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Sex Differences in Mood and Anxiety-Related Outcomes in Response to Adolescent Nicotine Exposure

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Abstract

Nicotine dependence is causally linked to increased risk of mood/anxiety disorders in later life. Females are reported to experience a higher prevalence of anxiety/depressive disorders and challenges in smoking cessation therapies, suggesting a potential sex-specific response to nicotine exposure and mood/anxiety disorder risk. However, pre-clinical evidence of sex-specific responses to adolescent nicotine exposure is unclear. Thus, to determine any sex differences in anxiety/depressive-related outcomes, adolescent male and female Sprague Dawley rats received nicotine (0.4 mg/kg; 3x daily) or saline injections for 10 consecutive days, followed by behavioural testing, *in-vivo* electrophysiology and Western Blot analyses. Our results revealed that adolescent nicotine exposure caused long-lasting anxiety/depressive-like behaviours, disrupted neuronal activity patterns and molecular signaling pathway targets in nicotine-treated male rats, but no significant effects in female cohorts, suggesting possible compensatory actions related to estrogen/progesterone signaling pathways in female. These novel results serve as a foundation for future investigations examining how adolescent nicotine exposure may differentially impact the male vs. female brains.

Keywords

Nicotine, Addiction, Adolescence, Neurodevelopment, Anxiety Disorders, Depressive Disorders, Sex Differences, Dopamine, Glutamate, GABA, BDNF, Prefrontal Cortex, Ventral Tegmental Area, Nucleus Accumbens, Estrogen, Sensitivity

Summary for Lay Audience

Smoking-related diseases remain the highest cause of preventable mortality worldwide. In recent years, the increase in teen vaping trends is particularly concerning since adolescence represents a critical stage of brain development. The main psychoactive component in these products, nicotine, can affect the brain's addiction pathways by changing different neurotransmitter levels, such as glutamate, GABA and dopamine. Many animal and human studies have demonstrated nicotine addiction could cause anxiety and depressive disorders. In human studies, females have a higher prevalence of anxiety and depression symptoms. They also experience more difficulty in quitting nicotine products and rehabilitation with nicotine replacement therapies, which suggests a potential sex-specific sensitivity to nicotine. However, most animal studies only used male animals for experiments. Sex-specific responses to nicotine exposure are therefore currently unclear. Hence, the objective of this thesis is to determine any sex differences in mood and anxiety-related outcomes following nicotine exposure during adolescence. Adolescent male and female Sprague Dawley rats received either nicotine or saline injections, 3 times daily for 10 consecutive days. Once animals reached adulthood, they performed a series of mood and anxiety-related behavioural tests, such as the elevated plus maze, forced swim test and novel object recognition task. Next, either *in-vivo* electrophysiology or Western Blot was conducted to investigate neuron firing patterns and changes in protein level. Our results revealed that nicotine-treated male rats displayed anxiety and depressive-like behaviours and cognitive deficits. Alterations in glutamate and dopamine signaling as well as brain oscillation waves were observed in this same group. Furthermore, there were changes in expression levels of anxiety and depressive-related molecular targets. On the contrary, the nicotine-treated female rats showed no significant changes in behaviours or neuronal activity states, but displayed opposite trends to the male cohorts in some molecular markers. This indicates possible interactions between estrogen/progesterone and nicotine exposure in the female body. To conclude, these results are consistent with reports suggesting female is less sensitive to the pharmacological effects of nicotine. The current project could also serve as a starting point for future studies to investigate how the adolescent female brain is protected against anxiety and depressive disorders following prolonged nicotine exposure.

Co-Authorship Statement

Mohammed H. Sarikahya, PhD (c) – Demonstrated different experimental techniques and data analysis procedures including behavioural paradigms, *in-vivo* electrophysiology and Western Blot. Assisted with *in-vivo* electrophysiology recordings, brain slicing with cryostat machine, and protein extraction protocol for molecular analyses. Provided valuable feedback and knowledge on various experimental protocols and topics regarding mood and anxiety disorders.

Enzo Pérez-Valenzuela, PhD - Demonstrated different experimental techniques and data analysis procedures including behavioural paradigms, *in-vivo* electrophysiology, Western Blot, and vaginal sample collection for estrous cycle assessment. Provided valuable feedback and knowledge on various experimental protocols and topics regarding mood and anxiety disorders.

Taygun C. Uzuneser, PhD - Demonstrated different experimental techniques and data analysis procedures including behavioural paradigms, *in-vivo* electrophysiology and Western Blot. Provided valuable feedback and knowledge on various experimental protocols and topics regarding mood and anxiety disorders.

Hanna J. Szkudlarek, PhD - Demonstrated different experimental techniques and data analysis procedures including behavioural paradigms and *in-vivo* electrophysiology. Assisted with *in-vivo* electrophysiology recordings. Provided valuable feedback and knowledge on various experimental protocols and topics regarding mood and anxiety disorders.

Marta De Felice, PhD - Demonstrated different experimental techniques and data analysis procedures including behavioural paradigms, *in-vivo* electrophysiology, and histological analyses for electrode placements. Provided valuable feedback and knowledge on various experimental protocols and topics regarding mood and anxiety disorders.

Emma Proud, MSc (c) - Demonstrated Western Blot experiment and data analysis procedures. Provided valuable knowledge on the relationship between prenatal nicotine exposure and anxiety/depressive disorders.

Dana Gummerson, MSc (c) - Demonstrated Western Blot experiment and data analysis procedures.

Matthew J. Jones, PhD (c) - Demonstrated different experimental techniques and data analysis procedures including behavioural paradigms, *in-vivo* electrophysiology and Western Blot. Provided valuable feedback and knowledge on various experimental protocols.

Walter J. Rushlow, PhD - Provided valuable feedback and knowledge on Western Blot protocols and topics regarding mood and anxiety disorders. Assisted with troubleshooting for molecular analyses.

Steven R. Laviolette, PhD – Created and supervised the current project. Provided valuable feedback and knowledge on various experimental protocols and topics regarding mood and anxiety disorders.

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Table of Contents

List of Figures

List of Appendices

ANOVA	Analysis of variance
AP	Anterior-posterior
BDNF	Brain-derived neurotrophic factor
BLA	Basolateral amygdala
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
CNS	Central nervous system
CPP	Conditioned place preference
D1R	Dopamine 1 receptor
D2R	Dopamine 2 receptor
DA	Dopamine
DLPFC	Dorsolateral prefrontal cortex
DSM-5	Diagnostic and Statistical Manual of Mental Disorders
DV	Dorsal-ventral
E-cigarette	Electronic cigarette
E/I	Excitatory/inhibitory
EPM	Elevated plus maze
EPSCs	Excitatory postsynaptic currents
ERK	Extracellular signal-regulated protein kinase

fMRI	Functional magnetic resonance imaging
FST	Forced swim test
GABA	γ-aminobutyric acid
GAD	Glutamate decarboxylase
Hipp	Hippocampus
HPA	Hypothalamus-pituitary-adrenal
HRT	Hormone replacement therapy
ICD-11	The 11th edition of International Classification of Diseases
IL	Infralimbic
IPSCs	Inhibitory postsynaptic currents
ISI	Interspike interval
ITI	Inter-trial interval
LDB	Light-dark box
LFP	Local field potential
LH	Luteinizing hormone
LSD	Least significant difference
LTP	Long-term potentiation
MAPK	Ras-mitogen-activated protein kinase
MDD	Major depressive disorder
ML	Medial-lateral

mPFC	Medial prefrontal cortex
MSN	Medium spiny neuron
Na^+	Sodium ion
NAc	Nucleus accumbens
nAChR	Nicotinic acetylcholine receptor
NaCl	Sodium chloride
NIC	Nicotine
NMDAR	N-methyl-d-aspartic acid receptor
NOR	Novel object recognition
OFT	Open field test
PFC	Prefrontal cortex
PI3K-Akt	Phosphatidylinositide 3-kinase-protein kinase B
PL	Prelimbic
PLCγ	Phospholipase Cγ
PND	Post-natal day
RSFC	Resting-state functional connectivity
S.C.	Subcutaneous
SA	Spontaneous alternation
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis

SI	Social interaction
SPT	Sucrose preference test
TBS-T	Tris buffered saline with Tween 20
TONOR	Temporal order novel object recognition
TrkB	Tyrosine kinase receptor B
VEH	Vehicle
VGLUT	Vesicular glutamate transporters
VTA	Ventral tegmental area

Chapter 1

1 Introduction

The nature of tobacco dependence has changed drastically over the decades, both in terms of delivery methods and relative content of nicotine, the primary psychoactive compound underlying its addictive liability. Smoking-related diseases have been one of the top global causes for preventable deaths associated with various diseases, such as cancer, respiratory diseases and cardiovascular problems (West, 2017). Besides its detrimental outcomes in the periphery, nicotine also impacts the central nervous system (CNS) by altering different neurotransmitter dynamics, including dopamine (DA), γ-aminobutyric acid (GABA), glutamate, serotonin, and acetylcholine (Le Houezec, 2003; Laviolette, 2021) and their associated molecular signalling pathways. Clinical studies have found that nicotine dependence is closely associated with a variety of psychiatric disorders, such as schizophrenia, anxiety disorders and depression (Martínez-Ortegaa et al., 2017; Boden et al., 2010; Moran et al., 2013; Sagud et al., 2019). However, such correlational studies are limited in terms of their ability to reveal underlying *causal* mechanisms. Accordingly, the use of controlled, pre-clinical research methods that allow for precise temporal and drug exposure controls may allow us to better understand the pathophysiological effects of nicotine on the vulnerable developing brain and reveal the underlying neuropharmacological substrates that increase risk for neuropsychiatric disorders.

Adolescence is a developmental period where smoking initiation usually occurs (Chambers et al. 2003; Crews et al. 2007; National Center for Chronic Disease Prevention, 2012). Despite overall reductions in tobacco use in the general population due to increased public awareness of tobacco's harmful effects, there has been a rise in electronic cigarette (e-cigarette) product consumption by adolescents, which were originally marketed as a nicotine titration tool to cease tobacco smoking (Jones and Salzman, 2020; Yoong et al., 2018). This relative increase in teen vaping, termed the new smoking epidemic, is particularly alarming since the adolescent brain is still premature and vulnerable to extrinsic drugs of abuse. Decades of pre-clinical and human studies

have documented the acute and chronic effects of nicotine dependence on the mesocorticolimbic circuitry, which involves brain regions such as the prefrontal cortex (PFC), nucleus accumbens (NAc) and ventral tegmental area (VTA). Influences of nicotine on these neural executive and emotional regulation pathways as well as their underlying molecular signaling cascades, have been shown to be associated with various psychiatric disorders (Boden et al., 2010; Moran et al., 2013; Jobson et al., 2019; Hudson et al., 2021). However, the majority of previous pre-clinical research heavily utilized male animals and thus focused exclusively on the developing male brain. Thus, the neurophysiological responses by the female brain to nicotine exposure remain unclear. This represents a serious gap in our translational understanding of neurodevelopmental nicotine impacts. For example, in clinical populations, female patients often experience challenges in quitting nicotine products and a higher prevalence of mood and anxiety disorders (McLean et al., 2011; Torres & O'Dell, 2016; Cepeda-Benito et al., 2004). Based on these findings, the current project aimed to explore potential sex differences in mood and anxiety-related outcomes using a previously established rodent model of adolescent nicotine exposure with male and female Sprague-Dawley rat cohorts (Jobson et al., 2019), with the goal of identifying potential divergent mechanisms underlying the differential sensitivity of the male vs. female brains during adolescent nicotine exposure.

1.1 Smoking Prevalence and the Rise of E-Cigarette Vaping

Although the rate of tobacco dependence has been steadily decreasing in recent years, tobacco-related diseases remain one of the top global causes of preventable deaths (Yoong et al., 2018; National Center for Chronic Disease Prevention, 2014). In the United States, an estimated 30.8 million (~12.5% of the population) adults aged over 18 years have reported currently smoking cigarettes, and more than 16 million Americans are diagnosed with smoking-related diseases (National Center for Chronic Disease Prevention, 2014; Cornelius et al., 2022); in Canada, the prevalence of cigarette smoking is approximately 3.2 million people (~10% of the population), where a higher percentage of men (~12%) smoke cigarettes than women (~10%) (Health Canada, 2020). Notably, trends in nicotine consumption among adolescents continue to raise alarm. Smoking

initiation of lifelong smokers typically begins during the stage of adolescence, despite the widespread imposition of age-restrictions on the purchase of tobacco products (Edvardsson et al., 2009; Crews et al. 2007). Over the past decade, the development of e-cigarettes has gained considerable popularity among adolescents – in the United States, more than 2.5 million middle and high school students have used e-cigarettes in the past 30 days, where ~380,000 middle school students and ~2.14 million high school students have reported current use of vape products (Cooper et al., 2022); in Canada, ~5.2 million Canadians (aged 15 years and older) have reported using e-cigarettes, where ~291,000 youths (aged 15-19) and ~312,000 young adults (aged 20-24) have consumed vape products in the past 30 days (Health Canada, 2020).

E-cigarettes were initially developed and marketed as a novel nicotine delivery tool to assist in the reduction of nicotine dependence (National Center for Chronic Disease Prevention, 2016), since it could replicate most sensory cues from cigarette smoking and slowly titrate nicotine exposure level over time (Siu et al., 2015). In addition to the addictive properties of nicotine in vaping products, the wide variety of flavours present is also believed to be a major driver of popular use among adolescents, since it provides users an extended range of appetitive and sensory experiences along with nicotine consumption, relative to traditional cigarette formats (Hefner et al., 2017). The availability of flavours ranges from candy-floss, chocolate, vanilla, to mint, and they can mask the harsh taste of regular tobacco products (Sapru et al., 2020; Pepper et al., 2016; Ambrose et al., 2015). Compared to traditional cigarettes, nicotine concentrations in e-cigarettes can also be controlled manually, and certain carcinogens existed in tobacco could be avoided (Fernández et al., 2015; Goniewicz et al., 2014). Nevertheless, this relative rise in teen vaping trends, despite the overall drop in use of traditional smoking formats, has been termed the new smoking epidemic (Jones and Salzman, 2020; Yoong et al., 2018).

From a neuropsychiatric perspective, the increase in teen vaping is particularly concerning since adolescence represents a critical stage of neurodevelopment, where complex changes occur at the molecular, synaptic and network level. Considerable evidence has demonstrated that the adolescent brain is more susceptible to the

dependence-producing effects of tobacco, given that adolescents may develop dependence at substantially lower concentrations of nicotine and over shorter periods of time (Kandel and Chen, 2000; O'Loughlin et al., 2003).

1.2 Neuropharmacology of Nicotine and the Mesocorticolimbic System

Nicotine is the main alkaloid found in tobacco and is believed to be responsible for the addictive potential of these products (Le Houezec, 2003; Benowitz, 2009). Upon smoking, nicotine is carried into the lungs along with smoke particles and absorbed rapidly into the pulmonary venous circulation, where it then enters the arterial circulation and reaches the brain within 20 seconds of inhalation. Because of its lipophilic nature, nicotine can diffuse readily into brain tissues and bind to nicotinic acetylcholine receptors (nAChRs) widely expressed in the CNS **(Figure 1A)**. nAChRs are ligand-gated ion channels located on cell bodies, nerve terminals, and axons, where the binding of nicotine results in the opening of the channel and influx of cations, such as sodium (Na^+) and calcium (Ca^{2+}) ions, from extracellular space to the intracellular space of neurons **(Figure 1B, 1C)**. As a result of this action, membrane depolarization occurs and it leads to the release of neurotransmitters **(Figure 1C, 1D)** (Le Houezec, 2003; Benowitz, 2009). Past research has demonstrated that nicotine can modulate different neurotransmitter dynamics through presynaptic nAChRs, including acetylcholine, DA, GABA, glutamate, and serotonin (Le Houezec, 2003; Laviolette, 2021; Hudson et al., 2021; Jobson et al., 2019).

nAChRs are pentameric in nature and consist of either homomeric (α7-α10) or heteromeric (α2-α6, β2-β4) subunits. The wide range of subunit combinations contribute to a diverse receptor pharmacology in terms of agonist affinity, desensitization, and downstream signaling cascades (Gotti et al., 2006; Dani & Bertrand, 2007). Throughout the CNS, each nAChR subunit exhibits different expression patterns and functions, where the two most common subtypes are the α4β2 nAChR and the homomeric α7 nAChR (Yuan et al., 2015; Perry et al., 2002; Gotti et al., 2006; Dani & Bertrand, 2007). α4β2 nAChRs are high-affinity receptors that desensitize at low nicotine concentrations

(Fenster et al., 1999; Dani & Bertrand, 2007). On the other hand, homomeric α7 nAChRs have low affinity for nicotine and desensitize only at high nicotine concentrations (Dani & Bertrand, 2007).

In the CNS, nAChRs are widely distributed in brain regions involved in the addiction pathways, also known as the mesocorticolimbic system. These areas include the PFC, NAc, VTA, hippocampus (Hipp), basolateral amygdala (BLA), and contain interconnected circuitries providing multi-directional control across the regions **(Figure 2)** (Yang et al., 2018; Yuan et al., 2015; Koob & Volkow, 2016; Arnsten, 2009). Overall, the mesocorticolimbic system plays a critical role in many addictive behaviours, such as risk-taking behaviours, drug withdrawal symptoms, positive reinforcement and craving (Cheron & Kerchove d'Exaerde., 2021; Koob & Volkow, 2016). For instance, the PFC is responsible for decision making and executive functions, where it processes information from the NAc, BLA and Hipp, evaluates probable outcomes of each action, as well as regulate emotions (Alexander & Brown, 2011; Arnsten, 2009). Activities of GABAergic and glutamatergic neurons in the PFC are also thought to be associated with impulse control and risk-taking behaviours (Yuan et al., 2015; Benes, 2015). With regards to the NAc and VTA, DAergic neurons originate from the VTA and project to the NAc, forming the mesolimbic pathway. It has been well established that DA activity in this circuitry underlies the acute rewarding and aversive effects of different drugs of abuse and goal-directed behaviours (Adinoff, 2004). For example, in several pre-clinical studies using a drug self-administration protocol, the amount of self-administered cocaine was positively correlated with the amount of extracellular DA released in the NAc, whereas the administration of DA receptor antagonists attenuated self-administration of cocaine (Pettit & Justice, 1991; Bergman et al., 1990). However, previous research has also revealed that DA transmission in the mesolimbic pathway can mediate the acute aversive stimulus properties of both systemic and intra-VTA nicotine (Grieder et al., 2012; Laviolette and van der Kooy, 2003; Laviolette et al., 2008). Indeed, DA transmission in the mesolimbic pathway has been shown to only mediate the appetitive, rewarding effects of nicotine following chronic exposure to nicotine, which sensitizes VTA DA neurons to the acute effects of nicotine (Tan et al., 2009).

Given the broad expression profile and the role of nAChRs on neuronal activity, nicotine exposure can affect various neurotransmitter levels in the mesocorticolimbic system. Previous studies have shown that α4β2 nAChRs are involved in spiking activity of DAergic neurons in the VTA (Livingstone et al., 2009; Zhao-Shea et al., 2011), and α7 nAChRs could modulate DA release in the PFC and NAc, as well as glutamate signaling (Mameli-Engvall et al., 2006, Livingstone et al., 2009; Mansvelder et al., 2009). Besides addiction, the mesocorticolimbic system also plays a pivotal role in mood and anxiety-related behaviours. The relationship between nicotine dependence and mood/anxiety disorders will be discussed further in subsequent sections.

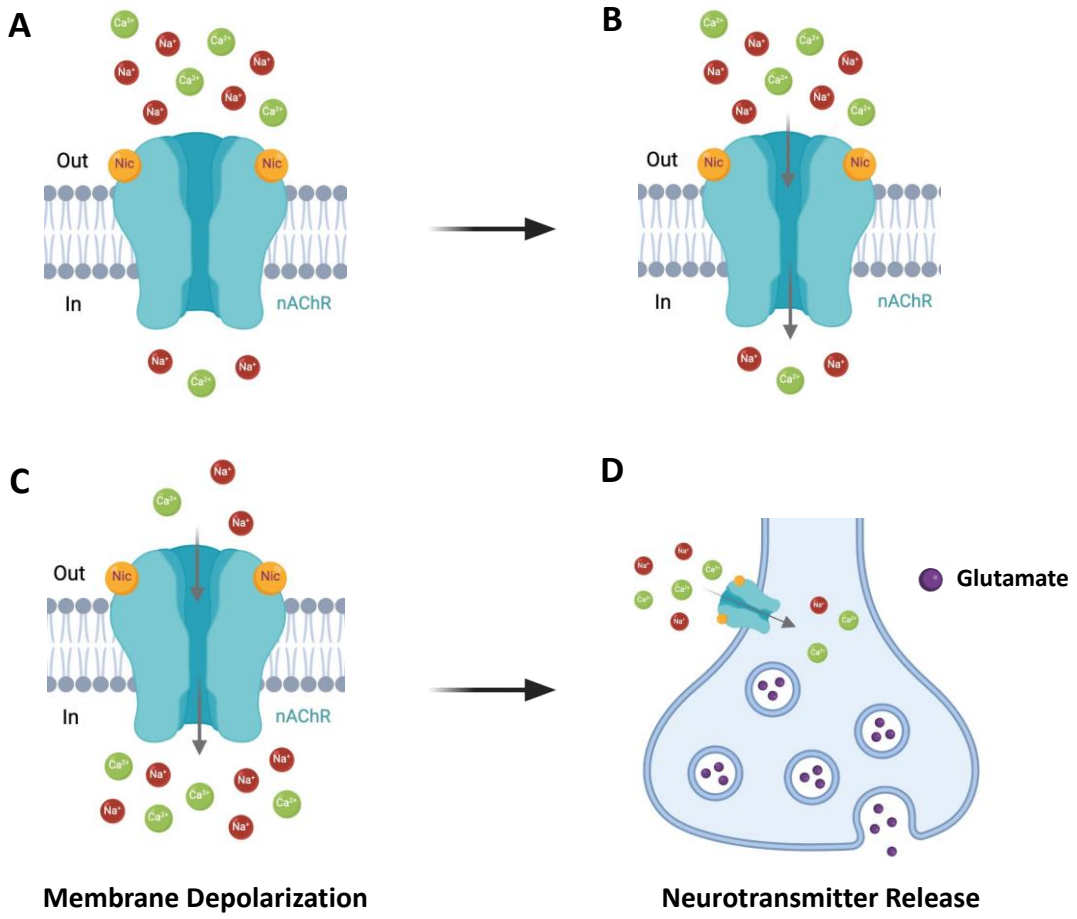

Figure 1. Neuropharmacodynamics of nicotine in the central nervous system. Green: calcium ion (Ca^{2+}); Red: sodium ion (Na^{+}); Yellow: nicotine; Purple: glutamate. **(A)** Nicotine binds to nAChRs and the ion channel is open. **(B, C)** Opening of ion channel allows the influx of Ca^{2+} and Na^{+} ions from extracellular space to the intracellular space of neurons. **(C, D)** Entry of Ca^{2+} and Na^{+} ions into neurons leads to membrane depolarization and release of different neurotransmitters (e.g., glutamate). Created with BioRender.com.

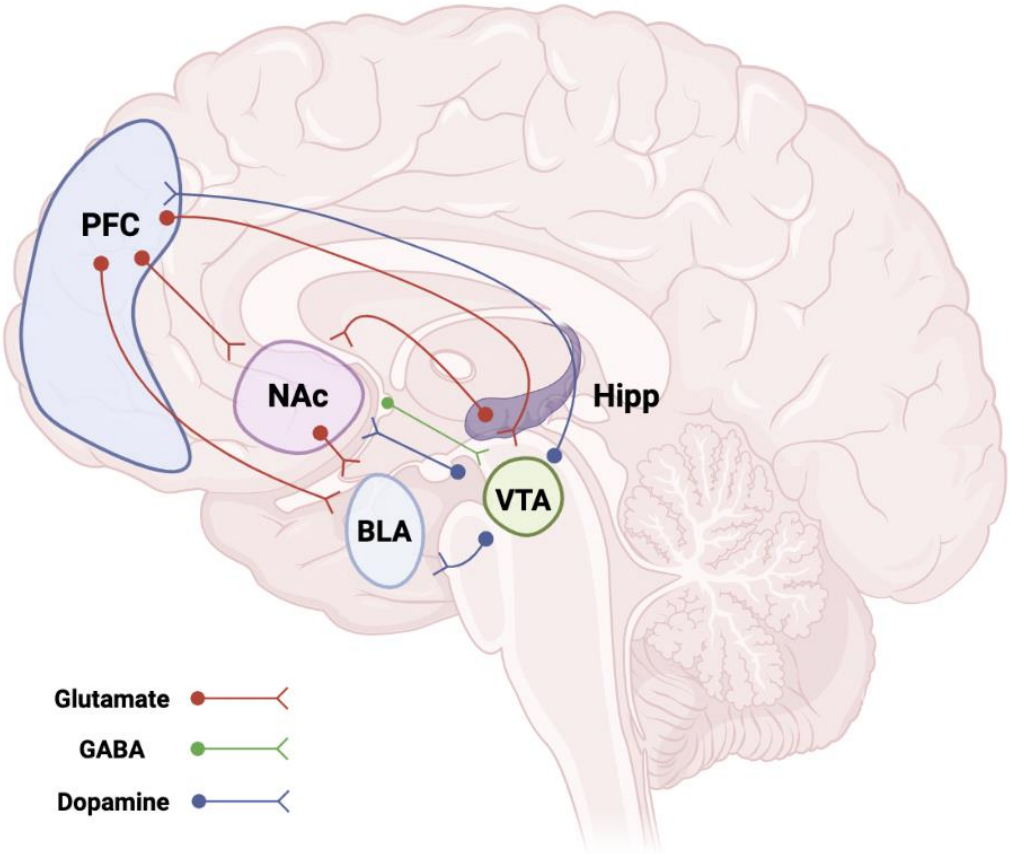

Figure 2. Neurocircuitries within the mesocorticolimbic system. Brain regions that are affected in addiction and mood/anxiety disorders, including the prefrontal cortex (PFC), nucleus accumbens (NAc), ventral tegmental area (VTA), basolateral amygdala (BLA), hippocampus (Hipp). Multi-directional connections exist between these areas to provide regulation over local activity. The mesocorticolimbic system is involved in impulse control, decision making, reward learning, motivation, cognitive function, memory formation, emotional regulation, and fear processing. Created with BioRender.com.

1.3 Vulnerability of the Adolescent Brain

Puberty refers to the process of sexual maturation (i.e., reactivation of hypothalamic–pituitary–gonadal axis and capability of reproduction); adolescence is a transition period from childhood to adulthood that generally spans from the age of 10-24 years, which is accompanied by increased risk-taking behaviours, peer associations, sensitivity to social cues, and maturing cognitive control (Arain et al., 2013; Spear, 2000; Spear, 2013). Adolescence represents a unique period of brain development where intricate neurocircuitries are still vulnerable to fluctuations of sex hormones and external environmental factors, and complex structural and functional reorganizations of the brain are occurring simultaneously (Yuan et al., 2015; Arain et al., 2013; Spear, 2000). First, white matter connectivity and network structures in the male and female brains are heavily influenced by hormonal changes in the body. For instance, studies have reported that testosterone levels are positively correlated with whole brain white matter volume in male but not females, and androgens are also capable of modulating axonal growth patterns (Perrin et al., 2008; Fargo et al., 2008). In addition, during the early pubertal phase, increased luteinizing hormone (LH) levels are associated with increased overall white matter volume and regional white matter density, specifically in prefrontal and temporal brain regions (Peper et al., 2008). It has also been suggested that estrogen and progesterone may enhance both cortical-cortical and subcortical-cortical (e.g., amygdala and PFC) functional connectivity, where testosterone may decrease subcortical-cortical but increase subcortical-subcortical functional connectivity (Peper et al., 2011; Arain et al., 2013), implicating the role of sex hormones on neurodevelopment during adolescence.

Synaptic pruning also occurs in this developmental period, which involves the refinement of dendritic branching and synaptic connections to ensure appropriate connectivity is established for adulthood, along with the loss of redundant synapses (i.e., those failed to make connections) (Spear, 2013; Brenhouse & Andersen, 2011; Arain et al., 2013). During adolescence, there is significant synaptic pruning in the human frontal cortex, where ~40% of synapses are lost between the age of 7-15 years. In particular, synaptophysin levels, a critical synaptic marker, in the dorsolateral PFC (DLPFC) rises

slowly between birth and 5 years of age, plateaus around the age of 10, and then falls back to adult levels by the age of 16 (Glantz et al., 2007).

Furthermore, in the adolescent brain, major changes in network connectivity are not homogenous temporally, but rather regional-based, with phylogenetically ancient brain regions (e.g., limbic system: amygdala, NAc, VTA) maturing earlier than frontal cortical regions (e.g., PFC), in a caudal to rostral fashion (Gogtay et al. 2004; Arain et al., 2013). It has been suggested that completion of frontal region development in late adolescence/early adulthood can result in a “top-down” control system over subcortical emotional/reward-focused systems (e.g., VTA, NAc, amygdala) (Casey et al., 2008). Hence, during adolescence, the differential maturation timepoints of subcortical areas and cortical executive/impulse control regions (e.g., PFC) are thought to underlie risk-taking behaviours in adolescents (Casey et al. 2011; Smith, 2013; Benes, 2015).

Within the frontal cortex, GABAergic neurons play a critical role in synchronizing cortical activity through feedforward and feedback mechanisms that regulate activities of pyramidal/glutamatergic neurons (Di Cristo et al., 2007; Constantinidis et al., 2002). By early adolescence, glutamatergic (excitatory) signaling is already established in the PFC, where GABA (inhibitory) neurotransmission is only premature at this stage (Arain et al., 2013; Li & Xu, 2008). As a result, this excitatory/inhibitory (E/I) imbalance in the PFC, are insufficient to provide tight regulation of frontal and sub-cortical cognitive/emotional regulation centers, potentially leading to the lack of impulse control and increased drug-seeking behaviours in teenagers (e.g., alcohol, tobacco, cannabis) (Chambers et al. 2003; Crews et al. 2007). Neuroimaging studies have also revealed that the PFC of adolescents is less activated than that of adults in decision-making tasks, and adolescents rely more on emotional processing/regulation areas when interpreting social cues (Arain et al., 2013). Taken together, ongoing development and structural reorganizations in the adolescent brain can result in impulsive and risky behaviours such as experiencing different drugs of abuse. Considering the impacts of nicotine on the CNS, nicotine exposure and subsequent chronic use by adolescents is very likely to disrupt such delicate and complex neurodevelopmental processes in the brain, and alter a variety of neurotransmitter

dynamics within the mesocorticolimbic system. This could lead to earlier onsets of cognitive deficits and mood/anxiety disorders in adulthood, given the role of the mesocorticolimbic system in higher-order cognitive function and emotional regulation.

1.4 Diagnosis of Anxiety and Depressive Disorders

Fear refers to the conscious feelings elicited by threat or imminent danger, whereas anxiety is the anticipation of real or perceived future threats or dangers (Crasket et al., 2011; Penninx et al., 2021). Both fear and anxiety are adaptive in nature and highly conserved across species, since they facilitate avoidance of danger and survival of the organism (Beesdo et al., 2009; Penninx et al., 2021; Steimer, 2002; Adolphs, 2013). However, it requires clinical attention when fear and anxiety become excessive and persistent, which interferes with normal functioning (e.g., social interactions, workplace productivity) (Beesdo et al., 2009; Penninx et al., 2021; DeMartini et al., 2019). Anxiety disorders can be classified into many types of disorders including specific phobias, panic disorder, social anxiety disorder, generalized anxiety disorder and other sub-variants. The diagnosis of anxiety disorders is based on two main classification schemes: the fifth edition of Diagnostic and Statistical Manual of Mental Disorders (DSM-5) and the 11th edition of International Classification of Diseases (ICD-11). For example, common symptoms of generalized anxiety disorders include uncontrollable worry and fear of daily activities, fatigue, sleeplessness, difficulty concentrating; whereas patients with social anxiety disorder exhibit excessive and unreasonable fear or anxiety of negative judgements by others, avoidance of social interactions and situations (Penninx et al., 2021). Distinctions between anxiety disorders requires clinical judgement of duration, severity, and degree of distress and impairment. Screening questionnaires such as the Hamilton Anxiety Scale and the Beck Anxiety Inventory are commonly used in diagnostic process as well. Risk factors for anxiety disorders include interactions between genetics and environmental factors, as well as epigenetic modulations. Anxiety disorders are also highly co-morbid with other psychiatric disorders, such as major depressive disorder, bipolar disorder, and obsessive-compulsive disorder. (Penninx et al., 2021; Thibaut, 2017). Globally, anxiety disorders account for 3.3% of global burden of disease and impose heavy economic burden on societies along with depression (~USD $1 trillion

per year) (Penninx et al., 2021; The Lancet Global Health, 2020). The life-time prevalence of anxiety disorders in the United States is approximately 34% for adults, whereas in females this prevalence is ~1-2.5 times greater (Szuhany & Simon, 2022; Penninx et al., 2021). Brain regions involved in fear and anxiety processing include the amygdala, hippocampus, stria terminalis and its bed nucleus, medial PFC (mPFC) and insula, where these circuitry components interact together and generate adaptive defensive responses depending on the nature of threats (Penninx et al., 2021).

Anxiety disorders are highly co-morbid with mood disorders, which involve emotional disturbances manifested as excessive and sustained feelings of depression, mania, or both (Penninx et al., 2021; Thibaut, 2017; Datta et al., 2021). Mood disorders are generally categorized into depressive disorders and bipolar disorders. Depressive disorders can be further classified into major depressive disorder (MDD), persistent depressive disorder, disruptive mood dysregulation disorder, and premenstrual dysphoric disorder; whereas bipolar disorders can be classified into bipolar I disorder, bipolar II disorder, cyclothymia, and substance-induced bipolar disorder (Datta et al., 2021). Depression is characterized by persistent sadness, anhedonia (i.e., loss of interest/pleasure), low energy, trouble with concentrating, sleep disturbance and worse appetite, which would disrupt daily activities and psychosocial functions (Li et al., 2021; Datta et al., 2021). Bipolar disorders involve biphasic mood episodes alternating between depression and mania/hypomania (Datta et al., 2021). Mania refers to a distinct period of persistent euphoria or irritable mood with hyperactivity or excessive energy (Jain & Mitra, 2023).

Similar to anxiety disorders, depressive disorders and bipolar disorders are diagnosed based on DSM-5 and ICD-11, requiring clinical judgement of duration, severity, and degree of distress and impairment (Malhi & Mann, 2018; Datta et al., 2021). Assessment questionnaires are often utilized as screening tool during the process of diagnosis as well, which for instance include the nine-item Patient Health Questionnaires, and the Beck Depression Inventory (García-Batista et al., 2018; Maurer et al., 2018). For the purpose of this study, anxiety and depressive disorders will be the main focus due to their high prevalence and association with substance abuse globally. Depressive disorders

are affecting approximately 280 million people globally (World Health Organization, 2023), and importantly, females are twice as likely to be affected vs. males (Malhi & Mann, 2018). Decades of research have discovered a wide range of factors that could contribute to the pathogenesis of depressive disorders (Li et al., 2021; Malhi & Mann, 2018), such as the monoamine hypothesis, aberrations in the hypothalamus-pituitary-adrenal (HPA) axis, neuroinflammation, disrupted synaptic plasticity and neurogenesis, structural and functional changes in brain regions, and interactions between genes and environment. Considering nicotine addiction, anxiety disorders, and depressive disorders, there are overlapping symptomologies across the three disorders, given the role of the mesocorticolimbic system in reward learning, motivation/goal-directed behaviours, cognitive function, emotion regulation and fear processing. Indeed, the PFC, NAc, VTA, Hipp and BLA are often recognized as primary neural regions of interest in these disorders.

1.5 Clinical Findings of Nicotine Dependence and Anxiety/Depressive Disorders

Given the influence of nicotine on neuronal activity states, and the role of the mesocorticolimbic system in addiction and mood/anxiety disorders, it is not surprising that nicotine dependence is associated with increased risk of mood and anxiety disorders (Laviolette, 2021; Moylan et al., 2013). Researchers have always questioned the direction of causality between smoking and these psychiatric disorders. For example, individuals with social anxiety have reported increased tobacco use, suggesting that tobacco products may serve as a medium to relieve anxiety in social situations (Sonntag et al., 2000). Alternatively, nicotine addiction could result in anxiety and depressive disorders by disrupting different neurotransmitter systems and underlying molecular pathways associated with these disorders (Moylan et al., 2013; Fluharty et al., 2017). Overall, both relationships appear not to be mutually exclusive and could co-exist in a positive feedback loop manner, indicating the complex interactions between these variables along with other confounding factors. For the purpose of this study, we will focus on the latter, wherein neurodevelepmental nicotine exposure could trigger pathophysiological

processes in the mesocorticolimbic system, setting the stage for the development of mood and anxiety disorders in later life.

Many clinical studies have demonstrated strong associations between nicotine addiction and the presence of mood and anxiety disorders. From prospective cohort studies, smoking behaviours have been shown to unidirectionally increase anxiety and mood disorder symptoms, as well as the likelihood and onset of panic attacks, even when controlling for socioeconomic factors and other confounding variables (Isensee et al., 2003; Brown et al., 1996; Cuijpers et al., 2007). Furthermore, causal relationships between smoking behaviours and depressive disorders have also been reported. For example, using a fixed-effects regression model and structural equation modelling methods, Boden et al. (2010) examined the causal relationship between smoking and depression at the age of 18, 21 and 25 years in a birth cohort. The fixed-effects regression model revealed persistent significant associations between nicotine dependence and depressive symptoms at each age point, even after adjustments for time-dynamic confounding factors such as stressful life events and other illicit drug uses. A series of structural equation models were then used to examine both unidirectional and reciprocal effects between smoking and depression at these time points, where the best-fitting causal model was indicative of nicotine addiction *causally* leading to increased risks of depression vs. a reverse causality effect in which the presence of depression led to increased smoking behaviours. Another similar study investigated the association between regular cigarette smoking and the subsequent new onset of mood and anxiety disorders (Mojtabai and Crum, 2013). This comprehensive review utilized logistic regression analysis to examine the relationship between smoking behaviours preceding the onset of MDD, persistent depressive disorders, manic episodes, generalized anxiety disorder, and panic disorders. Regular smokers displayed a higher likelihood of new onset mood and anxiety disorders, where this association was more significant in participants aged 18 to 49 years but not older adults, indicating age could be another factor in this relationship. Altogether, there is strong evidence suggesting nicotine dependence could result in the onset of mood and anxiety disorders. However, the following critical question would be – how does nicotine addiction affect the

mesocorticolimbic system and how might these impacts set up the brain for increased risk for mood/anxiety disorders in later life?

To answer this question, several human imaging studies have examined activation dynamics in the mesocorticolimbic system under cognitive tasks. For instance, Sweitzer et al. (2016) studied whether changes in striatal processing of rewards would predict relapse likelihood during smoking cessation period. Disordered reward processing within the mesolimbic dopamine circuitry has been implicated in addictive disorders including nicotine dependence. The incentive sensitization theory posits that repeated drug exposure (e.g., nicotine) would result in a sensitized mesolimbic system to drug-related cues, thus conferring motivational incentive properties to these stimuli and leading to drug-seeking behaviours (Robinson & Berridge, 1993). On the other hand, the opponent process theory postulates that chronic drug exposure would alter reward processing of the striatum, where non-drug rewards lose their incentive value and fail to motivate behaviours (Koob & Le Moal, 2005). In this study, participants attended two functional magnetic resonance imaging (fMRI) sessions before and after abstinence, where they completed a reward guessing task to earn smoking and monetary rewards. Next, they attempted smoking cessation by a 3-week contingency management plan to examine relapse behaviours. Statistical analyses revealed increased striatal activation in anticipation of smoking reward, but decreased activation for monetary reward. Furthermore, individuals with greater decrements in striatal activation to monetary reward were more likely to relapse during smoking cessation period. Another similar fMRI study has shown heightened activation pattern in the PFC and NAc in smokers when presented with smoking-related cues, but not with neutral cue (David et al., 2005). These findings provide significant clinical relevance since they might serve as possible explanations behind decreased motivation or goal-oriented behaviours in smokers with depressive disorders. Moreover, another study has investigated the functional role of frontal cortical regions in young smokers using the Stroop colour-word test (Yuan et al., 2016). The Stroop colour-word assesses cognitive control, which involves frontal cortical regions (Matsumoto & Tanaka, 2004). In this task, participants were presented with different colour-words (e.g., red, green, blue) and they were required to identify the ink colour of the word (e.g., the word “red” printed in green ink; the answer is green).

Resting-state functional connectivity (RSFC) was also conducted to evaluate interactions between frontostriatal circuitries. Young smokers exhibited reduced RSFC between the striatum, the PFC and limbic regions. Increased performance errors were also observed in young smokers, which were positively correlated with the number of cigarette packs smoked per year. Altogether, considerable evidence is alluding to the negative influence of nicotine dependence on the mesocorticolimbic system, by disrupting normal reward processing function and impairing higher-order cognitive abilities in regular smokers.

1.6 Pre-clinical Studies of Adolescent Nicotine Exposure

As discussed previously, it has been demonstrated that nicotine addiction can negatively affect the mesocorticolimbic system and result in mood and anxiety disorders. However, clinical studies present challenges in determining the underlying neural mechanisms and pathophysiological effects of nicotine dependence on the brain, due to ethical concerns and various confounding variables in human population. Hence, pre-clinical studies have been conducted to experimentally model nicotine exposure in humans, in attempts to examine relevant neurobiological changes that may place specific individuals and populations at risk. Although there are obvious differences in complexity between rodent and human brains, similarities in adolescent brain maturation exist across these two species with regards to synaptogenesis events, anatomical, neurochemical, and hormonal changes (Agoglia et al., 2017; Semple et al., 2013). Depending on sex and species, adolescence and sexual maturation could begin as early as post-natal day (PND) 21 in rats and last until PND 60 (Yuan et al., 2015; Brenhouse & Andersen, 2011; McCutcheon & Marinelli, 2009). Compared to human clinical studies, rodent modelling of adolescent brain development offers precise experimental manipulation of drug exposure temporally and concentration-wise, and controls for environmental differences, which are difficult to control for in epidemiology studies. Taken together, to gain a better understanding of the long-term effects of chronic nicotine exposure on increased vulnerability to mood and anxiety disorders, clinical and pre-clinical studies are both necessary and essential to draw mechanistic inferences about these observed associations in human populations.

Provided the immaturity of the mesocorticolimbic system during adolescence and the effects of nicotine on neuronal activity, a plethora of pre-clinical animal studies have investigated how chronic adolescent nicotine exposure could lead to anxiety and depressive-like behaviours by impeding different neurotransmissions in relevant brain regions. For example, Jobson et al. (2019) and Hudson et al. (2021) examined the neurobiological consequences of chronic adolescent nicotine exposure with anxiety and depressive-related behavioural paradigms, *in-vivo* electrophysiology and tissue molecular analysis via Western Blot. In this protocol, male, adolescent Sprague Dawley rats received either subcutaneous (S.C.) nicotine or saline injections 3 times daily, from PND 35 to PND 44 (i.e., 10 consecutive days); whereas male adult rats received S.C. nicotine or saline injections from PND 65 to PND 74 and served as the control group. After that, animals stayed in their home cages for a 30-day drug-free washout period until behavioural testing was carried out, to examine whether chronic adolescent nicotine exposure alone would be sufficient in inducing anxiety and depressive-like phenotypes. Results from both studies revealed only rats treated with nicotine during adolescence displayed anxiety and depressive-like behaviours. In the open field test (OFT) and light-dark box (LDB) test, adolescent-nicotine-treated rats spent less time in the central zone and the light side of the apparatus respectively, indicative of anxiety-like behaviours since normal, healthy rodents have a tendency to explore open spaces when they are less anxious (Heinz et al., 2021). In the sucrose preference test (SPT) and forced swim test (FST), the same group of animals exhibited reduced sucrose preference index and increased immobility time respectively. Furthermore, decreased sociability scores were observed in these animals as well. These behaviours resemble anhedonia and lower motivation states in depressive disorders, which are VTA- and NAc-dependent given the role of the VTA→NAc pathway in reward learning (Berhow et al. 1995; Supekar et al., 2018; Papakostas, 2020; Miyanishi & Nitta, 2021). Jobson et al. (2019) also reported that these animals experienced heightened sensitivity to non-salient fear stimuli, which could implicate abnormality in fear processing pathways common in individuals with anxiety and specific phobia (Shin & Liberzon, 2010). Beyond behavioural phenotypes, dysregulation in neuronal firing patterns was observed in cortical and motivation/emotional processing regions. In the mPFC and VTA respectively, pyramidal

and DA neurons both displayed hyperactivity in spontaneous firing frequency and burst activity (Jobson et al., 2019), whereas medium spiny neurons (MSNs) in the NAc showed increased tonic firing activity but decreased bursting rate (Hudson et al., 2021). These neurotransmission disturbances are consistent with other pre-clinical studies of anxiety and depressive-related phenotypes, where dysregulated E/I balance in the PFC and aberrant DA and GABA signaling in the VTA and NAc were observed (Zhu et al., 2017; Francis et al., 2015; Bi et al., 2013; Yorgason et al., 2013).

In addition to aberrations in behavioural and electrophysiological outcomes, disruptions in molecular targets were reported in the mPFC and NAc as well, where there was a decrease in dopamine 1 receptor (D1R) but no change in dopamine 2 receptor (D2R) expression level in both regions (Jobson et al., 2019; Hudson et al., 2021). D1R and D2R have been suggested to be involved in cognitive function and modulation of E/I balance in the PFC (Li et al., 2011; Robinson & Sohal, 2017; Abi-Dargham et al., 2002; Floresco & Magyar, 2006; Seamans & Yang, 2004; Xing et al., 2022), as well as reward and aversive learning in the NAc (Hikida et al., 2010; Kravitz et al., 2012; Soares-Cunha et al., 2020). Thus, alterations in either D1R or D2R level may indicate disturbances in downstream signaling cascades or pathological changes in behavioural-related circuitries in the mesocorticolimbic system. Finally, in another study, adolescent-nicotine-treated male Wistar rats displayed impaired attentional performance and increments in impulsive actions, which are both PFC-dependent cognitive abilities (Counotte et al., 2009). This deficit in cognitive performance was also associated with excessive DA release in the mPFC. Overall, it appears that chronic adolescent nicotine exposure could disrupt different neurotransmitter dynamics in the mesocorticolimbic system, and ultimately lead to anxiety/depressive-like behaviours as well as deficits in cognitive control.

1.7 Molecular Targets Relevant in Anxiety and Depressive Disorders

Apart from behavioural and neuronal aberrations, disturbances in molecular targets and signaling pathways have also been reported in anxiety and depressive disorders. Mentioned previously, two common nAChR subtypes in the brain are the α4β2 nAChRs and the homomeric α7 nAChRs (Yuan et al., 2015; Perry et al., 2002; Gotti et

al., 2006; Dani & Bertrand, 2007). The cholinergic system and nAChRs in the mPFC play a crucial role in attention and learning (Dalley et al., 2001; Hahn et al., 2003). In anxiety and depressive disorders, patients often experience difficulty in maintaining concentration and impairments in executive function, working memory and psychomotor processing speed (Lam et al., 2014; McIntyre et al., 2013; Murrough et al., 2011). In pre-clinical models, one previous study has reported that α7 nAChRs knockout mice displayed worse performances in the delayed matching-to-place task of the Morris water maze (Fernandes et al., 2006), and stimulation of these receptors enhanced persistent neuronal firing of N-methyl-d-aspartic acid receptor (NMDAR)-mediated/working memory-related circuits in the DLPFC (Yang et al., 2013). For the β2 subunit, Guillem et al. (2011) demonstrated that β2-knockout mice exhibited impaired attentional performance, and re-expression of β2-nAChRs in the prelimbic (PL) region of the mPFC was sufficient to reverse the attentional deficits in these mice. Hence, expression levels of the α7 and β2 subunits of nAChRs could serve as potential biomarkers to draw associations with cognitive performance in pre-clinical rodent models.

Besides cholinergic targets in the PFC, dysregulation of the E/I balance in the PFC has also been implicated in pre-clinical and clinical studies of anxiety and depression (Bi et al., 2013; Veeraiah et al., 2014; Czéh et al., 2018; Ghosal et al., 2017; Lener et al., 2017). For instance, infusion of a GABA-A receptor antagonist into the infralimbic (IL) region of the mPFC resulted in anxiety-like behaviours in mice in elevated plus maze (EPM) and OFT (Bi et al., 2013). This behavioural change was associated with smaller inhibitory postsynaptic currents (IPSCs) and larger excitatory postsynaptic currents (EPSCs) in pyramidal neurons in the IL cortex, suggesting the role of mPFC imbalanced E/I level in anxiety-related behaviours. For MDD patients, proton magnetic resonance spectroscopy revealed lower GABA level in the DLPFC (Yildiz-Yesiloglu & Ankerst, 2006), a cortical region functionally homologous to the mPFC in rodent (Uylings et al., 2003). Hyperactive glutamate signaling and reduced GABA level were also reported in pre-clinical rodent models of depression and depressed patients (Veeraiah et al., 2014; Czéh et al., 2018; Arnone et al., 2015; Jobson et al., 2019). Altogether, these findings are pointing to the involvement of PFC E/I balance in anxiety

and depressive disorders. Thus, glutamate and GABA-related targets such as vesicular glutamate transporters (VGLUT; transporting glutamate to synaptic vesicles) and glutamate decarboxylase (GAD; involved in GABA synthesis; Walls et al., 2011) could be used as indirect markers to estimate glutamate and GABA signaling activity (i.e., E/I balance) in the PFC in pre-clinical studies of anxiety and depressive-related outcomes.

The VTA→NAc DA pathway plays a pivotal role in motivation and reward learning behaviours, as mentioned previously. Therefore, expression levels of D1R and D2R in the NAc could act as indirect markers for dopamine signaling in pre-clinical models as well. It also appears that D1R and D2R are involved in distinctive roles in the NAc. For example, Francis et al. (2015) reported that optogenetic activation of D1R-MSNs in the NAc promoted social interaction and sucrose preference, whereas activating D2R-MSNs resulted in social avoidance in social defeat stress rodent model. Another study also demonstrated optogenetic stimulation of D1R-MSNs in the NAc enhanced cocaine preference in a conditioned place preference (CPP) test, which is typically used to study rewarding and aversive effects of different drugs; whereas activation of D2R-MSNs in the NAc suppressed cocaine preference (Lobo et al., 2010). Furthermore, GABA signaling in the NAc has been shown to modulate DA release from the VTA (Yang et al., 2018), suggesting GAD could be an indirect marker of local inhibition in the VTA→NAc circuitry. On the other hand, D1R and D2R in the PFC seem to play a role in cognitive functions, where blockade of D1R and D2Rs in the mPFC impaired performances in attention set-shifting tasks and working memory task (Ragozzino, 2002; Floresco et al., 2006; Sawaguchi and Goldman-Rakic, 1994). Hence, D1R and D2R expression level in the PFC could be another indirect proxy for cognitive functions in pre-clinical studies.

Furthermore, many pre-clinical and clinical studies have been investigating the role of brain-derived neurotrophic factor (BDNF) in depressive disorders. BDNF is an upstream signaling molecule that binds to tyrosine kinase receptor B (TrkB), and it has been shown to play a role in synaptic plasticity and structural integrity of synapses in the CNS (Minichiello et al., 2002; Panja et al., 2014; Bosch et al., 2014; Ji et al., 2010). Previous studies have demonstrated BDNF-TrkB signaling could mediate long-term

potentiation (LTP) in the Hipp by recruiting phospholipase Cγ (PLCγ) (Minichiello et al., 2002; Panja et al., 2014). BDNF-TrkB signaling could also maintain synaptic integrity through local interactions with cytoskeletal-related proteins, and facilitate neurite elongation, neurite branching, spine head enlargement and spine neck elongation (Bosch et al., 2014; Ji et al., 2010). In depressive disorders, reduced brain region volume has been observed in the mesocorticolimbic system, and this decrease in volume is thought to be associated with the loss of synaptic integrity (Yu & Chen, 2011; Belleau et al., 2019; McKinnon et al., 2009; Videbech & Ravnkilde, 2004; Wise et al., 2017; Treadway et al., 2015). Provided the role of BDNF in synaptic plasticity and integrity, it has been proposed that alterations in BDNF levels could be associated with disrupted synaptic integrity in depressive disorders. Human post-mortem studies have also reported lower BDNF levels in the PFC of suicide patients (Karege et al., 2005). Mice with loss of BDNF expression in forebrain regions also displayed depressive-like behaviours (Monteggia et al., 2007; Lindholm and Castrén, 2014). Interestingly, local BDNF infusion in the VTA induces depressive-like behaviours in the FST, where inhibition of BDNF in the NAc produces antidepressant effects in the social defeat paradigm (Berton et al., 2006; Eisch et al., 2003). Taken together, there appears to be region-specific effects of BDNF in the mesocorticolimbic system parallel to depressive-related outcomes. Thus, BDNF level could serve as an indirect marker for synaptic integrity which could be associated with behavioural phenotypes relevant to depressive disorders in animal models.

1.8 Literature Gap in Sex Differences in Nicotine Addiction

As discussed previously, the existing literature has demonstrated how repeated nicotine exposure during adolescence can induce pathological states in the mesocorticolimbic system and increase the vulnerability of developing mood and anxiety disorders. Nevertheless, despite these findings, there remains a substantial knowledge gap in how males vs. females may respond differently to nicotine exposure. The majority of pre-clinical studies to-date have only utilized male animals as experimental subjects, thus, findings from these studies cannot be generalized to female. Furthermore, limited studies have focused on the sex-specific responses to nicotine exposure in terms of mood

and anxiety-related outcomes; and even in available studies, reported results are currently mixed and show no clear signs of direction (Caldarone et al., 2008; Cheeta et al., 2001; Elliott et al., 2004; Faraday et al., 2001; Torres et al., 2013). For instance, Caldarone et al., (2008) reported anxiogenic effects of chronic nicotine exposure in female C57BL/6J mice, where they spent less time exploring the open arm in EPM. On the contrary, Faraday et al. (2001) demonstrated that female Sprague Dawley rats with chronic nicotine exposure displayed fewer anxiety-related behaviours compared to male rats, where they spent more time in the central zone of the OFT apparatus. However, importantly, most studies differ in nicotine administration protocols and experimental animal species/strains. These discrepancies in results could also be due to potential interactions between the estrus cycle and various neurotransmission systems in the brain. Taken together, more consistent nicotine administration protocols and experimental paradigms need to be developed to properly investigate how females vs. males may respond to chronic nicotine exposure in terms of anxiety and depressive-like phenotypes, as well as the potential interactions between estrogen/progesterone, nicotinic receptors in the CNS, and the mesocorticolimbic system.

In clinical populations, females are shown to have higher prevalence of mood and anxiety disorders (McLean et al., 2011; Ford & Erlinger, 2004). They also experience more difficulty in quitting nicotine products as well as lower efficacy in nicotine replacement therapies (Torres & O'Dell, 2016; Perkins, 2001; Perkins & Scott, 2008; Bohadana et al., 2003; Cepeda-Benito et al., 2004; Costello et al., 2011). Moreover, studies have suggested females are less sensitive to the pharmacological effects of nicotine, and they are more reinforced by sensory cues strongly associated with smoking in the absence of nicotine (e.g., greater craving relief from a denicotinized tobacco inhaler) (Perkins, 1999; Perkins et al., 1999; McBride et al., 2006; Barrett, 2010). Furthermore, there is evidence suggesting sex differences in motivation to smoke, where males smoke primarily for the rewarding effects of nicotine (i.e., dependent on mesocorticolimbic system), and females are more likely to smoke to relieve stress, anxiety, and negative withdrawal symptoms (Perkins et al., 1999; Perkins et al., 2012; Crisp et al., 1999). It has been reported that nicotine provides calming effects in young women as well (File et al., 2001). Female smokers also exhibited a higher response in the

dorsal striatum than in the ventral striatum to nicotine stimulation compared to male smokers, which could potentially underlie the greater resistance to nicotine cessation interventions in females, given the preferential role of dorsal striatum in habit formation vs the ventral striatum, which is more critical for reinforcement learning (Cosgrove et al., 2014). This could mean that smoking could become a habit sooner in females compared to males. Sex-specific differences in high-affinity β2 nAChR availability were also displayed in the striatum, where there was higher availability in male smokers compared to non-smokers, but no significant differences in the striatum between female smokers vs non-smokers (Cosgrove et al., 2012). These distinctive phenotypes are suggestive of fundamental differences in the neuropharmacology of nicotine between males and females, as well as the contrasting perceptions to smoking behaviours, and disparate outcomes in response to smoking-cessation therapies.

1.9 Research Objectives and General Hypothesis

From the extant literature, there is still a relatively limited knowledge on how females vs. males responds to chronic nicotine exposure, especially during adolescence, specifically in the context of mood and anxiety-related outcome measures. To answer these questions, the current project serves as a starting point that aims to characterize sex differences in anxiety and depressive-related outcomes in response to chronic adolescent nicotine exposure, in terms of behavioural, neuronal and molecular measures using a previously established pre-clinical rodent model (Jobson et al., 2018). This thesis has three primary objectives and associated hypotheses building upon existing clinical and pre-clinical findings:

Objective 1: Characterize the long-term effects of chronic adolescent nicotine exposure on anxiety and depressive-like behaviours as well as cognitive function in male vs. female Sprague Dawley rat cohorts.

- **Hypothesis:** Nicotine-treated male and female rats will display differential anxiety and depressive-like behaviours as well as cognitive deficits compared to vehicle male and female rats respectively. These behavioural phenotypes will be divergent across male vs. female cohorts.

Objective 2: Examine the long-term effects of chronic adolescent nicotine exposure on baseline neural activity dynamics in the PFC and VTA linked to emotional deficits using *in-vivo* electrophysiology and analyzing single unit neuronal activity patterns and local field potential (LFP) oscillatory powers.

- **Hypothesis:** Nicotine-treated male and female rats will exhibit hyperactive glutamatergic and DA signaling in the PFC and VTA respectively compared to vehicle male and female rats respectively. These neuronal/electrophysiological phenotypes will be divergent across male vs. female cohorts.

Objective 3: Determine the long-term effects of chronic adolescent nicotine exposure on cognitive-, anxiety- and depressive-related molecular targets in the PFC and NAc, including α7 nAChR, β2 nAChR, VGLUT1, GAD65, D1R, D2R and BDNF.

- **Hypothesis:** Nicotine-treated male and female rats will exhibit dysregulated expression levels of several molecular markers for nicotine dependence and mood/anxiety disorders, including: α7 nAChR, β2 nAChR, GAD65, D1R, D2R, BDNF, VGLUT1 in the NAc/PFC network. These molecular biomarker expression patterns will be divergent across male vs. female cohorts.

Chapter 2

2 Materials and Methods

2.1 Animal Model

2.1.1 Animals and Housing

Male and female Sprague-Dawley rats (n=88) were obtained at PND 28 from Charles River Laboratories (Quebec, Canada). Animals were pair-housed in a controlled 12-hour light-dark cycle facility (constant temperature and humidity) with access to food and water *ad libitum*. All experimental procedures were conducted in accordance with Canadian Council of Animal Care and institutional guidelines.

2.1.2 Adolescent Nicotine Exposure Protocol

The chronic nicotine exposure protocol was adapted from previous neurodevelopmental nicotine exposure models, and it was shown to induce behavioural, neuronal and molecular dysregulations resembling mood and anxiety-related phenotypes (Counotte et al., 2009; Jobson et al., 2019; Hudson et al., 2021). Rats were administered sub-cutaneous injections of 0.4 mg/kg of nicotine (NIC; nicotine bitartrate, Sigma) or phosphate buffered saline (VEH; control) 3x daily at (10:00/13:00/16:00) for 10 consecutive days (PND 35-44). Following nicotine exposure, rats were left in the home cage for a 30-day drug-free washout period until the start of experiments on PND 75. Although direct physiological comparison between human tobacco/vaping exposure and systemic nicotine exposure in rats is not possible, the total concentration of daily nicotine exposure (1.2 mg/kg) used in the present model corresponds to blood nicotine levels obtained from smoking ~1 pack of cigarettes per day in moderate/heavy smokers (Fung & Lau, 1989; Murrin et al., 1987). The age range of nicotine exposure (PND 35-44) corresponds to the mid-adolescence stage in rats (McCormick et al., 2013; Wilkin et al., 2012), a typical period where human adolescents are first exposed to tobacco-related products and other drugs of abuse (Edvardsson et al., 2009; Taioli and Wynder, 1991; Stanton et al., 1991). A schematic representation of the experimental procedures is presented in **Figure 3**.

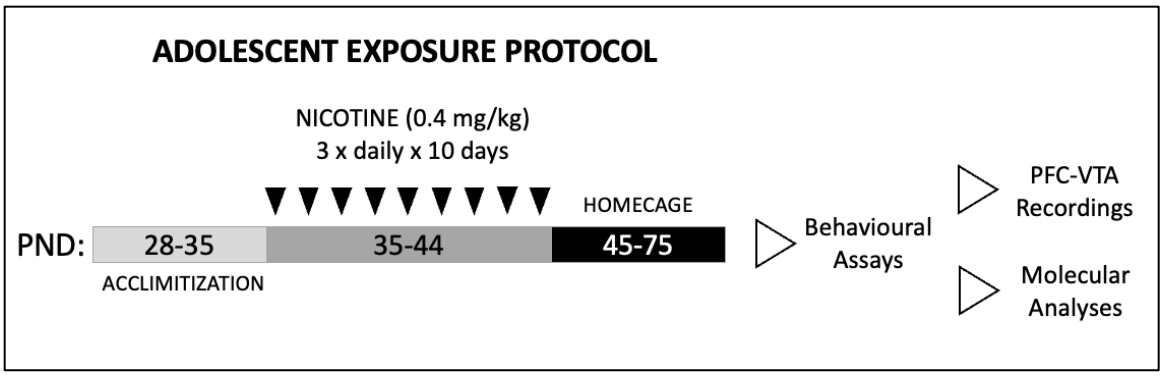

Figure 3. Adolescent nicotine exposure protocol. Schematic illustration showing chronic nicotine administration protocol for male and female adolescent rat cohorts, as well as associated behavioural, electrophysiological and protein analysis experiments.

2.2 Behavioural Testing

Starting on PND 75 and ending on PND 100, a series of behavioural assays were performed to examine the long-term effects of adolescent nicotine exposure on anxiety and depressive-like behaviours. Between experiments, rats were returned to the home cage for a minimum of 24-48 h. We conducted the following series of behavioural assays:

2.2.1 Open Field Test (OFT)

The open field test (OFT) functions as a measure of locomotion and anxiety. Rats were placed in an automated open field activity chamber (80x80×50 cm, Med Associates) for 15 mins. Locomotor activity was assessed as the total distance travelled in the entire apparatus, while anxiety was assessed as the time spent in the central zone of the chamber during the first 5 min of the test. The chamber was cleaned between sessions with a 70% ethanol solution to avoid olfactory cue bias. Time spent in the central zone relative to the peripheral area is a well-established index of anxiety as higher states of anxiety are associated with lower exploration times in the central region (Seibenhener & Wooten, 2015; Sarikahya et al., 2022).

2.2.2 Elevated Plus Maze (EPM)

The EPM is a measure of anxiety, and the apparatus [black acrylic, 4 arms (10 × 50 cm) stemming from a 10 × 10 cm platform forming a plus shape] is raised above the floor by 50 cm and dimly illuminated at 40 lux (Szkudlarek et al., 2019). Two opposing arms are enclosed with 40 cm high walls, while the other two arms are open with a 1-cm-high barrier. Rats were placed on the central platform facing a closed arm and explored the maze for 5 mins. The apparatus was cleaned between sessions with a 70% ethanol solution to avoid olfactory cue bias. Anxiety-like behaviours were defined as the number of entries (i.e., all four paws in arm) and the time spent in closed and open arms. Behaviours were video recorded and analyzed offline (Behaview software).

2.2.3 Spontaneous alternation (SA) task

The SA task is an assessment of spatial working memory (Kraeuter et al., 2019). The test apparatus is a Y-shaped maze with 3 arms 120° from each other (black non-reflecting acrylic; arm length: 50 cm; wall height: 40 cm) (Szkudlarek et al., 2019). Rats were placed in the center of the arm facing either arm A, B or C (counterbalanced between animals), and behaviours were recorded for 5 mins. The apparatus was cleaned between sessions with a 70% ethanol solution to avoid olfactory cue bias. An arm entry was counted only when all 4 paws were in the arm. The alternation score was calculated as (unique triplets)/(total arm entries – 2) x 100%, where unique triplets were considered as three consecutive arm entries (e.g., ABC, BCA) without re-entries (e.g., AAB) or entries to previously visited arms (e.g., ABA, CBC)

2.2.4 Novel object recognition (NOR)

The NOR task is a measure of recognition memory (Lueptow, 2017). The test apparatus is a 80×80×50 cm black acrylic box, and the test session consisted of two 3-min trials with an inter-trial interval (ITI) of 60 mins. In the first trial (training phase), rats were allowed to freely explore the arena containing a pair of identical objects for 3 mins (e.g., training phase: objects A|A). One hour later in the second trial (testing phase), rats were placed in the same chamber again but with 1 copy of the first (familiar) object and 1 copy of a novel object (e.g., testing phase: objects A|B). Object positions were counterbalanced and randomized. The chamber and objects were cleaned between trials with a 70% ethanol solution to avoid olfactory cue bias. Object exploration was defined as sniffing the object. Exploration time of each object was evaluated, and preference was expressed as object recognition score: (time spent with novel object/total time exploring both objects) × 100%.

2.2.5 Temporal order novel object recognition (TONOR)

The TONOR task is an extension of NOR that assesses the recency/temporal aspect of recognition memory (Barker et al., 2007; Morici et al., 2015). The same apparatus from NOR was used and the test session consisted of three 3-min trials with ITI of 60 mins. In the first trial (training phase), rats were allowed to freely explore the arena

containing a pair of identical objects for 3 mins (e.g., 1st training phase: objects A|A). In the second trial (training phase), rats were placed in the arena again for 3 mins but with a different pair of identical objects (e.g., 2nd training phase: objects B|B). In the third trial (testing phase), rats were allowed to explore the chamber again but with 1 copy of the first (more novel, or less familiar/recent) object and 1 copy of the second (less novel or more familiar/recent) object (e.g., testing phase: objects A|B). Object positions were counterbalanced and randomized. The chamber and objects were cleaned between trials with a 70% ethanol solution to avoid olfactory cue bias. Object exploration was defined as sniffing the object. Exploration time of each object was evaluated, and preference was expressed as object recognition score: (time spent with first object/total time exploring both objects) × 100%. A normal, healthy rat would explore the first object more since it is more novel (i.e., less familiar based on the order presented) relative to the second object.

2.2.6 Three-chamber sociability and social novelty test

The social interaction (SI) test evaluates two aspects of social behaviours: social motivation and social recognition memory (Kaidanovich-Beilin et al., 2011; Renard et al., 2017a,b; Szkudlarek et al., 2019). The test apparatus consisted of a transparent acrylic chamber divided into three equal compartments separated with guillotine doors. One day before the assessment, rats were room acclimated for 30 min and subsequently habituated to the apparatus (3 mins center + 12 mins entire apparatus). The social interaction test involves 2 stages. In stage 1 (social motivation test), following a 30-min acclimatization, rats were placed in the central compartment (3 mins; guillotine doors in place). Subsequently, 2 wire cages were placed in the side compartments (one contained a stranger male rat; the other one is empty) and the test rat could explore the entire apparatus for 6 mins. In stage 2 (social memory test), a novel, unfamiliar stranger rat was placed in the previously empty wire cage, and the test rat could explore both chambers (containing the previously encountered rat or the new stranger rat) for 6 mins. The position of empty cage and stranger rats were counterbalanced between sessions. The chamber was also cleaned between trials with a 70% ethanol solution to avoid olfactory cue bias. Behaviors were video recorded and analyzed offline. The total duration of exploratory bouts with the stranger vs. empty cage (stage 1) was calculated as sociability

index = $t_{stranger}/(t_{stranger} + t_{empty})$ x 100%. The time spent with the previously encountered vs. novel stranger rat in phase 2, was calculated as social recognition score = $t_{novel}/(t_{novel} + t_{familiar})$ x 100%.

2.2.7 Porsolt forced swim test (FST)

The FST is a measure of learned helplessness, a symptom in depression. Learned helplessness refers to the deficit in escape/coping behaviours after exposure to uncontrollable stress (Vollmayr & Gass, 2013). Rats were placed in a 70 × 19 cm cylinder filled 3⁄4 of the way with water (room temperature) for a 10-min test phase. Behaviors examined included average times spent climbing, swimming, and being immobile, as well as latency to immobility. (Can et al., 2012; Jobson et al., 2019; Hudson et al., 2021). Behaviours were recorded and analyzed offline (Behaview software). Decreased climbing time and increased immobility time are considered depressive-like behaviours since it indicates reduced motivation in escaping the water cylinder.

2.3 *In-vivo* Electrophysiology

2.3.1 Single Unit Recording

Neuronal recordings were performed unilaterally in the VTA or PFC, as previously described (Tan et al., 2009; Renard et al., 2017a,b; Jobson et al., 2019; Hudson et al., 2021). Rats were anesthetized with urethane (1.4 g/kg, i.p.) and placed in a stereotaxic frame with body temperature maintained at 37±1°C. A scalp incision was made and a hole was drilled in the skull overlaying the VTA or PFC. A glass microelectrode (with an average impedance of 6–10MΩ) filled with a 2% pontamine sky blue solution was slowly lowered using a hydraulic micro-positioner (Kopf 640) into the VTA or PFC regions. The coordinates of the recordings were from the (1) VTA, anterior-posterior (AP): −5.1 to −5.3 mm, medial-lateral (ML): ±0.7 to 1.0 mm from bregma, dorsal-ventral (DV): −7 to −9 mm from the dural surface; and the (2) mPFC, AP: +2.6 to +4.2 mm, ML: ±0.4 to 1.0 mm from bregma, DV: −1.8 to −3.6 mm from the dural surface. Single-unit neuronal activity was filtered (bandpass 0.3–5 kHz) and individual action potentials were isolated and amplified (MultiClamp 700B amplifier, Molecular Devices), digitized at 25 kHz and recorded using a Digidata 1440 A and pClamp software

(Molecular Devices). Recordings of putative DA and pyramidal neurons in the VTA and PFC were assessed respectively. Analyses were performed offline using Clampfit10 (Molecular Devices). Population spontaneous activity was determined in predetermined recording tracks separated by 200 μm and the activity of each neuron was recorded for 5 mins. Putative VTA DA and mPFC pyramidal neurons were identified according to previously established electrophysiological features: VTA DA neuron: (1) frequency <10 Hz, (2) action potential duration ≥2.2 ms (>1.1 ms from the start to the negative trough), (3) single irregular or bursting pattern; mPFC pyramidal neurons: (1) frequency <10 Hz, (2) action potential duration ≥2.5 ms (Ungless and Grace 2012; De Felice et al., 2021). The two parameters of activity that were analyzed were basal firing rates and burst frequency. The onset of a burst was defined as, (1) VTA DA neurons: 2 consecutive spikes with an interspike interval (ISI) of ≤80 ms; (2) mPFC pyramidal neurons: 3 consecutive spikes with an ISI of ≤45 ms.

2.3.2 Local Field Potential (LFP) Recording

Local field potential (LFP) signals were analyzed using NeuroExplorer (Nex Technologies). LFP signals were decimated to 1 kHz, and lowpass filtered (IIR Butterworth filter at 100 Hz; filter order set to 3). Subsequently, a spectrogram function was used to calculate the power of oscillations at frequencies between 0–100 Hz (window length 2 s; shift 0.5 s). Power values for a given frequency were averaged over time of the recording epoch and normalized so that the sum of all power spectrum values equals 1. The total power was calculated by adding all the power values at frequencies between 0–59 and 61–80 Hz. Power values at 60±1 Hz were excluded from all the calculations. Neural oscillations (i.e., LFP) were defined within specific frequency bands as follows: delta, 0-4 Hz; theta, 4-7 Hz; alpha, 7-14 Hz; beta, 14-30 Hz; gamma, 30-80 Hz. Following either VTA or PFC recordings, recording electrode locations were marked with iontophoretic deposit of Pontamine sky blue (–20 μA for 15 mins), and histological analyses were performed later to verify recording sites with light microscopy.

2.4 Tissue Molecular Analysis

2.4.1 Western Blots

After behavioural testing, rats received an overdose of sodium pentobarbital (240 mg/kg, i.p., EuthanylTM). Brains were rapidly removed and flash frozen at −80°C. Bilateral punchouts of mPFC and NAc were obtained and homogenized using a Dounce homogenizer containing protein extraction lysis buffer (NaCl, Tris pH 8.0, 1% NP-40, 10% glycerol, and 0.1% sodium dodecyl sulphate [SDS]) with 1:100 protease and phosphatase inhibitors included (Halt 100x inhibitor cocktail, ThermoFisher). Following homogenization and centrifugation (10000 RPM) at 4°C to remove insoluble material, most of the sample was mixed with an equal volume of 2× Laemmli loading buffer and heated to 95 °C for 5 min prior to storage at −80 °C. The remaining homogenate (~20 µl) was used to determine the concentration of protein in the sample using a Pierce BCA Protein Assay kit. The western blotting procedure was performed loading 12.5 or 25 µg of protein per well in 8%, 10% or 12% acrylamide denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Each blot only contained samples from the vehicle group and the nicotine group from one sex (e.g., male VEH and NIC samples in one blot; female VEH and NIC samples in another). Samples were subjected to electrophoresis in a Bio-Rad Mini Protein 3 Western blotting apparatus with Tris/glycine/SDS buffer (Bio-Rad Cube Solutions) at 125V for 1.5h. The protein transference into nitrocellulose membranes was performed using the Trans-Blot Turbo Transfer System (Bio-Rad) at 2.5 A for 10 min. Membranes were then blocked with either 5.0% non-fat dry milk or bovine serum albumin (BSA; Sigma) in Tris buffered saline with Tween 20 (TBS-T) for 1 hour with rocking, at room temperature. The membranes were then incubated overnight in a solution of TBS-T with a primary antibody of interest at 4°C with rocking. Primary antibody host species, dilutions and sources were as follows: α-tubulin (Mouse, 1:500, Santa Cruz Biotechnology; Rabbit, 1:1000, Sigma-Aldrich), β-actin (Mouse, 1:1000, Sigma-Aldrich), α7-nAChR (Rabbit, 1:1000), Alomone), β2-nAChR (Rabbit, 1:1000, NovusBio), VGLUT1 (Mouse, 1:1000, Abcam), GAD65 (Rabbit, 1:1000, Cell Signaling), D1R (Rabbit, 1:1000, EMD Millipore), D2R (Mouse, 1:500, EMD Millipore), BDNF (Rabbit, 1:1000, Abcam). Following primary antibody incubation, blots were

incubated for 1 hour at room temperature with species-appropriate secondary antibodies (LI-COR IRDye 680RD and IRDye 800CW; Thermo Scientific) at a 1:5000 dilution in either 2.5% non-fat dry milk or BSA in TBS-T. Proteins of interest were imaged using a LI-COR Odyssey Infrared Imaging System, and densitometry measurements were obtained using Image Studio analysis software. Relative band density was normalized to the intensity of the respective α-tubulin or β-actin band.

2.5 Vaginal Sample Collection and Estrous Cycle Assessment

To determine the stages of the estrous cycle and examine their influence on behaviours and electrophysiology, vaginal cytology samples were taken for 5 consecutive days to establish the length of the cycle, as well as immediately after individual behavioural test and upon completion of electrophysiological recordings. This protocol was adapted from a previously established method (Cora et al., 2015). 10 μL of saline solution was flushed in and out of the vagina of the rat with a pipette, and a small drop of the sample was placed on a glass slide under a microscope. Then, the stages of the estrous cycle were evaluated based on the cell density of neutrophils, nucleated and anucleated epithelial cells.

2.6 Statistical Analyses

Experimental data are presented as mean ± SEM, and statistical analyses were performed using GraphPad Prism 9 (San Diego, CA, USA). All datasets were tested for outliers. Comparisons between groups were determined using two-way analysis of variance (ANOVA) (sex x treatment) followed by Fisher's least significant difference (LSD) test for each behavioral assay and electrophysiology ($p<0.05$); whereas Mann-Whitney U test was used for Western blot protein analysis ($p<0.05$), since the expression level of protein targets in the NIC group was only compared within-group to the average, normalized values of the VEH group in each sex. Each blot also only contained samples from the vehicle group and nicotine group from one sex (e.g., male VEH and NIC in one blot; female VEH and NIC in another).

Chapter 3

3 Results

3.1 Behavioural Results

3.1.1 Adolescent nicotine exposure induces long-lasting anxiety-like behaviours in male but not female

3.1.1.1 Open Field Test

Repeated nicotine exposure during adolescence could lead to affective dysregulation such as increased anxiety (Mojtabai and Crum, 2013; Jobson et al., 2019; Hudson et al., 2021). Therefore, we first assessed anxiety-like behaviours and locomotor activity with OFT in adult male and female rats following chronic adolescent nicotine exposure (**Figure 4A, 4E, 4F**). Two-way ANOVA statistical analysis of center zone entries and total distance travelled demonstrated main effects of sex (total distance travelled: Sex: $F_{(1,60)} = 56.76$, $p < 0.0001$, Interaction: $F_{(1,60)} = 0.3420$, $p = 0.5609$, Treatment: $F_{(1,60)} = 0.3568$, $p = 0.5526$, **Figure 4B**; center zone entries: Sex: $F_{(1,58)} = 16.58$, $p = 0.0001$, Treatment: $F_{(1,58)} = 0.5756$, $p = 0.4511$, Interaction: $F_{(1,58)} = 1.464$, $p = 0.2313$, **Figure 4C**). *Post hoc* Fisher's LSD showed nicotine-treated female rats and female vehicle rats had more total distance travelled and center entries than their male counterparts respectively (total distance travelled: male VEH vs. female VEH: $p < 0.0001$, male NIC vs female NIC: $p < 0.0001$; center entries: male VEH vs. female VEH: $p = 0.0476$, male NIC vs female NIC: $p = 0.0004$). Statistical analysis also revealed main effects of treatment on the time spent in the central zone, ($F_{(1,54)} = 5.048$, $p = 0.0288$, **Figure 4D**), where *post hoc* Fisher's LSD showed that nicotine-treated male rats spent less time in the center compared to male vehicle rats ($p = 0.0124$), but there were no significant differences between female groups in both measures ($p = 0.6046$). Although there were no significant effects of interaction between sex and treatment ($F_{(1,54)} = 2.359$, $p = 0.1304$), the effect of sex was trending toward significance ($F_{(1,54)} = 3.020$, $p = 0.0879$), with *post hoc* comparisons revealing nicotine-treated female rats spent more time in the center than nicotine-treated male rats ($p = 0.0248$).

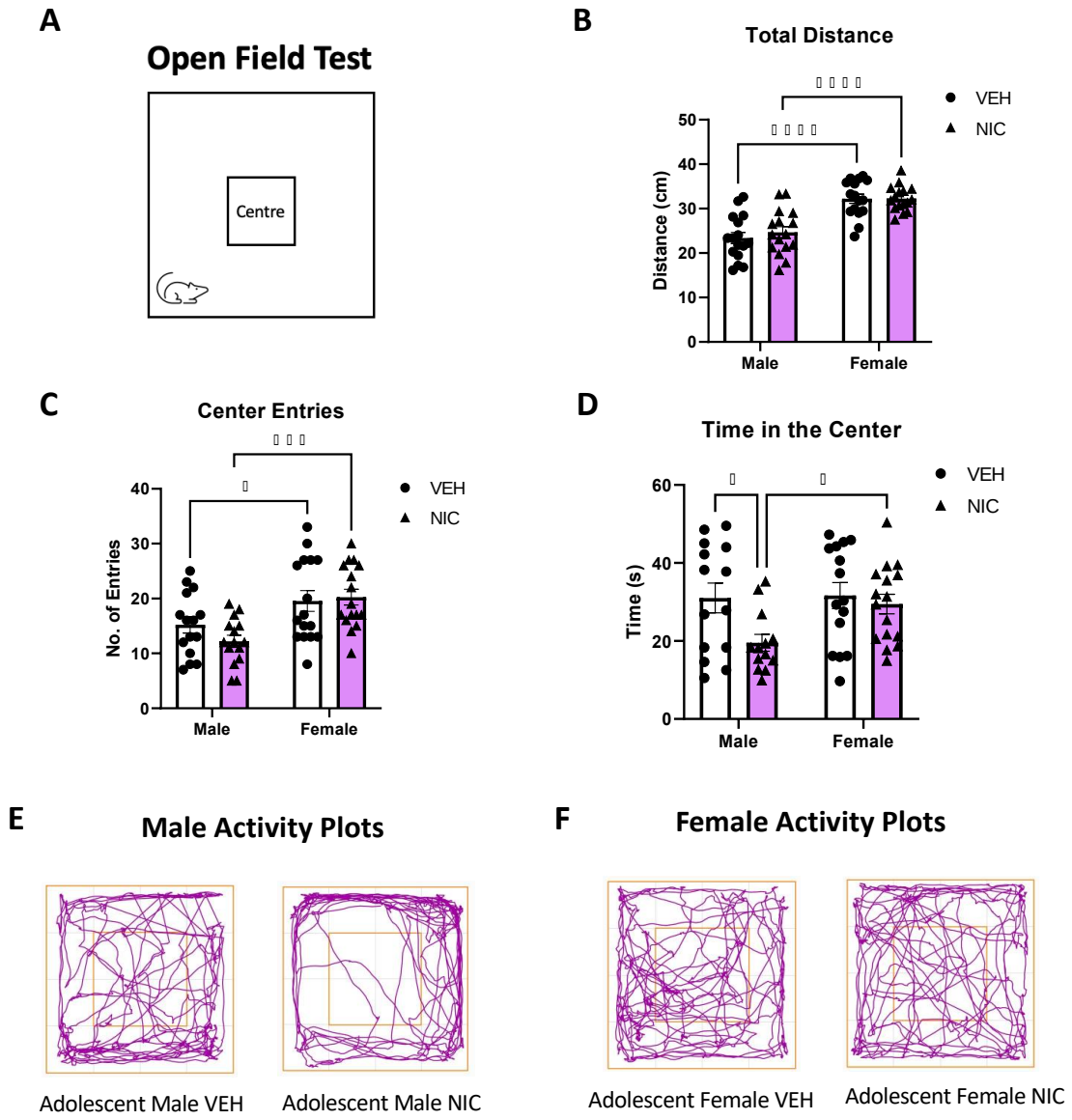

Figure 4. Sex-specific effects on locomotive activity and anxiety-related behaviours in OFT following chronic adolescent nicotine exposure. (A) Schematic representation of the OFT apparatus. **(B, C)** Adolescent nicotine treatment did not alter locomotive activity and center entries within-group, but both female groups had greater distance travelled and more center entries compared to their male vehicle and nicotine counterparts. **(D)** Adolescent-nicotine-treated male rats spent less time in the center than male vehicle rats, where both female groups performed similarly in this measure (*n* = 13-16/treatment/sex). **(E, F)** Representative activity traces of vehicle vs nicotine-treated rats

in each sex. Comparisons were made with two-way ANOVA followed by Fisher's LSD *post hoc* test, *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$, two-tailed.

3.1.1.2 Elevated Plus Maze

Besides OFT, we also examined the long-term sex-specific effects of chronic adolescent nicotine exposure on anxiety-like behaviours with EPM (**Figure 5A**). Two-way ANOVA analysis of open arm entries indicated main effects of sex, treatment, and interaction between both factors (Sex: $F_{(1,59)} = 4.259$, $p = 0.0434$; Treatment: $F_{(1,59)} = 5.770$, $p = 0.0195$; Interaction: $F_{(1,59)} = 4.836$, $p = 0.0318$; **Figure 5B**). *Post hoc* Fisher's LSD revealed nicotine-treated male rats had lower open arm entries than male vehicle rats ($p = 0.0020$), but no significant differences between female groups ($p = 0.8854$). Nicotine-treated female rats also had more open arm entries compared to nicotine-treated male rats ($p = 0.0035$). Statistical analysis demonstrated main effects of treatment and interaction between sex and treatment, but not sex, on closed arm entries (Sex: $F_{(1,59)} = 3.313$, $p = 0.0738$; Treatment: $F_{(1,59)} = 5.224$, $p = 0.0259$; Interaction: $F_{(1,59)} = 6.205$, $p = 0.0156$; **Figure 5C**). *Post hoc* comparisons showed that the nicotine-treated male group had fewer closed arm entries than the male vehicle group ($p = 0.0014$), but there were no significant changes between female groups ($p = 0.8841$). Nicotine-treated female rats also had more closed arm entries compared to nicotine-treated male group ($p = 0.0032$). Furthermore, two-way ANOVA analysis of open arm time revealed main effects of interaction between sex and treatment, but not sex and treatment independently (Sex: $F_{(1,53)} = 1.465$, $p = 0.2314$; Treatment: $F_{(1,53)} = 1.426$, $p = 0.2378$; Interaction: $F_{(1,53)} = 5.234$, $p = 0.0262$; **Figure 5D**). *Post hoc* comparisons indicated that nicotine-treated male rats spent less time in the open arm compared to male vehicle rats ($p = 0.0199$), but both female groups performed similarly in this measure ($p = 0.4308$). Nicotine-treated female rats spent more time in the open arm than nicotine-treated male rats as well ($p = 0.0193$). Statistical analysis also revealed main effects of treatment and interaction between sex and treatment on closed arm time (Sex: $F_{(1,55)} = 1.297$, $p = 0.2597$; Treatment: $F_{(1,55)} = 5.161$, $p = 0.0270$; Interaction: $F_{(1,55)} = 4.603$, $p = 0.0364$; **Figure 5E**), where *post hoc* comparisons showed that nicotine-treated male rats spent more time in the closed arm compared to male vehicle rats ($p = 0.0041$), and there were no significant differences between female groups ($p = 0.9258$). Nicotine-treated male rats also spent more time in the closed arm than nicotine-treated female rats ($p = 0.0253$).

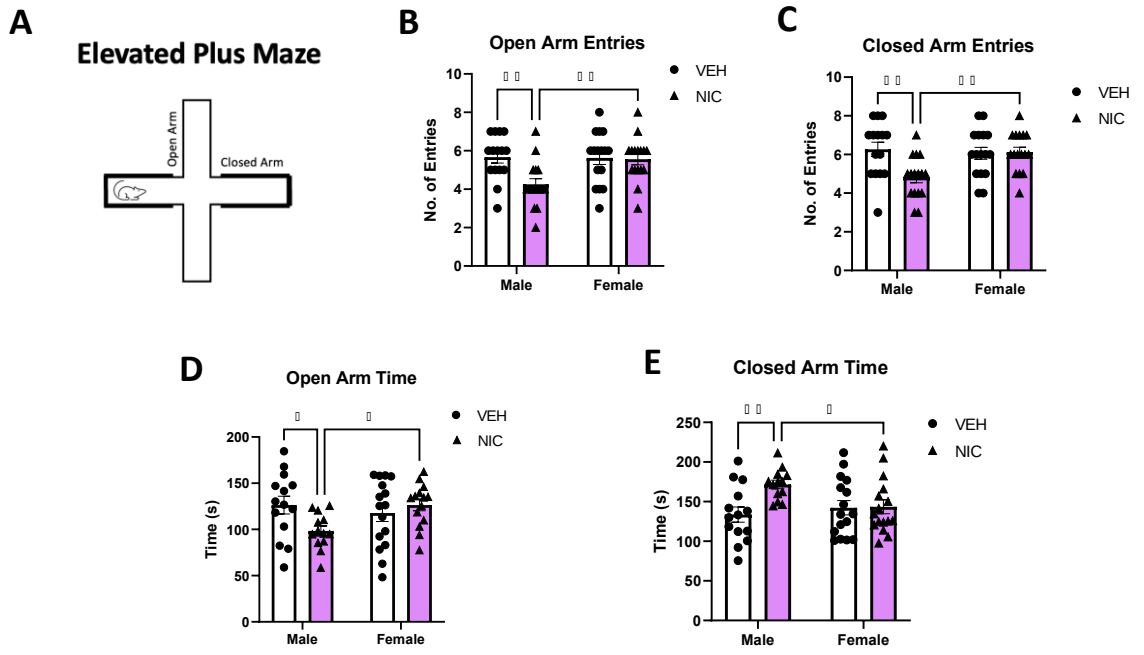

Figure 5. Chronic adolescent nicotine exposure results in sex differences in anxiety-related behaviours in EPM. (A) Schematic representation of the EPM apparatus for anxiety test. **(B, C)** Nicotine-treated male rats displayed lower open arm entries and closed arm entries than male vehicle rats, whereas both female groups performed similarly (n = 13-16/treatment/sex). **(D, E)** Adult male rats treated with nicotine during adolescence spent less time in the open arm, but more time in the closed arm compared to male vehicle group, where there were no significant differences between female groups. Comparisons were made with two-way ANOVA followed by Fisher's LSD *post hoc* test, $*p < 0.05$, $**p < 0.01$, two-tailed.

3.1.2 Adolescent nicotine exposure induces persistent depressive-like behaviours in male but not female

3.1.2.1 Forced Swim Test

In addition to anxiety disorders, nicotine dependence during adolescence could also result in depressive disorders (Boden et al., 2010; Mojtabai and Crum, 2013; Jobson et al., 2019; Hudson et al., 2021). Animal models of learned helplessness have been shown to be associated with symptoms of depressive disorders as they evaluate the motivational state/coping behaviours of animals under uncontrollable aversive events, which are often affected in depressed patients (Vollmayr & Gass, 2013; Alloy & Abramson, 1982; Maier, 1984; Song & Vilares, 2021). Thus, we assessed depressive-like behaviours via the FST in adult male and female rats following chronic adolescent nicotine exposure. (**Figure 6A**). Two-way ANOVA analysis of climbing time demonstrated main effects of treatment and interaction between sex and treatment (Sex: $F_{(1,60)} = 2.470$, $p = 0.1213$; Treatment: $F_{(1,60)} = 28.77$, $p < 0.0001$; Interaction: $F_{(1,60)} = 40.00$, $p < 0.0001$; **Figure 6B**). *Post hoc* Fisher's LSD revealed that nicotine-treated male rats exhibited decreased climbing time than male vehicle rats ($p < 0.0001$), but no significant differences between both female groups ($p = 0.4995$). Female vehicle rats had lower climbing time compared to male vehicle rats ($p = 0.0014$), whereas nicotine-treated female rats displayed increased climbing time than nicotine-treated male rats ($p < 0.0001$). Statistical analysis also revealed main effects of interaction between sex and treatment on immobility time (Sex: $F_{(1,60)} = 0.0475$, $p = 0.8283$; Treatment: $F_{(1,60)} = 2.730$, $p = 0.1037$; Interaction: $F_{(1,60)} = 10.40$, $p = 0.0020$; **Figure 6C**). *Post hoc* comparisons demonstrated that nicotine-treated male rats exhibited increased immobility time compared to male vehicle rats ($p = 0.0010$), and there were no significant differences between female groups ($p = 0.2704$). Female vehicle rats had higher immobility time than male vehicle rats ($p = 0.0179$), whereas nicotine-treated female rats displayed lower immobility time than nicotine-treated male rats ($p = 0.0376$). Two-way ANOVA analysis of swimming time showed no main effects of sex, treatment, and interaction between sex and treatment (Sex: $F_{(1,60)} = 0.9107$, $p = 0.3438$; Treatment: $F_{(1,60)} = 1.330$, $p = 0.2535$; Interaction: $F_{(1,60)} = 0.0568$, $p = 0.8124$; **Figure 6D**).

Moreover, statistical analysis demonstrated main effects of treatment and interaction between sex and treatment on latency to immobility (Sex: $F_{(1,60)} = 3.478$, $p = 0.0674$; Treatment: $F_{(1,60)} = 42.69$, $p < 0.0001$; Interaction: $F_{(1,60)} = 38.55$, $p < 0.0001$; **Figure 6E**). *Post hoc* comparisons revealed that nicotine-treated male rats exhibited decreased latency to immobility than male vehicle rats ($p < 0.0001$), meaning this treatment group reached the immobility state sooner than the male vehicle group; whereas there were no significant changes between female groups ($p = 0.8191$). Female vehicle rats also had decreased latency to immobility compared to male vehicle rats ($p = 0.0032$), whereas nicotine-treated female rats displayed increased latency than nicotine-treated male rats ($p < 0.0001$).

3.1.2.2 Social Interaction Test

Social phobia is common in anxiety disorders and it has been shown to be associated with depressive disorders (Ratnani et al., 2017; Beesdo et al., 2009; Penninx et al., 2021; DeMartini et al., 2019). Hence, we examined sex differences in social interaction behaviours in adult male and female rats following chronic adolescent nicotine exposure (**Figure 7A**). Two-way ANOVA analysis of sociability index indicated main effects of sex and interaction between sex and treatment (Sex: $F_{(1,59)} = 4.275$, $p = 0.0431$; Treatment: $F_{(1,59)} = 0.8416$, $p = 0.3627$; Interaction: $F_{(1,59)} = 5.843$, $p = 0.0187$; **Figure 7B**). *Post hoc* Fisher's LSD revealed that nicotine-treated male rats displayed lower sociability index than male vehicle group ($p = 0.0227$), but both female groups performed similarly ($p = 0.2893$). Nicotine-treated female rats exhibited lower sociability index compared to nicotine-treated male rats ($p = 0.0022$). For social recognition score, statistical analysis did not show any main effects of sex, treatment, and interaction between factors (Sex: $F_{(1,60)} = 1.257$, $p = 0.2667$; Treatment: $F_{(1,60)} = 0.0572$, $p = 0.8118$; Interaction: $F_{(1,60)} = 0.1773$, $p = 0.6752$; **Figure 7C**).

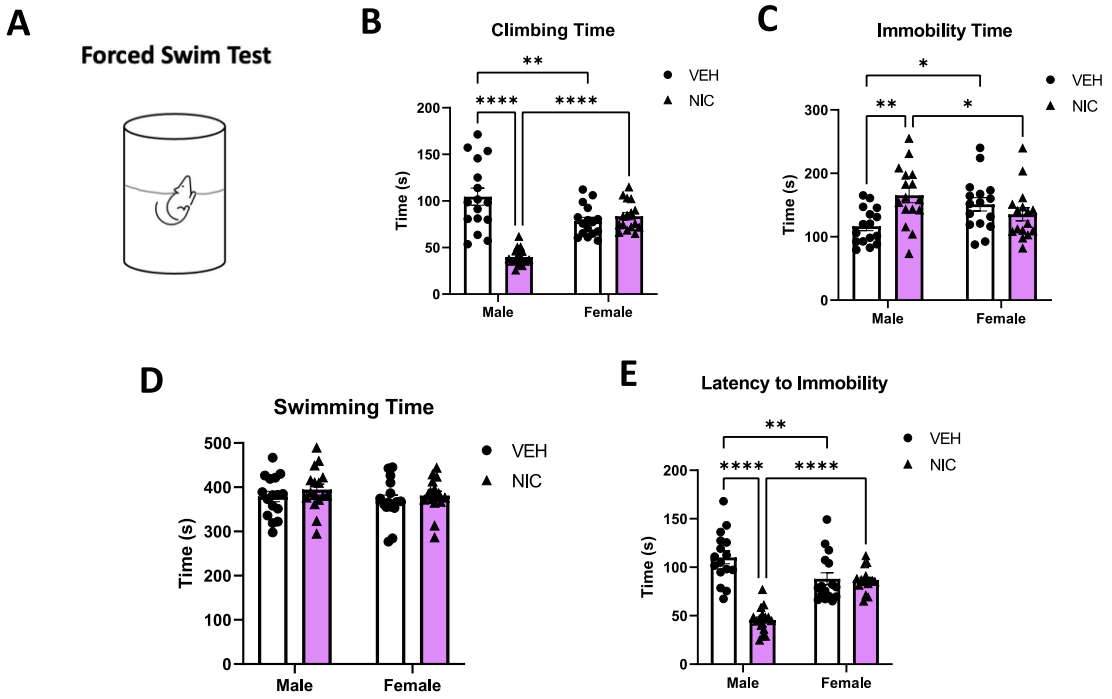

Figure 6. Sex differences in depressive-like behaviours in FST following adolescent nicotine exposure. (A) Schematic representation of the FST apparatus. **(B, C)** Nicotine-treated male rats displayed reduced climbing time and increased immobility time than male vehicle rats, whereas there were no significant changes between female groups (n = 16/treatment/sex). Nicotine-treated female rats exhibited increased climbing time and lower immobility time compared to nicotine-treated male rats. **(D)** There were no significant differences between treatment groups in each sex in swimming time. **(E)** Nicotine-treated male rats showed lower latency to immobility than male vehicle rats, and both female groups performed similarly in this measure. Comparisons were made with two-way ANOVA followed by Fisher's LSD *post hoc* test, *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, two-tailed.

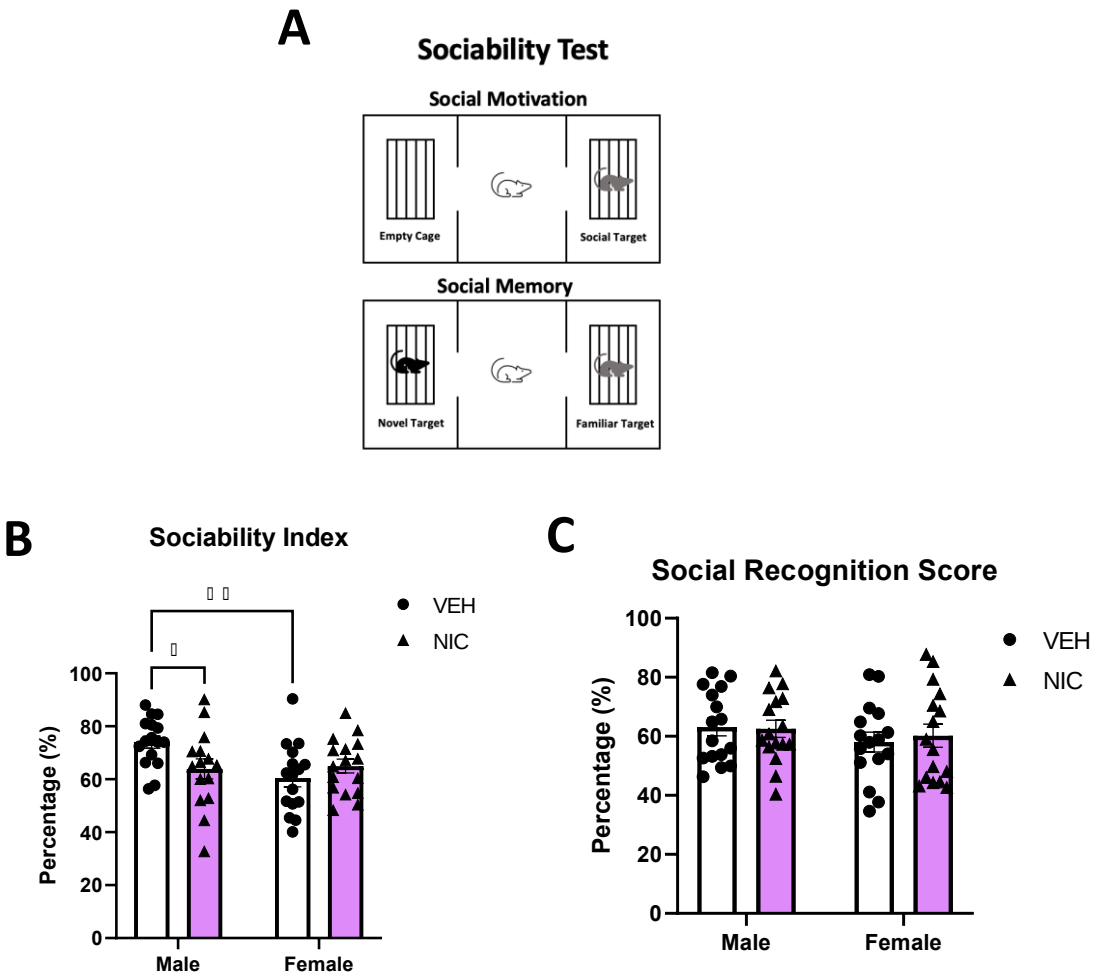

Figure 7. Chronic adolescent nicotine exposure results in sex-specific effects in social avoidance behaviours. (A) Schematic representation of the SI apparatus and set-up. **(B)** Nicotine-treated male rats exhibited reduced sociability index compared to male vehicle rats, whereas both female groups performed similarly. Female vehicle group displayed decreased sociability index than male vehicle group (n = 15-16/treatment/sex). **(C)** There were no significant differences between treatment groups in each sex. Comparisons were made with two-way ANOVA followed by Fisher's LSD *post hoc* test, $*p < 0.05$, $**p < 0.01$, two-tailed.

3.1.3 Adolescent nicotine exposure induces sex-specific working memory deficits in adulthood

3.1.3.1 Spontaneous Alternation Task

Chronic adolescent nicotine exposure could lead to cognitive deficit, which is also one of the symptoms in anxiety and depressive disorders (Counotte et al., 2009; Li et al., 2021; Datta et al., 2021; Penninx et al., 2021). Therefore, we first evaluated spatial working memory through the SA task in adult male and female rats following chronic adolescent nicotine exposure (**Figure 8A**). Two-way ANOVA analysis of total entries revealed main effects of sex (Sex: $F_{(1,59)} = 23.43$, $p < 0.0001$; Treatment: $F_{(1,59)} = 2.365$, $p = 0.1294$; Interaction: $F_{(1,59)} = 2.117$, $p = 0.1510$; **Figure 8B**). *Post hoc* Fisher's LSD indicated that both female groups displayed higher total entries compared to their male vehicle and nicotine counterparts (male VEH vs. female VEH: $p = 0.0209$; male NIC vs. female NIC: $p < 0.0001$). Nicotine-treated female rats exhibited higher total entries than female vehicle rats ($p = 0.0401$). Statistical analysis also demonstrated main effects of interaction between sex and treatment on alternation score (Sex: $F_{(1,60)} = 2.971$, $p = 0.0899$; Treatment: $F_{(1,60)} = 1.704$, $p = 0.1967$; Interaction: $F_{(1,60)} = 4.460$, $p = 0.0389$; **Figure 8C**). *Post hoc* comparisons showed that nicotine-treated male rats exhibited lower alternation score than male vehicle rats ($p = 0.0187$), but there were no significant differences between both female groups ($p = 0.5707$). Nicotine-treated female rats also displayed higher alternation score than nicotine-treated male rats ($p = 0.0087$).

3.1.3.2 Novel Object Recognition Task

Next, we assessed sex differences in recognition memory with NOR task following chronic adolescent nicotine exposure (**Figure 9A**). Two-way ANOVA analysis of recognition score revealed main effects of treatment (Sex: $F_{(1,28)} = 3.059$, $p = 0.0912$; Treatment: $F_{(1,28)} = 4.319$, $p = 0.0470$; Interaction: $F_{(1,28)} = 2.893$, $p = 0.1000$; **Figure 9B**). *Post hoc* Fisher's LSD showed that nicotine-treated male rats exhibited lower recognition score compared to male vehicle rats ($p = 0.0124$), whereas both female groups performed similarly in this measure ($p = 0.7917$). Female vehicle rats also displayed lower recognition score than male vehicle rats ($p = 0.0213$).

3.1.3.3 Temporal Order Novel Object Recognition Task

Last but not least, we examined the recency/temporal aspect of recognition memory in adult male and female rats following chronic adolescent nicotine exposure (**Figure 10A**). Two-way ANOVA analysis indicated main effects of interaction between sex and treatment on recognition score (Sex: $F_{(1,57)} = 0.0433$, $p = 0.8359$; Treatment: $F_{(1,57)} = 3.585$, $p = 0.0634$; Interaction: $F_{(1,57)} = 4.953$, $p = 0.0300$; **Figure 10B**). *Post hoc* Fisher's LSD demonstrated that nicotine-treated male rats exhibited lower recognition score than male vehicle rats ($p = 0.0054$), but there were no significant differences between female groups ($p = 0.8137$).

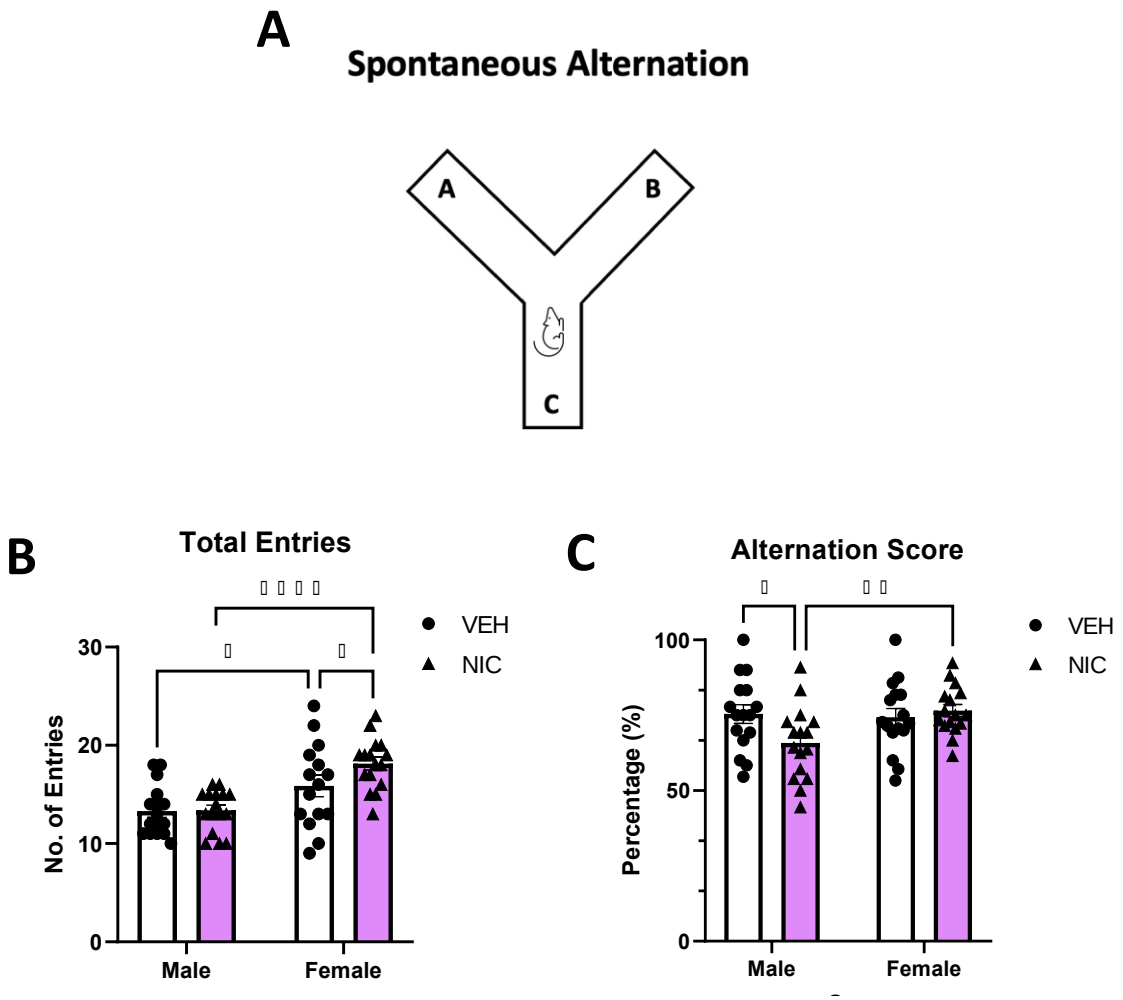

Figure 8. Sex-specific effects in spatial working memory following chronic adolescent nicotine exposure. (A) Schematic representation of the SA task apparatus. **(B)** Both female groups exhibited higher total entries compared to their male vehicle and nicotine counterparts. Nicotine-treated female rats also displayed increased total entries than female vehicle rats (n = 15-16/treatment/sex). **(C)** Nicotine-treated male rats demonstrated lower alternation score than male vehicle rats, and there were no significant differences between female groups. Nicotine-treated female rats had higher alternation score compared to nicotine-treated male rats. Comparisons were made with two-way ANOVA followed by Fisher's LSD *post hoc* test, *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, two-tailed.

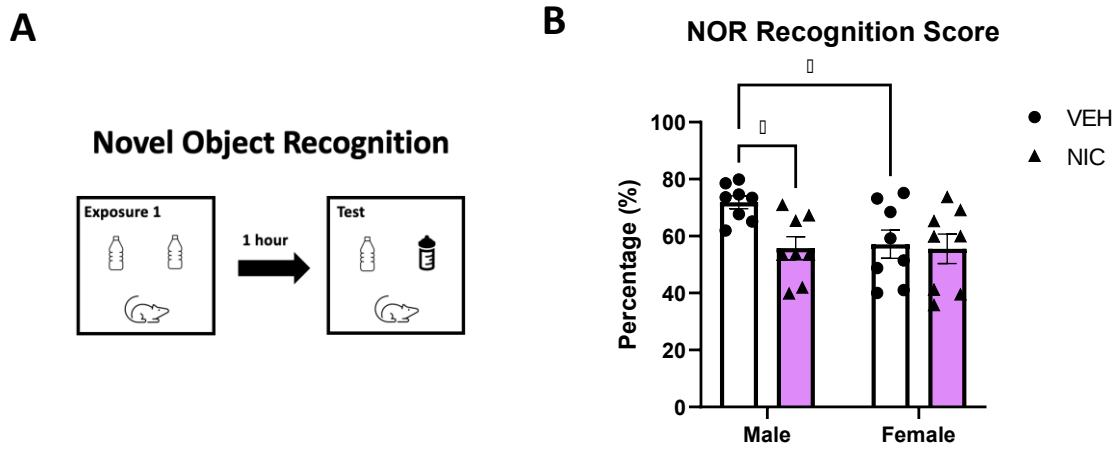

Figure 9. Chronic adolescent nicotine exposure leads to sex-specific deficits in recognition memory. (A) Schematic representation of the NOR task apparatus. **(B)** Nicotine-treated male rats displayed lower recognition score compared to male vehicle rats, whereas both female groups performed similarly. Female vehicle rats also exhibited lower recognition score than male vehicle rats (n = 8-10/treatment/sex). Comparisons were made with two-way ANOVA followed by Fisher's LSD *post hoc* test, $*p < 0.05$, two-tailed.

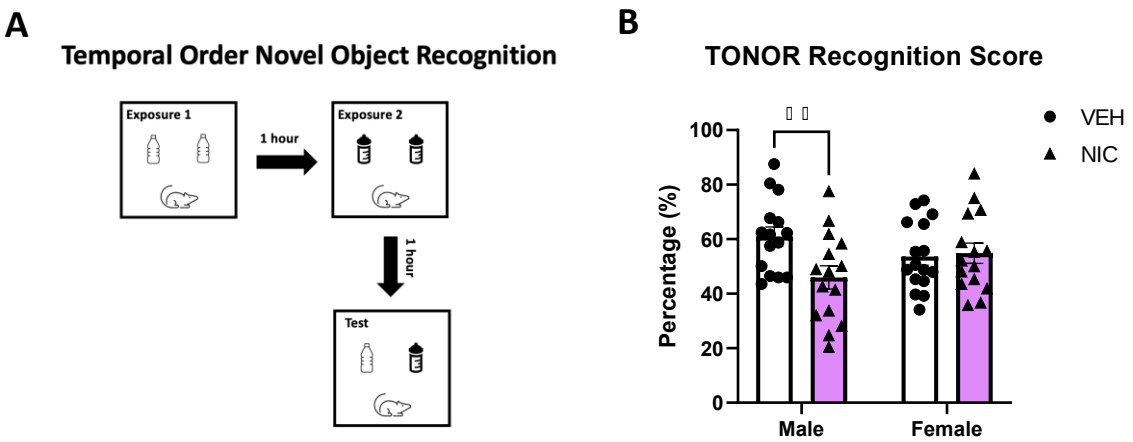

Figure 10. Sex-specific deficits in the temporal aspect of recognition memory following chronic adolescent nicotine exposure. (A) Schematic representation of the TONOR task apparatus. **(B)** Nicotine-treated male rats displayed lower recognition score compared to male vehicle rats, and there were no significant differences between female groups (n = 15-16/treatment/sex). Comparisons were made with two-way ANOVA followed by Fisher's LSD *post hoc* test, $^{**}p < 0.01$, two-tailed.

3.2 *In-vivo* Electrophysiology Results

3.2.1 Adolescent nicotine exposure induces sex-specific alterations to mPFC and VTA spontaneous neuronal spiking activity

3.2.1.1 Pyramidal Neurons in the mPFC

Nicotine exposure can alter neurotransmitter dynamics in the brain involving glutamate and DA signaling (Le Houezec, 2003), and disrupted neurotransmission in these systems is commonly reported in mood and anxiety disorders (Li et al., 2021; Malhi & Mann, 2018; Penninx et al., 2021). For instance, hyperactive glutamate and hypoactive GABA signaling, as well as aberrant DA signaling have been observed in patients and pre-clinical studies of anxiety and depressive disorders (Bi et al., 2013; Francis et al., 2015; Jobson et al., 2019; Hudson et al., 2021; Yildiz-Yesiloglu & Ankerst, 2006). Thus, following behavioural testing, we first examined sex-specific effects of chronic adolescent nicotine exposure on spontaneous firing activity of pyramidal (glutamatergic) neurons in the mPFC (**Figure 11A, 11B, 11F, 11G**). Two-way ANOVA analysis revealed main effects of treatment and interaction between factors on mPFC pyramidal neuron firing frequency (Sex: $F_{(1,196)} = 0.0656$, $p = 0.7981$; Treatment: $F_{(1,196)} = 8.792$, $p = 0.0034$; Interaction: $F_{(1,196)} = 7.874$, $p = 0.0055$; **Figure 11C**). *Post hoc* Fisher's LSD demonstrated that the nicotine-treated male group exhibited higher firing frequency than the male vehicle group ($p < 0.0001$), but there were no significant differences between both female groups ($p = 0.9106$). The female vehicle group also displayed elevated firing frequency compared to the male vehicle group ($p = 0.0316$). Statistical analysis of burst events indicated main effects of treatment (Sex: $F_{(1,194)} = 1.471$, $p = 0.2267$; Treatment: $F_{(1,194)} = 10.47$, $p = 0.0014$; Interaction: $F_{(1,194)} = 1.740$, $p = 0.1887$; **Figure 11D**). *Post hoc* comparisons showed that the nicotine-treated male group exhibited more burst events compared to the male vehicle group ($p = 0.0015$). No significant differences were observed between female treatment groups ($p = 0.1769$). Furthermore, analysis of burst frequency also revealed main effects of treatment (Sex: $F_{(1,194)} = 1.370$, $p = 0.2433$; Treatment: $F_{(1,194)} = 11.31$, $p = 0.0009$; Interaction: $F_{(1,194)} = 1.774$, $p = 0.1845$; **Figure 11E**). *Post hoc* comparisons demonstrated that nicotine-treated male rats displayed

increased burst frequency than male vehicle rats (p = 0.0011), and there were no significant differences between female treatment groups (p = 0.1526).

3.2.1.2 Dopaminergic Neurons in the VTA

Next, we investigated baseline spiking activity of VTA DA neurons in adult male and female rats following chronic adolescent nicotine exposure (**Figure 12A, 12B, 12F, 12G**). Two-way ANOVA analysis revealed main effects of treatment and interaction between factors on firing frequency (Sex: $F_{(1,178)}$ = 0.1687, p = 0.6818; Treatment: $F_{(1,178)}$ = 18.55, $p <$ 0.0001; Interaction: $F_{(1,178)}$ = 19.15, $p <$ 0.0001; **Figure 12C**). *Post hoc* Fisher's LSD indicated that nicotine-treated male rats exhibited higher firing frequency than male vehicle rats ($p <$ 0.0001), but there were no significant differences between both female groups (p = 0.9636). Female vehicle rats displayed increased firing frequency compared to male vehicle rats (p = 0.0017), whereas nicotine-treated female rats showed lower firing frequency than nicotine-treated male rats (p = 0.0031). Statistical analysis of burst events demonstrated main effects of treatment and interaction between factors (Sex: $F_{(1,147)}$ = 2.327, p = 0.1293; Treatment: $F_{(1,147)}$ = 6.789, p = 0.0101; Interaction: $F_{(1,147)}$ = 5.460, p = 0.0208; **Figure 12D**). *Post hoc* comparisons revealed that the nicotine-treated male group displayed increased burst events than the male vehicle group (p = 0.0002), but there were no significant differences between both female groups (p = 0.8593). The female vehicle group exhibited higher burst events than the male vehicle group as well (p = 0.0117). Moreover, analysis of burst frequency showed main effects of treatment and interaction between factors (Sex: $F_{(1,147)}$ = 2.071, p = 0.1523; Treatment: $F_{(1,147)}$ = 7.233, p = 0.0080; Interaction: $F_{(1,147)}$ = 5.731, p = 0.0179; **Figure 12E**). *Post hoc* comparisons indicated that nicotine-treated male rats exhibited higher burst frequency than male vehicle rats (p = 0.0002), but there were no significant differences between female treatment groups (p = 0.8455). Female vehicle rats also displayed elevated burst frequency compared to male vehicle rats (p = 0.0124).

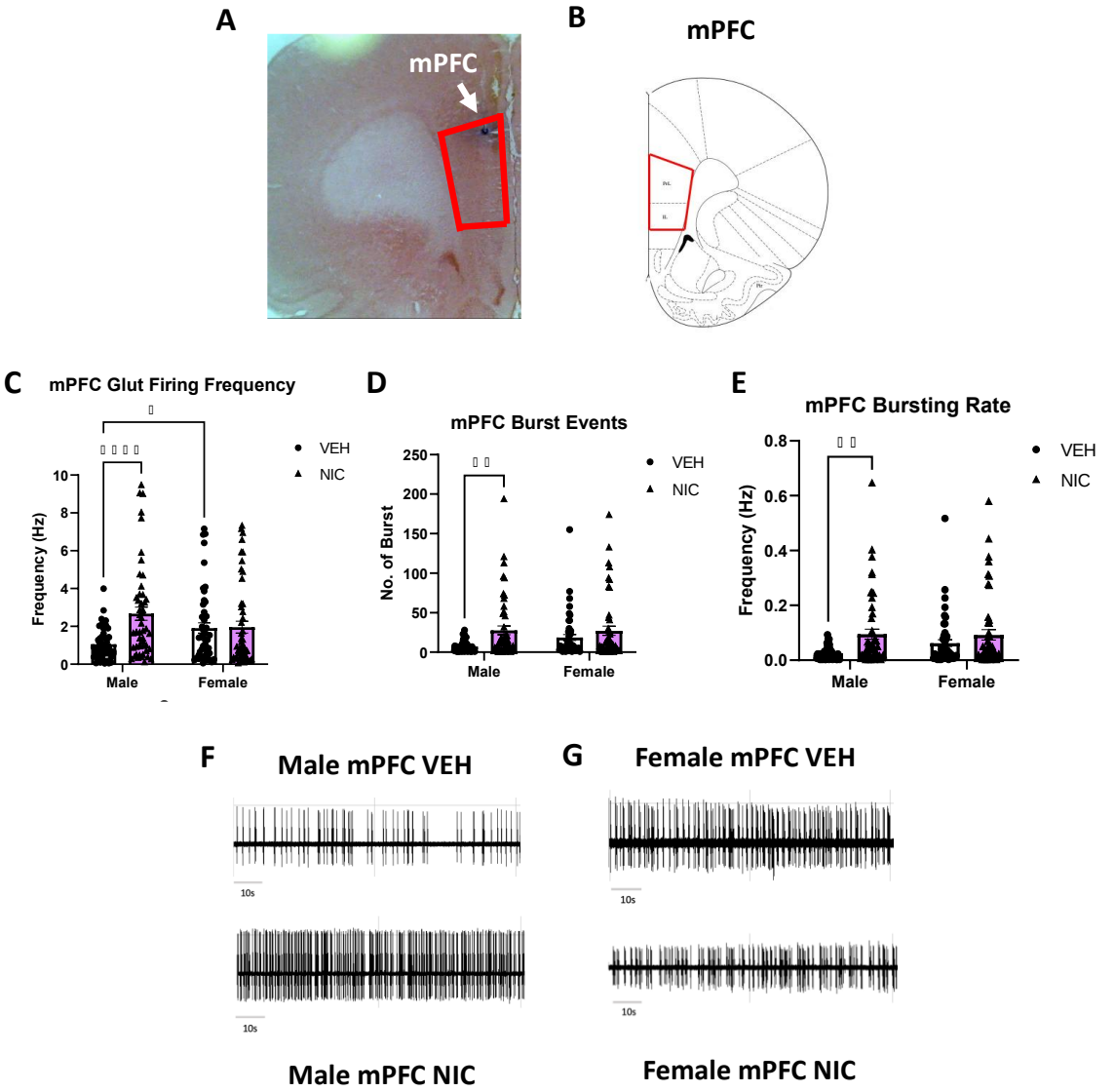

Figure 11. Chronic adolescent nicotine exposure results in dysregulated baseline spiking activity of mPFC pyramidal neuron in a sex-specific manner. (A) Microphotograph showing mPFC recording site with iontophoretic marking (indicated by white arrow); mPFC = medial prefrontal cortex, marked by red box. **(B)** Brain region of mPFC from rat brain atlas, indicated by red box. **(C, D, E)** Nicotine-treated male rats exhibited increased firing frequency, burst events, and bursting rate (*n* = 49-50 cells/treatment/sex from 11 rats) of mPFC pyramidal/glutamatergic neurons compared to male vehicle rats. No significant differences were observed between both female groups in these parameters. Female vehicle rats also displayed higher firing frequency than male vehicle rats. **(F, G)** Trace patterns of representative mPFC pyramidal neurons recorded

from vehicle and nicotine groups in each sex. Comparisons were made with two-way ANOVA followed by Fisher's LSD *post hoc* test, *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, two-tailed.

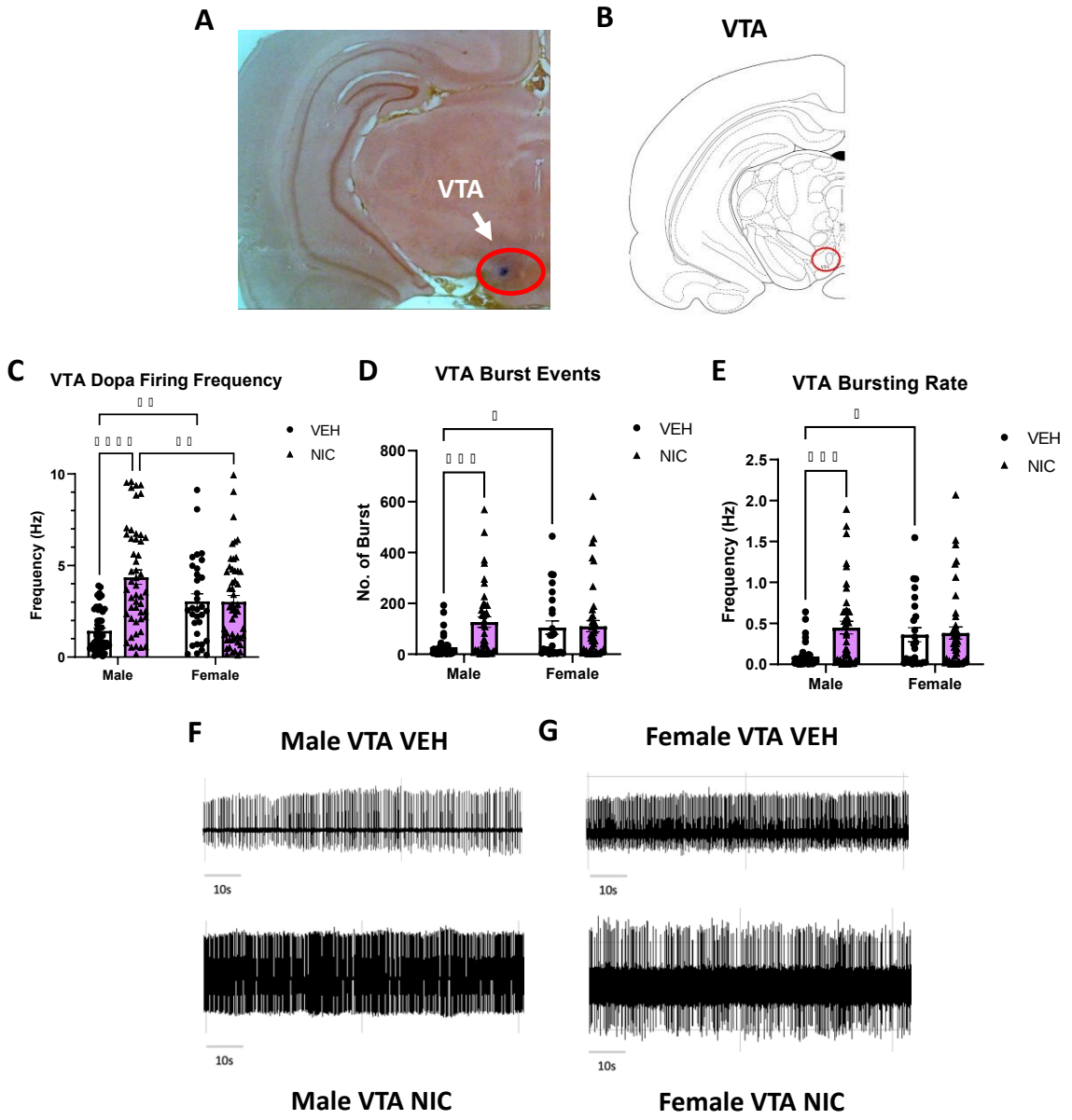

Figure 12. Sex-specific effects of chronic adolescent nicotine exposure on baseline spiking activity of VTA DA neurons. (A) Microphotograph showing VTA recording site with iontophoretic marking (indicated by white arrow); VTA = ventral tegmental area, marked by red circle. **(B)** Brain region of VTA from rat brain atlas, indicated by red circle. **(C, D, E)** Nicotine-treated male rats exhibited increased firing frequency, burst events, and bursting rate (*n* = 25-50 cells/treatment/sex from 11 rats) of VTA DA neurons compared to male vehicle rats. No significant differences were observed between both female groups in these parameters. Female vehicle rats also displayed elevated activity in

these measures than male vehicle rats. **(F, G)** Trace patterns of representative VTA DA neurons recorded from vehicle and nicotine groups in each sex. Comparisons were made with two-way ANOVA followed by Fisher's LSD *post hoc* test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, two-tailed.

3.2.2 Adolescent nicotine exposure leads to sex-specific disruption in mPFC oscillatory states

Alterations in oscillatory states in the brain have been reported following chronic adolescent nicotine exposure, as well as in patients with anxiety, depression, and cognitive deficits (Hudson et al., 2021; Oathes et al., 2008; Adhikari et al., 2010; Meerwijk et al., 2015; Grünewald et al., 2018; Proskovec et al., 2018). Hence, we examined mPFC oscillatory states with LFP signals in adult male and female rats following chronic adolescent nicotine exposure (**Figure 13A, 13B, 13C**). Two-way ANOVA analysis revealed main effects of sex and interaction between factors on delta wave activity (Sex: $F_{(1,193)} = 32.09$, $p < 0.0001$; Treatment: $F_{(1,193)} = 1.487$, $p = 0.2242$; Interaction: $F_{(1,193)} = 8.912$, $p = 0.0032$; **Figure 13D**). *Post hoc* Fisher's LSD indicated that nicotine-treated male rats exhibited decreased delta activity compared to male vehicle rats ($p = 0.0027$), but no significant differences between female treatment groups ($p = 0.2235$). Female vehicle rats also displayed reduced delta activity compared to male vehicle rats ($p < 0.0001$). Statistical analysis of theta activity demonstrated main effects of sex and interaction between factors Sex: $F_{(1,194)} = 24.34$, $p < 0.0001$; Treatment: $F_{(1,194)} = 3.391$, $p = 0.0671$; Interaction: $F_{(1,194)} = 9.179$, $p = 0.0028$; **Figure 13E**). *Post hoc* comparisons indicated that nicotine-treated male rats exhibited increased theta activity compared to male vehicle rats ($p = 0.0005$), but no significant differences between female treatment groups ($p = 0.4131$). Female vehicle rats also displayed elevated theta activity than male vehicle rats ($p < 0.0001$). Furthermore, analysis of alpha activity demonstrated main effects of sex, treatment, and interaction between factors (Sex: $F_{(1,183)} = 23.00$, $p < 0.0001$; Treatment: $F_{(1,183)} = 10.03$, $p = 0.0018$; Interaction: $F_{(1,183)} = 5.563$, $p = 0.0194$; **Figure 13F**). *Post hoc* comparisons showed that nicotine-treated male rats exhibited increased alpha activity compared to male vehicle rats ($p < 0.0001$), but no significant differences between female treatment groups ($p = 0.5855$). Female vehicle rats also displayed elevated alpha activity than male vehicle rats ($p < 0.0001$). Analysis of beta activity revealed main effects of sex (Sex: $F_{(1,191)} = 14.54$, $p = 0.0002$; Treatment: $F_{(1,191)} = 1.887$, $p = 0.1712$; Interaction: $F_{(1,191)} = 2.056$, $p = 0.1533$; **Figure 13G**). *Post hoc* comparisons showed that nicotine-treated male rats exhibited increased beta activity compared to male vehicle rats ($p = 0.0444$), but no significant differences between female

treatment groups (p = 0.9667). Female vehicle rats also displayed elevated beta activity than male vehicle rats (p = 0.0001). Last but not least, analysis of gamma activity showed main effects of sex (Sex: $F_{(1,193)}$ = 43.24, p < 0.0001; Treatment: $F_{(1,193)}$ = 1.098, p = 0.2961; Interaction: $F_{(1,193)}$ = 3.129, p = 0.0785; **Figure 13H**). *Post hoc* comparisons demonstrated that nicotine-treated male rats exhibited increased gamma activity compared to male vehicle rats (p = 0.0428), but no significant differences between female treatment groups (p = 0.6186). Female vehicle and nicotine-treated rats also displayed elevated gamma activity than their male counterparts respectively (male VEH vs. female VEH: p < 0.0001; male NIC vs. female NIC: p = 0.0015).

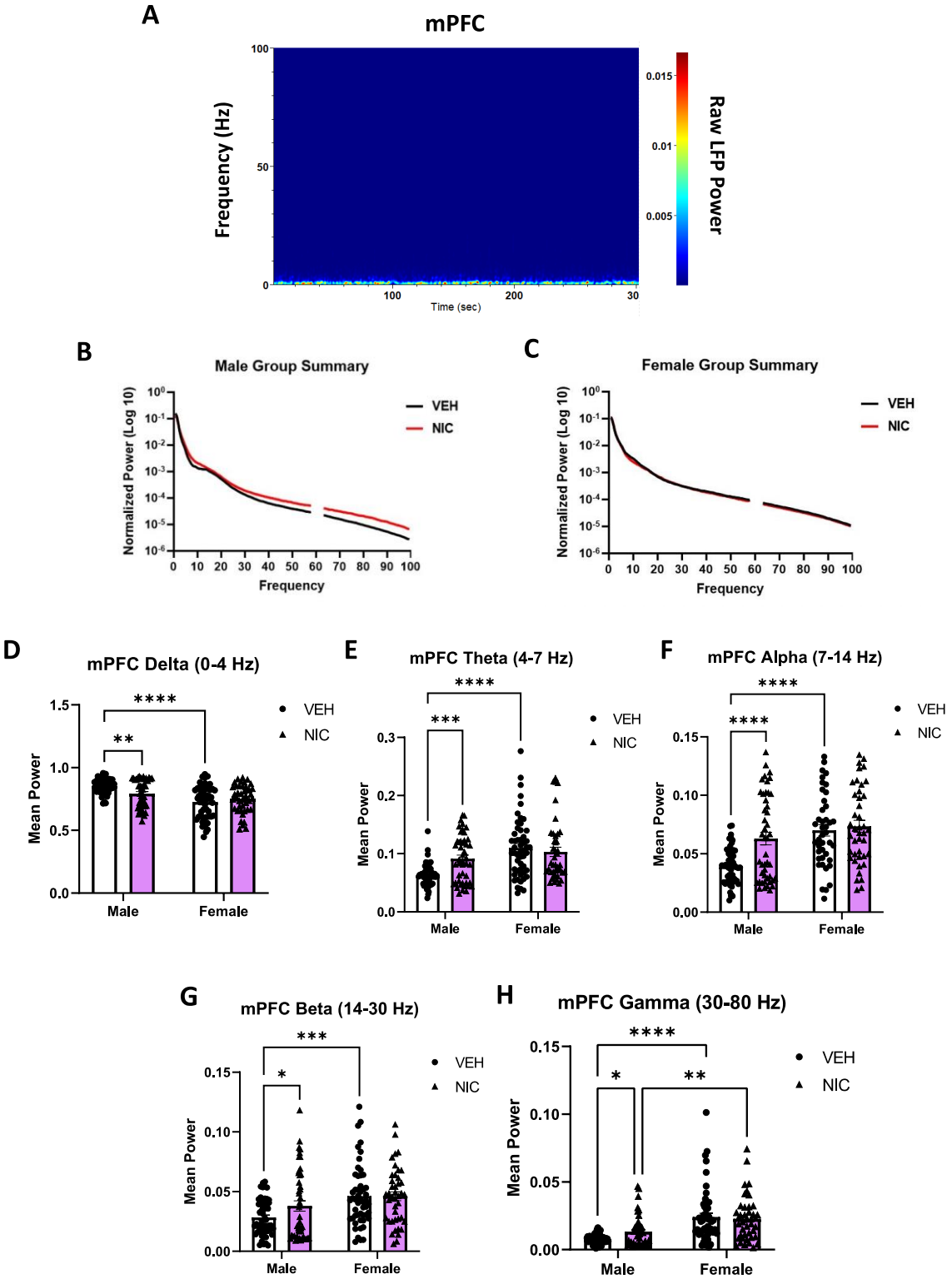

Figure 13. Sex differences in spontaneous mPFC oscillations in adulthood following chronic adolescence nicotine exposure. (A) Representative spectrogram of a 5-min

recording in mPFC. **(B, C)** Average normalized LFP power spectra in the mPFC between vehicle vs nicotine groups in each sex. **(D, E F, G, H)** Nicotine-treated male rats experienced a decrease in delta (0-4 Hz) oscillations but increases in theta (4-7 Hz), alpha (7-14 Hz), beta (14-30 Hz), and gamma oscillations (30-80 Hz) compared to the male vehicle rats, but no significant differences were observed between both female groups (n = 42-58 recording/treatment/sex from 11 rats). The female vehicle group exhibited decreased delta activity, but increased theta, alpha, beta and gamma activity compared to the male vehicle group. Nicotine-treated female rats also displayed elevated gamma oscillation than nicotine-treated male rats. Comparisons were made with two-way ANOVA followed by Fisher's LSD *post hoc* test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, two-tailed.

3.3 Tissue Molecular Analysis Results

3.3.1 Western Blots

Since nicotine exposure affects neurotransmission in the mesocorticolimbic system, and changes in neurotransmitter dynamics are shown to be associated with anxiety and depressive disorders (Le Houezec, 2003; Li et al., 2021; Malhi & Mann, 2018; Penninx et al., 2021), we also investigated protein expression levels in the mPFC and NAc of selected molecular targets relevant to nicotine exposure and neuropsychiatric disorders.

3.3.1.1 mPFC molecular targets

First, we assessed expression levels of α7 and β2 nAChR in the mPFC, which have been reported to play a role in attention, learning and working memory (Dalley et al., 2001; Hahn et al., 2003; McIntyre et al., 2013). Western blot analysis revealed that nicotine-treated male rats experienced reduction in α7 and β2 nAChR levels in the mPFC compared to male vehicle rats (α7: U = 10.00, p = 0.0016; β2: U = 10.00, p = 0.0400; **Figure 14B, 14C**), but there were no significant differences between female treatment groups (α7: U = 46.00, p = 0.7959; β2: U = 23.00, p = 0.9015; **Figure 14B, 14C**).

Next, we examined VGLUT1 and GAD65 levels in the mPFC, since dysregulated E/I balance in the mPFC has been indicated to be involved in anxiety and depressive disorders (Veeraiah et al., 2014; Czéh et al., 2018; Arnone et al., 2015; Jobson et al., 2019; Bi et al., 2013; Yildiz-Yesiloglu & Ankerst, 2006). Statistical analysis indicated no significant differences in VGLUT1 levels between treatment groups in each sex (male: U = 20.00, p = 0.6200; female: U = 24.00, p = 0.4418; **Figure 15B**). However, nicotine-treated male rats exhibited reduced GAD65 level in the mPFC compared to male vehicle rats (U = 10.00, p = 0.0401; **Figure 15C**), whereas nicotine-treated female rats displayed increased GAD65 level than female vehicle rats (U = 11.00, p = 0.0152; **Figure 15C**).

D1R and D2R in the mPFC have been reported to be involved in cognitive function and regulation of E/I balance (Floresco & Magyar, 2006; Seamans & Yang, 2004; Sawaguchi and Goldman-Rakic, 1994; Abi-Dargham et al., 2002; Li et al., 2011;

Robinson & Sohal, 2017). Therefore, we next investigated expression levels of D1R and D2R in the mPFC. Western blot analysis demonstrated that nicotine-treated male rats showed decreased D1R level in the mPFC compared to male vehicle rats (U = 7.00, p = 0.0262; **Figure 16B**), whereas nicotine-treated female rats exhibited increased D1R level than female vehicle rats (U = 5.00, p = 0.0111; **Figure 16B**). For D2R, nicotine-treated male rats displayed higher D2R level in the mPFC than male vehicle rats (U = 4.00, p = 0.0070; **Figure 16C**), whereas nicotine-treated female rats experienced reduced D2R level compared to female vehicle rats (U = 5.00, p = 0.0221; **Figure 16C**).

BDNF is involved in synaptic plasticity and integrity, and aberration in BDNF levels in the brain has been demonstrated in pre-clinical and clinical studies of mood and anxiety disorders (Minichiello et al., 2002; Panja et al., 2014; Bosch et al., 2014; Ji et al., 2010; Karege et al., 2005; Monteggia et al., 2007; Lindholm and Castrén, 2014). Thus, we also examined expression level of BDNF in the mPFC. Statistical analysis revealed that nicotine-treated male rats displayed lower BDNF level in the mPFC than male vehicle rats (U = 0.00, p = 0.0012; **Figure 17B**), whereas nicotine-treated female rats showed elevated BDNF level than female vehicle rats (U = 14.00, p = 0.0101; **Figure 17B**).

3.3.1.2 NAc Molecular Targets

In the NAc, we first examined expression levels of D1R and D2R, which have been demonstrated to be involved in motivation and reward learning behaviours (Hikida et al., 2010; Kravitz et al., 2012; Francis et al., 2015; Soares-Cunha et al., 2020). Deficits in these behaviours are often observed in depressive disorders (Li et al., 2021; Datta et al., 2021). Western blot analysis revealed that nicotine-treated male rats exhibited lower D1R level in the NAc than male vehicle rats (U = 1.00, p = 0.0013; **Figure 18B**), whereas nicotine-treated female rats displayed higher D1R level compared to female vehicle rats (U = 6.00, p = 0.0175; **Figure 18B**). For D2R, nicotine-treated male rats experienced increased D2R level in the NAc than male vehicle rats (U = 11.00, p = 0.0152; **Figure 18C**), whereas nicotine-treated female rats showed decreased D2R level compared to female vehicle rats (U = 5.00, p = 0.0221; **Figure 18C**).

Next, we investigated GAD65 level in the NAc, as GABA signaling in the NAc has been reported to modulate VTA DA release (Yang et al., 2018). Statistical analysis indicated that nicotine-treated male rats exhibited lower GAD65 level in the NAc compared to male VEH group (U = 6.00, p = 0.0350; **Figure 19B**), whereas there were no significant differences between female groups (U = 24.00, p = 0.4418; **Figure 19B**).

Last but not least, BDNF signaling in the NAc has been suggested to play a role in depressive disorders (Berton et al., 2006; Eisch et al., 2003; Krishnan et al., 2007; Taliaz et al., 2013). Thus, we last examined BDNF level in the NAc. Statistical analysis demonstrated that nicotine-treated male rats exhibited elevated BDNF level in the NAc than male vehicle rats (U = 4.00, p = 0.0080; **Figure 20B**), whereas nicotine-treated female rats experienced lower BDNF level compared to female vehicle rats (U = 5.00, p = 0.0411; **Figure 20B**).

3.4 Estrous Cycle Assessment

To investigate whether different stages of the estrous cycle exerted any effects on behavioural and electrophysiological outcomes, we attempted to collect vaginal samples and characterize estrous cycle stages for individual female rats. However, due to several technical difficulties and limitations, we could not establish a relationship between the estrous cycle and these corresponding experimental outcomes. Please refer to “Limitations” of the discussion section for further explanations.

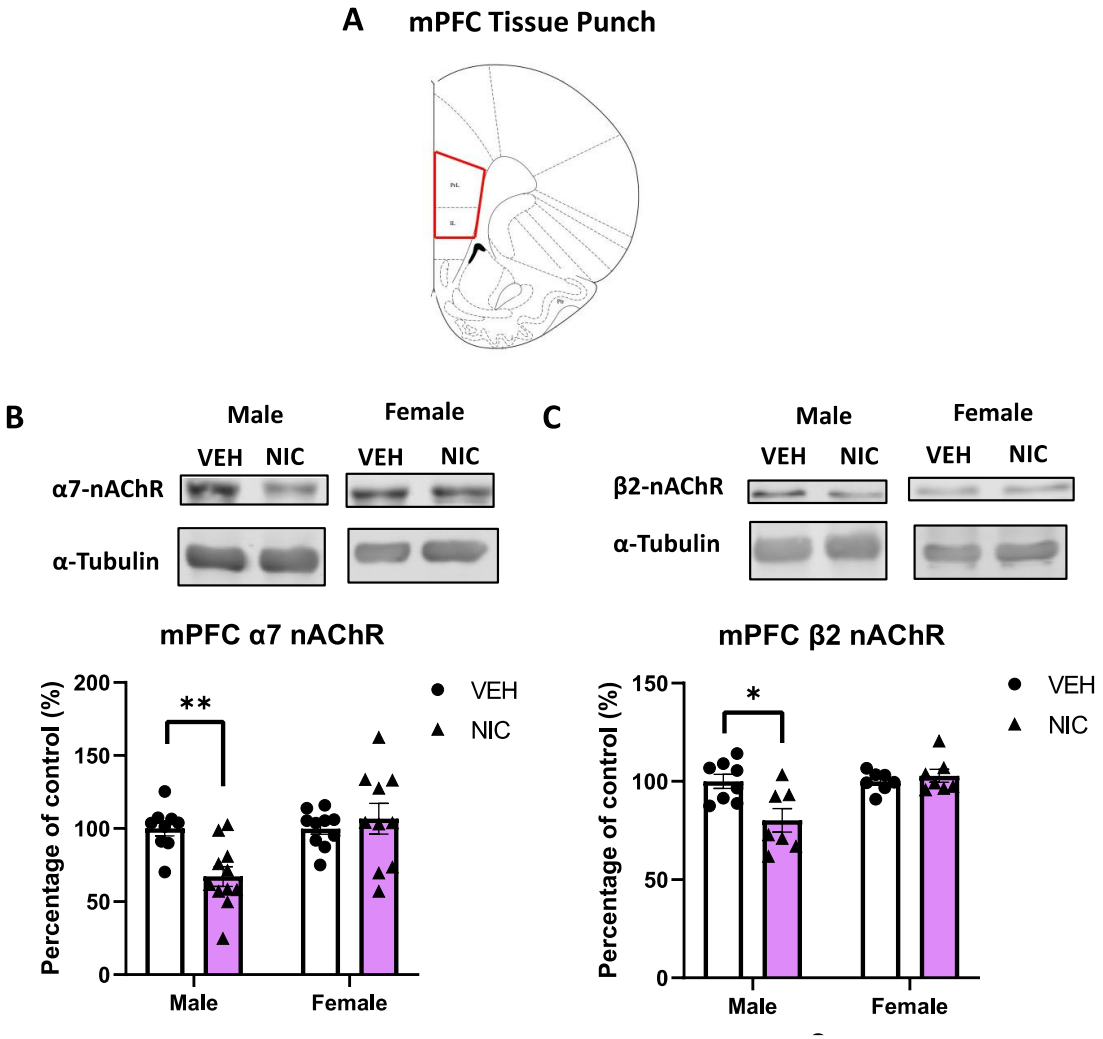

Figure 14. Chronic adolescent nicotine exposure induces sex-specific alterations in mPFC α7 and β2 nAChR expression levels. (A) Schematic representation of mPFC tissue punchout regions for molecular analyses. **(B, C)** *Top:* Representative western blots for α7 nAChR and β2 nAChR in the mPFC. *Bottom:* Densitometry analysis of protein expression levels between VEH and NIC groups in each sex. Nicotine-treated male rats experienced a reduction in α7 and β2 nAChR levels compared to male VEH group, whereas there were no significant differences between female groups (n = 7-11/treatment/sex). Within-group comparisons were made with Mann-Whitney U test, *$p < 0.05$, **$p < 0.01$.

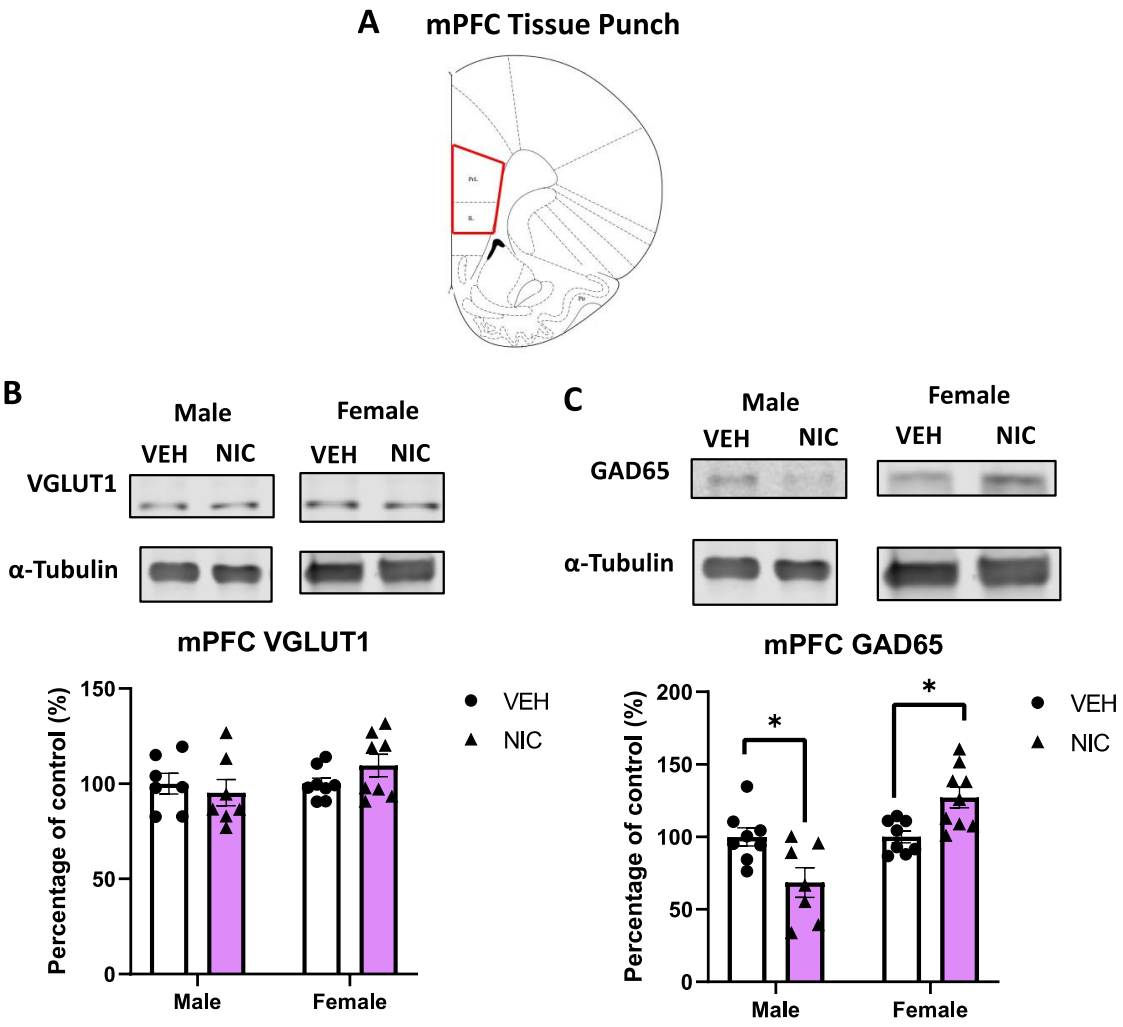

Figure 15. Sex differences in mPFC VGLUT1 and GAD65 levels following chronic adolescent nicotine exposure. (A) Schematic representation of mPFC tissue punchout regions for molecular analyses. **(B, C)** *Top:* Representative western blots for VGLUT1 and GAD65 in the mPFC. *Bottom:* Densitometry analysis of protein expression levels between VEH and NIC groups in each sex. **(B)** There were no significant differences in mPFC VGLUT1 levels between treatment groups in each sex. (n = 7-8/treatment/sex). **(C)** Nicotine-treated male rats exhibited lower GAD65 level in the mPFC compared to male vehicle rats, whereas nicotine-treated female rats displayed higher GAD65 level than female vehicle rats (n = 7-9/treatment/sex). Within-group comparisons were made with Mann-Whitney U test, *$p < 0.05$.

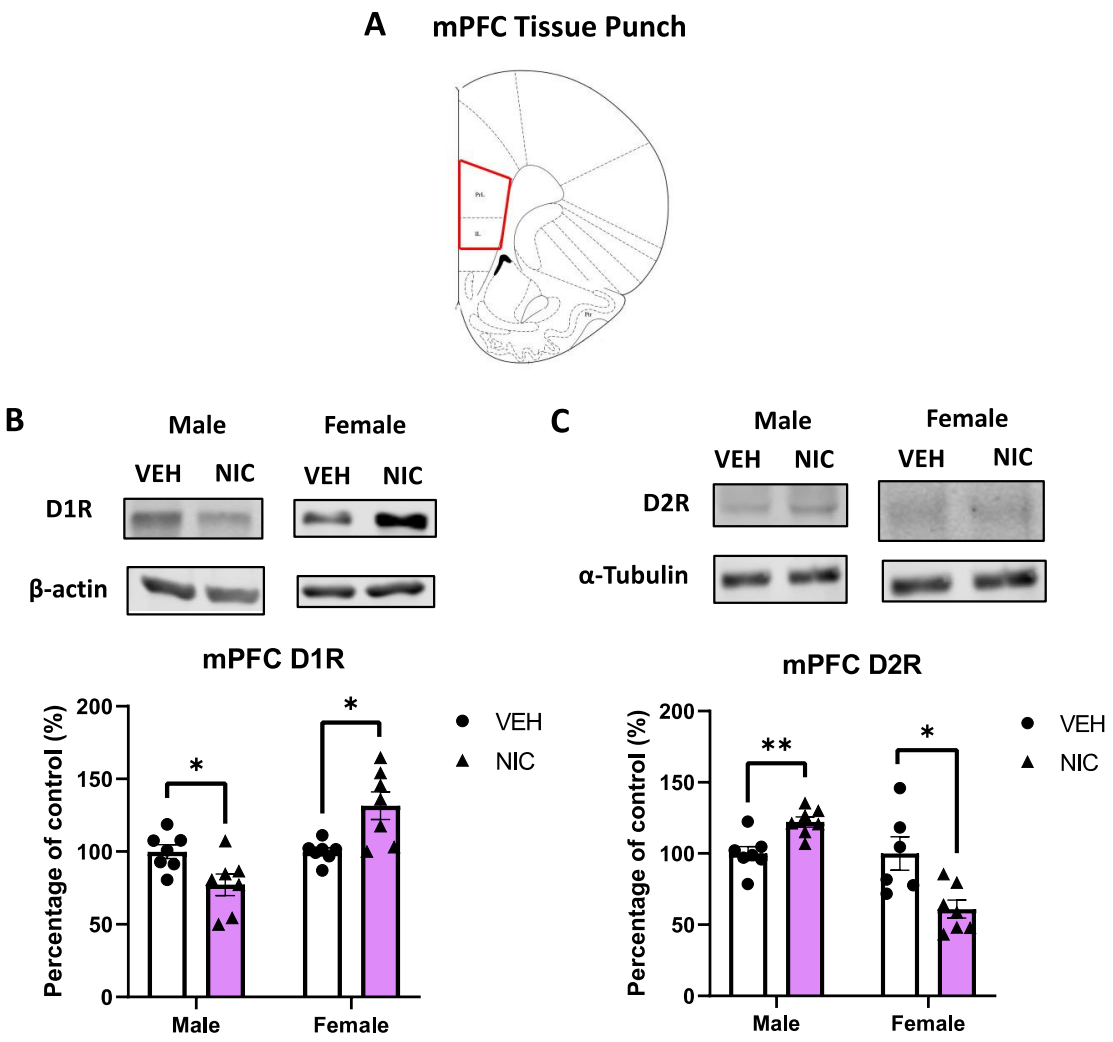

Figure 16. Chronic adolescent nicotine exposure leads to sex-specific changes in D1R and D2R expression levels in the mPFC. (A) Schematic representation of mPFC tissue punchout regions for molecular analyses. **(B, C)** *Top:* Representative western blots for D1R and D2R in the mPFC. *Bottom:* Densitometry analysis of protein expression levels between VEH and NIC groups in each sex. **(B)** Nicotine-treated male rats displayed lower D1R level in the mPFC than male vehicle rats, whereas nicotine-treated female rats displayed higher D1R level compared to female vehicle rats (n = 7/treatment/sex). **(C)** Nicotine-treated male rats experienced increased D2R level in the mPFC than male vehicle rats, whereas nicotine-treated female rats showed lower D2R level compared to female vehicle rats (n = 7-8/treatment/sex). Within-group comparisons were made with Mann-Whitney U test, *$p < 0.05$, **$p < 0.01$.

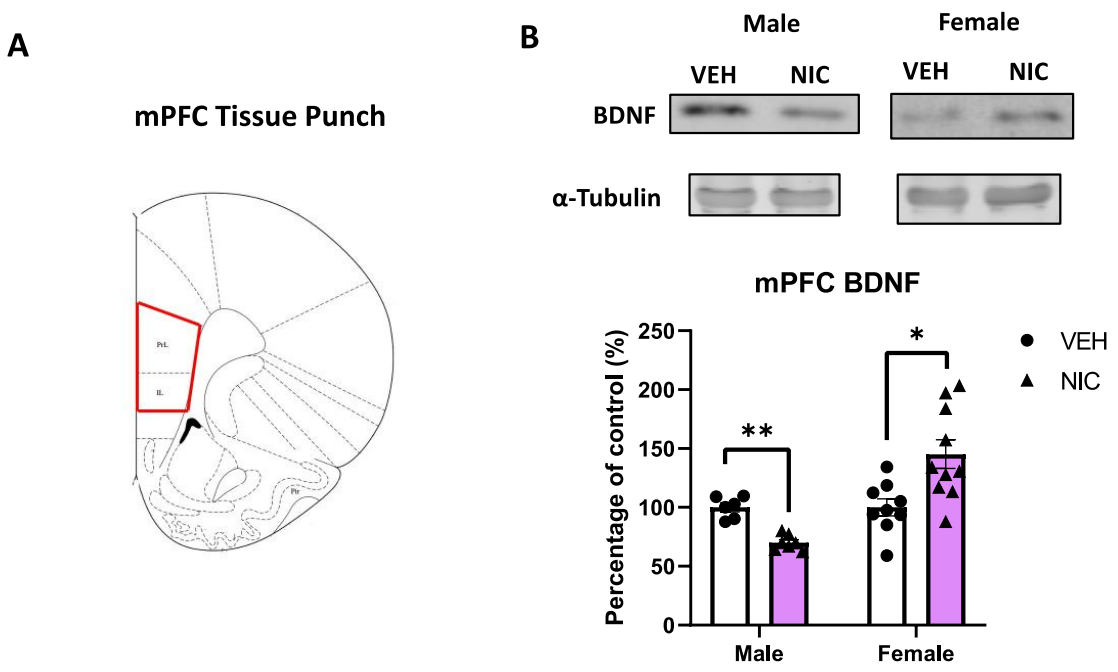

Figure 17. Sex-specific alterations in mPFC BDNF level following chronic adolescent nicotine exposure. (A) Schematic representation of mPFC tissue punchout regions for molecular analyses. **(B)** *Top:* Representative western blots for BDNF in the mPFC. *Bottom:* Densitometry analysis of protein expression levels between VEH and NIC groups in each sex. Nicotine-treated male rats exhibited lower BDNF level in the mPFC than male vehicle rats, whereas nicotine-treated female rats showed increased BDNF level compared to female vehicle rats (n = 6-10/treatment/sex). Within-group comparisons were made with Mann-Whitney U test, *$p < 0.05$, **$p < 0.01$.

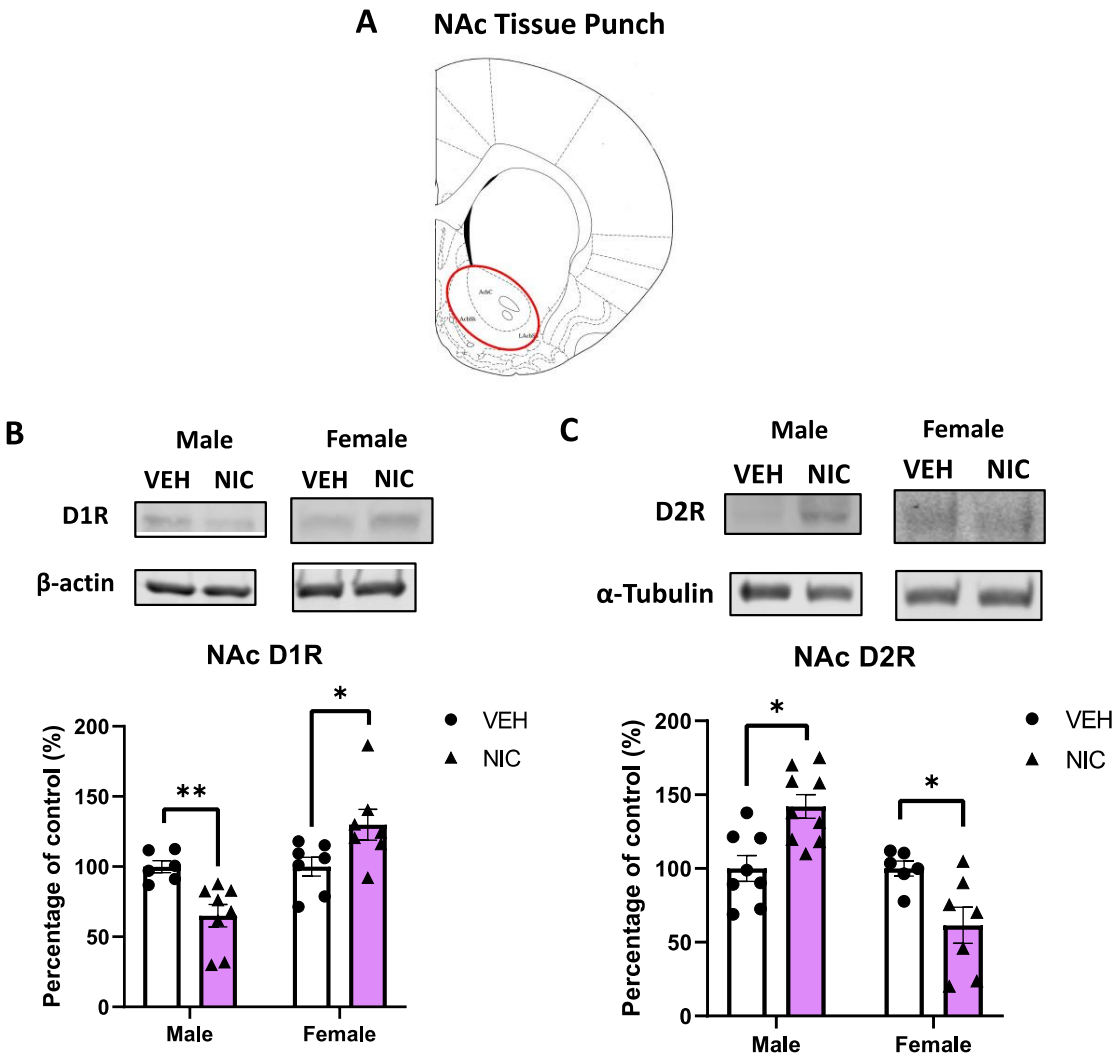

Figure 18. Chronic adolescent nicotine exposure induces sex-specific changes in D1R and D2R expression levels in the NAc. (A) Schematic representation of NAc tissue punchout regions for molecular analyses. **(B, C)** *Top:* Representative western blots for D1R and D2R in the NAc. *Bottom:* Densitometry analysis of protein expression levels between VEH and NIC groups in each sex. **(B)** Nicotine-treated male rats exhibited decreased D1R level in the NAc than male vehicle rats, whereas nicotine-treated female rats displayed higher D1R level compared to female vehicle rats (n = 6-8/treatment/sex). **(C)** Nicotine-treated male rats experienced increased D2R level in the NAc than male vehicle rats, whereas nicotine-treated female rats showed lower D2R level compared to female vehicle rats (n = 6-9/treatment/sex). Within-group comparisons were made with Mann-Whitney U test, *$p < 0.05$, **$p < 0.01$.

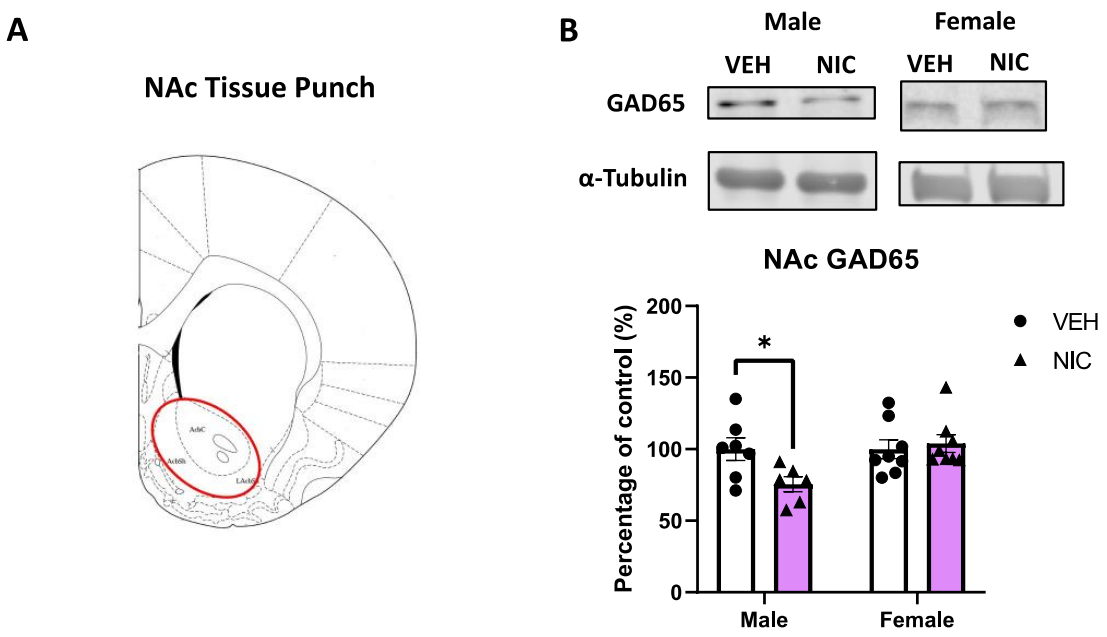

Figure 19. Sex-specific alterations in NAc GAD65 level following chronic adolescent nicotine exposure. (A) Schematic representation of NAc tissue punchout regions for molecular analyses. **(B)** *Top:* Representative western blots for GAD65 in the NAc. *Bottom:* Densitometry analysis of protein expression levels between VEH and NIC groups in each sex. Nicotine-treated male rats experienced reduced GAD65 level in the NAc compared to male VEH group, whereas there were no significant differences between female groups (n = 6-8/treatment/sex). Within-group comparisons were made with Mann-Whitney U test, $*p < 0.05$.

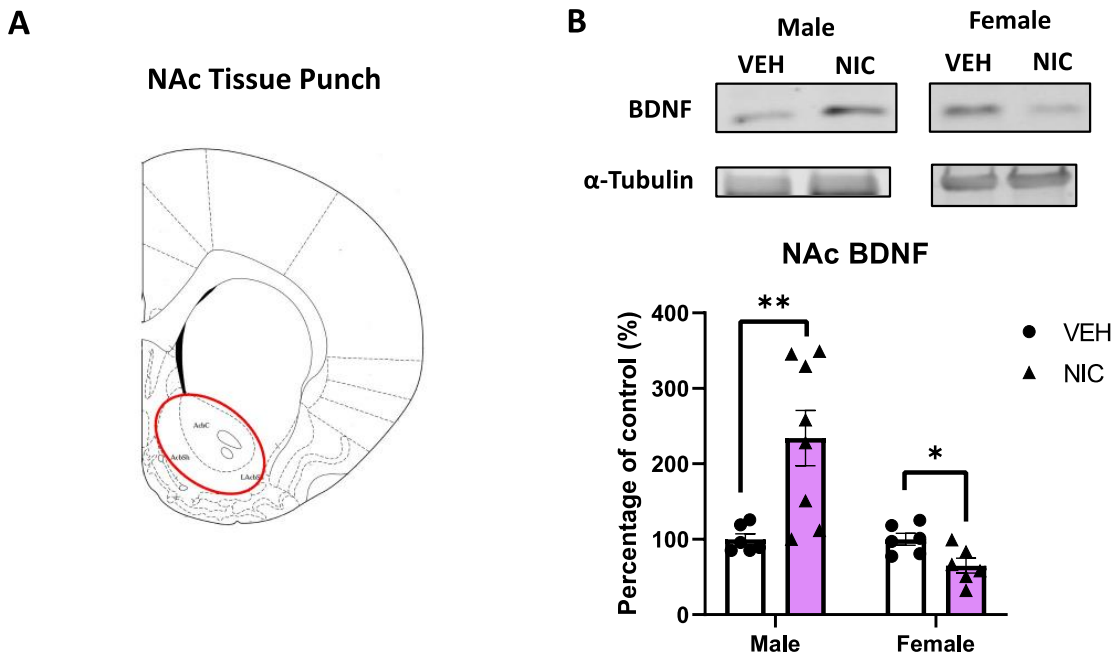

Figure 20. Chronic adolescent nicotine exposure results in sex-specific changes in BDNF expression level in the NAc. (A) Schematic representation of NAc tissue punchout regions for molecular analyses. **(B)** *Top:* Representative western blots for BDNF in the NAc. *Bottom:* Densitometry analysis of protein expression levels between VEH and NIC groups in each sex. Nicotine-treated male rats experienced elevated BDNF level in the NAc than male vehicle rats, whereas nicotine-treated female rats displayed decreased BDNF level compared to female vehicle rats (n = 6-8/treatment/sex). Within-group comparisons were made with Mann-Whitney U test, *$p < 0.05$, **$p < 0.01$.

Chapter 4

4 Discussion and Future Directions

The use of e-cigarettes among adolescents is currently rising, which presents a notable concern as the adolescent brain is highly vulnerable to the effects of psychotropic drug exposure. Clinical links between nicotine dependence and comorbidity with mood/anxiety disorders have been well-established (Martínez-Ortegaa et al., 2017; Boden et al., 2010; Moran et al., 2013; Sagud et al., 2019). Sex-specific trends in smoking behaviours and responses to nicotine replacement therapies have been reported as well (McLean et al., 2011; Ford & Erlinger, 2004; Torres & O'Dell, 2016; Perkins, 2001; Costello et al., 2011; Perkins et al., 1999; Barrett, 2010). The prevalence of mood and anxiety disorders is higher in female compared to male, and females experience greater difficulty in quitting nicotine products, as well as lower success with smoking cessation treatments. However, there remains a paucity in pre-clinical studies exploring how male and female respond differently to nicotine exposure. Sex-specific effects of adolescent nicotine exposure on the mesocorticolimbic system are also currently unclear in terms of behavioural, neuronal, and molecular outcomes. Hence, the present study examined sex differences in anxiety and depressive-related outcomes during adulthood following adolescent nicotine exposure.

In line with previous findings (Jobson et al., 2019; Hudson et al., 2021), we reported that nicotine-treated male rats displayed a host of anxiety and depressive-like behaviours, as well as spatial and recognition memory cognitive deficits. Importantly, these deficits were observed in early adulthood, underscoring the severity and endurance of these neurodevelopmentally-induced pathologies. In addition, they exhibited hyperactivity in spontaneous spiking events of pyramidal neurons and DA neurons in the mPFC and VTA respectively. Long-lasting alterations in mPFC baseline oscillation states were also observed in adult male rats following adolescent nicotine exposure. Last but not least, they experienced long-term changes in anxiety and depressive-related molecular markers in the mPFC and NAc. On the contrary, nicotine-exposed female cohorts did not demonstrate behavioural and neuronal abnormalities overall, but they

instead often exhibited opposing trends in several molecular targets compared to nicotine-treated male rats, indicative of potential compensatory interactions between estrogen/progesterone signaling pathways and the mesocorticolimic system, in response to chronic adolescent nicotine exposure.

4.1 Dysregulated Excitatory/Inhibitory Balance in Anxiety and Depressive Disorders

The anxiety and depressive-related phenotypes observed in the nicotine-treated male groups are consistent with previous reports, including reduced open arm time, central zone time, social motivation, cognitive deficits and increased immobility time (Jobson et al., 2019; Hudson et al., 2021; Counotte et al., 2011; Counotte et al., 2009). These anxiety and depressive-like behaviours were also accompanied by increases in baseline neuronal firing activity in the mPFC and VTA. Dysregulation of E/I balance in the PFC has been implicated in pre-clinical and clinical studies of anxiety and depression (Bi et al., 2013; Veeraiah et al., 2014; Czéh et al., 2018; Ghosal et al., 2017; Lener et al., 2017). For instance, Bi et al. (2013) reported that infusion of GABA-A receptor antagonist, bicuculine, into the IL region of the mPFC resulted in anxiety-like behaviours in adult male mice, including reduced center time in OFT and decreased open-arm exploration time in the EPM. This behavioural change was followed by smaller IPSCs and larger EPSCs in pyramidal neurons in the IL cortex, suggesting the role of mPFC imbalanced E/I level in anxiety-related behaviours. This proposition seems plausible given the connectivity between the IL cortex, limbic regions (e.g., NAc, amygdala) and the HPA axis in response to emotional stress (Radley et al., 2006; Bi et al., 2013; Page & Coutellier, 2019).

Apart from anxiety, aberrant glutamate and GABA neurotransmissions in the PFC are found in patients with MDD. Proton magnetic resonance spectroscopy revealed lower glutamate and GABA level in the DLPFC in MDD patients (Yildiz-Yesiloglu & Ankerst, 2006), a cortical region functionally homologous to the mPFC in rodent (Uylings et al., 2003). Arnone et al. (2015) also reported an association between decreased GABA levels and number of failed antidepressant treatments, which is a proxy for chronicity and severity of depressive illness course. Furthermore, dysregulations in glutamate and

GABA signaling have been shown in chronic stress and social defeat male rodent models of depression (Veeraiah et al., 2014; Czéh et al., 2018). Altogether, these results appear to be consistent with our findings, where we observed hyperactive mPFC pyramidal neurons and reduced mPFC GAD65 levels in the nicotine-treated male group, further implicating the involvement of PFC E/I balance in anxiety and depression-related behaviours.

4.2 Role of D1R and D2R in the PFC

Besides evidence for abnormal glutamate vs GABA balance in the mPFC, we observed significant changes in D1R and D2R levels, with a decrease in D1R but an increase in D2R level in nicotine-exposed male rats. Previous studies have indicated the role of these receptors in cognitive function and modulation of E/I balance in the mPFC (Floresco & Magyar, 2006; Seamans & Yang, 2004; Sawaguchi and Goldman-Rakic, 1994; Abi-Dargham et al., 2002; Li et al., 2011; Robinson & Sohal, 2017). For example, Sawaguchi and Goldman-Rakic (1994) performed local administration of D1R antagonist, such as SCH 23390 or SCH 39166, into the DLPFC of rhesus monkeys and reported pronounced deficits in an oculomotor delayed response task (for working memory). Other pharmacological studies have also demonstrated that blockade of D1R and D2Rs in the mPFC impaired performances in attention set-shifting tasks (Ragozzino, 2002; Floresco et al., 2006), suggesting a cooperative interaction between D1R and D2R that underlie behavioural flexibility in the PFC. Furthermore, D2R activation has been shown to enhance the excitability of mPFC pyramidal neurons through a stimulatory G-protein pathway (Robinson & Sohal, 2017). Thus, changes in D1R and D2R expression levels in the mPFC could be associated with the cognitive deficits and neuronal dysregulation observed in nicotine-exposed male rats. Nevertheless, future studies are required to further investigate the dynamics between DA signaling and glutamatergic activity in the PFC, as well as the connectivity between PFC and Hipp, provided the role of Hipp in learning and memory (Anand & Dhikav, 2012; Bird & Burgess, 2008).

4.3 Role of PFC α7 and β2 nAChR in Cognitive Function

The mPFC is involved in various cognitive processes, including goal-directed behaviours, working memory, and attention (Bloem et al., 2014). In particular, the

cholinergic system and nAChRs in the mPFC play a crucial role in attention and learning (Dalley et al., 2001; Hahn et al., 2003), specifically the α7 and β2 nAChRs (Valentine & Sofuoglu, 2018; Hoyle et al., 2006). Fernandes et al. (2006) reported that α7 nAChRs knockout male mice displayed worse performances in the delayed matching-to-place task of the Morris water maze, and stimulation of these receptors enhanced persistent neuronal firing of NMDAR-mediated/working memory-related circuits in the DLPFC (Yang et al., 2013). For β2-subunit, Guillem et al. (2011) demonstrated that β2 knockout male mice exhibited impaired attentional performance, and re-expression of β2 nAChRs in the PL region of the mPFC was sufficient to reverse the attentional deficits in these mice. Thus, reduction in both α7 and β2 nAChR expression levels in the mPFC could underlie the cognitive deficits in nicotine-exposed male rats, considering their established role in learning and attentional processing. In addition, the sensitivity of nAChRs varies across sub-units. For example, low-sensitivity α7-nAChRs are involved in modulating glutamate signaling and they desensitize slower than non-α7 subtypes that regulate GABA release (Mansvelder et al., 2009). This differential sensitivity in nAChR subtypes could result in greater glutamate vs GABA signaling following prolonged nicotine exposure, which could potentially be linked to the E/I imbalance seen in the nicotine-treated male group. Further investigations are necessary to study the role of α7 and β2 nAChR in cognitive ability following chronic adolescent nicotine exposure.

4.4 Brain Oscillation Activity in Anxiety and Depressive Disorders

Local field potentials (LFP) are the electric potentials generated from transient dipoles existing across the extracellular and intracellular space of neurons. These extracellular electric potentials change over time based on neuronal activity states, and form different wave or oscillatory patterns. Thus, LFP signals are thought to represent the aggregate changes in extracellular electric potentials of local populations of neurons, and they can be decomposed down to different frequencies of oscillations during analysis (Einevoll et al., 2013; Maling & McIntyre; 2016). Changes in oscillation states across various frequencies have been reported in patients with anxiety, depression and cognitive deficits. For example, higher levels of gamma activity were exhibited by patients with

general anxiety disorder during worry induction tasks, which were also negatively correlated with subjective emotional experience immediately following the task (Oathes et al., 2008). In a male mouse model of anxiety, theta activities and synchronization between the mPFC and the ventral hippocampus were also increased in the OFT and EPM, and the increase in mPFC theta oscillation was associated with inhibition of exploratory behaviours in both arenas (Adhikari et al., 2010). In addition, reduced delta activity was correlated with psychological pain in adults with a history of depression (Meerwijk et al., 2015). Grünewald et al. (2018) also reported contralateral asymmetry in resting frontal alpha oscillation in adolescents with unipolar depression. In another study, elevated beta activity was shown to be correlated with poorer performance in a spatial working memory task (Proskovec et al., 2018). Overall, we observed widespread alterations in mPFC oscillation states across different frequencies, including decreased delta activity, and increased theta, alpha, beta, gamma activity, which appears to be consistent with previous findings and possibly correlated with behavioural deficits displayed by the nicotine-treated male group. Nonetheless, most of these findings are associative in nature. Thus, caution should be taken when drawing mechanistic inferences for the roles of these patterns in the observed phenotypes. Future studies, perhaps employing awake neuronal recordings during behavioural tasks may be able to more precisely link these aberrant patterns with specific affective and/or cognitive task outcomes.

4.5 BDNF and Depressive Disorders

In recent years, BDNF has been a central point of discussion in terms of understanding the etiology of mood disorders, particularly due to its functional role in neurogenesis and synaptic plasticity in the CNS (Yang et al., 2020). BDNF is an upstream signaling molecule that binds to TrkB, and activates various downstream signaling cascades, including the Ras-mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositide 3-kinase-protein kinase B (PI3K-Akt) pathway and the PLCγ-Ca^{2+} pathway to carry out their corresponding function (Leal et al., 2017). For example, BDNF-TrkB signaling mediates LTP in the hippocampus through the recruitment of PLCγ, and the consolidation of LTP at hippocampal synapses requires

prolonged BDNF-TrkB signaling (Minichiello et al., 2002; Panja et al., 2014). BDNF was also shown to be involved in the increase of spine density of CA1 pyramidal neurons via the MAPK-extracellular signal-regulated protein kinase (ERK) pathway (Alonso et al., 2004). Furthermore, the effects of BDNF extend to the structural integrity of synapses as well, via its interaction with local cytoskeletal-related proteins (Bosch et al., 2014). Different downstream cellular responses also depend on the temporal aspect of BDNF-TrkB signaling, where transient BDNF-TrkB activation facilitates neurite elongation and spine head enlargement; and sustained activation promotes neurite branching and spine neck elongation (Ji et al., 2010). Besides synaptic plasticity and integrity, studies have reported that BDNF could stimulate neural progenitor cells proliferation and promote cell survival (Katoh-Semba et al., 2002; Sairanen et al., 2005; Scharfman et al., 2005). Overall, BDNF appears to be an upstream regulator of various cellular functions in the CNS.

In depressive disorders, dysfunction in neural plasticity, which can be defined as the capability of neuronal adaptation (e.g., generation of new cells) in response to a changing environment (Duman et al., 1999), has been proposed to be one of the underlying etiological mechanisms. Following exposure to environmental stressors, intracellular signaling cascades in brain circuitries are activated, and such signal transduction pathways have been shown to play a key role in modulating neuronal atrophy, neurogenesis and synaptic plasticity (Jeon & Kim, 2016). In the case of mood disorders, these signaling cascades are thought to be disrupted. Studies have demonstrated that negative stress and adverse experiences can result in significantly decreased proliferation of granule cells in the hippocampus, which could disrupt the process of memory formation and learning (Duman et al., 1999; Colla et al., 2007). Indeed, patients with MDD often experience reduced volume in regions of the mesocorticolimbic circuitry as well, such as the dorsal and ventral mPFC, the hippocampus and the amygdala (Yu & Chen, 2011; Belleau et al., 2019; McKinnon et al., 2009; Videbech & Ravnkilde, 2004; Wise et al., 2017; Treadway et al., 2015). This reduction in volume is thought to be associated with neuronal atrophy and the loss of synaptic integrity. Repeated stress could also reduce the dendritic complexity of PFC and hippocampal neurons (Radley et al., 2006a,b), and a decrease in synapse number in the

PFC has been shown in subjects with MDD (Kang et al., 2012). In addition, sub-anaesthetic dose of ketamine, which is an antagonist of NMDAR and plays a pivotal role in synaptic plasticity, can produce rapid antidepressant effects in patients with treatment-resistant depression (Berman et al., 2000; Zarate et al., 2006). Chronic administration of typical antidepressants can also enhance synaptic plasticity at several levels, by increasing neuronal proliferation in the hippocampus, upregulating neurotrophic factor expression, and increasing synapse formation (Castrén & Hen, 2013; Krishnan & Nestler, 2010; Duman & Aghajanian, 2012). Taken together, increasing evidence is pointing towards the involvement of disrupted neurogenesis and synaptic structure/plasticity in the pathology of depression.

Given the role of BDNF in neurogenesis and synaptic plasticity, it seems plausible that alterations in BDNF expression levels could be associated with disrupted synaptic integrity in depression pathophysiology. Post-mortem studies in humans have reported lower BDNF level in the PFC of suicide patients (Karege et al., 2005), and repeated stress in adult male rats led to dendritic shortening and decreased BDNF level in the mPFC (Brown et al., 2005). Male mice with the loss of BDNF expression in forebrain regions also displayed depressive-like behaviours (Monteggia et al., 2007; Lindholm and Castrén, 2014). Antidepressant drugs have been demonstrated to increase BDNF mRNA levels in the PFC after several days of treatment (Kozisek et al., 2008). Moreover, BDNF microinfusion into the mPFC also produced antidepressant effects, whereas anti-BDNF neutralizing antibody in the mPFC suppressed the antidepressant effect of ketamine (Kato et al., 2018). Altogether, these findings allude to the point that the therapeutic effects of antidepressant on synaptic plasticity in the PFC could be dependent on BDNF signaling to a certain extent.

On the other hand, BDNF signaling in the mesolimbic circuitry appears to have the opposite, pro-depressant effect. The VTA→NAc DA circuitry plays a critical role in drug addiction as well as motivation and reinforcing behaviours (Berhow et al. 1995; Supekar et al., 2018; Papakostas, 2020; Miyanishi & Nitta, 2021). Hence, abnormalities in the mesolimbic DA pathway are thought to be involved in anhedonia, a central feature of mania and depression (Nestler & Carlezon, 2006; Nestler, 2015; Nestler et al., 2002).

In contrast to the antidepressant effects of BDNF in the PFC, Berton et al. (2006) reported that local BDNF infusion in the VTA exerts depressive-like effects in the FST, where ablation of BDNF in the NAc induced antidepressant effects in the social defeat paradigm (Berton et al., 2006; Eisch et al., 2003). Clinical studies have also showed increased BDNF protein levels in the NAc of depressed patients, and reduction of BDNF expression in the VTA produced antidepressant effects (Krishnan et al., 2007; Taliaz et al., 2013). However, the precise mechanisms behind this opposite effect of BDNF in the NAc are currently unknown. Future studies are necessary to further investigate this relationship.

Overall, these data seem to be consistent with our findings in the nicotine-treated male group, with depressive-like behaviours in the FST and sociability test, as well as lower BDNF level in the mPFC and higher BDNF level in the NAc, suggesting regional-specific effects of BDNF in pathogenesis of depressive disorders.

4.6 Striatal Dopaminergic Circuitry in Depressive Disorders and Addiction

As mentioned previously, the VTA→NAc pathway is heavily involved in addiction and motivation-related behaviours (Berhow et al. 1995; Supekar et al., 2018; Papakostas, 2020). In the current study, we observed an increase in VTA DA neuron spontaneous firing activity in the nicotine-exposed male rats, which appears to be consistent with pre-clinical social defeat models of depression (Chaudhury et al., 2013; Yorgason et al., 2013) and previously observed following adolescent nicotine exposure (Jobson et al., 2018) and delta-9-tetrahydrocannabinol exposure (Renard et al., 2017a). Previous evidence has shown that optogenetic activation of VTA→NAc DA neurons rapidly induced depressive-like phenotypes in stress-resilient male mice; and inhibition of these neurons promoted resilience, whereas inhibition of VTA→mPFC DA projection induced susceptibility (Chaudhury et al., 2013), indicating pathway-specific function of DA neurons underlying depressive-like behaviours. Clinical studies have also demonstrated that lower striatal response to happy stimuli was positively correlated to higher level of anhedonia in depression, whereas increased striatal activity was seen in mania (Keedwell et al., 2005). Furthermore, in this study, lower D1R and higher D2R

level were observed in the nicotine-treated male group, where the nicotine-treated female group displayed opposite trends. Various reports have suggested distinctive roles of NAc D1R and D2R in anxiety/depressive-like behaviours and reward/aversive learning (Hikida et al., 2010; Kravitz et al., 2012; Francis et al., 2015; Soares-Cunha et al., 2020). For instance, Francis et al. (2015) utilized chemogenetic approach to inhibit NAc D1R-MSNs and observed depressive-like behaviours in resilient male mice in a chronic social defeat stress model. They also reported optogenetic activation of NAc D1R-MSNs reversed these depressive-like behaviours. In contrast, repeated activation of NAc D2R-MSNs induced social avoidance in this model. On the other hand, D1R and D2R appears to serve discrete roles in reinforcement and punishment as well. Reinforcement increases the frequency of a behaviour, while punishment decreases the frequency of specific behaviours (Kravitz et al., 2012). For example, Lobo et al. (2010) reported that optogenetic stimulation of NAc D1R-MSNs enhanced cocaine preference in a CPP paradigm, whereas activation of NAc D2R-MSNs suppressed this conditioned behaviour. Taken together, these findings are hinting at the interactions between NAc D1R and D2R behind different anxiety/depressive-like behaviours and reinforcing behaviours. Over-expression of D2Rs in the striatum has also been shown to increase EPSCs and decrease IPSCs in mPFC pyramidal neurons (Bi et al., 2013). Finally, our lab has reported previously that acute pharmacological blockade of DA receptors in the NAc can actually switch nicotine aversion into reward signals (Laviolette et al., 2008). This might suggest that chronic adolescent nicotine exposure might lead to long-term overstimulation of NAc DA receptors and subsequent downregulation of NAc DA receptors, as we report specifically for D1R levels in the present thesis. In turn, this state of intra-NAc D1R downregulation may ultimately lead to a pathological increase in nicotine's appetitive properties, similar to the effects of acute DA receptor antagonist blockade (Laviolette and van der Kooy, 2003; Laviolette et al., 2008) which could further worsen nicotine dependence into adulthood. Overall, these aberrations in the VTA→NAc mesolimbic DA pathway could underlie the behavioural, electrophysiological and molecular deficits observed in nicotine-exposed male rats in this study. Nonetheless, future studies are needed to further examine the roles of NAc D1R and D2R in the current adolescent

nicotine exposure model, and investigate downstream molecular signaling cascades behind these depressive-related phenotypes.

In the NAc, GABAergic MSNs expressing D1R project to the VTA and innervate local DA neurons (Van Bockstaele & Pickel, 1995; Yang et al., 2018). These GABAergic neurons have also been shown to directly regulate DA neurotransmission in the VTA by exerting inhibitory control over local DA neurons (Yang et al., 2018). Moreover, decreased GABA release from these neurons has been associated with depressive-like behaviours in pre-clinical rodent models (Zhu et al., 2017; Francis et al., 2015). In the present study, reduction in GAD65 expression level in the NAc was observed in nicotine-treated male rats, which possibly indicates the loss of local inhibitory control over VTA DA signaling. This seems to be in line with previous findings, as well as the hyperactivity in VTA DA neuron spiking events, and depressive-like behaviours experienced by the nicotine-treated male group. However, future investigations are still necessary to examine the mechanisms behind GABAergic signaling in the NAc and the connection to depression etiology.

4.7 Sex-Specific Responses to Nicotine Exposure

Nevertheless, despite the widespread aberrations in anxiety and depressive-related outcomes in the nicotine-treated male cohorts, no significant differences were observed within or across female treatment groups, apart from several opposing trends in GAD65, D1R, D2R, and BDNF expression levels. Here, we propose several possible explanations behind these sex-specific outcomes. First, female rats might exhibit lower sensitivity to the pharmacological effects of nicotine than male rats (Isiegas et al., 2009; Lopez et al., 2003). Pre-clinical studies have reported female mice exposed to nicotine were more sensitive to the conditioned rewarding effects of nicotine, where male mice were more directly responsive to nicotine concentrations in drinks (Isiegas et al., 2009). In human studies, male smokers were more accurate in discriminating placebo vs nicotine-containing nasal spray compared to females (Perkins, 1999; Perkins et al., 1994). Female smokers also experienced greater craving relief from a denicotinized tobacco, alluding to their preference towards the sensory aspects (e.g., puffs) of smoking (Perkins, 2009; Perkins, 2001; McBride et al., 2006; Barrett, 2010). Hence, a different exposure method

(e.g., vaping chamber) may present a more appropriate model for female Sprague-Dawley rats in the current protocol.

Second, prior studies have suggested sex differences in smoking behaviours, where females tend to smoke for non-nicotine-related factors (e.g., to relieve stress and negative moods) vs. male smokers who smoke primarily for the reinforcing effects of nicotine (which may also be more dependent on the mesolimbic DA pathway) (Perkins et al., 1999; Perkins et al., 2012). Female smokers also exhibited less DA response in the ventral striatum (including the NAc) than in the dorsal striatum to nicotine stimulation compared to male smokers, further implicating a lower sensitivity to the rewarding effects of nicotine in females (Cosgrove et al., 2014). Hence, the current nicotine-treated female group could have experienced lower sensitivity to the rewarding effects of nicotine. Third, sensitivity to nicotine exposure could be dependent on the strain of rat used. For example, Faraday et al. (2003) have reported that nicotine exposure induced greater locomotor activity in Long-Evans female rats than Sprague-Dawley female rats, which further suggests a lower sensitivity to nicotine in Sprague-Dawley female rats. Furthermore, the female brain could be more protected against nicotine-induced pathological changes compared to the male brain. For example, studies have indicated that the female brain in rodents and humans develops earlier than the male brain in terms of synaptic production and pruning, as well as neuronal myelination (Yuan et al., 2015; Brenhouse & Andersen, 2011). Thus, the female brain might have been more developed and protected during the nicotine exposure window in this protocol. In addition, regarding the dosage and method of nicotine exposure, regimens of nicotine administration for female animals are relatively mixed in the literature. The route of nicotine exposure ranges from oral administration to intraperitoneal injections. Thus, future studies are required to establish a more consistent model for investigating adolescent nicotine exposure in female Sprague-Dawley rats and to address alternative windows of exposure during neurodevelopment.

Moreover, the absence of anxiety/depressive-like behaviours and cognitive deficits in nicotine-treated female rats could be attributed to the presence of estrogen in the body during behavioural testing. In clinical settings, menopausal women (with low

circulating estrogen level) often experience anxiety and mood swings (Gleason et al., 2015; Sahingoz et al., 2011; Bloch et al., 2000; Bromberger & Kravitz, 2011; Bromberger et al., 2013; Freeman et al., 2005). Hence, they are prescribed with hormone replacement therapy (HRT), which involves a mix of estrogen and progesterone, to help alleviate these menopausal symptoms (Gleason et al., 2015; Đoković et al., 2015; Toffol et al., 2015; Zweifel & O'Brien, 1997; Gordon & Girdler, 2014). In particular, estrogen in HRT is shown to possess antidepressant effects (Grigoriadis & Kennedy, 2002), whereas progesterone is included to protect against the risk of hyperplasia and adenocarcinoma of the endometrium (Campagnoli et al., 2005). Estrogen has also been demonstrated to improve cognitive performance via spinogenesis and synaptogenesis in the PFC and hippocampus (Hara et al., 2015). Therefore, circulating estrogen in female rats might have provided protection against the anxiogenic and depressogenic effects of chronic adolescent nicotine exposure.

In addition, estrogen and progesterone appear to have contrasting roles in different neurotransmission and receptor dynamics as well (Barth, 2015). Studies have reported that estrogen can increase glutamate, α7 nAChR and BDNF expression levels, suppress GABA input, upregulate D1R but decrease D2R level in the striatum (Yokomaku et al., 2003; Centeno et al., 2006; Allen & McCarson, 2005; Murphy et al., 1998; Hruska & Nowak, 1988; Bazzett & Becker, 1994). Estrogen could also directly potentiate responses of α4β2 nAChR (Paradiso et al., 2001). On the contrary, progesterone could exert opposing effects on these biomarkers except BDNF and dopamine, where it increases BDNF level and acts synergistically with estrogen to modulate the release of dopamine (Smith et al., 1987; Czoty et al., 2009; Yang et al., 2010; Cabrera et al., 1993; Kaur et al., 2007). β2 nAChR availability in female has also been shown to be negatively correlated to progesterone level (Cosgrove et al., 2012). Moreover, beyond the traditional notion of nuclear localization, estrogen and progesterone receptors have been found at the cellular membrane level, where they might play a role in the synaptic regulation of GABA and DA transmission in different brain regions (Almey et al., 2015; Brinton et al., 2008; Herbison, 1997; Wang, 2001). Hence, in the current study, electrophysiological results and the opposing molecular trends in

nicotine-exposed female rats could be attributed to compensatory actions mediated by estrogen or progesterone on various signaling pathways in the mesocorticolimbic system. Altogether, the interplay between estrogen and progesterone on these molecular targets seems to be critical in maintaining behavioural and neuronal homeostasis in the female body. Unfortunately, the relationship between the estrus cycle and various experimental outcomes could not be established due to the small sample size of data available. Future studies should increase the number of experimental subjects per stage cycle or manipulate circulating levels of estrogen and progesterone via ovariectomized rats to examine this relationship.

In the current study, we observed baseline sex differences in several behavioural outcomes between male and female vehicle rats, such as the FST and social interaction test. This raises a very important question: are there fundamental sex differences in behavioural circuitries under different experimental circumstances? The answer to this question seems more complex than expected. Studies have proposed that, in some cases, differential signaling pathways are activated in the male and female brain in response to the same stimulus (Bangasser & Cuarenta, 2021). As a result, different signaling pathways and neuronal adaptations could lead to discrete cellular events and downstream cascades, and ultimately result in sexually divergent responses. The same concept could be applied to the current study, where the same behavioural paradigm activated different signaling cascades in male and female rats and led to differential responses. On the other hand, activation of the same circuitry could drive distinct behaviours in each sex. For instance, oxytocin signaling in the mPFC mediates prosocial behaviours in female mice but increases anxiety-like behaviours in male mice (Nakajima et al., 2014; Li et al., 2016). Thus, we cannot assume that the same brain circuitry in male and female possesses the same functionality under identical circumstances. Taken together, these findings highlight the complexity of brain circuitry between sexes. Due to the historical bias of excluding female animals in experimental studies, most behavioural paradigms relevant to anxiety and depressive disorders were developed for male rodents, which prevents our further understanding of the female brain in the context of neuropsychiatric disorders. Therefore, future studies may be required to develop behavioural tests

specifically for female rodents to properly characterize female behavioural outcomes in relation to anxiety and depressive disorders.

Overall, our current findings seem to contradict clinical findings where females experience higher prevalence of anxiety and depressive disorders, as well as challenges in smoking cessation therapies. Interestingly, previous studies have proposed sex differences in stress perception, where females report more subjective distress in stressful situations. For example, in the same stressful situation, females report heightened irritability and fear as well as decreased happiness vs. males, even though both have equivalent physiological responses (i.e., no significant differences in heart rate and plasma cortisol) (Kelly et al., 2007). Females have also been shown to possess higher emotional intelligence and be better at recognizing emotions (Poláčková Šolcová et al., 2017; Fischer et al., 2018). From these findings, it seems plausible that females may be more efficient in identifying their current emotional state, and smoking is utilized as a means to relieve their subjective distress and anxiety, rather than being more sensitive to the pharmacologically reinforcing effects of nicotine as reported in males. The ability in recognizing subjective emotional states could be a potential reason behind the high prevalence of mood and anxiety disorders in females. In addition, it has been shown that female smokers exhibit higher response in the dorsal striatum than in the ventral striatum to nicotine stimulation compared to male smokers (Cosgrove et al., 2014). This finding could possibly explain the obstacles experienced by female in nicotine cessation, given the role of the dorsal striatum in habit formation compared to the ventral striatum in reinforcing effects. Nevertheless, further investigations are necessary to determine the potential mechanistic factors behind these sex differences in nicotine sensitivity and anxiety/depressive disorders.

4.8 Limitations

There are several limitations in the present study. Most importantly, the observed alterations in behavioural, neuronal and molecular outcomes are correlational data, since they were collected at different timepoints during the experimental period. Hence, mechanistic and causal relationships between the experimental variables cannot be implied until future pharmacological, chemogenetic or optogenetic intervention studies

are conducted. Second, due to the possibility that female Sprague-Dawley rats possess lower sensitivity to the pharmacological effects of nicotine, and previous reports suggesting females are more sensitive to the sensory cues of smoking, a different nicotine exposure regime, such as a vaping chamber, would be a more appropriate model for female Sprague-Dawley rats. In addition, electrophysiology and molecular analysis only examined the PFC, VTA and NAc in the current study. Future studies could investigate how the Hipp and BLA are involved in chronic adolescent nicotine exposure and anxiety/depressive disorders, given their role in memory, learning (Bird & Burgess, 2008) and fear processing (Jhang et al., 2018).

Finally, several technical limitations exist in vaginal sample collections and estrous cycle interpretation. Microscopic evaluation of vaginal cytology may not be the most objective method for documenting stages of the estrous cycle. Under the microscope, cell density of vaginal smears could differ in the case of uneven sample volume. Assessment of the estrous cycle would thus be inferential, since the density of various cell types is the primary determinant of each stage using this technique (Cora et al., 2015). Therefore, measuring electrical impedance of the vaginal wall (based on thickness) would be a better approach to identify different stages of the cycle, without being confounded by inconsistent sampling method (Jaramillo et al., 2012; Chesney et al., 2020). In addition, transitions between stages present another challenge in categorizing each step of the estrous cycle, as they possess overlapping characteristics of two stages. Mentioned previously as well, the sample size in each stage of the cycle was not sufficient to conclude any relationships with behavioural and electrophysiological outcomes. Altogether, a more rigorous and objective method needs to be developed for estrous cycle assessment, and the sample size per stage should be increased in future studies to address these concerns.

Chapter 5

5 Conclusions

Smoking-related diseases remain one of the top global causes of preventable mortality. In recent years, the increase in teen vaping trends becomes particularly concerning since adolescence represents a critical stage of brain development, where complex changes occur at the synaptic and network level. The main psychoactive component in these products, nicotine, can affect the mesocorticolimbic circuitry by altering different neurotransmitter dynamics, such as glutamate, GABA and dopamine. Many pre-clinical and human studies have demonstrated that nicotine addiction is causally linked to mood and anxiety disorders. In clinical studies, female exhibits higher prevalence of mood and anxiety disorders. They also experience challenges in smoking cessation and lower efficacy in nicotine replacement therapies, suggesting a potential sex-specific response to nicotine. However, due to the historical bias of excluding female animals in pre-clinical studies, knowledge of sex-specific responses to nicotine exposure is limited in the extant literature. Thus, the present study aimed to determine any sex differences in mood and anxiety-related outcomes in response to chronic adolescent nicotine exposure using rodent model.

Our results indicated that nicotine-treated male rats displayed anxiety/depressive-like behaviours and cognitive deficits, such as reduced open arm time in the EPM, increased immobility time in FST, and lower recognition score in NOR. Aberrations in spontaneous neuronal activity and oscillation states were also observed in the PFC and VTA in this group. Furthermore, there were alterations in expression levels of anxiety and depressive-related molecular markers (e.g., α7 nAChR, VGLUT1, D1R/D2R, BDNF). Contrastingly, nicotine-treated female rats displayed no significant changes in behavioural and electrophysiological measures, but exhibited opposite trends in some molecular outcomes, indicating possible compensatory actions related to estrogen/progesterone signaling pathways in the female body. Overall, the current findings appear to be consistent with previous reports suggesting females are less sensitive to the pharmacological effects of nicotine. These corresponding results have

important implications in determining the sensitivity to nicotine in females, as well as exploring the interactions between nicotine, estrogen/progesterone, and different neurotransmitter dynamics in the female brain. Equally important is the question of why the male brain appears particularly vulnerable to adolescent nicotine. Are there neurobiological and/or hormonal factors that reduce protection against nicotine during adolescence, specifically in males? Regardless, the present data sets provide a viable platform for future research into sex-specific markers associated with anxiety and depressive disorders. Future studies could also investigate other brain regions within the mesocorticolimbic circuitry, including the Hipp and BLA, regarding their role in anxiety/depressive-related phenotypes following adolescent nicotine exposure. Finally, this study could serve as a therapeutic starting point for developing novel smoking intervention treatments, which would improve clinical outcomes in a sex-specific manner.

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