Pharma began a massive campaign to promote the prescription of their reformulated oxycodone pill “Oxycontin” (Alpert et al., 2022). Purdue falsely claimed that Oxycontin was much less addictive than other opioids and therefore provided a safer means by which doctors could help relieve chronic pain (Pappin et al., 2022). These claims, based on little to no scientific evidence, would have massive consequences in the long-term, leading to a near doubling of the prescription of opioids, from 43.8 million in 1999 to 89.2 million in 2010 and, consequently, during the same time, a doubling of the rate of opioid-related overdose deaths, from 2.9 to 6.8 per 100,000 (Congressional Research Service, 2022). Eventually, during the late 2000’s, when the true abuse liability of Oxycontin eventually came to light, the number of opioid prescriptions began to drop. However, this also meant that those who had developed an OUD because of prescription opioids were left desperate to find an alternative.

Due to its falling market price and widespread availability, heroin became the new opioid of choice for many individuals with OUD. This led to a “second wave” of the opioid epidemic, as heroin deaths increased from 1 to 4.9 per 100,000 between 2010 and 2016, surpassing prescription opioids as the leading cause of opioid-related deaths (Congressional Research Service, 2022). Shortly after, the introduction of illicit fentanyl engendered a “third wave” of the opioid epidemic, which would prove even more deadly than the preceding waves. Fentanyl is a synthetic opioid, 50 times more potent than heroin, typically mass manufactured in laboratories overseas and then smuggled into the United States (Kilmer et al., 2022; Pergolizzi et al., 2021). Because it can be mass produced, fentanyl is extremely cheap, and is therefore often mixed, without the knowledge of the individual taking the drug, into more expensive opioids like heroin, as well as into non-opioid drugs like cocaine (Duhart Clarke et al., 2022; Duncan Cance et al., 2023; LaForge et al., 2022). Unfortunately, because of its high potency, fentanyl is often fatal for

users, which led opioid-related overdose deaths to rise once again, from 10.4 to 21.4 per 100,000 between 2015 and 2020 (Spencer et al., 2022).

1.2 Nicotine Use and the Rise of E-Cigarettes in The United States

1.2.1 Smoking in the United States, a historical perspective

Though the U.S. has seen massive increase in opioid use in the last two decades, there has been, at the same time, a less drastic, but still meaningful, decline in the use of combustible cigarettes (Sun et al., 2021). Nicotine is the primary addictive compound found in tobacco, a plant which has been grown and smoked by indigenous tribes in South America as early as the first century BC (Mishra & Mishra, 2013). The invention of combustible tobacco cigarettes in the 19th century popularized smoking in the United States, as it provided a more compact and convenient way to consume nicotine, compared to more involved alternatives like chewing tobacco, or smoking cigars and pipes (Dani & Balfour, 2011). In the U.S., nicotine use peaked in the 1960's, when over 40% of Americans reported regularly smoking cigarettes (Cummings & Proctor, 2014).However, there rates began to fall shortly after when the Surgeon's General published its shocking findings on the negative health outcomes associated with cigarettes- reporting a 70% higher mortality rate for smokers compared to non-smokers (Cummings & Proctor, 2014). In fact, by 2005, smoking prevalence in the United States had dropped to 20.9%, nearly half of what it was at its peak (CDC, 2005). Today, only 11.5% of Americans report regularly smoking cigarettes, although, despite this decline, smoking is still responsible for 480,000 deaths per year in the U.S. (Cornelius et al., 2023) .

The decline in cigarette smoking in the United States- particularly during the late 1990's and early 2000's- can be attributed, in part, to anti-smoking campaigns aimed at preventing teens and young adults from initiating cigarette use (Meza et al., 2020). In 1998, the Master Tobacco Settlement Agreement was enacted, requiring the largest tobacco companies in the

U.S. to repay billions of dollars each year to states for increased health care costs associated with treating smoking-related illnesses (Jones & Silvestri, 2010). A significant portion of this payment was put toward creating policies and programs designed to reduce adolescent cigarette smoking. This included the establishment of the American Legacy Foundation, which began a massive media and youth education campaign- the “Truth Initiative”- that presented stark facts about the impact of cigarette smoking on physical health and unmasked the dubious marketing practices of tobacco companies (Rath et al., 2021). This initiative, and others like it, were massively effective; among high school students, smoking rates fell from 20% to 3% between 2000 and 2021 (Gentzke et al., 2022; Wang et al., 2019).

1.2.2 The birth of JUUL and the ensuing adolescent vaping ‘epidemic’

But then, just as the U.S. government was beginning to mobilize resources to fight the opioid epidemic, a new threat to public health, specifically the health of adolescents, emerged. In 2015, the first JUUL - a sleek, nicotine-containing electronic cigarette (e-cig) - was made commercially available. Though e-cigs had been on the market since 2007, older models were bulky and contained low concentrations of nicotine, making them inconvenient and unpalatable (Dinardo & Rome, 2019; Walley et al., 2019). Conversely, JUUL and other companies that followed suit, packaged their high-concentration nicotine products into tiny, inconspicuous devices, making ‘vaping’ both more appealing and more addictive (Nardone et al., 2019; R. Williams, 2020). These companies attempted to advertise their new e-cigarettes as both a safer alternative to combustible cigarettes and a means to quit smoking (Huang et al., 2019; R. Williams, 2020). However, mirroring the false marketing of Oxycontin by Purdue, these claims were misleading; in fact, though nicotine content was initially hidden from their label, JUUL’s products contained upwards of 5% nicotine, the equivalent of 20 combustible cigarettes, making them highly addictive (Prochaska et al., 2022).

Even more insidious, e-cigarette companies like JUUL also created a wide variety of flavored, nicotine-containing products- with names like Kool Aid and Cotton Candy- designed specifically to appeal to teenagers (Kong et al., 2019; Pepper et al., 2016; Vargas-Rivera et al., 2021). This tactic would prove frighteningly successful; by 2019, nearly 81% of middle and high school students who reported ever using a tobacco product said that the first product they used was flavored (Vallone et al., 2019; Walley et al., 2019). Yet, at the same time, because nicotine content of these products was not explicitly advertised, many adolescent users did not realize they were consuming nicotine. In fact, 39% of adolescents did not consider JUULs to be e-cigarettes and that 63% of adolescents did not know JUULs contained nicotine at all (Morean et al., 2019). In this way, the appeal of added flavoring, coupled with dubious marketing and limited regulation, led e-cigarettes to soar in popularity among teenagers. By 2019, 27.5% of high schoolers reported regularly using e-cigarettes, up from only 1.5% in 2011 (Huey & Granitto, 2020).

Vaping, though marketed as a safer alternative to smoking combustible cigarettes, presents significant consequences to users, especially adolescents. E-cigarette vapor contains numerous harmful chemicals including acetaldehyde, acrolein, and formaldehyde, which are known to cause adverse physical side effects (Cao et al., 2020; Goniewicz et al., 2014; Kosmider et al., 2014). Specifically, these chemical additives have been linked to acute lung injury as well as long-term lung and cardiovascular disease (Behar et al., 2018; Christiani, 2020; Gaur & Agnihotri, 2019; Qasim et al., 2017). Especially concerning for adolescents who prefer flavored e-cigarettes, cinnamon, strawberry, and menthol flavorants may dysregulate the cellular inflammatory response and increase cytotoxicity (Barrington-Trimis et al., 2014; Jabba & Jordt, 2019; Sundar et al., 2016). Behaviorally, nicotine exposure in adolescence has been associated with cognitive deficits, increased impulsivity and impairment in memory and

executive function (Demissie et al., 2017; Leslie, 2020; Livingston et al., 2022; Ren & Lotfipour, 2019).

Use of nicotine in adolescence can also promote the use of other substances, including combustible cigarettes, later in life. In fact, 90% of adult daily smokers initiate nicotine use before the age of 18 and using e-cigarettes makes an individual 3.6 times more likely to use combustible cigarettes in adulthood (Barrington-Trimis et al., 2020; Cantrell et al., 2018; Leventhal et al., 2015). Additionally, a comprehensive survey of over 50,000 adolescents conducted by the National Institute of Drug Abuse (NIDA) revealed that adolescents who vaped were 20 times more likely to use cannabis and 5.6 times more likely to have binge drank on at least one occasion (SAMHSA, 2019). This relationship between the use of nicotine and the use of other substances has also been observed in the adult population. Individuals who meet criteria for nicotine addiction are more likely to report past-year alcohol use disorder (16.5% vs 6.3%), as well as a past-year other substance use disorder (12.7% vs 3.0%) (SAMHSA, 2019).

1.3 Nicotine and Opioids: A Common Form of Polysubstance Use

Among polysubstance use disorders, the co-use of nicotine and opioids is one of the most widely and consistently reported. In fact, smoking prevalence among individuals who used opioids either illicitly or who are receiving methadone maintenance treatment (MMT), a type of medication-assisted therapy (MAT) for OUD, is between 74 and 97% (Guydish et al., 2011; Haas et al., 2008; Zirakzadeh et al., 2013). Moreover, smoking is a strong predictor of the non-medical use and misuse of prescription opioids (Zale et al., 2015). Polysubstance use is especially concerning because it leads to increased mortality and morbidity, in large part due to the additive effect of toxicity on the user's body, including the renal, cardiovascular, and pulmonary systems, among others (Compton et al., 2021; Gjersing & Bretteville-Jensen, 2018; A. Jordan et al., 2018; Meacham et al., 2018). Leading theories postulating the neurological

basis for polysubstance use generally focus on one of two hypotheses; 1) the use of one drug enhances the subjective positive experience or rewarding value of the other or 2) the use of one drug prevents or reduces withdrawal symptoms of the other drug.

Clinical studies have attempted to provide more insights into the biological factors that underlie the high incidence of nicotine and opioid co-use. Some studies have suggested a key role for chronic pain, finding that smoking increases the incidence of chronic pain and decreases pain tolerance, which may then promote the use of opioids as analgesics (Patterson et al., 2012; Zvolensky et al., 2009). Other studies have indicated that opioids may alter the rewarding properties of nicotine, demonstrating that heroin and methadone both increased number of cigarettes smoked, and methadone also increased the subjective experience of smoking (Pajusco et al., 2012a). At the same time, it is likely that nicotine also alters the rewarding properties of opioids, as a meta-analysis examining long-term outcomes for patients with SUD found that smoking cessation during treatment for OUD was associated with a 25% higher likelihood to abstain from opioids (Prochaska et al., 2004). Interestingly, smoking during opioid detoxification was associated with increased withdrawal discomfort, suggesting that nicotine enhances, rather than alleviates, opioid withdrawal (Mannelli et al., 2013). Together, these studies, and others reporting similar results, indicate that nicotine and opioid co-use results in a range of behavioral effects. Though limited preclinical data has analyzed co-use of these drugs, it is likely that the observed behavioral effects are mediated by complex alterations to the brain circuits that regulate reward and associated processes.

1.4 Mechanisms of Drug Reward and Aversion in the Ventral Tegmental Area (VTA)

1.4.1 Dopamine neurons are mesolimbic mediators of drug reward and aversion

Whereas clinical studies highlight the prevalence of, and provide some insight into, nicotine and opioid co-use, preclinical studies have attempted to further elucidate the neural

mechanisms underlying this particular form of polysubstance use. The ventral tegmental area (VTA) is one of the most well studied brain regions in the field of substance use disorders (Doyle & Mazei-Robison, 2021; Oliva & Wanat, 2016; Polter & Kauer, 2014; Sun, 2011; Volkow et al., 2019). The VTA was first identified in 1957 as a region with a dense population of dopamine (DA) neurons, a catecholamine neurotransmitter discovered by Arvid Carlsson the year prior (Yeragani et al., 2010). Subsequent work in the 1970's and 1980's would reveal the existence of two distinct pathways that arise from the dopaminergic (DAergic) neurons within the VTA. The mesocortical dopamine pathway comprises VTA DA neurons that project to the Prefrontal Cortex (PFC) and is critical for the regulation of cognitive processes and executive functions, including attention, working memory, inhibitory control, planning (Bannon & Roth, 1983; Douma & de Kloet, 2020; Lammel et al., 2008). The mesolimbic pathway, a dynamic mediator of both the rewarding and the aversive properties of drugs, consists of VTA DA neurons that project primarily to the nucleus accumbens (NAc), but also extend to an array of sites including bed nucleus of stria terminalis (BNST), amygdala, hippocampus, and lateral hypothalamus (LHA) (Maddaloni et al., 2022; Polter & Kauer, 2014; Sesack & Grace, 2009).

Work in animal models has revealed that almost all known drugs of abuse, either through direct or indirect effects on VTA DA neurons, increase DA release in the NAc (Di Chiara & Imperato, 1988). More specifically, it has been observed that many drugs of abuse promote a shift in VTA DA neuron firing, from a slower, tonic firing pattern (1-6 Hz) to a phasic, burst firing pattern (14-30 Hz) and that this shift is required for increased DA release in the NAc and subsequent drug reward (Floresco et al., 2003; Satoh et al., 2003; Schultz, 1998). The activation of phasic firing in VTA DA neurons, as well as the subsequent increase in DA release, can be observed with the unexpected delivery of a reward or with presentation of reward-predictive stimuli. Some theories hypothesize that the role of DA release is to facilitate

reinforcement learning via reward prediction error (RPE), when DA neuron firing increases in response to a reward that is better than expected and decreases when a reward is omitted (Budygin et al., 2020; Darvas et al., 2014; Day et al., 2007; Hart et al., 2014, 2015). Others suggest that DA release signals the incentive salience, or motivational value, of a reward or a reward-related cue, and thus dictates future reward-seeking behavior (Halbout et al., 2019; Phillips et al., 2003; Stuber et al., 2012; Wassum et al., 2013).

In addition to their crucial role in regulating many aspects of drug reward, VTA DA neurons also participate in the response to aversive stimuli. Both human and animal models have revealed that response to aversive stimuli and associated cues can be dysregulated by drug use and conversely, that stressful and aversive experiences can promote drug seeking and taking (Everitt & Robbins, 2013; Koob, 2009). Preclinical models of aversion, including fear-extinction and conditioned-place aversion, have shown that though most VTA DA neurons show a decrease in activity in response to aversive stimuli, a subset simultaneously show an excitatory response (Lammel et al., 2014; Pignatelli & Bonci, 2015; Yuan et al., 2019). It has been hypothesized that the VTA DA neurons that project to the amygdala, a brain region known to regulate emotion and affect, drive behavioral response to aversion. This notion was supported by recent work which showed that VTA DA neurons projecting to the basolateral amygdala (BLA) contribute to fear-memory formation by coding and signaling salience of a foot-shock event (Tang et al., 2020). Other studies have also demonstrated a role for VTA DA neurons in fear-memory extinction, revealing that VTA DA neurons encode an RPE-like signal during extinction, such that their activity increases when the foot-shock is omitted at the offset of the cue (Badrinarayan et al., 2012; Salinas-Hernández et al., 2018). Together these data indicate that VTA neurons show complex response to both rewarding and aversive stimuli, as

well as to the cues associated with those stimuli, and provide dynamic regulation of subsequent behavioral responses.

1.4.2 VTA DA neurons are heterogenous in structure and function

The complex response of VTA DA neurons to rewarding and aversive stimuli highlights the heterogeneity of this cellular population. Recent work has revealed that there are numerous subpopulations of VTA DA neurons, which can be identified based on their anatomical location, molecular signature, electrophysiological properties, as well as their afferent and efferent projections(Anderegg et al., 2015; Beier et al., 2015, 2019; Collins & Saunders, 2020; Farassat et al., 2019; Poulin et al., 2020). A variety of experimental tools has been used to target these unique subpopulations and study their specific functionality as it relates to regulation of reward and aversion. For example, a seminal paper by Lammel and colleagues (2012) identified and characterized microcircuit connectivity of VTA DA neurons with a combination of retrograde viral tracing, optogenetic, electrophysiological and behavioral techniques. Using this multifaceted targeting approach, they revealed that reward processing is modulated specifically by VTA DA neurons that receive input from the laterodorsal tegmental nucleus (LDTg) and rostromedial tegmental nucleus (RMTg) and project to the NAc shell. On the other hand, they showed that aversion-related behaviors are mediated by PFC-projecting VTA DA neurons, which receive extensive input from the lateral habenula (LHb) (Lammel et al., 2012).

Others have suggested that whether a specific VTA neuron signals reward or aversion depends on the dopamine receptor subtype (D1 or D2) expressed by the postsynaptic medium spiny neurons (MSNs) it targets within the NAc. Generally, it is thought that stimulation of the excitatory D_1 receptor on D_1-expressing MSNs (D_1-MSNs) drives reward-related behaviors, stimulation of the inhibitory D_2 receptor on D_2-MSNs drives aversion (Calipari et al., 2016; Lobo et al., 2010; Soares-Cunha et al., 2016; Verharen et al., 2019). However, it has recently been

demonstrated that that D1 and D2-MSN's can signal both reward and aversion, and that it may be the duration of receptor stimulation, rather than the receptor subtype, that determines behavioral response to a given stimulus (Soares-Cunha et al., 2020). This further highlights the complexity of the mesolimbic DA circuitry and the way that both the afferent and efferent projections of VTA DA neurons can regulate reward and aversion.

1.4.3. VTA GABA neurons provide inhibitory control of the mesolimbic system

Although the largest population of neurons in the VTA is dopaminergic (60-65%), there is also a substantial population of y-aminobutyric acid (GABA) neurons (20-25%) and a smaller number of glutamatergic neurons (3-5%) (Morales & Margolis, 2017). Anatomically, VTA GABA neurons are found interspersed throughout the VTA, though they are more abundant in the posterior and medial regions of the VTA, whereas DA cells are denser in the anterior and lateral regions (Ciccarelli et al., 2012; Morales & Margolis, 2017; Root et al., 2018). Functionally, VTA GABA neurons regulate reward-related behaviors via their modulation of VTA DA neurons, as well as their projections to distal brain regions (Bouarab et al., 2019). Many VTA GABA neurons form local, inhibitory synapses with VTA DA neurons, though the extent of this inhibitory effect is not completely understood (Creed et al., 2014; Margolis et al., 2012; Steffensen et al., 1998). One study reported that VTA GABA neuron firing results in near-complete suppression of VTA DA firing, although another reported only a partial decrease in DA firing with low frequency stimulation of VTA GABA neurons (Tan et al., 2012; Van Zessen et al., 2012).

Like VTA DA neurons, VTA GABA neurons respond both to rewarding stimuli and to the cues predicting those stimuli. With the onset of reward-predicting cues VTA GABA neurons show activation, which is then sustained during the delay between cue and reward-delivery, thereby signaling reward expectation and contributing to the RPE calculations of VTA DA neurons (Eshel et al., 2015). VTA GABA neurons also play a role in aversion as optogenetic

stimulation of this population is sufficient to both interrupt reward consumption and support real-time place aversion (Lowes et al., 2021; Tan et al., 2012; Van Zessen et al., 2012). The regulation of both reward and aversion by VTA GABA neurons is thought to be heavily influenced by the nature of their inhibitory and excitatory afferent projections, which stem from a variety of structures including BNST, LHA, NAc, dorsal raphe nuclue (DRN) and periaqueductal gray (PAG) (Bocklisch et al., 2013; Jennings et al., 2013; Morales & Margolis, 2017; Nieh et al., 2016; Yang, De Jong, et al., 2018). More specifically, the activation of inhibitory afferent projections, and the resulting inhibition of VTA GABA neurons, is necessary both for producing pro-reward behavior and for alleviating aversive behavioral states (Jennings et al.,2013; McHenry et al., 2017; Nieh et al., 2016).

1.4.4. Heterogeneity is also a key feature of VTA GABA neurons

Like VTA DA neurons, VTA GABA neurons are heterogenous, varying in baseline firing rates (2-20 Hz) and firing rates in response to stimuli (> 60 Hz) (Chieng et al., 2011; Steffensen et al., 1998; Tan et al., 2012). VTA GABA neurons are also variable in their anatomical and functional profiles. Although a large proportion of VTA GABA neurons synapse locally onto VTA DA neurons, others make synapses directly in the NAc (Bouarab et al., 2019; Morales & Margolis, 2017). Notably, via their inhibition of cholinergic interneurons in the NAc, VTA GABA neurons may enhance the ability to discriminate between conditioned and unconditioned stimuli (Brown et al., 2012).

There is also a subset of VTA GABA neurons that synapses with neurons in the DRN (Breton et al., 2018; Taylor et al., 2014). The anatomical location of VTA GABA neurons may dictate their efferent projection site and subsequent function: GABA neurons in the anterior VTA inhibit DRN serotonergic (5-HT) neurons and produce real-time place aversion, whereas activation of GABA neurons in the posterior VTA, which project to the DRN GABA neurons,

produces reward-like behavior (Li et al., 2019). Finally, a subset of VTA neurons that project to the LHb show neurochemical markers for both GABA and glutamate release. Stimulation of this population results in the co-release of both neurotransmitters and can produce conditioned-place aversion (Root et al., 2014). Other LHb-projecting VTA neurons show markers for GABA release and the DA precursor, tyrosine hydroxylase (TH), but only release GABA - not DA - upon activation. Interestingly, activation of this population produces real-time place aversion, the opposite behavioral effect seen in neurons that co-released glutamate and GABA (Stamatakis et al., 2013).

Due to their smaller population size, VTA glutamate neurons have been less extensively studied compared to DA and GABA neurons, although the existing literature indicates that this population of cells also regulates reward reinforcement and aversive behaviors (Flavia Barbano et al., 2020; Mangieri et al., 2019; Yu et al., 2019). As previously mentioned, a subset of these glutamatergic neurons also co-release GABA at synapses that are critical for the expression of conditioned place aversion (CPA) (Yoo et al., 2016). Similarly, other studies have indicated that a portion of glutamatergic VTA neurons also express TH and may also co-release DA (Hnasko et al., 2010; Morales & Root, 2014; Stuber et al., 2010; Tecuapetla et al., 2010). Though a complete overview of the anatomy and function of the VTA glutamate neurons can be found elsewhere (Cai & Tong, 2022), it is important to keep in mind that these sites of co-release are likely critical in modulating the balance between GABA and DA neurotransmission that may influence whether an experience is rewarding or aversive.

1.5. The Effects of Morphine on VTA DA and GABA Neurons

Now that we have a more complete understanding of the way in which VTA DA and GABA neurons signal reward and aversion, we will turn to drug-specific alterations to these cell types. Morphine is the archetypal opioid, as well as the opioid used in the experiments that

comprise this body of work, so we will focus specifically on morphine-induced alterations to VTA DA and GABA neurons. We then address nicotine-specific effects on these cell types before finally examining the limited literature related to the co-administration of nicotine and morphine. It should be noted that a complete review of all the drug-related neuroadaptations stemming from nicotine and/or morphine use, though fascinating, is beyond the scope of this thesis, though we do attempt to highlight other crucial circuits, brain regions and cell types.

1.5.1 VTA mechanisms of reward and aversion to acute morphine

Both reward and aversion to morphine have been well documented in animal models (Angst et al., 2012; Wang et al., 2021; Yu et al., 2021; Zhou et al., 2022). Rodents will self-administer morphine directly into the VTA and show conditioned-place preference (CPP) or conditioned-placed aversion (CPA) to morphine, depending on the dose administered (Bryant et al., 2009; Kirkpatrick & Bryant, 2015; Meisch, 2001; Ou et al., 2023). The primary neural mechanism by which acute morphine increases DA release in the NAc, and subsequently facilitates reward, is through disinhibition of VTA DA neurons. Within the VTA, morphine activates mu-opioid receptors (MORs), located presynaptically on GABA neurons that make local synapses with DA neurons (Bull et al., 2017; Johnson et al., 1992; Lüscher & Malenka, 2011; Reeves et al., 2022; White, 1996). Morphine also binds with lower affinity to the other opioid receptor subtypes, kappa opioid receptors (KORs) and delta opioid receptors (DORS), which are expressed on both GABA and DA neurons in the VTA but have a less clearly defined role in mediating reward (Al-Hasani & Bruchas, 2011; Jordan et al., 2000). Opioid receptors are G_i/G_o-coupled, G-protein coupled receptors (GPCRs) and their activation results in the inhibition of adenyl cyclase (AC), the modulation of calcium and potassium ion channels, and the mobilization of second messenger systems, resulting in cell hyperpolarization (Reeves et al., 2022; Shang & Filizola, 2015). At the cellular level, morphine binding to MORs on VTA GABA

interneurons results in GABA neuron hyperpolarization, depression of GABA release onto DA neurons, and subsequent disinhibition of VTA DA neuron firing.

Although the model of MOR-dependent disinhibition of VTA DA neurons may appear straightforward, recent evidence has demonstrated that acute morphine may exert distinct effects on the different neuronal subpopulations within the VTA. Morphine differentially alters the firing rate of VTA DA neurons such that VTA DA neurons that have a slower firing rate at baseline have larger percent increases in firing rate after morphine administration compared to cells that have a higher baseline firing rate (Gysling et al., 1983). Acute morphine may also affect neural plasticity at specific VTA DA synapses. A single injection of morphine can increase the AMPA/NMDA ratio in VTA DA neurons, reflecting an induction of long-term potentiation (LTP) at glutamatergic synapses (Authement et al., 2016; Brown et al., 2010; Saal et al., 2003). On the other hand, acute morphine may result in the MOR-dependent, inhibition of presynaptic GABAergic LTP, which could then disinhibit, and enhance the excitability of, VTA DA neurons (Dacher & Nugent, 2011; Niehaus et al., 2010).

Subpopulations of VTA GABA neurons may also be uniquely altered by - and mediate the rewarding or aversive behavioral effects of - acute morphine administration. In particular, the specific afferent or efferent connectivity of these subpopulations may dictate their functionality. It has been shown that morphine-dependent activation of VTA DA neurons relies on the inhibition of GABA interneurons in the RMTg, also known as the tail of the VTA, an area critical for RPE and drug aversion (Jalabert et al., 2011). Activation of VTA DA neurons by morphine also depends on the inhibition of VTA GABA neurons that form local synapses with glutamate neurons, whose subsequent disinhibition results in increased excitatory transmission at DAergic synapses (Chen et al., 2015; Yang et al., 2020). The functionality of VTA GABA neurons may also be indicated by their anatomical location within the VTA. MORs are selectively expressed

on GABA neurons in the anterior VTA, a subregion associated with CPA, indicating that this GABAergic subpopulation may drive the aversive properties of morphine (Li et al., 2019).

Whereas MORs on VTA GABA neurons play a prominent role in morphine reward and may modulate aversion, it is believed that a different receptor subtype, the kappa opioid receptor (KOR), is critical for expression of the dysphoric and negative affective manifestations that accompany opioid use (Bruijnzeel, 2009). Unlike MOR, KORs are expressed both presynaptically, on VTA GABA neurons, and postsynaptically on VTA DA neurons, thereby capable of driving inhibition of both cell types (Margolis & Karkhanis, 2019). The expression of KORs on VTA DA neurons is necessary for the expression of CPA, as the conditional knockout of KORs specifically in DA neurons can block KOR-agonist induced place aversion (Ehrich et al., 2015). As with MOR-mediated mechanisms of reward, it appears that the circuit-level effects of KOR activation vary between VTA subpopulations. For example, it was demonstrated that VTA DA neurons that project to the amygdala and PFC are sensitive to KOR-induced hyperpolarization, though NAc-projecting VTA DA neurons are not (Margolis et al., 2006, 2008). Further, KOR activation also inhibits GABA release onto VTA neurons that project to the amygdala, but not the NAc (Ford et al., 2006).

1.5.2 Chronic morphine use: VTA mechanisms of tolerance, dependence, and withdrawal

Chronic use of opioids, such as morphine, can result in the development of tolerance, where an individual may need progressively higher doses of the drug to attain the desired effect. Many of the physiological effects of morphine use can display tolerance including analgesia, respiratory depression, euphoria, and reward (Bekhit, 2010; Dumas & Pollack, 2008; Mayer et al., 1999; Morgan & Christie, 2011). In this way, tolerance can be challenging for doctors using opioids to treat pain and can also become a liability for people with substance use disorder. The development of morphine tolerance is thought to result from multiple, complex alterations at the

level of the μ-opioid receptor, as well as at the synaptic, cellular and circuit levels within varied brain regions (Al-Hasani & Bruchas, 2011). More specifically, tolerance to morphine has been linked to MOR desensitization, heterodimerization, and a general decrease in functionality, thought to result from increased adenylate cyclase (AC) activity and an elevated cyclic adenosine monophosphate (cAMP) level, uncoupling of the $G_{i/o}$ protein from the GTP complex and dysregulation of β-arrestin proteins, and other molecular alterations (Adhikary & Williams, 2022; Christie, 2008). Structural and functional changes to MORs subsequently dysregulates signaling at both pre- and post-synaptic sites within the VTA and the broader mesolimbic DA circuitry (Wang et al., 2023).

Chronic morphine treatment, like acute exposure, results in increased tonic and burst firing of VTA DA neurons (Koo et al., 2012; Mazei-Robison et al., 2011a). Morphine-tolerant rats also show increase VTA DA release in the NAc, suggesting that morphine induces an upregulation in mesolimbic DA signaling (Johnson and Glick, 1993). However, another group demonstrated that electrically-evoked DA release in the NAc decreases following chronic morphine, highlighting the differential regulation of subpopulations of VTA DA neurons (Mazei-Robison et al., 2011a). It is possible that increased or decreased DA release reflects anatomical changes to VTA DA neurons. Several groups have noted that, following chronic morphine administration, the soma size, and length and surface area of processes are decreased in VTA DA neurons (Chu et al., 2007; Mazei-Robison et al., 2011; Russo et al., 2007; Sklair-Tavron et al., 1996; Spiga et al., 2003). These changes also appear to be subpopulation specific, as decreased soma size was only seen in VTA DA neurons that projected to the NAc medial shell, although the soma size of NAc-core projecting VTA DA neurons was unchanged (Simmons et al., 2019). The same group also observed an increase in soma size of VTA DA neurons projecting to the PFC following chronic morphine, suggesting DA transmission may be

upregulated in other brain regions as well. A separate study also reported a difference between the VTA-PFC and VTA-NAc responses to chronic morphine, showing chemogenetic inhibition of the NAc-projecting VTA DA, but not the PFC-projecting VTA DA neurons, prevented the development of morphine tolerance (Sun et al., 2021).

VTA GABA neurons are also sensitive to the effects of chronic drug exposure, and in particular, prolonged morphine treatment leads to the desensitization of MORs on GABAergic neurons (Bergevin et al., 2002; Lowe & Bailey, 2015; Williams et al., 2013). Morphine tolerance is also exacerbated by the optogenetic inactivation of VTA GABA interneurons, suggesting that local inhibitory control of the VTA DA system may be dysregulated during chronic morphine use (Sun et al., 2021). Although GABAergic inhibitory tone appears to be decreased at synapses within the VTA, some distal VTA GABA projections have increased inhibitory transmission following chronic morphine, demonstrating that prolonged drug use, like acute use, can have varied effects on different subpopulation of neurons (Jolas et al., 1999).

Chronic morphine use can lead to physical and psychological dependence, which in addition to the development of tolerance, is indicated by the appearance of characteristic withdrawal symptoms upon cessation of drug administration. The physical component of morphine withdrawal typically appears around 8-12 hours - and peaks around 48-72 hours - after the last morphine dose, including symptoms such as pain sensitivity, chills, muscle aches, abdominal pain, diarrhea, nausea, and tremors, among other effects (Pergolizzi et al., 2020; Wallace & Papp, 2023). Morphine withdrawal also encompasses varied affective symptoms, like anhedonia, anxiety, depression, irritability, and restlessness, which may continue for days or weeks, even after the initial physical symptoms have subsided. Because of the unpleasant physical and psychological experience of withdrawal, there is often a strong desire to prevent

these symptoms, which may compel individuals to continue using opioids (Kosten et al., 2019; Pergolizzi et al., 2020).

It is hypothesized that both the somatic and affective components of withdrawal reflect, in part, the unmasking of various cellular and circuit-level neuroadaptations induced by chronic drug use (Koob, 2020; Koob & Volkow, 2016). In this way, many of the mechanisms that contribute to the development of morphine tolerance are also believed to play a compensatory role during periods of morphine abstinence. To this point, because chronic opioid use results in increases adenylate cyclase (AC) activity and cAMP levels, removal of the inhibitory opioid from the system can lead to an upregulation of AC activity and an overshoot of cAMP activity, resulting in cell hyperexcitability (Chan & Lutfy, 2016; Christie, 2008).

The expression of the physical signs of acute morphine withdrawal has been associated with neuroadaptations in both the peripheral and central nervous system, including hyperexcitability of the neurons involved in pain signaling, specifically those in the periaqueductal gray (PAG) and the dorsal horn of the spinal cord, as well as increased cholinergic signaling within the medial habenula (MHb) – interpeduncular nucleus (IPN) pathway (Bie et al., 2005; Boulos et al., 2020; Hao et al., 2011; Muldoon et al., 2014a; Neugebauer et al., 2013; Ouyang et al., 2012). In addition, leading theories indicate that the negative affective state experienced during protracted opioid withdrawal is heavily influenced by alterations to the mesolimbic DA circuitry (Koob, 2020; Koob et al., 1992; Radke et al., 2011).

Preclinical behavioral studies have consistently shown that anhedonia, the lack of reactivity to previously rewarding stimuli, is a symptom of drug withdrawal, indicating dysregulation of the reward system (Harris & Gewirtz, 2005; Harris & Aston-Jones, 2003; Holtz et al., 2015; Kiluk et al., 2019; Lubman et al., 2018; Swain et al., 2019). At the circuit level,

morphine withdrawal has been associated with decreased VTA DA neuron firing and subsequently, decreased DA release in the NAc (Diana et al., 1995, 1999). A reduction in DA signaling during morphine withdrawal may reflect increased activity of VTA GABA neurons. Enhanced GABAergic tone at synapses with VTA DA neurons during morphine withdrawal has been associated with elevations in cAMP at presynaptic GABAergic terminals and GABA release probability (Bonci & Williams, 1997; Madhavan et al., 2010). Increased activation of VTA GABA neurons during withdrawal may also reflect a shift, from inhibitory to excitatory, in the response of presynaptic receptors, increasing GABA release probability (Laviolette et al., 2004; Wittenberg et al., 2023).

1.6 Effects of Nicotine on VTA DA and GABA Neurons

1.6.1 Acute nicotine activates both DA and GABA neurons to produce reward and aversion

Like morphine, nicotine possesses both rewarding and aversive properties, which are dependent on mesolimbic DA mechanisms (Fowler & Kenny, 2014; Wills et al., 2022). Humans, non-human primates, and rodents will self-administer nicotine according to an inverted U-shaped dose-response curve, where a moderate dose produces a larger response than low or high doses (Ashton et al., 1980; Fattore et al., 2002; Herskovic et al., 1986; Natarajan et al., 2011). Nicotine can produce both CPP and CPA, though the extent to which it produces either response depends on length of exposure, route of administration, and dosage (Ahsan et al., 2014; Brielmaier et al., 2008; Fisher et al., 2021; Fudala et al., 1985; Fudala & Iwamoto, 1987; Le Foll & Goldberg, 2005). Both reward and aversion to nicotine involve, among other mechanisms, activation of the cholinergic system within the VTA, more specifically the binding of nicotine to nicotinic acetylcholine receptors (nAChRs), which are expressed on almost all VTA cell types including DA, GABA and glutamate neurons (Dani & De Biasi, 2001; De Biasi & Dani, 2011; Wills et al., 2022).

nAChRs are ligand-gated ion-channels composed of five subunits that combine to create a central pore, which is permeable to select cations (K^+, Na^+ and Ca^{2+}). Generally, the binding of nicotine to the nAChR results in a conformational change which opens the central pore and allows the flow of cations across the cell membrane, temporarily depolarizing the cell and exciting neurotransmitter release (Brunzell et al., 2015; Galzi et al., 1995; Letz et al., 1997; Miyazawa et al., 2003). A fundamental property of nAChRs is their susceptibility to desensitization, where repeated or prolonged stimulation results in decreased ability of receptor binding to elicit a cellular response (Giniatullin et al., 2005). Whereas acute, systemic injection of nicotine initially activates nAChRs and increases firing of both VTA DA and GABA neurons, repeated exposure and/or exposure to high concentrations of nicotine may result in desensitization of these nAChRs and diminished firing in response to nicotine (Dani & Bertrand, 2007; Pidoplichko et al., 1997). Activation and desensitization of nAChRs play distinct, and sometimes overlapping roles, in nicotine reward and aversion (Picciotto et al., 2008; Picciotto & Kenny, 2013).

Nicotinic receptors have an additional layer of complexity conferred by differences in their subunit composition. nAChR receptor subunits are divided into alpha (α 2-7, α 9, α 10) and beta (β 2-4) classes and can form either heteromeric - comprised of different subunits - or homomeric - comprised of the same subunit - pentamers (Dani & Bertrand, 2007). nAChR subunit composition is crucial to the functionality of the given receptor as it determines nicotine binding affinity and desensitization kinetics and is also indicative of expression pattern in the brain (Wooltorton et al., 2003; Wu & Lukas, 2011). It has also been suggested that nicotine produces its separate rewarding and aversive effects by activating different nAChR subtypes and/or by activating similar nAChR subtypes located on different cell populations within the VTA (Grunber, 1994; Laviolette et al., 2004).

There are multiple nAChR subtypes expressed on the cell bodies of VTA GABA and DA neurons, as well as on the presynaptic terminals of GABA and glutamatergic inputs. The most prominent nAChR subtype in the VTA is the α4β2 receptor, which binds nicotine with high affinity and shows rapid desensitization upon exposure to low or moderate doses of nicotine (Mansvelder et al., 2002; Picciotto et al., 1998). α4β2* nAChRs in the VTA are critical to the expression of nicotine reward; β2 or α4 null mutant mice do not show reward to nicotine, though reintroduction of the missing subunit into the VTA rescues the reward phenotype (Cahir et al., 2011; Maskos et al., 2005; Pons et al., 2008). The addition of an α5 subunit to this subtype - forming a α4β2α5 nAChR- can be a powerful modulator of nicotine intake in both rodents and humans (Fowler et al., 2013; Morel et al., 2014; Salas et al., 2003) and may also determine the effects of nicotine on DA neurons function (Yang et al., 2023). Finally, α7 homomeric nAChRs, which show much faster activation but are more resistant to desensitization than α4β2* nAChRs, are found presynaptically on glutamatergic afferents to VTA DA neurons (Klink, De Kerchove D'exaerde, et al., 2001; Maex et al., 2014; Mansvelder et al., 2002). α7 nAChRs are critical for the enhancement of DA neuron firing and the induction of long-term potentiation in response to nicotine (Mansvelder et al., 2000, 2002). A more complete overview of the nicotinic receptor subunits/subtypes and their involvement in reward and aversion within the VTA can be found elsewhere (Wills et al., 2022).

As previously mentioned, acute nicotine administration results in VTA DA phasic burst firing and a subsequent release of DA in the NAc (De Biasi & Dani, 2011; Grenhoff,et al., 1986). This shift from tonic to burst firing of VTA DA neurons is necessary for the development of nicotine reward (Grenhoff et al., 1986; Schultz, 2007; Tolu et al., 2013; Zhang et al., 2007; Zhang et al., 2009). At the cellular level, nicotine binding to α4β2* nAChRs mediates the initial shift from tonic to phasic DA neuron firing (Picciotto & Kenny, 2021; Zhang et al., 2009). α4β2*

nAChRs quickly desensitize, within seconds to minutes of nicotine exposure, after which time α4β2α6* receptors sustain VTA DA activation (Pons et al., 2008a, 2008b). β2* receptors appear especially critical for the expression of nicotine reward, as mice that lack this subunit do not show nicotine-induced phasic burst firing of DA neurons nor increased DA release in the NAc and nicotine does not support self-administration in these animals (Picciotto et al., 1998). As previously mentioned, the α5 subunit may also be a key regulator of nicotine-induced burst firing as VTA DA neurons of mice null for this subunit fail to respond to nicotine at doses that increase DAergic firing in wildtype cells (Yang et al., 2023).

Several circuit-level mechanisms have been proposed to explain this increase in VTA DA firing in response to acute nicotine, including enhanced excitation of the DA cells, decreased inhibition from local GABA neurons, or modification of excitatory glutamatergic inputs (D'souza & Markou, 2013; Li et al., 2014). Notably, even brief exposures to nicotine may alter plasticity at both excitatory and inhibitory synapses. For example, acute nicotine treatment resulted in an increase in the ratio of AMPA receptor (AMPAR) to NMDA receptor (NMDAR) at excitatory glutamatergic synapses with DA neurons, consistent with the induction of LTP (Mansvelder et al., 2000). LTP at these synapses was also demonstrated by reports of increased presynaptic glutamate release following acute nicotine exposure (Gao et al., 2010; Saal et al., 2003). At the same time, a single injection of nicotine can produce LTD at synapses between GABA and DA neurons in the VTA (Mansvelder et al., 2002a; Nugent & Kauer, 2008). It is likely that acute nicotine mediates changes in synaptic plasticity, potentially facilitating both increased excitatory input and decreased inhibitory input onto VTA DA neurons.

Recent evidence suggests that, in addition to mediating nicotine reward, VTA DA neurons also facilitate nicotine aversion. To that end, it has been demonstrated that subpopulations of VTA DA neurons are inhibited by acute nicotine, and that these

subpopulations may be critical in signaling nicotine aversion (Addy, 2021; Lammel et al., 2014; Liu et al., 2022; Nguyen et al., 2021). Demonstrating a role for VTA DA neurons in nicotine aversion, nicotine-induced CPA was blocked either by inhibiting the phasic firing of DA neurons (Grieder et al., 2012) or by inhibiting DA signaling in the NAc (Laviolette & Van Der Kooy, 2003). Like its crucial role in reward, the β2 subunit is also critical for DA-mediated nicotine aversion, as re-expression of β2 in β2 null mice restores the conditioned aversive effects of nicotine (Grieder et al., 2019). Two separate sets of elegant experiments have since revealed circuit mechanisms, at the level of the VTA, which may underlie aversion to nicotine.

First, Liu and colleagues showed that high doses of nicotine, which result in nicotine aversion, cause biphasic excitatory and inhibitory response in DA neurons, whereas low doses produce reward and a sustained excitatory DA response (Liu et al., 2022). They also demonstrated that the downstream effects of this biphasic VTA DA activation are subpopulation specific, DA release decreases in the lateral NAc, but not in the medial NAc, in response to aversive doses of nicotine. The second set of experiments by Nguyen and colleagues revealed that the effect of nicotine on VTA DA neurons was dependent on their efferent connectivity (Nguyen et al., 2021). Specifically, they reported that a low, reinforcing dose of nicotine resulted in the excitation of VTA DA neurons projecting to the NAc, but an inhibition of medially located, amygdala-projecting DA neurons. They also showed that inhibition of these amygdala-projecting DA neurons produced anxiety-like behavior, whereas their activation prevented the anxiogenic effects of nicotine. Together, these experiments highlight the ways in which acute nicotine differentially acts on VTA DA neurons to promote nicotine aversion in addition to reward.

Like DA neurons, VTA GABA neurons also express high levels of α4β2* nAChRs, which mediate increased GABAergic firing in response to acute nicotine (Mansvelder et al., 2002; Yin et al., 2000). Additionally, VTA GABA neurons express α4β2* nAChRs on their presynaptic

terminals, where nicotine binding increases GABA release, separate from increases in action potential firing (Fisher et al., 1998; Lu et al., 1999; Radcliffe et al., 1999). Preclinical models have attempted to determine a precise role for VTA GABA neurons in regulating the rewarding and/or aversive properties of nicotine. Although β2* nAChRs on VTA DA neurons mediate the conditioned aversive effects of nicotine, β2* nAChRs on VTA GABA neurons may facilitate the conditioned rewarding effects (Grieder et al., 2019). Selective optogenetic stimulation of VTA GABA neurons also appears to be sufficient to produce nicotine reward (Ngolab et al., 2015). However, a separate study reported that VTA GABA neurons display excitatory responses only to high, aversive doses of nicotine but do not respond to low, rewarding doses (de Jong et al., 2019).

There are also discrepancies regarding the desensitization kinetics of nAChRs on VTA GABA neurons. The prevailing literature indicates that α4β2* nAChRs on VTA GABA neurons show sustained desensitization after nicotine exposure, facilitating disinhibition of VTA DA neurons (Dani & Bertrand, 2007; Mansvelder et al., 2002). It has been hypothesized that this reflects a decreased in excitatory cholinergic input from LDTg/PPN neurons, which preferentially make synapses with GABA neurons in the VTA (De Biasi & Dani, 2011; Wills et al., 2022). Desensitization of nAChRs prevents endogenous ACh, released presynaptically from LDTg/PPN inputs, from exciting VTA GABA neurons, which diminishes their activity, sometimes below baseline (Mansvelder et al., 2002). Yet, subsequent reports contradict that notion and demonstrate that GABA neurons do not desensitize upon repeated stimulation with nicotine, confounding the notion that decreased inhibitory tone facilitates increases VTA DA activity (Taylor, 2011; Tolu et al., 2013).

It seems likely that nicotine's ability to confer both reward and aversion reflects the balance of VTA DA and GABA signaling, rather than the activity of a singular population. For

example, biphasic activity of VTA DA neurons in response to high doses of nicotine depends both on desensitization of α4β2* nAChRs on VTA DA neurons, as well as activation of α7 nAChRs on GABAergic afferents from the LDTg (Liu et al., 2022). Furthermore, Tolu et al. revealed that cholinergic modulation of VTA GABA neurons is necessary for the phasic burst firing of VTA DA neurons (Tolu et al., 2013). They also demonstrated that nicotine acting on both VTA DA and GABA is what drives increases or decreases in DA burst firing, in a way that correlates with the positive or negative motivational value of drug-associated cues. Hence, it is necessary to consider the dynamic balance between both DAergic and GABAergic signaling within the VTA and the way that the interplay between these two cell types drives nicotine reward or aversion.

1.6.2 Chronic nicotine upregulates nAChRS and dysregulates plasticity in the VTA

Chronic nicotine use, like the prolonged use of other drugs, can result in tolerance, dependence, and the emergence of a withdrawal syndrome upon drug abstinence (De Biasi & Dani, 2011). Nicotine withdrawal typically arises within 4-24 hours after nicotine cessation, and, like withdrawal from opioids, can be characterized by the emergence of both physical and affective symptoms that may persist for weeks (Hughes et al., 1994). Physical symptoms of nicotine withdrawal may include tremors, bradycardia, gastrointestinal discomfort, and increased appetite, whereas affective symptoms encompass wide ranging changes in mood and cognition like insomnia, irritability, anxiety, and fatigue (Hughes & Hatsukami, 1986; Le Foll & Goldberg, 2009; McLaughlin et al., 2015; West, 1984). As for opioid use, the aversive experience of the withdrawal syndrome and the desire to avoid these symptoms contributes to the perpetuation of nicotine use (Garvey et al., 1992; Hughes et al., 1992).

Repeated exposure to nicotine alters nAChRs structure and function and, subsequently, dysregulates nAChR-expressing brain regions (Govind et al., 2009; Nashmi et al., 2007; Wills &

Kenny, 2021). The emergence of the withdrawal syndrome reflects these complex neuroadaptations induced by chronic drug use (Paolini & De Biasi, 2011; Rahman et al., 2004). Though not discussed here, the MHb-IPN pathway, which densely expresses low-affinity α3β4* nAChRs, is especially critical for the expression of the physical symptoms of nicotine withdrawal, as well as other aversive components of nicotine use (Jackson, Sanjakdar, et al., 2013; Salas et al., 2004, 2009). A comprehensive overview of the role of this circuit in nicotine withdrawal and aversion can be found here (Dani & De Biasi, 2013; McLaughlin et al., 2017; Salas et al., 2009). On the other hand, it has been suggested that alterations to the mesolimbic dopamine system, specifically hypofunctionality of DAergic transmission, contributes to the negative affect, dysphoria and anhedonia experienced during nicotine withdrawal (Koob & Le Moal, 2008; Oliver et al., 2017; Pergadia et al., 2014)

Unlike chronic morphine, which does not alter the overall expression of MORs, chronic nicotine exposure upregulates nAChRs (De Biasi & Dani, 2011). This upregulation is likely a compensatory mechanism following the sustained receptor desensitization occurring with repeated nicotine exposure (Fenster et al., 1999; Govind et al., 2009; Ngolab et al., 2015). Desensitization is especially prominent with nicotine use because desensitized nAChR conformations have a higher affinity for agonists, so nAChRs will increasingly adopt desensitized conformations as nicotine use perpetuates (Ochoa et al., 1989; Quick & Lester, 2002). The brains of chronic smokers reveal both nAChR desensitization and upregulation, suggesting that these dual processes drive nicotine dependence and may contribute to the manifestation of reward deficits during withdrawal (Brody et al., 2014; Picciotto et al., 2008).

The precise cellular mechanisms behind nicotinic receptor upregulation are not entirely understood. It has been suggested that upregulation results from desensitization-dependent conformational modifications, alterations in subunit stoichiometry, accelerated assembly,

maturation and trafficking of nAChRs to the cell surface, or slow removal of nAChRs from the cell surface (Darsow et al., 2005; Kuryatov et al., 2005; Nashmi et al., 2003; Srinivasan et al., 2011). Other mechanisms which govern the rate of recovery from the desensitized state, like nAChR phosphorylation, are also thought to influence the rate of upregulation (Chrestia et al., 2023). Different nAChR subtypes, with varied desensitization kinetics and expression patterns throughout the brain, also show differences in the extent of their upregulation, suggesting that upregulation is also a property that is intrinsic to the receptor subtype. Receptors containing the β2 subunits (β2* nAChRs) are particularly sensitive to nicotine-induced upregulation. If β2 is replaced by β4 in either α3* or α4* nAChRs, receptor upregulation is significantly diminished (Govind et al., 2009; Sallette et al., 2005).

In theory, functional upregulation of nAChRs, specifically α4β2* nAChRs, on VTA DA neurons, should make this cellular population more sensitive to the effects of nicotine. In this way, it would be expected that chronic nicotine enhances the excitability of VTA DA neurons in response to nicotine. And yet, the effects of chronic nicotine on DA cell firing have been mixed. Some groups reported that chronic nicotine treatment, via osmotic minipump or intraperitoneal injection (i.p.), leads to decreased spontaneous firing of VTA DA neurons (Grieder et al., 2012; Rasmussen et al., 1995; Taylor, 2011). One study found that spontaneous DA neuron firing decreases following chronic nicotine administration, whereas nicotine-induced burst firing is enhanced (Grieder et al., 2012). Other work cited that chronic nicotine treatment, via injection (IP or subcutaneous) or drinking, does not alter the VTA DA neuron firing, nor does it alter ACh-mediated nAChRs currents on VTA DA neurons (Besson et al., 2007; Nashmi et al., 2003; Xiao, Nashmi, McKinney, et al., 2009; Yang et al., 2023). It has been suggested that the α7 (Besson et al., 2007) and α5 subunits (Yang et al., 2023), which both show resistance to desensitization and upregulation, are critical mediators of homeostatic VTA DA neuron firing, which are likely to

become dysregulated in the presence of chronic nicotine. For example, it has been demonstrated that, over time, nicotine can enhance the synchronous firing of a subset of VTA DA neurons (Li et al., 2011) and may also selectively increase the number and length of VTA DA burst, but not tonic, firing events (Zhang et al., 2009). Considering these findings, it seems likely that variability in the desensitization and upregulation properties of nAChR subtypes may result in the dysregulation of certain VTA DA subpopulations during periods of prolonged nicotine use.

During nicotine withdrawal and withdrawal from many drugs of abuse, there are consistent reports of decreased DA release in the NAc (McLaughlin et al., 2015; Miyata et al., 2011; Oliver et al., 2007; Pergadia et al., 2014). The role of VTA DA neuron activity in facilitating this reduction in DA release is less clear and has not been extensively studied. One group showed that DA neuron firing decreased in the 24 hours after nicotine cessation but returned to baseline levels after 48 hours (Rasmussen et al., 1995). A separate study indicated that the size of nAChR currents in VTA DA neurons decreased at 24 hours and lasted for more than 4 weeks after nicotine cessation (Yang et al., 2023). Importantly, the long-lasting reduction in VTA DA nAChR currents during nicotine withdrawal was absent in α5-null mice, further highlighting the importance of this subunit in mediating the VTA DA response to chronic nicotine treatment (Yang et al., 2023).

Like for mechanisms of acute nicotine reward and aversion, VTA GABA neurons also play a critical role in mediating the expression of nicotine withdrawal. In one study it was revealed that, following 12 days of nicotine injections, VTA GABA firing rate significantly increased (Taylor, 2011). This nicotine treatment paradigm also increased GABA-mediated IPSCs on VTA DA neurons but decreased them on VTA GABA neurons. Separately, it was demonstrated that chronic nicotine increased the spontaneous activity of VTA GABA neurons

(Grieder et al., 2019). It seems likely that the enhanced GABAergic inhibitory transmission seen during nicotine withdrawal mediates the hypodopaminergic state mentioned prior (Jalabert et al., 2011; Lobb et al., 2010). One possible mechanism to explain this effect is that, though α4β2* nAChRs are expressed by both VTA DA and GABA neurons, those expressed on GABA neurons may be more prone to nicotine-induced upregulation (Nashmi et al., 2007; Ngolab et al., 2015; Xiao, Nashmi, McKinney, et al., 2009). Increased sensitivity to upregulation may reflect a lack of the α5 subunit, which is expressed in only 20% of α4β2* nAChRs on VTA GABA neurons, whereas 80% of VTA DA α4β2* nAChRs contain the α5 (Klink et al., 2001). Together this data indicates that chronic nicotine and nicotine withdrawal mediates unique alterations to specific nAChRs and to the neuronal subpopulations that express those nAChRs.

1.7 Effects of Nicotine and Morphine Co-Exposure in Preclinical Models

1.7.1 Behavioral effects of co-administration

As detailed above, there is an extensive body of literature that examines the effects of separate nicotine or morphine exposure on VTA neurons. However, there are a relatively limited number of preclinical studies describing the effects of co-administration of these drugs, despite the prevalence of their co-use in the human population. The bulk of the preclinical literature examining exposure to both nicotine and morphine, or to any opioid, focuses mainly on behavioral outcomes, including reward- and withdrawal-related behaviors. In addition, rather than examining the effects of *concurrent* exposure to both drugs, these studies often entail pre-treatment with one drug prior to administration of the other. A large portion of this work forgoes dual drug exposure altogether, looking instead at the effect of nicotine on the endogenous opioid system or, conversely, of morphine on the cholinergic system.

According to the drug gateway theory, pre-exposure to one drug of abuse can make subsequent exposure to a different drug of abuse more rewarding, thereby perpetuating drug

use (Kandel et al., 1992; Levine et al., 2011). Morphine pre-treatment in rats shifts the dose response curve for nicotine down and to the left, suggesting morphine enhances the efficacy and potency of nicotine (Huston-Lyons et al., 1993). Similarly, systemic nicotine pre-treatment results in increased morphine self-administration in rats (Loney et al., 2021). Depending on the route and schedule of administration, nicotine and morphine can produce cross-tolerance or cross sensitization in CPP paradigms, where exposure to one drug increases or decreases the conditioned-place response to the other (Rezayof et al., 2007; Vihavainen, Relander, et al., 2008; Zarrindast et al., 2003). However, although morphine pre-treatment is capable of reinstating extinguished nicotine CPP (Biala & Budzynska, 2006) nicotine cannot reinstate morphine seeking following extinction of morphine CPP (Feng et al., 2011). Notably, Kota and colleagues demonstrated that a 7-day exposure to nicotine in adolescence significantly enhances morphine CPP during adulthood, highlighting the sensitivity of the adolescent brain and reward circuitry to drug exposure (Kota et al., 2018).

The prevalence of polysubstance use may also reflect the ability of one drug to lessen the severity of the withdrawal syndrome from the other, thereby making the drug use experience less aversive. In support of this notion, repeated nicotine treatment reduces the number of withdrawal signs, like CPA or jumping, displayed by morphine-dependent mice following precipitation of withdrawal with the MOR antagonist naloxone (Araki et al., 2004; Haghparast et al., 2008; Zarrindast et al., 2001). However, other studies report that nicotine exposure in adolescence does not alter withdrawal signs in adult, morphine-dependent mice (Kota et al., 2018). Similar to the effects of nicotine on morphine withdrawal, morphine decreases the severity of spontaneous or nAChR-antagonist precipitated nicotine withdrawal (Ise et al., 2000; Malin et al., 1993).

1.7.2 A role for the endogenous opioid and cholinergic systems

Leading theories suggest that the endogenous opioid system plays a key role in mediating behaviors associated with co-use of nicotine and opioids like morphine. This system comprises the opioid receptors described previously, mu (MOR), kappa (KOR) and delta (DOR), as well 3 classes of endogenous opioid peptides, β-endorphin, met- and leu-enkephalins, and dynorphins, that exhibit different affinities for each opioid. A fourth opioid peptide receptor, the N/OFQ (NOP) and its endogenous opioid ligand, nociceptin/orphanin FQ (N/OFQ) were subsequently discovered (Butour et al., 1997; Donica et al., 2013). The opioid receptors and the endogenous opioid peptides are expressed widely throughout the peripheral and central nervous system, including throughout the mesolimbic dopamine circuitry, and are involved in regulation of pain sensitivity, emotional states, and addiction-related behaviors (Bodnar, 2022; Corder et al., 2018; Emery & Akil, 2020; Kieffer & Gavériaux-Ruff, 2002) .

The acute administration of nicotine promotes the release of endogenous opioid peptides and induces changes in the expression of these peptides throughout the brain (Dhatt et al., 1995; Isola et al., 2009). In particular, the MOR is thought to be a site for overlapping mechanisms of reward and withdrawal following co-exposure to nicotine and morphine. For example, mice that lack MORs (MOR KO) or β-endorphin do not show the expression of nicotine CPP (Berrendero et al., 2002; Trigo et al., 2010; Walters, Cleck, & Kuo, 2005). Additionally, MOR KO mice do not show expression of somatic, mecamylamine-precipitated nicotine withdrawal (Berrendero et al., 2005), and null mutation of α5 and β4 nAChR subunits attenuates the manifestation of physical dependence to morphine (Muldoon et al., 2014), suggesting close interactions between nicotinic and endogenous opioid signaling at the cellular and circuit-level. Naloxone also blocks the expression of nicotine CPP and precipitates nicotine withdrawal in nicotine-dependent rodents (Biala & Budzynska, 2006; Malin et al., 1993; Walters,

Cleck, & Kuo, 2005; Zarrindast et al., 2003), and it seems to be more effective than mecamylamine at precipitating the affective component of nicotine withdrawal, as determined by increased expression of CPA (Balerio et al., 2004). Whereas antagonism of the MOR exacerbates nicotine withdrawal, the administration of MOR, DOR or KOR agonists reduces the capability of mecamylamine to produce CPA during withdrawal from nicotine (Ise et al., 2000, 2002).

The role of KOR, DOR and NOP in mediating nicotine reward and aversion are less clear. DOR KO mice do not show nicotine CPP and show decreased self-administration of nicotine compared to wild-type mice (Berrendero et al., 2012). KOR appears to be critical for modulating the effects of stress on nicotine related behaviors, as KOR agonists were sufficient to reinstate nicotine seeking, an effect diminished by KOR antagonists (Al-Hasani & Bruchas, 2011; Jackson, McLaughlin, et al., 2013; Smith et al., 2012). Finally, there is evidence that NOP agonists increase nicotine self-administration, whereas NOP antagonists decrease responding for nicotine (Cippitelli et al., 2016)

As previously mentioned, there is also evidence that morphine and other opioids interact with the cholinergic system, potentially contributing to the opioid-induced modulation of nicotine reward and withdrawal behaviors. nAChR antagonism can decrease morphine self-administration and morphine seeking during abstinence and also decreases CPP to morphine (Hall et al., 2011; Zarrindast et al., 2003). Additionally, specific antagonism of either β*containing nAChRs, or α7 homomeric nAChRs, reduces drug-primed reinstatement of morphine CPP (Feng et al., 2011; Natarajan et al., 2011). *In-vitro* work also suggests that morphine acts as a partial agonist at α4β2* nAChRs and as a weak antagonist at α3* nAChRs, providing a possible mechanism to explain the sensitivity of morphine reward to nAChR antagonism (Talka et al., 2013). The α5 subunit also appears to play a critical role in the

cholinergic modulation of opioid reward as expression of a hypofunctional variant of α5 in VTA DA neurons results in potentiation of the peak amplitude of ACh-gated nAChR currents following morphine conditioning (Yang et al., 2023).

Despite the evidence discussed above, there have been only a handful of studies examining overlapping mechanisms of nicotine and morphine reward and aversion specifically within the VTA. Two separate reports indicated that intra-VTA infusion of nicotine potentiated (De Rover et al., 2004), whereas mecamylamine inhibited morphine CPP (Rezayof et al., 2007). One neurochemical study in rodents revealed that nicotine-induced DA release in the NAc is modulated by the activation of MORs within the VTA (Tanda et al., 1998). To date, only a single study has looked at the way that nicotine vapor and morphine co-administration can affect behavior and may potentially alter VTA neurons (Avelar et al., 2022). Avelar and colleagues showed that the combination of nicotine vapor administration and morphine treatment (10mg/kg, i.p.) decreases reward-related behavior to both drugs, as evidenced by decreased self-administration for nicotine vapor and decreased CPP to morphine. Furthermore, morphine exposure reduced the upregulation of α4* nAChRs on both VTA GABA and DA neurons. Though this study examined nAChR expression in VTA neurons, it did not evaluate nAChR functionality or the effects of this reduced upregulation on neuronal activity. We build on the body of work detailed in this section and examine the way that both acute and chronic use of nicotine and/or morphine alters the natural dynamics of VTA DA and GABA neuron activity.

1.8.1 Overview of techniques used in preclinical models of drug abuse

The preclinical work referenced above has relied on a variety of different strategies to study both the behavioral and neuronal effects of nicotine and morphine use. The rewarding and/or aversive behavioral effects of these drugs have been evaluated largely via conditioned-place preference and self-administration paradigms. Both genetic mouse models (e.g.

knockouts, overexpression, hypofunctionality, etc.) and the use of receptor agonists or antagonists are widely employed to determine the role that a specific receptor, or receptor subunit plays, in mediating behaviors associated with drug reward, aversion, tolerance and withdrawal. The use of Cre-Lox mouse models in conjunction with optogenetic and chemogenetic tools allows for the excitation or inhibition of specific cell-types or neuronal populations, which may produce varying effects on behavior, including behaviors related to reward and aversion. Furthermore, the Cre-Lox system has been leveraged to perform viral tracing studies, which extensively map neural circuits and subcircuits. In addition, *in-vivo* and *ex-vivo* electrophysiology techniques have provided critical insight into the effects of drug use on precise patterns of neuron firing as well as on receptor-mediated currents. Though each of these tools has separately contributed to our fundamental understanding of addiction, when used together, they may offer an even greater understanding of neuroadaptations occurring at the cellular, circuit and population level.

Within the last decade, the advent of another preclinical tool, calcium imaging, has helped to provide even more insight into the natural dynamics of given neuronal populations. In brief, calcium imaging relies on the use of fluorescent genetically encoded calcium indicators (GECIs) to measure changes in neural activity in a specific population of neurons. The next section takes a deeper look at the history of calcium imaging and highlight its utility in studying neuroadaptations associated with addiction.

1.8 Calcium Imaging: A New Tool to Study Addiction

1.8.2 A historical look at calcium imaging

The recognition that calcium plays a crucial role in cell function dates back well before the advent of modern neuroscience. In 1883, British physician and physiologist Sydney Ringer published a series of papers in the *Journal of Physiology* detailing the importance of different

ions—sodium, potassium, calcium, and chloride—in sustained contraction of the frog heart (Miller, 2004; Ringer, 1882, 1883). Over the next century, thousands of studies would reveal that calcium acts as a critical regulator of ubiquitous cellular functions including contraction, gene expression, and secretion, among others (Berridge et al., 2003; Carafoli & Krebs, 2016; Bootman, 2002; Berridge, 2000). Calcium was first appreciated for its role in neuronal excitability following seminal experiments by Katz and Miledi, who demonstrated that increases in intracellular calcium ($[Ca^{2+}]_i$) correlated with presynaptic neurotransmitter release (Katz & Miledi, 1968). Following these revelations and with the hope of gaining a greater understanding about signaling and circuitry, the search began for ways to measure dynamic fluxes of intracellular calcium and the field of calcium imaging was born. A complete history of calcium imaging, though fascinating, is beyond the scope of this review, but has been documented elsewhere (Zhou et al., 2021; Zlatic et al., 2021).

In neurons, free calcium levels are extremely low in the cytosol while at rest (50-100nM), rise to (1uM) during an action potential through voltage gated calcium channels, and then—via various extrusion, buffering and pumping mechanisms—return to baseline, all in about 70ms seconds (Augustine et al., 1987, 2003). This process underlies the theoretical basis for calcium imaging, which continuously measures the concentration of intracellular calcium and uses this measurement as proxy for neural activity. Today, most neuronal calcium imaging studies rely on the expression of a genetically encoded calcium indicator in a genetically defined population of cells.

A GECI typically consists of a binding/sensing domain and a reporter, which is based either on a single-wavelength fluorescent protein (FP) or the Forster-resonance energy transfer (FRET) between two FPs (Broussard et al., 2014; Inoue, 2020). For GECIs with a single FP, the binding of Ca^{2+} results in a conformational change that subsequently causes a change in

fluorescence intensity. The most widely utilized GECI, GcAMP, belongs to this class. The GcAMP protein consists of circularly permuted green fluorescent protein (GFP), the calcium-binding protein calmodulin (CaM) and a CaM-interacting M13 peptide, often referred to as the calmodulin binding protein (CBP) (Akerboom et al., 2009; Ding et al., 2014). In the presence of Ca^{2+}, CaM binds the M113 peptide from the myosin light chain kinase, causing a conformational change in the protein, which ultimately increases fluorescence of the GFP moiety. In the absence of calcium, this process reverses and GFP fluorescence is quenched (Mao et al., 2008; Miyawaki et al., 1997; Nakai et al., 2001). Multiple iterations of the GcAMP protein have been constructed, varying in properties like intrinsic brightness, kinetic profile, and dynamic range, but all following the same basic principles outlined above.

Several systems exist for detecting, recording, and reporting changes in GECI fluorescence in neurons, the most popular being two-photon imaging and fiber photometry. For a more detailed and comprehensive review of various neuronal calcium imaging techniques, see (Corkrum et al., 2020; Girven & Sparta, 2017; Oh et al., 2019; Russell, 2011). In essence, two photon imaging uses a pulsed, low energy laser to confine GECI excitation to a specific focal plane. It is often used in conjunction with lightweight microendoscopes, which contain a self-focusing GRIN lens and a miniature microscope, allowing for single-cell resolution and real-time visual recording of calcium transients (Stosiek et al., 2003). Conversely, fiber photometry utilizes a single multimode fiber both to deliver excitation light from an LED driver and to collect emitted photons from a given population of cells. Although this technique is less invasive and less costly than two-photon imaging with microendoscopes, some spatial and temporal resolution is lost (Gunaydin et al., 2014).

Regardless of the technique used, due to the slower kinetics of the calcium transient (10ms) compared to the rapid time course of the action potential (2-3 ms), precise measurement

of spiking activity with calcium indicators cannot be directly measured, necessitating the use of complicated, post-experimental deconvolution and spike inference methods (Akerboom et al., 2012; Deneux et al., 2016; Pachitariu et al., 2018; Vanwalleghem et al., 2021). GcAMP6f, a popular GcAMP variant, demonstrates a linear relationship between number of action potentials and fluorescence and can detect *some* individual action potentials, but cannot reliably (> 50%) detect the highest frequency action potentials, such as those seen with phasic burst firing in dopaminergic neurons. For these reasons, the use of additional techniques (e.g optogenetics, electrophysiology, pharmacological manipulation) should be employed when attempting to make definitive statements about the function of a particular circuit and relationship to observed behavioral outcomes.

1.8.3 Calcium imaging to study addiction

As stated previously, addiction is a complex and chronic disorder characterized by cyclical behavioral states (i.e binge, withdrawal, preoccupation) and maladaptive changes to many brain regions, circuits, and neurochemical systems. Before the advent of calcium imaging, *in vivo* electrophysiological techniques were the most common tool available to study the real-time activity of neurons during specific behavioral states, like those relevant to addiction. *In vivo* electrophysiology involves inserting one or more glass pipettes or electrodes (wires) into the brain region of interest and recording changes in voltage, current or both from one, or multiple, neurons (Noguchi et al., 2021; O'Keefe, 1976; Sargolini et al., 2006; Taof et al., 2015; Zhou et al., 2021).

Although this approach provides high temporal (<μs) and spatial (<μm) resolution, the experimenter is unable to determine the morphological or genetic identity of the recorded cells. This can be confounding because there is now a substantial body of evidence demonstrating the existence of neuronal subpopulations and subnuclei—as well as microcircuits connecting

them—within nearly all brain regions (Albright et al., 2000; Beretta et al., 2012; Ciriachi et al., 2019; Imamura et al., 2020; Ma et al., 2021; Parker et al., 2016; Yang, de Jong, et al., 2018a). These neuronal subpopulations are functionally and anatomically distinct, unique in their regulation of and contribution to behavioral outcomes, including responses related to reward and aversion. For example, as previously discussed, the VTA contains three different types of neurons (dopaminergic, GABAergic and glutamatergic) which project to and receive input from specific nuclei and subnuclei, both local and distal (Beier et al., 2015; Watabe-Uchida et al., 2012; Yuan et al., 2019).

When utilized in conjunction with genetic tools and mouse models, calcium imaging allows for the examination of defined, cell-type specific circuits. As touched upon previously and as their name implies, GECI's are designed to be targeted to a genetically defined population of cells. This is typically achieved via stereotaxic injection of Cre-dependent virus encoding for a GECI in a rodent line expressing Cre-recombinase in the cell type of interest (e.g. Cre-GCaMP into the VTA of a Dopamine Transporter (DAT) Cre mouse). To examine a specific circuit or pathway, a dual viral strategy might be used, whereby the expression of a GECI injected into the target region is contingent upon activation by an antero- or retrograde Cre-virus injected into a specific afferent or efferent projection region. Other experimenters bypass stereotaxic viral injection and opt instead to bred novel rodent lines, crossing GCaMP reporter animals and the Cre-line of interest to obtain cell-type specific expression of a GECI (i.e Ai148 x DAT-Cre). In addition to offering genetic control and evaluation of defined neuronal circuits, calcium imaging is advantageous to study addiction because it can be performed chronically, *in vivo,* in freely behaving animals (Aramuni & Griesbeck, 2013; Broussard et al., 2014b; Mank et al., 2008; Stosiek et al., 2003). By allowing the study of the same population of neurons, in the same

animal, throughout the varied stages of drug dependence, calcium imaging provides critical insights into the time-course by which a given neuroadaptation occurs.

1.9 Rationale and Thesis Objectives

As discussed, prior, despite its predominance in the human population, polysubstance use remains relatively understudied in the preclinical literature. This is likely due to the unique challenges presented by studying more than one drug, including the possibility of drug-drug interactions, which may affect both physiological and behavioral effects of either drug, as well the requirement of additional controls. Likely due to these reasons, rather than studying the concurrent use of both drugs, most studies tend to give a pre-exposure to one drug before, subsequently, studying the effects of the other. The lack of preclinical polysubstance use paradigms may be especially detrimental to the study of nicotine and opioid co-use, considering most people with opioid use disorders, who regularly use opioids, are also daily cigarette smokers, and therefore frequently have both drugs on board (Guydish et al., 2011; Haas et al., 2008; Zirakzadeh et al., 2013).

To address this critical need as well as move towards more closely mimicking the human experience, we have developed a chronic, physiologically relevant model of nicotine and opioid co-use. We use a passive nicotine vapor administration system, maintaining the critical pharmacokinetic and pharmacodynamic properties imparted by nicotine inhalation (via combustible or electronic cigarettes) in humans (Le Houezec, 2003), in conjunction with a continuous two-bottle choice (C2BC) paradigm for morphine exposure, a voluntary form of drug administration that allows mice to titrate their preferred morphine dose (Fleites et al., 2022). We begin nicotine vapor exposures in adolescence, mirroring the time at which many humans initiate using e-cigarettes, and then progress to chronic, co-exposure to both nicotine and opioids, reflective of the experience of many adult polysubstance users (Barrington-Trimis et al.,

2014; Huey & Granitto, 2020). Finally, we use fiber photometry in conjunction with this novel drug treatment paradigm to monitor changes to two critical mesolimbic cell populations, VTA DA and GABA neurons, as animals progress throughout the co-exposure paradigm.

Specifically, this body of work is structured around the following aims: we seek to determine and compare how the activity of the **(1)** VTA-DA and **(2)** VTA-GABA neurons is affected by **(a)** exposure to acute nicotine **(b)** exposure to chronic nicotine and **(c)** co-exposure to nicotine and morphine. In line with the current literature, we hypothesized that acute nicotine exposure would increase activity in both DA and GABA neurons. For VTA DA neurons, we hypothesized that chronic nicotine treatment would augment the increase in activity in response to nicotine and finally, that nicotine and morphine co-exposure would furth augment the response further, resulting in the greatest nicotine-induced increase in neural activity. On the other hand, for VTA-GABA neurons we predicted that chronic nicotine exposure would dampen the neural response to nicotine, and that nicotine and morphine co-exposure would further dampen the GABAergic response to nicotine.

CHAPTER 2: CHRONIC CO-EXPOSURE TO NICOTINE AND MORPHINE DOES NOT ALTER VTA DA RESPONSE TO ACUTE NICOTINE

2.1 Introduction

Worldwide, nearly 1.3 billion people report regularly smoking tobacco cigarettes and smoking accounts for nearly 8 million deaths annually (Reitsma et al., 2021). In addition to widespread use of combustible cigarettes, electronic cigarettes (e-cigarettes) have become increasingly popular in the last decade, with an estimated global prevalence of vaping around 11% (Tehrani et al., 2022). Though e-cigarettes were initially marketed as a safer alternative to smoking and a smoking cessation tool, the high nicotine concentration of most e-cigarettes makes them addictive in their own right (Nardone et al., 2019; Williams, 2020). E-cigarette use also presents numerous health concerns as e-liquid vapor contains carcinogens such as formaldehyde, acetaldehyde, and nitrosamines, as well as other cytotoxic compounds like diacetyl and reactive oxidative species (ROS) (Canistro et al., 2017; Ebersole et al., 2020; Goniewicz et al., 2014; Jabba & Jordt, 2019; Ogunwale et al., 2017; Rouabhia et al., 2017).

Although e-cigarettes are commonly used by adults, they are even more popular among adolescents. In 2019, 27.5% of high schoolers and 10.5% of middle schoolers reported regular use of e-cigarettes (Huey & Granitto, 2020). Adolescents and young adults are also more likely to used flavored e-cigarette products, which contain additional cytotoxic chemicals and may be more appealing, sustaining exposure to both nicotine and other toxic compounds in e-liquid vapor (Barrington-Trimis et al., 2014; Patten & De Biasi, 2020; Sundar et al., 2016b, 2016a). Despite recent efforts to ban flavor additives from popular e-cigarette brands, as of 2022, 89.4% of teenagers that use e-cigarettes still report using a flavored product, highlighting the persistence of the adolescent vaping epidemic (Birdsey et al., 2023). Nicotine use during adolescence has been associated with various mental health disorders including depression, and anxiety, as well as a general reduction in cognitive function (Breslau et al., 1991; Counotte

et al., 2011; Cuijpers et al., 2007; Goriounova & Mansvelder, 2012; Ĩiguez et al., 2009; Jobson et al., 2019; Laviolette, 2021). Moreover, approximately half of youths who ever tried e-cigarettes, reports currently using them, indicating that adolescent e-cigarette usage may continue into adulthood, increasing the risk of developing nicotine use disorder (Birdsey et al., 2023). It has also been proposed that use of nicotine during adolescence can serve as a 'gateway' to future substance use in adulthood (Kandel et al., 1992; Levine et al., 2011). This theory is supported by clinical survey data which revealed that, compared to those who had never vaped, adolescents who regularly vaped were 20 times more likely to use cannabis, and 5.6 times more likely to have binge drank, on at least one occasion (SAMHSA, 2019a).

A relationship between the use of nicotine and the use of other substances appears to persist into adulthood, with the co-use of nicotine and opioids representing an especially common form of polysubstance use (SAMHSA, 2019b). Smoking prevalence among those who either use opioids illicitly, or who are receiving methadone maintenance treatment (MMT) for an opioid use disorder (OUD), is between 74 and 97% (Guydish et al., 2011; Haas et al., 2008; Zirakzadeh et al., 2013a). Though death associated with opioid overdose is a significant public health issue (Spencer et al., 2022), co-use of nicotine and opioids presents additional cause for concern due to the additive effects of toxicity from both drugs on multiple organ systems (Compton et al., 2021; Gjersing & Bretteville-Jensen, 2018; Jordan et al., 2018; Meacham et al., 2018). Leading theories hypothesize that high prevalence of polysubstance might reflect the enhancement of drug reward when both drugs are used or the mitigation of drug withdrawal when use of one drug stops but use of the other persists (Boileau-Falardeau et al., 2022; Compton et al., 2020; Crummy et al., 2020; Pan et al., 2022).

Preclinical models have uncovered some of the neural mechanisms underlying individual reward to nicotine or opioids. The mesolimbic dopamine system, comprising dopamine (DA)

neurons in the ventral tegmental area (VTA) that project primarily to the nucleus accumbens (NAc), is considered the canonical reward pathway. Nearly all drugs of abuse, including nicotine and opioids exert their rewarding effects by enhancing DA release in the NAc (Di Chiara & Imperato, 1988). Overall, nicotine facilitates DA release by binding to nicotinic acetylcholine receptors (nAChRs) within the VTA, which results in a shift in the activity of VTA DA neurons, from tonic firing to phasic, burst firing (Fisher et al., 2021; Gao et al., 2010; Liu et al., 2012; Pidoplichko et al., 1998; Rice & Cragg, 2004; Zhao-Shea et al., 2011). On the other hand, opioids increase DA release primarily via disinhibition, binding to μ-opioid receptors (MORs) on VTA GABA interneurons which decreases local, inhibitory GABAergic transmission onto VTA DA neurons (Bull et al., 2017; Johnson et al., 1992; Lüscher & Malenka, 2011; Reeves et al., 2022).

However, recent work revealed that dopaminergic mechanisms of nicotine and opioid reward are likely more complex than initially believed, due to the existence of subpopulations of VTA DA neurons with unique anatomical and functional properties (Barbano et al., 2016; Beier et al., 2015a; Brischoux et al., 2009; Eddine et al., 2015; Maddaloni et al., 2022; Vargas-Perez et al., 2014; Yang, de Jong, et al., 2018). For example, it was demonstrated that nicotine increases the activity of lateral VTA DA neurons that project to the NAc, leading to reward-related behavior, whereas it inhibits medial VTA DA neurons that project to the amygdala, producing anxiolytic effects (Nguyen et al., 2021; Reynolds et al., 2023). Increased understanding of the functionality of different subpopulations of VTA DA neurons will be critical to understanding the rewarding and aversive properties of both nicotine and opioids.

Despite its high prevalence in the human population, co-use of nicotine and opioids has not been extensively examined in the preclinical literature. The limited body of work which exists suggests that exposure to both nicotine and opioids may produce cross-sensitization or cross-

tolerance to the rewarding and aversive behavioral effects of each drug (Araki et al., 2004; Biala & Weglinska, 2005; Haghparast et al., 2008; Rezayof et al., 2007; Zarrindast et al., 2001, 2003; M.R. Zarrindast et al., 1999). Though insightful, these studies often used drug delivery paradigms where an animal is pre-treated with one drug prior to an acute exposure to the other drug, thus not truly modeling concurrent drug use or the chronic nature of addiction. Additionally, though alterations to DA signaling have been suggested by behavioral studies, to date no one has studied the effects of co-exposure to nicotine and opioids on the functionality of VTA DA neurons.

We first established a physiologically relevant model of polysubstance use, whereby nicotine vapor exposure is initiated in adolescence, followed by a period of co-treatment with both nicotine vapor and the opioid morphine, via the continuous two-bottle choice (C2BC) paradigm. We then used this co-drug exposure model in conjunction with fiber photometry to study the natural dynamics of VTA DA activity during periods of both acute and chronic drug administration.

2.2 Materials and Methods

2.2.1 Animals

This study utilized DAT-Cre (n=58) male and female mice between postnatal days 28 (PND 28) and 130 (PND 130). Animals were housed at the University of Pennsylvania with a reverse 12 h light/dark cycle in a temperature-controlled room (24 ± 2°C; relative humidity, 55 ± 10%) with *ad libitum* access to chow and water. All fiber photometry recordings and vapor exposures occurred in the dark phase of the light cycle. Homozygous IRES-DAT-Cre males (Slc6a3$^{tm1(cre)Xz}$/J; Strain #:020080) and were purchased from The Jackson Laboratory and bred with WT C57BL/6J females. DAT-Cre animals were used in all behavioral and photometry experiments. ChAT-Cre animals were used only to establish the co-drug exposure paradigm

(Figure 3) and were not used in the fiber photometry experiments. Prior to these experiments, DAT-Cre and ChAT-Cre mice were found to perform similarly to each other and to WT C57BL/6J mice in the open field area, elevated plus maze, and marble burying tests (data not shown). All procedures described here were approved by the Institutional Animal Care and Use Committee and followed the guidelines for animal research from the National Institutes of Health.

2.2.2 Drugs

E-liquids were prepared as previously described (Patten et al., 2023). Briefly, e-liquid components [Vegetable Glycerin (VG), Propylene Glycol (PG), NicSelect Nicotine (free-base; 100 mg/ml in VG)] were purchased from Liquid Barn (Simi Valley, CA). The vehicle (VEH) solution consisted of a 50:50 blend of VG/PG. Nicotine (NIC) solutions maintained a 50:50 blend of VG/PG and were adjusted to achieve the desired nicotine concentration (5, 10 or 30 mg/mL). NIC solutions were kept in the dark and made fresh every 3 days to prevent nicotine degradation (Patten et al., 2023).

For continuous two-bottle choice (C2BC) experiments, free-base morphine sulfate (Morphine Sulfate, Spectrum Chemical MFG, New Brunswick, NJ) was dissolved in a 2.0% saccharin + tap water to reach a concentration of 0.1 - 0.2 mg/mL morphine. Morphine solutions were kept in amber bottles and were replaced every 7 days (Fleites et al., 2022). For systemic administration, morphine was dissolved in 0.9% saline to a concentration of 1 mg/mL and administered intraperitoneally (i.p.) to achieve a 10 mg/kg dose, adjusting for animal body weight. Naloxone (Naloxone Hydrochloride, 0.4 mg/mL, Mylan Institutional LLC., Morgantown, WV) was dissolved in 0.9% saline to a concentration of 0.2 mg/mL and administered via i.p. injection, adjusting for animal body weight to achieve a dose of 1 mg/kg.

2.2.3 E-cigarette vapor delivery

E-cigarette vapor delivery was performed as previously described, with modification (Patten et al., 2023). Animals were transported to a designated room 30 min prior to vapor exposure to allow for habituation, followed by placement in an air-tight exposure chamber (dimensions = 11.15” x 6.75” x 4.5”, Allentown LLC, Allentown, NJ). Air-tight exposures chambers contained a vapor delivery port and a vacuum port to replace chamber air with clean room air (flow rate = 2.0 L/min). E-liquid was vaporized using a custom-made vapor generator controller (University of Pennsylvania, Electronic Design Shop) in conjunction with a SMOK Baby Beast Brother e-cigarette tank and V8 X-Baby-Q2 coils (0.4 Ω; SMOK). The vapor generator controller was operated at 75.0 W using the preset “nicotine” settings, with the temperature set to 400°F. A 1 s puff was delivered every 90 s for a total of 25 min, followed by a 5-min wash out. We previously found that this exposure pattern and duration are sufficient to deliver pharmacologically relevant doses of nicotine and produce plasma cotinine levels comparable to a 1 mg/kg i.p. injection of nicotine (Patten et al., 2023).

2.2.4 Morphine continuous two-bottle choice (C2BC)

In addition to DAT-Cre mice, this experiment also used IRES-CHAT-Cre mice (B6;129S6-Chat$^{tm2(cre)Lowl}$/J; Strain #: 006410), Jackson Lab) and the data were pooled, as morphine consumption was comparable in both strains. Adult, single-housed ChAT-Cre (n=42) or DAT-Cre (n=39) mice were provided with continuous access to two bottles in their home cage.One bottle contained a 2.0% solution of saccharin (a non-caloric sweetener that masks morphine’s bitter taste) dissolved in tap water (Horowitz et al., 1977). The other bottle contained a solution of free-base morphine dissolved in 2.0% saccharin + tap water. This bottle initially contained a 0.1 mg/mL morphine solution, which was then escalated to 0.2 mg/mL after the first week of exposure. Mice were then maintained on a 0.2 mg/mL morphine solution for 4-5 weeks,

during which time they consumed a steady dose (mg/kg) of morphine. Control animals received two bottles of 2.0% saccharin solution only. Both bottles were cleaned and then replaced with fresh solution every 7 days. Bottles were weighed daily to measure morphine solution intake (mL) and mice were weighed weekly to calculate morphine dose (mg/kg). Position of the bottles was alternated daily to control for any potential positional preference. Our lab and others have shown that this method generate levels of opioid consumption that are sufficient to produce opioid-dependence in rodents (Fleites et al., 2022; Zanni et al., 2020).

To confirm morphine dependence when establishing the co-exposure paradigm, morphine bottles were removed from the home cage and somatic signs of withdrawal were observed 24 h later. Briefly, animals were transported to a designated behavioral testing room with a low level of illumination (3 Lux) and were allowed to habituate for 30 min. Animals were then placed in a clean empty cage without bedding and a Plexiglas lid was placed on top of the cage. For 30 min, the expression of somatic signs was monitored and recorded, including wet-dog shaking, scratching, jumping, teeth chattering, and rearing. Our lab and others have previously used these signs to quantify the severity of morphine withdrawal (Kalamarides et al., 2023; Muldoon et al., 2014b). Somatic signs were measured in the morphine-sated state (Week 4-5 of the C2BC paradigm) prior to morphine bottle removal and served as a within subject's control.

2.2.5 Viral injections and fiber optic implants

We utilized 42 male and female DAT-Cre mice (PND 60 – PND 65). Mice were anesthetized with 3.0% isoflurane, administered a subcutaneous (s.c.) injection of extended-release (ER) Meloxicam (6 mg/kg, ZooPharm, Fort Collins, CO) and then placed into a stereotaxic frame (Angle Two, Leica Biosystems, Deer Park, IL) with continuous isoflurane delivery (1.0 - 2.0%). AAV2/9-hsyn-FLEX-GCaMP6f-WPRE-SV40 (#100843, titer: 1.0×10^{12}

Vector genomes (vg) /mL, Addgene, Watertown, MA) was injected unilaterally into the VTA (0.7µL, 0.07 µL/min; bregma -3.25 A/P, -0.5 M/L, -4.5 D/V) using an infusion pump (KD Scientific, Holliston, MA) and 700 series Hamilton Syringes (#80314, Franklin, MA). Syringes were left in place for 5 min following injection to allow for complete viral diffusion. Mono fiber-optic cannulas (400 um core, .48 NA, Doric Lenses, Québec City, Ont.) were implanted dorsal to the VTA virus injection site (-3.25 A/P, 0.5 M/L, -4.3 D/V) and secured with low auto-fluorescent Metabond cement (S380, Parkell, Edgewood, NY). Mice received a second dose of ER Meloxicam 24 h after surgery to provide post-surgery pain control. For all experiments described in this chapter, viral infusion and optical fiber implantation were performed between PND 60 and PND 65, in accordance with the parameters specified above. Photometry experiments were performed no earlier than 20 days post-surgery, to allow for complete physical recovery and sufficient viral GCaMP expression.

2.2.6 Fiber photometry

GCaMP6f was excited by two wavelengths, 465-nm (calcium-dependent excitation) and 405-nm (calcium independent excitation, isosbestic control), which were generated through fiber-coupled LEDs and modulated via a real-time amplifier (RZ10X, TDT, Alachua, FL). A fluorescence mini cube (4 FC connector ports, Doric) was used to filter and combine the two excitation wavelengths. The combined excitation light was then passed through a low-autofluorescence mono fiber patch cord (400um core, 0.57 NA, Doric) and attached to the implanted mono fiber-optic cannula (400um core, 0.48 NA, Doric) via quick-release interconnect clamp (ADAF2, THOR, Newtown, NJ). GCaMP6f fluorescence was then detected via the same mono fiber patch cord, passed back through the mini cube, and eventually demodulated by the photoreceiver (RZ10X, TDT). Fluorescence was sampled at 6 Hz and digitized at 100 Hz via Synapse Software (TDT). Timestamps for nicotine vapor puffs were also digitized in Synapse

Software via TTL input from the vapor generator controller (University of Pennsylvania, Electronic Design Shop, Philadelphia, PA).

2.2.7 Fiber photometry during nicotine vapor and co-drug exposures

Mice were habituated to handling and tethering prior to all fiber photometry experiments. For nicotine vapor-only experiments, mice were tethered and then placed inside an air-tight chamber (dimensions: 4.5” x 5.4” x 7.3”), fitted with a small opening for the fiber cord that was then sealed with a customized rubber stopper, allowing mice to move freely inside the chamber. Mice were recorded from for 5 min prior to each vapor exposure to establish a baseline signal using the same vapor delivery system described above in the “e-cigarette vapor delivery” section. Briefly, after the baseline recording, the vapor generator controller was turned on and 1 s vapor puffs were delivered at 90 s intervals for a total of 25 min, followed by a 5 min washout period. TTL from the vapor generator controller to the Synapse Software provided timestamps for vapor puff delivery on the digitized photometry recording. When photometry recordings also involved acute administration of either morphine (10 mg/kg) or naloxone (1 mg/kg), mice were recorded from for 5 min prior to drug administration and were then injected i.p. with the designated drug. The vapor generator controller was then turned on 1 min after the injection to initiate nicotine vapor exposure.

2.2.8 Photometry analyses

All photometry data were analyzed using pMAT v1.3 (Bruno et al., 2021, The Barker Lab). Change in fluorescence relative to baseline fluorescence (ΔF/F) was calculated by fitting the GFP fluorescent signal (465 nm) to the isosbestic signal (405 nm). Briefly, via pMAT v1.3, data from the signal and isosbestic channels was extracted and then smoothed using the LOWESS method, a type of local linear regression that also reduces high-frequency noise (Bruno et al., 2021). The scale of the channels was then normalized to control for differences in

power level and the ΔF/F was calculated from the scaled channel signals using the following equation:

$$\Delta F/F = \frac{(Signal\ Channel - Scaled\ Control\ Channel)}{Scaled\ Control\ Channel}$$

The final normalization of the ΔF/F (z-score, number of standard deviations from baseline) relative to the timestamped vapor puff (event) was carried out by calculating the ΔF/F during a 5 s baseline sampling window prior to vapor puff initiation and then during the 10 s event window after puff delivery. Z-scores for individual vapor puff events (i) were calculated using the following equation:

$$\frac{\Delta F}{F}\text{ Z-Score (i)} = \frac{\left(\frac{\Delta F}{F}Event\ (i) - median\frac{\Delta F}{F}baseline\right)}{median\ absolute\ deviation\ (MAD)\ of\ baseline}$$

Traces for individual events were then exported as CSV files for further analysis in Microsoft Excel. Area Under the Curve (AUC) for individual events was also extracted from the robust z-score calculation and exported from pMAT as CSV files. Visualization of photometry data was performed in GraphPad Prism 10.0.02® (GraphPad Software, San Diego, CA). Unless otherwise noted, the data shown here represent the change in fluorescence over baseline fluorescence (ΔF/F) of GCaMP-expressing VTA DA neurons, relative to the time (s) before and after each vapor puff, averaged across all 17 puffs a given vapor exposure session. We quantified these changes in fluorescence by calculating and comparing the AUC of the ΔF/F (z-score) trace during 2s intervals before and after vapor puff delivery.

2.2.9 GCaMP visualization and confirmation of fiber placement

At the end of the experiment, mice were deeply anesthetized with a 4:1 ketamine/xylazine mixture and then perfused with phosphate-buffered saline (PBS), followed by a 10% formalin solution. Brains were then extracted and fixed overnight in 10% formalin

followed by at least 24 h cryoprotection with 30% sucrose solution in PBS. Brains were then embedded in clear Tissue-Plus Optimal Cutting Temperature Compound (OCT; 23-730571, Fisher Scientific, Hampton, NH), and stored at -80°C. Brains were sectioned coronally at 50 µm via cryostat and slices were mounted to slides with VECTASHIELD Antifade Mounting Medium with DAPI (NC9524612, Fisher Scientific) to verify both accurate viral targeting and fiber optic placement using epifluorescence microscopy at 4X and 10X (BX63, Olympus, Waltham, MA). If fiber placement and/or viral expression were not accurately targeted to the VTA, data from that animal was removed prior to analysis.

2.2.10 Statistical analyses

Both ΔF/F traces and AUC values are expressed as mean +/- standard error of the mean (SEM) and were analyzed using GraphPad Prism 10.0.02® (GraphPad Software). A p-value of < 0.05 was considered statistically significant. Statistical outliers were identified with GraphPad Prism using robust regression and outlier removal (ROUT, Q=1%). A repeated-measures (RM) two-way ANOVA with Tukey's post-hoc analysis was used to assess both main effect and interaction between vapor type (VEH or 5, 10, 30 mg/mL NIC) and time relative to vapor puff (Figure 1). For all other photometry comparisons, a mixed-effects ANOVA with Sidak's post-hoc analysis was used to assess main effect of drug treatment and interaction between treatment and time relative to vapor puff. A mixed-effects ANOVA was also used to determine effect of vapor treatment on morphine consumption and interaction between vapor treatment and C2BC Week (Figure 3). A paired t-test was used to compare somatic signs expressed in the morphine-sated state versus morphine-withdrawal state. No significant sex effects were observe, so sexes were collapsed for all analyses presented here.

2.3 Results

2.3.1 Acute nicotine produces a transient decrease in VTA DA activity in nicotine-naïve mice

We first established the effect of nicotine exposure alone on VTA DA activity with a vapor exposure system that delivers physiologically relevant doses of nicotine to mice. This system was previously validated by our lab (Patten et al., 2023) and, importantly, we demonstrated that this method for nicotine vapor exposure reliably produces plasma cotinine levels comparable to those observed after i.p. injections of nicotine, which has long been considered the standard route of administration in the preclinical field. We connected the e-cigarette vapor controller, used to deliver timed nicotine vapor puffs, to our fiber photometry processor to provide timestamps for these vapor puffs on the digitized photometry recording (**Figure 2-1A**). A fiber optic patch cord was fed through a small hole at the top of the vapor exposure chamber, which was then further sealed with a rubber stopper, allowing mice to be tethered to the photometry system while simultaneously being exposed to vapor. We also demonstrated that infusion of a AAV2/9-hsyn-FLEX-GCaMP6f-WPRE virus into the VTA (bregma: -3.25 A/P, -.5M/L, -4.5 D/V) of DAT-Cre mice produced stable GCaMP expression in Cre^+ neurons and that changes in GCaMP fluorescence could be measured via an optical fiber implanted just above the infusion site (+0.2 D/V, **Figure 2-1B**).

Having established a method for performing fiber photometry recordings during vapor exposure sessions, we next tested the response of VTA DA neurons to both vehicle (VEH) vapor, which consists of a 50/50 blend of Propylene Glycol (PG) and Vegetable Glycerin (VG), and three different concentrations of nicotine vapor (5, 10, 30 mg/mL NIC in 50:50 PG/VG). To mimic the age at which humans might begin vaping (Birdsey et al., 2023; Hammond et al., 2017; Miech et al., 2019) and to habituate animals to the experience of being vaped, DAT-Cre mice (n=6) began exposure (5x week, 30 min/day) to VEH vapor in adolescence (PND 28) and

continued this treatment into adulthood (PND 80, **Figure 2-1C**). At PND 80, mice were randomly assigned to either VEH or NIC (5, 10 or 30 mg/mL) vapor for the first recording. Subsequent vapor exposures were randomized, via a Latin square design, to ensure that each mouse received every vapor type twice, with at least 48 h between recordings, totaling 8 recordings per animal.

Following assignment to a specific vapor type, mice were tethered to the photometry system and placed inside the vapor exposure chamber. Prior to vapor puff delivery, we recorded VTA DA neuron activity for 5 min to establish a baseline fluorescence signal. The vapor controller was then turned on and mice were exposed to 1 s vapor puffs every 90 s for a total of 17 puffs delivered over a 25 min period. Though the prevailing literature indicates that nicotine should produce an increase in phasic firing of VTA DA neurons (Floresco et al., 2003; Schultz, 1998), we observed a sharp decrease in VTA DA activity in response to all concentrations of NIC vapor, with the 10 and 30 mg/mL solutions producing a slightly larger reduction in activity than the 5 mg/mL solution (**Figure 2-1D**). Decreased VTA DA activity was noted ~1 s after the vapor controller was turned on, approximately the time it takes for the puff to travel from the e-cigarette to the chamber. This decrease in VTA DA activity was sustained for at least 10 s post-puff but recovered to baseline prior to delivery of the next vapor puff (not shown). Whereas NIC produced a decrease in VTA DA activity, we saw the opposite effect in response to VEH vapor, with a transient increase in activity starting 1 s after the vapor controller was turned on and lasting approximately 5 s post-puff. Importantly, VTA DA response to either NIC or VEH vapor was not dependent on the cues associated with a vapor puff (**Supplemental Fig. 2-1**). We then quantified these VTA DA responses via AUC analysis and determined a significant effect of vapor type (RM two-way ANOVA; $F(2.27, 11.37) = 20.22$, $p < .001$) and a significant interaction between vapor type and time relative to vapor puff (RM two-way ANOVA; $F(1.11, 5.28) =$

10.56, p = .019, **Figure 2-1E**). For each of the NIC vapors compared to VEH vapor, there was a significant difference in AUC at both the 1-3 and 3-5 s post-puff time points (Tukey's multiple comparisons, $p \leq 0.01$). In essence, contrary to the predominant preclinical literature, we show that acute nicotine produces a reduction in VTA DA activity.

2.3.2 Chronic exposure to nicotine mitigates the reduction in VTA DA activity produced by acute nicotine

Although it was important to establish the effects of acute vapor exposure on VTA DA activity, addiction is a chronic disease, with changes to behavior and neural circuitry progressing over the course of many weeks, months, and years (Koob & Volkow, 2009, 2016). Therefore, it is equally as important to investigate how chronic nicotine exposure might change VTA DA activity, in addition to studying the effects of an acute exposure. Importantly, the fact that chronic nicotine use often begins in adolescence, with 90% of daily adult smokers trying their first cigarette before the age of 18, prompted us to design a preclinical model that more closely resembles the human experience (Kong & Krishnan-Sarin, 2017). To mirror both the duration and age of initiation of nicotine use, we exposed DAT-Cre mice (n=29) to nicotine vapor (30 mg/mL) according to our standard vapor exposure schedule (5x week, 30 min/day), beginning in adolescence (PND 28) and continuing into adulthood (PND 80, **Figure 2-2A**). At this time, we recorded VTA activity in mice that had been chronically treated with nicotine (Chronic NIC) during a nicotine (30 mg/mL) vapor exposure session, and analyzed changes in VTA DA activity, before and after vapor puff delivery.

For Chronic NIC mice, we noted a mitigation of the initial decrease in VTA DA activity in response to NIC that we had observed in nicotine naïve (Acute NIC) animals (Figure 2-1D, **Figure 2-2B**). However, chronic nicotine treatment also appeared to prolong the time that VTA DA neuron activity remained depressed following a NIC puff, though, like Acute NIC animals,

activity recovered to baseline before the delivery of the next puff (data not shown). Quantification of changes in fluorescence signal via AUC analysis revealed a significant interaction between duration of treatment (Chronic vs. Acute) and time relative to vapor puff (mixed-effects ANOVA, $F(2, 58) = 3.84$, $p = 0.027$, **Figure 2-2C**). Post-hoc analysis revealed a significant difference in AUC between Chronic and Acute NIC mice specifically during the 1-3 s post-puff period, reflecting the mitigation of decreased VTA DA activity that we had observed qualitatively (Sidak's multiple comparison's, $p < 0.05$).

As noted previously, nAChR desensitization is one of the hallmarks of chronic nicotine use and contributes to nicotine dependence, so we next explored a potential role for this biological phenomenon in our photometry data (Ochoa et al., 1989; Quick & Lester, 2002; Wooltorton et al., 2003). To this end, we binned nicotine vapor puffs and separately analyzed puffs 1-5, puffs 6-11, and puffs 12-17, in both Acute NIC and Chronic NIC mice (**Supplemental Fig. 2-2**). Although Acute NIC animals showed a similar VTA DA response across each of the binned NIC vapor puff periods, Chronic NIC animals showed the largest VTA DA response to the first 5 puffs, and a notably attenuated response to the subsequent puffs, potentially indicating increased desensitization of these neurons following chronic exposure to nicotine.

2.3.3 Chronic co-exposure to nicotine and morphine does not further alter VTA DA response to acute nicotine

In general, polysubstance use is extremely common among those with substance use disorders, although it has historically received limited attention in the preclinical literature (John et al., 2018; Mojtabai, 2022). Because co-use of nicotine and opioids is a highly prevalent form of polysubstance use, we next explored a role for the mesolimbic DA system in mediating this dual drug use (Compton et al., 2020; Morris & Garver-Apgar, 2020; Yoon et al., 2015). We first established a preclinical model for co-drug exposure, taking into consideration specific aspects

of the human drug user experience (**Figure 3A**). Like all previous experiments described in this chapter, mice were vaped with NIC (30 mg/mL) according to our standard vapor schedule (5x week, 30 min day, 17 puffs/session), beginning in adolescence, and continuing into adulthood (PND 28 – PND 80). At PND 80, while continuing regular NIC vapor exposures, mice also began morphine exposure, via a continuous two-bottle choice (C2BC) paradigm, a method for opioid self-administration that promotes consumption of - and dependence to - opioids in rodents (Fleites et al., 2022; Zanni et al., 2020). This model also allows animals to titrate their daily dose of morphine, more closely mimicking the human experience, and potentially revealing individual differences in susceptibility to developing morphine dependence (Fleites et al., 2022).

In our co-exposure C2BC paradigm, mice were given 24-h access to two bottles in their home cage, one which contained a solution with 2.0% saccharin (a non-caloric sweetener that masks morphine's bitter taste) and one which contained a solution of morphine dissolved in 2.0% saccharin (Horowitz et al., 1977). After the first week of drinking, we escalated morphine concentration from 0.1 to 0.2 mg/mL to promote increased consumption. Accordingly, mice doubled their morphine intake (mg/kg) between the first week (mean: 12.73, SEM: ± 1.19) and the second week (mean: 23.27, SEM: ± 2.14, **Figure 2-3B**). Mice proceeded to consume a steady amount of morphine (24.26 – 28.29 mg/kg) as C2BC treatment continued for another 3-4 weeks, totaling 5-6 weeks of morphine exposure in total.

During Week 5 of the co-exposure paradigm, we observed mice for 30 mins in the home cage in the morphine-sated state and quantified the expression of various somatic signs (e.g., jumping, head scratching, wet-dog shakes, etc., **Figure 2-3C**). A week later, morphine bottles were removed from the home cage and, 24 h later, during the presumed morphine-withdrawal state, we again measured the expression of somatic signs. Compared to baseline (morphine-sated state), the number of somatic signs displayed increased significantly at the 24 h post-

bottle removal timepoint (paired t-test; $t = 5.80$, $df = 11$, $p < .001$). An increase in the number of somatic signs displayed upon cessation of drug treatment is indicative of drug dependence in rodents (Chellian et al., 2021), highlighting the effectiveness of the C2BC model to produce morphine dependence in our co-exposure paradigm.

After establishing this physiologically relevant model for chronic co-exposure to both nicotine and morphine, we studied whether co-exposure further alters the VTA DA neuronal response to nicotine. Following chronic exposure to nicotine throughout adolescence (PND 28) and young adulthood (PND 80), DAT-Cre (n=29) mice were exposed to an acute dose of NIC vapor while changes in VTA DA activity were recorded (Figure 2-2). Following this recording, a subset of these mice (n=16) also began the morphine C2BC paradigm, while maintaining regular NIC vapor exposures (NIC+MOR mice), effectively beginning a 5-week co-exposure period (**Figure 2-4A**). The remaining animals (n=13) served as controls and were exposed to two bottles containing 2.0% saccharin (NIC+SACC).

Once per week, VTA DA neuron activity was recorded while mice underwent a NIC vapor exposure session. We did not detect differences in VTA DA activity in response to the delivery of NIC vapor puffs between NIC+MOR and NIC+SACC mice at any week during the co-exposure period (**Figure 2-4 B-F**). Although the peak fluorescence signal did appear to shift slightly to the right in the NIC+MOR group during Week 5, the time to peak signal was not significantly different from the NIC+SACC response (data not shown).

2.3.4 Chronic morphine exposure does not alter VTA DA activity in response to acute nicotine in nicotine-naïve mice

Though chronic co-exposure to nicotine and morphine did not further alter VTA DA activity in response to nicotine, it has been demonstrated that pre-treatment with morphine can produce both cross-tolerance and cross-desensitization to a subsequent nicotine exposure

(Rezayof et al., 2007; Vihavainen, Relander, et al., 2008; Zarrindast et al., 2003). So, we next turned to examining the effects of chronic morphine treatment on acute exposure to nicotine in nicotine-naïve animals. For this experiment, mice were treated with VEH vapor throughout adolescence and young adulthood (PND 28 – PND 80, **Figure 2-5A**). VEH vapor exposures then continued for the next five weeks, during which time mice were also consuming morphine in the C2BC model. Notably, vehicle-treated (VEH+MOR) mice consumed less average daily morphine (mg/kg) then nicotine-treated (NIC+MOR) mice over the course of the entire 5-week co-exposure paradigm (mixed-effects ANOVA; $F(1, 61) = 4.67$, $p = 0.035$), suggesting that chronic nicotine exposure alters the rewarding properties of morphine (**Figure 2-5B**).

After the 5-week co-exposure, VEH+MOR underwent their first exposure to NIC while we recorded VTA DA neuron activity. In this paradigm, VTA DA neuron activity decreased sharply in response to NIC vapor, a nearly identical response to that seen in Acute NIC mice (Figure 2-2), suggesting that chronic morphine alone does not change VTA DA response to nicotine in nicotine-naïve animals (**Figure 2-5C**). This was, of course, in contrast to the increase in VTA DA activity following NIC vapor puff delivery that we observed NIC+MOR mice (Figure 2-4F, **Figure 2-5C**). When quantifying these changes in VTA DA activity via AUC analysis, we noted a significant interaction of vapor treatment (NIC vs. VEH) and time relative to vapor puff (mixed-effects ANOVA; $F(2, 50) = 6.47$, $p = 0.0032$, data not shown). Post-hoc analysis revealed a significant difference in AUC between NIC+MOR and VEH+MOR mice at the 1-3 s post-puff timepoint (Tukey's multiple comparisons, $p \leq 0.01$). Together, this data suggests that chronic nicotine alone is responsible for the increase in VTA DA activity following acute nicotine exposure, though the addition of chronic morphine does not appear to further alter this DAergic response.

2.3.5 Heterogeneity of VTA DA neurons may explain discrepancies in response to nicotine vapor

The decrease in VTA DA activity that we saw in response to acute nicotine exposure seemed at odds with the prevailing literature, which indicates that nicotine should produce an increase in phasic, burst firing of VTA DA neurons (Floresco et al., 2003; Schultz, 1998). However, recent work has demonstrated the existence of subcircuits within the VTA that may be segregated, anatomically, functionally, or by their specific afferent and efferent projections (Lammel et al., 2014; Nguyen et al., 2021; H. Yang, de Jong, et al., 2018). In particular, Nguyen et al., and others demonstrated that medial VTA DA neurons, via projections to the amygdala, show decreased activity in response to an injection of nicotine, whereas lateral VTA neurons, which project primarily to the NAc, show increased activity following acute systemic nicotine (Eddine et al., 2015; Nguyen et al., 2021; Reynolds et al., 2023).

Given that our viral infusion and recording sites were located more medially within the VTA (-0.5 M/L), we suspected that anatomical location may explain the decrease in VTA DA activity we observed in response to NIC vapor. To begin to assess this theory, we performed three surgeries in which we injected GCaMP6f virus, and implanted optical fibers, at a more lateral position within the VTA (-3.1 A/P, -1.0 M/L, -4.3 D/V). Three weeks later we recorded VTA DA activity in these mice during a standard NIC (30 mg/mL) vapor exposure session. Supporting our hypothesis, in 2/3 of the mice with GCaMP6f infused into in the lateral VTA, we noted a rapid and brief increase in VTA DA activity in response NIC vapor (**Supplemental Fig. 2-3**). Though we have yet to confirm anatomical fiber placement and GCaMP expression in these mice, this data does suggest that cellular heterogeneity may explain diverging VTA DA responses to nicotine vapor.

2.4 Discussion

The co-use of nicotine and opioids is a commonly reported form of polysubstance abuse, and it is suspected that overlapping mechanisms at the level of the mesolimbic dopamine system may mediate this behavioral phenomenon (Custodio et al., 2022; Yoon et al., 2015; Zale et al., 2015; Zirakzadeh et al., 2013). To investigate those mechanisms, we established a preclinical model for physiologically relevant co-drug administration, with chronic exposure to nicotine vapor beginning in adolescence, followed by a 5-week co-exposure to both nicotine vapor and oral morphine, via C2BC, in adulthood (**Figure 2-1C**). We then use this paradigm in conjunction with fiber photometry to study the activity of VTA DA neurons in response to acute nicotine, chronic nicotine, and chronic nicotine plus morphine.

We found that acute nicotine decreases in VTA DA neuron activity in nicotine-naïve mice (**Figure 2-1 D, E**). This decrease in DA activity was observed at all NIC vapor concentrations evaluated (5, 10 and 30 mg/mL) and contrasted with the increase in VTA DA activity we observed when mice were exposed to VEH-only vapor, which contains a blend of propylene glycol and vegetable glycerin. We first considered the possibility that the decrease in VTA DA activity in response to NIC vapor reflected a change in the characteristics of the expected rewarding stimulus (VEH vapor). It has been demonstrated that, via inhibition from RMTg GABA neurons, VTA DA neuron burst firing is decreased when an expected reward is omitted (Matsumoto & Hikosaka, 2007). Because this group of mice had been exposed to VEH vapor throughout adolescence, it seemed possible that they found VEH vapor inherently rewarding or had developed a conditioned reward response to vapor cues. However, we previously reported that mice do not express CPP to VEH vapor (Patten et al., 2023) and clinical data indicate that neither PG nor VG are reinforcing to human e-cigarette users in the absence of nicotine (Smith et al., 2020). We also showed that VTA DA activity does not change in response to the

presentation of the vapor cue alone (**Supplemental Fig. 2-1**), suggesting that VTA DA response to either VEH or NIC does not reflect the expectation of the subsequent vapor puff. Given the lack of a response to vapor cue but the robust response to VEH vapor itself, it seems likely that either PG or VG, or the combination of the two substances, acts on VTA DA neurons, either directly or indirectly, to increase their activity. To this point, PG can enhance dopamine release when applied extracellularly *in-vitro,* indicating that PG may increase VTA DA activity on its own (Hattori et al. 2000).

If not a reflection of reward omission, the decrease in VTA DA activity that we observed in response to NIC vapor seems at odds with the prevailing literature. Historically, it has been demonstrated that nicotine increases VTA DA neuron burst firing, facilitating increased DA release in the NAc, and ultimately producing reward to nicotine (De Biasi & Dani, 2011; Grenhoff, Aston-Jones, et al., 1986; Schultz, 2007). Yet, it is notoriously difficult to achieve stable nicotine self-administration in rodents, suggesting that nicotine reward may be more complicated than reward to other drugs of abuse. The complexity of nicotine reward is further demonstrated by the dose response curve for nicotine, which follows an inverted U-shape in both humans and rodents, indicating that nicotine becomes aversive at high doses (Fudala et al., 1985; Picciotto, 2003; Rose & Corrigall, 1997). One recent study (Liu et al., 2022) employed calcium imaging to reveal that high doses of nicotine not only produced behavioral aversion but also produced a biphasic neuronal response - an initial decrease in activity followed by a delayed increase - in a subset of VTA DA neurons. Thus, perhaps each of the NIC concentrations we tested was too high to facilitate nicotine reward, instead falling on the aversive side of the dose-response curve, reflected in the decreased VTA DA activity that we observed in response to NIC vapor puffs. This hypothesis is supported by the observation that there was a slightly smaller decrease in DAergic response to vapor puffs from the lowest

concentration of NIC (5 mg/mL) tested, compared to the higher NIC concentrations (10 and 30 mg/mL). Patten et al (2023) demonstrated that mice display the most robust CPP to NIC vapor when using a 2.5 mg/mL concentration, so future experiments should record VTA DA activity in response to this, and lower, nicotine concentrations.

As highlighted previously, recent work has revealed that nicotine may differentially affect subpopulations of VTA DA neurons, which vary in their afferent and efferent connectivity and may serve divergent functions. In this way, the ability of VTA DA neurons to signal nicotine reward and aversion may not only be a function of dose, but also of the intrinsic properties of the cell. Specifically, Nguyen et al. (2021) reported that nicotine depresses activity in more medially located VTA DA neurons, which project primarily to the amygdala and are responsible for regulating nicotine's anxiolytic properties. On the other hand, lateral VTA DA neurons show increased activity in response to nicotine and mediate nicotine reward via extensive efferent projections to the NAc. A similar effect has been demonstrated in a handful of other studies, where medial VTA DA neurons show an inhibitory response to nicotine but lateral VTA DA neurons are excited by the same nicotine exposure (Eddine et al., 2015; Reynolds et al., 2023). Given that we had recorded from VTA DA neurons in a more medial location (-0.5 M/L), we hypothesized that the decrease in VTA DA activity we saw in response to NIC vapor may reflect the anatomical location and functional connectivity of the subpopulation from which we were recording. To begin to probe this theory, we performed a pilot experiment in which we recorded from lateral (-1.0 M/L) VTA DA neurons. In accordance with the findings from Nyugen et al. and others, we show that DA neurons in this lateral VTA recording site display a rapid and brief increase in activity in response to puffs of NIC vapor (**Supplemental Fig. 2-3**). Though these preliminary results are intriguing, further experimentation, with more specific targeting of

purported subpopulations of VTA neurons, using retrograde or anterograde viral infusions, will be necessary to confirm that NIC vapor differentially regulates DAergic activity.

Though we saw a decrease in VTA DA activity in response to acute nicotine in nicotine-naïve animals (Acute NIC), this response was mitigated in animals that were chronically exposed to nicotine (Chronic NIC, **Figure 2-2**). In the Chronic NIC mice, we saw an early, small increase in VTA DA response to NIC vapor, which was followed by a sustained decrease in activity, more closely aligned with the DA response observed in Acute NIC mice. It is likely that this change in initial VTA DA response reflects both the age that mice began NIC vapor exposures and the length of NIC treatment prior to recording during an acute exposure session. Clinical studies report that exposure to nicotine during adolescence is associated with continued nicotine use in adulthood, suggestive of alterations to the mesolimbic reward circuitry (Barrington-Trimis et al., 2014; Cantrell et al., 2018; Leventhal et al., 2015). Work in preclinical models has further explored this phenomenon, demonstrating that, compared to adult animals, adolescent rodents are more sensitive to the rewarding effects of nicotine (Adriani et al., 2002; Faraday et al., 2001; Schassburger et al., 2016; Shram et al., 2006), as well as less sensitive to its aversive effects (Dannenhoffer et al., 2016; Elliott et al., 2004; Wilmouth et al., 2004), highlighting the increased sensitivity of the DA system to nicotine during adolescence.

Especially relevant for our findings, it was recently shown that, in adult mice who had been exposed to nicotine during adolescence, there was a stronger activation of DA neurons in response to nicotine, compared to controls, demonstrating that early life exposure to nicotine may ‘freeze’ VTA DA neurons in the hypersensitive, adolescent-like state (Reynolds et al., 2023). In this way, the mitigation of decreased VTA DA response to NIC that we observed in mice who had been chronically exposed to NIC since adolescence may be reflective of a shift in

the behavioral effects of NIC vapor exposure, away from nicotine aversion and toward nicotine reward.

Although plasticity is especially prominent in adolescent brains, prolonged exposure to nicotine in adulthood can also affect neural circuitry, offering another explanation for the mitigation in VTA DA response to NIC vapor that we observed after chronic nicotine exposure. Desensitization and upregulation of nAChRs, specifically α4β2* nAChRs, are two of the most well studied molecular effects related to chronic nicotine use (De Biasi & Dani, 2011; Fenster et al., 1999; Govind et al., 2009; Ngolab et al., 2015). However, the effects of nAChR desensitization and upregulation on VTA DA activity are less clearly defined. Both increased and decreased DAergic activity have been reported in response to chronic nicotine treatment, though other reports cite that VTA DA activity remains unchanged (Besson et al., 2007; Grieder et al., 2012; Nashmi et al., 2007; Rasmussen et al., 1005; Taylor, 2011; Xiao et al., 2009).

To begin to explore a role for desensitization and upregulation in our results, we binned NIC vapor puffs, separately examining the response of VTA DA neurons to puffs 1-5, 6-11 or 12-17. We revealed that, for Chronic NIC mice, NIC vapor produced the most robust increase in VTA DA activity during the first 5 vapor puffs, with a more muted DA response to subsequent vapor puffs (**Supplemental Fig. 2-2**) This is suggestive of both nAChR upregulation, with the initial increase in sensitivity of VTA DA neurons compared to control, and enhanced desensitization, with a diminished response to NIC vapor over the course of the exposure session. Interestingly, in nicotine-naive mice, the response of VTA DA neurons to acute NIC vapor did not change with subsequent vapor puffs. Together this indicates that, for mice chronically exposed to nicotine, the mitigation of the decrease in VTA DA activity in response to NIC vapor may reflect molecular alterations to nAChRs. To that end, we are in the process of

collecting electrophysiological data, measuring nAChR currents in both nicotine-naïve and chronically-exposed animals, further exploring this desensitization hypothesis (data not shown).

Having examined the VTA DA response to NIC in both nicotine-naïve and chronic nicotine-exposed mice, we finally turned to the effects of concurrent treatment with nicotine and morphine on VTA DA activity. We showed that chronic co-exposure to nicotine and morphine does not further alter the VTA DA response to nicotine, compared to chronic nicotine exposure alone (**Figure 2-4**). This was somewhat unexpected as the preclinical literature indicates that both nicotine and morphine can produce cross-sensitization or cross-tolerance to the rewarding effects of the other drug, seeming to indicate that co-exposure may uniquely alter the mesolimbic DA circuitry (Rezayof et al., 2007; Vihavainen, Relander, et al., 2008; Zarrindast et al., 2003). Interestingly, we revealed that mice exposed to NIC vapor throughout adolescence and adulthood consume more morphine in the C2BC during a period of co-exposure than mice exposed to VEH vapor (**Figure 2-5B**).

Despite the lack of apparent change to VTA DA signaling during chronic co-exposure, it is possible that the VTA DA neurons were in fact undergoing alterations, but that these changes were masked by modifications to other components of the circuit. In this way, homeostatic function of DA neurons may have been maintained during chronic drug use, though neuroadaptations may have eventually been revealed upon cessation of drug use, alongside the emergence of the behavioral withdrawal syndrome. Yet, when we evaluated the VTA DA response to NIC vapor in nicotine-naïve, morphine-dependent animals (VEH+MOR) during morphine-withdrawal we did not observe any significant differences in VTA DA activity compared to the morphine-sated state (**Figure 2-5E**). However, we did not perform the same experiment in mice chronically co-exposed to both nicotine and morphine (NIC+MOR), so it is

possible that co-exposure induces unique, subtle changes in DAergic signaling that may not be fully revealed until morphine-withdrawal.

As alluded to previously, we suspected that alterations to another component of the mesolimbic reward circuitry, VTA GABA neurons, may underlie the apparent lack of change to DA activity observed during co-exposure to both nicotine and morphine. Unlike VTA DA neurons, which minimally express the μ-opioid receptor (MOR), VTA GABA neurons show dense expression of MORs at both the soma and presynaptic terminals (Bull et al., 2017; Johnson et al., 1992; Lüscher & Malenka, 2011; Reeves et al., 2022; White, 1996). In this way, morphine binding to MORs on GABA neurons can directly alter both GABAergic firing and the presynaptic release of GABA. Whereas acute morphine binding to MORs leads to hyperpolarization of VTA GABA interneurons, thereby decreasing inhibition at local synapses with VTA DA neurons, chronic morphine may dysregulate this inhibitory control. MOR desensitization occurs with chronic morphine use, which may decrease the ability of VTA GABA neurons to both inhibit, but also to disinhibit, VTA DA neurons (Bergevin et al., 2002; Lowe & Bailey, 2015; Williams et al., 2013). Further, VTA GABA neurons make local synapses onto glutamatergic afferents, and disinhibition at these synapses is critical for increased excitability of VTA DA neurons observed in the presence of morphine (Chen et al., 2015; Yang et al., 2020). Therefore, though VTA DA neurons may be experiencing less inhibition at GABAergic synapses after chronic morphine, they may also be receiving less excitatory input at glutamatergic synapses, resulting in the lack of an apparent change in VTA DA activity.

In conclusion, we find that chronic nicotine exposure mitigates the reduction in activity we observed in VTA DA neurons in response to acute nicotine vapor, but that co-exposure to both nicotine and morphine does not further alter this DAergic response. However, given that we saw an increase in morphine consumption in animals who were concurrently treated with

nicotine vapor, we suspect that the mesolimbic dopamine system and the VTA are in fact altered by co-exposure, but perhaps in a more nuanced way. It is possible that if we recorded from specific subpopulations of VTA DA neurons, rather than from the population as a whole, we may have been better able to detect meaningful differences in activity following co-exposure. It is also feasible that changes to VTA DA activity were masked during chronic co-exposure, perhaps due to compensatory changes in other cell types, like GABA interneurons, but could have been unveiled during drug withdrawal. To address these concerns, future experiments will need to employ more advanced viral targeting techniques, to isolate and record from varied VTA neuronal subpopulations, making sure to record neuronal activity during both drug-sated and drug-withdrawal states.

2.5 Figures

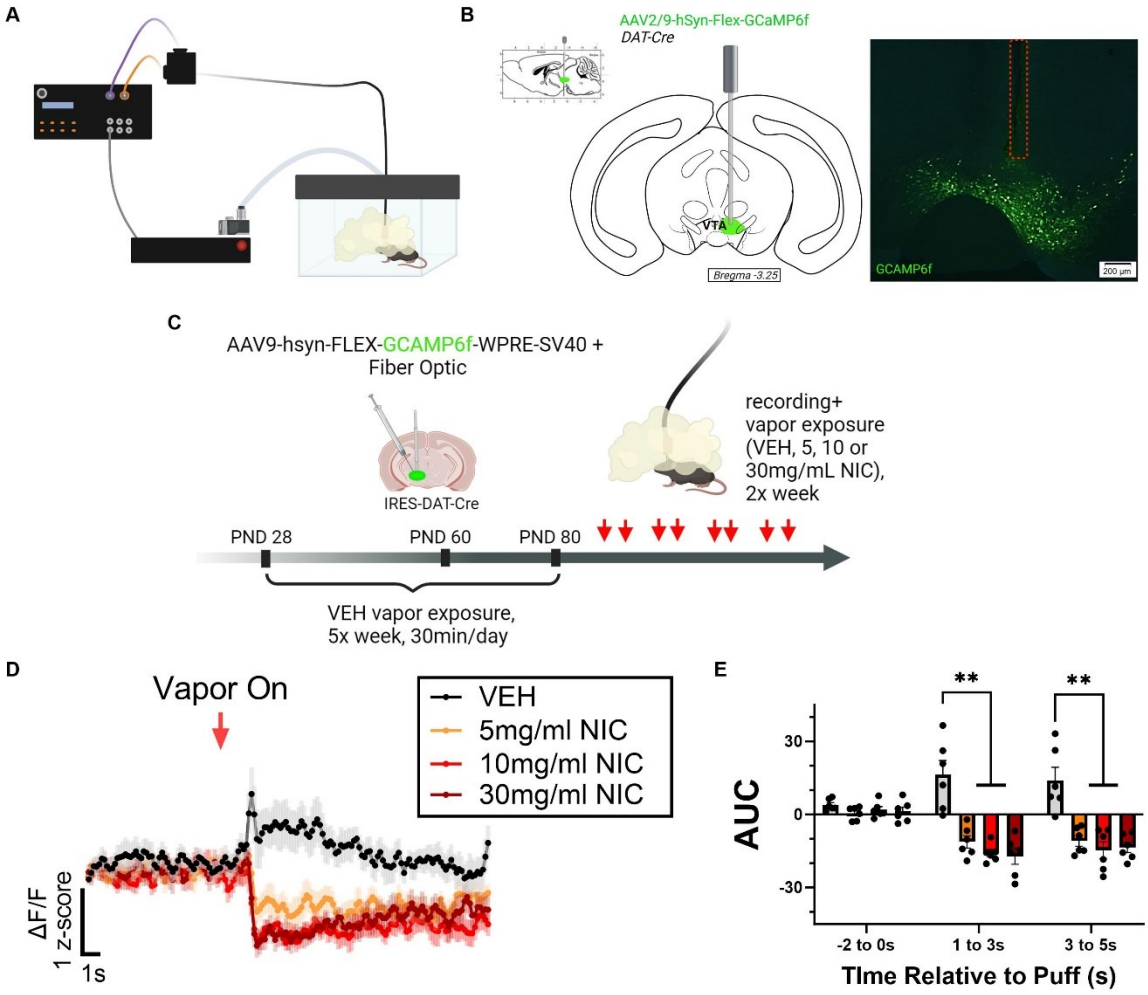


Figure 2-1. Acute nicotine produces a transient decrease in VTA DA activity in nicotine-naïve mice.

(A) Experimental set-up. Our validated, nicotine vapor administration system was connected to a fiber photometry processor to provide time-stamped recordings of VTA DA neurons during vapor exposures. (B) Confirming GCaMP expression in DAT-Cre mice. Infusion of AAV2/9-hsyn-FLEX-GCaMP6f-WPRE virus (0.6µL, 0.1µL/min) into the VTA (-3.25 A/P, -.5M/L, -4.5 D/V) of DAT-Cre mice produced stable GCaMP expression in Cre^{+} neurons within the VTA. An optical fiber was implanted just above the injection site (-3.25 A/P, -.5M/L, -4.3 D/V) to detect changes in GCaMP fluorescence (C) Experimental timeline. DAT-Cre mice *(n=6)* vaped with vehicle (VEH) vapor (5x week, 1x day, 17 puffs/session) from PND 28 to PND 80. At PND 60, AAV2/9-GcAMP6f was infused into the VTA, and optical fibers were placed above the injection site. At PND 80, VTA DA neuron activity was recorded during exposure to randomized vapor (VEH, or 5, 10 or 30 mg/mL NIC). (D) Average ΔF/F (z-score) of VTA DA neurons before (5s) and after (10s) exposure to vapor puffs. Response represents the average of 17 puffs administered 90 s apart over the course of a 25 min session. VEH vapor produced an increase in VTA DA activity. All NIC vapor puffs produced a decrease in VTA DA activity. (E) Area under the curve (AUC) of the Average ΔF/F (z-score) during 2 s intervals before and after vapor puff. Repeated measures (RM) two-way ANOVA revealed significant effect of vapor type on VTA DA activity (RM two-way ANOVA; $F(2.27, 11.37) = 20.22$, $p < .001$) and a significant interaction between vapor type and time relative to vapor puff (RM two-way ANOVA; $F(1.11, 5.28) = 10.56$, $p = 0.019$). Post-hoc analysis revealed a significant difference in AUC at both post-puff timepoints for each of the NIC concentrations compared to VEH (Tukey's multiple comparisons, $p \leq 0.01$).

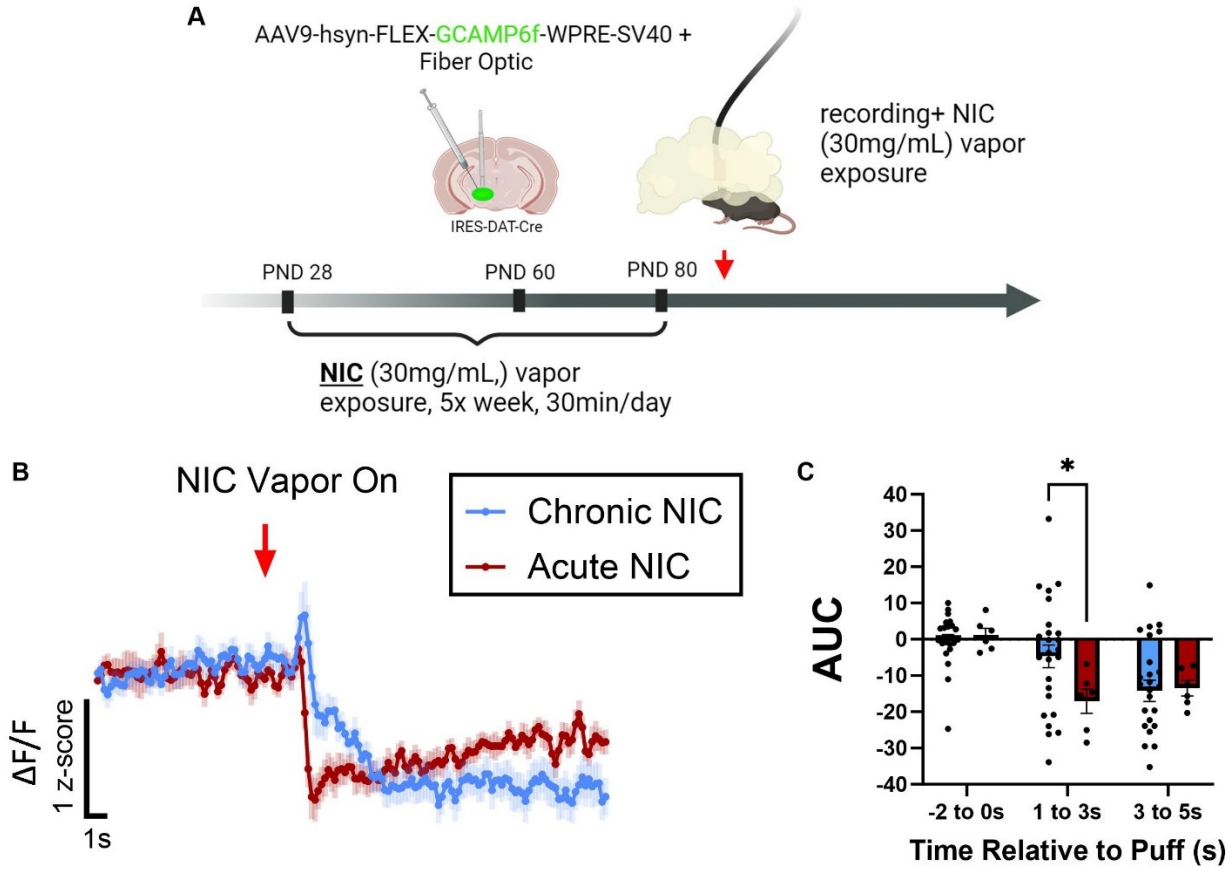


Figure 2-2. Chronic exposure to nicotine mitigates the reduction in VTA DA activity produced by acute nicotine.

(A) Experimental timeline. DAT-Cre mice (n=29) were exposed to nicotine (NIC, 30 mg/mL) vapor between PND 28 to PND 80. VTA DA neurons recorded from during exposure to NIC (30 mg/mL) vapor at PND 80. (B) Average ΔF/F (z-score) of VTA DA neurons before (5s) and after (10s) exposure to NIC (30 mg/mL) vapor puff. In mice chronically exposed to nicotine (Chronic NIC) prior to recording, the reduction in VTA DA activity in response to NIC vapor was diminished in comparison to acutely exposed mice (Acute NIC, Figure 1). (C) Area under the curve (AUC) of the Average ΔF/F (z-score) during 2s intervals before and after NIC vapor puff. Mixed-effects ANOVA reveals significant interaction between duration of treatment (Chronic vs. Acute) and time relative to vapor puff (mixed-effects ANOVA, $F(2, 58) = 3.84$, $p = 0.027$). Post-hoc analysis showed significant difference in AUC during the 1-3s post-puff period (Sidak's multiple comparison's, $p < 0.05$)

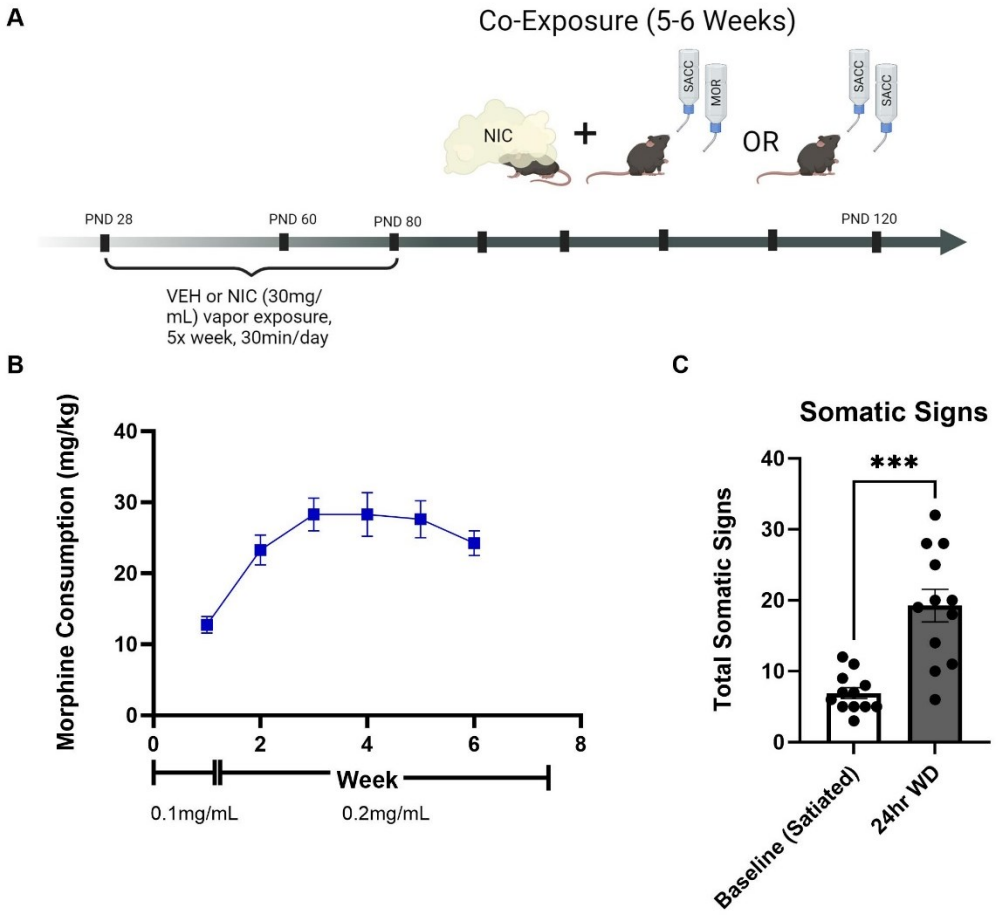


Figure 2-3. Morphine treatment via continuous two-bottle choice (C2BC) results in stable morphine consumption and morphine dependence in mice during chronic co-treatment with nicotine

(A) Co-exposure experimental paradigm. Adolescent mice (n=30) mice were exposed to nicotine vapor (NIC, 30 mg/mL) beginning at PND 28 and through PND 80. The co-exposure paradigm began at PND80, consisting of the continuation of NIC vapor treatment and morphine treatment via C2BC. Mice were given *ad libitum* access to 1 bottle with 2.0% saccharin and 1 bottle with morphine + 2.0% saccharin. Morphine concentration was escalated from 0.1 mg/mL during the first week to 0.2 mg/mL during the second week. (B) Morphine consumption (mg/kg) during 5-week C2BC. NIC-exposed mice doubled their morphine intake between the first week (mean: 12.73, SEM: ± 1.19) and the second week (mean: 23.27, SEM: ± 2.14). Mice proceeded to consume a steady amount of morphine (24.26 – 28.29 mg/kg) during weeks 3-6 of the co-exposure paradigm. (C) Quantification of somatic signs of morphine withdrawal (e.g.; wet-dog shaking, scratching, jumping, teeth chattering, and rearing) during sated and withdrawal states. Baseline somatic signs were observed during a 30 min period for a subset of mice (n=12) in the morphine-sated state during Week 5 of the co-exposure paradigm. Morphine bottles were removed during Week 6 and, 24 h later, somatic signs were observed again during the withdrawal state. The number of somatic signs at 24 h post-bottle removal was significantly higher than at baseline in the morphine-sated state (paired t-test; $t = 5.80$, $df = 11$, $p < 0.001$).

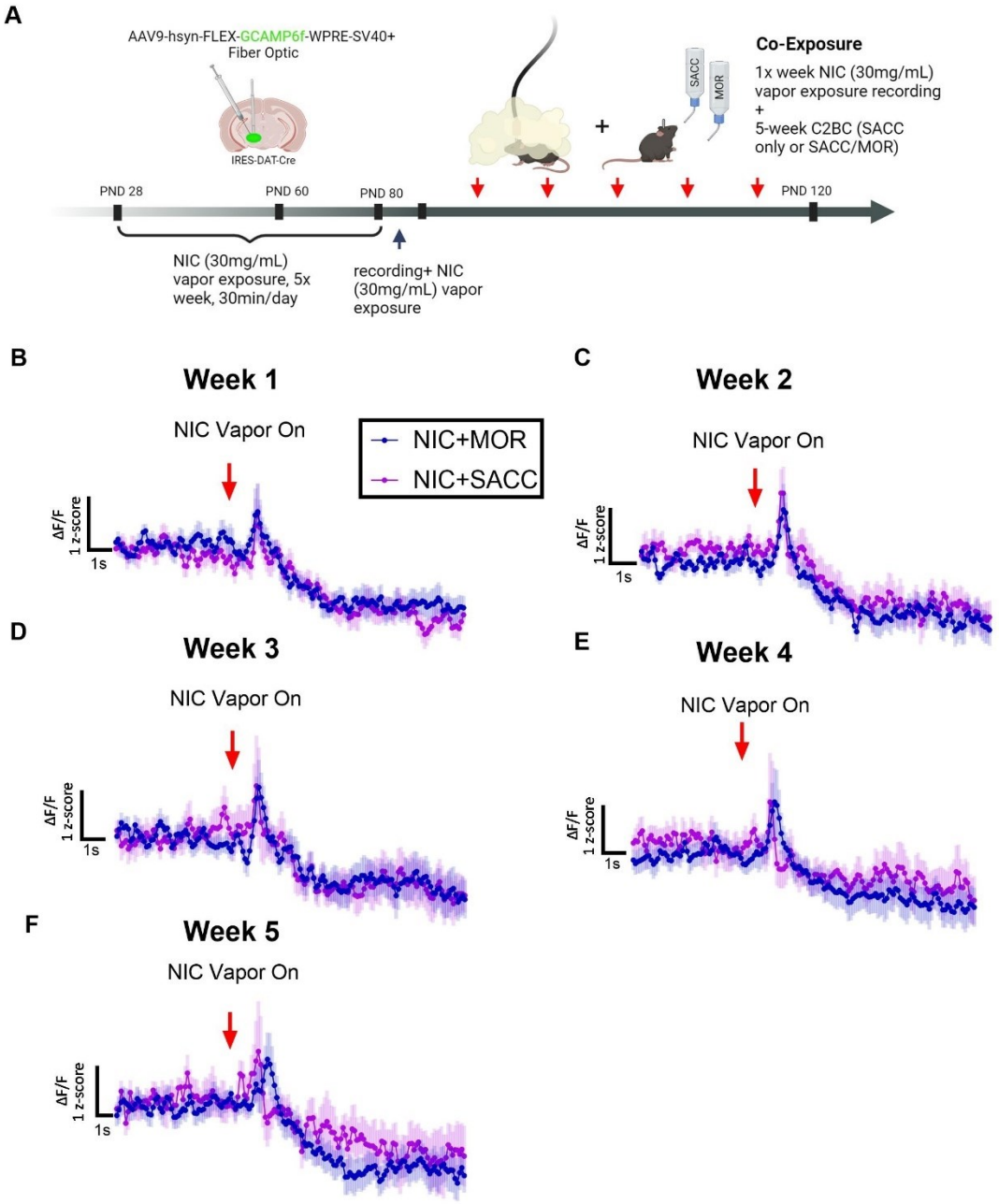


Figure 2-4. Chronic co-exposure to nicotine and morphine does not alter VTA DA response to acute nicotine.

(A) Experimental timeline. DAT-Cre mice (n=29) were exposed to nicotine (NIC, 30 mg/mL) vapor from PND 28 to PND 80. The 5-week co-exposure paradigm began at PND 80, with continuation of NIC vapor treatment and morphine (MOR) via a continuous 2-bottle choice (C2BC) paradigm. A subset of mice (n=16, NIC+MOR) was given *ad libitum* access to one bottle with 2.0% saccharin and one bottle with 2.0% saccharin + morphine (concentration escalated from 0.1mg/mL to 0.2mg/mL after the first week). Control animals (n=5, NIC+SACC) received two bottles of 2.0% saccharin solution. Photometry recordings during NIC vapor exposures were performed 1x week during the co-exposure period. (B-F) Average ΔF/F (z-score) of VTA DA neurons before (5s) and after (10s) exposure to NIC (30 mg/mL) vapor puff. No significant differences observed between NIC+MOR and NIC+SACC mice at any timepoint during the co-exposure period.

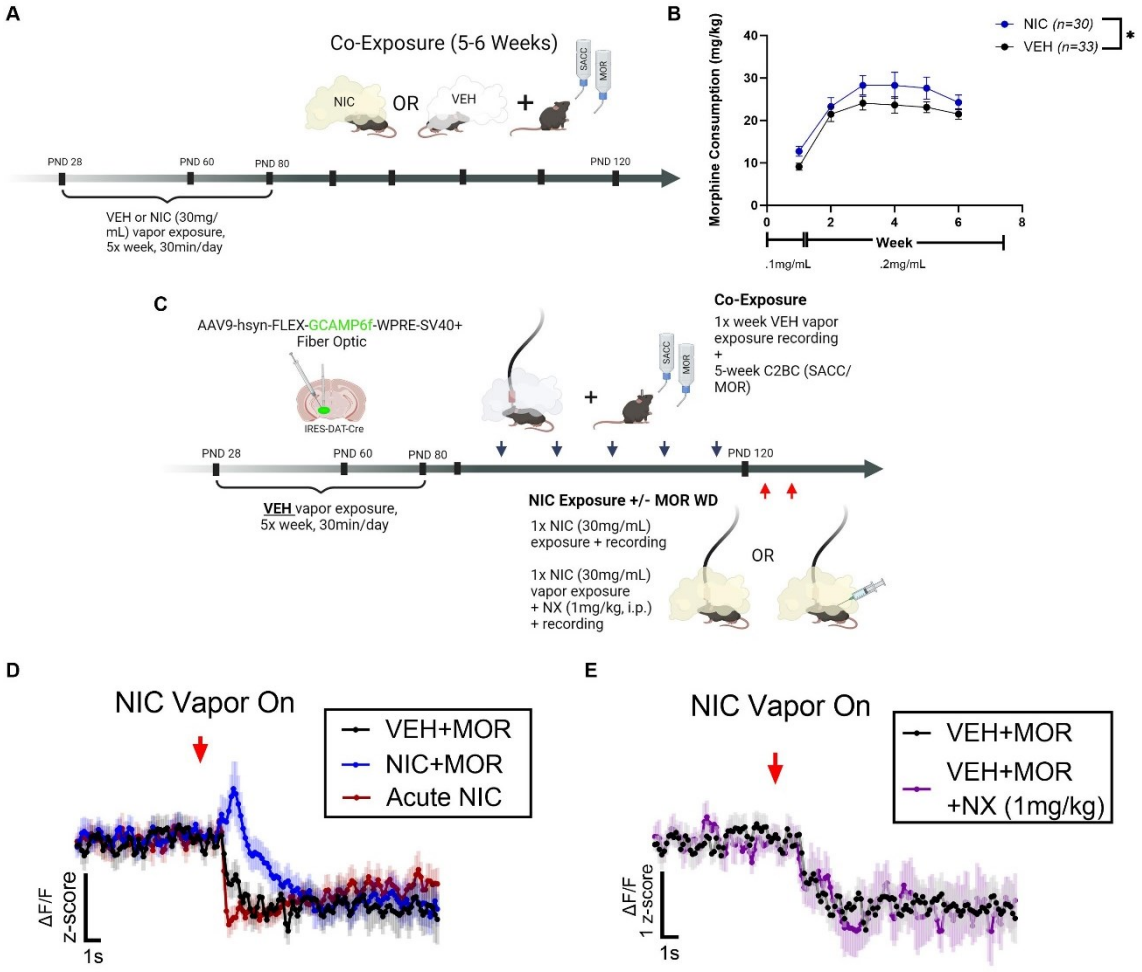
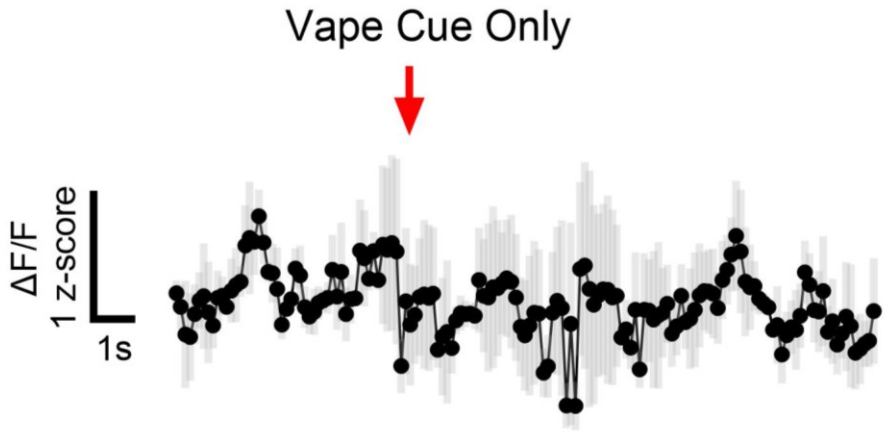


Figure 2-5. Chronic morphine exposure does not alter VTA DA activity in response to acute nicotine in nicotine-naïve mice.

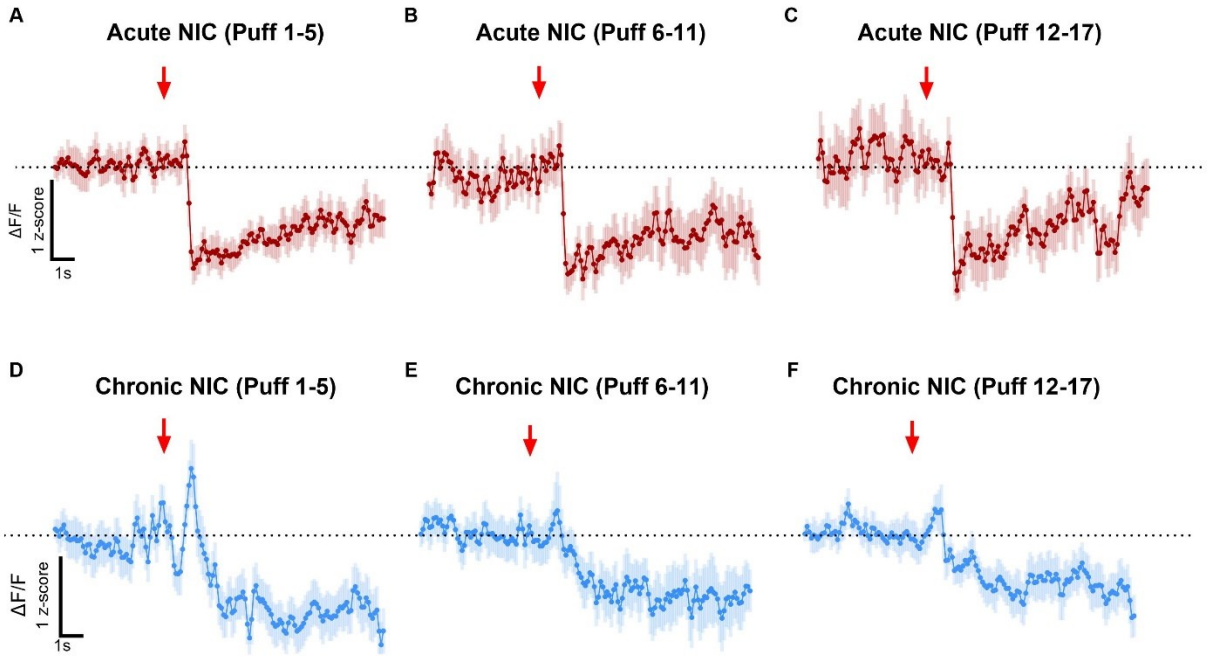
(A) Mice were exposed to nicotine (NIC, 30 mg/mL, n=30) or vehicle (VEH, n=33) vapor from PND 28 to PND 80. The co-exposure paradigm began at PND 80, with both vapor (NIC or VEH) treatment and morphine treatment via a continuous two-bottle choice (C2BC) paradigm. Mice were given *ad libitum* access to one bottle with 2.0% saccharin and one bottle with 2.0% saccharin + morphine (concentration escalated from 0.1mg/mL to 0.2mg/mL after Week 1). (B) Mice exposed to NIC throughout adolescence and the co-exposure period (NIC+MOR) consumed more average daily morphine (mg/kg) than VEH-exposed mice (VEH+MOR). Mixed-effects ANOVA revealed a significant effect of vapor treatment (mixed-effects ANOVA; $F(1, 61) = 4.67$, $p = 0.035$). (C) A subset of DAT-Cre mice (n=7) was exposed to VEH vapor starting in adolescence and continuing into adulthood (PND 28 – PND 80). Co-exposure began at PND 80 with continuation of regular VEH vapor treatment + morphine via C2BC. Once per week VTA DA activity was recorded during a VEH vapor exposure session. After Week 5, mice were exposed to NIC (30 mg/mL) vapor and VTA DA activity was recorded. 72 h later mice received an injection of naloxone (NX, 1 mg/kg, i.p.) and VTA DA activity was recorded again during a NIC vapor exposure session. (D) Average ΔF/F (z-score) of VTA DA neurons before (5s) and after (10s) exposure to NIC (30 mg/mL) vapor puff. VEH+MOR mice show similar VTA DA response to NIC as morphine-naïve animals (Acute NIC, see Figure 1). (E) Average ΔF/F (z-score) of VTA DA neurons before (5s) and after (10s) exposure to NIC vapor puff. Similar VTA DA activity in response to NIC observed in morphine-sated (VEH+MOR) and morphine-withdrawal (VEH+MOR+NX) states.

2.6 Supplemental Figures



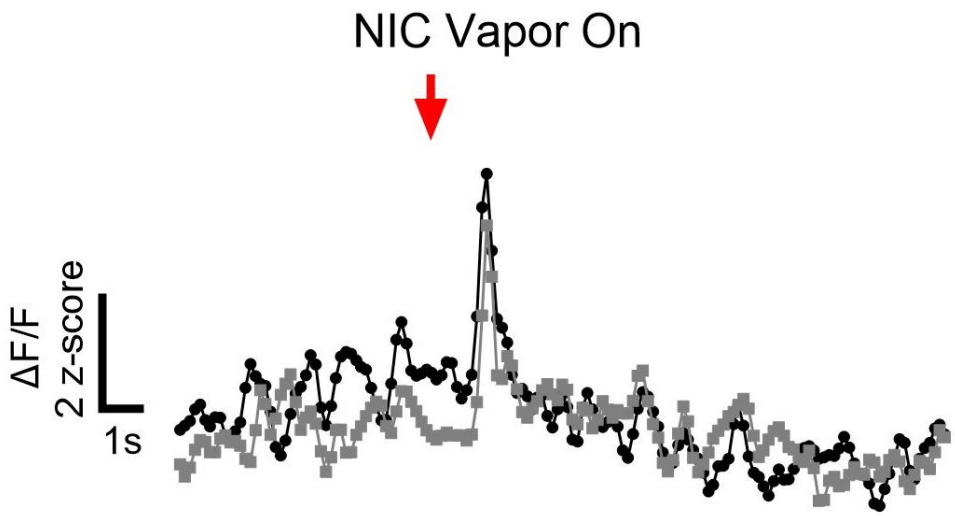
Supplemental Figure 2-1. VTA DA activity does not change in response to vapor cue alone.

Average ΔF/F (z-score) of VTA DA neurons before (5s) and after (10s) presentation of vapor cue alone. DAT-Cre mice (n=3) were tethered to a fiber optic patch cord and placed inside vapor exposure chamber. Vapor exposure session was set-up according to standard protocol (e.g., vacuum at 2.0 flow rate, vapor generator controller programmed to deliver 17 puffs, puffs triggered by controller, etc.) but tubing connecting the e-cigarette to the vapor exposure chamber was unhooked, preventing vapor from entering the chamber.



Supplemental Figure 2-2. Mice chronically exposed to nicotine show an increase in VTA DA activity in response to acute nicotine, but the response diminishes with successive vapor puffs.

(A-C) Average ΔF/F (z-score) of VTA DA neurons before (5s) and after (10s) nicotine (NIC, 30 mg/mL) vapor puffs, binned into Puff 1-5, 6-11, 7-12, in nicotine-naïve animals (Acute NIC, Figure 1). VTA DA response is similar across all binned vapor puffs. (D-F) Average ΔF/F (z-score) of VTA DA neurons before (5s) and after (10s) NIC vapor puffs, binned into Puff 1-5, 6-11, 7-12, in chronic-nicotine exposed animals (Chronic NIC, Figure 2). VTA DA response is largest during first 5 NIC vapor puffs and diminishes during vapor puffs 6-11 and 12-17.



Supplemental Figure 2-3. Lateral VTA DA neurons may show increased activity in response to acute nicotine.

Average ΔF/F (z-score) of lateral VTA DA neurons before (5s) and after (10s) nicotine (NIC, 30 mg/mL). AAV2/9-hsyn-FLEX-GCaMP6f-WPRE virus (0.7µL, 0.07µL/min) was infused into the lateral VTA (-3.1 A/P, -1.0 M/L, -4.3 D/V) of DAT-Cre mice (n=3). DAT-Cre mice (n=2) with GCaMP6f injected into in lateral VTA show rapid, brief increase in VTA DA activity in response to NIC vapor.

CHAPTER 3: EFFECTS OF NICOTINE AND MORPHINE CO-EXPOSURE ON GABAERGIC ACTIIVTY IN THE VTA

3.1 Introduction

Over the course of the 21st century, two distinct, drug-related 'epidemics' have developed in the United States. The opioid epidemic arose in the late 1990s and early 2000s due to the deceptive marketing and overprescription of Oxycontin. It was sustained by the increased availability of heroin in the mid-2000s and made even more prominent over the course of the last decade by the mass production and distribution of potent, synthetic opioids like fentanyl (Congressional Research Service, 2022; Jones et al., 2020; Pappin et al., 2022). Underscoring the severity of this public health crisis, 2.1 million Americans currently report having on opioid use disorder (OUD) (Chang et al., 2018). Drug overdoses, the majority of which involved opioids, surpassed vehicular accidents as the leading cause of injury mortality in 2019 (Schiller et al., 2023).

While opioid use and overdose-related deaths were skyrocketing in the United States, electronic cigarette (e-cigarette) use among teenagers and young adults was also increasing at a rapid rate, precipitating a 'vaping' epidemic among the adolescent population. The marketing of flavored e-cigarette products to teenagers drove the use of e-cigarettes among high-school students from just 1.5% in 2011 to a staggering 27.5% in 2019 (Huey & Granitto, 2020). Not only do e-cigarettes contain high concentrations of nicotine, making them particularly addictive, but e-liquid vapor can produce carcinogens and other cytotoxic compounds, presenting significant health risks to users (Canistro et al., 2017; Ebersole et al., 2020; Ogunwale et al., 2017; Rouabhia et al., 2017; Sundar et al., 2016; Wold et al., 2022). Like for smoking, exposure to nicotine during adolescence through e-cigarettes can negatively impact mental health and has been associated with variety of mental health disorders including depression, anxiety, suicidal ideation (Livingston et al., 2022; Nguyen & Mital, 2022; Taylor et al., 2014).

Though the opioid and ‘vaping’ epidemics are separate clinical phenomena, the co-use of nicotine and opioids is one of the most frequently reported types of polysubstance use. In the United States, smoking prevalence among people who use opioids illicitly or receive methadone maintenance treatment, a type of medication assisted therapy (MAT) for OUD, is between 74 and 97%, compared to just 14% of the general adult population who smoke (Pajusco et al., 2012; Zirakzadeh et al., 2013). The prevalence of nicotine and opioid co-use may reflect the bidirectional enhancement of the pleasurable aspects of drug use, or a reduction in the aversive symptoms of withdrawal that arise upon drug cessation (Elkader et al., 2009; Richter et al., 2007; Spiga et al., 2005). High incidence of nicotine and opioids co-abuse may also reflect the age at which drug use began. Polysubstance use has become increasingly common among adolescents and theories suggest that nicotine may act as a ‘gateway’ drug, facilitating changes in behavior and in brain circuitry which promote the use of other drugs later in life (Kandel et al., 1992; Levine et al., 2011; Thepthien et al., 2021; Zuckermann et al., 2019).

Adolescent initiation of drug use and polysubstance use may exert additive effects on drug reward and aversion by impinging upon the mesolimbic dopamine system. This pathway comprises dopamine (DA) neurons in the ventral tegmental area (VTA) that project to and release DA in the Nucleus Accumbens (NAc). Almost all drugs of abuse exert their rewarding effects by increasing DA levels in the NAc (Di Chiara & Imperato, 1988). Recent work has also demonstrated a role for the mesolimbic circuit in drug aversion, establishing the existence of subcircuits and subpopulations of neurons within the VTA with distinct connectivity and functionality (De Jong et al., 2022; Lammel et al., 2012, 2014; Yang, de Jong, et al., 2018; Yuan et al., 2019). Although 60-65% of neurons in the VTA are DAergic, there is also a sizeable population of y-aminobutyric acid (GABA) neurons (20-25%), as well as a smaller percentage of

glutamate neurons (3-5%), both of which influence DA signaling in the mesolimbic circuit (Collins & Saunders, 2020; Morales & Margolis, 2017).

VTA GABA neurons are of particular interest when considering the overlapping mechanisms underlying the high prevalence of nicotine and opioid co-use. Within the VTA, the μ-opioid receptor (MOR) is found primarily on VTA GABA neurons, where opioid binding results in decreased firing of the GABAergic neuron and decreased GABA release (Johnson et al., 1992; Lüscher & Malenka, 2011; Reeves et al., 2022; White, 1996). A large subset of VTA GABA neurons are interneurons, which make local inhibitory synapses with DA neurons. Decreased neurotransmission at these synapses in response to MOR activation results in the disinhibition of DAergic activity (Devine et al., 1993; Omelchenko et al., 2009; Van Zessen et al., 2012). GABA neurons in the VTA also express nicotinic acetylcholine receptors (nAChRs), specifically the α4β2* subtype, at pre- and post-synaptic sites, and nicotine binding to these receptors enhances both GABAergic activity and the release of GABA onto DA neurons (Fisher et al., 1998; Lu et al., 1999; Mansvelder et al., 2002; Radcliffe et al., 1999; Yin et al., 2000). Importantly, VTA DA neurons also show dense expression of α4β2* nAChRs and the concerted activity of both DA and GABA neurons is necessary for driving rewarding and aversive behavioral response to nicotine (Grieder et al., 2009; Liu et al., 2022; Nguyen et al., 2021; Tolu et al., 2013)

The fact that GABA neurons in the VTA express both MORs and nAChRs makes them a site of convergence between endogenous opioid and cholinergic inputs, suggesting they are important mechanistic mediators of nicotine and opioid co-use. Here, we employed our novel co-exposure paradigm, which involves pre-exposure to nicotine vapor during adolescence followed by co-exposure to both nicotine and morphine during adulthood, in conjunction with

fiber photometry in VGAT-Cre mice, to study the effects of nicotine and opioid co-use on VTA GABA neuron activity.

3.2 Materials and Methods

3.2.1 Animals

We studied male and female VGAT-Cre (n=25) mice between postnatal day 28 (PND28) and PND 130. Animals were housed at the University of Pennsylvania with a reverse 12 h light/dark cycle in a temperature-controlled room (24 ± 2°C; relative humidity, 55 ± 10%) with *ad libitum* access to chow and water. All fiber photometry recordings and vapor exposures occurred in the dark phase of the light cycle. IRES-VGAT-Cre (Slc32a1$^{tm2(cre)Lowl}$/J, Strain #: 016962) males were purchased from The Jackson Laboratory and bred with WT C57BL/6J females. All procedures described here were approved by the Institutional Animal Care and Use Committee and followed the guidelines for animal research from the National Institutes of Health.

3.2.2 Drugs

E-liquids were prepared as previously described (Patten et al., 2023). Briefly, E-liquid components [Vegetable Glycerin (VG), Propylene Glycol (PG), NicSelect Nicotine (free-base; 100 mg/ml in VG)] were purchased from Liquid Barn (Simi Valley, CA). Vehicle (VEH) solution consisted of a 50:50 blend of VG/PG. Nicotine (NIC) solutions maintained a 50:50 blend of VG/PG and were adjusted to achieve the desired nicotine concentration (5, 10 or 30 mg/mL). NIC solutions were kept in the dark and made fresh every 3 days to prevent nicotine degradation. For continuous two-bottle choice (C2BC) experiments, free-base morphine sulfate (Morphine Sulfate, Spectrum Chemical MFG, New Brunswick, NJ) was dissolved in a 2.0% saccharin + tap water to reach a concentration of 0.1 - 0.2 mg/mL morphine. Morphine solutions were kept in amber bottles and were replaced every 7 days (Fleites et al., 2022). For systemic

administration, morphine was dissolved in 0.9% saline to a concentration of 1 mg/mL and administered intraperitoneally (i.p.) to achieve 10 mg/kg dose, adjusting for animal body weight. Naloxone (Naloxone Hydrochloride, 0.4mg/mL, Mylan Institutional LLC. Morgantown, WV) was dissolved in 0.9% saline to a concentration of 0.2 mg/mL and administered via i.p. injection, adjusting for animal body weight to achieve a dose of 1 mg/kg.

3.2.3 E-cigarette vapor delivery

E-cigarette vapor delivery was performed as previously described (Patten et al., 2023), with some modifications. Animals were transported to a designated room 30 min prior to vapor exposure for habituation to the environment. After the habituation period, animals were placed in an air-tight exposure chamber (dimensions = 11.15” x 6.75” x 4.5”, Allentown LLC, Allentown, NJ) containing a vapor delivery port and a vacuum port to replace chamber air with clean room air (flow rate = 2.0 L/min). E-liquid was vaporized using a vapor generator controller (University of Pennsylvania, Electronic Design Shop) in conjunction with a SMOK Baby Beast Brother e-cigarette tank and V8 X-Baby-Q2 coils (0.4 Ω; SMOK). The vapor generator controller was operated at 75.0 W using the preset “nicotine” settings, with the temperature set to 400°F. For all vapor exposures, the “puff” from a single e-cigarette was transported via plastic tubing into 2 air-tight exposure chambers. A 1 s puff was delivered every 90 s for a total of 25 min, followed by a 5 min wash out. This exposure pattern and duration was found to be sufficient to deliver pharmacologically relevant doses of nicotine and produced plasma cotinine levels comparable to those achieved via i.p. injection of nicotine (Patten et al., 2023).

3.2.4 Morphine continuous two-bottle choice (C2BC)

Adult, single-housed VGAT-Cre (n=14) mice were provided with continuous access to two bottles in their home cage. One bottle contained a solution of 2.0% saccharin (a non-caloric sweetener that masks morphine’s bitter taste) dissolved in tap water (Horowitz et al., 1977). The

other bottle contained a solution of free-base morphine (0.1 - 0.2 mg/mL) dissolved in 2.0% saccharin. This bottle initially contained a 0.1 mg/mL morphine solution, which was then increased to 0.2 mg/mL after the first week. Subsequently, mice were maintained on a 0.2 mg/mL morphine solution for 4-5 weeks, during which time they consumed a steady dose (mg/kg) of morphine (mean: 21.99, SEM: ± 5.76). Control animals received two bottles of 2.0% saccharin solution only. Both bottles were cleaned and then replaced with fresh solution every 7 days. Bottles were weighed daily to measure morphine solution intake (mL) and mice were weighed weekly to calculate morphine dose (mg/kg). Position of the bottles was alternated daily to control for any potential positional preference. We and others showed that this method generates levels of opioid consumption that are sufficient to produce opioid-dependence in rodents (Fleites et al., 2022; Zanni et al., 2020). We had previously validated, via quantification of somatic signs in both the morphine-sated and morphine-withdrawal state, that our C2BC + vapor exposure paradigm produced morphine dependence in mice after 5-6 weeks (Chapter 2, Figure 3).

3.2.5 Viral injections and fiber optic implants

We studied male and female VGAT-Cre mice (n=25; PND 60 - 65). Mice were anesthetized with 3% isoflurane, administered a subcutaneous (SC) injection of extended-release (ER) Meloxicam (6 mg/kg, ZooPharm, Fort Collins, CO) and then placed into the stereotaxic frame (Angle Two, Leica Biosystems, Deer Park, IL) equipped for continuous delivery of isoflurane (1.0 - 2.0%). AAV2/9-hsyn-FLEX-GCaMP6f-WPRE-SV40 (Addgene # 100843, titer: 1.0 × 10^12 vg/mL) was injected unilaterally into the VTA (0.7µL, 0.07 µL/min; -3.25 A/P, -0.5 M/L, -4.5 D/V) using a 700 series Hamilton Syringes (#80314, Franklin, MA) and an infusion pump (KD Scientific, Holliston, MA). Syringes were left in place for 5 min following injection to allow for complete viral diffusion. Mono fiber-optic cannulas (400 um core, 0.48 NA,

Doric Lenses, Québec City, Ont.) were implanted dorsal to VTA injection site (+0.2 D/V) and secured with low auto-fluorescent Metabond cement (S380, Parkell, Edgewood, NY). Mice were given a second dose of ER Meloxicam 24 h after surgery to provide post-surgery pain control. For all experiments described in this chapter, viral infusion and optical fiber implantation were performed between PND 60 and PND 65, in accordance with the parameters specified above. Photometry experiments were performed no earlier than 20 days post-surgery, to allow for complete physical recovery and sufficient viral GCaMP expression.

3.2.6 Fiber photometry

GCaMP6f was excited by two wavelengths, 465-nm (calcium-dependent excitation) and 405-nm (calcium independent excitation, isosbestic control), which were generated through fiber-coupled LEDs and modulated via a real-time amplifier (RZ10X, TDT, Alachua, FL). A fluorescence mini cube (4 FC connector ports, Doric Lenses) was used to filter and combine the two excitation wavelengths. The combined excitation light was then passed through a low-autofluorescence mono fiber patch cord (400um core, 0.57 NA, Doric Lenses) and attached to the implanted mono fiber-optic cannula (400um core, 0.48 NA, Doric) via quick-release interconnect clamp (ADAF2, THOR, Newtown, NJ). GCaMP6f fluorescence was then detected via the same mono fiber patch cord, passed back through the mini cube, and eventually demodulated by the photoreceiver (RZ10X, TDT). Fluorescence was sampled at 6 Hz and digitized at 100 Hz via Synapse Software (TDT). Timestamps for nicotine vapor puffs were also digitized in Synapse Software via TTL input from the vapor generator controller (University of Pennsylvania, Electronic Design Shop, Philadelphia, PA).

3.2.7 Fiber photometry during nicotine vapor and co-drug exposures

Mice were habituated to handling and tethering prior to all fiber photometry experiments. For nicotine vapor-only experiments, mice were tethered and then placed inside an air-tight

chamber (dimensions: 4.5” x 5.4” x 7.3”), fitted with a small opening for the fiber cord that was then sealed with a customized rubber stopper, allowing mice to move freely inside the chamber. Mice were recorded from for 5 min prior to each vapor exposure to establish a baseline signal. Vapor was delivered using the same vapor delivery system described above in the “E-cigarette vapor delivery” section. Briefly, after the baseline recording, the vapor generator controller was turned on and 1 s vapor puffs were delivered at 90 s intervals for a total of 25 min, followed by a 5 min washout period. TTL from the vapor generator controller to the Synapse Software provided timestamps for vapor puff delivery on the digitized photometry recording. When photometry recordings also involved administration of either morphine (10 mg/kg) or naloxone (1 mg/kg), mice were recorded from for 5 min prior to drug administration and were then injected i.p. with the designated drug. Subsequently, the vapor generator controller was turned on 1 min after the injection to initiate vapor exposure.

3.2.8 Photometry analyses

All photometry data were analyzed using pMAT v1.3 (Bruno et al., 2021, The Barker Lab). Change in fluorescence relative to baseline fluorescence (ΔF/F) was calculated by fitting the GFP fluorescent signal (465 nm) to the isosbestic signal (405 nm). Briefly, via pMAT, data from the signal and isosbestic channels were extracted and then smoothed using the LOWESS method, a type of local linear regression that also reduces high-frequency noise (Bruno et al., 2021). The scale of the channels was then normalized to control for differences in power level and the ΔF/F was calculated from the scaled channel signals using the following equation:

$$\Delta F/F = \frac{(Signal\ Channel\ -\ Scaled\ Control\ Channel)}{Scaled\ Control\ Channel}$$

The final normalization of the ΔF/F (z-score, number of standard deviations from baseline) relative to the timestamped vapor puff (event) was carried out by calculating the ΔF/F

during a 5 s baseline sampling window prior to vapor puff initiation and then during the 10 s event window after puff delivery. Z-scores for individual vapor puff events (i) were calculated using the following equation:

$$\frac{\Delta F}{F}\text{ Z-Score (i)} = \frac{\left(\frac{\Delta F}{F}Event\ (i) - median\frac{\Delta F}{F}baseline\right)}{median\ absolute\ deviation\ (MAD)\ of\ baseline}$$

Traces for individual events were then exported as CSV files for further analysis in Microsoft Excel. Area Under the Curve (AUC) for individual events was also extracted from the robust z-score calculation and exported from pMAT as CSV files. Visualization of photometry data was performed in GraphPad Prism 10.0.02® (GraphPad Software, San Diego, CA). Unless otherwise noted, the data shown here represent the change in fluorescence over baseline fluorescence (ΔF/F) of GCaMP-expressing VTA GABA neurons, relative to the time (s) before and after each vapor puff, averaged across all 17 puffs a given vapor exposure session. We quantified these changes in fluorescence by calculating and comparing the AUC of the ΔF/F (z-score) trace during 2s intervals before and after vapor puff delivery.

3.2.9 GCaMP visualization and confirmation of fiber placement

Mice were deeply anesthetized with a 4:1 ketamine/xylazine mixture, and then perfused with phosphate-buffered saline (PBS), followed by 10% formalin. Brains were then extracted and fixed overnight in 10% formalin followed by at least 24 h cryoprotection with 30% sucrose solution in PBS. Brains were then embedded in clear Tissue-Plus Optimal Cutting Temperature Compound (OCT; 23-730571, Fisher Scientific, Hampton, NH), and stored at -80°C. Brains were sectioned coronally at 50 µm via cryostat and slices were mounted to slides with VECTASHIELD Antifade Mounting Medium with DAPI (NC9524612, Fisher Scientific) to verify both accurate viral targeting and fiber optic placement using epifluorescence microscopy at 4X

and 10X (BX63, Olympus, Waltham, MA). If fiber placement and/or viral expression were inaccurate, data collected from that animal were removed prior to analysis.

3.2.10 Statistical analyses

Both ΔF/F traces and AUC values are expressed as mean +/- standard error of the mean (SEM) and were analyzed using GraphPad Prism 10.0.02® (GraphPad Software, San Diego, CA). A p-value of < 0.05 was considered statistically significant. Statistical outliers were identified with GraphPad Prism using robust regression and outlier removal (ROUT, Q=1%). A repeated-measures (RM) two-way ANOVA with Tukey's post-hoc analysis was used to assess both main effect and interaction between vapor type (VEH or 5, 10, 30 mg/mL NIC) and time relative to vapor puff (Figure 1). For all other photometry comparisons, a mixed-effects ANOVA with Sidak's post-hoc analysis was used to assess main effect of drug treatment and interaction between treatment and time relative to vapor puff. No significant sex effects were observed, so sexes were collapsed for all data presented here.

3.3 Results

3.3.1 Acute nicotine produces a transient increase in VTA GABA activity in nicotine-naïve mice

We hypothesized that the decrease in VTA DA activity we observed in response to nicotine vapor (see Chapter 2) might be due, in part, to activation of VTA GABA interneurons, which also express nAChRs, and form local, inhibitory synapses with DA neurons (Creed et al., 2014; Mansvelder et al., 2002; Steffensen et al., 1998). With this in mind, we examined the effects of NIC vapor on VTA GABA neuron activity. Like the experiments described in Chapter 2, we used the vapor exposure method validated and detailed by Patten et al., 2023, in conjunction with our fiber photometry system, to deliver time-stamped vapor puffs while recording the activity of VTA GABA neurons (**Figure 3-1A**). We also demonstrated that infusion of a AAV2/9-hsyn-FLEX-GCaMP6f-WPRE virus into the VTA (bregma: -3.25 A/P, -.5M/L, -4.5

D/V) of VGAT-Cre mice produced stable GCaMP expression in Cre^+ neurons and that changes in GCaMP fluorescence could be measured via an optical fiber implanted just above the infusion site (+0.2 D/V, **Figure 3-1B**).

VGAT-Cre mice (n=7) were treated with vehicle (VEH) vapor, a 50:50 blend of propylene glycol and vegetable glycerin, beginning in adolescence and continuing into adulthood (PND 28 – PND 80, **Figure 3-1C**). Besides habituating mice to the experience, starting vapor exposures in adolescence models the time at which a sizeable percentage of the population begins experimenting with e-cigarettes (Birdsey et al., 2023; Hammond et al., 2017; Miech et al., 2019). At PND 80, we used a Latin square design to randomly designate which vapor type (VEH or 5,10, 30 mg/mL NIC) mice would receive during the first recording session and for all subsequent sessions. Each mouse received every vapor type twice, with recordings spaced at least 48 h apart, totaling 8 recordings per animal. After assignment to a specific vapor type, the mouse was tethered to the fiber optic patch cord and placed inside the vapor exposure chamber, followed by a 5 min recording to establish a baseline fluorescence signal of VTA GABA neurons prior to vapor puff delivery. The vapor controller was then turned on and mice were exposed to 1 s vapor puffs every 90 s for a total of 17 puffs delivered over a 25 min period.

In response to each of the NIC (5, 10 or 30 mg/mL) vapors, we noted a large increase in VTA GABA activity starting around 1 s after the vapor generator controller was turned on, corresponding to the approximate time that it takes the vapor puff to travel from the e-cigarette to the exposure chamber (**Figure 3-1D**). Notably, like for VTA DA neurons, there was no change in activity in response to presentation of a vapor cue alone, indicating that VTA GABA responses are dependent on vapor exposure (**Supplemental Fig. 3-1**). The increase in VTA GABA activity following NIC vapor exposure was sustained for approximately 6 s after puff initiation, before then returning to baseline. Though VEH vapor also produced an increase in

VTA GABA neuron activity, the maximal fluorescence peak was smaller, and the signal returned to baseline more quickly, compared to the response to NIC vapor. When examining the area under the curve (AUC) of the ΔF/F traces, we found a significant effect of vapor type (RM two-way ANOVA; $F(2.11, 12.65) = 13.27$, $p < .001$, **Figure 3-1E**) and a significant interaction of vapor type and time relative to vapor puff (RM two-way ANOVA; $F(1.38, 8.28)$, $p = 0.014$). Post-hoc analysis revealed a significant decrease in AUC at both the 1-3 and the 3-5 s post-puff timepoint for each of the NIC vapor concentrations compared to VEH vapor (Tukey's multiple comparisons, $p < 0.05$). Counter to the decrease in VTA DA activity in response to nicotine, these data reveal that nicotine increases VTA GABA activity to a greater extent than vehicle in nicotine-naïve mice.

3.3.2 Acute morphine dampens the VTA GABA response to acute nicotine

VTA GABA neurons express mu-opioid receptors (MORs) and morphine binding to these receptors results in hyperpolarization of the neuron and subsequently, decreased GABAergic activity (Reeves et al., 2022; White, 1996). However, to date, the effect of morphine-induced inhibition on VTA GABA neuron response to nicotine has not been tested. We hypothesized that acute morphine would reduce VTA GABA activity overall and, in turn, reduce the GABAergic response to NIC vapor. Following the experiment described above, in which VGAT mice were recorded from during NIC or VEH vapor exposures (Figure 3-1), we injected these same mice (n=7) with morphine (10 mg/kg, i.p.) and recorded VTA GABA neuron activity during a NIC (30 mg/mL) vapor exposure session (**Figure 3-2A**). As predicted, following delivery NIC vapor puffs, we observed a reduction in both the peak high of VTA GABA neuron response and the time it took for GABAergic activity to return to baseline (**Figure 3-2B**). Importantly, a saline injection did not alter VTA GABA response to vapor (**Supplemental Fig. 3-**

2), indicating that the change in VTA GABA activity that we observed was specific to morphine and not a reflection of stress induced by the injection.

Quantification of the ΔF/F traces via AUC analysis revealed a significant effect of morphine treatment (mixed-effects ANOVA; $F(1, 6) = 137.0$, $p = < 0.001$, **Figure 3-2C**) and a significant interaction between morphine treatment and time relative to vapor puff (mixed-effects ANOVA, $F(1.56, 7.02) = 37.10$, $p < .001$). Post-hoc analysis revealed that morphine injection significantly altered the AUC at both the 1-3 and 3-5 s post-puff timepoint (Tukey's multiple comparison's, $p < 0.01$). Thus, we determined that acute morphine reduces VTA GABA neuron response to acute nicotine.

3.3.3 Chronic nicotine treatment does not significantly alter the VTA GABA response to acute nicotine

Addiction is a chronic condition, with neuroadaptations often developing over time, as drug use progresses from casual or intermittent to chronic (Koob, 2009; Koob & Volkow, 2016). Using calcium imaging techniques, like fiber photometry, in combination with preclinical models of substance use can provide insight into the time course and extent of drug-induced changes to neural circuitry. In previous experiments, we observed that chronic exposure to nicotine (Chronic NIC) mitigated the reduction in VTA DA activity observed in response to acute nicotine (Acute NIC, Chapter 2, Figure 2-2). We hypothesized that this may reflect decreased VTA GABA activity and the subsequent disinhibition of VTA DA neurons.

To test this hypothesis, we employed the same chronic nicotine exposure paradigm we had established to study VTA DA response to NIC vapor. In brief, VGAT-Cre mice (n=12) were exposed to NIC (30 mg/mL) vapor starting at PND 28, corresponding to the age at which many humans try nicotine for the first time, followed by continued exposure during adulthood through PND 80 (Kong & Krishnan-Sarin, 2017, **Figure 3-3A**). At PND 80, VTA GABA neurons were

studied during an acute NIC (30 mg/mL) vapor exposure. Examination of ΔF/F traces following a puff of NIC revealed that, when compared to Acute NIC mice, Chronic NIC mice showed a slight decrease in the peak height of the VTA GABA response and slightly faster return to baseline (**Figure 3-3B**). However, quantification of these GABAergic responses via AUC analysis did not reveal any statistically significant difference between Acute NIC and Chronic NIC treatments (**Figure 3-3C**).

3.3.4. Morphine withdrawal reveals an enhanced VTA GABA response to nicotine in mice chronically co-exposed to nicotine and morphine

As previously stated, co-use of nicotine and morphine is extremely prevalent in the general population and the mesolimbic DA system is a site of overlapping, reward-related mechanisms between these two drugs (Avelar et al., 2022; De Rover et al., 2004; Rezayof et al., 2007; Tanda et al., 1998). Though chronic co-exposure to nicotine and morphine did not appear to alter VTA DA activity (Chapter 2, Figure 2-2), we hypothesized that VTA GABA neurons may be more susceptible due to the expression of both nAChRs and MORs (Galaj et al., 2020; Grieder et al., 2019). In this way, VTA GABA activity can be directly affected both by nicotine, through excitation via binding to nAChRs, and by morphine, through inhibition resulting from the activation of hyperpolarizing MORs. To study the effects of nicotine and morphine co-exposure on VTA GABA activity, we employed the same dual drug paradigm utilized to study VTA DA neuron activity (**Figure 3-4A**). VGAT-Cre mice were chronically exposed to NIC (30 mg/mL) vapor throughout adolescence and young adulthood (PND 28 – PND 80), followed by a single photometry recording during a NIC (30 mg/mL) vapor exposure session at PND 80, comprising the “Chronic NIC” exposure (Figure 3-3B).

After this initial recording, a subset of mice (n=7) began a 6-week co-exposure period where they continued regular NIC vapor exposures and were also given access to morphine via

the continuous two-bottle choice (C2BC) paradigm, as described previously (**Figure 3-4A**). In brief, mice were given 24 h access to two bottles in their home cage, one solution comprised of 2.0% saccharin and the other containing 2.0% saccharin + morphine (escalated from 0.1 mg/mL to 0.2 mg/mL after the first week). The remaining mice (n=5) served as controls and only received two bottles of 2.0% saccharin. We had previously validated that morphine treatment via the C2BC paradigm, during co-exposure with nicotine, was able to produce stable morphine consumption (mg/kg) and morphine dependence in mice (Chapter 2, Figure 2-3).

Once per week during the co-exposure period, we recorded from mice during a NIC (30 mg/mL) vapor exposure session. We did not detect significant differences in the VTA GABA response to NIC between co-exposed (NIC+MOR) mice or control (NIC+SACC) during any point during the co-exposure period (**Figure 4-4 B-G**). However, there was slightly greater intra- and inter-week variability in the VTA GABA response of NIC+MOR mice compared to the more consistent response of NIC+SACC mice, though variability between groups was not statistically significant. Importantly, we also did not detect any differences in the VTA GABA response to VEH at either Week 1 or Week 5, suggesting that co-exposure to nicotine and morphine does not alter the response to either VEH or NIC vapor (**Supplemental Fig. 3-3**). NIC+MOR mice did appear to show less variability in their VTA GABA response to VEH vapor compared to the GABAergic response to NIC vapor, suggesting increased variability in neural activity might be a product of co-drug exposure.

We hypothesized that this subtle variability in VTA GABA response to NIC may be reflective of drug-induced neuroadaptations, because although chronic drug use promotes alterations to neural circuitry, these changes may not become apparent until cessation of drug use, alongside the behavioral withdrawal syndrome (Besson et al., 2007b; Rahman et al., 2004; Yang et al., 2023). Therefore, at the end of the 6-week co-exposure paradigm, we injected both

NIC+MOR and NIC+SACC mice with naloxone (NX, 1 mg/kg, i.p.) to precipitate morphine withdrawal and then measured potential changes in VTA GABA neuron response to NIC vapor (**Figure 3-5A**). For NIC+SACC mice, NX did not alter VTA GABA response to NIC vapor (**Figure 3-5C**). However, in NIC+MOR mice, precipitated morphine-withdrawal with NX revealed a large increase in VTA GABA response to NIC vapor (**Figure 3-5B**).

Further analysis of the corresponding VTA GABA fluorescence traces in the NIC+MOR group revealed a significant effect of NX treatment (RM two-way ANOVA; $F(1, 6) = 11.00$, $p = 0.016$) and a significant interaction between NX treatment and time relative to vapor puff (RM two-way ANOVA; $F(1.41, 8.44) = 6.53$, $p = 0.026$, **Figure 3-5D**). In a separate analysis, we also compared AUC values between NIC+MOR and NIC+SACC animals and identified a significant interaction between NX treatment and time relative to vapor puff (mixed-effects ANOVA; $F(2, 20) = 5.08$, $p = 0.016$). Together, these data unveil an increase of VTA GABA activity during morphine withdrawal, an effect likely induced by chronic co-exposure to both nicotine and morphine.

3.3.5. Compared to acute morphine, chronic morphine treatment extends the VTA GABA response to acute nicotine

We next wanted to determine if co-exposure to nicotine and morphine is necessary for the alteration to VTA GABA activity observed during morphine withdrawal, or if this effect could be attributed to morphine treatment alone. To this end, we treated mice with VEH vapor throughout adolescence and young adulthood (PND 28 – PND 80, **Figure 3-6A**). VEH vapor exposures then continued for the next 6 weeks, during which time mice were also consuming morphine in the C2BC model. At the start (Week 1) and the end (Week 5) of the co-exposure period we recorded from mice during a VEH vapor exposure session and did not note any effect of chronic morphine on VTA GABA response to VEH (**Supplemental Fig. 3-4**). After the 6-week

co-exposure, VEH-treated mice underwent their first exposure to NIC vapor while we recorded from VTA GABA neurons.

When comparing VTA GABA activity of these VEH-treated, chronic-morphine exposed mice (VEH+MOR) to the response of mice who received an acute injection of morphine (Acute NIC+ MOR, Figure 2) we saw similar peak height of the GABAergic response to NIC vapor (**Figure 3-6B**). However, VEH+MOR mice displayed a prolonged elevation of VTA GABA activity compared to the more transient increase seen in the Acute NIC+MOR (10 mg/kg i.p.) mice (Figure 2). AUC analysis of these fluorescence traces revealed a significant interaction between length of morphine treatment (chronic vs. acute) and time relative to vapor puff (mixed-effects ANOVA; $F(2, 22) = 5.22$, $p = 0.014$), with post-hoc analysis revealing a significant difference in AUC at the 3-5 s post-puff timepoint (Sidak's multiple comparisons, $p < 0.05$, **Figure 3-6C**). This finding highlights the way that chronic exposure to morphine may induce unique and varied neuroadaptations, ultimately producing an entirely different GABAergic response compared to acute morphine exposure.

3.3.6 Chronic morphine alone is not sufficient for the enhancement of VTA GABA response to acute nicotine during morphine-withdrawal

Following examination of the GABAergic response to NIC vapor in the morphine-sated state, we injected VEH+MOR mice with NX (1 mg/kg, i.p) to precipitate morphine withdrawal, and then recorded VTA GABA activity during a NIC vapor exposure session (**Figure 3-7A**). Although NX treatment in VEH+MOR produced an increase in VTA neuron GABA activity in response to NIC vapor, this increase did not appear to be of the same magnitude as the response observed in the NIC+MOR+NX mice (**Figure 3-7 B, C**). Quantification of these differences in response revealed no significant effect of NX on VTA GABA activity in VEH+MOR mice. This contrasted with the statistically significant, withdrawal-induced upregulation of VTA

GABA activity we had observed in response to NIC vapor in co-treated, NIC+MOR mice (Figure 3-5D, **Figure 3-7D**). Hence, we have effectively demonstrated that co-treatment with both nicotine and morphine is necessary for the enhanced VTA GABA response to acute nicotine during morphine withdrawal.

3.4 Discussion

Polysubstance use, including the co-use of nicotine and opioids, is extremely common among those with substance use disorders and yet remains understudied in the preclinical literature (Compton et al., 2020; Gjersing & Bretteville-Jensen, 2018; Steinhoff et al., 2022). A limited body of work in rodent models suggests that the high prevalence of nicotine and opioid co-use may be the result of overlapping mechanisms at the level of the mesolimbic dopamine system, which may subsequently alter reward-related behavior (Araki et al., 2004; Avelar et al., 2022; Rezayof et al., 2007; Talka et al., 2013; Vihavainen, Relander, et al., 2008; Zarrindast et al., 2003). Though DA neurons within the VTA have historically received the most attention in the addiction-research field, a growing body of evidence suggests that VTA GABA neurons also play a crucial role in regulating both reward and aversion to various drugs of abuse (Bonci & Williams, 1997; Bouarab et al., 2019; Tan et al., 2012; Van Zessen et al., 2012). To study drug-induced alterations to GABA neurons within the VTA, we combined our previously established model for chronic co-exposure to nicotine and opioids with fiber photometry.

Like VTA DA neurons, VTA GABA neurons express α4β2* nAChRs and are activated by acute exposure to nicotine (Grieder et al., 2019; Mansvelder et al., 2002; Tolu et al., 2013). We further corroborated this finding, reporting that exposure to NIC vapor produces a robust and transient increase in VTA GABA activity in nicotine-naïve mice (**Figure 3-1**). Though we tested 3 different concentrations of NIC vapor (5, 10 and 30 mg/mL), we did not see any corresponding differences in the magnitude of the VTA GABA response. Given the similar responses at each

of the 3 concentrations, it is possible that there was a ceiling effect, whereby the maximal number of VTA GABA neurons had been activated. Although the concentrations of NIC we tested produce plasma cotinine levels comparable to i.p. injections of rewarding doses of nicotine (Patten et al., 2023), we did not separately test if these specific vapor exposures produce rewarding or aversive behavioral responses. One group found that VTA GABA neurons only display excitatory response to high, aversive doses of nicotine, but do not respond to low, rewarding doses (de Jong et al., 2019). Therefore, it is possible that each of the NIC concentrations we tested was aversive, as Patten et al. (2023) report that mice only show CPP to 2.5 mg/mL NIC vapor, but not to 10 mg/mL or 25 mg/mL NIC vapor. In future experiments, correlating increases in VTA GABA activity to, for example, CPP or CPA to a given concentration of NIC vapor, would provide powerful insight into the functionality of this observed GABAergic response.

The prevailing literature indicates that α4β2* nAChRs on VTA GABA neurons show sustained desensitization, leading to decreased GABAergic transmission at local synapses with DA neurons, which facilitates a hyperdopaminergic state during periods of prolonged exposure to nicotine (Dani & Bertrand, 2007; Mansvelder et al., 2002). However, other studies report that the response of VTA GABA neurons to nicotine does not desensitize after repeated nicotine exposures (Taylor, 2011; Tolu et al., 2013). In mice that had been chronically exposed to nicotine, we observed a slight, non-significant dampening of the VTA GABA response to NIC vapor, compared to the GABAergic response in nicotine-naïve mice (**Figure 3-3**). Moreover, for both nicotine-naïve and chronically exposed animals, we noted that, over the course of a given NIC exposure session, the response of VTA GABA neurons to a NIC vapor puff diminished only slightly, suggesting minimal desensitization of nAChRs (data not shown). Discrepancies between our observation and the findings in the seminal paper by Mansvelder et al. (2002)

highlight how differences in experimental techniques and drug exposure paradigms, can influence the way a given drug appears to alter neuronal activity. Mansvelder et al. (2002) used *ex-vivo* electrophysiology to measure GABAergic release at presynaptic terminals with VTA DA neurons, where they observed rapid desensitization of α4β2* nAChRs and decreased GABAergic release after a 10-min bath application of nicotine. In contrast, we used *in-vivo* fiber photometry to record population-level VTA GABA activity, showing only minimal desensitization of the GABAergic response to NIC vapor in mice who had been exposed to nicotine regularly, beginning in adolescence, for nearly 2 months.

These differences in methodology may explain variability in the extent of VTA GABA desensitization following nicotine exposure. For example, both presynaptic terminals and cell bodies of VTA GABA neurons express α4β2* nAChRs, a receptor subtype known to rapidly desensitize in response to nicotine (De Biasi & Dani, 2011; Pons et al., 2008). However, VTA GABA neuron cell bodies also express α7 nAChRs, which are more resistant to desensitization and upregulation (Besson et al., 2007; Liu et al., 2022). Therefore, it is possible that, with prolonged exposure to nicotine, α4β2* nAChRs at presynaptic terminals desensitize, preventing GABA release, despite α7* nAChR activation maintaining persistent activity at the level of VTA GABA soma.

In addition to reflecting differences in the recording site, diverging results may also stem from differences in recording technique. *In-vivo* recording methods, like fiber photometry, ensure that VTA GABA neurons maintain their regular function within the wider mesolimbic circuitry, receiving excitatory input from glutamatergic and cholinergic afferents and sending inhibitory afferents to a variety of brain regions (Bouarab et al., 2019; Feduccia et al., 2012). Though *ex-vivo* electrophysiology allows for more experimental control than fiber photometry, recording from VTA GABA neurons in isolation negates the influence that excitatory or inhibitory afferents

may have on natural neuronal activity. Thus, when recorded from *ex-vivo*, nAChRs on GABAergic terminals may quickly desensitize to nicotine, but, perhaps, *in-vivo*, compensatory upregulation of excitatory glutamatergic and cholinergic input maintains homeostatic firing at the soma of VTA GABA neurons. It is interesting to note that the studies which found no desensitization of VTA GABA neurons in response to nicotine (Taylor, 2011; Tolu et al., 2013) were, like our work, also conducted *in-vivo* and measured GABAergic activity at the soma, further highlighting the need to carefully consider methodology when studying addiction-related neuroadaptations.

Our findings may be indicative of the unique neuroadaptations induced by chronic, as opposed to acute, exposure to drugs of abuse. Though we did not detect an effect of length of nicotine exposure (chronic vs. acute) on the VTA GABA response to NIC vapor, when we moved to study co-exposure to nicotine and morphine, we noted a significant effect of the length of morphine treatment. Specifically, in mice that received a singular injection of morphine (10 mg/kg, i.p.) before a NIC vapor exposure, we noted a significant reduction in the VTA GABA response to NIC vapor, compared to acute NIC vapor exposure in these same mice (**Figure 3-2**). On the other hand, in a separate cohort of mice that had been treated with morphine for 6 weeks via the C2BC, we saw a prolonged VTA GABA response to NIC vapor, compared to the response following a singular injection of morphine (**Figure 3-6**).

It is likely that these differences in GABAergic response to NIC vapor in the co-exposure paradigm reflect morphine-induced alterations to MORs, which are densely expressed on VTA GABA cell bodies. Acute morphine binds to and activate MORs on VTA GABA neuron cell bodies, thereby hyperpolarizing and decreasing the activity of these cells (Johnson et al., 1992; Reeves et al., 2022; White, 1996). Regarding a specific interaction between MOR function and response to nicotine, it was demonstrated that nicotine-induced DA release in the NAc is

modulated by the activation of MORs within the VTA (Tanda et al., 1998). Therefore, it is possible that during acute exposure to both NIC vapor and morphine, morphine-induced hyperpolarization competes with nicotine induced-depolarization (via nAChR activation), shifting the VTA GABA neuron toward a less excitable state, thus reducing the ability of the GABAergic population to respond to NIC vapor puffs.

Interestingly, compared to mice acutely treated with morphine, mice that had been chronically exposed to morphine via the C2BC showed a similar peak height - but prolonged elevation - of VTA GABA activity in response to NIC vapor. Chronic morphine results in desensitization and decreased cell surface expression of MORs at the VTA GABA cell body (Bergevin et al., 2002; Lowe & Bailey, 2015; Williams et al., 2013). This phenomenon might indicate that VTA GABA cells are under less inhibitory control, both from morphine and from endogenous opioids, and are thus able to sustain activation in the presence of NIC vapor puffs. Chronic morphine also shifts MOR signaling, from the predominantly inhibitory G_i/G_o adenylyl cyclase to the stimulatory G_s adenylyl cyclase through the upregulation of different MOR receptor variants, which could further promote excitability of VTA GABA neurons (Chakrabarti et al., 2019). Furthermore, the altered shape of the GABAergic response to NIC following chronic morphine treatment likely reflects changes not only to MORs, but also to the nicotinic cholinergic system. One study demonstrated that chronic morphine exposure decreases levels of acetylcholinesterase and upregulates nAChRs in the midbrain (Neugebauer et al., 2013), which may further enhance VTA GABA excitability and prolong GABAergic response to NIC vapor. Together, these findings highlight how the length of drug exposure can have wide and varied consequences on drug-induced neuroadaptations, especially within the mesolimbic dopamine system.

Finally, we reveal that chronic co-exposure to nicotine and morphine results in enhanced VTA GABA neuron response to NIC vapor. Although we did not detect differences in VTA GABA activity during the 6-week co-exposure period (**Figure 3-4 B-G**) while the mice were drug sated, when morphine withdrawal was precipitated with naloxone, there was a significant increase in VTA GABA response to NIC vapor only in mice co-treated with both nicotine and morphine (NIC+MOR), but not in mice only exposed chronically to nicotine (NIC+SACC, **Figure 3-5 B-D**). This is in line with theories that suggest that chronic drug exposure induces changes to neural circuitry, but that compensatory mechanisms maintain homeostatic function of the system until cessation of drug use, at which time drug-induced neuroadaptations may be revealed (Besson et al., 2007; Christie, 2008; De Biasi & Dani, 2011; Koob & Volkow, 2016). The increase in VTA GABA activity in response to NIC vapor we observed during morphine withdrawal could reflect augmented cAMP signaling. *In-vivo* studies have shown that though morphine initially decreases adenyl cyclase (AC) thereby reducing production of cAMP, AC activity returns to normal as morphine exposure becomes chronic (Watts et al., 2005.; Williams et al., 2001). However, morphine withdrawal unmasks neuroadapations that lead to increased AC activity and cAMP levels, and consequent cell hyperexcitability. Specific to VTA GABA neurons, it has been demonstrated that morphine withdrawal is associated with elevations in cAMP at presynaptic GABAergic terminals and enhanced GABAergic tone at synapses with VTA DA neurons (Bonci & Williams, 1997; Madhavan et al., 2010).

Although morphine-withdrawal significantly increased VTA GABA response to NIC vapor in co-exposed mice, we did not observe the same effect in nicotine naïve, morphine dependent mice (**Figure 3-7**). This indicates that morphine-induced enhancement of the cAMP signaling pathway cannot fully explain the effect on GABAergic activity that we observed in co-exposed mice. Rather, the specificity of this GABAergic effect is likely indicative of extensive crosstalk

between the nicotinic and the endogenous opioid systems. Nicotine administration can increase the release of endogenous opioids and increase MORs expression (Berrendero et al., 2010; Davenport et al., 1990.; Houdi et al., 1991.; Pierzchala et al., 1987.; Walters et al., 2005). In this way, nicotine exposure could enhance the morphine-induced inhibition of VTA GABA neurons. Like the adaptive response within the cAMP pathway, upregulation of MORs and endogenous opioids may be masked by compensatory downstream mechanisms during chronic drug exposure, but then unveiled by MOR-receptor antagonism during precipitated morphine withdrawal. Hence, the increased response of VTA GABA neurons to NIC vapor during morphine-withdrawal likely reflects both changes in cAMP signaling, resulting from chronic morphine treatment, and alterations at the level of the MOR, resulting from chronic nicotine treatment.

In summary, our study highlights the importance of employing chronic models of drug exposure and suggests that differences in methodology can influence drug-induced changes to neural circuitry. We demonstrate the unique neuroadaptations to VTA GABA neurons induced by chronic co-exposure to nicotine and morphine, which may reflect changes to both the endogenous opioid and cholinergic systems. Future studies will be needed to further understand how these modifications in GABAergic activity may affect VTA DA signaling and may ultimately modify drug-related behaviors.

3.5 Figures

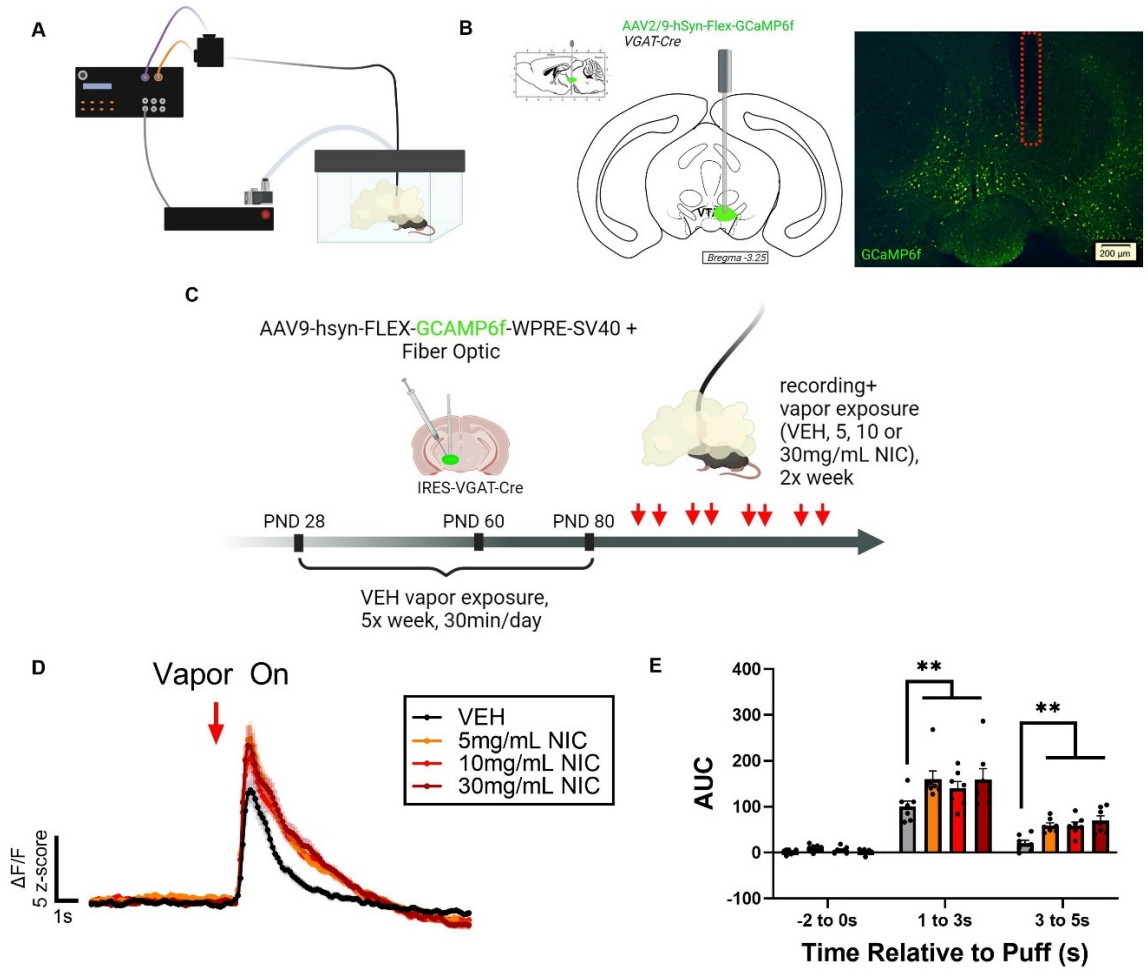


Figure 3-1. Acute nicotine produces a transient increase in VTA GABA activity in nicotine-naïve mice.

(A) General experimental techniques. Our validated, nicotine (NIC) vapor administration system was connected to a fiber photometry processor to provide time-stamped recordings of VTA GABA neurons during passive vapor exposures. (B) Confirming GCaMP expression in VGAT-Cre mice. Infusion of AAV2/9-hsyn-FLEX-GCaMP6f-WPRE virus (0.7µL, 0.07µL/min) into the VTA (-3.25 A/P, -.5M/L, -4.5 D/V) of VGAT-Cre mice produced stable GCaMP expression in Cre^+ neurons within the VTA. An optical fiber was implanted just above injection site (-3.25 A/P, -.5M/L, -4.3 D/V) to detect changes in GCaMP fluorescence (C) Experimental timeline. VGAT-Cre mice (n=7) vaped with vehicle (VEH) vapor (5x week, 1x day, 17 puffs/session) from PND28 – PND 80. At PND 60, AAV2/9-GcAMP6f was infused into the VTA and optical fibers were placed above the injection site. At PND 80, VTA GABA neurons were recorded from during exposure to randomized vapor (VEH, or 5, 10 or 30 mg/mL NIC). (D) Average ΔF/F (z-score) of VTA GABA neurons before (5s) and after (10s) exposure to vapor puffs. Response represents average of 17 puffs administered 90 s apart over the course of a 25 min session. VEH vapor produces an increase in VTA GABA activity. All NIC vapor puffs produce a larger increase in VTA GABA activity. (E) Area under the curve (AUC) of the Average ΔF/F (z-score) during 2 s intervals before and after vapor puff. Repeated measures (RM) two-way ANOVA reveals significant effect of vapor type on VTA GABA activity (RM two-way ANOVA; $F(2.11, 12.65) = 13.27$, $p < 0.001$) and a significant interaction between vapor type and time relative to vapor puff (RM two-way ANOVA; $F(1.38, 8.28)$, $p = .014$). Post-hoc analysis revealed a significant difference in AUC at both post-puff timepoints for each of the NIC concentrations compared to VEH (Tukey's multiple comparisons, $p < 0.05$).

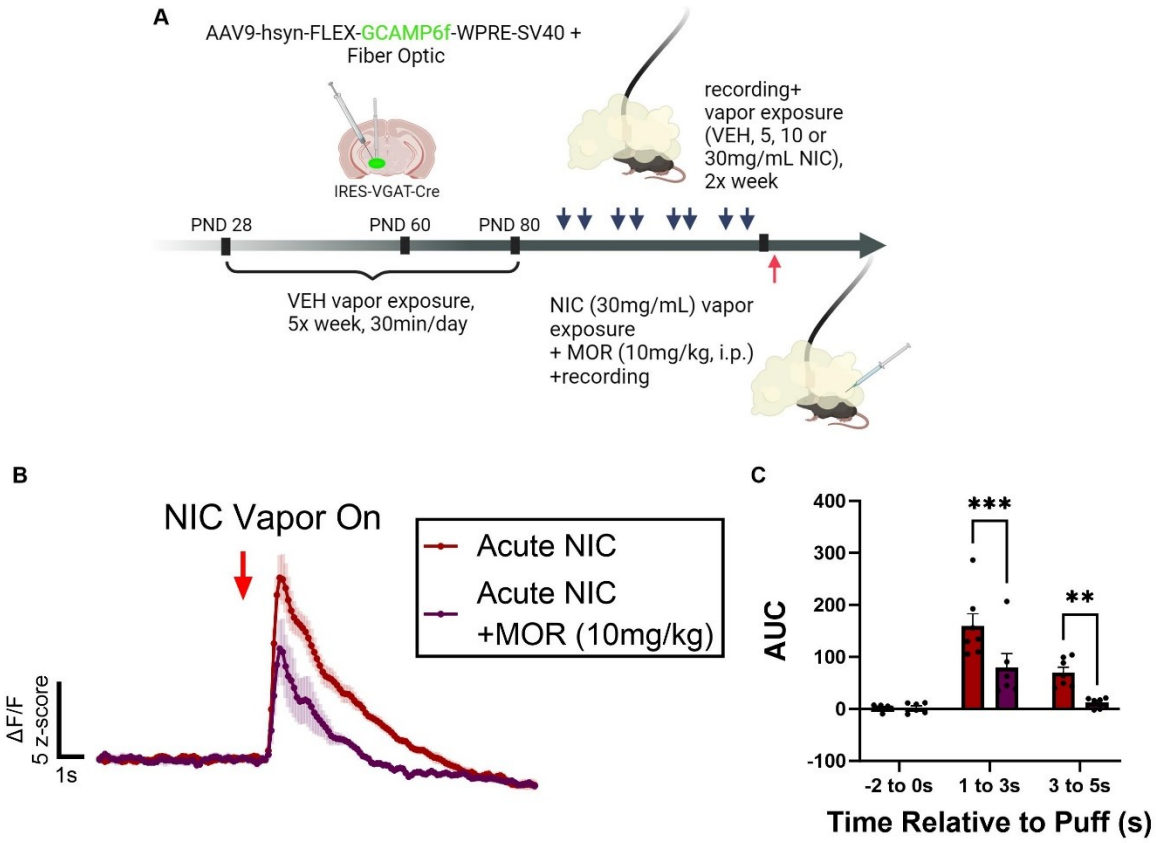


Figure 3-2. Acute morphine dampens the VTA GABA response to acute nicotine.

(A) Experimental timeline. VGAT-Cre mice (n=7) were exposed to vehicle (VEH) vapor from PND 28 – PND 80. VTA GABA neurons expressing GcAMP6f were recorded from during nicotine (NIC, 5, 10 or 30 mg/mL) or VEH vapor exposures between PND 80 – PND 110. AT PND 110, mice were injected with morphine (10 mg/kg, i.p.) and recorded from during a NIC (30 mg/mL) vapor exposure. (B) Average ΔF/F (z-score) of VTA GABA neurons before (5s) and after (10s) exposure to NIC (30 mg/mL) vapor puff. Response represents average of 17 puffs administered 90 s apart over the course of a 25 min session. Systemic, acute morphine dampens the increase in VTA GABA activity in response to NIC compared to morphine-naïve mice. (C) Area under the curve (AUC) of the Average ΔF/F (z-score) during 2 s intervals before and after NIC vapor puff. Mixed-effects ANOVA reveals a significant effect of morphine treatment (mixed-effects ANOVA; $F(1, 6) = 137.0$, $p = < 0.001$) and a significant interaction between morphine treatment and time relative to vapor puff (mixed-effects ANOVA, $F(1.56, 7.02) = 37.10$, $p < 0.001$). Post-hoc analysis revealed that morphine injection significantly altered the AUC value at both post-puff timepoints (Tukey's multiple comparison's, $p < 0.01$).

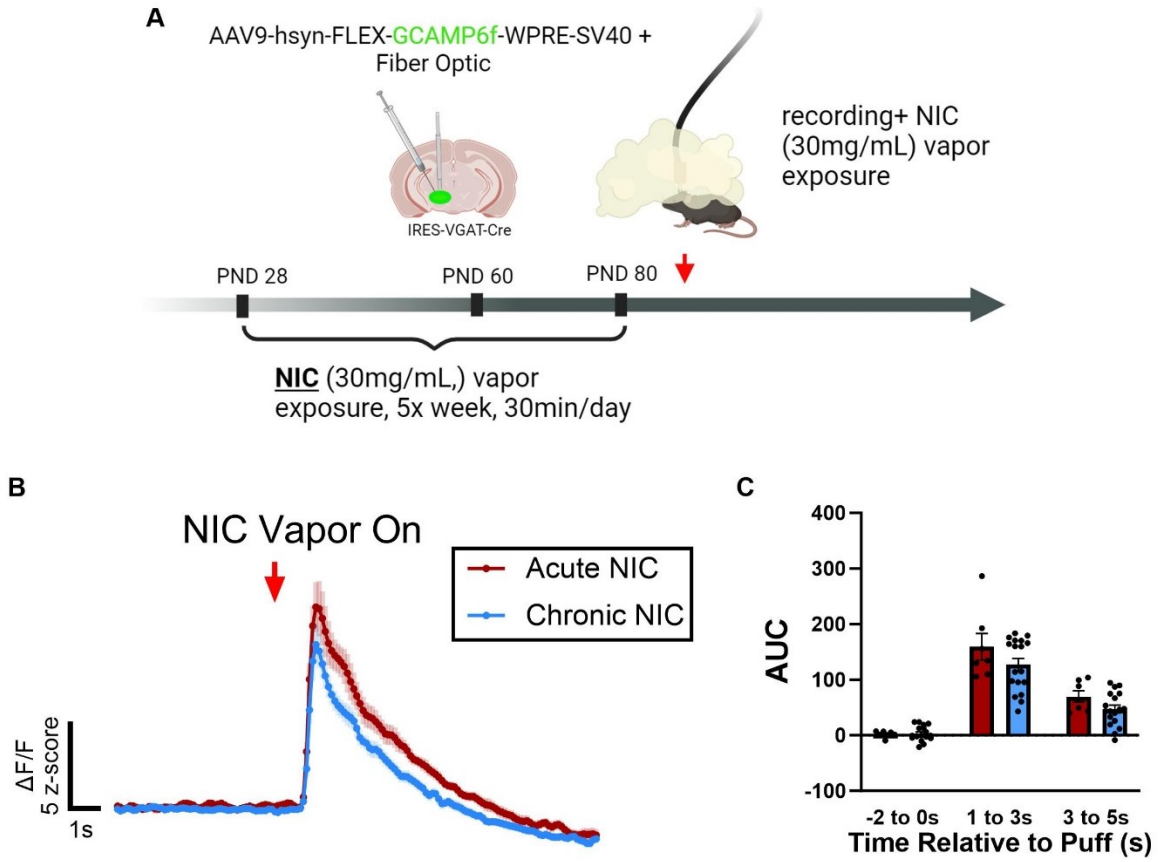


Figure 3-3. Chronic nicotine treatment does not significantly alter the VTA GABA response to acute nicotine.

(A) Experimental timeline. VGAT-Cre mice (n=12) were exposed to nicotine (NIC, 30 mg/mL) vapor from PND 28 – PND 80. VTA GABA neurons recorded from during exposure to NIC (30 mg/mL) vapor at PND 80. (B) Average ΔF/F (z-score) of VTA GABA neurons before (5s) and after (10s) exposure to NIC (30 mg/mL) vapor puffs. Response represents average of 17 puffs administered 90 s apart over the course of a 25 min session. Mice chronically exposed to nicotine (Chronic NIC) prior to recording show no significant decrease in response to NIC vapor compared to acutely exposed mice (Acute NIC, Figure 1). (C) Area under the curve (AUC) of the Average ΔF/F (z-score) during 2 s intervals before and after NIC vapor puff. No significant effects between Acute and Chronic NIC groups were detected.

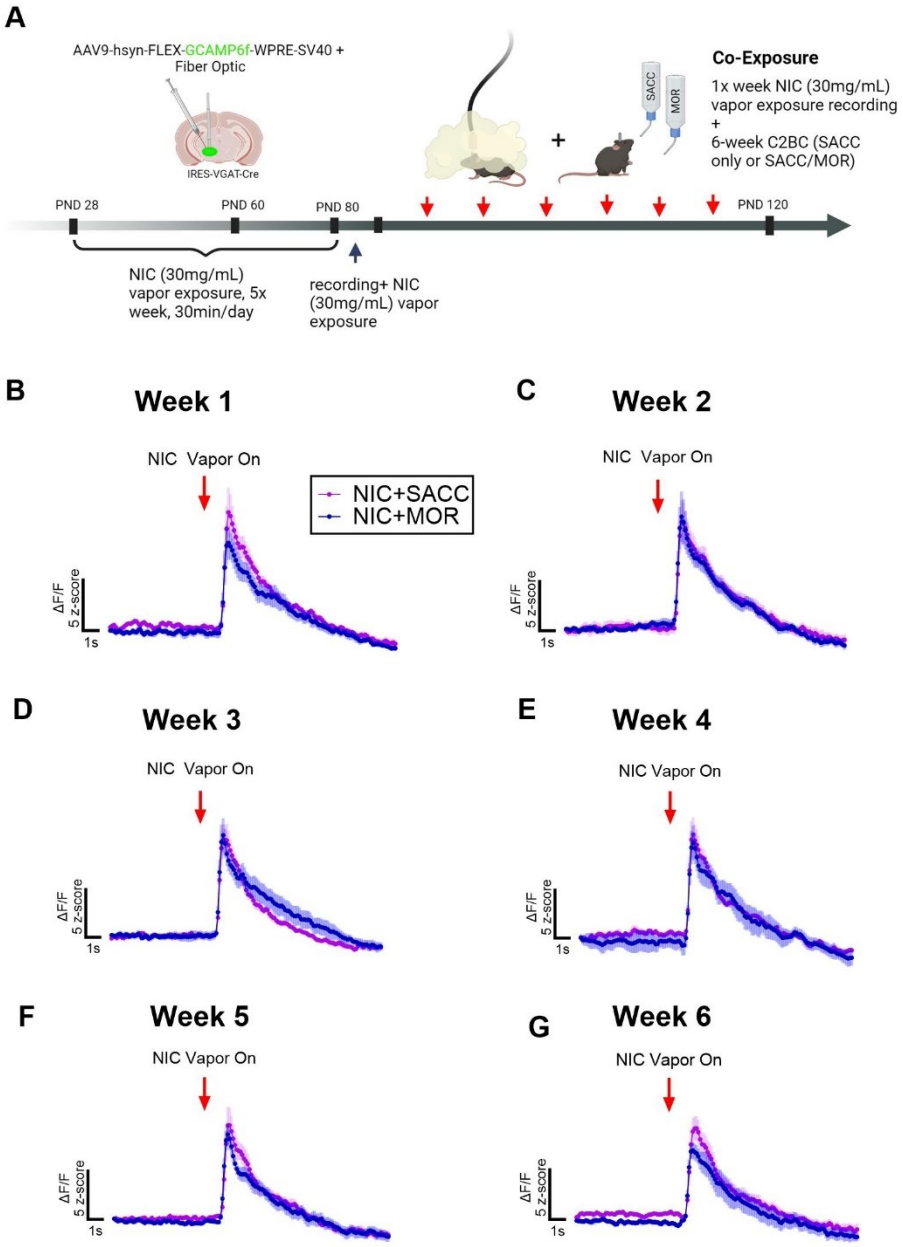


Figure 3-4. Chronic co-exposure to nicotine and morphine does not alter VTA GABA response to acute nicotine.

(A) Experimental timeline. VGAT-Cre mice (n=12) were exposed to nicotine (NIC, 30 mg/mL) vapor from PND 28 – PND 80. The 6-week co-exposure paradigm began at PND 80, with continuation of NIC vapor treatment and morphine (MOR) via a continuous 2-bottle choice (C2BC) paradigm. A subset of mice (n=7, NIC+MOR) were given *ad libitum* access to one bottle with 2.0% saccharin and one bottle with 2.0% saccharin + morphine (concentration escalated from 0.1mg/mL to 0.2mg/mL after the first week). Control animals (n=5, NIC+SACC) received two bottles of 2.0% saccharin solution. Photometry recordings during NIC vapor exposures were performed 1x week during the co-exposure period. (B-G) Average ΔF/F (z-score) of VTA-GABA neurons before (5s) and after (10s) exposure to NIC (30 mg/mL) vapor puff. Response represents average of 17 puffs administered 90 s apart over the course of a 25 min session. No significant differences observed between NIC+MOR and NIC+SACC mice at any timepoint during the co-exposure period.

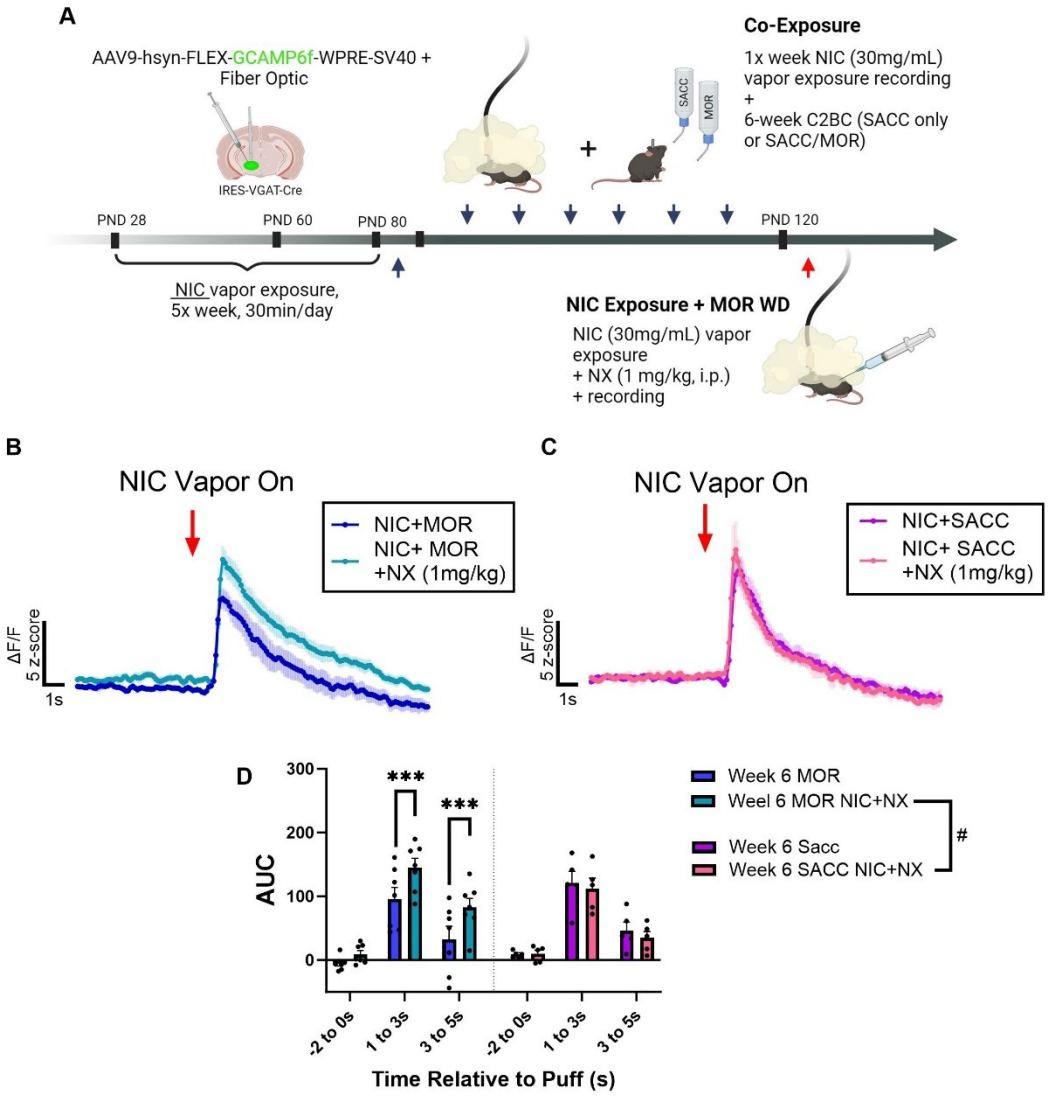


Figure 3-5. Morphine withdrawal reveals enhancement of VTA GABA response to acute nicotine in mice chronically co-exposed to both nicotine and morphine.

(A) Experimental timeline. VGAT-Cre mice (n=12) were exposed to nicotine (NIC, 30mg/mL) vapor from PND 28 – PND 80. The 6-week co-exposure paradigm began at PND 80, with continued NIC vapor treatment and morphine (MOR) via a continuous 2-bottle choice (C2BC) paradigm. NIC+MOR mice (n=7) have access to 2.0% saccharin and 2.0% saccharin + morphine (concentration escalated from 0.1mg/mL to 0.2mg/mL after the first week). Control animals (n=5, NIC+SACC) have two bottles of 2.0% saccharin. Photometry recordings during NIC vapor exposure session performed 1x week. After Week 6, mice were injected with naloxone (NX, 1 mg/kg, i.p.) and VTA GABA activity was recorded during a NIC vapor exposure session. (B-C) Average ΔF/F (z-score) of VTA GABA neurons before (5s) and after (10s) exposure to a NIC (30 mg/mL) vapor puff. Response represents average of 17 puffs administered 90 s apart over the course of a 25 min session. NX treatment increased VTA GABA activity in response to NIC in NIC+MOR mice but not in NIC+SACC mice. (D) Area under the curve (AUC) of the Average ΔF/F (z-score) during 2 s intervals before and after a NIC vapor puff. Repeated measures (RM) two-way ANOVA revealed there was a significant effect of NX treatment in the NIC+MOR group (RM two-way ANOVA; $F(1, 6) = 11.00$, $p = 0.016$) and a significant interaction between NX treatment and time relative to vapor puff (RM two-way ANOVA; $F(1.41, 8.44) = 6.53$, $p = 0.026$). Mixed-effects ANOVA between NIC+MOR and NIC+SACC animals also revealed a significant interaction between NX treatment and time relative to vapor puff (mixed-effects ANOVA; $F(2, 20) = 5.08$, $p \leq 0.01$).

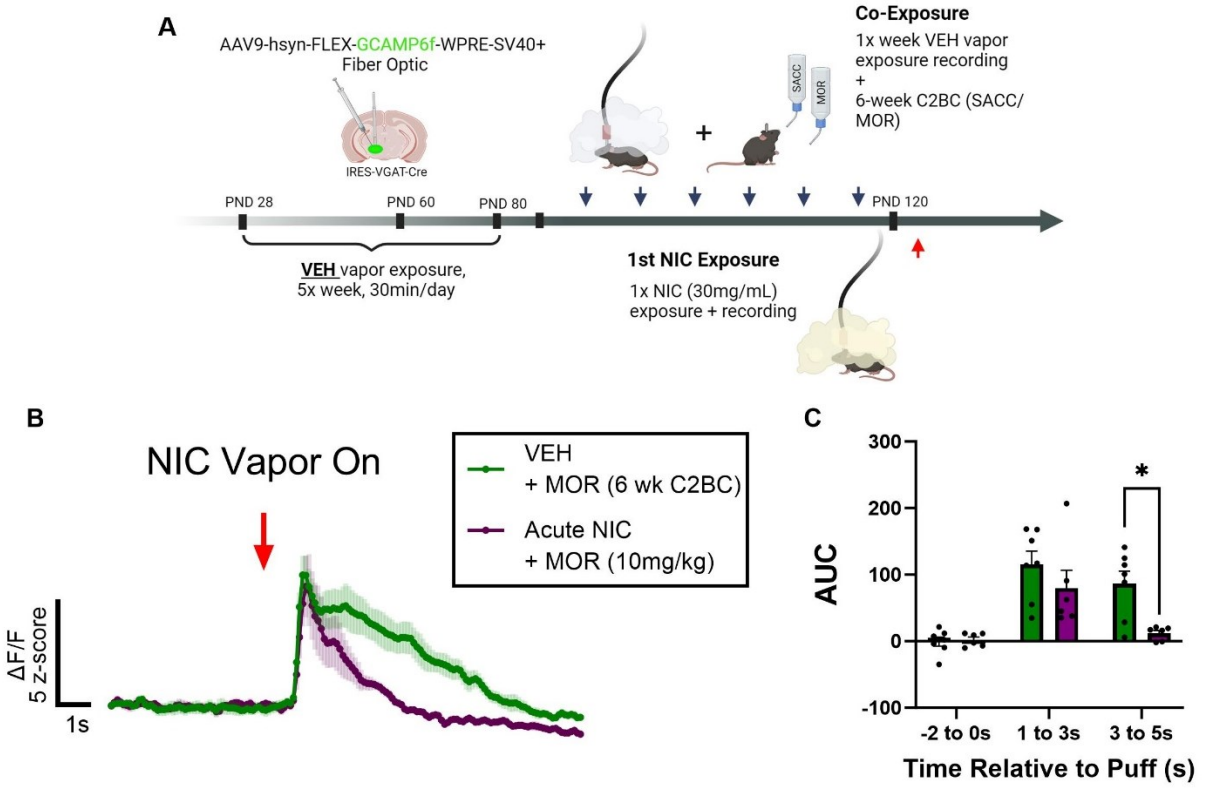


Figure 3-6. Compared to acute morphine, chronic morphine treatment prolongs the VTA GABA response to acute nicotine.

(A) Experimental timeline. VGAT-Cre mice (n=7) exposed to vehicle (VEH) vapor from PND 28 – PND 80. The 6-week co-exposure paradigm began at PND 80, consisting of continuation of VEH vapor treatment and morphine (MOR) via a continuous 2-bottle choice (C2BC) paradigm. In the C2BC, mice given *ad libitum* access to one bottle with 2.0% saccharin and one bottle with 2.0% saccharin + morphine (concentration escalated from 0.1mg/mL to 0.2mg/mL after the first week). Photometry recordings during VEH vapor exposures were performed 1x week during the C2BC period. At Week 6, VTA GABA activity recorded during a nicotine (NIC, 30 mg/mL) vapor exposure session. (B-C) Average ΔF/F (z-score) of VTA GABA neurons before (5s) and after (10s) exposure to NIC (30 mg/mL) vapor puff. Response represents average of 17 puffs administered 90 s apart over the course of a 25 min session. VEH+MOR mice show prolonged elevation in VTA GABA activity in response to NIC compared to mice that received a singular injection of morphine before NIC vapor exposure (Acute NIC + MOR, From Figure 2D). (D) Area under the curve (AUC) of the Average ΔF/F (z-score) during 2 s intervals before and after NIC vapor puff. Mixed-effects ANOVA revealed a significant interaction between of length of morphine treatment (chronic C2BC or acute injection) and time relative to vapor puff (mixed-effects ANOVA; $F(2, 22) = 5.22$, $p = 0.014$). Post-hoc analysis revealed a significant difference in AUC specifically at the 3-5 s post-puff timepoint (Sidak's multiple comparisons, $p < 0.05$).

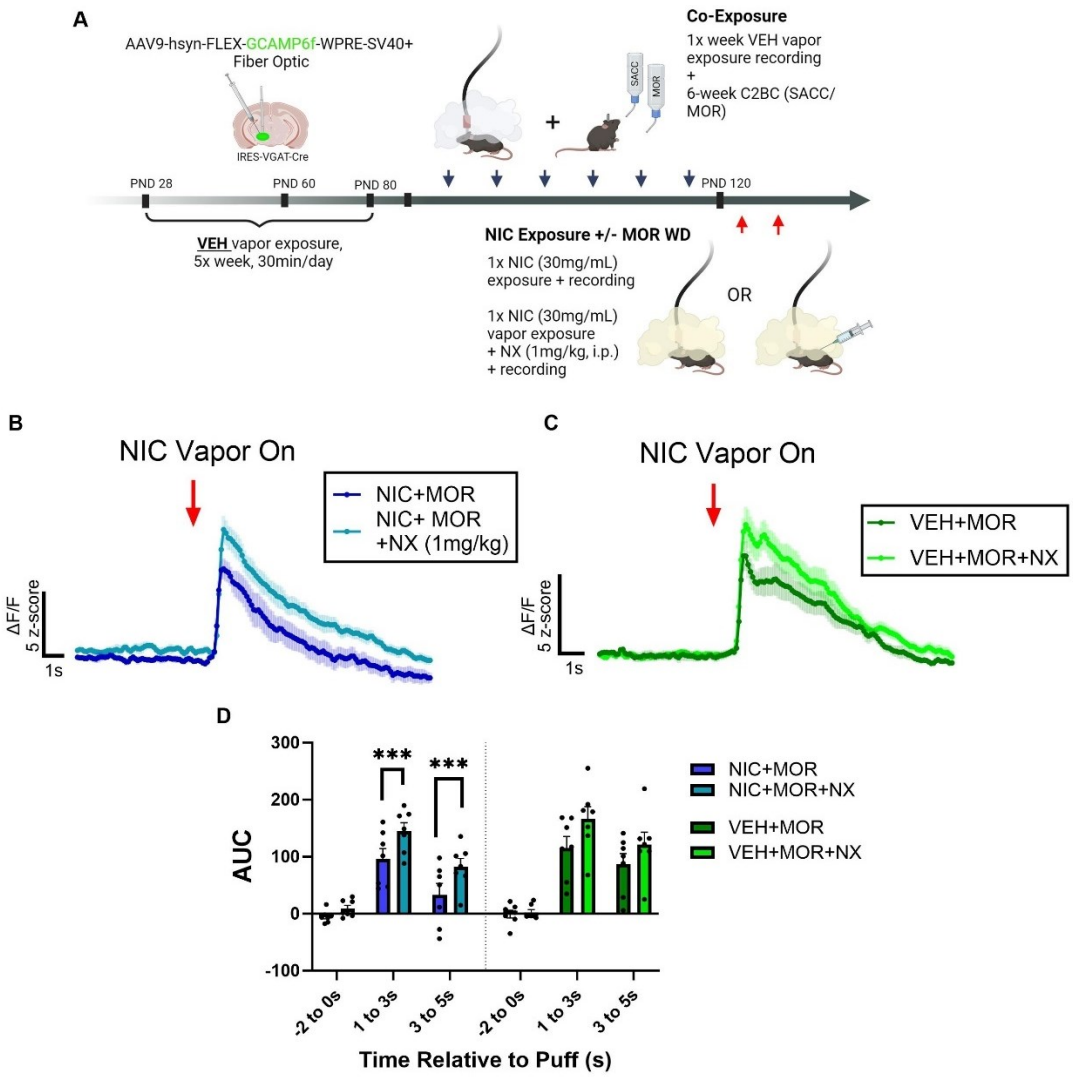
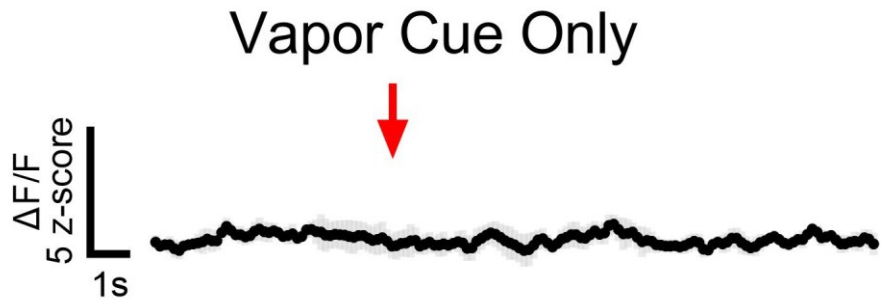


Figure 3-7. Chronic morphine alone is not sufficient for the enhancement of VTA GABA response to acute nicotine seen during morphine-withdrawal.

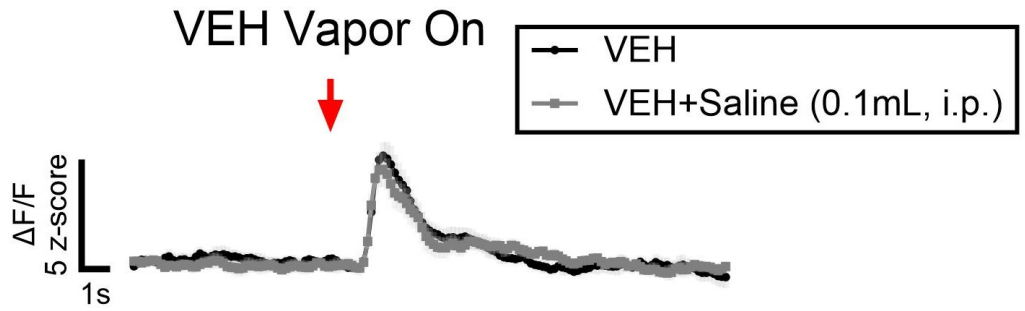
(A) Experimental timeline. VGAT-Cre mice (n=7, Figure 6) were exposed to vehicle (VEH) vapor from PND 28 - 80. The 6-week co-exposure paradigm began at PND 80, with continuation of VEH vapor treatment and morphine (MOR) via a continuous 2-bottle choice (C2BC) paradigm. In the C2BC, mice given ad-libitum access to one bottle with 2.0% saccharin and one bottle with 2.0% saccharin + morphine (concentration escalated from 0.1mg/mL to 0.2mg/mL after the first week). Photometry recordings during VEH vapor exposures were performed 1x week during the C2BC period. At Week 6, mice were recorded from during a nicotine (NIC, 30 mg/mL) vapor exposure session. Four days later, mice were injected with naloxone (NX, 1 mg/kg, i.p.) and then again recorded from during a NIC vapor exposure session. (B, Figure 5B) Average ΔF/F (z-score) of VTA-GABA neurons before (5s) and after (10s) exposure to NIC (30 mg/mL) vapor puff. NX increases VTA GABA response to NIC in mice co-exposed to nicotine and morphine (C) Average ΔF/F (z-score) of VTA-GABA neurons before (5s) and after (10s) exposure to NIC (30 mg/mL) vapor puff. NX treatment results in non-significant increase in VTA GABA response to NIC in nicotine-naïve, morphine dependent (VEH+MOR) mice. (D) Area under the curve (AUC) of the Average ΔF/F (z-score) during 2 s intervals before and after a NIC vapor puff. Repeated measures (RM) two-way ANOVA reveals a significant effect of NX treatment in NIC+MOR (RM two-way ANOVA; $F(1, 6) = 11.00$, $p = 0.016$, Figure 5D) but not VEH+MOR mice.

3.6 Supplemental Figures



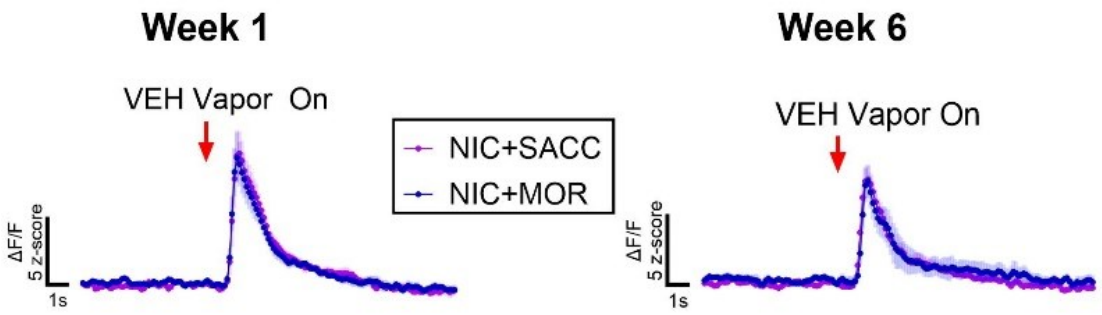
Supplemental Figure 3-1. VTA GABA activity does not change in response to vapor cue alone.

Average ΔF/F (z-score) of VTA GABA neurons before (5s) and after (10s) presentation of vapor cue alone. VGAT-Cre mice (n=3) were tethered to the fiber optic patch cord and placed inside the vapor exposure chamber. Vapor exposure session was set up according to standard protocol (e.g., vacuum at 2.0 flow rate, vapor generator controller programmed to deliver 17 puffs, puffs triggered by controller etc.) but tubing connecting the e-cigarette to the vapor exposure chamber was unhooked, preventing vapor from entering the chamber.



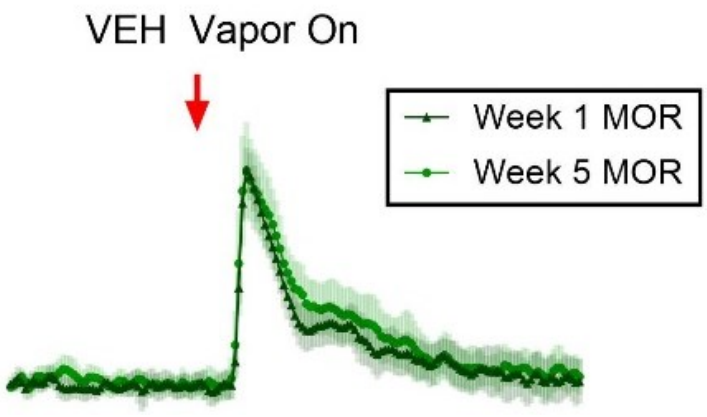
Supplemental Figure 3-2. Saline injection does not alter response of VTA GABA neurons to vapor puff.

Average ΔF/F (z-score) of VTA GABA neurons before (5s) and after (10s) vehicle (VEH) vapor puff with or without saline injection (0.1mL, i.p.). VEH + Saline mice received an injection of saline (0.1mL, i.p.) following a 5 min baseline recording and were then recorded from during a standard VEH vapor exposure session (17 puffs, 25 min). VEH mice were recorded from during a standard VEH vapor exposure session. Treatments were counterbalanced such that mice were randomly assigned to a treatment for the first session and were recorded from during the opposite treatment two days later.



Supplemental Figure 3-3. Chronic morphine does not alter VTA GABA response to vehicle (VEH) vapor in nicotine-treated mice.

Average ΔF/F (z-score) of VTA GABA neurons before (5s) and after (10s) vehicle (VEH) vapor puff during Week 1 and Week 6 of co-exposure to both nicotine (NIC) and morphine (MOR) or saccharin (SACC). NIC+MOR and NIC+SACC mice show similar VTA GABA response to VEH at both the start (Week 1) and the end (Week 6) of the co-exposure period.



Supplemental Figure 3-4. Chronic morphine does not alter VTA GABA response to vehicle (VEH) vapor in VEH-treated mice.

Average ΔF/F (z-score) of VTA GABA neurons before (5s) and after (10s) vehicle (VEH) vapor puff during Week 1 and Week 5 of the morphine continuous two-bottle choice (C2BC) paradigm. VTA GABA response to VEH is similar at both the start (Week 1) and end (Week 5) of morphine C2BC.

CHAPTER 4: GENERAL DISCUSSION, CONCLUSIONS, AND FUTURE DIRECTIONS

4.1 General Overview

Opioid dependence has emerged as one of the leading public health concerns of the 21st century, with an estimated 2.1 million Americans suffering from opioid use disorder (OUD, Chang et al., 2018). This has been paralleled by a rise in the popularity of e-cigarettes, especially among adolescents (Cullen et al., 2018). Though use of traditional tobacco cigarettes has been steadily declining for the last 60 years, 34 million Americans still report daily smoking and 500,000 deaths per year can be attributed to smoking or second-hand smoke (Wang et al., 2018). Co-dependence of nicotine and opioids is very common, in fact 75-94% of people on methadone maintenance therapy (MMT) also report smoking cigarettes (Zirakzadeh et al., 2013). Smoking is also a strong predictor of both opioid craving and withdrawal severity during methadone detoxification and, more generally, of prescription opioid misuse (Yoon et al., 2015; Zale et al., 2015).

Both clinical and preclinical researchers have worked to uncover the neuronal mechanisms that underlie nicotine and opioid co-dependence. The bulk of their work has centered on the canonical reward pathway- the mesolimbic circuit- which primarily comprises DAergic projections from the VTA to the NAc. The VTA also contains a substantial population of GABAergic neurons which project both locally, to DA neurons and remotely, to several other regions (Bouarab et al., 2019; Tan et al., 2012). Animal models have revealed that activating μ-opioid receptors (MORS), which are located predominantly on VTA GABA neurons, modulates nicotine-induced dopamine (DA) release in the NAc and, conversely, that chronic nicotine administration augments morphine-induced DA release in the NAc (Berrendero et al., 2010; Vihavainen, Relander, et al., 2008; Walters, Cleck, & Kuo, 2005). Other studies suggest chronic nicotine use can alter the endogenous opioid system, while at the same time chronic morphine

may modify the cholinergic system, both of which are mechanisms that would likely affect the mesolimbic circuitry (Bajic et al., 2015; Jensen et al., 2018; Kiguchi et al., 2008; Kishioka et al., 2014; Kuwabara et al., 2014; Ueno et al., 2014).

Though nicotine and opioid co-use is frequently reported in the clinical literature, there has been a lack of preclinical studies examining the neuroadaptations underlying this behavioral phenomenon. In this dissertation, we utilized calcium imaging (fiber photometry) in conjunction with a physiologically relevant co-drug delivery paradigm to uncover nicotine- and opioid-induced alterations to the activity of DA and GABA neurons within the VTA. This work was sought to determine and compare how the activity of the **(1)** VTA DA and **(2)** VTA GABA neurons is affected by **(a)** exposure to acute nicotine **(b)** exposure to chronic nicotine and **(c)** co-exposure to nicotine and morphine. We revealed that VTA DA neurons are especially sensitive to the effects of chronic nicotine, whereas VTA GABA neurons are uniquely altered after chronic, co-exposure to nicotine and morphine. In the following paragraphs we consider the relationship between the observed VTA DA and GABA responses, elaborate on the potential mechanisms underlying changes in neural activity, reflect on the behavioral implications of these findings, and contemplate the future directions for this body of work.

4.2 Development of a physiologically relevant mouse model to study the co-use of nicotine and opioids

For decades, mice and rats have represented the most popular species used in preclinical models of drug abuse (Baptista et al., 2021; Johanson et al., 1976; Spanagel, 2017). Much of what is known about the behavioral, neurobiological, and pharmacological mechanisms that underlie substance use disorders comes from work in rodent models. Although these studies have provided invaluable insight into the consequences and biological underpinning of drug use, they have not always accounted for critical aspects of the human drug user experience. In our

work, we attempted to more closely model drug use in the human population, developing a drug administration paradigm that considers several key factors, including the route of administration, the age at which drug use typically initiates, the length of time that drug use may persist, and the high prevalence of polysubstance use.

4.2.1 Consideration for the route of administration

Historically, preclinical studies examining nicotine or morphine use have relied on drug delivery via intraperitoneal (i.p.) and subcutaneous (s.c.) injections. Although injection does allow for more precise control over the dose of drug the animal receives, it does not reflect the typical routes of administration for these two drugs in the human population. For example, nicotine is most often inhaled, via use of combustible or electronic cigarettes, although it may also be ingested orally, via chewing tobacco or nicotine gum. Not only does inhalation of tobacco smoke or e-cigarette vapor encompass a multi-sensory experience, but, when inhaled, nicotine evades first-pass metabolism in the liver, so that, almost instantaneously, nearly the entire dose reaches the brain (Le Houezec, 2003; Olsson Gisleskog et al., 2021). Importantly, both the rate and extent of drug delivery can impact it's rewarding and reinforcing properties, indicating that the slower delivery and more extensive metabolism of nicotine conferred by an i.p. or s.c. injection could alter nicotine's behavioral effect (Dong et al., 2010; Jensen et al., 2020; Matta et al., 2006). For these reasons, in our work, we employed an adapted version of the model for nicotine vapor exposure that we had previously validated produces plasma cotinine levels comparable to an i.p. injection of nicotine (Patten et al. 2023).

The typical route of administration for opioids among individuals with OUD tends to be more varied as it may entail intravenous (i.v.) injection, oral consumption, or inhalation of a smoked form of the drug. For our paradigm, instead of an IP or SC injection, we administered morphine orally, via a continuous two-bottle choice (C2BC) model where mice are given *ad*

libitum access to morphine in the home cage. This preclinical method has previously been used by our lab (Fleites et al., 2022) and others (Zanni et al., 2020; Zhang et al., 2018), as an opioid self-administration method, capable of producing steady opioid consumption and dependence in rodents. Although metabolism of morphine tends to be slower when taken orally, the C2BC model is advantageous in that it is a voluntary form of drug administration, which allows rodents to titrate their dose, potentially revealing differences in susceptibility to developing morphine dependence (Fleites et al., 2022). Moreover, it has been suggested that the nature of the drug exposure paradigm (passive versus active), may not only influence the behavioral effects, but also the neuroadaptive effects of addictive drugs (Fernàndez-Castillo et al., 2012; Francis et al., 2022; Jacobs et al., 2003). Finally, *ad libitum* access to morphine via C2BC is likely less stressful to animals than daily injections. This is especially critical given that stress can impact both addiction-related behaviors and the functionality of the mesolimbic dopamine system (Aydin et al., 2015; Drude et al., 2011; Du Preez et al., 2020; Haass-Koffler & Bartlett, 2012; Koob, 2009; Ostroumov et al., 2016; Polter & Kauer, 2014). Thus, by treating mice with morphine via the C2BC we aimed to minimize the effect of stress, while also imparting an active component to our drug administration paradigm.

4.2.2 Consideration for the age of initiation of nicotine use

For our drug exposure paradigm, we chose to begin nicotine vapor exposures during adolescence (PND 28), corresponding to human age of approximately 12 years old (M. Yuan et al., 2015). This is in line with epidemiological literature suggesting that most adult smokers try their first cigarette before the age of 18 and that the use of e-cigarettes during adolescence may be associated with the future use of combustible cigarettes (Barrington-Trimis et al., 2020; Cantrell et al., 2018; Leventhal et al., 2015). Modeling the age at which most people initiate nicotine use may be especially important when studying alterations to neural circuitry because

the adolescent brain is in a critical stage of development and drug use during this time may induce unique neuroadaptations, potentially contributing to future drug use (Chambers et al., 2003; Goriounova & Mansvelder, 2012; Yuan et al., 2015). The developing mesolimbic DA circuitry may be particularly sensitive to the effects of nicotine as it has been demonstrated that adolescents more readily experience nicotine reward, but are less susceptible to nicotine aversion, compared to adults (Adriani et al., 2002; Ahsan et al., 2014; Doura et al., 2010; Shram et al., 2006; Spear, 2000; Wilmouth et al., 2004). Particularly relevant for our co-exposure paradigm, it has also been demonstrated that nicotine use during adolescence, but not adulthood, enhances morphine preference and fentanyl self-administration, further demonstrating the unique susceptibility of the adolescent mesolimbic system (Cardenas et al., 2021; Kota et al., 2018). By beginning vapor exposures in adolescence, we hoped both to capture the time that most humans begin nicotine use and to reveal the way that VTA DA and GABA neurons may be specifically vulnerable to nicotine-induced alterations during development.

4.2.3 Consideration for the length of time that drug use persists

Although the route of drug administration and age of drug use initiation are critical factors governing the translatability of preclinical models of substance use disorders, the length of drug treatment should be given equal consideration. Historically, animal models of addiction have tended to use acute drug exposure paradigms, sometimes exposing rodents to as little as a single dose of a drug, to determine the behavioral and cellular effects of drug treatment. However, as previously mentioned, addiction is a chronic disease, involving the continued use of a substance for an extended duration. In humans, addiction involves progressive changes in both behavior, including reward and aversion responses, and the mesolimbic neural circuitry governing those processes, which may be revealed slowly over the course of days, weeks,

months, and years (Detar, 2011; Heilig et al., 2021; Koob & Volkow, 2016). More recent preclinical evidence suggests that, like in humans, chronic drug exposure in rodents may produce entirely different effects on behavior and may induce unique neuroadaptations, compared to acute exposure (Lynch et al., 2010; Sinha, 2008; Spanagel, 2017). Thus, to better model the chronic nature of addiction, and capture the neuroadaptations associated with prolonged drug use, we established a nicotine vapor exposure paradigm that spans 3 months and a morphine C2BC paradigm that lasts 6 weeks.

4.2.4 Consideration for the high prevalence of polysubstance use

Having established physiologically relevant models for both nicotine and morphine use separately, we turned to combining these paradigms to fill a critical gap in the preclinical literature. As already discussed, despite its predominance in the human population, polysubstance, and specifically nicotine and opioid co-use, remains relatively understudied in the preclinical literature. This may be reflective of the practical difficulty of treating animals concurrently with more than one drug, or a desire to avoid the possibility of drug-drug interactions, which may affect drug metabolism and the dose of drug delivered. As we highlight in this work, studying polysubstance use also requires extensive controls, to concretely determine if one or both drugs mediates the observed effect.

Likely due to these reasons, most “co-exposure” studies entail pre-exposure to one drug, before subsequently studying the effects of the other, rather than studying the concurrent use of both drugs. The lack of preclinical paradigms for polysubstance use may be especially detrimental to the study of nicotine and opioid co-use, considering most people with opioid use disorders, who regularly use opioids, are also daily cigarette smokers, and therefore frequently have both drugs in their system (Guydish et al., 2011; Haas et al., 2008; Zirakzadeh et al., 2013a). With this in mind, along with each of the considerations discussed previously, we

bridged our chronic drug treatment paradigms, establishing a model for co-exposure to nicotine and morphine that begins with nicotine vapor exposure in adolescence and continues with concurrent nicotine vapor and morphine exposure (via C2BC) in adulthood. To our knowledge, we are the first to use a chronic (> 2 weeks) paradigm to study the effects of simultaneous exposure to nicotine and morphine on VTA DA and GABA activity.

4.3 E-cigarette vehicle vapor activates the mesolimbic reward circuitry

In our NIC vapor exposure paradigm, we used a 50:50 blend of propylene glycol and vegetable glycerin as our vehicle vapor control, which we expected would not elicit a neuronal response. Yet, surprisingly, we found that VEH vapor activated both VTA DA and VTA GABA neurons. We habituated mice to VEH vapor exposures starting in adolescence, which should negate the novel, unexpected quality of the vapor exposure, making it seem unlikely that these increases in neuronal activity were reflective of reward prediction error. Moreover, when we exposed mice to vapor cues alone, without the subsequent delivery of vapor, there was no change in either VTA DA or GABA activity, indicating that the response to VEH vapor was likely not a product of conditioned reinforcement. For these reasons, we suspect that PG or VG may be either directly or indirectly stimulating the mesolimbic circuitry.

Whereas a growing body of evidence suggests that PG/VG vapor affects peripheral cells, particularly the lung epithelium, there are only a handful of studies that have examined the effects of PG/VG vapor on nervous system cells (Madison et al., 2020; Pisinger & Døssing, 2014; Woodall et al., 2020). Clinical reports have demonstrated that the ratio of PG to VG can affect both taste sensation, as well as the “pleasant” or “satisfactory” feelings associated with using e-cigarettes, suggestive of the activation of the reward system (Harvanko et al., 2019; Kosmider et al., 2014; Spindle et al., 2018). In addition, a recent preclinical study showed that ICSS thresholds were the same across nicotine or vehicle (50:50 PG/VG) vapor-treatment

groups (Martínez et al., 2023) and preliminary research in our lab demonstrates that mice may self-administer VEH vapor alone (data not shown), further suggesting that VEH vapor exposure may be reinforcing. These behavioral effects may stem from activation of a variety of different brain regions, including for instance, the taste and smell processing systems in the olfactory bulb and gustatory cortex. However, PG may stimulate DA release *in-vitro,* highlighting the way that PG and VG may directly interact with the mesolimbic system (Hattori 2000). Future experiments will be needed to correlate the VEH vapor-induced activation of VTA DA or GABA neurons to potential rewarding or reinforcing behavioral effects of PG/VG.

4.4 Acute nicotine vapor produces divergent response in VTA DA and GABA neurons

Though VEH vapor activated both DA and GABA neurons within the VTA, we report an opposing effect of NIC vapor on these two cell populations. In response to NIC vapor in nicotine naïve mice, we noted a small magnitude decrease in VTA DA activity, and a large magnitude increase in VTA GABA activity. Although seemingly at odds with the prevailing literature, which indicates that VTA DA and GABA neurons are both activated by nicotine, differences in experimental technique may explain these discrepancies. Prior to the last decade, *ex-vivo* electrophysiology was the primary method employed to measure neuronal activity and, more specifically, the technique used to determine the effects of nicotine on DA neuron firing (Fisher et al., 1998; Mansvelder et al., 2002; Pidoplichko et al., 1997). Although *ex-vivo* electrophysiology can determine spiking activity with high signal-to-noise ratio and temporal fidelity, it typically only offers access to a sparse number of relatively active neurons, and also effectively isolates the recorded neuron from any afferent input. Though newer methods for measuring neural activity *in-vivo*, like fiber photometry, may forgo the temporal and spatial resolution of a single-cell recording, they do maintain the integrity the system, ensuring that the recorded population of cells continue to serve its functional role within the larger circuit. These

new *in-vivo* techniques, along with advances in other techniques like viral targeting, have helped to reveal the existence of subpopulations of VTA DA neurons, which vary in their afferent and efferent connectivity, and serve diverse functions (Anderegg et al., 2015; Collins & Saunders, 2020; Eddine et al., 2015; Lammel et al., 2014; Morales & Margolis, 2017; Yang, De Jong, et al., 2018).

Contrary to historic electrophysiological findings, recent work has revealed that nicotine may differentially affect subpopulations of VTA DA neurons (Eddine et al., 2015; Lammel et al., 2014; Liu et al., 2022). Specifically, it was shown that nicotine activates DA neurons in the lateral VTA, which project primarily to the NAc, whereas it inhibits DA neurons in the medial VTA, which project primarily to the amygdala (Nguyen et al., 2021). There is reason to speculate that *ex-vivo* electrophysiologists may be biased toward recording preferentially from lateral VTA DA neurons, which might begin to explain seemingly contradictory reports. Identification of VTA DA neurons during an *ex-vivo* electrophysiology recording has traditionally relied on both the expression of tyrosine hydroxylanse (TH), the precursor for DA, and the detection of a hyperpolarization activated (I_h) current. However, it was demonstrated that DA neurons from the lateral VTA generally display larger amplitude I_h, relative to DA neurons located in the medial VTA (Zhang et al., 2010). For this reason, electrophysiologists may be more inclined to record from lateral VTA DA neurons, which appear more active and “DA-like” at baseline, and to avoid less “DA-like” medial VTA DA neurons, subsequently drawing the conclusion that nicotine exclusively increases activity of VTA DA neurons.

Considering the recent evidence highlighting the differential effects of nicotine on subpopulations of VTA DA neurons, we hypothesize that the decrease in DAergic activity we observed in response to NIC vapor may be reflective of our more medial (-0.5 M/L) recording location within the VTA. To begin to probe this theory, we injected our Cre-dependent GCaMP6f

virus in a more lateral location within the VTA (-1.0 M/L) of DAT-Cre mice (n=3). In 2/3 of the DAT-Cre mice expressing GCaMP6f in lateral VTA DA neurons, we noted a brief (~1s), sharp increase in DAergic activity following a NIC vapor puff. The stark contrast between this response and the response we had recorded from more medial VTA DA neurons suggests that NIC vapor, like nicotine administered via injection, also differentially affects subpopulations of VTA DA neurons. It seems most probable that, in our main experiments, we were recording mainly from medial VTA DA neurons, but also from some lateral VTA DA neurons, and that the noise in our signal may reflect the divergent response to NIC in these two subpopulations.

It has recently been demonstrated that the concerted activity of VTA DA and GABA neurons is responsible for regulating reward and aversion to nicotine, so it is intriguing to consider the VTA DA response to NIC vapor in relation to the VTA GABA response to the same stimuli (Grieder et al., 2019; Lobb et al., 2010; Tolu et al., 2013). In our work, we observed that the magnitude of the increase in VTA GABA activity (~15 z-scores) in response to NIC vapor was substantially larger than the magnitude of the decrease in VTA DA activity (~1 z-score). The ratio of GABA to DA neurons may be higher in medial and posterior sections of the VTA, compared to lateral and anterior sections (Ciccarelli et al., 2012; Morales & Margolis, 2017; Nair-Roberts et al., 2008; Root et al., 2018). Given our more medial and posterior recording site (-3.25 A/P) it is possible that we were recording from a portion of the VTA where GABAergic activity dominates, thereby suppressing the activity VTA DA neurons.

Yet, in the absence of more precise targeting of subpopulations of VTA DA and GABA neurons, it can be difficult to infer the relationship between the opposing responses to NIC vapor that we observed in these cell types. For example, the difference in the magnitude of VTA GABA, compared to the VTA DA, response to NIC vapor may reflect the extent to which we recorded primarily from either a single, or multiple, cellular subpopulations. As suggested

previously, it is possible that though we were recording from multiple subpopulations of VTA DA neurons, we were recording predominantly from a single subtype of VTA GABA neuron, which displayed a more synchronous neural response, resulting in the larger magnitude of the GABAergic response to NIC vapor, compared to the DAergic response. This theory is supported, in part, by work showing that afferents to VTA GABA neurons have a higher degree of convergence than input to VTA DA neurons, with one presynaptic cell synapsing onto, and uniformly controlling the activity of, many VTA GABA neurons (Faget et al., 2016).

It is also important to consider that although many VTA GABA neurons are interneurons, there is a substantial portion that projects outside of the VTA (Bouarab et al., 2019; Omelchenko et al., 2009). Though the activity of GABAergic interneurons increases in response to nicotine, resulting in a decrease in VTA DA firing, it is unclear if we were recording from VTA GABA neurons with distal or local projections (Tolu et al., 2013). Therefore, we cannot conclude that the increase in VTA GABA activity we observed would have a direct effect on VTA DA neuron firing. Given that there has been relatively little work examining different subpopulations of VTA GABA neurons, it can be difficult to infer what the GABAergic response to NIC vapor tells us about, for example, the afferent or efferent connectivity of these neurons. To move toward more clearly defining the relationship between GABAergic and DAergic responses to NIC vapor, it will be necessary to specifically target the various subpopulations of VTA DA and GABA neurons.

In addition to more refined targeting of neural subpopulations, we plan to further test the rewarding or aversive behavioral effects of these concentrations (5, 10, 30 mg/mL) of NIC vapor, which may provide even greater insight into the relationship between VTA DA and GABA activity. Two separate studies using optogenetic stimulation have shown that activation of VTA GABAergic neurons can lead to disruption of reward and induce aversion (Tan et al., 2012; Van Zessen et al., 2012). However, more recent work has suggested that β2* nAChRs on VTA DA

neurons regulate nicotine aversion, whereas β2* or α4* nAChRs on VTA GABA neurons regulate nicotine reward (Grieder et al., 2019; Ngolab et al., 2015). Interestingly, as demonstrated previously in our lab (Patten et al., 2023), only vapor from a 2.5 mg/mL solution of NIC, and not from 10 or 25 mg/mL NIC vapor solutions, produces CPP in mice. Notably, despite its ability to produce nicotine CPP, a 2.5 mg/mL NIC vapor produces plasma cotinine levels comparable to a sub-rewarding dose (0.1 mg/kg) of nicotine when administered i.p., whereas the higher concentration NIC vapors produce cotinine levels in line with rewarding i.p. doses (Patten et al., 2023).

It is possible that, with the addition of the sensory experience that accompanies nicotine vapor inhalation, which is absent when nicotine is injected, the dose-response curve for nicotine shifts down and to the right, making only low doses of nicotine rewarding. In turn, this could imply that each of the concentrations of NIC vapor we used were aversive to mice, supporting the theory that VTA GABA neuron activity mediates nicotine aversion. If each of these concentrations produced a similar behavioral response, this may help to explain the ceiling effect we observed, whereby DAergic or GABAergic response to NIC vapor remained similar across each of the three concentrations of NIC tested. It should also be noted that, if transformed to a logarithmic scale, the difference between each of these concentrations becomes quite small, suggesting that, perhaps, the NIC vapors were so similar that they elicited nearly identical neuronal responses. In future experiments, we plan to further assess this theory, recording from VTA DA and GABA neurons in response to much lower, or much higher, concentrations of NIC vapor. We also intend to use CPP and CPA to probe the rewarding or aversive properties of these 3 NIC vapor concentrations, to ultimately gain a better understanding of the way that VTA DA and GABA neurons work in concert to mediate the behavioral response to NIC vapor.

4.5 VTA DA neurons may be especially sensitive to chronic nicotine exposure during adolescence

When we transitioned to studying long-term effects of NIC vapor on neuronal activity within the VTA, we noted that DAergic neurons seem particularly sensitive to prolonged, adolescent nicotine exposure. In mice that had been chronically exposed to nicotine beginning in adolescence, we observed a smaller decrease in VTA DA activity in response to NIC vapor puffs, compared to the DAergic response of nicotine-naïve mice. In contrast, chronic nicotine exposure produced a nonsignificant dampening of the VTA GABA response to NIC vapor. It has been demonstrated that the DAergic system undergoes dramatic and dynamic changes in activity across development and well into adolescence (Bissonette & Roesch, 2016; Galvan, 2010; Wahlstrom et al., 2010). For example, compared to VTA DA activity in adult rodents, the VTA DA neurons of adolescent rodents show higher tonic firing rate and longer phasic bursts (Mccutcheon et al., 2012; Philpot et al., 2009; Placzek et al., 2009; Yuan et al., 2015). The developing mesolimbic DA circuit may be particularly responsive to nicotine exposure, as nicotine-induced DA release in the NAc is greater in adolescents than in adults (Corongiu et al., 2007). Enhanced DA tone in response to nicotine may reflect the higher expression of many nAChR subunits, including α7, α4 and β2, in the VTA of adolescent, compared to adult, brains (Azam et al., 2007; Doura et al., 2008; Renda et al., 2016).

It has also been suggested that nicotine exposure during adolescence may have long-lasting consequences on VTA DA neuron activity. In a recent study, Reynolds et al., 2023 observed that nicotine produced a strong, adolescent-like, activation of DA neurons, but only in adult mice who had been treated with nicotine (2BC) during early adolescence and not in control mice who were treated with nicotine during adulthood. Interestingly, they also report that this “freezing” of DAergic response to nicotine in an immature state was subpopulation specific. In agreement with other work cited here, Reynolds et al., 2023 reiterate that lateral VTA DA

neurons are activated by nicotine, whereas medial VTA DA neurons are inhibited by the same stimuli. They proceed to demonstrate that only DA neurons in the lateral VTA DA, and not those in the medial VTA, experience the “freezing” effect following adolescent nicotine exposure, such that adolescent nicotine exposure only appears to alter the nicotine-induced excitation, but not the nicotine-induced inhibition, of VTA DA neurons. It seems possible that if, as previously speculated, we were recording from both medial and lateral VTA DA neurons, then an increase in excitation of lateral DA neurons could shift the overall DA neuron response to NIC, from inhibitory to excitatory. In this way, the mitigation of the inhibitory VTA DA response to NIC vapor that we observed may reflect the differential effects of adolescent nicotine exposure on VTA DA subpopulations.

We were somewhat surprised by the lack of significant alteration to the VTA GABA response to NIC vapor following chronic adolescent nicotine treatment. It had previously been documented that a 14-day adolescent nicotine exposure results in impaired chloride extrusion from VTA GABA neurons and depolarizing shifts in $GABA_A$ reversal potentials (Thomas et al., 2018). A separate study in adult mice showed that chronic nicotine treatment leads to an upregulation of α4* nAChRs specifically on VTA GABA neurons, which facilitated enhanced VTA GABA response to nicotine (Ngolab et al., 2015). Yet, as highlighted previously, the discrepancies between these findings and our results may reflect differences in recording techniques, namely *ex-vivo* electrophysiology versus fiber photometry. It is critical to reiterate that VTA GABA neurons receive extensive excitatory and inhibitory input from a variety of brain regions, including from the LH, DRN, BNST, and LHb, all of which can be altered by nicotine (Bubser et al., 2005; Chang et al., 2011; Hernández-López et al., 2016; Himmi et al., 1993; Jadzic et al., 2021; Pierucci et al., 2022; Reisiger et al., 2014; Zuo et al., 2016). We theorize that, though VTA GABA neurons may in fact be altered by chronic adolescent nicotine

exposure, an effect which can be captured *ex-vivo*, concurrent increases in excitatory input, or decreases in inhibitory input, may result in the appearance of an unchanged GABAergic response to NIC vapor when examined *in-vivo*. Future experiments, targeting the afferent structures of these VTA GABA neurons will be needed to further understand the effects of chronic adolescent nicotine on GABAergic function.

It is interesting to consider changes in DAergic or GABAergic activity in relation to the behavioral effects of adolescent nicotine exposure. It has been well documented that, compared to adults, adolescents experience increased reward and reinforcement to nicotine, but at the same time are less sensitive to nicotine's aversive properties (Adriani et al., 2002; Renda et al., 2016; Shram et al., 2006; Torres et al., 2008; Wilmouth & Spear, 2004). Moreover, like the DA response to nicotine, Reynolds et al., 2023 also report that the behavioral response to nicotine "freezes" following adolescent nicotine exposure, such that adult mice who were exposed to nicotine during adolescence are more sensitive to the rewarding, but less sensitive to the anxiogenic, properties of nicotine. Similar behavioral effects have observed been when chronic nicotine treatment begins in adulthood; adult rats chronically exposed to nicotine show CPP to a dose of nicotine that induces CPA when infused into the VTA of nicotine-naïve rats(H. Tan et al., 2009). We suspect that the increased VTA DA activity, coupled with the slight dampening of VTA GABA activity, may reflect a shift in the balance between mesolimbic excitation and inhibition which could subsequently alter the behavioral response to NIC vapor. We plan to further investigate the way that chronic nicotine exposure, beginning in adolescence, alters the rewarding or aversive behavioral response to NIC vapor exposure, and how relative changes in VTA DA or GABA activity may influence these behaviors.

4.6 Chronic co-exposure to both nicotine and morphine uniquely alters VTA GABA neurons

Having established the response of VTA DA and GABA neurons to both acute and chronic nicotine, we turned to examining the effects of co-exposure to nicotine and morphine. First, we report that chronic nicotine exposure beginning in adolescence results in significantly greater consumption of morphine in the C2BC paradigm during a 6-week co-exposure to both nicotine and morphine during adulthood. This finding is in line with recent studies showing that early adolescent nicotine exposure increases both morphine preference in a CPP paradigm and fentanyl self-administration (Cardenas et al., 2021; Kota et al., 2018; Vihavainen, Piltonen, et al., 2008). Interestingly, we found that, without prior adolescent nicotine exposure, nicotine and morphine co-exposed adult animals did not consume more morphine than controls (data not shown), suggesting that adolescence is a critical time for the development of dependencies to these drugs. This notion is further supported by work from Kota et al. (2018), who show a similar effect, reporting that nicotine pretreatment in adult mice does not alter the subsequent expression of morphine place-preference. In future experiments, we plan to test the behavioral response to acute injection of morphine both before and after the period of chronic co-exposure, to understand how co-treatment, separate from adolescent nicotine exposure, may further alter reward to morphine.

In our paradigm, it seems most probable that chronic adolescent nicotine treatment initiates changes to VTA DA activity, which are maintained, or perhaps enhanced, during the period of chronic co-exposure to both nicotine and morphine, effectively sustaining the shift in excitatory /inhibitory signaling within the mesolimbic circuitry, and ultimately facilitating an increased consumption of morphine. In support of this hypothesis, we showed that mice treated chronically with nicotine during adolescence continue to show enhanced VTA DA activity in response to NIC vapor during the 6-week co-exposure to both nicotine and morphine. Although the

enhanced response to NIC vapor was sustained during the co-exposure period, the addition of morphine did not further alter DAergic sensitivity to nicotine, compared to controls (nicotine + saccharin mice). This was surprising considering behavioral data indicating that morphine treatment can augment the rewarding properties of nicotine (Huston-Lyons et al., 1993) and electrophysiological data showing that chronic morphine treatment results in enhanced VTA DA neuronal firing (Mazei-Robison et al., 2011b). Considering our subsequent findings in VTA GABA neurons, we believe it is likely that co-exposure to nicotine and morphine did in fact induce alterations to VTA DA neurons, but that these changes were concealed during the period of chronic co-exposure, perhaps to due compensatory changes in cellular signaling pathways or altered input from afferent structures. Had we precipitated morphine withdrawal in these animals, effectively removing the chronic inhibitory stimulus from the mesolimbic circuit, it is possible that alterations to VTA DA activity may have been revealed. As such, in upcoming experiments, we are planning to record from VTA DA neurons in the morphine-withdrawal state, as well as in the morphine-sated state, following chronic co-exposure to nicotine and morphine.

During the period of co-exposure to nicotine and morphine, we found that the VTA GABA response to NIC vapor, like VTA DA response, is unchanged compared to nicotine + saccharin-treated mice. However, during naloxone-precipitated morphine-withdrawal, we found that VTA GABA neuron activity in response to NIC vapor significantly increases in co-exposed mice, compared to the response in the morphine-sated state and compared to nicotine + saccharin mice. Naloxone can precipitate a withdrawal syndrome in nicotine-dependent mice (Biała et al., 2005; Malin et al., 1993), but we did not observe any change in VTA GABA activity in nicotine + saccharin mice following naloxone injection, indicating that chronic nicotine treatment alone could not reproduce the GABAergic effect we observed in co-exposed mice. Additionally, we showed that precipitated morphine withdrawal produces a nonsignificant increase in VTA GABA

response to NIC vapor in vehicle + morphine mice, effectively demonstrating that chronic morphine treatment alone is not sufficient to enhance the VTA GABA response to NIC vapor. In effect, we reveal that the chronic co-exposure to both nicotine and morphine is necessary for the increase in VTA GABA activity in response to NIC vapor seen during precipitated morphine-withdrawal.

We hypothesize that the specificity of the VTA GABA response following co-exposure to nicotine and morphine reflects changes to both the endogenous opioid and cholinergic systems. Studies have shown that acute morphine decreases adenyl cyclase (AC) activity and the subsequent production of cAMP via activation of the MOR signaling cascade (Listos et al., 2019; Sharma et al., 1977). These effects disappear upon prolonged morphine exposure, with the cAMP signaling pathway returns to baseline (Watts et al., 2005.; Williams et al., 2001). Upon cessation of morphine exposure, the chronic inhibitory stimulus is effectively removed from the system, revealing an augmented cAMP signaling pathway, which facilitates cell hyperexcitability. Morphine withdrawal has been specifically associated with elevations in cAMP in VTA GABA cells and enhanced GABAergic tone at synapses with VTA DA neurons (Bonci & Williams, 1997; Madhavan et al., 2010). It follows then that hyperexcitability of VTA GABA neurons during morphine withdrawal may predispose them to fire in the presence of an excitatory stimulus such as NIC vapor.

In addition to hyperactivation of cAMP signaling, chronic morphine may also modify the cholinergic system, further contributing to the effect we observed in VTA GABA neurons. Chronic morphine has been linked to changes in cholinergic transmission in varied brain regions, including afferent and efferent structures of the VTA like the LDT, NAc and mPFc, and it may also alter nAChR expression in the midbrain (De Rover et al., 2005; Jiang et al., 2021; Mortazavi et al., 1999; Neugebauer et al., 2013; Rada et al., 1996). In this way, if morphine also

serves as a chronic inhibitory stimulus on the cholinergic system, withdrawal from morphine would reveal increased cholinergic tone, which, in turn, could further enhance the excitability of VTA GABA neurons.

Although morphine-induced alterations to the cAMP signaling pathway and cholinergic system likely play integral roles in the augmentation of VTA GABA activity, they cannot fully account for this GABAergic effect, as we demonstrate that chronic morphine alone is not sufficient to elicit this neuronal response. In light of this finding, we suggest that chronic nicotine is also altering the endogenous opioid system, in particular modifying signaling at the level of the MOR on VTA GABA neurons. Nicotine treatment promotes the release of endogenous opioids and increases the expression of MORs (Berrendero et al., 2010; Davenport et al., 1990.; Houdi et al., 1991.; Pierzchala et al., 1987.; Walters et al., 2005). We speculate that nicotine may enhance the morphine-induced inhibition of VTA GABA neurons via increased activation of upregulated MORs on GABAergic cells. Like the adaptive neuronal response to chronic morphine, nicotine-enhanced inhibition of VTA GABA neurons may be concealed by compensatory cellular or circuit-level mechanisms, but then unveiled by MOR receptor antagonism during precipitated morphine withdrawal. Hence, we suggest that chronic co-exposure to both nicotine and morphine results in morphine-dependent modulation of cholinergic signaling, as well as nicotine-dependent modifications to the endogenous opioid system, which ultimately culminates in increased VTA GABA response to NIC vapor during morphine-withdrawal.

4.7 Clinical Implications and Concluding Remarks

By demonstrating that chronic co-exposure to nicotine and opioids, but not to either drug alone, alters the GABAergic neurons of the mesolimbic circuitry, we highlight the critical need to study polysubstance use in preclinical models. Polysubstance use is the norm, and not the

exception in the human population, with drug-dependent individuals reporting an average use of 3.5 substances (Leri et al., 2004; Lorvick et al., 2018; Onyeka et al., 2012). In America, the high prevalence of OUDs, in conjunction with the rising popularity of e-cigarettes, may precipitate an even greater increase in the number of individuals with co-dependence to nicotine and opioids, as smoking may increase opioid use and, conversely, opioid use may reinforce smoking patters (Elkader et al., 2009; Richter et al., 2007; Spiga et al., 2005). Though studying individual drugs offers greater experimental control, it also risks overlooking the interactions between commonly used substances, like nicotine and opioids, which may have synergistic, or antagonistic, effects on behavior as well as on the functioning of key brain regions.

In this dissertation, I have established a physiologically relevant, co-drug administration paradigm, with chronic nicotine vapor exposure beginning in adolescence followed by a 6-week co-exposure to both nicotine vapor and morphine (C2BC) during adulthood. I have also effectively demonstrated the feasibility and utility of using calcium imaging to the study long-term neuroadaptations associated with chronic drug use. Using these techniques, we reveal the sensitivity of VTA DA neurons to chronic, adolescent nicotine exposure and position VTA GABA neurons as key mediators of nicotine and opioid co-dependence. Establishing paradigms to study the concurrent use of multiple drugs increases the translatability of preclinical research and may also improve the efficacy of treatment for individuals with multiple SUDs.

Within the clinical community, there has been a growing call to implement co-treatment as the standard of care for patients with concurrent SUDs (Morris & Garver-Apgar, 2020). Polysubstance use has repeatedly been linked to worse treatment outcomes, including higher rates or relapse and three-fold higher mortality rates, compared to use of only one substance, highlighting the lack of effective treatments for these individuals (de la Fuente et al., 2014; Staiger et al., 2012; Williamson et al., 2006). Co-treatment may be especially critical for treating

co-dependence to nicotine and opioids as clinical evidence suggests that treating both OUD and nicotine-dependence leads to a 25% greater likelihood of sustained abstinence from either substance (Prochaska et al., 2004). Clinical surveys also cite that many individuals being treated for OUD have a strong desire to quit smoking (Clarke et al., 2001; Vlad et al., 2020). Yet, despite the benefit of, and expressed desire for, co-treatment, less than half of OUD treatment centers offer tobacco cessation services (Knudsen et al., 2015).

It seems likely that this reflects both a lack of information on the clinical benefit of co-treatment, as well as a lack of targeted therapeutics designed to specifically help with cessation of concurrent nicotine and opioid use. Given their unique alteration by concurrent drug exposure, we point to VTA GABA neurons as one potential therapeutic target for the treatment of nicotine and opioid co-dependence. Future studies will be needed to explore the way that nicotine and/or morphine may differentially affect subpopulations of VTA neurons, and to understand the relationship between changes in neural activity and alterations to the rewarding or aversive properties of these drugs. Together, this work and future experiments will facilitate increased understanding of the mesolimbic mechanisms that mediate the co-use of nicotine and opioids, thereby filling a substantial gap in the preclinical literature and potentially offering new insights into therapeutic targets for polysubstance use disorders.

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