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Impact of vaporized nicotine and cannabis co-exposure in adolescence on sex-specific behavioural and neural effects in adulthood

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Abstract

Nicotine and cannabis are commonly co-used during adolescence, yet there is limited research on their long-lasting impacts on behavior and neural connectivity. This study examines the long-term effects of adolescent exposure to nicotine and high Δ9-tetrahydrocannabinol-cannabis (THC) flower vapor on brain development and reward-learning and seeking behavior. Male and female adolescent Sprague-Dawley rats received daily exposure to vaporized nicotine, THC, both nicotine and THC together, or vehicle vapor. In adulthood, behavioral tests and MRI scans were conducted. Female adult rats co-exposed to both nicotine and THC in adolescence demonstrated a heightened sensitivity to reward, while males exposed to THC displayed learning impairments. Males exposed to THC also expressed one network with increased functional connectivity compared to the co-exposed nicotine and THC group. Our results indicate that co-exposure to vaporized cannabis and nicotine during adolescence produces long-term sex-specific effects in reward- and cognition-related behavior.

Keywords

Cannabis, tetrahydrocannabinol, nicotine, locomotion, cognition, addiction, MRI

Summary for Lay Audience

Nicotine and cannabis are commonly used substances by adolescents in Canada, with their dual use being on the rise among Canadian high school students in recent years. Limited research exists surrounding the consequences of nicotine and cannabis co-use in adolescence on subsequent behavior and brain development in adulthood. Evidence suggests that exposure to nicotine or cannabis in isolation during early stages of brain development, such as in adolescence, alters brain activity and behavioral and cognitive outcomes. However, the long-term outcomes of adolescent nicotine vapor and cannabis co-exposure remain unclear, and this lack of information may facilitate the continual use of both substances by adolescents under the guise of no harmful outcomes. Thus, this study aimed to characterize the long-term behavioral and neural effects of nicotine and cannabis vapor co-exposure using male and female adolescent rats. Adolescent rats were administered either nicotine vapor, cannabis vapor, both vapors, or air and vapor containing no active substances daily for 14 days. After reaching adulthood, exposed rats then underwent behavioral tests that explored reward learning and reinforcing behavior. We found that cannabis impaired reward-learning in males only; an effect that seemed to be neutralized by co-exposure to nicotine. We also found that the co-exposure of cannabis and nicotine in females induced hyper-reactivity to a new setting, which may be indicative of increased reward sensitivity. We then explored the effects of adolescent cannabis and nicotine exposure on adult brain activity. Brain activity was increased in a network involved in sensory processing, responsible for smell and hearing, in adult male rats exposed to cannabis, compared to males co-exposed to cannabis and nicotine. In contrast, females exhibited no alterations in brain network connectivity across groups. This study's findings suggest that adolescent nicotine and cannabis exposure may lead to opposing or additive long-term behavioral and neural effects, which differ across sexes.

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1 Introduction

1.1 Nicotine and Cannabis Co-use

Nicotine and cannabis are frequently used by adolescents; from 2013-2018, the dual use of nicotine and cannabis has increased among Canadian high school students (Zuckermann et al., 2019). Additionally, Rao et al. (2023) reported that 13% of American middle and high school students under 18 used nicotine e-cigarettes and cannabis, according to the 2020 National Youth Tobacco Survey. Among Canadian adolescents, the most common methods of nicotine consumption include electronic cigarette (e-cigarette) vaping (Rotermann & Gilmour, 2022), and of cannabis use include smoking dried cannabis flower. Ample research exists investigating the effects of both adult and adolescent exposure to either nicotine or cannabis (Belluzzi et al., 2004; Schneider & Koch, 2003; Schuster et al., 2016). Yet there is limited research surrounding the effects of nicotine and cannabis co-use in adolescence on subsequent reward-related behaviors and functional neural connectivity in adulthood. This research is further limited by the ethical consequences preventing drug exposure and subsequent examination in humans, necessitating the use of preclinical models to investigate possible relationships between the co-use of these substances and resultant consequences on brain development and behavior (Nelong et al., 2019; Renda et al., 2020). Understanding the implications of co-exposure could inform strategies to reduce the co-use of nicotine e-cigarettes and cannabis among adolescents.

1.2 Adolescent Neurodevelopment

Adolescence is a period of life characterized by developmental changes that occur when transitioning from childhood and adulthood, including ages around 10-19 years (Spear, 2000). One of the main changes that take place during this period is puberty, or the onset of sexual maturation which occurs at around 13 years of age in females, and 15 years in males (Spear, 2000). The critical period of neurodevelopment during adolescence involves the reduction of gray matter and an increase in white matter volume via the reorganization and refinement of neuron synapses in cortical and limbic brain regions (Jaworska & MacQueen, 2015; Schneider, 2013). These processes lead to the manifestation of behaviors that characterize adolescence such as increased novelty- and reward-seeking behaviors which can contribute to the increased tendency to experiment with recreational drugs (Schneider, 2013). Since adolescence is a critical period of

neurodevelopment, it is also a time when the brain is particularly sensitive to exogenous substances, including psychoactive drugs like nicotine and THC (Thorpe et al., 2020). Thus, adolescents are both at increased risk for initiating recreational substance use and are highly vulnerable to their neuropsychological effects.

Rats are often used as animal models in neurodevelopmental research, due to their physiological and behavioral parallels with humans (Clancy et al., 2007). For example, female rats typically reach sexual maturation earlier than males which is similar in humans (Schneider, 2013). These physiologic and behavioral similarities are important when studying the adolescent phase which is marked by significant neurobiological and behavioral transformations (Spear, 2000). While adolescence in humans approximately spans the ages of 10-19 years, in rats this period has been estimated to correspond to post-natal days (PND) 28-42 which includes their pre-pubertal and pubertal periods (Spear, 2000). Beyond sexual maturation, the period of adolescence in rats is also characterized by changes in neurodevelopmental processes and behavioral phenotypes. For example, critical brain development processes such as myelination, synaptic pruning, and neurotransmitter receptor level alterations in rats during this period are comparable to those observed in human adolescents (Schneider, 2013). Behavioral traits common during human adolescence, such as increased novelty seeking, risk-taking, and exploratory behaviors, are also prominently observed in adolescent rats (Spear, 2000; Stansfield & Kirstein, 2006). These similarities in rats allow for more translatable conclusions when studying the neural and behavioral impacts of adolescent drug exposure in an animal model.

1.3 Nicotine

Nicotine, a psychoactive substance found primarily in tobacco products, has long been a subject of public health concern due to its addictive nature and adverse health effects (Becker & Rice, 2022). More recently, the use of electronic cigarettes, or vaping, has become the most common method of use (Rotermann & Gilmour, 2022). Among adolescents, the prevalence of vaping nicotine in Canada has become a significant issue, with 14% of youth aged 15-19 years old reporting vaping in the past 30 days in 2022 (*Canadian Tobacco and Nicotine Survey (CTNS)*, 2023). Nicotine exerts its psychoactive effects primarily through its interaction with nicotinic acetylcholine receptors (nAChRs) in the brain which are distributed throughout the peripheral and central nervous systems (Verplaetse et al., 2018). Investigating the effects of nicotine vapor

exposure on adolescent brain development and behavior is critical for elucidating its long-term and short-term consequences.

1.3.1 Neurobiological Effects of Nicotine

Nicotine is a potent cholinergic stimulant that exerts its main effects through its interaction with nAChRs in the brain composed of alpha and beta subunits. The interaction between nicotine and its receptor leads to the activation of various neurotransmitter systems, with profound implications for neuronal function and behavior (Dani & Bertrand, 2007). Upon entering the brain, nicotine binds to nAChRs, a family of ligand-gated ion channels, facilitating the influx of cations such as calcium and sodium, which in turn modulates neuronal excitability (Gotti et al., 2006). This influences dopaminergic pathways associated with reward and addiction (Picciotto et al., 1998) and affects glutamatergic and GABAergic systems, critical for cognitive processes and neural inhibition, respectively (Mansvelder et al., 2002). In the CNS, nAChRs mediate processes including cell excitability, neuronal integration, and neurotransmitter release of acetylcholine, dopamine (DA), serotonin, gamma-aminobutyric acid (GABA), and glutamate (Gotti et al., 2006; Levin et al., 2006). The effects of nicotine interacting with nAChRs thereby influence physiological functions such as arousal, fatigue, anxiety, central processing of pain, and several cognitive functions (Gotti et al., 2006). These receptors are widely distributed throughout the central and peripheral nervous systems, and nAChRs carrying the β2 subunit play a critical role in mediating the rewarding and reinforcing properties of nicotine (Verplaetse et al., 2018).

The acute effects of nicotine on nAChRs enhance neurotransmitter release, promoting attention, learning, and memory (Levin et al., 2006). However, chronic nicotine exposure leads to neuroadaptive changes, including receptor desensitization and upregulation, contributing to dependence and withdrawal symptoms upon nicotine cessation (Changeux, 2010). In adolescent rodents, chronic nicotine exposure has been shown to upregulate nAChRs that contain α4β2 subunits that mediate the reinforcing effects of nicotine (Counotte et al., 2012). This nicotine-induced upregulation interferes with the normal developmental decrease of these receptors from early adolescence to adulthood in the medial PFC which may contribute to the increased sensitivity to nicotine experienced in adolescence (Counotte et al., 2012). Increased expression of nAChRs has also been found in the brains of human smokers post-mortem, indicating long-term neurochemical changes (Perry et al., 1999). In addition, adolescent nicotine exposure has been

shown to cause persistent alterations in the dopamine (DA) system via increased activity in dopaminergic neurons in the mesolimbic ventral tegmental area (VTA), and the downregulation of DA receptors in the PFC (Jobson et al., 2019). Daily injections of nicotine during adolescence in rats alters GABA signaling in the VTA, and subsequently increases reward-seeking tendencies in adulthood via upregulated alcohol self-administration (Thomas et al., 2018). Functional Magnetic Resonance Imaginb (fMRI) studies have shown that nicotine administration in adolescence leads to reduced functional connectivity between the hypothalamus and cortical regions (Frie et al., 2024). Understanding the lasting neurobiological effects of nicotine is crucial for elucidating the mechanisms underlying its cognitive alterations in isolation and concurrently with other substances, providing a foundation for developing interventions to mitigate the adverse health impacts.

1.3.2 Behavioral Effects of Nicotine

Nicotine exerts differing behavioral effects between adolescent and adult rats. For example, nicotine exposure in adolescence leads to increased locomotor activity, while in adults it results in reduced locomotion (Cao et al., 2010). Evidence suggests the reinforcing effects of nicotine in rodents are more intense during adolescence compared to adulthood. In a conditioned place preference task, used to assess rewarding effects of stimuli, adolescent rats showed enhanced sensitivity to the rewarding effects of nicotine (Belluzzi et al., 2004; Brielmaier et al., 2008; Vastola et al., 2002), yet they also displayed reduced sensitivity to the aversive effects of nicotine (Shram et al., 2006; Torres et al., 2008; Wilmouth & Spear, 2004). The increased tendency of adolescent rats to self-administer nicotine more readily than adults may stem from the heightened rewarding sensations and diminished withdrawal symptoms experienced in adolescence (Gellner et al., 2016; Levin et al., 2006).

Research has consistently shown that exposure to nicotine during adolescence leads to persistent deficits in critical executive functions, such as impaired attention, impulsivity (Counotte et al., 2009), and reduced capacity for learning complex sequences (Fountain et al., 2008). Beyond these cognitive impairments, nicotine use during this developmental stage significantly influences behaviors involved in reward learning. A previous study further elucidates the heightened risk-taking and novelty-seeking behaviors observed in adolescent rodents exposed to nicotine (Adriani & Laviola, 2004), suggesting a broad spectrum of behavioral changes. Similar behavioral effects

of nicotine are found in humans as well. Human adolescents who smoke are more likely to seek new experiences and exhibit a greater tendency to discount delayed rewards compared to non-smokers (Peters et al., 2011). Heightened affinity for reward learning after adolescent nicotine exposure has been found in two previous studies investigating the effects of nicotine exposure on appetitive Pavlovian learning, suggesting that nicotine exposure enhances the motivational value of stimuli indicative of reward (Quick et al., 2014; Ruffolo et al., 2022). These studies highlight the cognitive deficits and behavioral changes that can arise from early nicotine exposure, painting a comprehensive picture of the altered novelty seeking, and reward-learning implications posed by adolescent nicotine use.

1.4 Cannabis

Cannabis is a substance that is commonly used recreationally among adolescents and adults, with approximately 18% of Canadian adolescents aged 12-18 reporting use in the past 12 months in 2021-2022 (Canada, 2023). Cannabis is comprised of many components including flavonoids, terpenoids, and cannabinoids (Turgeman & Bar-Sela, 2017). The main psychoactive component of cannabis is Δ9- tetrahydrocannabinol (THC), which is primarily responsible for the effects of cannabis on the brain (Urits et al., 2020).

1.4.1 Neurobiological Effects of Cannabis

The neurobiological impact of cannabis, particularly evoked through its psychoactive component, THC, on the adolescent brain involves complex interactions with the body's endogenous endocannabinoid system (Jacobus & Tapert, 2014). The endocannabinoid system is a neuromodulatory system that plays a role in regulating cognitive and emotional functions to influence behavior, and is dysregulated in mood disorders and neurological conditions like epilepsy and schizophrenia (Capodice & Kaplan, 2021). THC acts as a partial agonist of the cannabinoid receptor type 1 (CB1R) and CB2R, both G-protein coupled receptors (GPCR). While CB1R is the most prominent subtype, both types of cannabinoid receptors are predominantly located within the CNS (Zou & Kumar, 2018). CB1R activation has been found to inhibit GABA and glutamate release from presynaptic terminals, which facilitates its ability to modulate neurotransmission (Zou & Kumar, 2018). The normal functioning of endocannabinoids (eCBs), particularly the CB1R full-agonist, 2-arachidonoylglycerol (2-AG), modulates synaptic activity by

regulating neurotransmitter release at both excitatory and inhibitory synapses (Araque et al., 2017). Such mechanisms of eCBs are critical for maintaining homeostasis within the central nervous system, influencing various physiological and cognitive processes including pain perception, mood regulation, and memory formation (Meyer et al., 2018; Prini et al., 2020). However, THC from external sources like cannabis binds to CB1 receptors across the brain, disrupting the homeostasis maintained by eCBs (Prini et al., 2020). This disturbance in synaptic regulation can lead to impairment of the brain's circuit networks' functional connectivity, manifesting in the cognitive deficits commonly associated with cannabis use such as memory and motor impairment (Prashad & Filbey, 2017; Prini et al., 2020).

Adolescent cannabis exposure results in altered endocannabinoid signaling which alters the structure and function of the brain, including a downregulation of CB1R expression in the PFC, VTA, and hippocampus. Studies have found that these alterations may either be persistent into adulthood (Cuccurazzu et al., 2018; Zamberletti et al., 2014;), or simply a transient effect (Behan et al., 2012). Cannabis exposure may also affect other neurotransmitter systems, including glutamatergic, GABAergic, and dopaminergic systems (Bloomfield et al., 2014; Gunasekera et al., 2022; Raymundi et al., 2023; Zamberletti et al., 2014). Previous studies have displayed that intraperitoneal (IP) injection of THC decreased glutamatergic receptor expression of GluN2A-NMDA in the dorsal hippocampus (Raymundi et al., 2023). Chronic THC injections in adolescence also led to a reduction in the depolarizing response to NMDA in deep-layer PL-PFC neurons, suggesting a reduction in neuronal excitation in this region (Pickel et al., 2020). In addition, chronic adolescent exposure to THC in rats led to alterations in the PFC including a reduction in GABAergic neurotransmission, and a decrease in the enzyme, GABA synthesizing enzyme glutamate decarboxylase 67 (GAD67), that produces GABA (Jobson et al., 2019; Zamberletti et al., 2014). While a long-term reduction in GABA was also found in the hippocampus, levels of the GAD67 enzyme remained unchanged (Zamberletti et al., 2014). In terms of dopaminergic effects, acute cannabis use is associated with increased dopaminergic function, while regular cannabis users display a reduction in DA synthesis capacity in the striatum over time (Bloomfield et al., 2014; Gunasekera et al., 2022). Additionally, increased excitability of neurons in the VTA in response to substances such as morphine, cocaine, and amphetamine was noted in subjects that had been administered a WIN55212.2 (WIN) cannabinoid agonist during their adolescent phase. This indicates that cannabinoid-induced changes in the dopaminergic

system might lead to a tolerance effect towards the immediate effects of various other addictive substances (Pistis et al., 2004). A neuroimaging study also found that in healthy occasional cannabis users, there is decreased connectivity in the hippocampus-midbrain-striatum network after inhalation of vaporized THC (Pelgrim et al., 2023).

1.4.2 Behavioral Effects of Cannabis

The behavioral consequences of cannabis use, particularly when initiated during adolescence, have been extensively studied, revealing significant impacts on cognitive and neuropsychological outcomes, including impairments in memory, attention, learning, and executive functioning (Battisti et al., 2010; Savulich et al., 2021; Schoch et al., 2018; Schuster et al., 2016). Battisti et al. (2010) found that while male cannabis users had worse visual recognition memory, female users had worse attention and executive function capacity. In addition, increased use of cannabis was more strongly associated with worsened episodic memory in male users compared to females (Savulich et al., 2021).

Early-onset cannabis use, initiated before age 16, has been shown to weaken learning significantly, reflected in an impaired ability to recall information after a delay (Schuster et al., 2016). These results suggest that the primary cognitive impairment associated with early-onset cannabis use lies in the initial learning or encoding of information, rather than in the consolidation or retrieval stages of memory (Schuster et al., 2016). In terms of learning reward-related tasks, exposure to cannabinoids during puberty was found to significantly impair reward-seeking behavior in adult rats, as evidenced by a reduction in breakpoints during a Progressive Ratio (PR) schedule, suggesting less motivation to work for a reward (Schneider & Koch, 2003). Notably, this effect was specific to cannabinoid exposure during puberty, highlighting the critical impact of adolescent cannabis use on long-term behavioral outcomes related to reward learning (Schneider & Koch, 2003). A subsequent study demonstrated that adolescent THC vapor exposure led to impaired learning in adulthood in the delay discounting task, indicating a long-term impact on the ability to make decisions based on future rewards (Hamidullah et al., 2021). It has also been reported that adolescent rats repeatedly exposed to cannabinoids exhibit greater memory impairment compared to those exposed as adults (Schneider & Koch, 2003). These studies highlight the potential for persistent cognitive impairments and altered reward processing in adulthood.

1.5 Interactions Between Nicotine and Cannabis in the Brain

While research exploring the long-term effects of nicotine and cannabis vapor co-use is limited, the current literature suggests that co-use may produce opposing (Jansma et al., 2013; Manwell et al., 2019) or synergistic (Hernandez Mejia et al., 2024) effects on behavioral and neural outcomes. As previously mentioned, the acute and chronic neurobiological effects of both THC (Bloomfield et al., 2014; Raymundi et al., 2023; Renard et al., 2017) and nicotine (Gotti et al., 2006; Levin et al., 2006; Mansvelder et al., 2002) result in their respective glutamatergic, GABAergic, and dopaminergic effects via different signaling pathways.

The co-use of nicotine and cannabis has been associated with domain-specific effects on cognitive functions such as memory, attention, and impulsivity which has been previously discussed. For instance, while nicotine may enhance attentional processes (described in 1.3.2), cannabis use has been linked to impairments in memory and increased impulsivity (described in 1.4.2). The interaction between these substances could therefore result in differing outcomes, depending on the balance of their neurochemical effects.

1.5.1 Adult Co-Exposure of Nicotine and Cannabis

Chronic nicotine exposure in adult Sprague-Dawley rats has been shown to attenuate the anxiety-related effects of THC (Manwell et al., 2019). In this study, an IP injection of THC led to anxiogenic effects in an elevated plus maze task, where THC-treated rats spent increased time in the closed arm of the maze, indicating increased anxiety-like behavior compared to controls. When rats were treated with daily IP injections of nicotine for 2-weeks, the THC-induced anxiogenic effects were ameliorated (Manwell et al., 2019). In humans, acute administration of THC in males with nicotine addiction reduced the increased activity in the nucleus accumbens (NAcc) in response to reward anticipation, while healthy controls showed an opposing response to THC administration (Jansma et al., 2013). This study concluded that nicotine addiction may be associated with altered eCB regulation in the NAcc (Jansma et al., 2013). A previous study in humans also found that tobacco seemed to mask cannabis-induced impaired memory (Schuster et al., 2015).

1.5.2 Adolescent Co-Exposure of Nicotine and Cannabis

Fewer studies have investigated the effects of concurrent nicotine and cannabis use in adolescence. However, investigations into cortical thickness have shown that adolescents who use either nicotine or THC alone display thinner brain cortices compared to those who co-use both nicotine and cannabis who exhibit increased cortical thickness (Hernandez Mejia et al., 2024). These patterns suggest that nicotine might modulate the impact of cannabis on certain areas of the brain, such as the frontal cortex, an area pivotal for executive functions and decision-making processes (Hernandez Mejia et al., 2024). In addition, a study looking at combined substance use in adolescents aged 13-15 found that regular cannabis and nicotine use was associated with an additive risk for psychiatric disorders (Boys et al., 2003), while another study found that their co-use has been associated with increased measures of nicotine addiction (Rubinstein et al., 2014). In contrast a neuroimaging study by Karoly and colleagues found that adolescents that used nicotine alone had decreased activity in the NAcc during a monetary incentive delay task compared to non-using peers (Karoly et al., 2015). However, this decrease was not seen in those using both cannabis and nicotine (Karoly et al., 2015). Thus, cannabis may play a role in counteracting the effects of nicotine on the NAcc. The emerging evidence from the limited studies exploring nicotine and cannabis co-use shows the importance of considering their combined effects on the brain, highlighting the need for further research to elucidate the mechanisms underlying these interactions and their long-term implications for cognitive development.

1.6 Reward-Learning and Reinforcement

The intricate relationship between reward learning, incentive, and associative prediction plays a role in understanding substance use and its neural underpinnings (Boys et al., 2003). Reward learning, a fundamental aspect of behavioral neuroscience, involves the ability to learn the association between a stimulus and its rewarding outcome, often studied through Pavlovian conditioning paradigms (Flagel et al., 2011). Impulsivity, characterized by actions that are premature, or risky, is closely linked to reward-learning processes, as both are mediated by overlapping neural circuits, including the mesolimbic DA pathway (Berridge & Robinson, 1998; Flagel et al., 2011). This section delves into the effects of nicotine and cannabis on reward learning and impulsivity, with a particular focus on Pavlovian conditioned approach tasks and locomotor reactivity to novel settings.

1.6.1 Pavlovian Conditioned Approach

The Pavlovian Conditioned Approach (PCA) task serves as a measure of appetitive learning, where animals learn to associate a neutral cue with a rewarding outcome (Fitzpatrick & Morrow, 2016; Flagel et al., 2011). This form of associative learning, while encompassing the anticipation of rewards, primarily sheds light on the broader spectrum of appetitive learning processes. This paradigm has been instrumental in investigating the neural and behavioral dynamics of reward processing and learning (Fitzpatrick & Morrow, 2016). By employing the PCA task, researchers can dissect the multifaceted aspects of how nicotine and cannabis affect the brain's reward system, providing a nuanced understanding of the substances' influence on reward-related learning and behavior (Fraser et al., 2016).

1.6.1.1 Nicotine and PCA

Studies suggest that nicotine can acutely enhance the salience of reward-predictive cues, thereby facilitating Pavlovian appetitive learning in both males and females (Stringfield et al., 2019). Nicotine's action on nAChRs in the brain's reward circuits, such as the VTA and NAcc enhances DA release in response to reward-predictive cues (Benowitz, 2009). This dopaminergic surge reinforces the association between the cue and the reward and increases the motivational value of the cue, making the animal more likely to perform approach behaviors towards it (Stringfield et al., 2019). A previous study done in our lab also found that adolescent males, but not females, exposed to nicotine displayed enhanced appetitive Pavlovian learning later in adulthood, indicating a long-term sex-specific nicotine-induced enhancement in learning reward tasks (Ruffolo et al., 2022).

1.6.1.2 Cannabis and PCA

Studies have demonstrated that adolescent exposure to THC can lead to impairments in reward learning, characterized by a reduced preference for tasks requiring higher cognitive effort despite the animals' capability to complete these tasks accurately (Silveira et al., 2017). This impairment correlates with the density of CB1 receptors in the medial prefrontal cortex, a region implicated in effortful decision-making (Silveira et al., 2017). This correlation highlights the significant role of the endocannabinoid system in influencing cognitive functions related to reward and decision-making processes.

Moreover, investigations into the voluntary consumption of THC by adolescent rats have identified sex-specific effects on reward-related behaviors in adulthood. Specifically, adult males, but not females, exhibited impairments in behaviors associated with Pavlovian reward-predictive cues (Kruse et al., 2019). This impairment was attributed to the selective loss of CB1R glutamatergic terminals in males, underscoring the complex interplay between the endocannabinoid system, neurodevelopmental timing, and reward learning (Kruse et al., 2019). These age-related and sex-specific effects emphasize the necessity for a deeper understanding of how cannabis impacts the neural mechanisms underlying reward learning and behavior.

1.6.2 Reactivity to Novel Settings

The assessment of locomotor reactivity to novel environments provides valuable insights into the behavioral phenotypes associated with substance exposure, particularly in the context of reward sensitivity (Vanhille et al., 2015). Increases in locomotor responses to novelty have previously been linked to mechanisms of stress (Piazza et al., 1991) and self-administration of drugs (Deminiere et al., 1989; Klebaur et al., 2001), corresponding with altered dopaminergic neurotransmission (Deminiere et al., 1989). An increased locomotor response to novelty has also been found to predict the propensity to self-administer nicotine in rats (Suto et al., 2001). Animals displaying increased locomotor activity in novel settings have also shown elevations in both stress-mediated or cocaine-mediated DA release in the NAcc (Hooks et al., 1992; Piazza et al., 1991; Rougé-Pont et al., 1993).

1.6.2.1 Nicotine and Novelty-Induced Activity

There are limited studies on the long-term effects of nicotine on locomotor reactivity to a novel environment. Yet one study found that adolescent rats exhibiting high locomotor activity in the novel open field at baseline displayed a greater tolerance to the locomotor depressant effects of nicotine compared to adolescents with low locomotor activity at baseline (Philpot et al., 2012). This may suggest that reactivity to novel settings, specifically during the adolescent period, may predict sensitivity to the effects of nicotine, underlying likelihood of progression to addiction (Philpot et al., 2012). Another study found that male rats exposed to prenatal nicotine have enhanced locomotor responses to acute nicotine administration on PND14 (Shacka et al., 1997).

Still, there is a lack of research investigating the long-term effects of nicotine on locomotor reactivity to a novel environment (LRNE) in particular.

1.6.2.2 Cannabis and Novelty-Induced Activity

In contrast to the long-term locomotor effects of nicotine exposure, one previous study in male rats reported that chronic late adolescent administration of the cannabinoid agonist, WIN, decreased locomotor activity in adulthood (Wegener & Koch, 2009), while another study found that THC exposure in utero led to hyperactivity in an open-field test during adolescence (Brancato et al., 2020). These opposing results may be due to the different developmental periods in which rats were exposed to cannabis. More research is needed to understand the long-term effects of adolescent cannabis use on novelty-induced and reward-seeking activity.

1.7 Neuroimaging

Preclinical neuroimaging plays a crucial role in neuropsychopharmacology by providing insights into the effects of drug use on brain structure and activity (Jonckers et al., 2015). These studies help to elucidate the direct impacts of substances on brain structure and function in non-human subjects and serve as a bridge for translating findings to human research, thereby enhancing our mechanistic understanding of drug effects. By identifying how specific drugs alter functional connectivity between brain regions in animal studies, researchers can infer potential effects in humans, guiding the development of targeted therapies for cognitive and behavioral disorders (Balleine & O'Doherty, 2010). This approach enables a detailed exploration of drug interactions with neural pathways, shedding light on the neurobiological underpinnings of substance use.

Understanding the neural impacts of polysubstance use presents a complex challenge, necessitating an understanding of how multiple drugs interact to exert their influences on the brain. It is especially difficult to study interactions of polysubstance use in humans due to the many confounds that exist between participants, and the limitations in controlling for doses. Preclinical neuroimaging can unravel these complexities by demonstrating the dose-dependent interactions between different substances within the animal brain's circuitry (Murnane et al., 2023). Findings from controlled preclinical neuroimaging studies can help inform human studies when formulating hypotheses. Moreover, the findings from animal neuroimaging studies can advise further research on mechanistic underpinnings and human associations.

Recent advancements in neuroimaging, particularly through functional magnetic resonance imaging (fMRI), have significantly enhanced our understanding of the neurobiological impacts of substance use, including cannabis and nicotine (Farra et al., 2020; Frie et al., 2024). These studies serve as a crucial translational bridge, offering insights from human clinical observations that can be replicated and further explored in animal models. This approach not only elucidates the direct impacts of substances on brain structure and function but also informs the development of targeted interventions for addiction and its related disorders.

1.7.1 Substance Effects on Functional Connectivity

Previous neuroimaging studies implicate both cannabis and nicotine exposure during adolescence in causing differing neural changes observed in adulthood (Farra et al., 2020; Frie et al., 2024). Blood Oxygen Level Dependent (BOLD) MRI analysis has shown that rodent brains are acutely affected by cannabis exposure, particularly in areas rich in CB1 receptors, such as the olfactory system, cortex, and amygdala (Farra et al., 2020). These findings indicate significant activation and resultant functional alterations within the brain after cannabis exposure.

Similarly, research on nicotine, especially delivered through e-cigarettes, has uncovered effects on brain connectivity and function that vary by sex and age. A study conducted in our lab investigated the impact of nicotine vapor on rats and found, through fMRI analysis, that exposure to nicotine led to reduced connectivity between the hypothalamus and cortical regions, with the effects significantly modulated by sex differences (Frie et al., 2024).

These diverse neuroimaging findings highlight the multifaceted effects of cannabis and nicotine use on the brain. By leveraging advanced imaging techniques, such as fMRI, we can uncover the functional alterations induced by these substances, providing a deeper understanding of their long-term consequences.

1.8 Sex-Differences

A plethora of existing research has uncovered sex differences in pharmacological, behavioral, and neural effects of both nicotine and cannabis use. Evidence suggests pharmacological differences and behavioral changes attributable to nicotine exposure differ between sexes (Harrod et al., 2007; Ruffolo et al., 2022; Verplaetse et al., 2018). Female rats have been shown to exhibit higher plasma

nicotine concentrations 1 min after an intravenous injection (Harrod et al., 2007) and 10 min after nicotine vapor exposure (Frie et al., 2024). As previously mentioned, the reinforcing effects of nicotine are mediated by nAChRs containing the β2 subunit (Verplaetse et al., 2018). While the β2-nAChR availability between male and female human nonsmokers is similar (Cosgrove et al., 2007), nicotine exposure results in upregulated β2-nAChR expression in males only (Verplaetse et al., 2018). A previous study conducted by our lab also found that male, but not female, rats displayed enhanced learning of a Pavlovian appetitive learning task (Ruffolo et al., 2022). In contrast, adolescent female rats displayed increased sensitivity to the rewarding effects of nicotine compared to males (Xue et al., 2018). In humans, greater withdrawal symptoms have also been observed among women following attempts to quit using nicotine (Verplaetse et al., 2018).

Similar to the effects of nicotine, cannabis exposure has been shown to induce pharmacological and behavioral alterations that differ between sexes (Harte & Dow-Edwards, 2010; Torrens et al., 2022). Female rats administered THC during both adolescence and adulthood exhibit the potent 11-OH-THC metabolite in double the concentrations of male rats between 1-8 hours after injections (Torrens et al., 2022). Additionally, peak levels of CB1 receptors are reached earlier in female rats (PND 30) than in male rats (PND 40), further validating that there are sex-dependent differences in THC pharmacokinetics (Craft et al., 2013). Behaviorally, sex-specific differences also exist where cannabis induces differential domain-specific impairments across sexes. For example, when assessing spatial learning through a Morris water maze task, adolescent male rats showed less THC-induced impairments compared to age-matched females (Cha et al., 2007). In contrast, another study found that chronic exposure to THC during early adolescence (PND 22-40) decreased retention in male rats only for an active avoidance task, suggesting a sex-specific impairment in avoidance learning (Harte & Dow-Edwards, 2010).

Due to the vast array of sex-specific effects induced by nicotine and cannabis across pharmacokinetic and behavioral domains, especially during the development of the adolescent brain, it is crucial to elucidate the impacts of concurrent use of nicotine and cannabis during adolescence across these domains in adulthood in both sexes.

1.9 Objectives and Hypothesis

Significant advancements have been made in understanding the impacts of nicotine and cannabis used in isolation, yet further investigation is necessary to uncover potential combined effects of their co-use, given the widespread occurrence of their dual usage among adolescents. Thus, the objective of this present study is to investigate the short- and long-term effects of adolescent nicotine and cannabis co-use on behavior, cognition, and functional connectivity. We hypothesize that a) nicotine will increase reward-learning behavior, b) cannabis will reduce reward-learning behavior, and c) the co-exposure of nicotine and cannabis in adolescence will not alter reward-related behavior relative to the control group. To investigate the effects of adolescent nicotine and cannabis exposure on long-term alterations in functional connectivity, we use an exploratory non-hypothesis driven approach to identify all connections that may be altered.

2 Materials and Methods

2.1 Animals

All animal procedures were approved by the University of Guelph Animal Care Committee and the University of Western Ontario Animal Use Subcommittee and were consistent with guidelines established by the Canadian Council on Animal Care.

Male (n = 32) and female (n = 32) Sprague-Dawley rats were obtained from Charles River Laboratories at post-natal day (PND) 23. Animals were housed in same-sex groups of 4 and kept in a room with standard conditions of temperature and humidity with a 12-h light-dark cycle (lights on 7:00AM-7:00PM). Laboratory rats, particularly Sprague-Dawley strains, are validated models for studying the effects of nicotine and cannabis and have been used in our lab (Nelong et al., 2019; Renda et al., 2020). Rats had ad libitum access to food and water unless otherwise specified during behavioral testing in adulthood. Rats were randomly assigned to one of four exposure groups (N=8 per group per sex): 1) Control (Vehicle Vapor/Air); 2) Nicotine (Nicotine Vapor/Air); 3) THC (Vehicle Vapor/Vaporized Cannabis Flower); 4) Nicotine-THC (Nic-THC) (Nicotine Vapor/Vaporized Cannabis Flower). Air was used to control for cannabis vapor, while the control for nicotine was vehicle vapor made of propylene glycol (PG) and vegetable glycerin (VG) without any active substances. This ensures that any observed effects are due to the specific compounds being tested (nicotine, THC, or both) rather than the vehicle itself. The experimental design is outlined in Figure 1. Equipment was cleaned between each rat for each experiment with a hydrogen peroxide solution.

2.2 Drug Preparation

2.2.1 Nicotine

Commercially available STLTH unflavored 2% nicotine e-liquid pods (20mg/ml), or vehicle STLTH 0% unflavored e-liquid (0mg/ml) was used. The e-liquid contained a mixture of PG and VG.

2.2.2 THC

The THC-treated group was administered THC-rich dried cannabis flower (Truro Wedding Mint; 270-300mg/g THC, 0-1 mg/g CBD) obtained from the Ontario Cannabis Store. Rats were exposed

to 0.25g of vaporized dried cannabis flower administered in this experiment which corresponds with the mass of one standard joint (Casajuana Kogel et al., 2017).

2.3 Experiment 1: Drug Plasma Concentrations from Vapor Exposures

2.3.1 Blood Collection

Blood was collected on the final day of exposure (day 14; PND 41) immediately after the respective exposure sessions. Before their last exposure, the hind legs of the rats were shaved. After exposures, the rats were placed on heat pads for 5 min. Vaseline was applied to skin covering the saphenous vein, and a 22G needle was used to puncture the vein. Blood drops were collected in a capillary blood collection tube with a maximum volume of 300 µL (Microvette CB300, Sarstedt, Germany). Samples were kept on ice and then centrifuged at 8000 RPM for 5 min. The blood plasma was transferred to microcentrifuge tubes and stored at −80°C until serum analysis.

2.3.2 Serum THC, CBD, and 11-OH-THC Quantification

Serum quantification of THC, CBD, and 11-OH-THC followed our laboratory protocols used in previous studies. Reference standards of THC, CBD, and 11-OH-THC, and their deuterated internal standards THC-D3, CBD-D3, and 11-OH-THC-D3 were purchased from Sigma-Aldrich Canada (Oakville, ON). To extract the cannabinoids, THC, CBD, and 11-OH-THC, from rat serum, Captiva enhanced matrix removal lipid (EMR-Lipid) 96-well plate was used (Agilent, Santa Clara, CA, USA). Briefly, 250 µL of acetonitrile (acidified with 1% formic acid) was added to each well, then 50 µL of rat serum and 20 µL of internal standard solution were added. After the sample passed through under positive pressure at 3 psi, the extraction plate was washed with 150 µL of a mixture of water/acetonitrile (1:4, v:v) solution. The effluent was evaporated under nitrogen at 40 °C, and the residual was reconstituted with the mobile phase for subsequent LC-MS/MS analysis. Calibration Standards (2-1000 ng/mL) and quality controls (3 ng/mL and 800 ng/mL) were prepared on the day of analysis by spiking standard working solutions into blank rat serum. The liquid chromatography separation was achieved on a Thermo Scientific Vanquish Flex UHPLC system. Five µL of plasma extracts were injected and separated on an ACQUITY UPLC BEH C18 Column (1.7 µm, 2.1 mm × 50 mm; Waters, Ireland) connected with a VanGuard UPLC BEH C18 Pre-Column (Waters, Ireland). The auto sampler was kept at 4 °C and the column

temperature was at 35 °C. The mobile phase consisted of 10 mM ammonium formate with 0.1% formic acid aqueous solution, and acetonitrile with 0.1% formic acid. The flow rate was 400 µL/min under a gradient mode. The gradient conditions were sustained as follows: Mobile phase B linearly ramped up from 40% to 95% from 0.1 to 4 min, and maintained at 95% for 2 min, then ramped back to 40%. 11-OH-THC, CBD and THC were eluted at 3.5, 4.0 and 4.6 minutes, respectively, with a total run time of 7 min. MS analysis was conducted with a Thermo Q Exactive Focus Orbitrap mass spectrometer equipped with an Ion Max source in positive electrospray ionization (ESI) mode. The source conditions were optimized as the spray voltage of 3.5 kV, the capillary temperature of 300 °C, and aux gas heater temperature of 425 °C. Data were acquired and processed in parallel-reaction monitoring (PRM) mode using Thermo Scientific™ TraceFinder™ software. In this PRM mode, protonated 11-OH-Δ9-THC ion (m/z 331.23), CBD ion (m/z 315.23) and Δ9-THC ions (m/z 315.23) were selected as precursors, then fragmented in the higher-energy C-trap dissociation (HCD) cell at collision energy of 20 eV for 11-OH-THC and 25 eV for CBD and THC. The resulting MS/MS product ions were detected in the Orbitrap at a resolution of 17,500 (FWHM at m/z of 200) with AGC target set at 1e5. The most abundant fragments from the MS/MS spectra (m/z 313.22 for 11-OH-THC and m/z 193.12 for CBD and THC) were selected as the quantifying ions. Other specific fragments, m/z 193.12 for 11-OH-THC and m/z 259.17 for CBD and THC, were selected as the confirming ions. The resulting chromatograms were extracted and reconstructed with a mass accuracy of 5 ppm for quantification and confirmation. The optimized MS/MS compound parameters are summarized in Table 1.

Table 1: Optimized LC-MS/MS compound parameters for quantitation of 11-OH-THC, CBD, and THC using PRM mode (CE: collision energy, m/z: mass/charge ratio, RT: retention time).

Analyte & Internal Standard	Precursor Ion *(m/z)*	CE	Quantitation ion (*m/z)*	Confirming Ion (*m/z)*	RT (min)
11-OH-Δ^9-THC	331.23	20	313.22	193.12	3.5
11-OH-Δ^9-THC–D3	334.24	20	316.23	196.14	3.5
CBD	315.23	25	193.12	259.17	4.0
CBD-D3	318.25	25	196.14	262.19	4.0
Δ^9-THC	315.23	25	193.12	259.17	4.5
Δ^9-THC–D3	318.25	25	196.14	262.19	4.5

2.4 Experiment 2: Short-term Behavioral Cannabinoid-Mediated Effects of Adolescent Nicotine and Cannabis Co-Exposure

2.4.1 Vapor Administration Procedures

2.4.1.1 Nicotine

Nicotine vapor was administered to rats, starting at PND28, via the OpenVape exposure device (Frie et al., 2020). Rats in both the Nicotine and the Nicotine-THC (Nic-THC) groups received 20mg/mL nicotine vapor, while the rats in the control and THC groups received 0mg/mL nicotine vapor. All rats were exposed to their respective vapors for 10 min daily for 14 days. Animals from each exposure group and sex were placed in the exposure chamber together. For the entire duration of the exposure period, the apparatus produced a 2-sec puff of vapor every 4-sec, leading to 10 puffs per min (Frie et al., 2020). In total, rats received 100 2-sec puffs each day, resulting in the vaporization of 0.5 mL of liquid per group per day. The nicotine administration procedure was adapted from previous studies in our lab which caused pharmacological and behavioral outcomes that validated this exposure protocol in studying behavioral and neural effects of nicotine in adolescence (Frie et al., 2020, 2024). Due to the recent change in Canadian regulations regarding legal nicotine concentrations for vape pods, we used 20mg/mL nicotine pods while our previous studies have used 59mg/mL pods (Canada, 2021). Since then, a recent study with female rats have shown similar behavioral and pharmacological effects from this lower concentration of nicotine e-liquid (Roeder et al., 2023).

2.4.1.2 THC

Vaporized dried cannabis flower was administered via a Volcano® Vaporizer (Storz and Bickel, GmbH and Co., Tuttlingen, Germany) as previously described by Hazekamp et al., (2006). Dried cannabis flower (0.250 g) was ground and loaded into the vaporizer and then vaporized at 200°C. The fan of the vaporizer was then turned on for 5 min, and the cannabis vapor was pumped to rats through a tube into an enclosed box (48×39×20 cm). The box contained a small breathing hole for the animals and a plastic divider. The divider split the box into 4 quadrants which were used to keep the rats from different cages separated from one another. The 16 control and nicotine rats were exposed to air simultaneously, similarly to the 16 rats in the THC and Nic-THC groups. The box and divider were cleaned between each exposure. Separate, clean tubing delivered the air to

the control group. Cannabis vapor filled the box with either cannabis vapor or air for 5 min while the fan was turned off. After the fan was turned off, rats remained in the box for 10 more minutes to continue inhaling the cannabis vapor left in the box. Thus, every rat was exposed to either cannabis vapor or air for 15 minutes daily for 14 days starting PND 28.

2.4.2 Cannabinoid Tetrad Test

The cannabinoid tetrad test assesses acute behavioral outcomes of cannabis administration. The tetrad test measures locomotor activity, catalepsy, body temperature, and analgesia, performed in this order from least to most stress-inducing. All rats underwent a baseline tetrad test on PND 27 before their first vapor exposure, immediately after their first exposure (PND 28), and immediately after their 10th exposure (PND 38). Locomotor activity was assessed using open field boxes (50×50×34 cm), by recording the distance moved in 10 minutes. Activity was tracked using CCD cameras mounted 2.6 m above the floor and was analyzed using a video tracking software (EthoVision XT v.16.0.1538, Noldus, Wageningen, NL). Catalepsy was measured using an open-source automated bar test as previously described (Luciani et al., 2020). Body temperature was determined using a Digi-Sense WD-20250-91 digital thermometer with a T-type thermocouple rectal probe (Physitemp Ins. NJ). Lubrication was used to reduce discomfort and stress. The latency of the tail flick response was assessed using a tail-flick meter (Columbus Instruments, Columbus, OH).

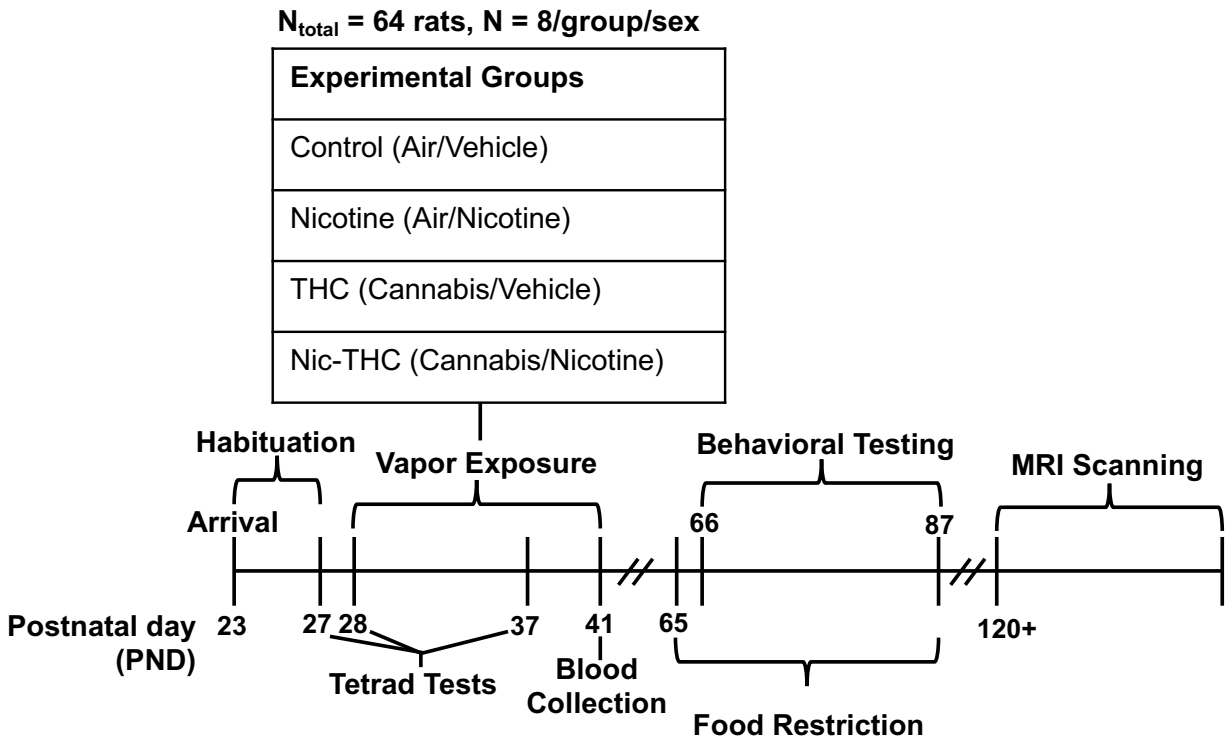

Figure 1: Experimental design timeline. Daily vapor exposures began on PND28 and continued for two weeks until PND 41. Tetrad tests were conducted PND 27, PND 28, and PND 37, and blood was collected on PND 41 immediately after exposures. Rats were then left to grow into adulthood. Starting PND 65, rats were food restricted and underwent behavioral testing starting PND 66 to assess the long-term effects of nicotine, THC, and their combination on behavior using the locomotor reactivity to a novel environment task, and the Pavlovian Conditioned Approach task. MRI scans were then acquired.

2.5 Experiment 3: Effects of Adolescent Nicotine and THC Co-Exposure on Behavioral Outcomes in Adulthood

After the vapor administrations in adolescence, rats were left to grow to adulthood (PND 65) with ad libitum access to food and water. Food restriction started on PND 65 until PND 87 to reduce body weights to 85% of baseline during the Pavlovian Conditioned Approach task. Behavioral testing, including Locomotor Reactivity to A Novel Environment and Pavlovian Conditioned Approach, commenced on PND 66.

2.5.1 Locomotor Reactivity to A Novel Environment

This task measures the locomotor reactivity to a novel environment in adult rats which is represented by their total distance moved after being introduced to the new setting.

2.5.1.1 Apparatus

The arena used was a Plexiglas cage (100 × 100 × 40 cm) with a gray floor and black walls. Activity of each rat was recorded with a GigE camera mounted 210 cm from the floor, and a computer running video tracking software (Ethovision v15.0.1418, Noldus, NL, USA). The arena was cleaned using a hydrogen peroxide solution before each test.

2.5.1.2 Behavioral Procedure

Rats were placed in an open field arena and were recorded for a 10 min period. Video recordings were used to calculate the distance moved in cm for each rat using the tracking software (Ethovision v15.0.1418, Noldus, NL, USA).

2.5.2 Pavlovian Conditioned Approach

The motivational significance of reward-related cues can be assessed through Pavlovian conditioned approach (PCA) tasks. In these tasks, a previously neutral stimulus, such as a lever, becomes a conditioned stimulus (CS+) through its repeated association with an unconditioned stimulus (CS-), such as a food reward (Meyer et al., 2012). A preference to exhibit the conditioned response (CR) of approaching the CS+ is known as sign-tracking behavior. Conversely, a tendency to approach the location where the reward is delivered upon cue presentation is indicative of goal-tracking behavior. While both behaviors demonstrate associative learning, the extent of sign-tracking behavior indicates the motivational significance, or incentive salience, assigned to the cue.

2.5.2.1 Apparatus

Behavioral training and testing procedures were carried out in eight standard HABITEST Operant Cages (24 × 30.5 × 29 cm; Coulbourn Instruments) enclosed in HABITEST sound attenuating isolation cubicles (76.2 x 46.99 x 44.96 cm; model: H10-24; Coulbourn Instruments). The operant chambers contained a food cup connected to an external food dispenser in the center of the right sidewall. Two retractable levers were on the left and right of the food cup. The cubicles also contained an exhaust fan for background noise and ventilation. Lever presses and food cup entries were automatically measured and recorded via a photocell using Graphic State software (v.3.03). Responses were processed using a customized Microsoft Excel macro. The reinforcing

unconditioned stimulus was 45 mg banana-flavored sucrose pellets (Bio-Serv, Flemington, NJ, product #F0024).

2.5.2.2 Behavioral Procedure

The PCA protocol for this study was adapted from a previous publication from our laboratory (Meyer et al., 2012). Food restriction started on PND65 to reduce body weights to 85% of baseline body weight. All rats remained food restricted until the completion of the experiment and were fed at least one hour after each testing session. Banana flavored sucrose pellets were placed in their home cages one day prior to the training session to reduce potential neophobia. The first day of the experiment (PND71), each rat was assigned to a chamber and underwent one 30-minute magazine training session to acclimatize rats to the operant chamber and the food reward. For this day only, both levers were retracted, and one banana pellet was freely delivered randomly approximately once every 30 s (± 30 s).

PCA testing lasted for the next 12 days and started 24-hours after magazine training. The rats were randomly assigned to a left or right conditioned stimulus (CS+) and unconditioned stimulus (CS-) lever. The left and right assignment of the CS+ and CS- lever was counterbalanced within exposure groups and across animals. Each testing session was 60 min and included 25 CS+ trials and 25 CS- trials. For the CS+ trials, the CS+ lever was presented for 10 sec immediately followed by a sucrose pellet, regardless of any interaction with the lever. For CS- trials, the other CS- lever was extended for 10 sec, but no reinforcer was delivered upon retraction. The testing session consisted of 25 CS+ and 25 CS- trials with an intertrial interval of 60 sec ± 15 sec. Lever presentation was pseudorandomized, with the same lever being presented no more than two times in a row. Food cup entries were only recorded when a CS+ lever was presented.

2.6 Experiment 4: Effects of Adolescent Nicotine and THC Co-Exposure on Neural Functional Connectivity in Adulthood

2.6.1 Magnetic Resonance Imaging

This study's protocol for functional magnetic resonance imaging (fMRI) closely followed a recent study by our laboratory for functional connectivity analysis in the rat brain (Frie et al., 2024). Prior to scanning, anesthesia was initiated by placing rats in an induction chamber with 4-5% isoflurane

and an oxygen flow rate of 1-1.5 L/min. Following induction, isoflurane was maintained between 2.0-2.5% with an oxygen flow rate of 1-1.5 L/min through a custom-built nose cone, and an IP injection of 0.018 mg/kg dexmedetomidine was administered (Wallin et al., 2021). Once the animal was positioned in the MRI scanner, a constant rate infusion of 0.018 mg/kg/hr dexmedetomidine was administered for the duration of the scan (Wallin et al., 2021). Following initiation of the dexmedetomidine infusion, isoflurane was reduced over a 15-minute period to between 0.8-1.0% with the same oxygen flow rate of 1-1.5 L/min. The rectal temperature was kept at 37.0 ± 0.5 ºC using an air heater system (Wallin et al., 2021).

2.6.2 Image Acquisition

Images were acquired using a 9.4 T Bruker small animal MRI scanner at the Centre for Functional and Metabolic Mapping in the Robarts Research Institute at Western University.

2.6.2.1 Anatomical Acquisition

T2 Anatomical images were acquired for each subject at the beginning of each session using a T2-weighted TurboRARE pulse sequence (Hennig et al., 1986). Total acquisition time for anatomical images was 14 min (8 averages, 35 slices, slice thickness = 400 μm; Field of View, FOV 38.4 x 38.4 mm; matrix size 192 x 192, in-plane resolution = 200 x 200 μm; Echo time, TE = 44.0 ms, Repetition time, TR = 7.0 s, Echo Spacing = 11.00 ms, Rare Factor 8).

2.6.2.2 Functional Acquisition

Resting-state fMRI images were acquired based on optimized scan parameters (Gilbert et al., 2019), using a gradient echo-planar-imaging (EPI) sequence (2 runs, 400 volumes each; TE = 15.0 ms; TR = 1.5 s; FOV 38.4 x 38.4 mm; matrix size 96 x 96, isotropic resolution = 400 μm, bandwidth 280 kHz).

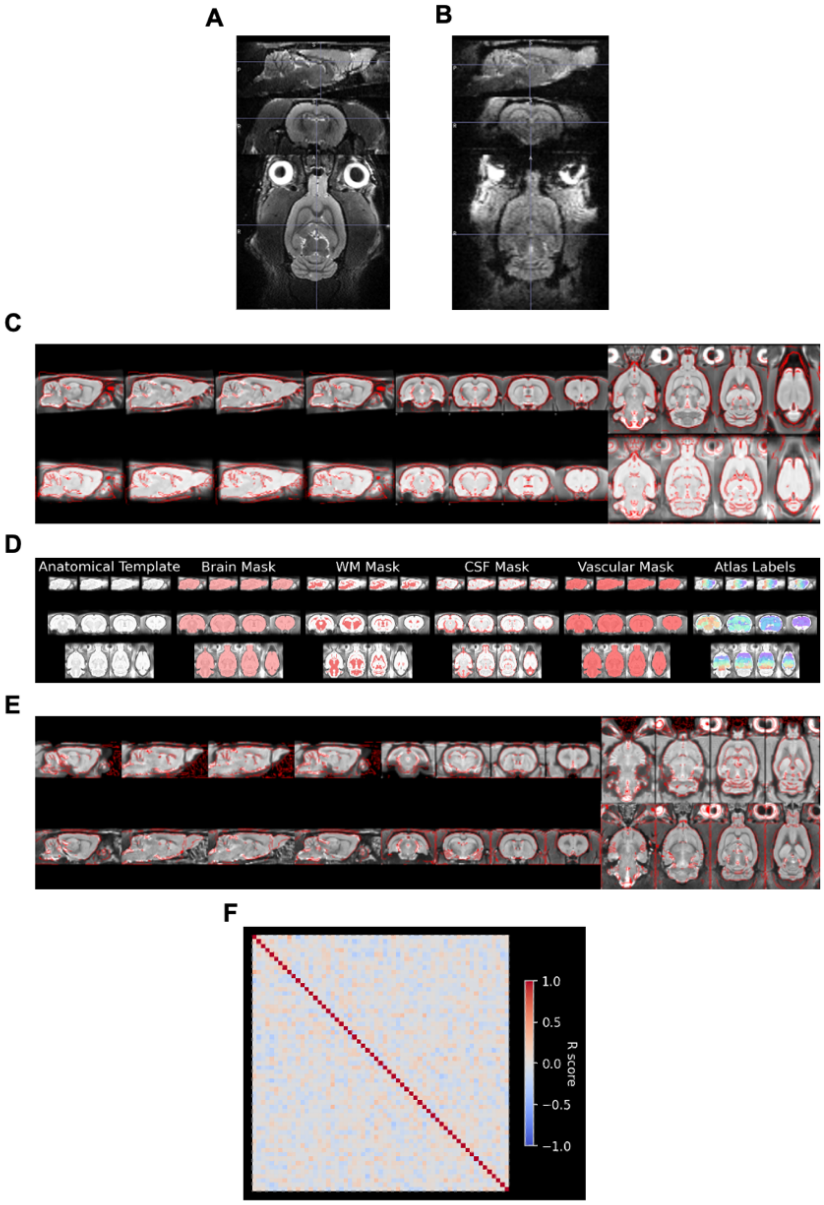

Figure 2: Functional MRI processing. Anatomical (A) and functional (B) MRI images. Anatomical images were registered together creating an unbiased common-space template of their anatomical brain (C) which was then aligned to an external rat brain atlas template (D). Individual functional images were then registered to the anatomical scan from the same rat (E). Whole-brain connectivity matrices were then generated for each rat to represent the connectivity between every corresponding region pair identified in the anatomical atlas (F).

2.6.3 Functional Image Processing

Image processing was conducted using open-source RABIES software (https://github.com/CoBrALab/RABIES) and the functional SIGMA rat brain template (Barrière et al., 2019; Desrosiers-Gregoire et al., 2022). Image processing consisted of preprocessing,

confound correction, and functional connectivity analysis using RABIES. For both the anatomical (Figure 2A) and functional (Figure 2B) images, extra space around the brain was automatically cropped, and temporal spikes were corrected for at each voxel (Cox, 1996). To conduct common space alignment, structural images were corrected for inhomogeneities, and then registered together to allow the alignment of different MRI acquisitions (Figure 2C). This registration was conducted by generating an unbiased data-driven template through the iterative nonlinear registration of each image to the dataset consensus average, where the average gets updated at each iteration to provide an increasingly representative dataset template (Avants et al., 2011). The finalized anatomical template after the last iteration provides a representative alignment of each MRI session to a template that shares the acquisition properties of the whole dataset, which makes it a stable registration target for cross-subject alignment. After aligning the MRI sessions, this newly generated unbiased template was then registered, using a nonlinear registration, to the SIGMA rat brain template in Figure 2D (Barrière et al., 2019). To correct for EPI susceptibility distortions, the volumetric EPI image was also subjected to inhomogeneity correction, and then registered using a nonlinear registration to the anatomical scan from the same MRI session (Wang et al., 2017), displayed in Figure 2E. After calculating the transformations required to correct for head motion and susceptibility distortions, transforms were concatenated into a single resampling operation (avoiding multiple resampling) which is applied at each EPI frame, generating the preprocessed EPI timeseries in native space (Esteban et al., 2019). Preprocessed timeseries in common space were also generated by further concatenating the transforms, allowing resampling to the reference atlas at a voxel resolution of 0.3x0.3x0.3 mm. We conducted quality control of the intermediate preprocessing images to ensure the appropriate execution of the pipeline workflow based on provided RABIES documentation.

Following preprocessing, confound correction was executed on the EPI timeseries resampled to common-space using RABIES to prevent the re-introduction of confounds to the fMRI images. Voxelwise linear detrending was first applied to remove first-order drifts and the average image. Motion sources were then automatically removed using a modified version of the ICA-AROMA classifier used by (Pruim et al., 2015) where classifier parameters and anatomical masks are instead adapted for rodent images. Low pass filtering (0.1Hz) and high pass filtering (0.01Hz) were then applied (Abraham et al., 2014). The mean signal from white matter and cerebrospinal fluid and the global signal was modelled at each voxel and regressed from the data. Lastly, a Gaussian

smoothing filter was applied at 0.3mm full-width at half maximum (Abraham et al., 2014). After the confound correction was complete, we conducted a functional connectivity analysis in RABIES, which generated a whole-brain matrix for each rat representing the connectivity between every corresponding region pair identified in the SIGMA anatomical atlas (Barrière et al., 2019).

After the confound correction step, whole-brain connectivity matrices were generated in common-space for each subject individually using the rat SIGMA functional template (59 total Regions of Interest) by extracting the seed time-course for every parcel and then measuring the cross-correlation (Pearson's r) between every region pair. The correlation values were then reorganized into a whole-brain matrix representing the connectivity strength between every corresponding region pair (Figure 2F).

2.6.4 Network-Based Statistical Analysis

Network-based statistic (NBS; v.1.2) for MATLAB (v.R2023b; MathWorks Inc., 2023) was used to identify significantly different subnetworks comprised of nodes (brain regions) and edges (connections) between experimental groups (Zalesky et al., 2010). NBS first identifies edges that surpass a given threshold (suprathreshold links), followed by identification of connected nodes within this subnetwork and finally permutation testing to assign a p-value (controlled for the family-wise error rate, FWER) to each subnetwork based on its size. Using NBS, all matrices were entered into a one-way ANOVA ($p = 0.05$, F-threshold = 5). Statistically significant networks identified by NBS ($p < 0.05$) were extracted for post-hoc analysis of between-group differences using two-sample one-tailed t-tests ($p < 0.004$, T-threshold = 2.5). Post-hoc statistical significance was Bonferroni corrected (4 groups, twelve contrasts =0.05/12 = 0.004).

2.7 Statistical Analyses

All data analyses were performed using the IBM SPSS Statistics 28 (Armonk, New York, United States) software. Data from the body temperature, locomotor activity, and tail-flick tests for the cannabinoid tetrad test were all analyzed using a two-way ANOVA for repeated measures. Blood concentrations of both THC and the 11-OH-THC metabolite were analyzed with two-way t-tests between sexes and drug treatment groups. The locomotor activity data were evaluated through a two-way between-subjects ANOVA, with drug treatment and sex serving as the independent variables.

For the PCA task, measures of behavior that were used to calculate the PCA index score included: the number of lever presses per session, the number of food cup entries per session, the probability of pressing the lever, the probability of entering the food cup during a trial, the latency to press the lever, and the latency to enter the food cup during presentation of the CS+. Given previously observed sex differences in the impact of adolescent nicotine and cannabis exposure on behavior (Fountain et al., 2008; Kruse et al., 2019), males and females were analyzed separately using a two-way-repeated measures ANOVA with drug treatment and day as the between-subjects and within-subject factor respectively. PCA index scores were calculated to classify goal-tracking, sign-tracking, or intermediate behavior based on performance during all 12 sessions (Meyer et al., 2012). The PCA Index score was calculated as the sum of response bias (contacting the CS+ lever or food cup entries in relation to the total number of CS+ or food cup responses), probability Difference (difference between the probability of pressing the CS+ lever and probability entering the food cup) and latency score (the difference between latency to contact the CS+ lever and latency to enter the food cup), which was then divided by 3. A PCA index score of -1.0 to -0.3 classified as goal-tracking behavior, and scores of +0.3 to +1.0 indicated sign-tracking behavior. A PCA index score in the range of -0.29 to +0.29 indicated intermediate behavior.

3 Results

3.1 Experiment 1: THC Plasma Concentrations Immediately After Vapor Exposures

The serum concentrations of THC, CBD and 11-OH-THC were identified in the rats immediately after vapor exposures. No significant levels of CBD were detected in any of the samples. The control groups also had no amounts of THC, CBD nor 11-OH-THC.

3.1.1 Serum Levels of THC

A univariate ANOVA revealed that there was no difference in serum THC levels between THC and Nic-THC treated groups and between males and females. There was also no interaction between treatment groups and sexes. Among the males, the THC group had a serum concentration of THC of 21.4ng/mL while the co-exposed Nic-THC group had a THC serum concentration of 22.0ng/mL. The female THC group had a serum THC concentration of 23.1ng/mL while the female co-exposed Nic-THC group had a concentration of 18.8ng/mL (Fig 2A).

3.1.2 Serum Levels of 11-OH-THC

A univariate ANOVA revealed that there was no difference in serum THC levels between THC and Nic-THC treated groups [$F(1,24)= 0.084$; $p= 0.774$], and no interaction between treatment group and sex [$F(1,24)= 0.090$; $p= 0.767$]. However, there was a significant difference in serum 11-OH-THC levels between males and females. Specifically, females exhibited higher serum 11-OH-THC concentrations than males [$F(1,24) = 11.111$; $p = 0.003$], indicating a sex-based variation in 11-OH-THC serum levels post-exposure. The male THC group had 2.8ng/mL while the male co-exposed Nic-THC group had 3.7ng/mL. Females exposed to THC alone had 7.8ng/mL. Similarly, females co-exposed to Nic-THC also had 7.8ng/mL 11-OH-THC in their plasma (Fig 2B).

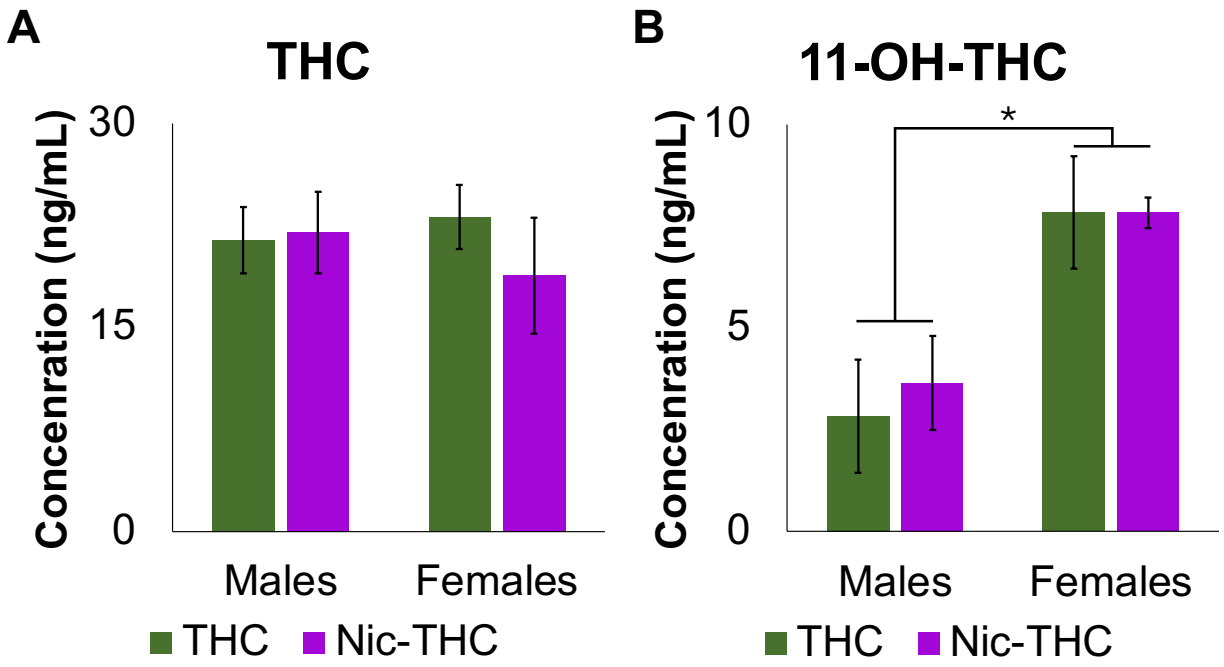

Figure 3: (A) Serum concentrations of THC are similar across sexes and exposure groups, (B) Increased serum 11-OH-THC in female rats. Data are mean ± standard error of mean (*p=0.003, comparing to males and females, univariate ANOVA).

3.2 Experiment 2: Short-term Behavioral Cannabinoid-Mediated Effects of Adolescent Nicotine and Cannabis Co-Exposure

To assess cannabinoid-receptor mediated effects throughout their exposure period, rats underwent tetrad tests at baseline, immediately after their first exposure, and on the 10th day of the 2-week exposure period. This consisted of measuring catalepsy, locomotion, body temperature and analgesia. No animals experienced catalepsy at any timepoint.

3.2.1 Acute THC Vapor Exposure Induced Hypothermia in Males on Day 10 of Exposures

A two-way ANOVA for repeated measures was utilized to evaluate the effects of drug treatments on body temperature across three key time points during the 2-week exposure period: baseline, day 1 of exposure, and day 10 of exposure. In males, the analysis indicated a trend towards differences in body temperature among the treatment groups, though this did not reach statistical significance [$F(3,28) = 2.586$; $p = 0.073$]. Furthermore, changes in body temperature over the specified time points did not significantly differ across groups [$F(2,56) = 0.810$; $p = 0.450$], indicating that repeated sessions did not significantly alter body temperature across exposure groups. However, a one-way ANOVA comparing differences between groups at this final time point, revealed a significant effect of treatment on body temperature activity [$F(3,28) = 4.091$, $p = 0.016$]. Post-hoc analyses revealed that the high-THC treated group displayed a decrease in body temperature compared to the control group on the 10^{th} day of exposures (p - 0.019 Bonferroni test, Fig 3A). There were no significant differences in body temperature over the three sessions across exposure groups in females.

3.2.2 Tail-Flick Latency was Increased in THC-exposed Males Compared to Nicotine-Exposed Males on Day 10 of Exposures

A two-way ANOVA for repeated measures was conducted to assess the impact of drug treatments on tail-flick latency in rats over a series of days. The analysis of between-subject effects indicated that the treatments did not significantly alter tail-flick latency overall [$F(3,28) = 1.778$; $p = 0.174$]. However, within-subject effects showed a significant change in tail-flick latency over the days since baseline [$F(2,56) = 12.249$; $p < 0.001$]. Importantly, there was a significant interaction between treatment and days [$F(6,56) = 5.114$; $p < 0.001$]. Post-hoc analysis revealed that rats

exposed to nicotine exhibited significantly lower tail-flick latency compared to those exposed to THC ($p = 0.005$ Bonferroni test, Fig 3B). In females, the analysis revealed a significant main effect of treatment [$F(3,28) = 3.353$; $p = 0.033$], and days on tail-flick latency [$F(2,56) = 5.227$; $p = 0.008$], showing that tail-flick latency varied over the course of the study. Post-hoc Bonferroni analyses revealed distinct differences only on day 1 of exposures: the nicotine group and the THC group demonstrated significantly lower tail-flick latency in comparison to the nicotine-treated group ($p < 0.05$) and the nicotine-treated group also showed lower tail-flick latency relative to the group co-exposed nicotine-THC treatment ($p < 0.05$) on day 1. Importantly, by day 10, these initial differences in pain sensitivity ameliorated, as indicated by the absence of significant differences between any of the groups at the final timepoint.

3.2.3 Locomotor activity

In assessing the effects of various treatments on locomotor in male rats, a two-way ANOVA for repeated measures was utilized. This analysis identified a main effect of treatment [$F(3,24) = 5.935$; $p = 0.004$] and repeated sessions [$F(3,72) = 61.261$; $p < 0.001$], with locomotor activity showing variability over the study duration. Initial observations suggested variations in tail-flick latency among treatment groups at baseline and day 1, with the nicotine group showing a tendency towards hyperlocomotion compared to controls ($p < 0.05$) However, it is important to note that baseline differences reflect pre-existing variability. Post-hoc Bonferroni adjustments also found significant differences between nicotine and THC groups on subsequent days 1 and 10 of exposures ($p < 0.05$; Fig 3C)

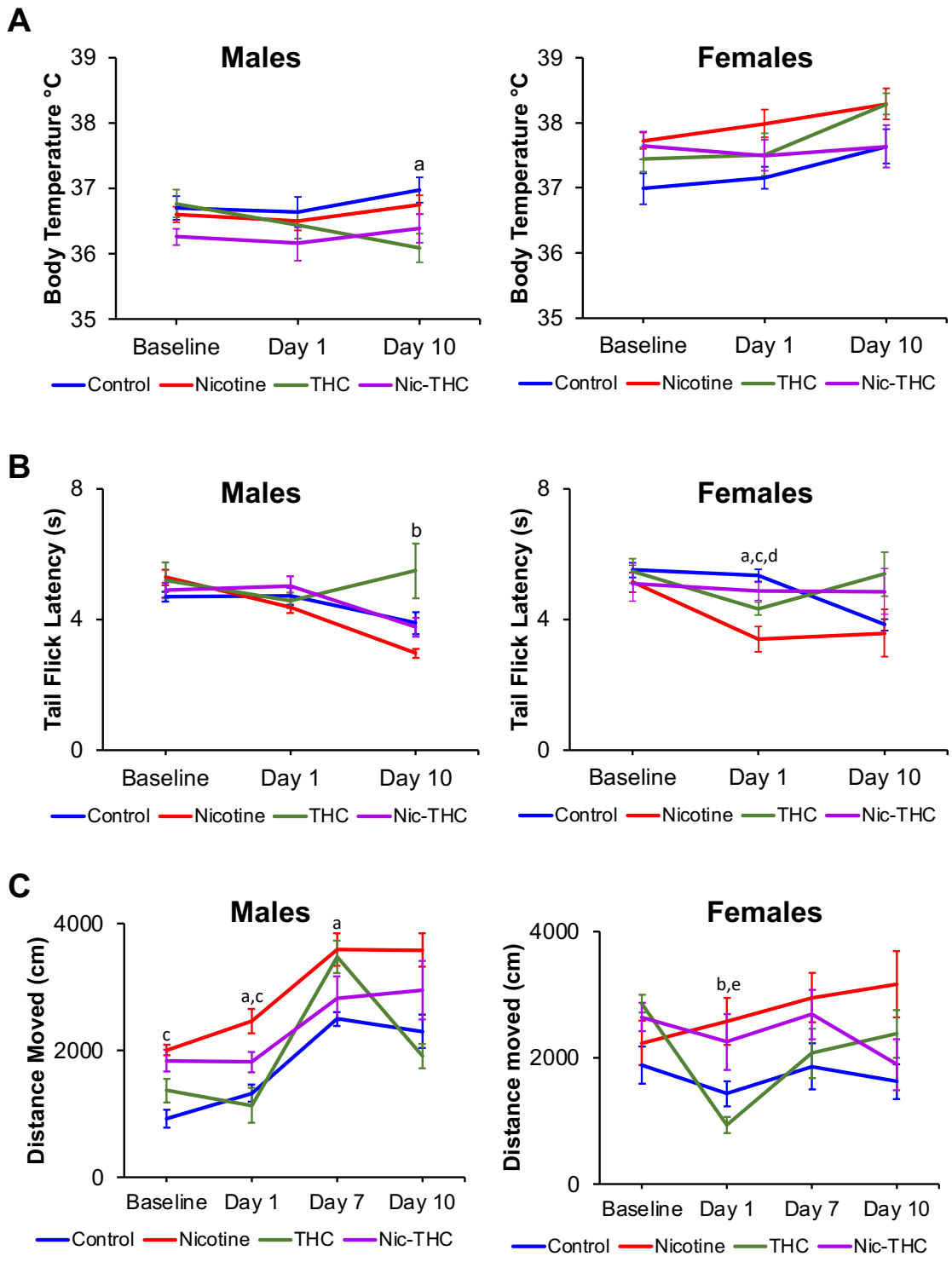

Figure 4: Cannabinoid Tetrad Test: (A) Body Temperature indicated chronic THC exposure induced hypothermia in males only. (B) THC group displayed increased tail flick latency compared to nicotine group in males only. (C) locomotor activity results of males and females following vapor exposure. Data are mean ± standard error of mean ([a]$p<0.05$, THC vs. Control; [b]$p< 0.05$, THC vs. Nicotine; [c] $p< 0.05$, Nicotine vs. Control; [d]$p<0.05$ Nicotine vs. Nic-THC; [e] $p<0.05$ THC vs. Nic-THC; Bonferroni post-hoc).

3.3 Experiment 3: Effects of Adolescent Nicotine and THC Co-Exposure on Behavioral Outcomes in Adulthood

Starting PND 65, rats were put on food restriction and underwent two behavioral tasks (PND 66-83) to investigate the long-term effects of adolescent nicotine and cannabis co-exposure on reward-learning behaviors. Between experiments, rats were returned to the home cage for a minimum of 24 hours. The following behavioral assays were conducted:

3.3.1 Locomotor Reactivity to a Novel Environment

To analyze locomotor reactivity to a novel environment across different drug exposure groups, a one-way ANOVA was conducted for each sex. A significant effect of drug treatments on distance moved were revealed in females [$F(3,28) = 4.032$; $p = 0.017$], while no significant effects were found in males. Further analysis using Bonferroni's multiple comparisons test a notable difference between the control and THC-Nicotine groups in females (Bonferroni, $p = 0.009$, Fig. 5), suggesting that the co-exposure to THC and nicotine significantly increases locomotor activity in females compared to control conditions. This effect was not paralleled in the male subjects, nor were significant differences observed between other treatment comparisons within each sex.

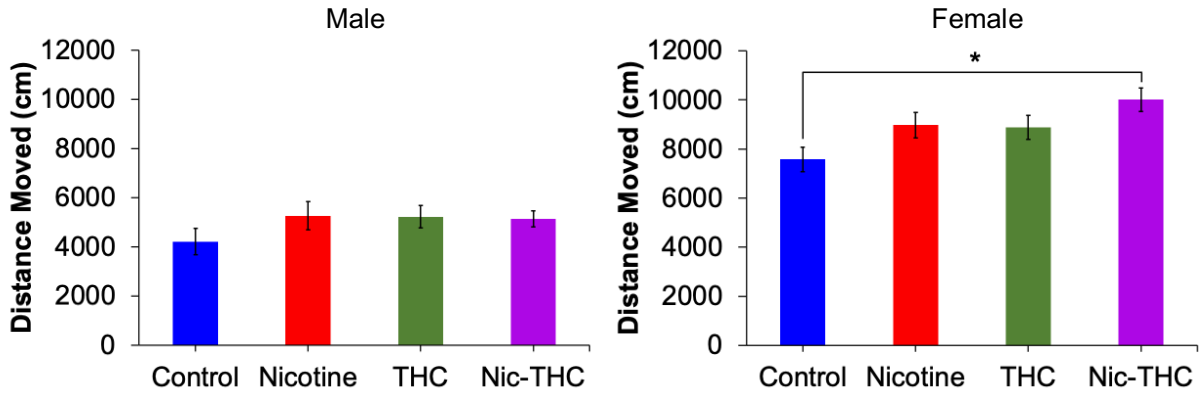

Figure 5: Locomotor reactivity in a Novel Environment indicated that only females co-exposed to Nic-THC exhibit hyperactivity to a novel environment compared to controls. (Female One-way ANOVA p=0.017; post-hoc Bonferroni *p=0.009)

3.3.2 Exposure in adolescence to either nicotine or cannabis vapor alone altered reward-associated learning in adulthood, while co-exposure ameliorated those alterations in males.

A two-way ANOVA for repeated measures indicated a significant effect of adolescent exposure to nicotine, THC, or their co-exposure on PCA index scores during adulthood in males [treatment effect: $F(3,280)= 9.628$; $p<0.0001$, Fig 5]. PCA scores significantly changed over the sessions [time effect: $F(11,280)= 54.31$; $p<0.0001$], but the effects of treatment did not interact with those changes over the sessions [interaction: $F(33,280)= 1.255$; $p=0.1672$]. Post-hoc analysis revealed distinct patterns in PCA index scores among male rats exposed to nicotine, THC, or their co-exposure during adolescence. On day 2, rats co-exposed to Nic-THC exhibited higher PCA scores than those exposed to nicotine alone ($p = 0.0302$). By day 3, controls displayed higher PCA scores compared to nicotine-only exposed rats ($p = 0.0293$), and both nicotine-only and THC-only exposed rats showed lower PCA scores than rats co-exposed to nicotine and THC ($p = 0.0002$ and $p = 0.0138$, respectively). This trend continued into day 4, with THC-only exposed rats maintaining lower PCA scores compared to their co-exposed counterparts ($p = 0.0267$). By day 5, significant contrasts were noted where the THC-only exposed rats exhibited lower PCA scores than vehicle-treated rats, nicotine-only exposed rats, and Nic-THC co-exposed rats ($p = 0.0481$; $p = 0.0054$; $p = 0.0119$, Fig 6). There were no significant differences between treatment groups in female rats in the Pavlovian Conditioned Approach task.

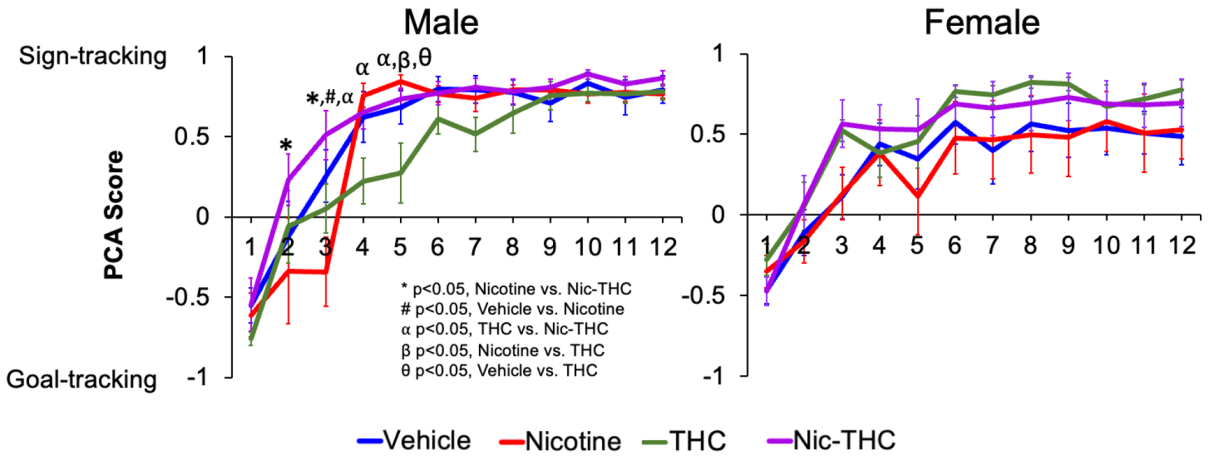

Figure 6: Pavlovian Conditioned Approach (PCA) scores indicate adolescent THC exposure impairs appetitive Pavlovian learning in males only. Data are mean ± standard error of mean (*$p< 0.05$, Nicotine vs Nic-THC; #$p< 0.05$, Vehicle vs Nicotine, α$p<0.05$, THC vs. Nic-THC, β$p<0.05$, Nicotine vs. THC, θ$p<0.05$ Vehicle vs. THC)

3.4 Experiment 4: Effects of Adolescent Nicotine and THC Co-Exposure on Functional Neural Connectivity in Adulthood

Data were successfully collected from 32 male and 32 female rats. Figure 7 shows a representative single subject T2 anatomical image, and the first volume of an fMRI dataset.

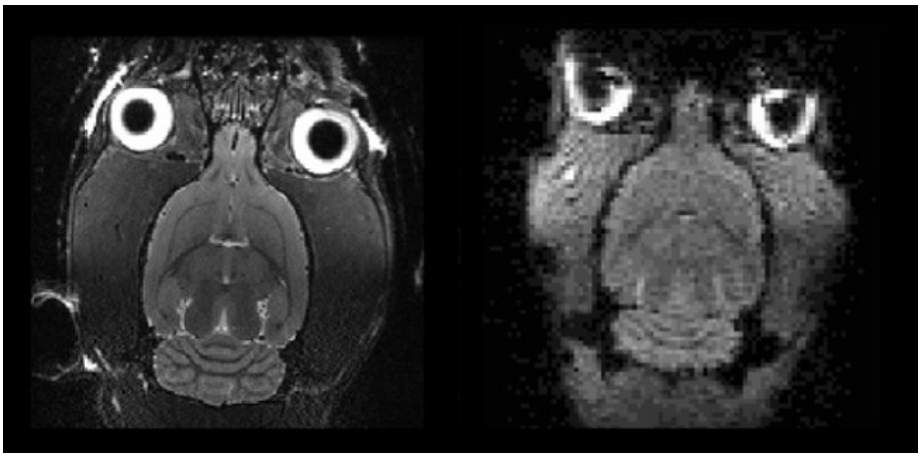

Figure 7: Representative single subject T2 anatomical image (left) and the first volume of an fMRI dataset (right)

NBS statistics revealed a single network with altered functional connectivity in males amongst the four groups using an ANOVA (p = 0.027, 8 edges, 9 nodes). The affected connections included the Cingulate Cortex (interhemispheric), Parietal Cortex (right), Dorsal Dentate Gyrus (left), Dorsal Hippocampus (right), Insular Cortex (right), External Colliculus (VI) (left), Piriform Cortex (left), Endo/Piriform Cortex (left), and the External Cortex of the Inferior Colliculus (right).

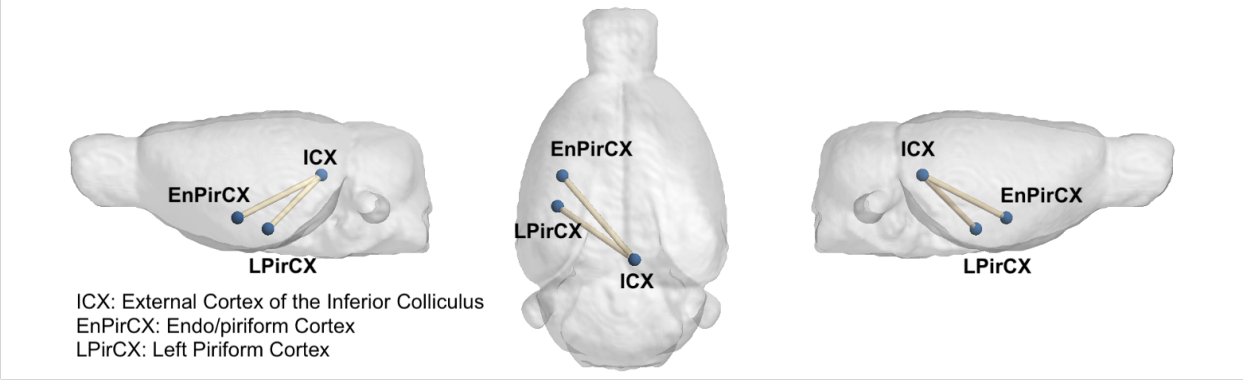

Figure 8: NBS statistics revealed a single network with altered functional connectivity (p = 0.0026, 2 edges, 3 nodes). The affected connections comprised the Piriform Cortex (Left), Endo/piriform cortex (Left), and the External Cortex of the Inferior Colliculus (Right).

Post-hoc t-tests revealed a single network, comprised of the Piriform Cortex (left), Endo/Piriform Cortex (left), and the External Cortex of the Inferior Colliculus (right) with increased functional connectivity in the THC group in comparison with the combined Nic-THC group ($p = 0.0026$, 2 edges, 3 nodes, Table 1, Fig 8).

NBS statistics revealed no networks with altered structural connectivity amongst the four female groups using an ANOVA.

Table 2: All connections identified by NBS statistics to have statistically significantly higher functional connectivity in the THC group as opposed to the combined Nic-THC group. ($p = 0.0026$, 2 edges, 3 nodes).

Node 1	Hemisphere	Node 2	Hemisphere
Piriform Cortex	Left	External Cortex of the Inferior Colliculus	Right
Endo/piriform cortex	Left	External Cortex of the Inferior Colliculus	Right

4 Discussion

Amidst the rising trend of nicotine vaping and cannabis smoking among adolescents (Canada, 2022; Rao et al., 2023; Zuckermann et al., 2019), the long-term effects of their co-exposure on behavior and neural connectivity remain largely unexplored. This study aims to investigate these impacts through passive exposure to both nicotine and cannabis flow vapor during adolescence, utilizing a vapor administration method reflective of prevalent trends of use, which addresses potential pharmacokinetic differences associated with alternative routes of administration (Rotermann, 2021; Rotermann & Gilmour, 2022). This approach enhances the translational relevance of our findings, to better align with the current landscape of adolescent substance use. Our results indicate sex-dependent impacts on incentive and associative learning processes, highlighting the need for further research into the complex consequences of dual substance exposure during a critical neurodevelopmental period.

4.1 Plasma Concentrations Following Vapor Exposure

Adolescent males and females exposed to THC in isolation or co-exposed with nicotine exhibited similar plasma THC concentrations 15 min post-exposure, indicating that nicotine co-exposure does not substantially alter THC's pharmacokinetic profile. These results contribute to the broader understanding of poly-substance use, indicating that nicotine may not significantly influence the initial absorption and distribution of THC in the body. Moreover, the absence of sex-based differences in serum THC levels is consistent with human findings in the literature (Sholler et al., 2021).

In contrast, the analysis of 11-hydroxy-THC (11-OH-THC) levels reveals significant sex-dependent differences in the THC-only exposure group, and a trend towards significance in the Nic-THC co-exposure group, where females displayed increased plasma levels of 11-OH-THC compared to males. Our findings also suggest that co-exposure to nicotine does not affect the sex-specific differences in THC metabolism. The higher levels of 11-OH-THC found in female plasma agree with previous literature (Sholler et al., 2021; Torrens et al., 2022), which may support underlying differences in the metabolism and disposition of THC between sexes. The potential influence of hormonal variations on 11-OH-THC pharmacokinetics highlights the need for sex-specific considerations when addressing cannabis use and designing intervention strategies.

4.2 Immediate Behavioral Effects of Combined Nicotine and Cannabis Use in Adolescents

By the tenth day of THC vapor exposure, males exhibited hypothermia. This significant and temporally isolated finding suggests that the hypothermic effects of THC may become more pronounced or sensitized after repeated exposure. Notably, in the male group co-exposed to nicotine and THC, there were no differences compared to the control group, suggesting a potential compensatory effect of nicotine on body temperature in adolescent males. Interestingly, the absence of significant alterations to body temperature across exposure groups in females indicates a potential sex difference in the thermoregulatory response to THC. This observation aligns with previous studies indicating that repeated vapor exposures to THC in females induce tolerance to THC-induced hypothermia (Nguyen et al., 2020). Such tolerance could be attributed to biological differences, including hormonal influences on cannabinoid receptor sensitivity and body temperature regulation. Specifically, estrogen has been shown to modulate the endocannabinoid system and could play a role in mediating the body's response to THC (Craft et al., 2013). The lack of significant body temperature changes in females following THC exposure supports potential biological differences in how THC affects physiology.

This study's findings on the antinociceptive effects of THC, as measured by tail-flick latency, indicate significant opposing effects of THC and nicotine exposure. In males, exposure to THC led to an increase in tail-flick latency compared to nicotine by day 10 of exposures, indicating an enhanced analgesic effect of THC. Conversely, females showed a variation in pain sensitivity over the course of the study, with initial differences between nicotine and THC exposure diminishing by day 10. This indicates that the acute effects of these substances on pain sensitivity may lessen with repeated exposure in females, indicating an increased tolerance to alterations in pain.

Variations in locomotor activity were also observed, with some initial indications of increased movement in the male nicotine group when compared to controls, possibly reflecting pre-existing variability within the subjects. In both sexes, the nicotine group trends upwards in locomotor activity throughout the study, although not statistically significant. This trend has been seen in previous literature, as low doses of acute nicotine exposure has induced hyperlocomotion (Lallai et al., 2021).

The differential response to THC and nicotine in males and females may be explained by various physiological and biological factors. For instance, differences in cannabinoid receptor density and distribution between males and females could influence how each sex responds to THC (Fattore & Fratta, 2010). Research has indicated that cannabinoid receptors, specifically CB1 receptors, are more densely populated in some areas of the male brain, which could potentially enhance the physiological effects of cannabinoids like THC (Fattore & Fratta, 2010).

The findings from this study highlight the importance of considering the effects of both sex and substance interactions when examining the behavioral effects of substance exposure during adolescence. The sex-dependent responses to THC and nicotine, particularly in terms of thermoregulation and pain sensitivity, highlight the complex interplay between biological sex and polysubstance use. These findings align with emerging research suggesting that females may exhibit different physiological and behavioral responses to cannabinoids, potentially due to differences in hormone levels, cannabinoid receptor density, or other sex-specific physiological factors (Craft et al., 2013; Fattore & Fratta, 2010; Kennedy, 2008; Nguyen et al., 2020; Waxman & Holloway, 2009). Moreover, the differential impact of THC and nicotine on body temperature regulation and locomotor activity emphasizes the need for a deeper understanding of how these substances interact and the unique risks they may pose to adolescent development.

4.3 Long-term Behavioral Effects of Nicotine and Cannabis Co-Exposure During Adolescence

Adolescent THC vapor administration led to impaired reward-learning in a Pavlovian Conditioned Approach (PCA) task in adulthood, where THC-exposed rats took longer to learn the task compared to those exposed to vehicle, nicotine, and co-exposed to Nic-THC in adolescence. Yet by the end of the two-week task, the THC-exposed group showed similar sign-tracking behavior to the rest of the groups, indicating their slower ability to learn the reward task. Previous studies in rats have investigated long-term effects of exposure to THC or other cannabinoids in adolescence on reward learning. One study in adulthood found that rats injected with THC had impairments in impulse control and attentional function in a five-choice serial reaction time task (Irimia et al., 2015). The contrast of these findings with the results of our PCA task, where THC-exposed rats were less inclined to interact with the reward cue at earlier timepoints, suggests that the observed effects are mediated by impaired reward learning, rather than decreased impulsivity

or motivation to receive reward. Another study, consistent with our results, found that rats exposed to THC in adolescence exhibited learning impairments upon learning a simple operant task in adulthood (Abela et al., 2023). In humans, a longitudinal study associated cannabis use with a blunted neural response during reward anticipation in the NAcc in a monetary incentive delay task (Martz et al., 2016), consistent with our behavioral findings in rats. Our results found that nicotine co-exposure seemed to neutralize the effects of THC on reward-related behavior. A previous study by our lab displayed that a higher concentration of adolescent nicotine exposure in isolation led to a persistent increase in sign-tracking behavior in adult male rats (Ruffolo et al., 2022), suggesting that nicotine may have ameliorated the THC-induced learning impairments in the Pavlovian Conditioned Approach task by increasing sign-tracking behavior similar to that of controls.

Interestingly, our results also indicated that co-exposure to cannabis and nicotine in adolescent female rats led to a hyperactive locomotor response in a novel environment. Hyperlocomotion in a novel environment has been associated with hyperdopaminergic activity (Vanhille et al., 2015). The dopaminergic system is involved in the control of movement for reward prediction, motivation, and cognition (Olguín et al., 2016). The increased locomotor activity in a novel environment could be a manifestation of an enhanced dopaminergic tone, which is known to increase the salience of novel stimuli and environments (Vanhille et al., 2015). This is consistent with findings that dopaminergic pathways, particularly those projecting from the VTA to the NAcc, are involved in the modulation of locomotion and exploration (Nicola, 2007). Our findings suggest the dopaminergic system may be modulated by the concurrent use of nicotine and cannabis during the adolescent neurodevelopmental period. The heightened locomotor response in a novel environment observed in our female rats, therefore, may not only reflect an increased exploratory drive but also an elevated reward sensitivity, suggesting that these animals may have a greater propensity to seek out and respond to rewarding stimuli (Kalinichev et al., 2004).

The implications of these findings are significant, especially when considering the long-term behavioral and neural consequences of adolescent exposure to nicotine and cannabis. The impairment in reward-learning found in THC-exposed male rats, and the suggested hyperdopaminergic state found in female rats co-exposed to nicotine and THC sheds light on the sex differences in long-term behavioral outcomes of nicotine and THC vapor exposure. The altered reward-learning and sensitivity we found between sexes could predispose individuals to a range

of behavioral phenotypes in adulthood, including learning impairments in males and increased risk-taking behaviors in females. This is particularly concerning given that adolescence is a critical period for neural development and the establishment of neural circuits governing reward and motivation (Spear, 2000).

4.4 Long-term Neural Connectivity Changes Due to Adolescent Nicotine and Cannabis Use

Our study revealed increased functional connectivity in male THC groups compared to Nic-THC in brain regions critical for sensory integration. In male rats, the observed changes in the Piriform Cortex (Left), Endo/piriform Cortex (Right), and the External Cortex of the Inferior Colliculus (Right), suggests a targeted impact of adolescent exposure to nicotine and THC on regions associated with sensory processing (Barrière et al., 2019). The Piriform Cortex and endopiriform cortex, integral to olfactory processing, and the External Cortex of the Inferior Colliculus, a key component of the auditory pathway, are crucial for the integration and processing of sensory information (Gruters & Groh, 2012). The observed alterations in connectivity within this network could imply a long-term modulation of sensory processing and integration mechanisms, potentially affecting sensory perception and related behaviors in adulthood. This suggests that THC exposure, either in isolation or in conjunction with nicotine, may have a distinct influence on the development and maintenance of neural circuits involved in sensory processing. The differential impact of nicotine and THC on neural connectivity highlights the complexity of substance interactions and their potential to induce lasting changes in brain function. Interestingly, a study conducted in adult male mice found that acute injections of both nicotine and THC lead to an increase in c-fos expression which correlates with neuronal activity (Valjent et al., 2002). This suggests that while nicotine and THC alter activity in the piriform cortex, they display differing effects depending on the type of exposure (chronic or acute) and the age of exposure. It is also important to consider that the study by Valjent et al. (2002) was done in adult mice and used injections as the route of administration compared to our study in adolescent rats with passive vapor administration as our method of drug exposure.

The inferior colliculus receives glutamatergic input from several sources for auditory processing (Robinson et al., 2019). The piriform cortex also receives substantial glutamatergic inputs which are essential for the processing and integration of olfactory information, as well as for the

modulation of olfactory-induced behaviors (Wilson & Sullivan, 2011). Glutamatergic neurotransmission in the piriform cortex is involved in the encoding of olfactory memories and the associative learning processes linking odors to rewarding or aversive outcomes (Wilson & Sullivan, 2011). The increase in functional connectivity between the piriform cortex and the inferior colliculus in males may then be consistent with our behavioral findings of impaired reward-learning in males since they may have suffered from impaired encoding of olfactory memories, resulting in decreased performance of associative learning in the Pavlovian Conditioned Approach task.

Our findings display the impact of adolescent nicotine and THC exposure on sensory processing neural circuits in male rats. The observed alterations in functional connectivity within key sensory regions, alongside behavioral impairments in reward learning, underscores the complex effects of early substance use on neural development and function. These findings emphasize the need for further exploration into the long-term sex-dependent consequences of adolescent exposure to nicotine and THC, to better understand and mitigate its impacts on brain and behavior.

4.5 Limitations and Future Directions

One notable limitation of the study is the uniform exposure period of PND 28-42 for both male and female rats, despite known differences in the timing of adolescent development between sexes. This approach does not fully account for the variations in the onset and progression of adolescence in male and female rats, which can influence susceptibility to substance exposure effects (Spear, 2000). The adolescent period in rats is characterized by significant neurodevelopmental changes, including synaptic pruning, myelination, and hormonal fluctuations (Spear, 2000). The timing of these processes is earlier in females than males (Spear, 2000). By applying a uniform exposure window, the study may not capture the critical periods of vulnerability or resilience specific to each sex. The onset of puberty, marked by surges in sex hormones, occurs at different times for male and female rats (Schneider, 2013). These hormonal changes play a crucial role in brain development and behavior and can modulate the effects of psychoactive substances. The fixed exposure period may overlook the impact of these hormonal shifts, particularly in females, whose earlier puberty onset could coincide with the latter part of the exposure window. Specifically, estrogen has been known to induce alterations in cytochrome P450 enzyme expression, which can

both act on and be acted upon by THC, thereby possibly altering plasma drug concentrations (Choi et al., 2013). Furthermore, the influence of growth hormones, which exhibit sex-dependent expression during the onset of adolescence and have sex-dependent effects on CYP enzymes, may further modulate these interactions and contribute to the observed sex differences in drug metabolism and response (Kennedy, 2008; Waxman & Holloway, 2009). The differential timing of adolescent development between sexes limits the ability to discern whether observed sex differences in outcomes are attributable to the substances' effects, differences in developmental timing across sexes, or a combination of both. Future research should consider staggered or sex-specific exposure periods that align with the developmental milestones of male and female rats (Schneider, 2013; Spear, 2000). This approach would enable a more precise assessment of the effects of nicotine and THC exposure during adolescence and improve the generalizability of findings to human populations.

A limitation of passive cannabis and nicotine exposure is that vapor is left remaining within the chamber after the duration of administration. Even though the concentration of the cannabis and STLTH pod is pre-established, a proportion of vapor in the chamber is not being inhaled, leading to ambiguity in precisely measuring total consumption. In addition, increased variability could potentially arise across subjects, due to the difference in breathing rate among the rats (Frie et al., 2020). While we obtained plasma samples to analyze THC concentration, future studies may also analyze plasma concentrations of nicotine.

Our study also measures adult behavior from PND 66 - PND 83, while MRI scans were done after PND 120 because rats needed to be transferred from the University of Guelph Central Animal Facility to Robarts Research Institute at Western University after behavioral testing. Thus, we can only compare our neural and behavioral outcomes as correlational since they were collected at different timepoints during the experimental period. While using fMRI to analyze neural connectivity offers a structured approach to understanding the brain's complex networks, it also comes with its own set of limitations. A functional atlas divides the brain into regions based on neural activity that happens near anatomical landmarks (Barrière et al., 2019). This division can lead to a generalization where diverse neural activities within a region are aggregated under a single label. Such generalization may mask the diversity of localized neural activities and their specific contributions to observed behaviors or physiological responses. To confirm neural activity

in specific brain regions in the future, it would be beneficial to obtain c-fos levels in altered brain regions to confirm differences in neural activity (Lazovic et al., 2005).

Addressing the limitations identified in this study, future research should prioritize the development of experimental designs that account for the differential timing of adolescent development between sexes, ensuring that exposure periods are aligned with the specific developmental stages of male and female rats. This approach would facilitate a more accurate assessment of the neurobehavioral consequences of nicotine and cannabis exposure during adolescence. Additionally, enhancing the precision of substance exposure measurements and incorporating methodologies to directly measure inhalation rates could significantly reduce variability and improve the reliability of dose-response relationships. The integration of other neuroimaging techniques, such as diffusional MRI to investigate structural brain changes, alongside molecular markers of neural activity, would be beneficial in elucidating the causal pathways linking substance exposure to observed outcomes. By refining experimental protocols and expanding the scope of analyses, future studies can overcome the current limitations, offering deeper insights into the complex interplay between substance exposure, neurodevelopment, and long-term behavioral and neural consequences. Such research is essential for informing public health strategies and interventions aimed at mitigating the adverse effects of substance use during the critical developmental window of adolescence.

5 Conclusion

Studies investigating the effects of adolescent cannabis and nicotine vapor co-use are limited despite the increasing prevalence of dual usage of these substances. This study was the first to look at the long-term effects of combined adolescent passive cannabis and nicotine vapor on behavior and cognition in a rat model. The results indicated THC in isolation and co-used with nicotine, impacted the behavioral measures we investigated in a sex-dependent manner. Further research is needed to investigate the interactions between different doses of nicotine and cannabis to further understand the long-term synergistic and neutralizing effects of their combined use.

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Appendices

Appendix A: Ethics Approval Letter for the Animal Use Protocol at the University of Guelph.

Tuesday, June 18, 2024 at 15:09:08 Eastern Daylight Time

Subject: AUP #4789 has been approved
Date: Monday, October 30, 2023 at 11:32:12 AM Eastern Daylight Saving Time
From: [redacted]
To: Jibran Khokhar
CC: Animal Care Services

Hello PI,

Your AUP has been approved. If you would like to review your aup, please visit the following link to view your AUP.

[redacted]

Please note that under the current COVID 19 restrictions, further authorization is necessary in order to conduct research at this time. Please see the Office of Research COVID-19 information page here for details:
[redacted]

Thanks!

Appendix B: Ethics Approval Letter for the Animal Use Protocol at Western University.

Tuesday, June 25, 2024 at 08:23:16 Central Daylight Time

Subject: eSirius3G Notification -- 2020-145 Annual Renewal Approved
Date: Thursday, March 21, 2024 at 8:51:14 AM Central Daylight Saving Time
From: eSirius3GWebServer
To: Miranda Bellyou, Jibran Khokhar, acc
CC: Jennifer McInnis

Western

2020-145:17

AUP Number: 2020-145
PI Name: Khokhar, Jibran
AUP Title: Rat models to study co-occurring mental illness and substance use disorders, and the impact of adolescent drug use on these disorders
Yearly Renewal Date: 03/01/2025

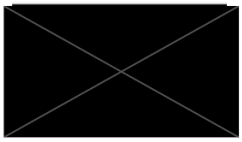
The **annual renewal** to Animal Use Protocol (AUP) 2020-145 has been approved by the Animal Care Committee (ACC), and will be approved through to the above review date.

Please at this time review your AUP with your research team to ensure full understanding by everyone listed within this AUP.

As per your declaration within this approved AUP, you are obligated to ensure that:

1. This Animal Use Protocol is in compliance with:
   - Western's Senate MAPP 7.12 [PDF]; and
   - Applicable Animal Care Committee policies and procedures.
2. Prior to initiating any study-related activities—as per institutional OH&S policies—all individuals listed within this AUP who will be using or potentially exposed to hazardous materials will have:
   - Completed the appropriate institutional OH&S training;
   - Completed the appropriate facility-level training; and
   - Reviewed related (M)SDS Sheets.

Submitted by: McInnis, Jennifer on behalf of the Animal Care Committee



Dr. Arthur Brown,

Animal Care Committee Chair

Animal Care Commitee
The University of Western Ontario
London, Ontario Canada N6A 5C1

*** THIS IS AN EMAIL NOTIFICATION ONLY. PLEASE DO NOT REPLY ***

7 Curriculum Vitae

Name:	Iman Sheikh Aziz
Post-secondary Education and Degrees:	University of Guelph Guelph, Ontario, Canada 2018-2022 B.Sc. The University of Western Ontario London, Ontario, Canada 2022-2024 M.Sc.
Honours and Awards:	Canadian Institutes for Health Research: CGSM Award 2023-2024 Andrea Leger Dunbar Award Summer Research Assistantship 2019, 2020
Related Work Experience	Research Student University of Guelph 2019, 2020

Publications:

Aziz, Iman S. et al. (2021). Circadian Influence on Inflammatory Response During Cardiovascular Disease. Current Opinion in Pharmacology, 57, 60-70