UNIVERSITY OF CALIFORNIA,
IRVINE

Persistent Effects of Adolescent THC Exposure on Behavior and the Brain

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Science

by

Maricela X. Martinez

Dissertation Committee:
Professor Stephen V. Mahler, Chair
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DEDICATION

To
my parents, brother, cat and friends –

for their undying support and love.

'It was written I should be loyal to the nightmare of my choice."
-Joseph Conrad, *Heart of Darkness*

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Butcher, G. M.[1], Ramirez, E. M.[1], Ruiz, C. M.[1], Castillo, E., Farrell, M.R., **Martinez, M. X.**, Mahler, S.V. (2026). Unpacking Ventral Pallidum GABA Neuron Contributions to Opioid Drug Reward in Rats. Journal of Neuroscience.

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ABSTRACT OF THE DISSERTATION

Persistent Effects of Adolescent THC Exposure
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Adolescence is a sensitive period for development of addiction-relevant brain circuits that regulate cognition, drug reinforcement, and motivation. These processes must be carefully balanced to guide adaptive behavior, yet they are particularly vulnerable to disruption during development. Cannabis is one of the most commonly used illicit substances among adolescents, and early-life exposure to Δ9-tetrahydrocannabinol (THC) is associated with an increased risk for psychiatric disorders and later-life substance use. However, the mechanisms by which adolescent THC (AdoTHC) exposure produces persistent alterations across cognitive and motivational domains remain incompletely understood.

Here, I explore whether AdoTHC exposure persistently alters brain circuitry and subsequent behavior across three domains: cognition, drug reinforcement, and motivation. Using a suite of behavioral paradigms assessing decision-making, drug reinforcement, and motivation in rats, combined with cell-type specific chemogenetics and immunohistochemistry approaches, I examine the behavioral and circuit-level consequences of AdoTHC exposure. I demonstrate that 1) AdoTHC potentiates amphetamine-induced risky decision-making independent of stimulation of ventral tegmental area dopamine neurons or their projections to prefrontal cortex, 2) AdoTHC promotes a persistent pro-opioid phenotype, especially in females, as well as region- and network-level changes in the brain contributing to distinct neural profiles under heroin in each sex, and that 3) AdoTHC sex-dependently alters responding to aversive stimuli, stressor-modulated motivation for opioid-drug seeking, and neural activity within the prefrontal cortex under pharmacological stress.

Together, these findings support the conclusion that AdoTHC exposure produces enduring alterations in brain circuitry that reshape adult cognitive, motivational, and drug reinforcement processes in male and female rats. By integrating these three domains within a unified framework, this work provides experimental evidence for the causal effects of AdoTHC exposure and offers mechanistic insights into how the convergence of these alterations induced by early-life cannabis exposure may increase vulnerability to addiction in humans.

INTRODUCTION

Adolescence as a Critical Period for Development of Addiction-Relevant Brain Circuits.

Adolescence is a dynamic period in the development of frontal brain circuits, and for one's life-long cognitive, emotional, and motivational traits and proclivities. During adolescence, the brain undergoes remodeling of circuits relevant to these behavioral processes, which normally leads to a stable personality and behavioral tendencies by adulthood. For example, prefrontal cortical (PFC) systems continue maturing until early adulthood, so during adolescence, 'top-down' control over earlier-developing 'bottom-up' motivational and emotional systems is still relatively weak (Gogtay et al., 2004; Casey et al., 2008; Spear, 2013). This differential maturation supports social learning, development, and exploration, essential for fostering independence and facilitating the transition to adulthood (Spear, 2000; Crone and Dahl, 2012). However, this combination of heightened subcortical responsiveness and immature top-down regulation may bias adolescents toward emotionally driven, impulsive, or risky behaviors, including experimentation with addictive drugs (Spear, 2000; Galvan et al., 2007; Casey et al., 2008; Somerville et al., 2010; Crone and Dahl, 2012; Spear, 2013). Unfortunately, there is considerable correlational evidence that early drug users have worse outcomes on a range of measures including an increased propensity for later-life drug problems (Kandel, 1975; Palmer et al., 2009; Moss et al., 2014). It is likely that underlying risk factors contribute to both later-life outcomes and early onset of drug use, but it is also plausible that drugs directly alter adolescent neurodevelopmental processes, and thus permanently alter the adult brain.

The adolescent brain undergoes several developmental changes that may be disrupted by drugs of abuse, especially in late-maturing brain regions like PFC. These include increases in myelination (Durston et al., 2001; de Faria Jr et al., 2021), decreases in synapse number (Huttenlocher, 1979, 1984), maturation of neuromodulatory systems including endocannabinoids and dopamine (Freund et al., 2003; Tseng and O'Donnell, 2007; O'Donnell,

2011; Caballero et al., 2016; Caballero and Tseng, 2016; Klune et al., 2021), and the continued development of cortical GABAergic interneurons (Caballero and Tseng, 2016; Caballero et al., 2021) that contribute to the refinement of excitation/inhibition (E/I) balance. These dynamic changes are thought to underlie emerging adolescent behavioral capabilities such as cognitive control, response inhibition, and social and emotional cognition (Jentsch and Taylor, 1999; Casey and Jones, 2010; Galván, 2010; Caballero et al., 2016; Hoops and Flores, 2017).

Preclinical studies show that various drugs of abuse can alter neurodevelopmental processes occurring in the adolescent brain (Spear, 2016; Salmanzadeh et al., 2020; Steinfeld and Torregrossa, 2023), and that adolescent drug exposure can lead to long-lasting changes in biological and behavioral phenotypes. Dopamine, a neurotransmitter that is intimately linked to reward learning and motivation, is potentiated by nearly all addictive drugs and is one system that may be affected by adolescent drug use (Wise, 2002; Pierce and Kumaresan, 2006; Koob and Volkow, 2010). Ventral tegmental area (VTA) dopaminergic axons innervate PFC during adolescence in rodents (Wahlstrom et al., 2010; Manitt et al., 2011; Hoops and Flores, 2017; Reynolds et al., 2018), and since activity in these developing circuits is likely related to their mature connectivity (Naneix et al., 2012), excessive drug-induced activation of dopamine signaling might alter the functional connectivity of these dopamine systems with other concurrently-developing PFC circuits involving glutamate, GABA, and others.

While most drugs of abuse influence developing addiction-relevant circuits in adolescence through dopamine, other neurotransmitter systems, such as the endocannabinoid system, can modulate dopamine signaling and thereby shape adolescent behavior. The endocannabinoid system indirectly regulates dopamine transmission by modulating GABAergic and glutamatergic inputs onto dopamine neurons within mesocorticolimbic circuits (Bossong and Niesink, 2010; Fitzgerald et al., 2012). Exogenous cannabinoids, like Δ9-tetrahydrocannabinol (THC), can therefore influence reward-related behaviors governed by dopaminergic activity, potentially altering circuit development and adult behavior.

The Endogenous Cannabinoid System and Impacts of THC. Cannabis is one of the most commonly used illicit drugs among adolescents (Johnston et al., 2022) and the ongoing significant neurodevelopmental changes and maturation in the adolescent brain render it susceptible to the effects of cannabis. Human studies have shown that early exposure to cannabis is associated with cognitive impairments, risky decision-making, and increased incidence of neuropsychiatric disorders, including schizophrenia (Schneider, 2008; Malone et al., 2010; De Bellis et al., 2013; Curran et al., 2016; Volkow et al., 2016). Notably, teenage cannabis use is associated with greater propensity of subsequently experimenting with other drugs of abuse (Yamaguchi and Kandel, 1984; Fergusson and Horwood, 2000; Lynskey et al., 2003; Agrawal et al., 2004; Hall and Lynskey, 2005; Fergusson et al., 2015; Wilson et al., 2022; Albaugh et al., 2023). A comprehensive understanding of the impact of adolescent cannabis use on the brain and its long-term risks is necessary for uncovering the underlying mechanisms that can inform preventative strategies and reduce the public burden of mental health issues.

The endocannabinoid (eCB) system, comprised of cannabinoid receptor types 1 and 2 (CB_1R and CB_2R), its endogenous ligands, anandamide (AEA) and 2-arachidonoyl glycerol (2-AG), and the enzymes responsible for their synthesis and degradation (Mechoulam and Parker, 2013; Lu and Mackie, 2021), plays an important role in shaping neuronal communication during development. CB_1Rs are highly expressed on GABAergic and glutamatergic presynaptic terminals throughout mesocorticolimbic circuits (Fitzgerald et al., 2012), where retrograde eCB signaling regulates neurotransmitter release, fine-tunes E/I balance, and supports synaptic plasticity and circuit maturation (Alger, 2002; Freund et al., 2003; Chevaleyre et al., 2006; Kano et al., 2009; Castillo et al., 2012; Ohno-Shosaku and Kano, 2014). Importantly, components of the eCB system dynamically change throughout one's life, as levels of CB_1R expression peak during adolescence (de Fonseca et al., 1993), and ligand levels fluctuate before stabilizing in adulthood, as AEA gradually increases throughout adolescence while 2-AG declines during early- to mid-adolescence before increasing prior to adult onset (Ellgren et al., 2008; Bara et al.,

2021; Peters et al., 2022; Rodrigues et al., 2024). Thus, impacts to the eCB system, such as with exogenous cannabinoid receptor stimulation with drugs like THC during adolescence, may potentially disrupt not only normative eCB signaling, but also the refinement of developing neural circuits.

Indeed, adolescent THC (AdoTHC) exposure disrupts normative endocannabinoid signaling in a sex- and region-specific manner. Chronic AdoTHC exposure produces broad CB_1R downregulation and desensitization across prefrontal and limbic regions which is more persistent and pronounced in females (Rubino et al., 2008; Burston et al., 2010; Rubino et al., 2015; Silva et al., 2015; Farquhar et al., 2019). While AdoTHC-induced CB_1R downregulation is consistent across studies, region-specific effects vary and may reflect differences in AdoTHC exposure protocols. In females, CB_1R density levels are reduced in the VTA, amygdala, and nucleus accumbens (Rubino et al., 2008), whereas males exhibit increases in CB_1R density within the nucleus accumbens shell and PFC with reductions in the amygdala and VTA CB_1R-expressing glutamatergic synaptic terminals (Ellgren et al., 2008; Rubino et al., 2008; Kruse et al., 2019; Poulia et al., 2021). Additionally, AdoTHC exposure may perturb endogenous eCB levels in a sex-specific manner, as AdoTHC exposure in females persistently reduces AEA levels in the PFC, while no such effect has been observed in males (Ellgren et al., 2008; Rubino et al., 2015).

These molecular changes in eCB signaling are accompanied by disruptions in eCB-mediated long-term plasticity mechanisms. AdoTHC exposure in both sexes persistently reduces eCB-mediated long-term depression in the PFC (Rubino et al., 2015; Shi et al., 2024), therefore disrupting the PFC's ability to regulate E/I balance and potentially impairing top-down control over subcortical motivational and emotional circuits. Thus, AdoTHC-induced disruptions to the eCB system may induce long-lasting consequences in behavioral domains that are supported by these networks, including cognition, drug reinforcement, and motivation.

Behavioral Consequences of Adolescent THC Exposure. *Cognitive Control Following Adolescent THC Exposure.* The prefrontal cortex is critical for adaptive, goal-directed behavior guided by the integration of information about the external world and our internal states, including motivation, affect, and memories (Miller and Cohen, 2001; Ott and Nieder, 2019; Howland et al., 2022). The more information encountered about the world, the greater the competing interpretations and actions that will require coordinated executive control to guide effective decision-making. Executive control processes encompassing cognitive flexibility, inhibitory control, attention, and working memory rely heavily on the PFC, which undergoes protracted maturation during adolescence. Given the ongoing maturation of the PFC, including the eCB system, AdoTHC can perturb these developing systems potentially disrupting cognitive control processes important for flexible decision-making.

A history of adolescent cannabis use is associated with impairments in top-down cortical cognitive control functions, including attention (Ehrenreich et al., 1999; Tapert et al., 2002), working memory (Meier et al., 2012), executive function (Pope Jr et al., 2003; Gruber et al., 2012; Meier et al., 2012; Auer et al., 2016), and decision-making (Alameda-Bailén et al., 2018; Ferland et al., 2023a). While accounting for social, environmental, and genetic factors reduces the severity of some of these cognitive impairments, these factors do not fully explain all observed cognitive deficits (Lorenzetti et al., 2020). The multitude of external environmental factors that influence human behavior makes it hard to disentangle pre-existing vulnerabilities from cannabis-induced impairments. Thus, animal models offer the ability to investigate the causal biological effects of AdoTHC exposure that would be otherwise difficult to establish in humans.

Consistent with human studies, rodent models of AdoTHC have likewise demonstrated long-term impairments in working memory. Across non-operant paradigms, AdoTHC exposure has been robustly shown to lastingly impair working memory, including on novel object recognition tasks in both sexes (Rubino et al., 2009a; Realini et al., 2011; Zamberletti et al.,

2014; Zamberletti et al., 2015; Murphy et al., 2017; Renard et al., 2017b; Prini et al., 2018; but see Bruijnzeel et al., 2019), and a delayed alternating T-maze task in males (Chen and Mackie, 2020). However, operant studies have observed persistent AdoTHC-induced enhancements in working memory on a delayed response working memory task (Hernandez et al., 2021) and delayed match-to-sample task (Stringfield and Torregrossa, 2021b) in males. Notably, AdoTHC-exposed females did not exhibit any effects on the delayed match-to-sample task, indicating a male-specific enhancement (Stringfield and Torregrossa, 2021b). Together, these studies suggest that task demands (i.e., active responding for rewards versus exploration) may shape how AdoTHC-induced PFC perturbations manifest behaviorally in a sex-dependent manner.

AdoTHC exposure does not produce uniform deficits across all aspects of PFC cognitive control, as evidence suggests sex-, task-, and context-dependent effects. Sex-specific effects of AdoTHC are observed on cognitive flexibility, as AdoTHC-exposed females show persistent impairments on an operant set-shifting task (Freels et al., 2024), whereas males are not affected (Hernandez et al., 2021; Poulia et al., 2021; Halbout et al., 2023; Freels et al., 2024). However, across most decision-making paradigms, including operant effort- and probability-based tasks in either sex (Freels et al., 2024; Martinez et al., 2025) and goal-directed and non-operant risk-based decision-making in males (Chen and Mackie, 2020; Halbout et al., 2023), AdoTHC exposure does not induce lasting impairments. Importantly, the lack of an observable AdoTHC-induced effect may not reflect an absence of AdoTHC impact. In an operant rat gambling task, AdoTHC-exposed adult males adopt a ‘risky’ decision-making profile early on, but by the end of training do not differ from controls (Ferland et al., 2023a). This ‘risky’ behavioral phenotype re-emerges following acute THC re-exposure (Ferland et al., 2023a). Consistent with this, AdoTHC exposure does not persistently alter basal probability-based decision-making, however pharmacological challenge with amphetamine reveals AdoTHC potentiation of ‘risky’ decision-making across sexes (Martinez et al., 2025). These studies suggest that AdoTHC exposure may produce latent vulnerabilities in decision-making that

emerge when the system is challenged. Together, these findings indicate that the impact of AdoTHC on PFC cognitive control is sex-, task-, and context-dependent, rather than robust and uniform.

Although nuanced, AdoTHC exposure persistently disrupts cognitive processes important for decision-making, suggesting that during this critical developmental period, THC may hijack still-maturing circuits that govern motivation and cognitive control. These alterations can increase the propensity to engage in risky decision-making, such as experimenting with other substances.

Effects of Adolescent THC Exposure on Opioid Drug Reinforcement. The reinforcing properties of drugs of abuse drive drug-taking behavior, and heightened sensitivity to the rewarding effects may increase propensity toward drug misuse. Longitudinal studies indicate that early-life cannabis exposure often precedes illicit drug use, giving rise to the so-called cannabis 'gateway effect' (Yamaguchi and Kandel, 1984; Fergusson and Horwood, 2000; Lynskey et al., 2003; Agrawal et al., 2004; Hall and Lynskey, 2005; Fergusson et al., 2015; Wilson et al., 2022; Albaugh et al., 2023). Notably, early cannabis initiation is associated with an increased likelihood to initiate and develop an opioid use disorder, particularly among those initiating at a younger age (Lynskey et al., 2003; Fergusson et al., 2015). Yet in humans it is difficult to determine the relative impacts of AdoTHC itself, versus comorbidities or societal, legal, and cultural factors that also contribute to this association.

Preclinical studies have shown that, indeed, AdoTHC conveys later-life sensitivity to opioid rewards in rodents. For example, male rats with prior chronic THC exposure in adolescence exhibit increased heroin consumption during self-administration (Solinas et al., 2004; Ellgren et al., 2007; Spano et al., 2010; Tomasiewicz et al., 2012; Lecca et al., 2020; Ferland et al., 2023b), and increased reinstatement of heroin drug seeking elicited by stress (Stopponi et al., 2014) in adulthood, while in adult female rats, AdoTHC history increases fentanyl self-administration (Nguyen et al., 2020). Additionally, genetic factors may play a role in AdoTHC-induced opioid

sensitivity. For instance, “addiction-prone” AdoTHC-exposed male Lewis rats exhibit increased heroin intake during self-administration, greater motivation for heroin, and enhanced heroin drug primed reinstatement (Cadoni et al., 2015; Lecca et al., 2020), whereas “addiction-resistant” male Fischer 344 rats exhibit increased heroin conditioned place preference and cue-induced reinstatement of heroin drug seeking (Cadoni et al., 2015; Lecca et al., 2020). Consistent with these findings, AdoTHC exposure enhances drug-induced reinstatement of morphine conditioned place preference in both adult male and female mice (Hubbard et al., 2024). Collectively, these studies suggest that across sex, strains, and species AdoTHC exposure consistently induces later-life vulnerability to opioids.

While the underlying biological disposition toward later-life opioid vulnerability is still not fully known, one potential mechanism may be the intimate relationship between the endocannabinoid and endogenous opioid systems. During adolescent neurodevelopment, both systems shape maturing brain reward and emotion circuits (Bossong and Niesink, 2010; Lutz and Kieffer, 2013; Kudrich et al., 2022; Marusak, 2022), and interact in a bidirectional manner to modulate the reinforcing effects of the other instead of acting as isolated systems (Vigano et al., 2005; Lopez-Moreno et al., 2010; Scavone et al., 2013; Befort, 2015; Mohammadkhani and Borgland, 2022). AdoTHC may therefore induce lasting alterations across both systems, and indeed chronic AdoTHC exposure in male rats reduces mu opioid receptor density during late adolescence in the nucleus accumbens shell, while increasing mu opioid receptor coupling in the VTA and substantia nigra in young adulthood (Ellgren et al., 2007; Ellgren et al., 2008). Moreover, heroin-experienced chronic AdoTHC-exposed adult males show a correlation between heroin intake and agonist activation of mu opioid coupling specifically in the nucleus accumbens shell (Ellgren et al., 2007), suggesting that these AdoTHC-induced alterations may underlie the reinforcing properties of opioids later in life. Thus, identifying how AdoTHC-induced cellular and molecular disruptions that may underlie circuit-level dysfunction contributing to opioid vulnerability may provide greater insight into how AdoTHC confers long-term risk.

Effects of Adolescent THC Exposure on Aversive Motivational Learning. Substance use disorder is characterized by recurrent drug use despite negative consequences (Association, 2013), and disregard of these negative outcomes can often aid in misuse and perpetuation of a cycle of relapse and continued use. While individuals with substance use disorder can achieve periods of abstinence, stressors and environmental cues previously associated with drug use can trigger craving and relapse (Sinha, 2001, 2007; Serre et al., 2012; Fatseas et al., 2015; Sinha, 2024). Notably, AdoTHC exposure has been reported to heighten vulnerability to stress-, cue-, and drug-primed reinstatement of opioid-seeking in adulthood (Stopponi et al., 2014; Lecca et al., 2020; Hubbard et al., 2024), suggesting that AdoTHC exposure can persistently modify the impact of relapse triggers, and raises the question of whether AdoTHC is also altering the perception of these triggers.

Environmental cues and the associated context are assigned salience and valence, which help guide adaptive selection of action. Aversive cues drive avoidance behaviors to get away from aversive stimuli or the resulting negative consequences (Elliot, 1999; Pessoa and Adolphs, 2010). The aversive motivational system can become dysregulated when perception of aversive stimuli or the integrated contextual information is disrupted, for example due to altered sensitivity to stressors, impaired threat discrimination, or maladaptive coping processes as seen in people with substance use disorder (Cheetham et al., 2010; Edwards and Koob, 2010). Stress is one way the aversive motivational system becomes disrupted, influencing how cues are perceived and thereby altering decision-making processes (De Kloet et al., 2005; Giovanniello et al., 2023). As such, stress is a critical modulator of addiction risk, capable of triggering relapse and drug-seeking (Sinha, 2001; Weiss et al., 2001; Goeders, 2003; Sinha, 2007, 2024).

Literature suggests that AdoTHC exposure produces persistent disruptions in aversive motivational learning rather than broadly heightening reactivity to aversive stimuli. For example, chronic exposure to THC vapor in adolescence accelerates acquisition of a repeated tone-shock

pairing during auditory fear conditioning in males, despite no adolescent treatment group differences on the first acquisition day (Smiley et al., 2021). Chronic AdoTHC exposure alone does not appear to alter the ability to extinguish a conditioned auditory fear response, as AdoTHC-exposed males exhibit comparable rates of extinction compared to controls in adulthood (Jouroukhin et al., 2019; Saravia et al., 2019). However, when chronic AdoTHC exposure is combined with concurrent adolescent stressors, including forced swim, tail suspension, and restraint, stressed-AdoTHC animals are more resistant to extinction than adolescent stressed controls (Saravia et al., 2019). Together, these findings suggest that AdoTHC produces latent vulnerabilities in aversive learning and motivational circuits that are not detectable under baseline conditions, but are unmasked with further developmental insults, like stressors.

Beyond aversive motivational learning, AdoTHC exposure persistently alters stress reactivity and how stressors modulate natural and drug reward-seeking behaviors. Chronic AdoTHC exposure in males increases basal levels of corticosterone in adulthood (Ferland et al., 2023b), suggesting a dysregulated stress system. As the hypothalamic-pituitary-adrenal axis and corticosterone can modulate reward circuit function, this observed change in brain stress pathways may not only alter reactivity to stressors, but how they may influence reward-seeking as well (Koob, 2008; Sinha, 2008; Koob, 2015; Sinha, 2024). Consistent with this, chronic AdoTHC-exposed adult males, following overnight social isolation stress, consume more sucrose in adulthood compared to controls (Ferland et al., 2023b). Likewise, AdoTHC-exposed males exhibit stress-sensitivity to opioid drug reward, as they exhibit enhanced reinstatement of heroin-seeking following acute pharmacological stress with yohimbine compared to controls (Stopponi et al., 2014). Together, these findings reflect enhanced stress-sensitivity to natural and drug reward processing, though AdoTHC exposure may not alter stress responsivity uniformly, such as in a purely stressful context when natural and drug rewards are not present. For example, AdoTHC-exposed mice do not exhibit the typical social withdrawal behavior

observed following repeated social defeat stress (Lee et al., 2022), suggesting that AdoTHC-induced stress-sensitivity may be context-dependent.

Together, these studies suggest that AdoTHC exposure persistently alters reactivity to aversive stimuli- and stressor-induced behaviors, as well as altering how stressors drive natural and drug reward-seeking behaviors. To better understand how AdoTHC is producing persistent behavioral disruptions in cognition, drug reinforcement, and motivation, we turn to the neural circuits that integrate these three domains.

Integrated Neural Circuits Underlying Adolescent THC-Induced Behavioral Effects. To achieve flexible, goal-directed behavior, the PFC integrates external/contextual information with internal states (Miller and Cohen, 2001; Ott and Nieder, 2019; Howland et al., 2022), and the amygdala provides internal state representations via rapid detection and assignment of emotional and motivational significance to environmental stimuli (Baxter and Murray, 2002; LeDoux, 2007). However, during adolescence, the amygdala is relatively mature compared to the PFC, leaving top-down regulation of emotional and motivational circuits highly reactive (Etkin et al., 2015; Goetschius et al., 2019). Possible disruption of the endocannabinoid system during this labile stage may cause enduring maladaptive effects on the balance between top-down and bottom-up processing, rendering the brain particularly susceptible to AdoTHC insults.

Effects of Adolescent THC Exposure on PFC Structure and Function. AdoTHC exposure disrupts the developmental trajectory of the medial prefrontal cortex (mPFC) by producing enduring structural and dynamic changes. For example, chronic AdoTHC exposure has been shown to reduce dendritic pyramidal cell complexity and induce premature spine pruning and maturation (Rubino et al., 2015; Prini et al., 2018; Miller et al., 2019). Additionally, anatomical connectivity from PFC to VTA dopamine neurons are also persistently altered by chronic AdoTHC exposure. Using a rabies virus-based circuit mapping, Hubbard et al. (2024) found that chronic AdoTHC exposure increased the anatomical connectivity of PFC projections onto VTA dopamine neurons indicating an enduring circuit-level reorganization. AdoTHC effects also

extend to lasting functional circuit changes, as chronic AdoTHC exposure reduces GAD67 protein expression, the primary GABA synthesizing enzyme, within the PFC resulting in PFC disinhibition due to a reduction of inhibitory transmission onto prefrontal pyramidal neurons (Renard et al., 2017a; Renard et al., 2017b). Further, the observed disinhibition in top-down PFC control led to a hyperactive dopaminergic state within VTA dopamine neurons, and GABA activation within the PFC was able to reverse this disruption in top-down control reining in VTA dopamine (Renard et al., 2017a; Renard et al., 2017b), indicating a causal relationship between weakened PFC inhibitory control and the hyperactive dopaminergic state.

AdoTHC exposure persistently alters PFC connectivity with reward circuitry following opioid exposure. Following acute morphine administration in adulthood, chronic AdoTHC-exposed mice exhibited higher levels of correlated cFos, a marker for neural activity, between frontal cortical regions and VTA compared to control mice, indicating enhanced functional connectivity under morphine (Hubbard et al., 2024). The elevated functional connectivity between PFC and VTA observed in AdoTHC-exposed animals may promote greater dopamine release in reward circuits compared to controls and may be one mechanism by which AdoTHC induces lasting sensitivity to opioid reward.

Together, these studies suggest that AdoTHC-induced structural and functional disruptions in PFC regulatory control over midbrain dopamine reward-circuitry persist under basal conditions and opioid insult and may extend to other limbic structures such as the amygdala.

Effects of Adolescent THC Exposure on Amygdala Structure and Function. Compared to the PFC, less is known about the persistent effects of AdoTHC exposure on the amygdala, with existing work largely focused on the basolateral amygdala (BLA). AdoTHC exposure persistently alters the BLA transcriptome and gene co-expression networks in males in a dose-dependent manner, as chronic AdoTHC exposure (1.5mg/kg) enriches gene-networks related to GABAergic signaling and neuronal morphology, while chronic AdoTHC exposure (5mg/kg)

enriches gene-networks related to glutamate transmission, dopamine receptors, and stress-related signaling (Ferland et al., 2023b). The BLA transcriptome is further altered following overnight social isolation stress in adulthood, as chronic AdoTHC exposure (1.5mg/kg) drives upregulation of genes related to the endogenous opioid system, including *Penk*, which is implicated in opioid vulnerability (Ellgren et al., 2007; Tomasiewicz et al., 2012; Ferland et al., 2023b), whereas 5mg/kg chronic AdoTHC exposure amplifies recruitment of stress-related gene networks (Ferland et al., 2023b). These transcriptional changes suggest that AdoTHC induces an underlying molecular priming in the brain that is transcriptionally unmasked following stress in adulthood, raising the question of whether these changes manifest behaviorally.

AdoTHC-induced alterations in amygdala function have been observed following multiple developmental insults across the lifespan, paralleling the alterations in the BLA transcriptome following adulthood stress. Concurrent AdoTHC exposure and multiple stressors in adolescence (forced swim, tail suspension, and restraint stress) impaired cued fear extinction following auditory fear conditioning in adulthood, with resistance to fear extinction associated with attenuated neuronal activity within the BLA compared to controls (Saravia et al., 2019). The observed amplification of transcriptional vulnerability following AdoTHC exposure and adulthood stress in males, may therefore manifest behaviorally with stress insults across development. Collectively, these findings indicate that AdoTHC induces persistent, dose- and stress-dependent molecular, functional, and behavioral changes within the BLA.

Effects of Adolescent THC Exposure on Glial Changes within the PFC and Amygdala.

Central nervous system glial cells, including microglia and astrocytes, aid in sculpting the developing adolescent brain into its adult configuration (Clarke and Barres, 2013; Dziabis and Bilbo, 2021; Martinez and Mahler, 2025); as such glial perturbation may be one mechanism by which AdoTHC exerts its effects on the developing brain. Following chronic exposure to AdoTHC, lasting morphological changes consistent with a pro-inflammatory microglial state are observed in females (Zamberletti et al., 2015; Gabaglio et al., 2021; Freels et al., 2023) and in

males (Hasegawa et al., 2023). Astrocytes are similarly altered, with changes in astrocytic markers (GFAP), astrocyte number, and structural hypotrophy observed in the BLA of adult AdoTHC-exposed males (Ferland et al., 2023a; Ferland et al., 2023b), indicative of a reactive pro-inflammatory state. Exposure to acute adulthood social isolation stress further exacerbates this pro-inflammatory phenotype, recruiting astrocytes and downregulating astrocyte transcripts related to GABA and glutamate signaling (Ferland et al., 2023b). Together, these persistent AdoTHC-induced glial alterations within the mPFC and amygdala suggest possible disruptions to the normal-developmental processes these cells are involved in, with lasting circuit-level consequences.

Effects of Adolescent THC Exposure on PFC-Amygdala Circuitry. AdoTHC exposure produces persistent neurobiological alterations across the mPFC, amygdala, and their interactions with reward circuitry, and these disruptions extend to glial cells that aid in the shaping and remodeling of the adolescent brain. Taken together, these findings raise the possibility of disruptions at the intersection of PFC executive functions/cognitive control and amygdala-dependent emotional and motivational processing. As such, this dissertation examines how these neurobiological disruptions may manifest behaviorally specifically in cognition, opioid reinforcement, and aversive motivation.

Across Adolescent THC Exposure Models. Here, I employ our well-characterized, translationally relevant AdoTHC exposure protocol in rats (Torrens et al., 2020; Ruiz et al., 2021a; Ruiz et al., 2021b; Torrens et al., 2022), in which male and female rats are exposed to daily intraperitoneal injections of THC (5mg/kg) from postnatal days 30-43 falling within the prototypic adolescent period in rodents (postnatal days 28-42; Spear, 2000). This 5mg/kg dose was used, as a single administration of this dose approximates the total drug exposure seen in the blood after voluntary cannabis inhalation in humans (Huestis et al., 1992).

Notably, AdoTHC exposure protocols vary across labs, and this likely contributes to observed sex differences. Across labs, protocol factors vary from THC dose, route of

administration (e.g., vaporized, subcutaneous, intraperitoneal; Miller et al., 2019; Saravia et al., 2019; Freels et al., 2024), exposure schedule (e.g., escalating twice-daily versus every three days; Zamberletti et al., 2015; Miller et al., 2019), and targeted adolescent period (Ferland et al., 2023a; Ferland et al., 2023b). Importantly, the prototypic adolescent period that protocols often target captures different pubertal windows for males and females (Spear, 2000). Additionally, females metabolize THC differently than males, exhibiting greater plasma concentrations of active THC metabolites in the blood and brain that persist longer than in males (Torrens et al., 2020; Ruiz et al., 2021a; Ruiz et al., 2021b; Torrens et al., 2022). Consequently, the same dose of THC may elicit stronger effects in females than males. Despite differences in adolescent pubertal timing and THC metabolism, most studies typically only include one sex, which precludes sex comparisons, necessitating the inclusion of both sexes. Taken together, the variability across AdoTHC exposure models, and sex differences in pubertal timing and THC metabolism, likely contribute to the varying neurobiological effects seen across AdoTHC exposure studies.

Overview of the Present Studies. The literature reviewed above establishes adolescence as a critical developmental period during which THC exposure can hijack circuits important for decision-making and motivation. To characterize the persistent behavioral and neural effects of AdoTHC exposure: 1) I quantify performance on two mPFC dopamine-dependent reward-based tasks—strategy set shifting and probabilistic discounting and also determine how acute dopamine augmentation with amphetamine, or specific chemogenetic stimulation of VTA dopamine neurons and their projections to mPFC impacts probabilistic discounting, 2) I examine the acute impact of our AdoTHC exposure model on mPFC microglia morphology, and the persistent effects on behavioral responses to two classes of opioid drugs, heroin and remifentanil, using a battery of drug-related behaviors in adulthood, and neural responses to an acute administration of heroin, and 3) I examine the lasting effects on aversive motivational learning to aversive stimuli, stressor-modulated motivation for opioids, and neural responses under acute pharmacological stress.

Thereby, I provide a framework for how AdoTHC exposure may persistently remodel the brain and behavior, and offer insight into how early-life cannabis use may produce dysfunction across cognition, drug reinforcement, and motivational domains that together confer addiction vulnerability.

CHAPTER 1: Adolescent THC impacts on mPFC dopamine-mediated cognitive processes in male and female rats

Introduction

Cannabis is one of the most widely used drugs among adolescents, and its availability is increasing around the world. Human studies show that early exposure to cannabis, and especially its main psychoactive constituent Δ^9-tetrahydrocannabinol (THC), is associated with later-life cognitive impairments, and increased risk for psychiatric disorders including schizophrenia and addiction (Ehrenreich et al., 1999; Schneider, 2008; Malone et al., 2010; Curran et al., 2016; Rubino and Parolaro, 2016; Volkow et al., 2016; Jenni et al., 2017; Murray et al., 2022). However, in humans it is difficult to dissect whether THC exposure causes these associations, or whether early cannabis use and long-term deficits both result instead from other underlying comorbidities or risk factors. Rodent models are thus essential for establishing casual effects of THC on the developing adolescent brain.

Neurodevelopmental disruptions persisting long after adolescent cannabis use are plausible because adolescence is a dynamic critical period for structural and functional brain remodeling, especially in late-developing structures like the prefrontal cortex (PFC) (Casey et al., 2000; Andersen, 2003). Some of this age-dependent plasticity seems to involve the endocannabinoid system, with dynamic changes in cannabinoid receptors (CBRs) and endocannabinoids (ECB) occurring across adolescence (Ellgren et al., 2008; Heng et al., 2011; Lee et al., 2016; Bara et al., 2021; Simone et al., 2022). Might THC, which also acts via CBRs, disrupt this age-dependent ECB signaling system and thus leave long-lasting consequences on the brain? If so, the adolescent-developing PFC (Spear, 2000; Peters et al., 2022; Scheyer et al., 2023), and its dopaminergic inputs from ventral tegmental area (VTA), which are actively innervating during this period (Manitt et al., 2011; Hoops and Flores, 2017; Reynolds et al., 2018), are a likely candidate for cognition-relevant neurodevelopmental insults caused by adolescent THC (Renard et al., 2017a; Renard et al., 2017b; Molla and Tseng, 2020).

The adult PFC is crucial for purposeful, goal-directed behaviors driven by the ability to flexibly converge our internal states, like reward motivation, with outside external information about contexts, cues, and rules (Miller, 2000; Miller and Cohen, 2001; Ott and Nieder, 2019). Executive functions like working memory, attention, rule shifting, and decision-making require PFC-dependent cognitive control. Rodent studies show adolescent cannabinoid drug exposure can cause persistent deficits in working memory (Schneider and Koch, 2003; O'Shea et al., 2004; De Melo et al., 2005; Quinn et al., 2008), social cognition (O'Shea et al., 2004; Zamberletti et al., 2014; Renard et al., 2017a; Renard et al., 2017b), and cognitive flexibility (Egerton et al., 2005; Gomes et al., 2015; Jacobs-Brichford et al., 2019; Szkudlarek et al., 2019) that may depend upon PFC.

Furthermore, dopamine in PFC plays a major role in decision making, working memory, cognitive flexibility, and goal-directed behaviors (Goldman-Rakic, 1995; Seamans and Yang, 2004; Goto et al., 2007; Floresco, 2013). PFC dopamine dysfunction has also been implicated in schizophrenia symptoms (Davis et al., 1991; Howes and Kapur, 2009), and there is a clear link between recent cannabis use and onset of psychosis in humans (Andreasson et al., 1987; Hambrecht and Hafner, 2000; D'Souza et al., 2004), though it is not clear that this link is causal in nature (Fergusson et al., 2005; Henquet et al., 2005; Sewell et al., 2009). Since VTA dopamine neuron axons actively infiltrate mPFC during adolescence (Manitt et al., 2011; Hoops and Flores, 2017; Reynolds et al., 2018) and are thus subject to disruption by THC exposure, and since THC impacts dopamine signaling at several key nodes in reward and salience circuits (Behan et al., 2012; Renard et al., 2017a; Renard et al., 2017b; Corongiu et al., 2020; Ferland et al., 2023b), it is plausible that adolescent THC might exert some of its neurodevelopmental disruptions by impacting the function of cognitive flexibility-relevant dopaminergic inputs to mPFC.

We therefore explored this possibility in a series of experiments quantifying effects of a well-characterized, translationally relevant adolescent THC exposure protocol in rats (Torrens et

al., 2020; Ruiz et al., 2021a; Ruiz et al., 2021b; Torrens et al., 2022) upon adulthood mPFC dopamine-dependent cognition.

Materials and Methods

Subjects. Long Evans rats (n = 29 males, 25 females) were used for set shifting, probabilistic discounting and amphetamine experiments, and transgenic TH:Cre (n = 19 males, 9 females) and wildtype littermates (n= 14 males, 4 females) were used for chemogenetic experiments. Rats were pair or triple-housed in same-sex groups at weaning (postnatal day; PD21), in a temperature, humidity, and pathogen-controlled colony under a 12:12hr reverse light/dark cycle. Water was provided ad libitum and food was restricted to ~85% of free-feeding weight during behavioral testing, starting at ~PD70. Experiments were approved by University of California Irvine's Institutional Animal Care and Use Committee and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Drugs. THC was provided in ethanol by the NIDA Drug Supply Program. For injection, THC was prepared fresh each day; ethanol is evaporated under N2, and THC is reconstituted in 5% Tween-80 in saline, with heat and sonication, to 2 ml/kg for intraperitoneal (IP) injection. D-amphetamine hemisulfate salt (amphetamine) was attained from Sigma and mixed in saline at 0.5 mg/ml for injection. Clozapine-N-oxide (CNO) was obtained from the NIDA Drug Supply Program, stored at 4°C in powder aliquots with desiccant, protected from light. For systemic injection, CNO (5 mg/kg) was dissolved daily for IP injection in 5% dimethyl sulfoxide (DMSO; Sigma Aldrich) in 0.9% saline. For microinjections, CNO (1mM; 0.5 µl/side over 60s) was dissolved in artificial cerebrospinal fluid (Fisher) with 0.5% DMSO, stored in aliquots at -20°C, and thawed/vortexed just before use.

Adolescent THC and Washout. All rats received daily IP injections of THC (5 mg/kg) or vehicle (VEH; 5% Tween-80 in saline) from PD30-43, followed by a washout period of 21+ days, allowing full THC clearance in both sexes (Lee et al., 2022) prior to behavioral testing in adulthood (**Fig. 1.1A**).

Experimental Design. Following adolescent THC/VEH treatment, 48 adult (PD 70+) rats (*n* = 29 males, 21 females) underwent strategy set-shifting training, followed by training on probabilistic discounting until a group displayed stable levels of choice for 3 consecutive days, determined with previously established criteria (St. Onge and Floresco, 2009). Thereafter they underwent 3 counterbalanced amphetamine challenge tests, each (0, 0.25, 0.5 mg/kg IP) delivered 5 min before behavioral testing. Following the challenge rats were retrained for at least 2 days before receiving their next challenge.

For chemogenetic experiments, another adolescent THC/VEH-treated group (*n* = 33 males, 13 females; *n* = 28 TH:Cre+,18 wildtype (WT)) underwent stereotaxic VTA virus injection of a Cre-dependent hM3Dq vector at ~PD65, and intra-mPFC bilateral cannulae implantation at least 45 days later, at least 8 days prior to the first microinjection. Following recovery from surgery, they were trained on the probabilistic discounting task to stability, then subjected to a series of counterbalanced tests, again with re-stabilization training occurring between them. First, rats received 4 counterbalanced IP injections of the DREADD agonist CNO (5 mg/kg) or VEH, 30 min prior to behavioral testing. Both CNO and VEH were administered twice on separate days, and data was combined between both tests for analysis. Next, they underwent 4 additional counterbalanced tests of probabilistic discounting, each held 5 min after intra-mPFC microinjections of CNO or VEH. Results from intra-mPFC VEH and CNO tests were again averaged to increase reliability of findings. For choice data, the raw number of risky choices in each block made on both VEH or CNO tests were summed and divided by the total number of choices made in those respective blocks on the two VEH/CNO tests, thereby factoring out trial omissions. Win-stay/lose shift values were combined in a similar manner, dividing the sum total of “stays” or “shifts” by the sum total of “wins” and “losses” on the two respective tests. Latency and omission values were averaged across the tests.

Behavioral Methods. *Operant Boxes*. Training and testing took place in Med-Associates rat operant conditioning chambers (30.5 x 24 x 21 cm; St Albans, VT) within sound-attenuating boxes, equipped with two retractable levers with white lights above them, and white house light. *Operant Pretraining.* Methods for both strategy set shifting and reward probabilistic discounting tasks closely followed prior reports (Floresco et al., 2008a; St. Onge and Floresco, 2009). Rats were first homecage-habituated to highly palatable, 45 mg banana-flavored reward pellets (Bio-Serv catalogue #: F0059), then given 2 days of magazine training in the operant boxes, where they received 38 pellets at variable intervals over a 60 min session. They were next sequentially trained to press each of two levers to receive a pellet over 2-5 days. On each lever training day, a lever extended into the chamber (side counterbalanced across rats), and one pellet was delivered on a fixed ratio 1 (FR1) schedule. Once they reliably pressed 50+ times in 30 min on a lever, they were transitioned to learning to press the other lever on the subsequent day, and training continued until meeting this criterion. Thereafter, they entered the next phase of the task, in which each lever was periodically extended into the chamber throughout a 30 min session. Levers extended in a pseudorandom order such that there were 45 left-lever trials and 45 right-lever trials, but no more than two consecutive trials on which the same lever was extended. Each lever extension was accompanied by illumination of a house light signaling the start of a trial (90 trials/session), and trials occurred every 20s. If the rat pressed the lever within 10s of the start of a trial, the lever was retracted and a pellet was delivered, and the house light remained on for 4s. If the rat failed to press in 10s, the lever retracted and the house light extinguished until the next trial, and the trial was considered an omission. Importantly, cue lights present above each lever were never illuminated during this training phase. Rats were trained in this manner for at least 5 days, or until they omitted less than 5 trials per session. Since individual rats frequently display idiosyncratic lever position biases that can influence interpretation of subsequent behavior (Brady and Floresco, 2015), and to minimize such

impacts of stochastic side-preference on subsequent tests, lever side preference was next assessed using a published protocol (Floresco et al., 2008a; Brady and Floresco, 2015).

Visual Cue Discrimination Training. Next, rats were trained to respond on only one of the two levers extended on each trial—whichever was signaled as the correct response by illumination of a cue light just above it (**Fig. 1.1B**). Sessions began with both levers retracted and the house light off. Every 20s, one of the two stimulus cue lights was illuminated in a randomized order, 3s later both levers extended, and the house light turned on. If the rat pressed the lever that had a cue light illuminated above it within 10s of lever insertion, it received a pellet, the levers retracted, the stimulus light was extinguished and the house light remained illuminated for 4s. If the rat did not choose a lever in 10s, the trial ended and levers retracted until the next trial, and the trial was scored as an omission. This training proceeded for at least 30 trials, ending when rats either made 10 consecutive correct responses, or after 150 trials had elapsed. If rats did not achieve 10 consecutive correct responses, they received a second identical training session on the following day.

Strategy Set Shifting Test. After learning to follow a cue light to respond for reward in the prior training phase, rats then underwent a single 40 min session on which the response rule was suddenly shifted; a procedure analogous to the Wisconsin Card Sorting Task used in humans to aid diagnosis of PFC dysfunction (Owen et al., 1991; Pantelis et al., 1999). On this day, the non-preferred lever (determined in side-bias training described above) became the correct response, and pressing it during trials yielded a pellet and commencement of the next inter-trial interval (Fig. 1h). Light cues above one of the levers were presented just before and during trials as in the prior visual cue discrimination training, but now their location was irrelevant to the receipt of reward. Instead, rats needed to recognize that this old rule (follow the cue light) no longer worked, and that pressing of the previously less-preferred lever, regardless of cue light position, was now the correct strategy for obtaining reward. Trials continued until rats performed 10 consecutive correct responses, or after 150 trials had occurred. Errors during set-shift were categorized into two

subtypes as in (Floresco et al., 2008a): perseverative errors, where rats responded on the incorrect lever when the previously-relevant visual cue was illuminated above it, and never-reinforced (or non-perseverative) errors, where rats pressed the incorrect lever despite when the visual cue was illuminated above the correct lever.

Probabilistic Discounting. In this task rats chose between a lever that always delivers a small (1 pellet) "certain" reward, or another that delivers a large (4 pellet) "risky" reward, delivered at various probabilities throughout the 52.5 min session (**Fig. 1.2A**). The probability of receiving 4 pellets upon pressing the risky lever decreases in each of five sequential choice blocks during each session (100%, 50%, 25%, 12.5%, 6.25%). Each block began with 8 forced trials (35s apart), in which the houselight was illuminated and 3s later, one of the two levers extended one at a time for 10s (4 trials for each lever, randomized in pairs). This permitted rats to sample the probability of reward receipt upon pressing of the risky lever in the upcoming certain/risky lever choice phase of the block. During the subsequent choice phase of each block, both levers were extended, and after rats pressed one of them, both levers retracted. Certain lever presses always yielded 1 tasty banana pellet, and risky lever choices delivered 4 or 0 banana pellets, at a probability that varied across blocks. Failure to respond on a lever within 10s on any trial resulted in lever(s) retraction and extinguishing of the house light, and the trial was scored as an omission. Animals were trained on this task for 21 days, at which point choice behavior had stabilized across groups. For rats tested with systemic amphetamine during probabilistic discounting, training on this task commenced following strategy set shifting training and testing described above. Rats in chemogenetic experiments did not undergo visual cue discrimination or set shifting tests. Instead, these rats received pellet habituation, magazine training, initial FR1 training, and retractable lever training prior to training on the probabilistic discounting task.

For rats that received acute amphetamine challenges (0, 0.25, 0.5 mg/kg IP) on risky decision making, 3 counterbalanced tests were conducted on separate days, with re-training between tests to reestablish stable baseline responding. When rats in chemogenetic experiments

achieved stable baseline responding, they similarly received 4 counterbalanced tests, 2 with CNO and 2 with VEH (data from both tests combined for analysis as described above), all injected IP 30 min prior to probabilistic discounting testing, with retraining between tests. After completing these systemic CNO/VEH tests, rats were implanted with mPFC cannulae, allowed to recover, and re-stabilized on discounting performance for 2+ days. They were then tested on the discounting task in 4 additional counterbalanced tests with intra-mPFC microinjections of CNO (1mM/0.5 µl; 2 tests) and VEH (2 tests).

Chemogenetic Methods. *Virus Surgery*. Rats were anesthetized with ketamine (56.5 mg/kg) and xylazine (8.7 mg/kg) and given meloxicam (1.0 mg/kg) for pain prophylaxis. An AAV2 vector containing a Cre-dependent, mCherry-tagged hM3Dq excitatory DREADD (hSyn-DIO-hM3Dq-mCherry; titer: 6×10^{12} vg/ml; Addgene catalog #: 44361) was injected bilaterally into VTA (relative to bregma (mm): AP: -5.5, ML: ±0.8, DV: -8.1; 0.75 µL/hemisphere) using a Picospritzer and glass micropipette (Martinez et al., 2023). Injections occurred steadily over 1 min, and the pipette was left in place for 5 min after injection to limit spread. Both TH:Cre and WT rats were injected with the active hM3Dq DREADD virus. Colocalization of mCherry expression to TH+ VTA neurons was verified in each TH:Cre rat, and lack of mCherry expression was confirmed in each WT rat. At least 3 weeks elapsed between virus injections and the first CNO administration. In a second surgery held following behavioral training and systemic CNO tests (to maximize accuracy of placement) guide cannulae (22 ga, 2MM, Plastics One) were implanted bilaterally in the mPFC (relative to bregma (mm): AP: 2.7, ML: ±1, DV: -3.1) to allow intra-mPFC CNO injection upon VTA dopamine neuron axon terminals, and occluded with steel stylets between tests.

Histological Validation. Following behavioral testing, chemogenetic rats were perfused with chilled 0.9% saline and 4% paraformaldehyde, brains were cryoprotected in 20% sucrose-azide, and they were sectioned coronally at 40 µm. VTA virus expression was amplified with mCherry immunohistochemistry, and expression verified to be in dopamine neurons via co-staining of

tyrosine hydroxylase. VTA sections were then blocked in 3% normal donkey serum PBST, tissue was incubated overnight at room temperature in rabbit anti-DSred (Clontech; 1:2500) and mouse anti-TH (Immunostar; 1:2000). After washing, sections were incubated in dark at room temperature for 4 hours in AlexaFluor-donkey anti-Rabbit 594 and donkey anti-Mouse 488 (Thermo Fisher Scientific; 1:500). Sections were mounted and cover slipped (Fluoromount; Thermo FisherScientific), mCherry/TH expression was imaged at 10X on a Leica DM4000 epifluorescent scope, and expression or lack thereof in VTA was verified in each animal. To verify cannula placement within mPFC, sections were nissl stained with cresyl violet, and cannula tracks were mapped using a rat brain atlas (Paxinos and Watson, 2006).

Data Analysis. Effects of adolescent treatment (AdoTX: THC or VEH) and Sex (M or F) on learning and cognition employed 2 x 2 ANOVA and Sidak posthoc tests. Effects of amphetamine (0 x 0.25 x 0.5mg/kg) on probabilistic discounting were tested with repeated measures ANOVA, and interactions of these acute treatements with AdoTX and Sex were examined by adding these between subjects variables to multivariate General Linear Model ANOVAs. Simple-main effects analyses conducted after observing staticially significant interactions included one-way ANOVA models using the appropriate error term from the overal multifactor analyses. Effects of CNO versus VEH were treated as within-subjects variable, while Genotype (TH:Cre x WT), AdoTX, and sex were treated as between subjects variables in multivariate General Linear Model ANOVAs. Prior to testing, rats were verified to display stable patterns of risky choice by examining discounting performance over at least two consecutive prior training days. Rats of both sexes were tested in chemogenetic experiments, but sample sizes for each sex were insufficient to allow formal analysis of this variable (**Fig. S1.1**). Six rats (*n* = 2 males, 4 females; *n* = 1 VEH, 5 THC) were excluded from set shifting analyses for failure to meet training critera for instrumental or visual cue rule performance, and nine rats (*n* = 9 females; *n* = 2 VEH, 7 THC) tested with amphetamine were excluded for failing to achieve stable performance on the probabilistic discounting task. Nine rats (*n* = 3 males, 6 females; *n* =

4 VEH, 5 THC) were excluded from the DREADD experiments for failure to stabilize on the probabilistic discounting, death, or inability to confirm virus expression.

Results

Effects of Adolescent THC on Strategy Set Shifting in Each Sex. *Initial Instrumental Training:* AdoTHC did not affect acquisition of instrumental food seeking behavior during initial training (no main effect of AdoTX: $Fs_{(1, 45)} < 0.40$, *ps* > 0.53), and acquisition was similar in both sexes (No main effect of sex: $Fs_{(1, 45)} < 3.60$, *ps* > 0.06; no Sex x AdoTX interaction: ($Fs_{(1, 45)} < 1.12$, *ps* > 0.30).

Visual Cue Discrimination: Rats were next trained to press whichever lever that had a cue light illuminated above it. AdoTHC exposed rats were more likely to learn the visual cue rule in one session rather than two, relative to AdoVEH rats (X^2: $p = 0.04$). This was driven by AdoTHC females, as evidenced by these animals requiring fewer trials and making fewer errors to reach criterion performance of 10 consecutive correct choices (**Fig. 1.1C-G**, AdoTX x Sex interaction; trials to criterion: $F_{(1,44)} = 5.31$, $p = 0.03$; errors to criterion: $F_{(1,44)} = 5.03$, $p = 0.03$; Šídák's posthoc for trials $p = 0.02$, errors $p = 0.02$). In addition, relative to controls, AdoTHC females were slower to respond ($F_{(1, 44)} = 4.55$, $p = 0.04$) but also made fewer omissions: ($F_{(1, 44)} = 8.46$, $p = 0.01$). In contrast, no differences were observed on these measures between AdoTHC males and controls (trials to criterion: $p = 0.87$; errors: $p = 0.89$; latency: $p = 0.68$, omissions: $p = 0.72$). More generally, we also saw some sex-differences on performance measures irrespective of treatment, as females were slower to respond (Sex: $F_{(1,44)} = 13.02$, $p < 0.001$) and omitted more trials ($F_{(1,44)} = 5.60$, $p = 0.02$) compared to males.

Strategy Set Shifting: Following training on the initial visual cue discrimination, rats were then trained to use an egocentric spatial rule (always press the left/right lever regardless of cue location), and tested in a single-session (Fig. 1I-M; Brady and Floresco, 2015). AdoTHC had few effects on the ability to learn this new response rule with no change in either sex seen on

the number of trials to reach criterion on the new rule (No main effect of AdoTX: $F_{(1,44)} = 0.42$, $p = 0.52$; or Sex: $F_{(1,44)} = 0.01$, $p = 0.92$; or interaction: $F_{(1,44)} = 0.80$, $p = 0.38$). Total errors to criterion were also unaffected (AdoTX: $F_{(1,44)} = 0.97$, $p = 0.33$; Sex: $F_{(1,44)} = 0.32$, $p = 0.57$; AdoTX x Sex interaction: $F_{(1,44)} = 0.19$, $p = 0.66$), as were errors of either a perseverative or never-reinforced subtype (AdoTX: $F_{(1,44)} = 1.56$, $p = 0.22$; Sex: $F_{(1,44)} = 0.13$, $p = 0.72$; AdoTX x Sex: $F_{(1,44)} = 0.58 \times 10^3$, $p = 0.98$). Additionally, we saw no effect of AdoTX on response latency or on omissions (AdoTX: Fs $_{(1,44)} < 2.62$, *ps* > 0.11; AdoTX x Sex: Fs $_{(1,44)} < 0.01$, *ps* > 0.94). Again, we saw that females took longer to respond (Sex: $F_{(1,44)} = 7.52$, $p = 0.01$) and omitted more trials ($F_{(1,44)} = 6.20$, $p = 0.02$) compared to males. Since visual discrimination learning was more efficient in AdoTHC females than in AdoVEH females, but shifting to a spatial rule was equivalent in both groups, we wondered if performance on rule #1 could have led to more robust or persistent learning that could have indirectly impacted performance on the set shift. We therefore performed a secondary analysis where we matched the performance of the two female AdoTX groups on the visual discrimination rule #1. We removed the 4 AdoTHC female rats that learned rule #1 quickest, and the 5 AdoVEH females that learned rule #1 slowest, thus yielding equivalent rule #1 performance in the retained rats from both groups (n=6/group;

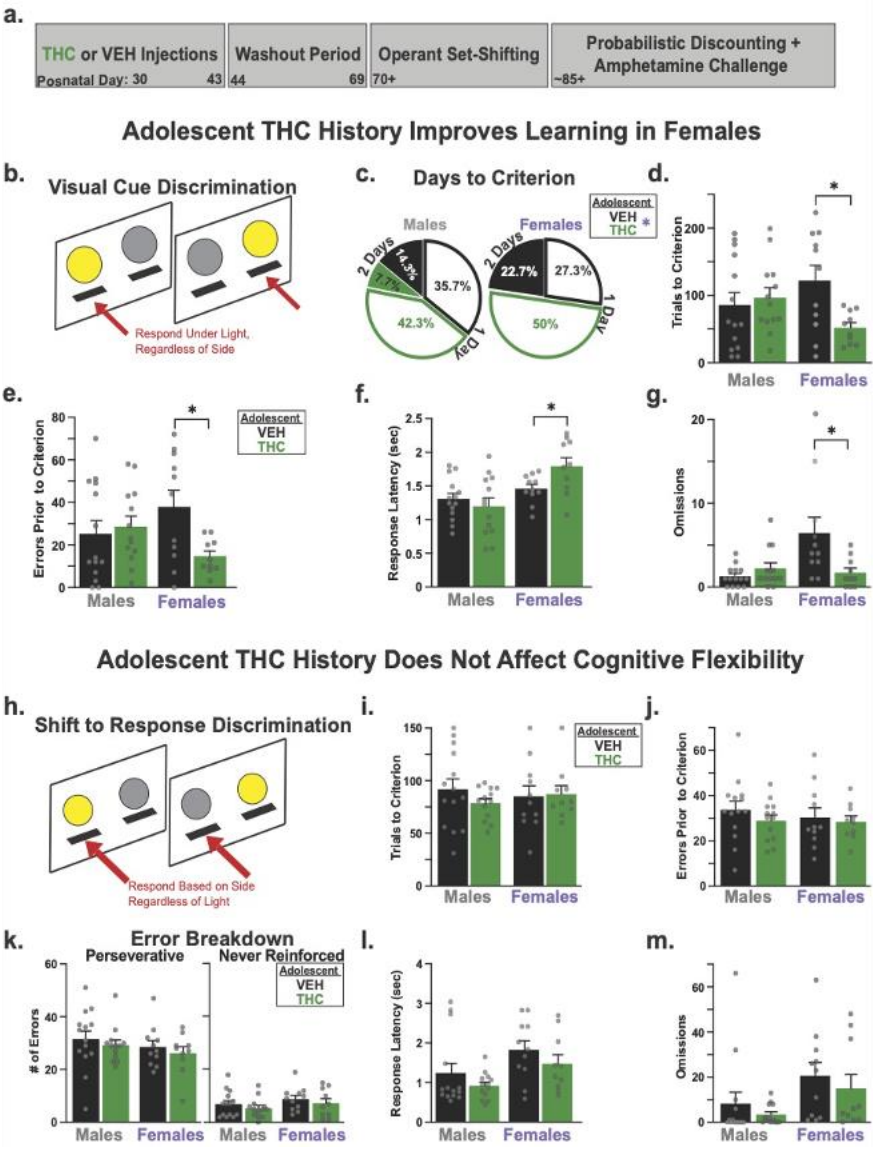


Figure 1.1 Adolescent THC history selectively impacts adulthood learning and cognition.
A) Experimental timeline. **B**) Schematic of visual cue discrimination task. **C**) AdoTHC rats (green wedges) were more likely than AdoVEH rats (black wedges) to acquire the visual cue task to criterion in only one training session (unfilled wedges), rather than requiring 2 sessions to acquire (filled wedges). Both sexes showed similar patterns. **D, E**) AdoTHC females took fewer trials to meet criterion, and made fewer errors when learning visual cue discrimination than AdoVeh females, no such effects were seen in males. **F, G**) AdoTHC females took longer to respond, and made fewer errors than AdoVEH females during cue discrimination training, without effects in males. **H**) Schematic of the subsequently tested strategy set-shifting task. **I**) AdoTHC did not alter the number of trials to learn the new rule to criterion in either sex. Likewise, AdoTHC did not alter in either sex **J**) the number of errors, **K**) the types of errors, **L**) latency to respond, or **M**) omitted trials during set shifting training. Individual rats shown as grey dots in each graph: AdoVEH (n = 14 males, 11 females), AdoTHC (n = 13 males, 10 females). X^2 $p^* < 0.05$ and repeated measure two-way ANOVA, Data represented as mean + SEM, Sidak post hoc: $p^* < 0.05$.

Errors: AdoVEH: M (SEM) = 17.83 (6.18) AdoTHC: M (SEM) = 19.00 (2.94); t(10) = 0.17, p = 0.87; Trials: AdoVEH: M (SEM) = 16.83 (22.83); AdoTHC: M (SEM) = 64.67 (9.58); t(10) = 0.17, p = 0.870). Additionally, in this subset, performance on the shift to rule #2 remained equivalent (Errors: AdoVEH: M (SEM) = 79.33 (16.07); AdoTHC: M (SEM) = 91.50 (13.04); t(10) = 0.22, p = 0.83; Trials: AdoVEH: M (SEM) = 27.17 (6.58); AdoTHC: M (SEM) = 28.83 (3.95); t(10) = 0.59, p = 0.57), rather than showing an AdoTHC-induced deficit as would be expected if rule #1 performance impacted subsequent shift to rule #2.

Effects of Adolescent THC on Probabilistic Discounting in Each Sex: *Acquisition of Probabilistic Discounting:* The same rats were next trained on a probabilistic discounting task for 21 days. All rats had acquired stable performance by the last three days of training (no block X day interaction: $F_{(5.498,241.89)} = 1.70$, $p = 0.13$). No effect of AdoTX ($F_{(1, 44)} = 0.82$, $p = 0.37$), Sex ($F_{(1,44)} = 0.12$, $p = 0.74$), or AdoTX x Sex interactions ($F_{(1, 44)} = 0.59$, $p = 0.45$) were found, suggesting that rats of both sexes and AdoTX histories acquired the discounting task at similar rates.

Stable Probabilistic Discounting: Following training, all rats exhibited stable and comparable performance on the task by the last three days, with decreasing choice of the high-reward lever as delivery of this reward became increasingly unlikely or "risky" (main effect of Block: $F_{(4,176)} = 75.01$, $p < 0.001$). No effect of AdoTX ($F_{(1,44)} = 0.85$, $p = 0.36$), Sex ($F_{(1,44)} = 0.10$, $p = 0.75$), or AdoTX x Sex interactions ($F_{(1, 44)} = 0.62$, $p = 0.44$) were found, suggesting that rats of both sexes and AdoTX histories displayed comparable levels of risky choice across blocks (**Fig. 1.2B**). Likewise, neither AdoTX nor Sex affected win stay or lose shift choice strategies (no main effect of AdoTX: $Fs_{(1,44)} < 1.64$, $ps > 0.21$; no main effect of Sex $Fs_{(1,44)} < 0.55$, $ps > 0.46$; no AdoTX x Sex $Fs_{(1,44)} < 1.51$, $ps > 0.23$; data not shown).

With respect to other performance measures, AdoTX did not affect choice latencies (no AdoTx: $F_{(1, 44)} = 0.16$, $p = 0.69$; no AdoTX X Sex interaction: $F_{(1, 44)} = 0.1.29$, $p = 0.26$). Additionally, we found that AdoVEH rats omitted more on the "riskier" probability blocks compared to AdoTHC animals (AdoTX x Sex x Block: $F_{(4, 176)} = 3.95$, $p = 0.004$, AdoTX x Block: $F_{(4, 176)} = 4.39$, $p = 0.002$; no AdoTx: $F_{(1, 44)} = 0.94$, $p = 0.34$), an effect that was particularly apparent in females (AdoTX x Block: $F_{(4, 68)} = 3.19$, $p = 0.02$; data not shown). Overall, we saw that females took longer to respond than males (Sex: $F_{(1,44)} = 40.03$, $p < 0.0001$) and omitted more trials ($F_{(1,44)} = 63.83$, $p < 0.0001$), especially in low-probability training blocks at the end of the session (Sex x Block: Latency: $F_{(4,176)} = 4.57$, $p = 0.002$; Omissions: $F_{(4,176)} = 26.57$, $p < 0.0001$).

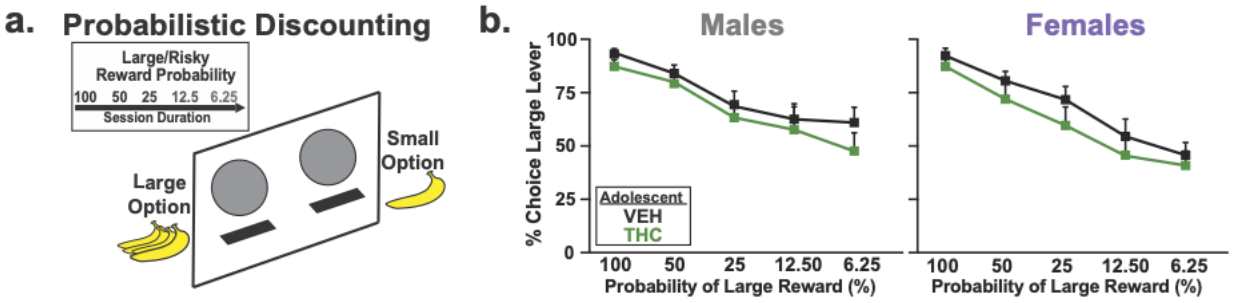


Figure 1.2. AdoTHC history does not affect baseline probabilistic discounting. **A**) Schematic of the probabilistic discounting task. Sessions consisted of 5 training blocks, in which 2 levers are presented, and rats must choose either a small reward lever that always delivers one pellet, or a large reward-delivering lever which becomes increasingly unlikely to deliver any reward as the session progresses. **B**) Data is shown for males and females, indicating that AdoTHC rats did not differ from their AdoVEH counterparts in probabilistic discounting, with both shifting from nearly exclusively preferring the large reward lever, but appropriately shifting away from it as it became less likely to deliver reward. AdoVEH (n = 14 males, 11 females), AdoTHC (n = 15 males, 8 females). Data represented as average within each probability block from three consecutive days of stable performance. Mean + SEM.

Effects of Acute Amphetamine on Probabilistic Discounting: As previously reported (St. Onge et al., 2010), amphetamine increased choice of the large/risky option in a dose dependent manner when analyzed across both groups and sexes (**Fig. 1.3A**, amphetamine x Block interaction: $F_{(8,328)} = 10.02$, $p < 0.0001$; main effect of amphetamine: $F_{(2,82)} = 9.88$, $p < 0.0001$). However, the effects of different doses of amphetamine varied as a function of AdoTX treatment and sex.

Analysis of the choice data also revealed significant amphetamine x AdoTX ($F_{(2,82)} = 3.33$, $p = 0.04$) and amphetamine x AdoTX x Sex interactions ($F_{(2,82)} = 3.20$, $p = 0.05$). Partitioning this latter interaction by AdoTX group revealed that for control rats, amphetamine exerted different effects on choice in males vs females (amphetamine x Sex: $F_{(2,44)} = 3.73$, $p = 0.03$). Post-hoc comparisons showed that, in males, amphetamine increased risky choice following treatment with the 0.5 mg/kg dose ($p < 0.02$) but not the lower, 0.25 mg/kg dose ($p = 0.19$). In contrast, the 0.5 mg/kg dose had more deleterious effects on choice in females, reducing risky choice in the high probability blocks and increasing it in the lower ones, while the 0.25 mg/kg dose induced a modest increase in risky choice in the latter block. This yielded an overall lack of effect of amphetamine in control females (**Fig. 1.3B**, amphetamine: $F_{(2,18)} = 0.57$, $p = 0.57$). On the other hand, amphetamine induced a more reliable and pronounced increases in risky choice in both males and females AdoTHC animals, with analysis of these data producing significant main effects of amphetamine treatment (**Fig. 1.3A,B**, $F_{(2,38)} = 12.07$, $p < 0.0001$) in the absence of an interaction with the sex factor ($F_{(2,38)} = 1.56$, $p = 0.22$). When collapsed across sex, post hoc comparisons showed that both the 0.25 and 0.5 mg/kg dose increase risky choice (both ps < 0.05), although inspection of Fig. 3b indicates that the effect of the 0.25 mg/kg dose was driven primarily by females. From these data, we conclude that AdoTHC treatment makes rats more sensitive to the ability of amphetamine to increase risky choice, and this effect appears to be more prominent in females.

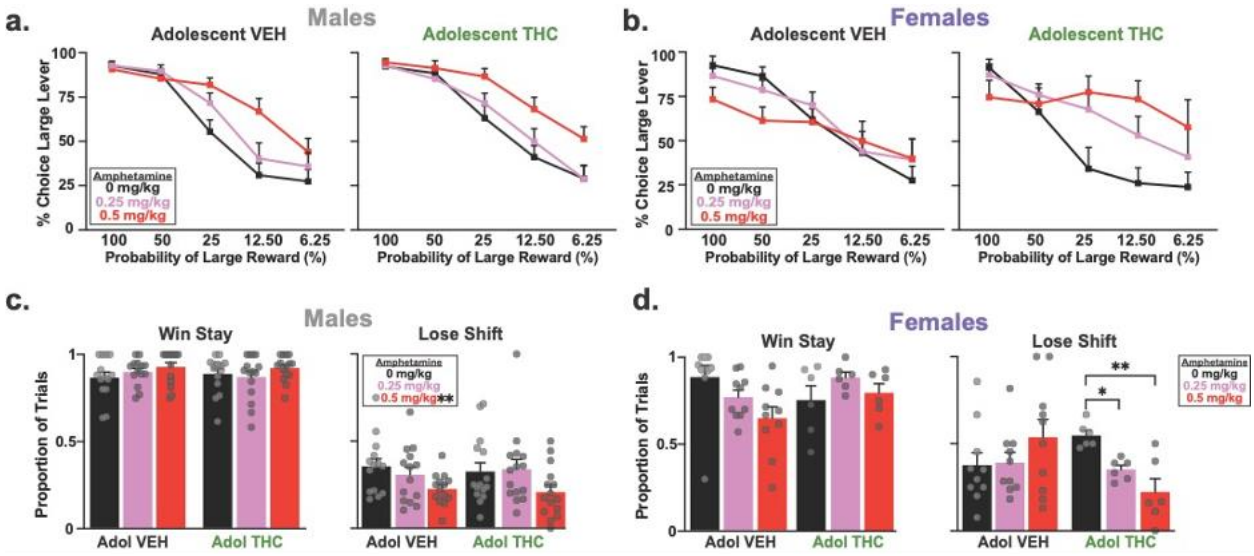


Figure 1.3. Amphetamine-induced 'risky' responding is potentiated after adolescent THC.
A) Data from saline (black line/bar), low dose (0.25mg/kg; pink line/bar) and high dose (0.5mg/kg; red line/bar) amphetamine tests in each sex are shown. When performance patterns were further interrogated, we found that high dose of AMPH in Males increased inflexibility across both AdoTX groups, however in **B**) females, the high dose of AMPH in AdoVEH rats reduced risky responding at high probability blocks not seen in AdoTHC females. **C**) AMPH did not alter win-stay,but reduced lose-shift in AdoTX males. **D**) In AdoVEH females, AMPH decreased win-stay and increased lose-shift, while in AdoTHC AMPH had no effect on win-stay but decreased lose-shift. AMPH = amphetamine. AdoVEH (n = 14 males, 10 females), AdoTHC (n = 15 males, 6 females). Repeated measure three-way ANOVA; Sidak post hoc: p* < 0.05, p** < 0.01. Data represented as mean + SEM, individual animals shown as grey dots.

Subsequent analyses examined how amphetamine alters sensitivity to recent rewarded or non-rewarded choices by comparing win-stay and lose shift ratios. Analysis of the win-stay data yielded a significant amphetamine x AdoTX x Sex interaction (**Fig. 1.3C,D**, $F_{(2,82)} = 4.34$, $p = 0.02$). This was driven by the fact that in control rats, the 0.5 mg/kg dose resulted in lower win-stay values in females vs males ($p < 0.01$), although neither group showed significant changes in these values relative to saline (both Fs < 2.5, both *p*s > 0.10). Win-stay behavior was unaltered in AdoTHC rats (all *F*s < 2.4, all $p > 0.10$). In contrast, amphetamine had more pronounced effects on sensitivity to reward omissions, as indexed by changes in lose shift behavior. The analyses here revealed a significant amphetamine x AdoTX interaction ($F_{(2,38)} = 4.84$, $p = 0.01$) and a three-way interaction with the sex factor ($F_{(2,38)} = 5.15$, $p = 0.01$). In controls, amphetamine reduced lose

shift behavior in males, but actually increased it in females (**Fig. 1.3C,D**, amphetamine x Sex: $F_{(2,44)} = 3.86$, $p = 0.03$, whereas in AdoTHC rats, these treatments uniformly reduced lose-shift behavior across sexes (amphetamine: $F_{(2,38)} = 8.87$, $p < 0.001$; amphetamine x Sex: $F_{(2,38)} = 2.61$, $p = 0.08$). From these data, we conclude that AdoTHC treatment makes rats more sensitive to the ability of amphetamine to increase risky choice and reduce sensitivity to losses, and this effect appears to be more prominent in females.

With respect to other performance measures, amphetamine increased choice latency and number of omissions across all probability blocks (amphetamine: $Fs_{(2,82)} < 15.19$, $ps < 0.001$, no amphetamine x Block interaction: $Fs_{(8,328)} < 0.98$, $ps > 0.45$). Analysis of the latency data revealed a significant amphetamine x AdoTX x Block ($F_{(8,328)} = 2.27$, $p = 0.02$) and amphetamine x AdoTX x Sex x Block interaction ($F_{(2,82)} = 2.67$, $p = 0.01$). Partitioning this latter interaction by AdoTX group revealed that in AdoVEH rats, females took longer to respond than males across the session (Sex x Block: $F_{(4,88)} = 3.02$, $p = 0.02$, Sex: $F_{(1,22)} = 44.13$, $p < 0.001$). In AdoTHC rat, females took longer to respond compared to males (Sex: $F_{(1,19)} = 21.44$, $p < 0.001$; no Sex X Block: $F_{(4,76)} = 2.41$, $p = 0.06$). Further analysis of the omission data revealed that overall females omitted more on trials compared to males (Sex x Block: $F_{(4,164)} = 12.93$, $p < 0.001$; Sex: $Fs_{(1,41)} < 71.39$, $p < 0.001$).

Chemogenetic Dopamine Neuron Stimulation During Probabilistic Discounting. The above experiments showed that AdoTHC enhances visual cue learning in females but had few other effects on cognitive flexibility or probabilistic discounting under basal conditions. However, when treated with the monoamine-enhancing drug amphetamine, we found evidence for stronger enhancement of “risky” responding in AdoTHC rats, relative to AdoVEH controls. We therefore next asked whether this effect relates to changes in the functions of VTA dopamine neurons in particular, by using Gq-coupled DREADDs to acutely stimulate VTA dopamine neurons or VTA dopamine neuron projections to mPFC in rats with both AdoTX histories.

Initial Training: Rats in this experiment did not undergo strategy set shifting training prior to probabilistic discounting training, so we confirmed that AdoTX again did not affect initial

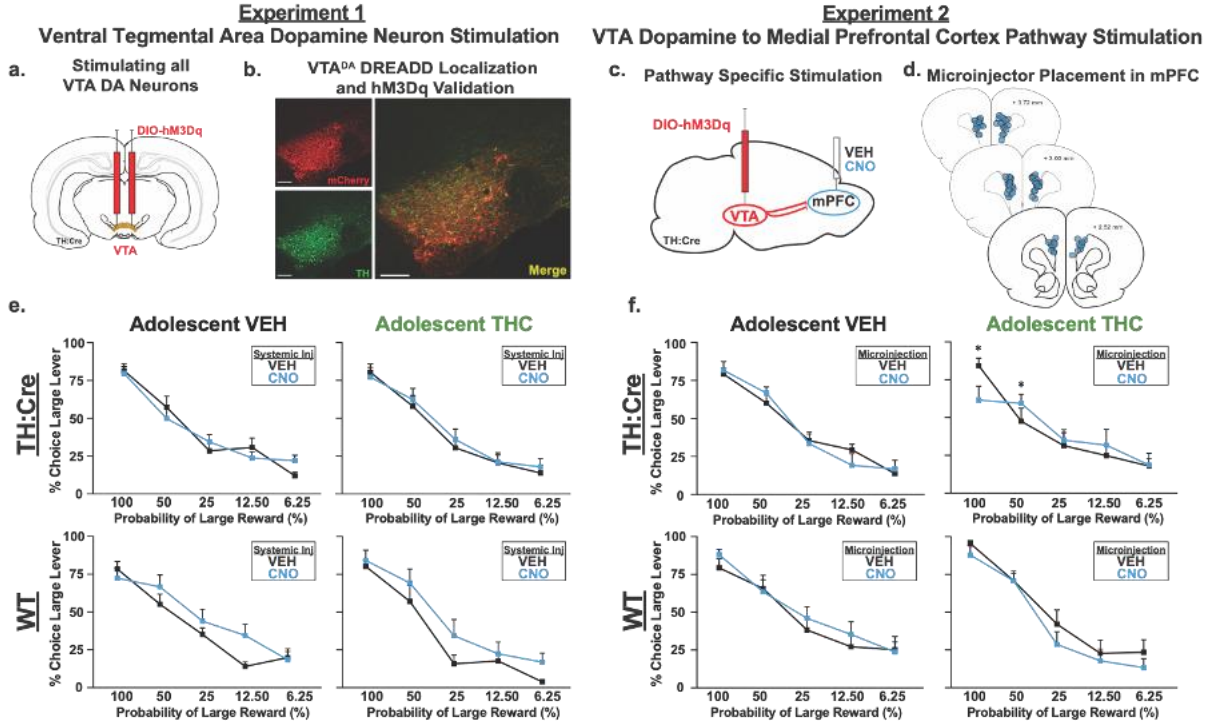


Figure 1. 4. Stimulation of VTA dopamine neurons, or VTA dopamine projections to mPFC does not affect probabilistic discounting.
A) Bilateral Cre-dependent hM3Dq DREADD AAV injections were made in VTA of TH:Cre rats, and of wildtype (WT) littermates. **B**) Example hM3Dq DREADD expression (red) is localized to tyrosine hydroxylase+ (TH; green) neurons within VTA (yellow=merge). Scale bar, 300 µm. **C**) For pathway-specific stimulation of VTA dopamine projections to mPFC, Cre-dependent hM3Dq DREADDs were injected into VTA as in Experiment 1, and cannulae targeting mPFC allowed CNO microinjection (1mM, 0.5 µl) upon DREADD-expressing dopamine neuron axons in this pathway. **D**) Cannula placements of each rat in pathway stimulation Experiment 2 is shown. **E**) Neither VTA dopamine neuron stimulation induced by systemic CNO in TH:Cre rats, nor **F**) stimulation of the VTA dopamine projection to mPFC induced by mPFC CNO microinjections in TH:Cre rats robustly altered probabilistic discounting in AdoTHC or AdoVEH rats. Likewise, lower panels of **E,F**) indicate that CNO did not have robust effects on WT rats without DREADDs. Data is represented as average % choice of the large reward lever in each probability block across the two CNO, and two VEH tests conducted in each rat, mean ± SEM, $p^* < 0.05$. TH:Cre+: VTA dopamine stimulation; AdoVEH (n = 8M, 3F) AdoTHC (n = 8M, 4F); VTA dopamine to mPFC: AdoVEH (n = 10M, 4F) AdoTHC (n = 9M, 5F). Wildtype: VTA dopamine stimulation; AdoVEH (n = 5M, 3F) AdoTHC (n = 5M); VTA dopamine to mPFC: AdoVEH (n = 9M, 4F) AdoTHC (n = 8M).

acquisition of instrumental food seeking behavior during initial training (no main effect of AdoTX: $Fs_{(1, 46)} < 0.78$, $ps > 0.38$). We also confirmed that genotype (Geno: TH:Cre+ or WT littermate)

did not alter instrumental training ($Fs_{(1,46)} < 2.55$, *ps* > 0.12; No AdoTX x Geno interaction: $Fs_{(1,46)} < 0.63$, *ps* > 0.43).

Probabilistic Discounting Training: Likewise, all included rats (TH:Cre and WT) successfully learned the discounting task (Main effect of Block: $F_{(4,184)} = 129.26$, $p < 0.001$, no main effects of, or interactions involving AdoTX or Geno: $Fs_{(1,46)} < 0.88$, *ps* > 0.35). Additionally, AdoTX nor genotype affected latency, omissions, or win-stay/lose-shift choice strategies (no main effects of, or interactions involving AdoTX or Geno: $Fs_{(1,46)} < 3.34$, *ps* > 0.07).

Impact of Acutely Activating VTA Dopamine Neurons on Probabilistic Discounting. Despite the robust changes in locomotion and reward seeking that is seen following chemogenetic VTA dopamine neuron manipulations in TH:Cre rats (Boekhoudt et al., 2016; Boekhoudt et al., 2018; Runegaard et al., 2018; Halbout et al., 2019; Mahler et al., 2019; Lawson et al., 2023), we found few effects of VTA dopamine neuron stimulation on the probabilistic discounting task. First, we looked at VTA dopamine neuron stimulation in only TH:Cre rats. All rats displayed normal discounting profiles (TH:Cre+: main effect of Block: $F_{(4, 84)} = 105.93$, $p < 0.001$), and as in the prior experiment, no effect of AdoTX alone was seen on discounting (AdoTX x Block: $F_{(4, 84)} = 0.91$, $p = 0.46$). Moreover, when we tested the effects of systemic CNO to activate DA neurons, we did not observe any effects of this treatment in either control or AdoTX groups (**Fig. 1.4E**, no main effect of, or interactions involving AdoTX or CNO: Fs < 2.27, *ps* > 0.07). Additionally, we did not see any effects of CNO, AdoTX, nor interactions therein on choice latency, omissions, or win-stay/lose-shift choice strategy ($Fs_{(1,21)} < 3.00$, *ps* > 0.10; data not shown). These findings are inconsistent with our hypothesis that increased excitability of mesolimbic dopamine neurons would be sufficient to recapitulate the ability of amphetamine to increase risky/perseverative responding in either control or in AdoTHC-experienced rats.

Impact of Selectively Activating VTA Dopamine Projections to mPFC. We next asked whether selectively stimulating VTA dopamine neuron projections to mPFC would recapitulate

the potentiation of amphetamine effects in AdoTHC rats. We did so by locally applying CNO upon DREADD-expressing axons of VTA dopamine neurons in mPFC. We have shown that this pathway-specific stimulation approach is capable of potentiating both axonal dopamine release and motivated reward seeking (Mahler and Aston-Jones, 2012; Halbout et al., 2019; Mahler et al., 2019). We found few effects of this manipulation on probabilistic discounting. On the probabilistic discounting task, "risky" responding across the session varied across CNO and AdoTX (**Fig. 1.4**; AdoTX x CNO x Block interaction: $F_{(4,104)} = 2.99$, $p = 0.02$; no main effect of AdoTX: $F_{(1,26)} = 0.15$, $p = 0.70$; no main effect of CNO: $F_{(1,26)} = 0.000$ $p = 0.10$), in AdoVEH animals there were no measurable effects of or interactions with CNO (all Fs < 1.80, *ps* > 0.14). However, in AdoTHC rats CNO modestly reduced risky responding at the 100% probability block followed by an increase at the 50% probability block (CNO x Block: $F_{(4,52)} = 2.99$, $p = 0.03$). Rats performed similarly on choice latency, omissions, and win-stay/lose-shift regardless of AdoTX, CNO, or interactions therein with block (All Fs < 2.00, *p*s > 0.10).

Minimal DREADD-independent Effects of CNO. Neither systemic nor intra-mPFC CNO had major behavioral effects in WT rats lacking DREADD expression (**Fig. 1.4E,F**). Systemic CNO in WT rats seemed to promote a more risk-prone phenotype, based on increased preference for the risky lever across all blocks (Main effect of CNO in WT rats: $F_{(1, 11)} = 9.42$, $p = 0.01$; no interactions of CNO, AdoTX, or Block: $Fs_{(4, 44)} < 2.04$, *ps* > 0.11), but did not affect response latency, omissions, or win-stay/lose-shift choice strategies ($Fs_{(1,11)} < 2.96$, *ps* > 0.11). Intra-mPFC CNO in WT rats altered risk responding such that "risky" choice was decreased in AdoTHC rats, whereas it was increased in AdoVEH animals (AdoTX x CNO: $F_{(1,16)} = 5.40$, $p = 0.03$). These effects were driven by changes in reward sensitivity, as CNO differentially affected win-stay behavior in the two groups ($F_{(1,160} = 5.78$, $p = 0.03$; no main effect of CNO $F_{(1,16)} = 0.73$, $p = 0.41$). Thus, CNO in AdoTHC WT rats were less likely to follow a risky win with another risky choice, while CNO had the opposite effects in in AdoVEH WT (data not shown). Additionally, we saw that AdoTHC animals had lower risky responding in the higher probability blocks, that then

increased in the latter probability blocks (AdoTX x Block: $F_{(4,64)} = 2.52$, $p = 0.05$). We did not see any effect of AdoTX or CNO on lose-shift strategies (CNO x AdoTX: $F_{(1,16)} = 0.67$, $p = 0.43$; no main effect of CNO $F_{(1,16)} = 1.71$, $p = 0.21$) or response latency and omissions ($Fs_{(1,16)} < 3.74$, $ps > 0.07$; data not shown).

Discussion

Here we show that administration of a well-characterized, human-relevant dose of THC (Torrens et al., 2020; Ruiz et al., 2021a; Torrens et al., 2022) during adolescence has subtle effects on behavioral tests of instrumental learning, without measurably altering performance on mPFC dopamine-dependent strategy set shifting or risk/reward decision making assessed with a probabilistic discounting task. However, when monoamine signaling was acutely enhanced with systemic amphetamine, an underlying effect of AdoTHC history was revealed. Relative to AdoVEH controls, amphetamine in AdoTHC rats were more sensitive to the ability of lower doses of amphetamine to increase perseverative responding for a large reward option when this choice was unlikely to result in reward. However, this potentiation of amphetamine effects in AdoTHC rats was not recapitulated by more specific chemogenetic stimulation of VTA dopamine neurons, or of their projections to mPFC in particular. This could suggest non-VTA dopaminergic mechanisms underlying potentiation of this cognitive effect of amphetamine in AdoTHC rats, suggesting potential impacts of THC on adolescent development of other systems upon which amphetamine acts. Alternatively, the enhanced effect of amphetamine reported here may be driven by alterations at the level of the dopamine terminal rather than changes in dopamine cell excitability, as dopamine receptor antagonism is able to block this amphetamine-induced risky responding (St. Onge and Floresco, 2009). Further, we thoroughly characterize sex differences in decision-making across these behavioral tasks, some of which mediate the persistent impacts of AdoTHC on behavior. Results open new directions for investigating long-term impacts of AdoTHC on non-VTA dopaminergic modulation of cognition, and may inform associational studies of the long-term impacts of adolescent cannabis use in humans.

Adolescent THC Exposure Enhances Initial Discrimination Learning. AdoTHC rats learned a visually cued instrumental response rule quicker than AdoVEH-treated controls, and this effect was most robust in females. This finding adds to the literature on potentially pro-learning/cognitive effects of AdoTHC (Hernandez et al., 2021; Stringfield and Torregrossa, 2021b). This said, previous studies using other adolescent cannabinoid exposure models have not found analogous increases in initial rule discrimination learning (Gomes et al., 2015; Hernandez et al., 2021; Freels et al., 2024), though this might be due to the fact that few studies included female subjects, and the THC administration protocols employed were quite different.

We found no clear effects of AdoTHC across our set shifting performance metrics, including trials to criterion, number of errors made, or error types on the set shift day. These findings are consistent with others that found no major AdoTHC-induced changes in cognitive flexibility, as quantified in with different set shifting tasks, including the intra/extra dimensional "digging" task and an operant-based task similar to the one used here (Gomes et al., 2015; Hernandez et al., 2021; Poulia et al., 2021). However, in a study utilizing the same strategy set-shifting task as used here, females exposed to self-administered cannabis extract vapor during adolescence took longer to learn the new rule, and made more errors on the set-shift day, though this deficit was not seen after experimenter-administered cannabis vapor (Freels et al., 2024). It is presently unclear whether differences in patterns of results reflect differences in route of THC administration or dose, the specific timing of THC exposure during adolescence, or other experimental details. As such, this remains an important topic for future research intended to probe how adolescent cannabinoid exposure may alter executive functioning.

Though untested here, it is possible that AdoTHC altered other related cognitive processes such as transitioning between tasks, mental sets, or rule structures, which depend upon more lateral PFC subregions such as orbitofrontal cortex (OFC; Birrell and Brown, 2000; McAlonan and Brown, 2003; Floresco et al., 2008a). For example, adolescent pubertal administration of the potent CBR agonist WIN 55,212 alters OFC-dependent reversal learning in

and attentional set-shifting task (Gomes et al., 2015), though adolescent vaporized cannabis or cannabis smoke did not impact this same type of reversal learning (Hernandez et al., 2021; Freels et al., 2024). Again, discrepancies between studies may reflect differences in effects of cannabinoid drugs, doses, exposure timing, and washout period; further underscoring the need for a consistent, rationally-designed AdoTHC exposure model in the field—we argue that the model used here is the best-characterized to date in the field (Torrens et al., 2020; Ruiz et al., 2021a; Lee et al., 2022; Torrens et al., 2022; Halbout et al., 2023; Lin et al., 2023b; Lee et al., 2024). This said, the possibility that OFC is even more sensitive to disruption by AdoTHC than mPFC should be directly tested in future studies.

Adolescent THC Does Not Alter Basal Probabilistic Discounting. In the mPFC-dependent probabilistic discounting task (St. Onge and Floresco, 2010), rats choose between a small reward that is always delivered when chosen (1 palatable banana pellet), and a larger reward that becomes increasingly unlikely to be delivered over the course of the ~1h session (providing either 4 or 0 pellets). Efficient performance on this task demands evaluation of both risk and opportunity, and is dependent on intact functioning of both the mPFC and mesocortical and mesoaccumbens dopamine transmission (St. Onge et al., 2010; St. Onge et al., 2012; Stopper et al., 2013; Jenni et al., 2017). Impaired PFC activity results in deficits in adjusting choice in response to changes in reward probabilities, loss assessment, and a diminished ability to appropriately compare and favor larger rewards even when the probability of receiving them is higher (St. Onge and Floresco, 2010; Bercovici et al., 2023). We found no major impacts of AdoTHC history on acquisition of, or stable performance on this task, as measured by choices of the ‘risky’ lever, choice strategies following rewarded vs unrewarded trials, latency to decide, and decisions to omit trials. One prior study (Jacobs-Brichford et al., 2019) found that administration of WIN 55,212 in both sexes during adolescence elevated preference for the ‘risky’ lever at lower reward probabilities, which we did not see in either sex as a result of our AdoTHC exposure model. Though several other differences exist between this study and the

present one, we note that several prior reports have also shown more severe lasting effects of synthetic CBR agonists versus THC when administered in adolescents (Renard et al., 2014; Higuera-Matas et al., 2015; Stringfield and Torregrossa, 2021a).

Adolescent THC History Potentiates Amphetamine Effects on Probabilistic Discounting. Dopamine markedly influences mPFC-dependent cognition (Goldman-Rakic, 1995; Seamans and Yang, 2004; Floresco and Magyar, 2006; Goto et al., 2007), including in the probabilistic discounting task employed here (Floresco and Whelan, 2009; St. Onge and Floresco, 2009; St. Onge et al., 2010; St. Onge et al., 2012; Jenni et al., 2017; Islas-Preciado et al., 2020). In the present study we replicated prior findings that pharmacologically challenging monoamine systems with amphetamine increased perseveration of responding for a large reward in both males and females (St. Onge and Floresco, 2009; Islas-Preciado et al., 2020). This change was accompanied by a reduction in lose-shift behaviors following 'risky' losses, indicating that enhancing DA levels attenuates the impact that non-rewarded actions exert over subsequent choice. Interestingly, we saw that amphetamine led to an overall increase in choice latency, and omitted trials. This may have been driven in part by increased psychomotor activity induced by amphetamine that may have displaced animals from the levers, thus delaying choice.

Moreover, we found that this effect of amphetamine was more potent in AdoTHC, relative to AdoVEH rats, especially in females. At the highest dose of amphetamine, AdoTHC rats chose the 'risky' lever more when probabilities of that reward were low. These effects were also markedly sex-dependent. In males, amphetamine reduced overall lose-shift behavior. In females, the highest dose of amphetamine induced a disruptive reduction in win-stay behavior only in AdoVEH females, whereas it reduced lose-shift behaviors only in AdoTHC females, contributing to the heightened increased 'risky' responding observed in the AdoTHC groups. While we refer to this behavior as 'risky' responding (St. Onge et al., 2010), this behavior may also be interpreted as amphetamine causing AdoTHC animals to be less adaptive to changing contingencies, or more liable to perseverate on the initially prepotent large reward lever.

Regardless, these results suggest that although probabilistic discounting under basal conditions is not altered by AdoTHC, underlying differences were nonetheless revealed upon acute activation of monoaminergic signaling. Amphetamine blocks and reverses the transporter for dopamine, norepinephrine, serotonin, and changes in one or more of these systems could be responsible for the potentiated response to amphetamine we see in this task in AdoTHC rats (especially females).

Effects of Chemogenetic VTA Dopamine Neuron, and mPFC Dopamine Projection Stimulation on Probabilistic Discounting. In our next experiments, we sought to determine whether, as predicted, changes in VTA dopamine neuron signaling are responsible for the potentiated effect of amphetamine in AdoTHC rats. We therefore prepared transgenic TH:Cre rats with excitatory DREADDs, allowing stimulation of either all VTA dopamine neurons, or of dopamine neuron projections to mPFC in particular. Surprisingly, no major effects of chemogenetically stimulating all VTA dopamine neurons were found on probabilistic discounting. In this regard, it bears mentioning that pharmacological stimulation of dopamine D2 or D1 receptors in the nucleus accumbens (a main target of the VTA dopamine projection) either had no effect on risky choice or led to more optimal decision making, respectively- an effect distinct from that induced by amphetamine (Stopper et al., 2013). Furthermore, when we stimulated VTA dopamine neuron projections to mPFC, this did not markedly affect probabilistic discounting in AdoVEH rats, which is similar to a prior finding examining manipulations of VTA dopamine projections to PFC (Verharen et al., 2018). On the other hand, chemogenetic stimulation of mPFC dopamine terminals in AdoTHC rats attenuated risky responding during the 100% probability block, but then increased it in the subsequent 50% block. This effect loosely resembles that induced by intra-PFC D_2 receptor stimulation on probabilistic discounting, which flattened the discounting curve (St. Onge et al., 2011). This is consistent with the possibility that AdoTHC exposure causes a slight enhancement in mesocortical dopamine D_2 receptor signaling—a possibility that should be directly investigated. Moreover, no other differential

effects of DREADD stimulation were seen in AdoTHC versus AdoVEH groups. The absence of significant effects in this experiment may also relate to the relatively small number of TH:Cre females, especially since amphetamine effects on the same behaviors seem to be driven by females. Additionally, the lack of behavioral effects of chemogenetic stimulation is unlikely to have resulted from inefficient DREADD stimulation or infection. Extent of viral expression, and selectivity of expression in TH+ VTA neurons was equivalent to our prior reports using these transgenic rats and viral vectors. In these reports we have shown this DIO-hM3Dq vector has >97% selectivity to TH+ neurons, and it is capable of driving dopamine neuron firing rates in vitro, increasing the number of them active in vivo, and increasing cFos expression in them (Mahler et al., 2019). Accordingly, we and others have found very robust behavioral effects of VTA dopamine neuron stimulation in a range of tasks, including cocaine intake and seeking, food reward intake, locomotion, and motivation (Boekhoudt et al., 2016; Boekhoudt et al., 2018; Runegaard et al., 2018; Halbout et al., 2019; Mahler et al., 2019; Lawson et al., 2023). We also previously confirmed that pathway-specific stimulation of VTA dopamine projections enhances dopamine release, and stimulation of mPFC projections is behaviorally efficacious for stimulating cue-induced cocaine seeking as well (Mahler et al., 2019). Therefore, it was particularly striking that we did not see any notable behavioral effects of dopamine neuron or dopamine projection stimulations on probability discounting, despite the fact that this task is sensitive to mPFC dopamine receptor manipulations. This may imply that alterations of VTA and non-VTA dopamine caused by amphetamine might simply be qualitatively distinct from manipulations of VTA dopamine neuron activity and dopamine release, as were conducted here chemogenetically (Mahler et al., 2014; Mahler et al., 2019). In this regard, performance of this probabilistic discounting task is associated with fluctuations in mesocortical and mesoaccumbens dopamine that relate to changes in the amount of rewards received and adjustments in risky choice biases (St. Onge et al, 2012). Amphetamine would cause robust increases in tonic dopamine levels, leading to a more static dopamine signal that impedes

flexible adjustments in choice biases, whereas chemogenetic increases in dopamine neuron excitability would be a comparatively more subtle manipulation that could still permit variations in dopamine signaling over a session. Alternatively, amphetamine-induced changes in risk taking seen in AdoTHC rats may not solely depend upon VTA dopamine neurons themselves, but may instead depend upon other neural systems targeted by amphetamine, such as nigrostriatal dopamine projections to the dorsomedial striatum (Schumacher et al., 2021), other non-mPFC targets of VTA dopamine neurons, or other monoamine systems affected by amphetamine. Regardless, this intriguing finding refines our understanding of the neural substrates of probability-based decision making and underscores the need for further exploration to identify the specific mechanisms involved.

Sex Differences. We conducted these studies in female as well as male rats, and found several consistent sex differences, both overall and in interaction with other experimental variables. Consistent with existing literature (Islas-Preciado et al., 2020; Gargiulo et al., 2022), we saw sex differences in response characteristics during operant set-shifting as well as probabilistic discounting, with females showing longer deliberation times (response latency), and more omitted trials than males. Strikingly, these specific sex differences consistently surfaced in the probabilistic discounting task across various experiments and cohorts, underscoring the robustness of the response latency and trial omission effects in females. Some of these sex differences may derive from females attaining satiety on the palatable reward quicker than males, but since both omissions and longer latencies at least partially emerged in females early in sessions during otherwise apparently vigorous reward seeking, this finding may also reflect different strategies taken by females relative to males (Orsini and Setlow, 2017; Chen et al., 2021). For example, female rats have previously been shown to be more risk-averse than males on average (Orsini et al., 2016), and may exhibit prolonged response times and higher omission rates due to aversion to the risk of losing a reward, as demonstrated by a heightened sensitivity to loss (van den Bos et al., 2012). Alternatively, extended choice latencies and omissions may

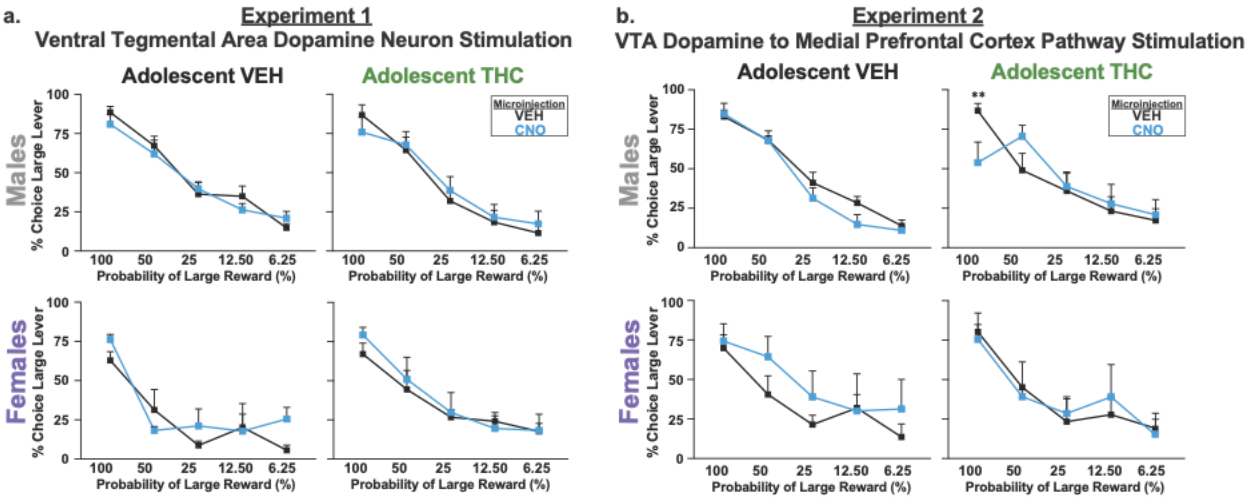
signify females' tendency to take more time in learning about the probability distribution of the outcomes. This aligns with both rodent and human findings, indicating that females take longer to develop a preference for the more advantageous option when learning about probability distributions of reward versus punishment (van den Bos et al., 2012; van den Bos et al., 2013). Both possibilities warrant further exploration in future.

Limitations. The present report has a number of limitations that should be considered. A single dose of THC (5mg/kg IP) was administered to adolescents daily from postnatal days 30 to 43, and persistent effects of THC are known to be dose-dependent (Amal et al., 2010; Irimia et al., 2015; Freeman-Striegel et al., 2023), and dependent upon the specific developmental stage at which it is experienced (Cha et al., 2006; Schramm-Sapyta et al., 2007; Mokrysz et al., 2016; Gorey et al., 2019; Torrens et al., 2020; Murray et al., 2022; Torrens et al., 2022). The low-dose THC regimen used here may capture the effects of frequent, low-potency cannabis use (Huestis et al., 1992; Cooper and Haney, 2009) rather than high-intensity or chronic cannabis exposure, use of high-potency cannabis products, or escalating use over time. Further work should examine how THC dose, dosing pattern, and means of administration (i.e. oral vs intraperitoneal vs inhalation) impact long-term function of dopamine-dependent cognition. Also relevant to dose, we and others often observe more potent behavioral effects of THC in female, relative to male adolescent rats, which is likely attributable in part to differences in THC metabolism, in particular with regard to a higher blood and brain levels in female adolescent and adult rats and mice of the THC metabolite 11-OH-THC, a CB1 agonist (Tseng et al., 2004; Wiley and Burston, 2014; Craft et al., 2019; Torrens et al., 2020; Baglot et al., 2021; Ruiz et al., 2021a; Ruiz et al., 2021b; Torrens et al., 2022), and this sex difference is also seen in humans after cannabis use (Matheson et al., 2020; Sholler et al., 2021; Arkell et al., 2022). This metabolic sex difference may at least in part account for the more robust persistent effects of adolescent THC in females than males seen here, and in numerous prior reports (Tseng et al., 2004; Cha et al., 2007; Rubino et al., 2009b; Rubino and Parolaro, 2011; Ruiz et al., 2021a; Ruiz et al., 2021b; Le et al.,

2022). We found that in females, AdoTHC had more pronounced effects than in males, including enhancement of visual cue learning, and stronger potentiation of amphetamine's effects on probability discounting behavior—but no effect on other behaviors like set shifting and baseline probability discounting. These results support the increasingly apparent possibility that AdoTHC's effects are both highly sex- and task-dependent. Further studies investigating the reasons why AdoTHC seems to influence some behaviors and not others are needed, and may provide important new evidence relevant to cognitive and anatomical development. Another limitation of this report is the lack of data on complementary cognitive tasks, which are dependent upon other cortical regions like OFC that are notably sensitive to adolescent cannabinoid exposure (Egerton et al., 2005; Klugmann et al., 2011; Gomes et al., 2015), and which are mediated by ECB-dependent processes that may be particularly sensitive to AdoTHC's long-lasting impacts.

Summary. In sum, this paper provides novel information about the impact of AdoTHC on the developing brain of males, and especially of females. Results point to long-lasting changes that influence cue learning, and the ability of acute amphetamine to alter risky decision making. A key finding is the pervasive nature of sex-differences—both in behavioral strategies employed by rats in these tasks, and in the severity of long-lasting consequences of AdoTHC.

Supplementary Figure 1



Supplementary Figure 1.1. Stimulation of VTA dopamine neurons, or VTA dopamine projections to mPFC does not affect probabilistic discounting in either sex
A) VTA dopamine neuron stimulation induced by systemic CNO in TH:Cre AdoTX female and male rats **B**) stimulation of the VTA dopamine projection to mPFC induced by mPFC CNO microinjections in TH:Cre AdoTX female and male rats. TH:Cre+: VTA dopamine stimulation; AdoVEH (*n* = 8M, 3F) AdoTHC (*n* = 8M, 4F); VTA dopamine to mPFC: AdoVEH (*n* = 10M, 4F) AdoTHC (*n* = 9M, 5F).

CHAPTER 2: Persistent effects of adolescent THC on behavioral and neural responses to opioid drugs in male and female rats

Introduction

Substance use by teenagers is a phenomenon that is very common, and very concerning to many adults. In particular, cannabis use has long been stigmatized, including in an overtly racialized manner (Wheeldon and Heidt, 2023) and amongst its putative harms was the claim that “marijuana” serves as a “gateway” to use of harder drugs like opioids (Yamaguchi and Kandel, 1984; Fergusson and Horwood, 2000; Agrawal et al., 2004; Hall and Lynskey, 2005; Wilson et al., 2022; Albaugh et al., 2023). Despite this political baggage, there is reason for legitimate concern that cannabis use by adolescents could have long-lasting, or even permanent effects on brain development, including by inducing brain changes that increase the addictive potential of other drugs (Hurd et al., 2014; Volkow et al., 2016). Indeed, adolescent cannabis use is empirically associated with increased incidence of subsequent opioid use and opioid use disorder, with those initiating cannabis use earliest having the greatest risk (Lynskey et al., 2003; Fergusson et al., 2015). Yet such correlation does not imply causation, especially considering the societal, legal, and cultural factors at play in human populations.

Therefore, animal studies are required to evaluate the causal impacts of adolescent drug use, and a consistent finding across labs and experimental procedures is that AdoTHC indeed leads to a pro-opioid behavioral phenotype that lasts long after the pharmacological effects of THC itself. In adult male rats, AdoTHC history increases self-administered heroin consumption (Solinas et al., 2004; Ellgren et al., 2007; Spano et al., 2010; Tomasiewicz et al., 2012; Lecca et al., 2020; Ferland et al., 2023b) and stress-induced reinstatement (Stopponi et al., 2014), and in adult females AdoTHC increases fentanyl self-administration (Nguyen et al., 2020). Strain-dependent effects have also been observed in rats, as AdoTHC selectively increases heroin consumption, effortful seeking, and primed reinstatement in “addiction-prone” male Lewis rats, but instead increases heroin conditioned place preference (CPP) and cue-induced

reinstatement in “addiction-resistant” male Fischer 344 rats (Cadoni et al., 2015; Lecca et al., 2020). In mice of both sexes, AdoTHC also enhances morphine-primed reinstatement of morphine CPP (Hubbard et al., 2024). These findings suggest a robustly pro-opioid effect of AdoTHC that interacts with, but ultimately transcends genetic influences on opioid seeking and taking.

Interestingly, AdoTHC seems to cause vulnerability to opioid drugs in particular, as psychostimulant reward is not similarly consistently augmented (Friedman et al., 2019; Orihuel et al., 2021). This likely reflects the close neurobiological connection between endogenous opioid and cannabinoid signaling systems in the brain (Scavone et al., 2013; Aghaei et al., 2023), which both participate in shaping the neurodevelopment of brain reward and emotion circuits (Bossong and Niesink, 2010; Kibaly et al., 2019; Marusak, 2022; Spodnick et al., 2025). These systems interact closely in the brain (Navarro et al., 2001; Fattore et al., 2004; Robledo et al., 2008; Parolaro et al., 2010), and both mediate the acute effects of opioid and cannabinoid drugs (Robledo et al., 2008; Solinas et al., 2008; Wenzel and Cheer, 2018), as well as the addiction-relevant neuroadaptations that occur with chronic use of either drug class (Navarro et al., 2001; Hoffman et al., 2003; Robledo et al., 2008; Mohammadkhani and Borgland, 2022).

Another mechanism by which AdoTHC might disrupt adolescent development of opioid addiction-relevant neural circuits is via direct or indirect actions of THC on microglia, which are long-lived glial cells that help sculpt neural circuits during development, including in adolescence (Frost and Schafer, 2016; Dziabis and Bilbo, 2021; Guedes et al., 2022). For example, microglia are involved in adolescent refinement of prefrontal cortex (PFC) circuits (Mallya et al., 2019; Dziabis and Bilbo, 2021; Schalbetter et al., 2022; von Arx et al., 2023), which are crucial for reward processing and decision making processes that underlie aspects of addiction (Koob and Volkow, 2016; Volkow et al., 2019).

The present studies aimed to determine the persistent effects of our well-characterized, translationally-relevant AdoTHC exposure protocol (Torrens et al., 2020; Ruiz et al., 2021a; Ruiz

et al., 2021b; Torrens et al., 2022) upon opioid reward behaviors in rats of both sexes, and to explore potential cellular mechanisms that might participate in this phenomenon. Results provide both causal and correlational evidence that AdoTHC alters addiction-relevant neurodevelopmental processes in a sex-dependent manner, and set the stage for further investigation of the underlying neural and glial mechanisms.

Materials and Methods

Subjects. A total of 91 Long Evans rats (*n*=45 females, 46 males) were born and reared in-house, weaned at postnatal day (PD) 21 into cages with 1-2 same-sex littermates, and treated with VEH or THC daily from PD30-43. All rats in a cage received the same adolescent drug treatment (AdoTX; AdoVEH or AdoTHC). Housing was under 12:12hr reverse light/dark cycle, with *ad libitum* water and food throughout. Experiments were approved by University of California, Irvine's Institutional Animal Care and Use Committee, and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

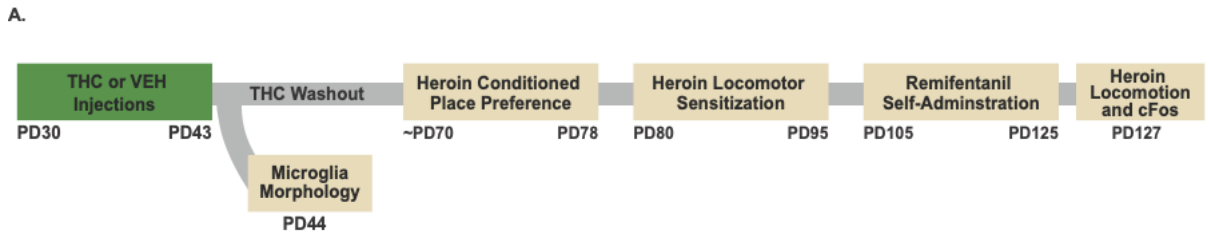


Fig. 2.1. Experimental Timeline.
Female and male rats were given 14 daily injections of THC or VEH during adolescence (PD30-43). Microglia morphology was examined in some rats at PD44, and others were allowed to grow to adulthood in a drug-free washout period. In adulthood (PD70+), they underwent behavioral tests relevant to opioid addiction. A subset of these were used to analyze cFos expression in 13 brain regions following a final experimenter-administered injection of heroin.

Drugs. THC was provided in ethanol by the National Institute on Drug Abuse (NIDA) Drug Supply Program (Research Triangle Park, NC, USA), and was freshly prepared for injection each day (ethanol was evaporated under N_2, and THC was reconstituted with heat and

sonication in 5% Tween-80 saline solution). Heroin (diacetylmorphine HCl) was provided by NIDA or Cayman (Ann Arbor, MI, USA), and remifentanil HCl was provided by NIDA. Both opioid drugs were dissolved to dose in 0.9% saline solution for intraperitoneal (IP), subcutaneous (SC), or intravenous (IV) administration.

Experimental Design and Group Sizes. Experimental design is summarized in **Fig. 2.1**. Most rats [*n*=74 total; *n*=37 females (*n*=21 AdoTHC), *n*=37 males (*n*=18 AdoTHC)] underwent behavioral testing with opioids as adults, starting 26d+ after AdoTX. Heroin CPP training and testing occurred first in these rats, over 8d starting at ~PD70. Because of COVID-19 and other disruptions, not all rats underwent all the other behavioral tests. Fifty CPP-tested rats [*n*=28 females (*n*=16 AdoTHC), *n*=22 males (*n*=10 AdoTHC)] next underwent heroin locomotor sensitization training over the next 15d. Forty-two of these [*n*=20 females (*n*=12 AdoTHC), *n*=22 males (*n*=10 AdoTHC)] next went on to remifentanil self-administration experiments.

All rats, 2d+ after their last tested behavior, were given a final test for locomotor activity after a heroin injection before they were sacrificed [*n*=37 females (*n*=21 AdoTHC), *n*=36 males (*n*=18 AdoTHC)]. Eleven rats [*n*=2 females (*n*=1 AdoTHC), *n*=9 males (*n*=4 AdoTHC)] underwent only heroin CPP testing prior to locomotion testing, eight (all females; *n*=4 AdoTHC) underwent heroin CPP and sensitization only, and thirteen [*n*=7 females (*n*=4 AdoTHC), *n*=6 males (*n*=4 AdoTHC)] underwent heroin CPP and remifentanil self-administration only. A total of fifty-five rats [*n*=27 females (*n*=16 AdoTHC), *n*=28 males (*n*=14 AdoTHC)] were tested in remifentanil self-administration experiments. One male AdoVEH rat died following catheter surgery, and one male AdoTHC rat was excluded from behavioral economic analyses due to catheter failure before completion of training on that task.

A subset of rats that underwent all behavioral tests [CPP, sensitization, and self-administration; *n*=20 females (*n*=12 AdoTHC), *n*=15 males (*n*=7 AdoTHC)] were used to examine cFos expression—these were perfused following the final heroin locomotion test. A separate group of adolescent rats [*n*=8 females (*n*=4 AdoTHC), *n*=9 males (*n*=5 AdoTHC)] were

used to determine how AdoTHC affects microglial morphology—their brains were collected at PD44, 24hr after the last AdoTX injection.

Adolescent THC Exposure Protocol. Following our established protocol (Torrens et al., 2020; Ruiz et al., 2021a; Ruiz et al., 2021b; Torrens et al., 2022; Martinez et al., 2025) rats received daily IP injections of THC (5mg/2ml/kg) or VEH from PD30-43. Behaviorally tested rats were undisturbed during a home cage washout period of 21d+, allowing for full THC clearance (Torrens et al., 2020; Lee et al., 2022; Lin et al., 2023b), and for rats to mature to adulthood (PD70+).

Behavioral Testing Methods. *Heroin Conditioned Place Preference:* A three-chamber CPP box (Med Associates, St Albans, VT), consisting of two main chambers (black walls + bar floor, or white walls + grid floor; 21 x 27 x 20cm each chamber) and a smaller central chamber (grey walls, smooth floor, 21 x 12 x 20cm) was used, and rat location was recorded using Ethovision software (Noldus, Leesburg, VA). Rats were first allowed to explore the entire apparatus for 15min (baseline), and whichever of the two main chambers they spent less time in during this session was assigned as the heroin context for that rat (the opposite chamber assigned to saline). Three rats (*n*=2 females: 1 AdoVEH, 1 AdoTHC; and *n*=1 male AdoTHC) spent >70% of the baseline session in the central chamber that was not subsequently paired with either heroin or saline, so they were excluded from CPP analyses. 24hr after the baseline session, rats received 40min training sessions daily for the next 6d. Immediately before each training session they received IP saline (training days 1, 3, 5), or heroin (0.25mg/kg; training days 2, 4, 6), and then were confined within the appropriate compartment. After all 6 training days, they were allowed to explore the entire 3 chamber apparatus again for 15min on the following day (CPP test). CPP data were analyzed as change in time spent in each chamber from baseline to CPP test.

Heroin Locomotor Sensitization: Sensitization to the locomotor-activating effects of drugs with repeated administration may relate to excessive incentive motivational “wanting” of drugs and

their cues which people with addiction often experience (Robinson and Berridge, 1993; Vanderschuren and Pierce, 2010), so we examined how AdoTHC affects heroin's locomotor sensitizing effects. Locomotion was tracked for 1hr after heroin injections (1mg/kg, SC) on each of 7 consecutive days, conducted in 43 × 43 × 30.5cm, initially novel, bedding-free chambers. Rats were then given 7 consecutive days off in their home cages, followed by another 1hr session after the 8th such injection of heroin. Locomotion in the first versus eighth heroin injection was analyzed (Liu et al., 2005; Jing et al., 2014).

Remifentanil Self-Administration: The short-acting fentanyl analogue remifentanil was chosen because it yields robust self-administration in short-access (2hr) training sessions, and is thus useful for studying the acutely reinforcing effects of opioid drugs, in the absence of long-lasting sedation that can interfere with measurement of a drug's acutely rewarding actions (Michelsen and Hug Jr, 1996; Glass et al., 1999). This is particularly relevant when conducting the within-session economic demand protocol we employ, which requires numerous drug infusions to be administered in short (~2hr) sessions (Porter-Stransky et al., 2017; Levis et al., 2021; Levis et al., 2022; Levis et al., 2024).

Rats were implanted with jugular catheters under isoflurane (3-3.5%) anesthesia, meloxicam (1mg/kg, IP) and cefazolin (10mg, IV) were administered intraoperatively, and heparin lock solution (10 U/0.1ml) was used to maintain catheter patency. After 6d recovery, rats began remifentanil self-administration training in Med Associates operant chambers (30.5 x 24 x 21cm) within sound-attenuating boxes, equipped with two retractable levers with white lights above them, and a white house light. Acquisition training occurred in daily 2hr sessions where pressing an active lever once (FR1) yielded a remifentanil infusion (3.2µg/kg/infusion, IV) and a 2.9kHz tone + lever light for 3.6sec, followed by a 20sec timeout period signaled by house light extinction. Lever presses during timeout, and all inactive lever presses, were recorded but inconsequential. After each session catheters were flushed with cefazolin and heparin lock.

Acquisition training continued for at least 5d, or until acquisition criterion was met (2 consecutive sessions with >20 infusions).

Next, rats were trained for 14d in the same chambers on a within-session economic demand thresholding procedure (Oleson and Roberts, 2009; Bentzley et al., 2013) using remifentanil (Porter-Stransky et al., 2017; Levis et al., 2021; Levis et al., 2022; Levis et al., 2024). Active lever presses activated a pump delivering IV remifentanil solution (0.0137mg/ml) and a coincident tone/light cue for a period of time that decreased across each of eleven, 10min blocks during the session (without timeouts). Presses delivered 1.92, 1.08, 0.608, 0.342, 0.192, 0.108, 0.0609, 0.0341, 0.0193, 0.0108, 0.00611µg remifentanil/infusion in the eleven blocks. Therefore, the number of presses required to achieve preferred levels of remifentanil intake increased block to block, as the dose delivered for each press decreased.

Remifentanil intake at each "price" was recorded daily, and demand parameters were calculated by fitting daily consumption to an exponential demand equation: $\ln Q = \ln Q_0 + k\,(e^{-\alpha Q_0 C} - 1)$; where Q = consumption, C = unit cost, k is a scalar constant for consumption range, α = demand elasticity, and Q_0 = extrapolated intake at price zero effort (Levis et al., 2021; Levis et al., 2024). The key variables α (reflecting demand elasticity, which is inversely related to motivation to receive the preferred amount of drug), and Q_0 (reflecting "hedonic setpoint," or preferred drug consumption at extrapolated price zero) were averaged for the last 7 training days, when behavior had stabilized in all rats, for analysis. We also analyzed intake (infusions) in each price bin, and Pmax (the bin in which the maximum infusions were delivered; Oleson and Roberts, 2009), as additional indices of demand elasticity.

Since some rats received substantial heroin exposure during the sensitization experiment while others did not, we asked whether this additional opioid drug exposure impacted remifentanil self-administration behaviors. We found that heroin sensitization did not affect acquisition of remifentanil self-administration in either sex (no main effect of Sensitization

on remifentanil infusions taken in 5d, or interaction of Sensitization with AdoTX or Day in females: Fs<0.48, *p*s>0.49, or males: Fs<3.49, *p*s>0.08). Likewise, Sensitization did not affect stable remifentanil demand in either sex (α: females: $F_{(1, 23)}$=0.62, *p*=0.44; males: $F_{(1, 22)}$=0.04, *p*=0.85; Q_0: females: $F_{(1, 23)}$=2.31, *p*=0.14; males: $F_{(1, 22)}$=1.88, *p*=0.18; Pmax: females: $F_{(1, 23)}$=3.54, *p*=0.07; males: $F_{(1, 22)}$=2.51, *p*=0.13). Therefore, rats with or without sensitization history were combined for subsequent analyses of self-administration data.

Locomotor Behavior After a Final Heroin Injection: At least 2d after their behavioral test, rats were injected with heroin (1mg/kg, SC), and placed for 1hr in a locomotor testing chamber (the same chamber used for heroin sensitization training of that rat, when applicable). Locomotion data for two male AdoVEH rats was lost due to camera failure. A subset of rats that had underwent all the above behavioral tests (CPP, sensitization, self-administration) were sacrificed after this session to examine cFos. These rats returned to their home cages for 1hr after the 1hr locomotor test, then they were deeply anesthetized with ketamine/xylazine, perfused, and brains were processed for cFos immunohistochemistry.

Immunohistochemical Experiments. *Microglial Morphology Analysis:* Our AdoTHC protocol transiently alters microglia morphology in male adolescent mice, and this may set the stage for longer-lasting changes in microglial responses to homeostatic challenges that are encountered later in life (Lee et al., 2022). Here we analogously investigated microglial morphology shortly after AdoTHC/VEH, in adolescent rats of both sexes.

We quantified microglia morphology 24hr after (PD44) the last AdoVEH (*n*=8, 4 females), or AdoTHC injection (*n*=9, 4 females), a timepoint when microglia are known to be altered in male mice by 14 daily injections of 5mg/kg THC (Lee et al., 2022). We visualized microglia using immunofluorescent staining for ionized calcium-binding adapter molecule 1 (IBA1), a protein that fills microglial soma and processes allowing morphological quantification (Ito et al., 1998; Lee et al., 2022; Green and Rowe, 2024). Rats were perfused with chilled

saline then 4% paraformaldehyde, and brains were removed and postfixed in 4% paraformaldehyde overnight, cryoprotected in 20% sucrose-azide, and coronally sliced at 40μm on a cryostat. Sections containing mPFC were blocked in 3% normal donkey serum (NDS) in PBST for 2hr, incubated overnight at RT in 1:1000 rabbit anti-IBA1 (Wako, #019-19741) with 3% NDS in PBST, then washed and incubated in a donkey anti-Rabbit Alexafluor-488 secondary antibody for 4hr. Sections were mounted on slides, coverslipped with Fluoromount (Fisher Scientific, Hampton, NH), and imaged at 20x magnification using a 0.75 NA oil immersion objective on a Lecia SP8 confocal microscope (488nm laser excitation, emission collected between ~500–550nm). In 2 slices/rat, bilateral regions of interest were chosen within the borders of the prelimbic (PLC) subregion of mPFC, from deep layers centered on layer V. 1μm z-step stacks were acquired, and processed using Imaris software (Bitplane, Zurich, Switzerland), with identical display settings used for all samples. The “Surfaces” tool was used to reconstruct microglia soma features, and “Spots” quantified microglia number. “Filaments” was used to initially skeletonize microglia processes, which were then manually curated by a trained observer blind to experimental conditions. The length, volume, and complexity (i.e. number of branch points/process and Sholl analysis) was computed for each imaged cell. Sholl analysis quantified intersections of processes with concentric spheres centered upon the soma, with radii from 5-40μm, in 5μm steps. Since processes travelling in the z dimension through the 40μm section were likely truncated, we also examined process complexity using a Sholl analysis restricted from 5-20μm from the soma, helping to minimize this potential artifact. Statistical analyses were based on per-rat averages calculated from all 4 imaged regions in 2 bilateral mPFC samples. Since male and female rats were stained in separate staining runs, morphology metric data from AdoTHC rats were normalized based on AdoVEH rats of the same sex, facilitating comparison between the sexes.

cFos Neural Activity Quantification: Two hours after injection of heroin and 1hr after the end of the locomotor test, rats were perfused and processed as described above before sectioning

coronally at 40μm. Sections were stained for cFos protein using a diaminobenzadrine (DAB)-based protocol described in detail elsewhere (Mahler et al., 2007; Ruiz et al., 2021a; Levis et al., 2022), here using 1:10,000 rabbit anti-c-Fos primary (Abcam, #ab190289), and 1:500 biotinylated donkey anti-rabbit secondary antibodies (Jackson ImmunoResearch, #711-065-152), with an avidin–biotin complex (ABC)-amplification (Vector Laboratories), and 0.04% nickel ammonium chloride DAB reaction to visualize nuclear cFos protein in blue-black. Sections were mounted on slides, dehydrated, coverslipped in Permount (Fisher Scientific, Hampton, NH), and regions of interest were imaged at 5x magnification on a Leica DM4000B microscope. cFos+ cells were quantified in each region of interest using the StereoInvestigator (Microbrightfield) particle counting tool, by a trained investigator blind to experimental groups. For each region, two sections at consistent positions in the brain were analyzed bilaterally, and mean cFos density (cFos/mm^2) for the 4 samples/region was computed for each rat. The regions quantified were mPFC [prelimbic (PLC) and infralimbic (ILC) subregions], orbitofrontal cortex [lateral (LOFC) and ventral (VOFC) subregions], anterior agranular insular cortex (insula), claustrum (CL), nucleus accumbens [core (NAcCo) and shell (NAcSh) subregions], lateral septum (LS), amygdala [basolateral (BLA) and central (CEA) subregions], paraventricular nucleus of the thalamus (PVT), and pedunculopontine tegmental nucleus (PPTg). Insula samples were lost for 1 AdoVEH female, and PPTg was lost for 1 AdoVEH male during staining.

Data Analysis. *Behavioral and Regional cFos Analyses:* GraphPad Prism was used to conduct analyses. We were interested in effects of AdoTHC that exist in females, in those that exist in males, and in those which occur in both sexes. Primary analyses thus consisted of ANOVAs and t-tests comparing AdoVEH to AdoTHC rats separately in each sex, and also with both sexes combined in the same analysis (i.e. main effect of AdoTX). Although the study was not specifically powered to detect sex differences, secondary analyses included Sex as a between-subjects factor, and sex differences (i.e. main effects or interaction involving Sex) were reported when statistically robust. Analyses also included repeated measures variables within mixed-

model ANOVAs for analysis of body weight during AdoTX [administration days 1-14 (PD30-43)], CPP [Chamber (change from baseline time spent in heroin or saline side)], sensitization (first versus 8th heroin injection), acquisition of remifentanil self-administration (training days 1-5), and remifentanil intake across the 2hr behavioral economic tests (average remifentanil infusions taken in each of the 11 price bins during stable responding). Effects of AdoTX on cFos were analyzed with t-tests (for brain areas in which we did not parse subregions), or mixed model AdoTX x Subregion ANOVAs (with Subregion treated as a within-subjects variable). Greenhouse-Geisser and Welch's corrections were applied when normality assumptions of parametric tests were violated, and Sidak or corrected t-test posthocs were used to determine the nature of significant ANOVA results.

cFos Functional Connectivity Analysis: To determine how AdoTHC impacts heroin-related activity at the level of neural networks, we examined the correlational structure of cFos expression patterns across quantified brain regions, using a previously-published approach (Ruiz et al., 2021a), using igraph, qgraph, and DescTools packages in R. We imputed two missing cFos values (insula in 1 AdoVEH female, and PPTg in 1 AdoVEH male) by using a k-nearest neighbors algorithm to define the 10 most similar rats based on the full cFos dataset, and averaging their values to replace the missing ones. Regional data were then normalized for interregional comparison by calculating z-scores reflecting the difference between cFos density in a given region/rat from the mean value of other rats of the same group (same Sex and AdoTX). Then, the degree of correlation between activity in the 13 quantified regions was examined for each Sex and AdoTX condition using Spearman rank correlations, and positive interregional correlations (representing functional connectivity between two regions) were quantified.

Our main goal was to determine how AdoTHC alters functional connectivity patterns relative to those seen after AdoVEH, so we next conducted a Fisher Z transformation of inter-regional correlations for each group, to normalize the distribution of correlation values between

groups. The difference between AdoTHC and AdoVEH conditions (ΔZ) for each pairwise connection was then calculated [pairwise correlations were capped at Z=4 (corresponding to r=0.9993) to prevent near-perfect correlations from producing mathematically unstable Fisher Z values, which can occur in small samples (Fisher, 1921; Perugini et al., 2025). Therefore, positive ΔZ values indicate strengthened interregional functional connectivity due to AdoTHC, and negative ΔZ values indicate weakened connectivity. Such AdoTHC-induced changes were statistically evaluated using two-tailed Z tests comparing each Z-statistic against the standard distribution for that pairwise comparison (Fisher, 1915, 1921; Perugini et al., 2025). To ask whether AdoTHC-induced changes at specific connections differed between the sexes, we computed a z-interaction test for each pairwise connection, which was calculated as the difference in ΔZ between males and females, divided by the pooled standard error accounting for all four groups.

In addition to analyzing bi-regional functional connectivity changes, we also examined effects of AdoTHC on the global connectivity of each node (region) with the entirety of the rest of the network. We did so by averaging the Fisher Z-transformed correlation values of each node's full set of connections with all other nodes, then comparing in each sex AdoTHC to AdoVEH rats using Welch's t-test. To ask whether such node-level effects differed between the sexes, we used a Sex X AdoTX ANOVA for each region.

AdoTHC-induced changes in region-region functional connectivity is visually represented in **Fig. 2.6.** by scaling thickness of the lines connecting regions based on ΔZ, and by coloring the lines to indicate the direction of change, relative to AdoVEH (blue = strengthened connectivity, pink = weakened connectivity), and AdoTX effects that were statistically robust are indicated by adding a border to the lines connecting regions. Regions in which AdoTHC significantly affected functional connectivity of a node with the entirety of the network (are indicated with a bolded circle around the region's name, and the direction of change is color-coded using the same logic).

Results

AdoTHC Treatment Transiently Suppresses Body Weight. As previously reported (Lin et al., 2023a), AdoTHC suppressed body weight gain during treatment, relative to AdoVEH; both in females (main effect of AdoTX: $F_{(1,43)}$=6.99, p=0.01), and in males ($F_{(1,44)}$=24.44, p<0.0001; both sexes: $F_{(1,87)}$ =25.17, p<0.0001; **Supplementary Figure 2.1A**). This AdoTHC-induced suppression of body weight was transient, since both sexes attained comparable body weights by adulthood (~PD70), when behavioral testing began (no effect of AdoTX in females: t_{35}=1.22, p=0.23, or males: t_{35}=0.46, p=0.65; **Supplementary Figure 2.1B**).

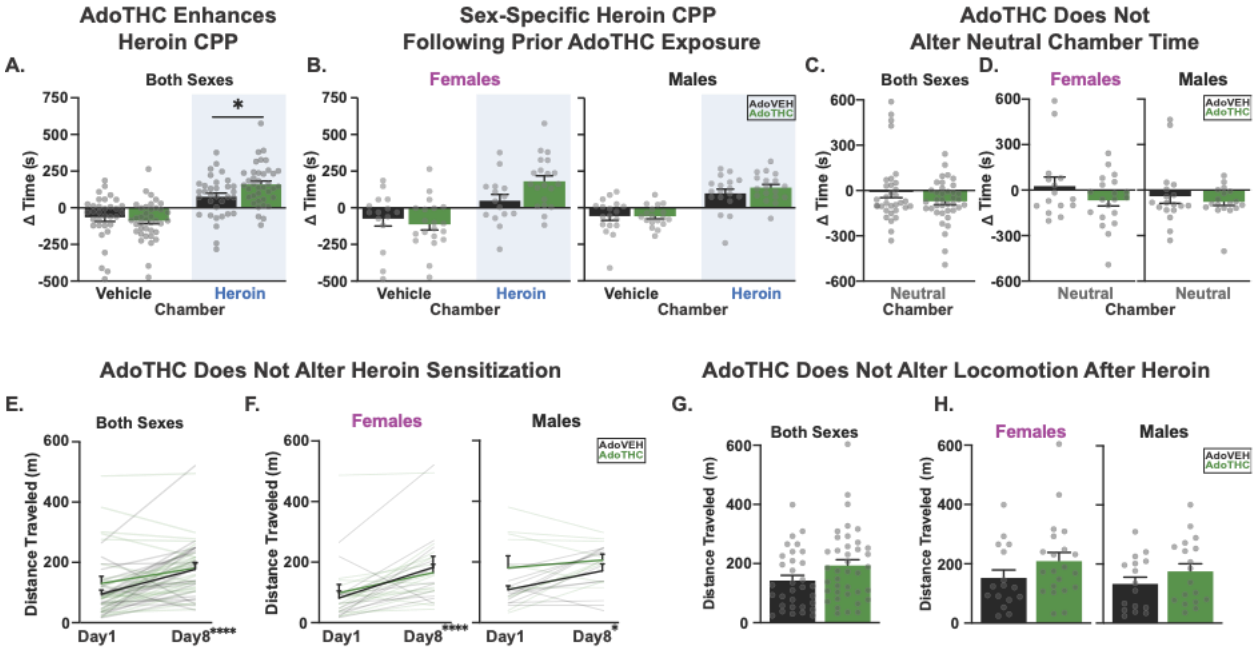


Figure 2.2. AdoTHC history alters heroin reward.
A) AdoTHC-exposed rats exhibited enhanced CPP for heroin (0.25 mg/kg) compared to AdoVEH (black) animals (both sexes combined; AdoTHC = green, AdoVEH = black). Data for the vehicle-paired, and heroin-paired chambers are shown as a change from time spent in the chamber on test, relative to baseline (Δ time). **B**) CPP data are shown for both sexes separately. **C**) AdoTHC had no effect on time spent in the unpaired neutral chamber of the CPP apparatus in all rats, or in **D**) either sex separately. **E**) AdoTHC-exposed rats displayed comparable locomotor sensitization to heroin, from the first to the 8th injection of heroin (1mg/kg, SC). **F**) Locomotor sensitization to heroin data is shown for both sexes separately AdoTHC/VEH females (left) and males (right). AdoTHC **G**) did not alter locomotion seen in the 1hr following a final injection of heroin (1mg/kg, SC), relative to AdoVEH counterparts, or **H**) in either sex separately. Data represent mean ± SEM. * p < 0.05, **** p < 0.0001.

AdoTHC History Augments Heroin Conditioned Place Preference. A modest CPP for this 0.25 mg/kg dose of heroin was seen in both females (main effect of heroin versus saline Side (change from baseline): $F_{(1,33)}$=18.24, p=0.0002), and in males ($F_{(1,34)}$=60.99, p<0.0001). AdoTX did not significantly augment this heroin CPP when either females (AdoTX x Side interaction: $F_{(1,33)}$=3.05, p=0.09; **Fig. 2.2B**) or males were analyzed separately (AdoTX x Side interaction: $F_{(1,34)}$=0.79, p=0.38), but when both sexes were combined for analysis, a significant augmentation of CPP by AdoTHC was detected (AdoTX x Side interaction: $F_{(1,67)}$=3.93, p=0.05; Sidak posthoc for AdoTX on heroin side: t_{138}=2.48, p=0.03; saline side: t_{138}=0.65, p=0.77; **Fig. 2.2A**). AdoTHC did not alter time spent in the unpaired center chamber in either sex, or when both sexes were combined (AdoTX effect on change from baseline time in center; females: t_{33}=1.38, p=0.18; males: t_{34}=0.70, p=0.49; both: $F_{(1,67)}$ =2.32, p=0.13; **Fig. 2.2C-D**).

AdoTHC History Does Not Alter Heroin Locomotor Sensitization. Both females and males showed heroin locomotor sensitization, in that locomotion after heroin was greater on the eighth, relative to the first injection (main effect of Day; females: $F_{(1,26)}$= 24.91, p<0.0001; males: $F_{(1,20)}$= 5.99, p=0.02; both sexes: $F_{(1,46)}$=26.71, p<0.0001; **Fig. 2.2F**). AdoTX did not affect heroin sensitization in females (no AdoTX x Day interaction: $F_{(1,26)}$=1.04, p=0.32), nor did it affect heroin-induced locomotion overall (main effect of AdoTX: $F_{(1,26)}$=0.0001, p=0.99), or after the initial injection (t_{26}=0.45, p = 0.66). In males, AdoTX also did not affect sensitization (no AdoTX x Day interaction: $F_{(1,20)}$ =1.06, p=0.32), and the trends toward AdoTHC males showing more locomotion than AdoVEH males on both tests (main effect of AdoTX: $F_{(1,20)}$ =3.22, p=0.09), and after the first injection ($t_{10.8}$=1.74, p=0.11) were not statistically significant. When both sexes were examined together, we again failed to find an effect of AdoTHC on sensitization (no AdoTX x Day interaction: $F_{(1,46)}$=2.07, p=0.16; **Fig. 2.2E**), locomotion across both tests ($F_{(1,46)}$=1.13, p=0.29), or locomotion after the first injection ($F_{(1,46)}$=2.67, p=0.11).

AdoTHC History Potentiates Remifentanil Self-Administration in Females. *Acquisition of Remifentanil Self-Administration:* Both females and males acquired remifentanil self-

administration over the first five FR1 training days, in that infusions [main effect of Day (1-5); females: $F_{(2.2,54.9)}$=12.58, p<0.0001; males: $F_{(2.3,56.6)}$=14.37, p<0.0001; both sexes: $F_{(2.3,113.2)}$=25.66, p<0.0001) and active lever presses (females: $F_{(2.0,48.8)}$=5.88, p=0.01; males: $F_{(2.5,61.7)}$=5.80, p=0.003; both sexes: $F_{(2.3,114.2)}$=11.42, p<0.0001) increased daily, and inactive lever presses decreased (females: $F_{(2.0,49.2)}$=10.40, p=0.0002; males: $F_{(2.6,65.8)}$=10.89, p<0.0001; both sexes: $F_{(2.9,144.2)}$=20.63, p<0.0001). In females, the number of remifentanil infusions self-administered over the 5 day acquisition training was significantly enhanced by AdoTHC (AdoTX

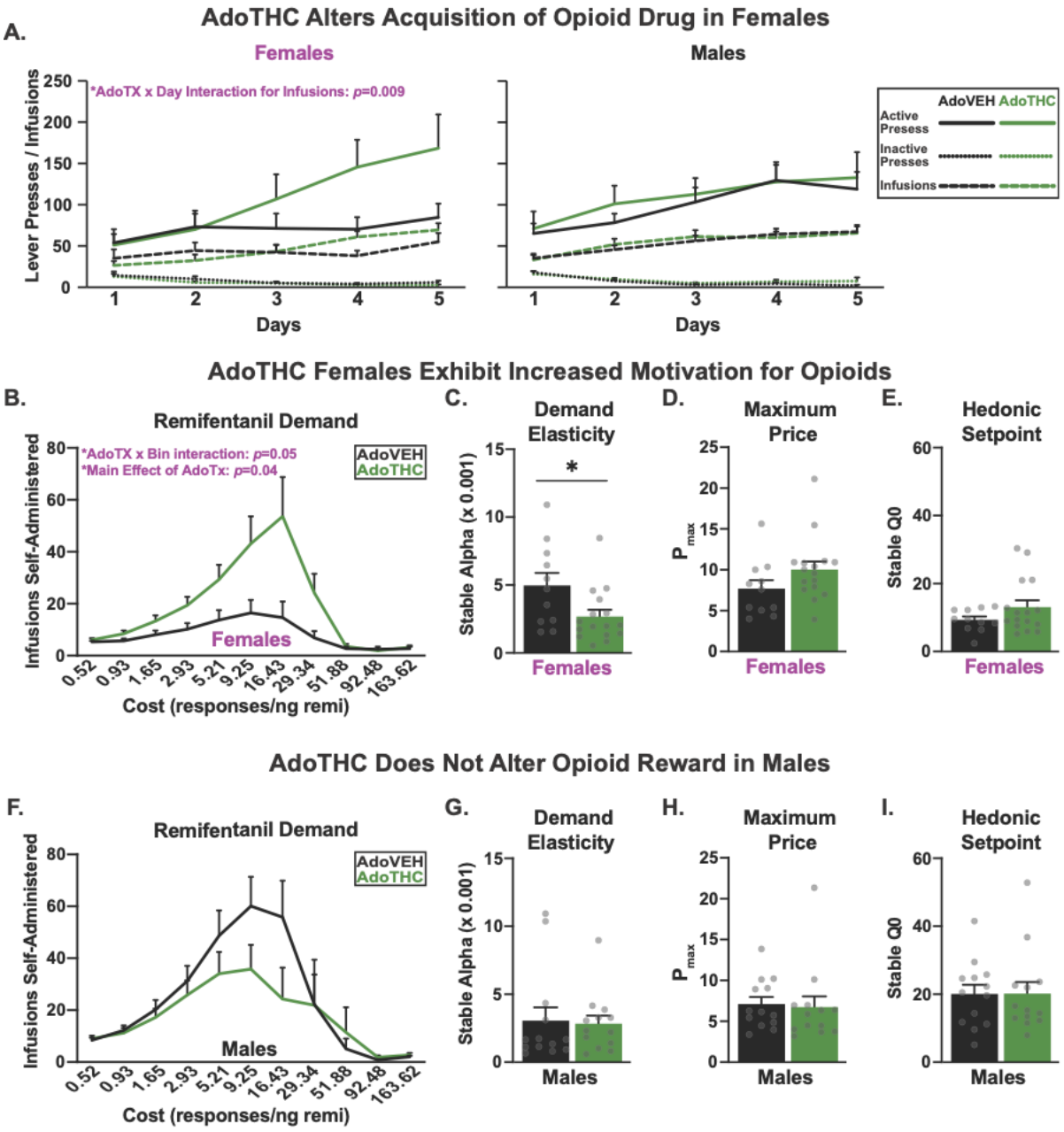


Figure 2.3. AdoTHC history in females enhances remifentanil reward.
A) AdoTHC (green) females, relative to AdoVEH (black) females (left) acquired self-administration of remifentanil on an FR1 schedule more rapidly, but this was not seen in males (right). Lines= active lever presses, dashed lines=infusions, dotted lines=inactive lever presses. **B**) In a behavioral economic demand test, the number of remifentanil infusions taken at each price bin is shown. AdoTHC females consumed more remifentanil at higher-effort bins than AdoVEH females, and they exhibited **C**) reduced demand elasticity (α, indicative of a heightened motivation for remifentanil), but no change in **D**) Pmax (the price bin in which maximal infusions were taken) or **E**) hedonic setpoint (Q_0, or preferred intake at zero cost). In males, no significant differences due to AdoTX were seen in **F**) remifentanil consumption at each price, **G**) demand elasticity, **H)** Pmax or **I)** hedonic setpoint. Data represent mean ± SEM. * $p < 0.05$

x

Day interaction: $F_{(2.2,54.9)}$=4.89, *p*=0.009; **Fig. 2.3A**), with a corresponding trend toward increased active lever presses ($F_{(2.0,48.8)}$=2.94, *p*=0.06), but no effect on inactive lever presses ($F_{(2.0,49.2)}$=0.45, *p*=0.64). In males, remifentanil acquisition was similar regardless of AdoTX AdoTX x Day interaction for infusions: $F_{(2.3,56.6)}$=0.48, *p*=0.64; active lever: $F_{(2.5,61.7)}$=0.19, *p*=0.87; inactive lever: $F_{(2.6,65.8)}$=0.61, *p*=0.59). AdoTX did not enhance acquisition when both sexes were analyzed together (no AdoTX x Day interaction for infusions: $F_{(2.3,113.2)}$=1.64, *p*=0.20; active lever: $F_{(2.3,114.2)}$=1.45, *p*=0.24; inactive lever: $F_{(2.9,144.2)}$=0.33, *p*=0.80). Furthermore, a significant Sex x AdoTX x Day interaction was observed for infusions ($F_{(2.3,113.2)}$=3.70, *p*=0.02), indicating that the effect of AdoTHC on acquisition was specific to females. No interactions involving Sex were seen for active or inactive lever pressing (Fs<2.05, *p*s>0.13).

Remifentanil Economic Demand Elasticity: Following acquisition of remifentanil self-administration, rats were trained on a behavioral economic demand elasticity task that allows estimation of both "hedonic setpoint" for remifentanil (represented by Q_0, an estimate of intake at zero cost; **Fig. 2.3E,I**), and motivation to exert increasing effort to obtain this desired amount of drug (represented by α, reflecting demand elasticity with increasing cost, and P_{max}, or the price bin at which the maximum number of infusions were taken in a session; **Fig. 2.3C,G,D,H**). In females, hedonic setpoint was unchanged by AdoTHC (Q_0; $t_{21.4}$=1.67, *p*=0.11; **Fig. 2.3E**), but demand elasticity was decreased (α; t_{25}=2.37, *p*=0.03; **Fig. 2.3C**). Accordingly, AdoTHC females pressed more in higher-effort than lower-effort bins (AdoTX x Bin: $F_{(1.3,33.2)}$=3.77, *p* =0.05; main effect of AdoTX: $F_{(1,25)}$=4.48, *p*=0.04; **Fig. 2.3B**), though P_{max} was not significantly altered by AdoTHC (t_{25}=1.59, *p*=0.13; **Fig. 2.3D**). No similar effects of AdoTHC on remifentanil demand were seen in males (Q_0: t_{24}=0.03, *p*=0.98; α: t_{24}=0.20, *p*=0.84; AdoTX x Bin: $F_{(2.0,48.9)}$=1.65, *p*=0.20; P_{max}: t_{24}=0.24, *p*=0.81; **Fig. 2.3F-I**). When both sexes were combined for analysis, AdoTHC again did not affect hedonic setpoint (no main effect of AdoTX on Q_0: $F_{(1,49)}$=0.59, *p*=0.44), and the decrease in α and P_{max} did not reach statistical significance (α: $F_{(1,49)}$=2.83, *p*=0.10; P_{max}: $F_{(1,49)}$ =0.82, *p*=0.37). No significant sex differences in AdoTHC's

effect on Q_0, α, or P_{max} were seen (no Sex x AdoTX interaction: Q_0: $F_{(1,49)}$=0.52, p=0.47; α: $F_{(1,49)}$=1.90, p=0.17; P_{max}: $F_{(1,49)}$=1.57, p=0.22), though we did find that AdoTHC increased intake selectively in high-effort bins to a greater extent in females than in males (AdoTX x Sex x Bin interaction: $F_{(2.1,103.1)}$=4.57, p=0.01).

AdoTHC History Does Not Alter Heroin Locomotion in Opioid-Experienced Rats. Following other behavioral tests, rats were given a final dose of heroin, and locomotion was measured for 1hr (**Fig. 2.2H**). AdoTHC did not alter locomotion in females (t_{35}=1.39, p=0.17), males (t_{32}=1.22, p=0.23), or when both sexes were analyzed together (main effect of AdoTX; $F_{(1,67)}$=3.36, p=0.07), and no sex difference was seen (no AdoTX x Sex interaction: $F_{(1,67)}$=0.07, p=0.79; **Fig. 2.2G**).

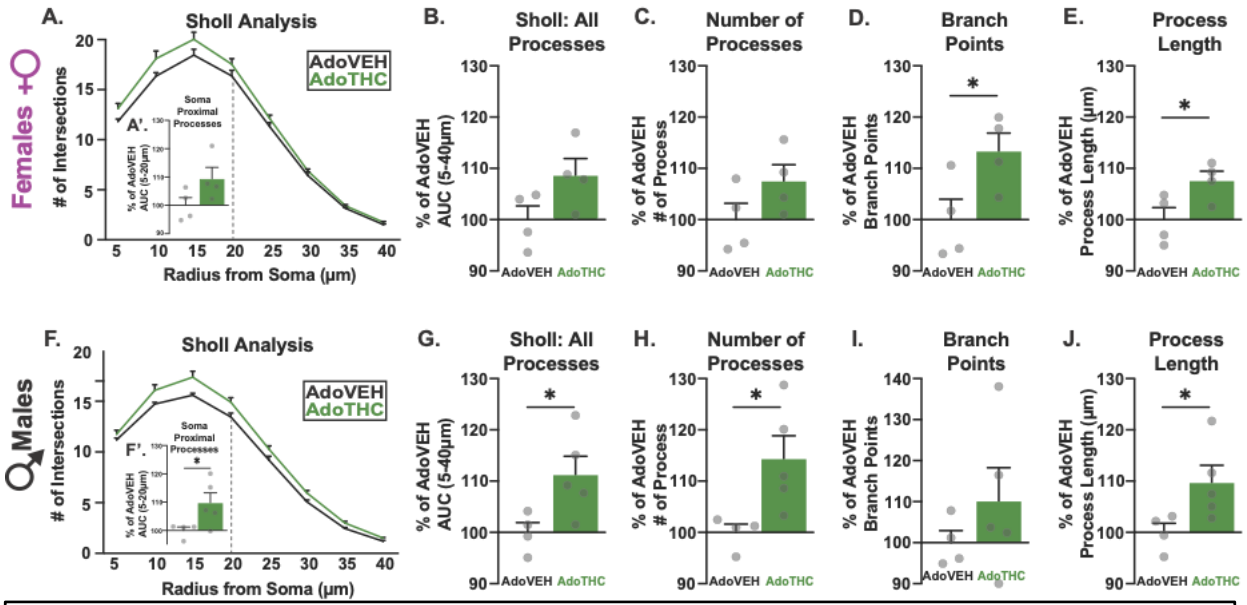


Figure 2.4. AdoTHC exposure alters microglial ramification.
Morphology of IBA1-visualized mPFC microglia was assessed 24hr after the 14th AdoTHC or AdoVEH injection (on PD44). In females (top row) and males (bottom row), a Sholl analysis was conducted to determine the number of microglial processes that intersected a series of concentric spheres centered on the soma of each quantified cell. **A,F**) The number of intersections processes made with spheres with the radii indicated on the x-axis are shown. Sholl analyses were quantified using area under the curve (AUC) for **A',F'**) processes proximate to cell soma (5-20μM, which were less subject to z-dimension truncation artifacts resulting from slice thickness), and for **B,G**) all visualized processes up to 40μM distal from soma. The **C,H**) number of processes, the **D,I**) number of branch points in them, and **E,J**) their length were also quantified. AdoTHC = green lines, AdoVEH = black. Data represent mean ± SEM, mean value for each individual rat is represented with grey circles. *p < 0.05

Effects of AdoTHC on mPFC Microglia Morphology. To explore the possibility that AdoTHC's persistent effects on behavior or neural activity might relate to THC-induced microglial changes that occur just after adolescent treatment (Lee et al., 2022), we quantified mPFC microglia number and morphology the day after the last AdoTHC/VEH injection, in a separate group of rats. In females, AdoTHC increased the average length of microglial processes (t_6=2.54, *p*=0.04; **Fig. 2.4E**). The complexity of these processes was also greater in AdoTHC females, in that the number of branch points was increased (t_6=2.51, *p*=0.05; **Fig. 2.4D**), though a Sholl analysis of these processes did not capture this increase (area under the curve: 5-20μm: t_6=1.93, *p*=0.10; 5-40μm: t_6=2.05, *p*=0.09; **Fig. 2.4A',B**). AdoTX did not affect the number (t_6=1.68, *p*=0.14; **Fig. 2.4C**) or volume of processes in females (t_6=1.87, *p*=0.11; data not shown), nor did it affect the number of microglia, or the volume or sphericity of their soma (ts_6 <0.86, all *ps*>0.42; data not shown). In males, AdoTHC also increased the length (t_7=2.38, *p*=0.05; **Fig. 2.4J**), number (t_7=2.70, *p*=0.03; **Fig. 2.4H**), and complexity of microglial processes (here determined by Sholl analysis AUC: 5-20μm: t_7=2.31, *p*=0.05; 5-40μm: t_7=2.55, *p*=0.04; **Fig. 2.4F',G**, but not number of branch points: t_7 = 1.06, *p*=0.32; **Fig. 2.4I**). AdoTX did not affect the volume of processes (t_7=1.54, *p*=0.17; data not shown), the number of microglia, or the volume and sphericity of their soma in males (ts_7<0.64, all *ps*>0.54; data not shown). When both sexes were combined for analysis, we confirmed that AdoTHC increased the complexity of processes (as determined by Sholl analysis AUC: 5-20μm: $F_{(1,13)}$=8.93, *p*=0.01; 5-40μm: $F_{(1,13)}$=10.49, *p*=0.007; branch points: $F_{(1,13)}$=4.27, *p*=0.06), as well as the number ($F_{(1,13)}$=9.63, *p*=0.008) and length ($F_{(1,13)}$=11.19, *p*=0.005) of processes, but not their volume ($F_{(1,13)}$=3.85, *p*=0.07). AdoTHC again did not affect the number of microglia, or the volume or sphericity of their soma (no main effect of AdoTX: all $Fs_{(1,13)}$<0.88, all *p*s>0.37). No significant sex differences were found on any of these measures (no AdoTX x Sex interaction: $Fs_{(1,13)}$<0.92, *p*s>0.35).

AdoTHC History Alters Neural Activity Seen After Heroin. *Region-Based Analyses:* We examined patterns of cFos expression in 13 brain regions following heroin injection in a subset of rats that had underwent all the above heroin and remifentanil behavioral tests. In females, differences in cFos expression were seen between AdoTHC and AdoVEH rats in several analyzed brain regions. AdoTHC females showed marked cFos increases in amygdala (main effect of AdoTX: $F_{(1,18)}$=15.07, *p*=0.001; CEA: t_{36}=3.35, *p*=0.004; BLA: t_{36}=3.29, *p*=0.005), with similar effects in CEA and BLA subregions (no AdoTX x Subregion interaction: $F_{(1,18)}$=0.004, *p*=0.95; **Fig. 2.5G**). In contrast, AdoTHC females instead showed less cFos in insula than AdoVEH females (t_{17}=2.86, *p*=0.01; **Fig. 2.5C**). No AdoTX effects were seen in females in frontal cortical regions (no main effect of AdoTX, or AdoTX x Subregion interactions: Fs<2.24, *p*s>0.15; **Fig. 2.5A-B,D**), accumbens (Fs<1.34, *p*s>0.26; **Fig. 2.5E**), lateral septum ($t_{9.4}$=0.71, *p*=0.50; **Fig. 2.5F**), PVT (t_{18}=0.26, *p*=0.80; **Fig. 2.5H**), or PPTg ($t_{16.1}$=1.98, *p*=0.07; **Fig. 2.5I**). In males, AdoTHC altered post-heroin brain activity primarily in cortical regions. Specifically, AdoTHC males had more cFos than AdoVEH males in mPFC ($F_{(1,31)}$=4.60, *p*=0.05; **Fig. 2.5J**), with similar patterns in PLC and ILC subregions (no AdoTX x mPFC Region interaction: $F_{(1,13)}$=0.06, *p*=0.81), though posthoc analyses of each mPFC subregion individually were not significant (PLC: t_{26}=1.90, *p*=0.13; ILC: t_{26}=2.08, *p*=0.09). AdoTHC males also had more cFos in insula (t_{13}=2.19, *p*=0.05; **Fig. 2.5L**), but OFC ($F_{(1,13)}$=1.35, *p*=0.27; **Fig. 2.5K**) and claustrum (t_{13}=1.25, *p*=0.23, **Fig. 2.5M)** were unaffected. No AdoTX differences were seen in amygdala ($F_{(1,13)}$=0.32, *p*=0.58; **Fig. 2.5P**), accumbens ($F_{(1,13)}$=0.001, *p*=0.97; **Fig. 2.5N**), lateral septum (t_{13}=0.71, *p*=0.49; **Fig. 2.5O**), PVT (t_{13}=1.22, *p*=0.25, **Fig. 2.5Q**), or PPTg (t_{12}=0.28, *p*=0.78, **Fig. 2.5R**). When regional cFos was examined with both sexes combined, no main effects of AdoTX reached significance ($Fs_{(1,31)}$<3.31, all *ps*>0.08), and sex differences in AdoTX cFos effects were statistically confirmed in insula (Sex x AdoTX: $F_{(1, 30)}$=12.59, *p*=0.001), claustrum (Sex x AdoTX: $F_{(1,30)}$=4.24, *p*=0.05), and mPFC (Sex x AdoTX: $F_{(1,30)}$=6.52, *p*=0.02). No other significant sex differences were found ($Fs_{(1,31)}$<3.77, all *ps*>0.06).

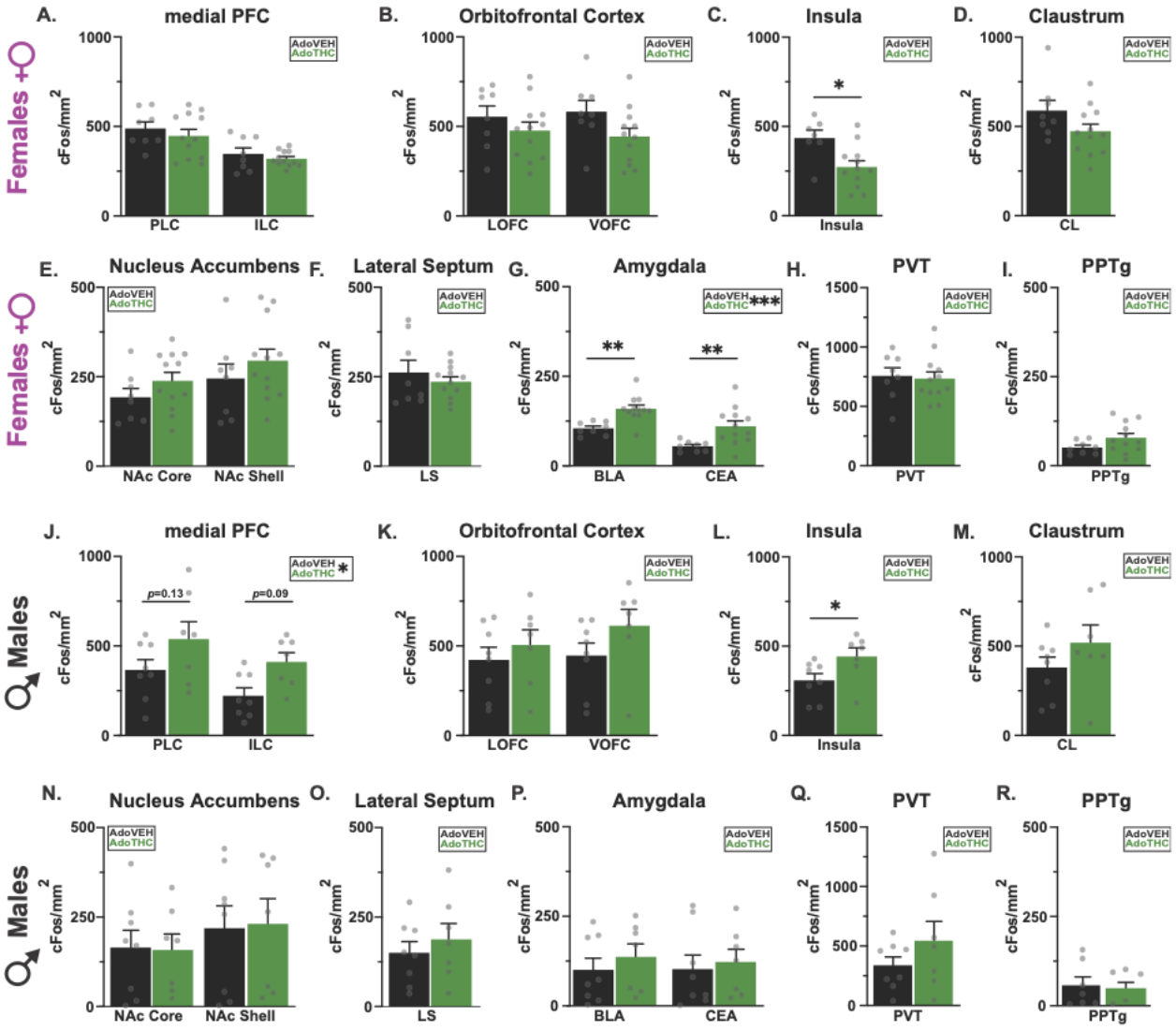


Figure 2.5. AdoTX effects on regional cFos expression seen after a final heroin injection.

Density of cFos (cFos+ cells per mm2) is shown for females (**A-I**), and males (**J-R**) in each measured region. **A,J**) medial prefrontal cortex (containing prelimbic cortex: PLC, and infralimbic cortex: ILC), **B,K**) orbitofrontal cortex (containing lateral and ventral orbitofrontal cortex: L/VOFC), **C,L**) insula, **D,M**) claustrum, **E,N**) nucleus accumbens (containing nucleus accumbens core: NAc Core, Shell), **F,O**) lateral septum (LS), **G,P**) amygdala (containing basolateral: BLA and central amygdala: CEA), **H,Q**) paraventricular thalamus (PVT), and **I,R**) pedunculopontine tegmental nucleus (PPTg). Bars represent group mean ± SEM. AdoVEH = black, AdoTHC = green. Mean cFos for each individual rat is represented with grey circles * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Asterix next to AdoTX condition in legend indicates main effect of AdoTX across both subregions, asterix above bars indicates AdoTX effect in the subregion in posthoc analysis.

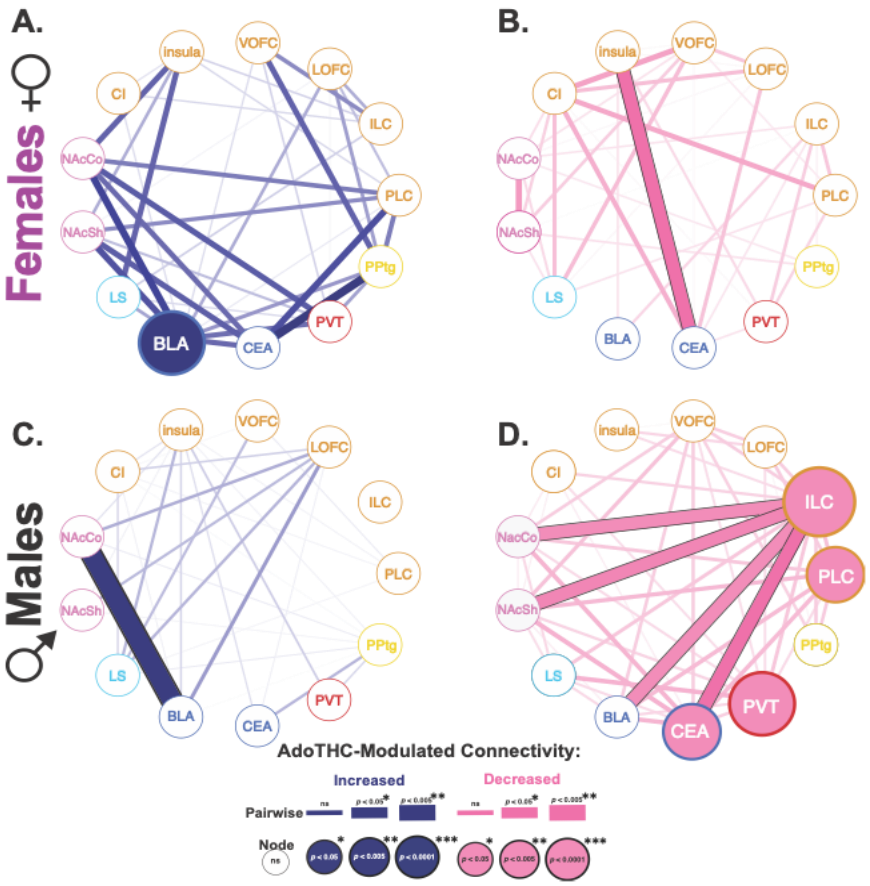


Figure 2.6. Impacts of AdoTHC on functional connectivity of regional nodes within wider brain networks in each sex.
Differences in correlated activity (cFos) across brain regions in AdoTHC rats, relative to AdoVEH rats of the same sex are represented. The top row (A-B) represents effects of AdoTHC in females, and the bottom row (C-D) in males. A) increases in co-activation between two individual regions (ΔZ = Fisher Z-transformed correlation in AdoTHC minus AdoVEH) are represented with lines connecting regions, scaled based on the magnitude of the increase (positive ΔZ; significantly altered connections are represented with bolded lines, p values for these changes are defined in the legend at bottom). Brain regions that showed increases in functional connectivity with the entire network (i.e. node-level changes; Welch's t-test average of one region with all its connections to other regions between AdoVEH and AdoTHC) are represented by blue color, and size of the circle representing the region, with p values for these changes defined in the legend at bottom. B) Using the same symbol logic, decreases in functional connectivity between two individual nodes (negative ΔZ; pink lines), and for node-level connectivity with the entirety of the network (pink circles) are likewise shown for females. Using the same symbol logic to show data for males, D) increases (blue) and E) decreases (pink) in functional connectivity due to AdoTHC, relative to AdoVEH, are shown. PLC=prelimbic cortex, ILC=infralimbic cortex, LOFC=lateral orbitofrontal cortex, VOFC=ventral orbitofrontal cortex, insula=anterior agranular insular cortex, CI=claustrum, NAcCo=nucleus accumbens core, NAcSh=nucleus accumbens shell, LS=lateral septum, BLA=basolateral amygdala CEA=central amygdala, PVT=paraventricular nucleus of the thalamus, PPTg= pedunculopontine tegmental nucleus.

Neural Network-Based Analyses: Since behavior-relevant effects of AdoTHC on brain responses to heroin presumably depend upon changes in neural networks rather than individual brain regions, we next analyzed regional cFos data based on differential patterns of correlated activity, or "functional connectivity," seen between AdoTHC and AdoVEH rats of either sex amongst the 13 quantified brain regions.

In females, AdoTHC increased the overall coupling of BLA with the entirety of the quantified network relative to AdoVEH ($t_{18.1}$=3.20, ΔZ=0.28, *p*=0.005; **Fig. 2.6A**), indicating that the BLA became more broadly coupled with other network nodes following AdoTHC exposure. This increased BLA network coupling was not accounted for by increases in any single pairwise connection (all *ps*>0.18), suggesting a distributed increase in BLA connectivity across multiple regions, rather than increased coupling between individual pairs of regions. In contrast, AdoTHC decreased functional connectivity of insula with CEA in females (ΔZ=-1.21, *p*=0.03), but other pairwise region-region comparisons showed only modest AdoTX effects that did not reach significance (e.g., CEA-PPTg: ΔZ=0.91, *p*=0.10; NacCo-NAcSh: ΔZ=-0.85, *p*=0.13; other comparisons: *ps*>0.17).

In males, AdoTHC decreased coupling of multiple regional nodes with the entirety of the wider network, relative to AdoVEH (**Fig. 2.6C**). ILC showed the most dramatic reduction after AdoTHC ($t_{16.8}$=-5.23, ΔZ=-0.92, *p*<0.0001), followed by PVT ($t_{18.6}$=-3.46, ΔZ=-0.38, *p*=0.003), CEA ($t_{17.2}$=-2.41, ΔZ=-0.51, *p*=0.03), and PLC ($t_{18.9}$=-2.37, ΔZ=-0.43, *p*=0.03). In pairwise region-region connectivity analyses, AdoTHC males showed decreased ILC coupling with CEA (ΔZ=-1.79, *p*=0.008), NacSh (ΔZ=-1.52, *p*=0.02), NacCo (ΔZ=-1.43, *p*=0.03), and BLA (ΔZ=-1.43, *p*=0 .03). Though PVT and PLC nodes showed significant decoupling with the wider network after AdoTHC, no pairwise comparisons of these regions with other individual nodes were significantly altered (PVT: all *ps*>0.17; PLC: all *ps*>0.22). A selective increase in functional connectivity in AdoTHC, relative to AdoVEH males was also observed between NAcCo and BLA

(ΔZ=2.14, p=0.001). No other region-region coupling changes were statistically significant (ps>0.17).

Sex differences in the effects of AdoTHC on connectivity of nodes with the rest of the network (Sex x AdoTX interactions) were found for four regions: ILC ($F_{(1,44)}$=19.27, p<0.0001), PVT ($F_{(1,44)}$=9.82, p=0.003), PLC ($F_{(1,44)}$=5.28, p=0.03), and CEA ($F_{(1,44)}$=4.56, p=0.04), confirming that AdoTHC differentially affected network interactions of these regions in males and females. These sex-dependent effects of AdoTHC on network nodes were not recapitulated in analyses of coupling between pairs of individual regions (ps>0.06).

Discussion

Here we show that a well-characterized, translationally-relevant AdoTHC exposure protocol (Torrens et al., 2020; Ruiz et al., 2021a; Torrens et al., 2022) persistently alters opioid reward in rats, with an especially pronounced pro-opioid phenotype in females. AdoTHC augmented heroin CPP without affecting heroin sensitization or locomotion, and AdoTHC females, but not males, acquired remifentanil self-administration faster, and worked harder for self-administered remifentanil than their AdoVEH counterparts. To generate hypotheses about the potential mechanisms by which AdoTHC alters neurodevelopment to achieve these effects, we also examined microglial morphology shortly after AdoTHC treatment, finding increased complexity of microglial processes that were present in both sexes. Finally, following behavioral testing we administered heroin to all rats once more, and examined neural activity (cFos expression) in 13 brain regions relevant to opioid addiction. We found notably distinct patterns of brain activity in AdoTHC and AdoVEH rats, both in individual brain regions, and in the coordination of activity between multiple regions within the wider network. Notably, AdoTHC effects on neural activity was markedly sex-dependent; with AdoVEH-relative changes centered on amygdala in females, but on frontal cortex in males. In sum, these results confirm prior reports that chronic AdoTHC causes long-lasting changes in opioid reward, and extends them by showing some of these effects are stronger in females than males, and by pointing to novel

short-term (microglial changes) and long-term (neural network response to heroin) mechanisms that may be related to this “biological gateway effect” of AdoTHC.

Adolescent THC Exposure Persistently Enhances Specific Aspects of Opioid Reward. There have been several prior reports that AdoTHC enhances opioid reward later in life, and this effect is observed across AdoTHC protocols, experimental species and strains, and research groups. Here we replicated this basic finding with our well-characterized, moderate-dose, translationally-relevant chronic AdoTHC protocol in Long-Evans rats, using multiple behavioral assays, and two classes of rewarding opioid drugs.

Rats and mice, like humans (Childs and de Wit, 2009, 2016; Palmisano et al., 2018), readily learn to associate rewarding drug effects with the environments in which they experience drug effects, so the formation of drug CPP is a commonly-used assay of the rewarding effects of abused drugs like heroin. We found that relative to AdoVEH controls, AdoTHC rats showed stronger CPP for a low-dose (0.25mg/kg) of heroin. The effect was modest in magnitude, and though it appeared to be more robust in females than males, the sample sizes used did not allow us to demonstrate a sex difference statistically, or even to show a significant enhancement in either sex examined independently—only when both sexes were combined for analysis was the AdoTHC augmentation apparent. Regardless, the overall finding aligns with a prior report in male rats, which reported that a different AdoTHC exposure protocol (twice daily, escalating-dose THC injections from PD43-45) altered adulthood CPP formed for a higher (0.5mg/kg) dose of heroin (Cadoni et al., 2015). Interestingly, that effect was seen only in “addiction-resistant” Fischer 344, but not “addiction-prone” Lewis rats, which differ in drug reward sensitivity, and also in HPA response to stress, and mesocorticolimbic dopamine system activity and response to addictive drugs (Cadoni, 2016). AdoTHC also enhanced the persistence of heroin CPP in Fischer 344 rats in the face of extinction training, but it did not affect the ability of a heroin priming injection to reinstate CPP. In contrast, neither initial heroin CPP nor extinction were altered in Lewis rats, but they did show augmented heroin-primed reinstatement of CPP

following extinction. This suggests that AdoTHC interacts with genetics to influence the specific behavioral effects induced, but also that AdoTHC has broadly pro-opioid effects across strains selectively bred to vary in their “addiction susceptibility.” Likewise, another study showed that AdoTHC enhanced morphine-primed (5mg/kg) reinstatement of high-dose (10mg/kg) morphine CPP in female and male C57BL/6J mice without affecting initial CPP or extinction, the same pattern as seen with heroin in male Lewis rats (Hubbard et al., 2024). Coupled with the present finding in outbred Long-Evans rats of enhanced low-dose (0.25mg/kg) heroin CPP after AdoTHC (we did not test extinction or reinstatement here), a clear pattern of AdoTHC-enhancement of the rewarding and motivation-enhancing effects of opioid drugs begins to emerge.

In addition to causing CPP, most rewarding drugs induce locomotor sensitization, or an enhanced locomotor response to drugs like heroin after repeated administration. Locomotor sensitization has been argued to be a proxy for the incentive motivational “wanting” of rewards, which become increasingly strong as repeated drug use transitions into addiction (Kalivas and Stewart, 1991; Robinson and Berridge, 1993; Robinson and Berridge, 2001, 2004; Vanderschuren and Pierce, 2010; Stewart and Vezina, 2024). Therefore we asked whether AdoTHC alters the locomotor sensitizing effects of heroin (1mg/kg). Although the procedure we employed induced robust sensitization in both male and female rats, we found no clear differences between AdoTHC and AdoVEH rats in locomotion after either initial, or repeated heroin injections, nor did we see changes in locomotion after the same dose of heroin weeks later following additional opioid exposure, namely 14d of remifentanil self-administration. These results replicate a similar negative finding in male and female mice using the same AdoTX protocol employed here, which showed no clear effect of AdoTHC on locomotor activity after morphine (Hubbard et al., 2024). This finding suggests that, at least under the experimental conditions and heroin doses tested here, AdoTHC does not alter the locomotor-activating effects of heroin in the same manner that it enhances the drug’s ability to induce CPP.

It has also been previously reported that AdoTHC persistently augments measures of self-administration of opioids in rats, and our results confirm and extend these findings. In male rats, prior reports showed that AdoTHC enhances acquisition of heroin self-administration as well as stable self-administration of the drug following acquisition, and that this enhancement is observable across a range of fixed-ratio operant reinforcement schedules in outbred rat strains (Solinas et al., 2004; Ellgren et al., 2007; Tomasiewicz et al., 2012; Ferland et al., 2023b). Likewise, when AdoTHC effects on heroin self-administration was tested in "addiction-prone" Fisher 344, or "addiction-resistant" Lewis rat strains (Lecca et al., 2020), an increase in motivated seeking under a progressive-ratio schedule, as well as enhanced heroin-primed reinstatement of extinguished heroin seeking was found in Lewis rats only. In contrast, a selective increase in cue-induced reinstatement of heroin seeking was seen in Fischer 344 rats only. Another study also showed (using a chronic inhaled vaping model), that AdoTHC aerosol increased fentanyl self-administration in female, but not male rats (Nguyen et al., 2020).

Here, we extended these prior findings to show that AdoTHC also augments the acquisition of, and motivated responding for the short-acting, but highly-reinforcing opioid drug remifentanil. These experiments further support the notion that AdoTHC preferentially enhances effortful seeking of remifentanil (i.e. intake when many lever presses are required to achieve desired intoxicating effects), without clear effects of AdoTHC on intake under lower-effort conditions (i.e. pressing for the drug when it was "cheap"). Some of these AdoTHC effects appeared to be more robust in females than males; for example there was a robust sex-difference in responding across effort bins in the economic demand task. This experiment indicates a previously-unknown selectivity in the effects of AdoTHC to enhance motivated seeking of opioids, rather than opioid "hedonic setpoint."

Overall, the present results provide additional, converging evidence supporting the conclusion that AdoTHC affects the developing adolescent brain in a manner that yields a robust, reproducible pro-opioid phenotype that manifests across behavioral paradigms, classes

of opioid drugs, and types of rodents. This seems to carry clear implications for the much discussed “gateway effect” of early cannabis use to facilitate use and abuse of harder drugs like opioids when they are subsequently encountered later in life.

AdoTHC Exposure Acutely Alters Microglial Morphology in Both Sexes. AdoTHC Exposure Acutely Alters Microglial Morphology in Both Sexes. The ability of AdoTHC to augment specific aspects of opioid reward is clear, but the mechanisms by which THC acts in the developing brain to achieve this are less so. One intriguing possibility is that direct or indirect actions of THC upon microglia are involved. Microglia are the brain’s resident immune cells, but they also play key roles in sculpting neural circuits during development (Frost and Schafer, 2016; Dziabis and Bilbo, 2021; Guedes et al., 2022) including adolescent development (Mallya et al., 2019; Dziabis and Bilbo, 2021; Schalbetter et al., 2022; von Arx et al., 2023; Stowell and Wang, 2025). A prior report from our group showed that our AdoTHC protocol in male mice increased ramification of microglia processes in the hippocampus of male mice (Lee et al., 2022), and we broadly replicated this finding here in mPFC of male and female rats, with clear increases in the length and complexity of processes after AdoTHC. This ramified morphology is classically associated with a surveillant, non-activated microglial state (Nimmerjahn et al., 2005; Wake et al., 2009; Tremblay et al., 2010; Vidal-Itriago et al., 2022), and the Lee et al report (Lee et al., 2022) accordingly interpreted enhanced ramification in relation to the persistently attenuated inflammatory response of microglia to homeostatic challenges from social stress, or bacterial insult with lipopolysaccharide they also observed after AdoTHC.

Since microglia are also involved in homeostatic responses to opioid drugs (Lacagnina et al., 2017; Linker et al., 2019; Green et al., 2022), and since they may importantly influence development of addiction-relevant neural circuits (Martinez and Mahler, 2025), it is possible that the microglial changes seen here shortly after AdoTHC also set the stage for neurodevelopmental changes ultimately resulting in the pro-opioid phenotypes we and others find after AdoTHC. This said, we did not ask here whether AdoTHC alters adulthood responses

of microglia to opioids, nor did we test whether the short-term changes in microglia are responsible for the later-life pro-opioid phenotype—these would be fascinating questions to ask in future studies.

It is also important to note that microglial hyper-ramification does not necessarily indicate hypoactive response to a challenge—for example ramification of mPFC microglial processes also results from stressors like acute traumatic shock, and chronic restraint or forced swimming stressors (Hinwood et al., 2012; Hinwood et al., 2013; Hellwig et al., 2016; Smith et al., 2019). Furthermore, AdoTHC can also induce a very different, “activated” phenotype in PFC microglia, characterized by enlarged soma and reduced process complexity (Zamberletti et al., 2015; Gabaglio et al., 2021; Freels et al., 2024)—neither of which we saw here. This discrepancy may be due to the different AdoTHC protocols tested across these studies (twice-daily, escalating-dose THC injections from PD35 to PD45 in (Zamberletti et al., 2015; Gabaglio et al., 2021), or self-administered cannabis extract vapor exposure from PD35-55 in (Freels et al., 2024)), or to the timepoint at which microglia were examined (the prior studies looked at microglia in adulthood long after AdoTHC, while here we examined morphology shortly after AdoTX). Further work will be required to understand the variables affecting how AdoTHC impacts microglia, and how such changes relate to the persistent effects of AdoTHC on opioid addiction vulnerability.

Impacts of AdoTHC on Neural Network Activity Seen After Heroin in Opioid-Experienced Adults. We also sought to explore the impacts of AdoTHC on neural activity in adults that had experienced rewarding opioid effects that yielded a pro-opioid phenotype. To do so, we examined cFos in 13 brain regions of behaviorally-tested male and female rats given a final injection of heroin after other opioid behavioral testing, and determined how AdoTX impacted regional neural activity, and activity within wider brain reward networks in which these regions function as nodes.

In Females, AdoTHC Preferentially Altered Activity in Amygdala and Associated Networks. In females, the most notable effect of AdoTX on regional brain activity seen after heroin was in amygdala, where activity was far greater in both BLA and CEA of AdoTHC, relative to AdoVEH females. Amygdala is best known for its role in aversive learning (Fanselow and LeDoux, 1999; Maren, 2001; McGaugh, 2004; Phelps and LeDoux, 2005; LeDoux, 2007; Janak and Tye, 2015), but is also key for addiction-relevant processes like salience attribution to motivationally-relevant learned and unlearned stimuli (Baxter and Murray, 2002; Wassum and Izquierdo, 2015; Smith and Torregrossa, 2021). Opioids in amygdala are particularly important for reward, as μ opioid stimulation there potently enhances food intake and pursuit (Kim et al., 2004; Wassum et al., 2011; Smith et al., 2016; Wassum et al., 2016), as well as cue-triggered reward seeking and incentive salience (Mahler and Berridge, 2009; DiFeliceantonio and Berridge, 2012; Mahler and Berridge, 2012; Lichtenberg and Wassum, 2017). Morphine is self-administered by rats directly into amygdala (David and Cazala, 1994), and chronic opioid intake also induces neuroplasticity in amygdala opioid signaling that may contribute to either the rewarding effects of opioid drugs, or the affective dysregulation that emerges after chronic opioid drug use (Brady et al., 1989; Sim-Selley et al., 2000; Befort et al., 2008; Koob, 2009, 2020; Kaplan and Thompson, 2023). It is not presently clear how the present results relate to the actions of heroin or remifentanil directly upon opioid signaling systems in amygdala, or how AdoTHC might alter these to generate the pro-opioid effects seen here.

In addition to changes in amygdala, AdoTHC females also showed reduced activity in insular cortex after heroin, relative to AdoVEH females. Insula has direct, reciprocal connections with amygdala (Nieuwenhuys, 2012; Gogolla, 2017), and it is thought to play a role in integrating interoceptive signals with emotional and motivational information (Craig, 2002, 2009; Craig, 2010). Insula may also be particularly important for drug addiction (Naqvi et al., 2007; Naqvi and Bechara, 2010; McGregor and LaLumiere, 2023), including for behaviors relevant to opioid addiction (Zhang et al., 2019; Joshi et al., 2020; Sun et al., 2020; McGregor et al., 2024; Clarke

et al., 2026). For example, reversibly inactivating insula disrupts expression of morphine CPP (Sun et al., 2020) and cue-induced reinstatement of heroin seeking (McGregor et al., 2024), and insula projections to NacCo are necessary for reinstatement of heroin seeking following heroin self-administration (Clarke et al., 2026) and reinstatement of morphine CPP (Zhang et al., 2019). Furthermore, insula lesions differentially affect heroin self-administration and reinstatement in heroin-experienced, relative to heroin-naïve rats (Joshi et al., 2020), suggesting a role in addiction-relevant plasticity. These findings suggest that the diminished insula activity seen in females after heroin in AdoTHC rats here might relate to the pro-opioid phenomenon under investigation here, but this is a possibility that also requires further investigation.

We also found that the role of amygdala nuclei within wider networks appeared to be reorganized by AdoTHC in females. In an analysis of node-level connectivity with the entirety of the quantified network, BLA stood out has having increased network connectivity in AdoTHC females. This effect was not dominated by increased connectivity with any other specific node, suggesting widespread enhancement of the integration of BLA with the entirety of the addiction-relevant network after AdoTHC. CEA also appeared to be altered in its connectivity with other network nodes, though this effect was in the opposite direction to BLA, and it involved a specific decrease in coordinated activity between CEA and insula. The specific implication of this difference in network-level interactions between BLA and CEA is unclear, but it fits within a broader conceptualization of these amygdala subregions having distinct functions in addiction-relevant behaviors (Baxter and Murray, 2002; LeDoux, 2003, 2007; Murray, 2007), including the generation of reward-seeking behaviors elicited by direct amygdala opioid receptor stimulation (Kim et al., 2004; Mahler and Berridge, 2009; Wassum et al., 2011; DiFeliceantonio and Berridge, 2012; Mahler and Berridge, 2012; Smith et al., 2016; Lichtenberg and Wassum, 2017). Furthermore, this finding of qualitatively distinct effects of AdoTHC on BLA versus CEA also demonstrates that such network analyses can uncover hidden changes that cannot be

discovered using regional analysis of cFos alone, where we found that both regions were more active in AdoTHC, relative to AdoVEH females.

In Males, AdoTHC Preferentially Potentiates Prefrontal Cortex and Associated Networks. The impacts of AdoTHC on cFos expression patterns seen after heroin were dominated by markedly increased activity in prefrontal cortical regions, with region-level cFos increases seen in ILC, PLC, and insula of AdoTHC, relative to AdoVEH males. Prefrontal cortex in general plays a key role in addiction susceptibility and addiction-associated plasticity, especially with regard to the impaired decision making, impulsivity, and altered attentional processes that characterize many with the disorder. Therefore, it is intriguing that following heroin injection in opioid-experienced males we found enhanced, rather than suppressed activity in these cortical regions. Notably, a similar potentiation of morphine-induced PFC cFos was also seen in male AdoTHC mice (Hubbard et al., 2024). Since adolescence is a key period for PFC development, it is not surprising that AdoTHC causes major changes there, and this phenomenon has been widely demonstrated in prior reports (Renard et al., 2018; Fischer et al., 2020; Scheyer et al., 2023). We found it surprising that similar effects of AdoTHC were found in PLC, ILC, and insula activity after heroin, as these cortical areas have distinct behavioral functions and anatomical connectivity (Vertes, 2004; Gogolla, 2017; Howland et al., 2022), though all have been reported to be affected by adolescent cannabinoid exposure (Lopez-Larson et al., 2011; Hurd, 2025; Nosko et al., 2025).

At the level of neural networks, AdoTHC potentiation of PFC activity corresponded with a global reduction in connectivity of these regions with the wider network, especially in mPFC. For ILC, this was dominated by decoupling of the region with amygdala (both BLA and CEA), as well as nucleus accumbens core and shell. ILC projects directly to both accumbens and amygdala, and there is an important reciprocal projection back from amygdala to ILC as well (Hurley et al., 1991; Vertes, 2004; Floresco, 2015; Yizhar and Klavir, 2018; Howland et al., 2022). These connections play key roles in numerous aspects of reward learning and seeking,

including seeking of opioid drugs (Cardinal et al., 2003; Ceceli et al., 2022; Howland et al., 2022). For PLC, it appeared that AdoTHC-induced network decoupling may have been less specific to any other specific region, as we saw a reduction there at the level of PLC as a network node, but no changes in its functional connectivity with any other specific region. We also found that AdoTHC suppressed node-level CEA functional connectivity with the network, which likely reflects, at least in part, its decoupling with ILC in particular. Likewise, PVT network connectivity was also suppressed in AdoTHC males. PVT directly projects to mPFC, nucleus accumbens, and amygdala, and it importantly mediates both appetitive and aversively motivated behaviors (Kirouac, 2015; Millan et al., 2017; Choi et al., 2019; Barson et al., 2020; Penzo and Gao, 2021), including those relevant to opioid addiction (Zhu et al., 2016; Keyes et al., 2020; Vollmer et al., 2022; Paniccia et al., 2024).

In addition to the network-level decoupling of these regions caused by AdoTHC, we also found that in the same rats NAcCo and BLA became more coordinated in their post-heroin activity. This pair of regions are directly connected, and this connectivity is well-known to be important for reward seeking, including for opioid rewards (Cardinal et al., 2003; Ambroggi et al., 2008; Stuber et al., 2011; Floresco, 2015; Wassum and Izquierdo, 2015; Keefer et al., 2021). One possibility is that these effects reflect a shift in the balance of cortical, reward-inhibitory processes (such as those subserved by regions like ILC and insula), which are suppressed by AdoTHC, in favor of motivation-promoting circuits like BLA-NAc, which are instead augmented. This said, the fact that these changes occur in males, in which AdoTHC had relatively modest pro-opioid behavioral effects, challenges this hypothesis. Clearly, the present findings pose more questions than are answered by them, and we hope that in pursuing some of these may focus ongoing and future research aimed at understanding how AdoTHC alters neural networks relevant to opioid addiction.

A key takeaway of the present neural activity analyses is that AdoTHC effects on post-heroin brain activity is markedly sex-dependent. These changes occur within individual brain

regions, but involve also altered activity across wider networks in the brain. A key outstanding question regards the behavioral significance of these changes, as we did not directly test here whether AdoTHC effects on brain activity underlie any of the behavioral changes we also observed after AdoTHC. It is also presently unclear how cFos findings relate to functionally-distinct subpopulations of neurons, such as inhibitory versus excitatory cortical cells that are differentially impacted by AdoTHC (Renard et al., 2017b; Renard et al., 2018). Another limitation is that we did not establish whether the neural activity observed here was specifically induced by heroin, or whether it depended upon prior opioid exposure, as we did not examine saline-injected AdoTHC/VEH rats, or rats without extensive prior opioid drug exposure in these experiments.

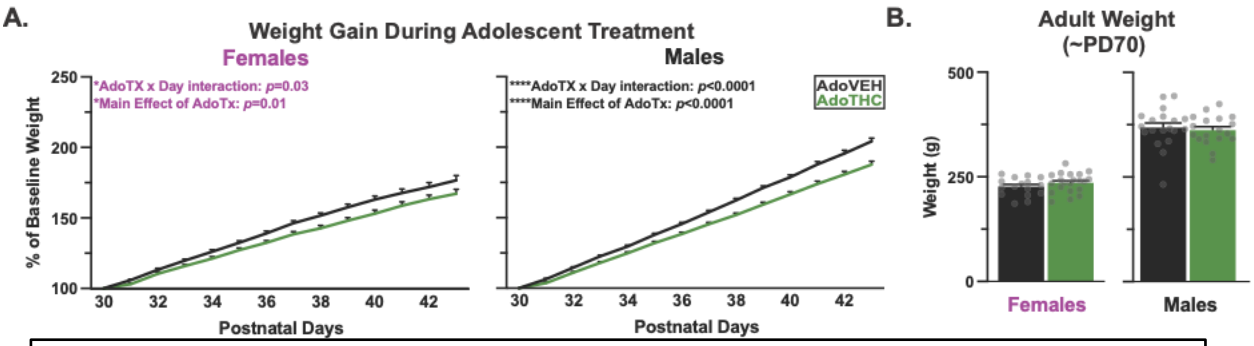
Another key outstanding question involves the mechanisms that mediate AdoTHC's ability to alter neural activity seen later in life. We propose that part of the answer may involve AdoTHC effects on microglia, but other changes are also possible. For example, the disruption by AdoTHC of normal functioning of endocannabinoid signaling, which undergoes dynamic developmental changes during adolescence (Ellgren et al., 2008; Lee et al., 2013), is a likely candidate for involvement (Ellgren et al., 2007; Rubino et al., 2008; Rubino and Parolaro, 2011; Rubino et al., 2012; Tomasiewicz et al., 2012; Ferland et al., 2023a; Ferland et al., 2023b). It would be interesting to determine whether opioid receptor distribution, localization, or function was altered by AdoTHC in a manner that related to neural activity elicited by heroin. Ultimately, however, the present cFos results are preliminary, and there are many key questions it raises that are currently unanswered.

Additional Limitations of the Present Findings. There are also other limitations of the present report that should be considered. We examined a specific model of AdoTHC exposure (Ruiz et al., 2021a; Torrens et al., 2022; Lin et al., 2023b) and although it is well-characterized, and was designed with translational-relevance in mind (Huestis et al., 1992; Cooper and Haney, 2009), its generalizability is not guaranteed. Indeed, persistent AdoTHC effects vary as a

function of dose (Amal et al., 2010; Irimia et al., 2015; Freeman-Striegel et al., 2023), developmental timing (Cha et al., 2006; Schramm-Sapyta et al., 2007; Mokrysz et al., 2016; Gorey et al., 2019; Torrens et al., 2020; Murray et al., 2022; Torrens et al., 2022), and route of administration (Ruiz et al., 2021a; Ruiz et al., 2021b; Torrens et al., 2022; Torrens et al., 2023). Likewise, the opioid behavioral tests we employed tested only a single dose of heroin and concentration of remifentanil—other doses, opioid drugs, or behavioral tests could have yielded different results, as has been seen in prior reports (Solinas et al., 2004; Ellgren et al., 2007; Tomasiewicz et al., 2012; Stopponi et al., 2014; Cadoni et al., 2015; Lecca et al., 2020; Nguyen et al., 2020; Ferland et al., 2023b).

Summary. Despite its limitations, this report replicates and extends prior preclinical work demonstrating that AdoTHC exposure conveys later-life vulnerability to opioid reward, and suggests some potential new mechanisms that may be involved in this effect, such as the potential involvement of AdoTHC-induced changes in microglia, and the markedly sex-dependent changes in neural network activation seen following heroin. Further work is clearly needed to pursue the broader implications of these preliminary findings. In the meantime, they contribute to an increasing body of literature supporting a biological component to the “gateway effect” of THC leading to harder drug use, and thus warrant caution when it comes to use of cannabis use by teenagers.

Supplementary Figure 2



Supplementary Figure 2.1. Chronic THC transiently attenuates body weight gain. **A**) Adolescent administration of THC from postnatal days 30-43 (green) suppresses weight compared to VEH treatment in the same period (black) in females (left) and males (right). **B**) By adulthood (PD80), body weight no longer differs between rats that received AdoVEH and AdoTHC. Data represents mean ± SEM.

CHAPTER 3: Adolescent THC Exposure Persistently Alters Defensive Responding and Opioid Motivation in Male and Female Rats

Introduction

The ability to flexibly select appropriate defensive behaviors is critical for survival and depends on the neural circuits that integrate motivational and emotional information. Animals are constantly inundated by cues in the environment signaling potential rewards and threats, and adaptive responses to either approach or avoid these cues depend on integration of related information and the significance of the cue (Elliot, 1999; Pessoa and Adolphs, 2010). The prefrontal cortex exerts top-down regulatory control over subcortical motivational and emotional circuits, while integrating external sensory/contextual information and internal sates, to guide flexible selection of defensive responding (Moscarello and LeDoux, 2013; Moscarello and Maren, 2018; Jercog et al., 2021). Notably, adolescence is a critical developmental period marked by protracted maturation of top-down prefrontal control (Spear, 2000; Gogtay et al., 2004; Casey et al., 2008; Spear, 2013), resulting in heightened activity of subcortical emotional and motivational regions, including the amygdala (Etkin et al., 2015; Goetschius et al., 2019). This imbalance can favor maladaptive defensive responses, which may bias adolescents toward affectively driven, impulsive, or risky behaviors typical in adolescence (Spear, 2000; Gogtay et al., 2004; Casey et al., 2008; Spear, 2013).

Importantly, the endocannabinoid system has emerged as an important regulator of defensive responding, aiding in the selection of appropriate defensive strategies toward threats (Loomba et al., 2025). The exogenous cannabinoid, Δ^9-tetrahydrocannabinol (THC), interacts with the endocannabinoid system, which plays an important role in circuit refinement and maturation during adolescence (Kano et al., 2009; Castillo et al., 2012; Klune et al., 2025). Consequently, impacts to the endocannabinoid system during this critical period, such as adolescent THC (AdoTHC) exposure, may result in persistent disruptions in the neural systems that govern defensive behaviors later in life.

Alterations in threat responding can indicate dysregulation in aversive motivational systems, which may suggest disruptions in other processes important aversive motivation, including sensitivity to stressors (Cheetham et al., 2010; Edwards and Koob, 2010). Stress is associated with substance use and misuse as both acute and chronic stress can shift decision-making and motivation toward maladaptive outcomes (Sinha, 2001; Goeders, 2003; Sinha, 2007, 2024), especially in people with opioid use disorder, who exhibit greater perceived stress that is linked to stronger cravings and poorer treatment outcomes (MacLean et al., 2019). Additionally, our lab and others have shown that AdoTHC exposure induces a propensity toward later-life opioid vulnerability (Solinas et al., 2004; Ellgren et al., 2007; Spano et al., 2010; Tomasiewicz et al., 2012; Stopponi et al., 2014; Cadoni et al., 2015; Lecca et al., 2020; Nguyen et al., 2020; Ferland et al., 2023b), suggesting that AdoTHC may have enduring effects on stress driven opioid-taking. Indeed, animals with prior AdoTHC exposure have heightened transcriptional stress vulnerability (Ferland et al., 2023b) and reinstatement of heroin seeking following a pharmacological stressor (Stopponi et al., 2014). Thus, interactions between AdoTHC exposure, later-life defensive responding to threats, and stress responsivity may be particularly consequential for understanding trajectories of addiction vulnerability.

We previously showed that our well-characterized, translationally relevant AdoTHC exposure protocol in rats (Torrens et al., 2020; Ruiz et al., 2021a; Ruiz et al., 2021b; Torrens et al., 2022) induced a pro-opioid phenotype in adulthood, especially in females (see chapter 2). However, these findings made us question how AdoTHC induces later-life opioid vulnerability. Therefore, our current study aims to determine whether AdoTHC exposure alters threat responding and stress-modulated opioid-taking, processes that may contribute to opioid vulnerability.

Materials and Methods

Subjects. Long Evans rats (*n* = 19 males, 20 females) were subjected to AdoTHC or adolescent vehicle (AdoVEH) as described below, then in adulthood (at least 21d after

adolescent treatment) underwent a shock rod task, fear conditioning, elevated plus maze (EPM), and remifentanil behavioral economics test with footshock and pharmacological stress challenge. A separate group (*n* = 12 males, 12 females) was treated in adolescence, then in adulthood neural activity assessed, via cFos activation, after acute administration of the pharmacological stressor yohimbine. All rats were pair or triple-housed in same-sex groups at weaning (postnatal day; PD21), in a temperature, humidity, and pathogen-controlled colony under a 12:12h reverse light/dark cycle. Water and food was provided *ad libitum* throughout behavioral testing. Experiments were approved by University of California, Irvine's Institutional Animal Care and Use Committee and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Drugs. THC was provided in ethanol by the National Institute on Drug Abuse (NIDA) Drug Supply Program (Research Triangle Park, NC, USA). THC was prepared fresh daily; ethanol was evaporated under N_2, and THC reconstituted in 5% Tween-80 in 0.9% saline, with heat and sonication to 2ml/kg for intraperitoneal (IP) injection of a 5mg/kg dose. Remifentanil HCl was provided by the NIDA Drug Supply Program and dissolved in sterile 0.9% saline. Yohimbine HCL was purchased from Sigma Aldrich (Cat# Y3125-5G) and dissolved in sterile water.

Adolescent THC and Washout. Rats received daily IP injections of THC from PD30-43, followed by a 21+ day washout (~PD70) to ensure full THC clearance (Lee et al., 2022), and for rats to mature to adulthood before starting their behavioral tasks.

Experimental Design. *Defensive Threat Behaviors and Stressor-Induced Opioid-Taking:* Following AdoTHC/VEH treatment, rats underwent a series of behavioral tasks (**Fig. 4.1A)**. 39 rats (*n* = 19 males: 8 VEH, 11 THC, and *n* = 20 females: 9 VEH, 11 THC) underwent a shock rod task first (four days), followed by fear conditioning (three days), and EPM. Beginning around PD90, rats then underwent remifentanil self-administration training, starting with 3d on a fixed-ratio 1 (FR1) schedule, then training on a within-session behavioral economic demand elasticity task (Levis et al., 2021; Levis et al., 2022; Levis et al., 2024). Once rats were stable, they were

tested on separate sessions with either three footshocks (0.5mA) delivered at random intervals across a 10min session without predictive cues or administration of the acute pharmacological stressor, yohimbine (2.5mg/kg, IP) or sterile water (Yveh) 30min prior to starting their behavioral economics session

cFos Following Yohimbine: Separate AdoTHC/VEH treated animals (*n* = 12 males: 6 VEH, 6 THC, and *n* = 12 females: 6 VEH, 6 THC) received an acute injection of yohimbine (2.5mg/kg, IP) in adulthood, and locomotion and ultrasonic vocalizations (USVs) were recorded for 30min prior to perfusion and cFos staining.

Behavioral Methods. *Shock Rod Task.* Rats were habituated for 30min on two consecutive days to a plexiglass tub cage (25 x 46 x 20cm) filled with 5cm of corncob bedding. On the following day, rats underwent a shock rod fear acquisition session in which the tub cage now had an uninsulated copper-wrapped glass stirring rod (5mm diameter x 200mm length; 1mm diameter wire) inserted through a 1cm diameter hole in the wall, located 8cm above the cage floor, which delivered a 1.5mA shock upon contact (Coulbourn Precision Animal Shocker), as previously described (Fucich and Morilak, 2018; Ramirez et al., 2026). Rats were placed opposite the shock rod, and contact was voluntary. Shock rod acquisition lasted 20min following the first shock. Twenty-four hours later, rats were reintroduced to the same tub cage for a 20min expression test with a dormant shock rod.

Sessions were recorded, and scored by an observer blind to adolescent treatment, as in (Ramirez et al., 2026). Briefly, instances of, and total duration of *defensive burying* was of primary interest, defined as forepaw movements directing bedding toward the shock rod, or backward toward the rat's tail to gather bedding for subsequent displacement toward the rod (Pinel and Treit, 1978; Berridge et al., 1988; Craft et al., 1988). Additional behaviors included freezing, escape attempts, shock rod investigations, latency to initiate burying, latency to first shock, and bedding pile height, defined in (Ramirez et al., 2026). Five rats (*n* = 3 male THC, and *n* = 2 females: 1 VEH, 1 THC) were excluded for failing to contact the rod and thus not

receiving a shock. Two additional AdoTHC rats (*n* = 1 male, 1 female) were excluded as statistical outliers (≥3 SD above the mean).

Fear Conditioning Task. *Auditory Cued Fear Acquisition.* Acquisition took place in Med Associates operant chambers (30.5 x 24 x 21cm; St Albans, VT) within sound-attenuating boxes, standard metal bar flooring, and a peppermint scent (McCormick). Acquisition day consisted of a 3min baseline period with the house light illuminated after which a 20s auditory cue (continuous white noise: 80dB) was played, followed by a high intensity footshock (0.75mA, 2s). Three total cue/shock pairings were delivered with 90s elapsing between each.

Context Fear Expression. Twenty-four hours later, rats were returned to the same context for an 8min session with house light illuminated, but void of cues or shock. Contextual fear expression was assessed by comparing freezing during the first 3min of this session to baseline freezing in a novel context on cued fear expression/extinction day.

Auditory Cued Fear Expression/Extinction. Forty-eight hours following auditory fear acquisition (24h after contextual fear expression), rats were placed into a different chamber with a metal grid floor, and an orange scent (McCormick) for a 47min cued fear expression/extinction session. Following a 3min baseline with house light illuminated, the previously shock-paired 20s white-noise cue was presented 24 times (90s intertrial interval) without shock.

Videos from all sessions were scored by an observer blind to adolescent treatment using a sampling method, as in (Ramirez et al., 2026). During baseline and 90s post cue periods, the predominant behavior observed every 8th second was scored. During 20s auditory cue periods, behavior was scored every 2s. Primary analyses were conducted on the percentage of collected samples in which behaviors occurred during baseline, 20s cue periods, and 90s post cue periods, including freezing, grooming, rearing, sniffing, head shakes, locomotion, and escape attempts, as defined in (Ramirez et al., 2026). For extinction, cue and post cue periods were grouped into bins of three cues or post cue periods and averaged.

Elevated Plus Maze. Rats were placed in an EPM apparatus for 5min and percentage of time spent in the open/close arms, and open/close arm entries were quantified using Ethovision software (Noldus, Leesburg, VA).

Behavioral Economic Thresholding Procedure. Remifentanil was chosen rather than heroin because it produces robust, repeated reinforcement in short-access (2h) conditions and is therefore ideal for studying the acutely reinforcing effects of opioid drugs (Michelsen and Hug Jr, 1996; Glass et al., 1999; Porter-Stransky et al., 2017; Levis et al., 2021; Levis et al., 2022; Levis et al., 2024).

Following shock rod task, fear conditioning, and EPM (at approximately PD79), rats were implanted with jugular catheters under isoflurane (3-3.5%) anesthesia, meloxicam (1mg/kg, IP) and cefazolin (10mg, IV) were administered intraoperatively, and heparin lock solution (10 U/0.1ml) used to maintain catheter patency. Rats then began remifentanil self-administration training in Med Associates operant chambers (30.5 x 24 x 21cm; St Albans, VT) within sound-attenuating boxes, equipped with two retractable levers with white lights above them, and a white house light. Acquisition training occurred in daily 2h sessions where pressing an active lever once (FR1) yielded a remifentanil infusion (3.2µg/kg/infusion, IV) and a 2.9kHz tone + lever light for 3.6s, followed by a 20s timeout period signaled by house light extinction. Lever presses during timeout, and all inactive lever presses were recorded, but inconsequential. After each session catheters were flushed with cefazolin and heparin lock solution. Acquisition training continued for at least 3d, or until acquisition criterion was met (>20 infusions in a session for at least 2d).

Next, rats began training on a within-session economic demand thresholding procedure (Oleson and Roberts, 2009; Bentzley et al., 2013), adapted for remifentanil (Porter-Stransky et al., 2017; Levis et al., 2021; Levis et al., 2022; Levis et al., 2024). Active lever presses activated a pump delivering IV remifentanil solution (0.0137mg/ml) and a coincident tone/light cue for a period of time that decreased across each of eleven 10min blocks during the session, without

timeouts after each infusion/cue. Presses delivered 1.92, 1.08, 0.608, 0.342, 0.192, 0.108, 0.0609, 0.0341, 0.0193, 0.0108, 0.00611µg remifentanil/infusion in subsequent blocks. Therefore, the number of presses required to achieve preferred levels of intoxication increased across the 11 blocks as the dose delivered for each press decreased.

Remifentanil intake at each "price" was recorded daily, and average intake in each block was averaged for the last 3 training days before animals first stressor-induced test day was analyzed, when behavior had stabilized in all rats. Consumption was then fit to an exponential demand equation: $\ln Q = \ln Q_0 + k\,(e^{-\alpha Q_0 C} - 1)$; where Q = consumption, C = unit cost, k is a scalar constant for consumption range, α = demand elasticity, and Q_0 = extrapolated intake at price zero effort (Levis et al., 2021; Levis et al., 2024). The key variables α (reflecting demand elasticity, which is inversely related to motivation to receive preferred amounts of drug), and Q_0 (reflecting "hedonic setpoint," or preferred drug consumption at an estimated cost of zero) were calculated for each rat and analyzed. We also analyzed intake (infusions) across Cost bins, and Pmax (the bin in which the maximum infusions were delivered; Oleson and Roberts, 2009), as additional indices of demand elasticity.

Animals were exposed to two stressors, footshock and the pharmacological stressor yohimbine, an alpha-2 adrenergic receptor antagonist that increases norepinephrine release and engages stress circuitry (Wang et al., 2017; Porrill et al., 2024), in a counterbalanced manner prior to their behavioral economics session. For footshock (0.5mA), rats were placed in the operant chamber with the house light illuminated and received three random, uncued shocks (1s each) over 10min before the start of their session. Behavior on footshock test days was compared to the average of the last 2-3 stable sessions. For pharmacological stress, rats received an acute injection of the adrenergic antagonist yohimbine (2.5mg/kg, IP) or Yveh (sterile water) 30min prior to the session.

Locomotion and USVs Following AdoTHC/VEH and Adult Yohimbine. A separate cohort of behaviorally naïve AdoTHC/VEH-treated animals were used to examine cFos in 5 brain regions

after a final acute injection of yohimbine (2.5mg/kg, IP). Thirty minutes following yohimbine injection, rats were placed into plexiglass tub cages (25 x 46 x 20cm) with paper fiber bedding for a 30min locomotion and USV session, as in (Lawson et al., 2021). Briefly, tub cages were enclosed within a wood sound-attenuating box (120 x 60 x 60cm), divided into four bays separated by wood walls, each equipped with a web camera (ZIQIAN; 1080p) and an ultrasonic sensitive microphone (Avisoft Bioacoustics; model CM16/CMPA; frequency range: 10-200kHz) positioned ~33cm above cages. Avisoft Bioacoustics receivers (model 816H; sampling rate 250kHz;16-bit resolution) and PC software (V3.4.4) were additionally employed to capture USVs. Locomotion and USVs were quantified using SLEAP (V1.3.3) and Deepsqueak (V3.1.0), respectively, by an observer blind to adolescent treatment. USV data from 4 rats (*n* = 2 males: 1 VEH, 1 THC, and *n* = 2 females: 1 VEH, 1 THC) were lost due to file corruption. Rats returned to their home cages for 60min, then were perfused and brains were processed for cFos immunohistochemistry.

Immunohistochemical Analyses. Rats were perfused with ice-cold saline then 4% paraformaldehyde, then brains were removed and postfixed in 4% paraformaldehyde, cryoprotected in 20% sucrose-azide, and coronally sliced at 40µm on a cryostat. Sections were stained for cFos protein using a diaminobenzadrine (DAB)-based protocol described in detail elsewhere (Mahler et al., 2007; Ruiz et al., 2021a; Levis et al., 2022). Briefly, endogenous peroxidase was quenched for 15min in 1% H_2O_2, blocked in 2% normal donkey serum (NDS) PBST for 2h, then incubated at RT in 1:10,000 rabbit anti-c-Fos primary antibody (Abcam, #ab190289) with 2% NDS for 16h. After washing, sections incubated in biotinylated donkey anti-rabbit secondary (Jackson ImmunoResearch, #711-065-152), followed by ABC amplification for 90min (Vector Laboratories). c-Fos was visualized with a 0.01% H_2O_2 and 0.04% Nickel Ammonium Chloride DAB reaction, yielding a nuclear blue-back reaction product. Slides were dehydrated in ethanol and xylenes, and coverslipped with Permount (Fisher Scientific, Hampton, NH). Samples were imaged at 5x magnification on a Zeiss Axio Observer 7 and

quantified using ImageJ software by a trained observer blind to experimental groups. For each quantified region, two sections were analyzed bilaterally at consistent positions in the brain, and mean density of cFos (Fos/mm^2) in each of the 4 samples/region was averaged to attain a per-rat value for the medial prefrontal cortex (mPFC; prelimbic; PLC and infralimbic; ILC) and amygdala (basolateral: BLA, central: CeA, and medial: MeA). Samples were lost for mPFC (in 1 AdoTHC male) and amygdala (in 1 AdoTHC female) during staining.

Data Analysis. GraphPad Prism was used for all statistical analyses. Because we were inherently interested in the effects of AdoTHC separately in each sex, primary analyses for all measures were conducted separately within each sex and supplemental analyses incorporating sex as a between-subjects factor were also examined. Effects of adolescent treatment (AdoTX: AdoTHC or AdoVEH) were assessed using independent samples t-test for adult body weight, shock rod task, cued fear expression, and demand elasticity measures (averaged across last 3d of stable training). Two-way mixed ANOVAs with Sidak posthocs tests for body weight (AdoTX x postnatal days: within-subject factor: days 30-43), fear acquisition and extinction (AdoTX x Cue/Post Cues or Cue Bin/Post Cue Bins: within subject factor: cues/post cues 1-3; cue/post cue bins 1-24), contextual fear expression (AdoTX x Context: within subject factor: context A/B), elevated plus maze (AdoTX x arm: within subject factor: open/closed arm), remifentanil self-administration (AdoTX x Day: within subject factor: days 1-3), and stressor-induced opioid taking (AdoTX x stressor: within subjects factor: stable pre-foot shock vs. footshock; Yveh vs. yohimbine) were used to assess effects of AdoTX. cFos data were analyzed with mixed model AdoTX x Subregion ANOVA in each sex (AdoTX x Region: within subject factor: mPFC: PLC, ILC; amygdala: BLA, CeA, MeA). Geisser-Greenhouse and Welch's correction were applied when applicable.

Results

Adolescent THC Transiently Suppresses Body Weight. Our AdoTHC protocol suppressed body weight gain during treatment, relative to AdoVEH, in females ($F_{(1.7, 52.2)}$ =13.38, p<0.0001)

and in males ($F_{(1.9,\ 54)}$=6.66, p=0.003) during adolescent treatment, with no significant sex difference in this effect (Main effect of AdoTX: $F_{(1,\ 59)}$=17.70, p<0.0001; no Sex x AdoTX interaction: $F_{(1,\ 59)}$=1.02, p=0.32; **Supplementary Figure 3.1A**). This suppression of adolescent body weight by THC was transient, as both sexes attained comparable body weights by adulthood, prior to commencement of behavioral testing (No effect of AdoTX at PD~70 in females: t_{29} =1.42, p=0.17, or males: t_{30}=0.70, p=0.49, no Sex x AdoTX interaction: $F_{(1,\ 59)}$ =1.15, p=0.29; **Supplementary Figure 3.1B**).

AdoTHC Exposure Attenuates Active Defensive Responses to a Localized Threat. We first asked whether AdoTHC exposure persistently alters responses to a localized threat, using the shock rod task **(Fig. 3.1B)**. All animals experienced a similar number of shocks and had similar latency to their first shock (independent samples t-test: Shock #: Females: t_{15}=0.46,

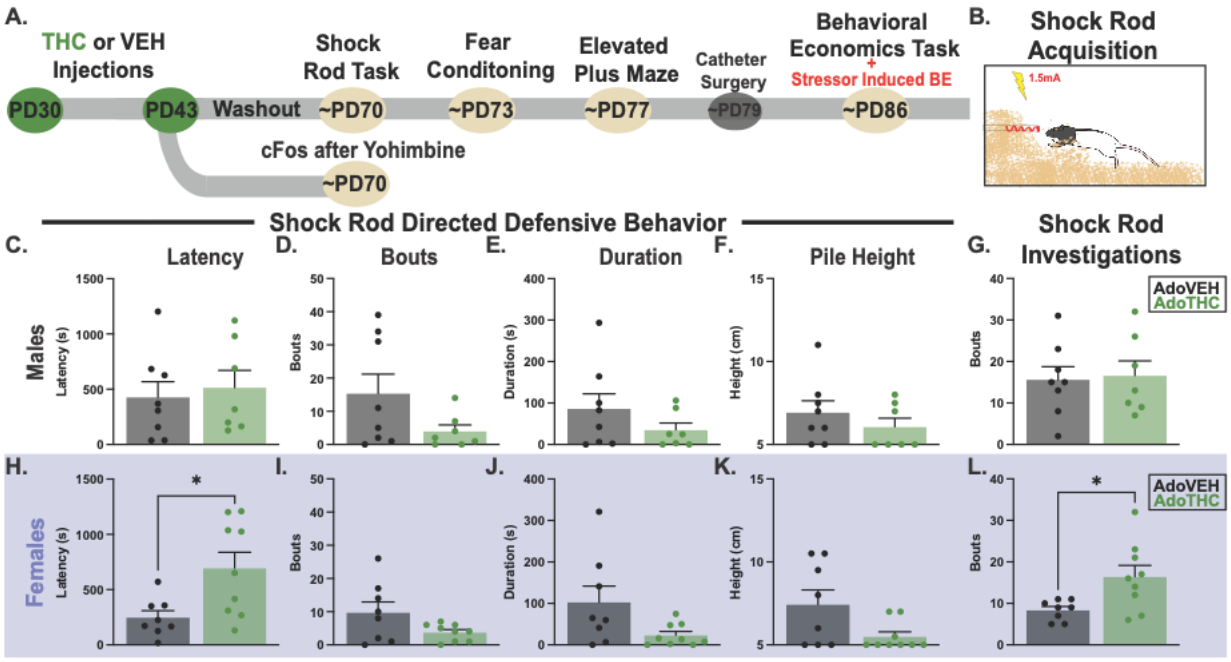


Figure 3.1. AdoTHC persistently reduces active defensive burying in response to a localized threat in females.
A) Experimental timeline. **B)** Schematic of defensive burying task on acquisition day. **C-G)** In males, AdoTHC (green) exposure had no effect on defensive burying behaviors compared to AdoVEH (black) controls. **H-L)** In females, AdoTHC exposure increased latency and rod investigations, with trends toward reduced bouts, bout duration, and pile height. Data represented as mean + SEM. *p < 0.05.

p=0.66; Males: t_{13}=0.84, p=0.42; Latency to Shock: Females: $t_{7.1}$=1.57, p=0.16; Males: $t_{6.2}$=1.21, p=0.27).

In females, AdoTHC exposure increased the number of shock rod investigations (**Fig. 3.1L**; $t_{9.6}$=2.83, p=0.02), although the total time investigating did not differ ($t_{9.6}$=1.49, p=0.17). Despite this, AdoTHC-exposed females showed reduced engagement in active defensive responses, including longer latency to initiate burying (**Fig. 3.1H**; $t_{10.8}$ = 2.87, p = 0.02), and trends toward shorter duration of burying (**Fig. 3.1J**; $t_{7.8}$=2.01, p=0.08), smaller bedding piles (**Fig. 3.1K**; $t_{8.5}$=2.10, p= 0.07), and fewer bouts of burying (**Fig. 3.1I**; t_{8}=1.81, p=0.11). In males, AdoTHC exposure did not alter shock rod investigations, time investigating, or defensive burying behaviors (**Fig. 3.1C-G**; Investigations: t_{13}=0.20, p=0.85; Investigation Time: t_{13}=0.39, p=0.70; Latency to Bury: t_{13}=0.41, p=0.69; Bouts: $t_{8.5}$=1.86, p=0.10; Duration: t_{13}=1.23, p=0.24; Pile Height: t_{13}=0.97, p=0.35). In contrast to these active defensive responses to the shock rod, AdoTHC exposure did not alter other behaviors such as escape attempts or freezing (Freezing: Females: t_{15}=0.18, p=0.86; Males: t_{13}=0.54, p=0.60; Escape: Females: t_{15}=0.55, p=0.59; Males: $t_{9.1}$= 1.30, p=0.23).

At the group level, a 2-way ANOVA revealed that prior AdoTHC exposure markedly reduced active defensive behaviors across sex (main effect of AdoTX: Bouts: $F_{(1, 28)}$=6.26, p=0.02; Duration: $F_{(1, 28)}$=5.48, p=0.03; Pile Height: $F_{(1, 28)}$=4.98, p=0.03; Latency to Bury: $F_{(1, 28)}$=4.14, p=0.05; AdoTX x Sex interactions: all $Fs_{(1, 28)}$<1.88, all ps>0.18), without affecting shock rod investigation (main effect of AdoTX: Investigations: $F_{(1, 28)}$=2.72, p=0.11; Investigation Time: $F_{(1, 28)}$=1.45, p=0.24).

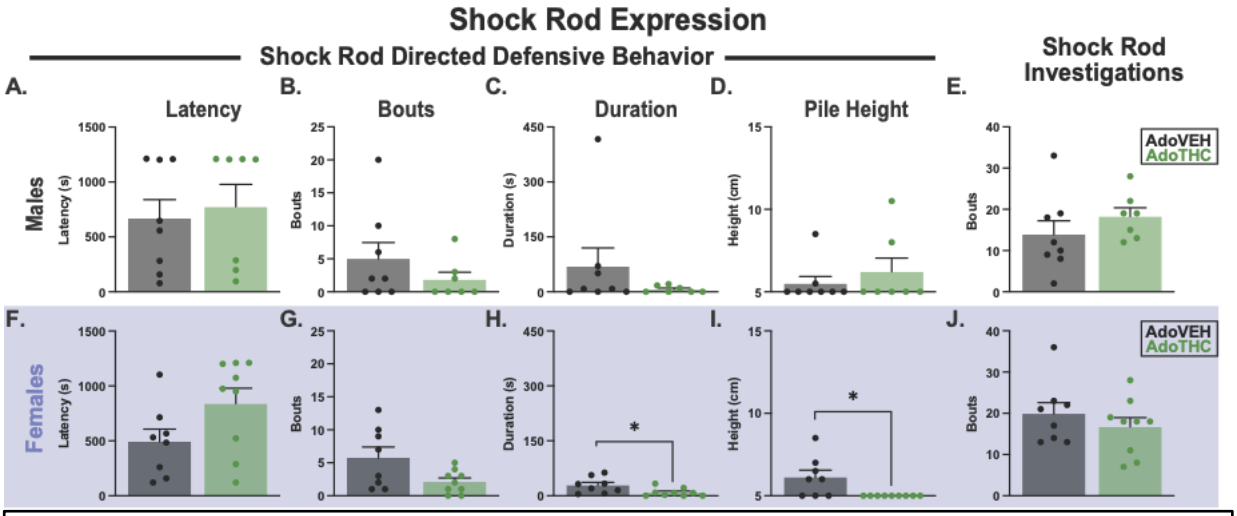


Figure 3.2. AdoTHC exposure in females attenuates active defensive burying to a previously learned localized threat in adulthood.
A-E) In males, AdoTHC (green) had no effect on defensive burying behaviors relative to AdoVEH (black) controls. **F-G)** In females, AdoTHC exposure reduced number of defensive burying bouts and bedding pile height but did not affect other defensive burying behaviors. Data represented as mean + SEM. *p < 0.05.

Active Defensive Responding to a Previously Learned Localized Threat. On expression day, animals were reintroduced to the tub cage containing a now dormant shock rod to assess expression of defensive responses to the previously learned threat.

In females, AdoTHC exposure did not alter exploratory interactions with the shock rod (**Fig. 3.2J**; independent samples t-test: Investigations: t_{15}=0.91, *p*=0.38; Investigation Time: t_{15}=0.91, *p*=0.38). However, AdoTHC-exposed females showed reduced engagement with the shock rod, including less time spent actively burying (**Fig. 3.2H**; t_{15}=2.40, *p*=0.03), smaller bedding piles (**Fig. 3.2I**; t_{7}=2.61, *p*=0.04), and trends toward fewer bouts of burying (**Fig. 3.2G**; $t_{8.8}$=2.10, *p*=0.07) and longer latency to initiate burying (**Fig. 3.2F**; t_{15}=1.89, *p*=0.08). In males, AdoTHC exposure did not alter shock rod investigation or defensive burying behaviors (**Fig. 3.2A-E**; Investigations: t_{13}=1.08, *p*=0.30; Investigation Time: t_{13}=0.003, *p*=1.00; Latency to Bury: t_{13}=0.39, *p*=0.70; Bouts: t_{13}=1.10, *p*=0.29; Duration: $t_{7.1}$=1.23, *p*=0.26; Pile Height: t_{13}=0.79, *p*=0.44). Additionally, AdoTHC exposure did not alter escape attempts or freezing (Escape:

Females: t_{15}=0.33, *p*=0.75; Males: $t_{8.1}$=1.27, *p*=0.24; Freezing: Females: t_{15}=0.39, *p*=0.70; Males: t_{13}=0.33, *p*=0.75).

At the group level, a 2-way ANOVA revealed that prior AdoTHC exposure reduced active defensive behaviors across sex, as AdoTHC-exposed animals exhibited fewer burying bouts (main effect of AdoTX: $F_{(1, 28)}$=4.47, *p*=0.04; no AdoTX x Sex interaction: $F_{(1, 28)}$=0.02, *p*=0.88). Other measures of defensive burying behavior were not altered, including total time spent treading, latency to initiate treading, pile height, or investigative behaviors (no main effect of AdoTHC: Duration: $F_{(1, 28)}$=2.54, *p*=0.12; Latency to Bury: $F_{(1, 28)}$=2.03, *p*=0.17; Pile Height: $F_{(1, 28)}$=1.88, *p*=0.67; Rod Investigations: $F_{(1, 28)}$=0.05, *p*=0.83; Investigation Time: $F_{(1, 28)}$=0.28, *p*=0.60; no AdoTX x Sex interaction: all $Fs_{(1, 28)}$<3.77, all *ps*>0.06).

AdoTHC Alters Passive Defensive Freezing to a Shock-Predictive Cue in Adulthood. Next, we asked whether AdoTHC exposure persistently alters passive defensive responses, specifically freezing behavior, to an auditory cue predictive of an aversive shock (**Fig. 3.3A**).

Cued Fear Acquisition. Rats first learned to associate a 20s auditory cue with a 0.75mA, 2s footshock, presented 3 times. Baseline freezing prior to cue presentation did not differ between AdoTHC and AdoVEH animals (independent samples t-test: Females: t_8=1.00, *p*=0.35; Males: t_7=1.00, *p*=0.35), indicating animals were not afraid of the context prior to cue-shock pairings. During acquisition, freezing increased across cue presentations (2-way mixed ANOVA: main effect of Cue: Females: $F_{(1.5, 27.3)}$=11.21, *p*=0.001; Males: $F_{(1.8, 30.8)}$=28.19, *p*<0.0001), indicating that all animals learned the cue-shock association.

In females, AdoTHC exposure enhanced freezing during cue presentations (**Fig. 3.3E**; 2-way mixed ANOVA: main effect of AdoTX: $F_{(1, 18)}$=4.62, *p*=0.05; no AdoTX x Cue interaction: $F_{(1.5, 27.3)}$=1.82, *p*=0.19), especially to the first auditory cue (post hoc Sidak Cue 1: $t_{17.5}$=2.91, *p*=0.03). We also examined the 90s periods immediately following each cue, and freezing was unaffected by AdoTHC (no main effect of AdoTX: $F_{(1, 18)}$=0.48, *p*=0.50; no AdoTX x Cue interaction: $F_{(1.9, 34.3)}$=0.08, *p*=0.91). In males, AdoTHC exposure did not alter freezing during

cue presentations (**Fig. 3.3B**; no main effect of AdoTX: $F_{(1, 17)}$=0.03, p=0.86, no AdoTX x Cue interaction: $F_{(1.8, 30.8)}$=1.81, p=0.18), but decreased freezing immediately following cues (**Supplementary Figure 3.2A**; main effect of AdoTX: $F_{(1, 17)}$=6.02, p=0.03; no AdoTX x Cue interaction: $F_{(1.3, 22)}$=1.20, p=0.30).

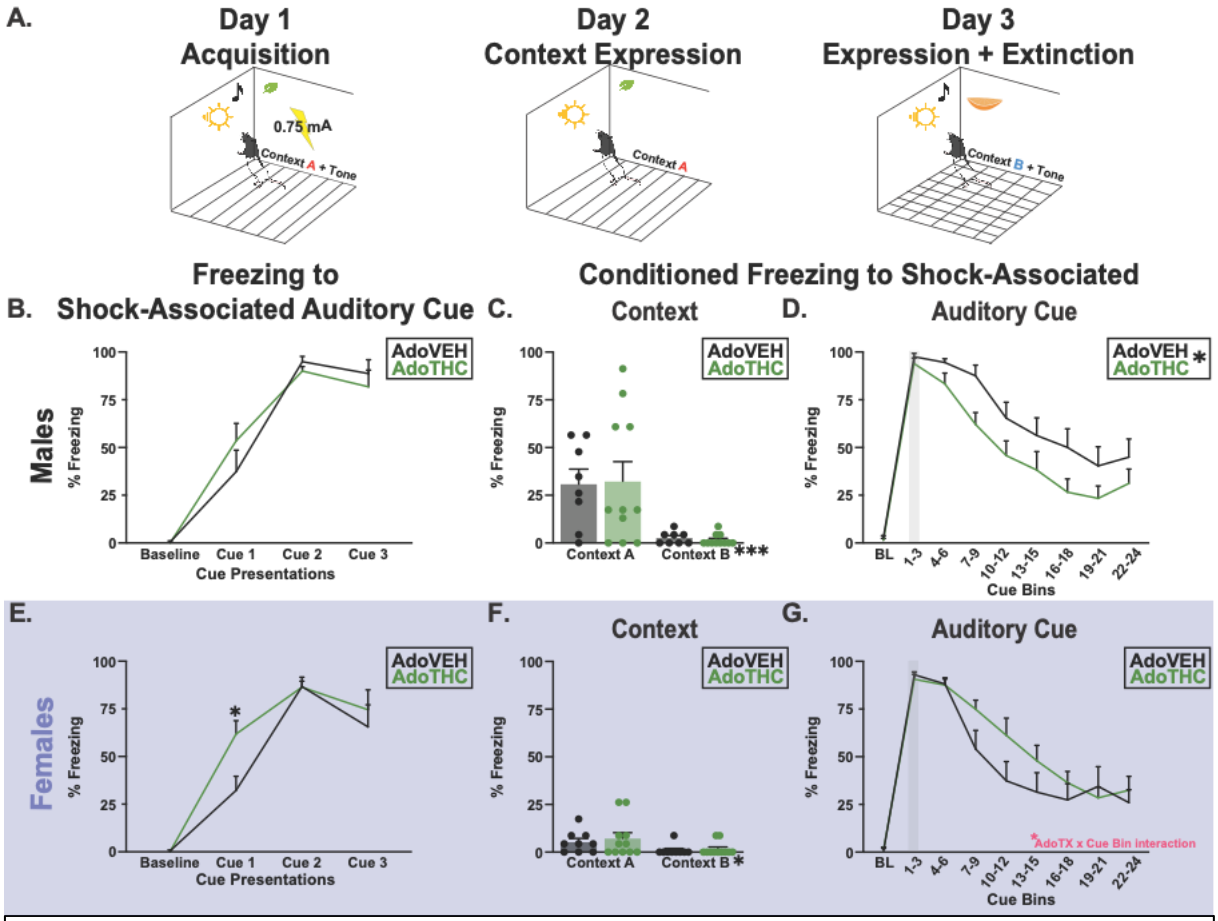


Figure 3.3. AdoTHC exposure alters passive defensive freezing in adulthood. **A)** Fear conditioning timeline. AdoTHC-exposure (green) in males had no effect on **B)** auditory cued fear acquisition, but **D)** exhibited reduced freezing during extinction compared to AdoVEH-exposed (black) males. AdoTHC-exposed females exhibited **E)** enhanced reactivity to the initial auditory cue presentation, and **G)** sustained extinction of cued fear expression compared to AdoVEH-exposed females. No difference between AdoTHC or AdoVEH were seen on contextual fear expression in **C)** males or **F)** females. Data represented as + SEM. BL = Baseline. Shaded area indicates cued fear expression bin. *p < 0.05, ***p < 0.001.

Broader 3-way mixed ANOVA confirmed that freezing during the first auditory cue presentation was enhanced in AdoTHC-exposed animals (AdoTX x Cue interaction: $F_{(1.7, 60.3)}$=3.42, p=0.05), driven by an increase in freezing to the first cue presentation (Sidak Cue 1:

$t_{34.4}$=2.78, *p*=0.03).Freezing immediately following cues was dependent on sex and AdoTHC-exposure (AdoTX x Sex interaction: $F_{(1, 35)}$=5.32, *p*=0.03), driven by the reduction in freezing in AdoTHC-exposed males.

Contextual Fear Expression. To assess whether AdoTHC exposure alters expression of a previously learned contextual fear memory, rats were returned 24h after acquisition to the original training context (Context A) without shock or cues. Freezing during the first 3min was compared to baseline freezing in a novel context (Context B). Animals froze significantly more in Context A than in Context B (2-way mixed ANOVA: main effect of Context: Females: $F_{(1, 18)}$=7.91, *p*=0.01; Males: $F_{(1, 17)}$=19.11, *p*=0.0004), indicating intact contextual fear memory, and this effect was not altered by adolescent treatment (no main effect of AdoTX: Females: $F_{(1, 18)}$=0.29, *p*=0.60; Males: $F_{(1, 17)}$= 0.0004, *p*=0.98; no main effect of AdoTX x Context: Females: $F_{(1, 18)}$=0.11, *p*=0.74; Males: $F_{(1, 17)}$=0.04, *p*=0.85).

Cue Fear Expression and Extinction. 24h following contextual fear expression, rats were placed in a novel context (Context B) where the auditory cue was presented 24 times without shock. Baseline freezing prior to cue onset did not differ between groups, indicating animals did not generalize fear to a new context (independent samples t-test: Females: t_{18}=0.42 *p*=0.68; Males: t_{17}=0.80, *p*=0.44). During the first cue bin (cues 1-3), when recall of the cue-shock association was expected to be highest, adolescent treatment groups did not differ in expression of fear during or following cue presentations (**Fig. 3.3D,G**; main effect of AdoTX: Cue: Females: t_{18}=0.74, *p*=0.47; Males: t_{17}=0.94, *p*=0.36; Post Cue: Females: t_{18}=0.17, *p*=0.87; Males: t_{17}=0.84, *p*=0.41).

Extinction learning was assessed across 24 unreinforced cue presentations in the same context. In females, AdoTHC-animals extinguished somewhat slower than VEH counterparts (**Fig. 3.3G**; 2-way mixed ANOVA: AdoTHC x Cue Bin interaction: $F_{(3.5, 62.5)}$=3.01, *p* = 0.030; no main effect of AdoTX: $F_{(1, 18)}$=1.09, *p*=0.31), although post hoc tests did not convey this at any given cue bin (Sidaks: all *ps*>0.49). Additionally, freezing immediately following cue bin

presentations was not affected by AdoTHC (main effect of AdoTX: $F_{(1, 18)}$=0.33, *p*=0.58; no AdoTHC x Cue Bin interaction: $F_{(1.6, 29.1)}$=0.26, *p*=0.73). In males, AdoTHC exposure reduced freezing across extinction (**Fig. 3.3D**; 2-way mixed ANOVA: main effect of AdoTX: $F_{(1, 17)}$=4.53, *p*=0.05; no AdoTHC x Cue Bin interaction: $F_{(3.7, 63.3)}$=0.89, *p*=0.47), including in bins immediately following cue presentations (**Supplementary Figure 3.2B**; main effect of AdoTX: $F_{(1, 17)}$=5.12, *p*=0.04; no AdoTHC x Cue Bin interaction: $F_{(2.7, 46)}$=0.70, *p*=0.54).

A broader 3-way mixed ANOVA confirmed that AdoTHC altered extinction-related freezing in a sex-dependent manner (AdoTX x Sex X Cue Bin: $F_{(4,142)}$=2.88, *p*=0.02; AdoTX x Sex interaction: $F_{(1, 35)}$=4.93, *p*=0.03), with AdoTHC exposed females extinguishing slower, while AdoTHC-exposed males exhibited an overall reduction in freezing during extinction. Analysis of freezing immediately following cue bins also revealed a sex-dependent effect of AdoTHC (AdoTX x Sex interaction: $F_{(1, 35)}$=4.74, *p*=0.04), driven by reduced freezing in AdoTHC-exposed males and no effect in females.

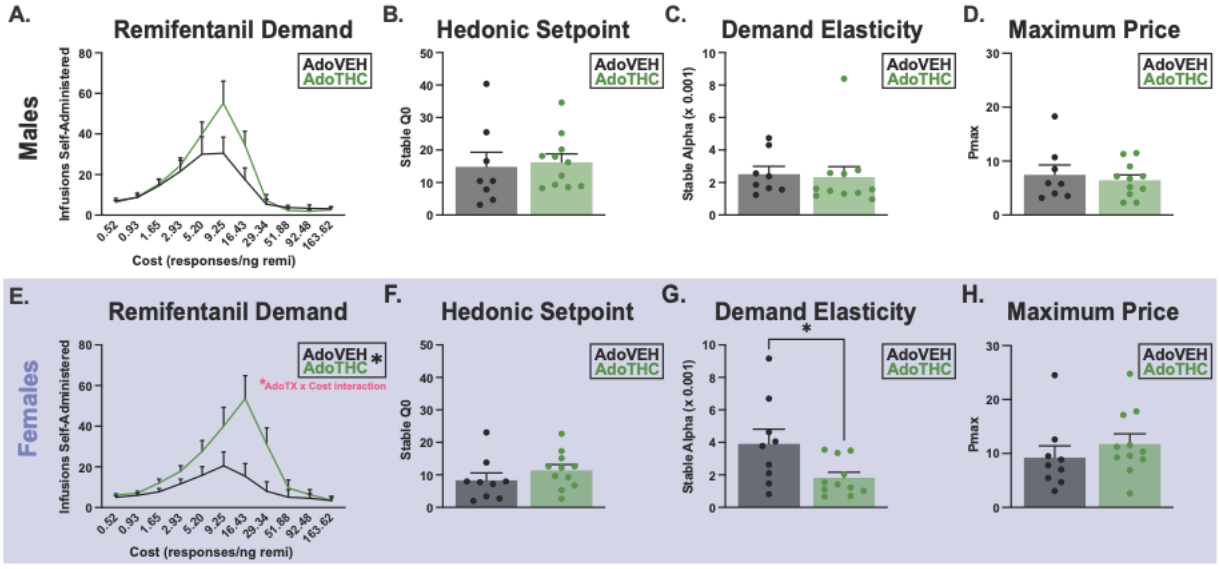


Figure 3.4. AdoTHC exposure persistently increases motivation for remifentanil in females.
In males, AdoTHC (green) exposure had no effect on **A)** remifentanil self-administered, **B)** intake (hedonic setpoint), **C)** motivation (demand elasticity) or **D)** Pmax (the price bin in which maximal infusions were taken). **E,G)** AdoTHC-exposed females exerted more effort for remifentanil at higher costs than AdoVEH-exposed females, **F)** despite no difference in consumption when the cost of remifentanil cost was low or in **H)** Pmax. Data are mean ± SEM. *p < 0.05.

AdoTHC Does Not Persistently Alter Anxiety-Like Response. All animals preferred the closed arms of the elevated plus maze over open arms (2-way mixed ANOVA: main effect of Arm: Females: time in arm: $F_{(1, 18)}$=212.1, p<0.0001; arm entries: $F_{(1, 18)}$=35.11, p<0.0001; Males: time in arm: $F_{(1, 17)}$=40.29, p<0.0001; arm entries: $F_{(1, 17)}$=14.33, p=0.002). AdoTHC did not affect these measures (no main effect of AdoTX: Females: time in arm: $F_{(1, 18)}$=0.94, p=0.35; arm entries: $F_{(1, 18)}$=0.0007, p=0.98; Males: time in arm: $F_{(1, 17)}$=3.17, p=0.09, arm entries: $F_{(1, 17)}$=0.04, p=0.84; data not shown), indicating that AdoTHC history does not persistently alter anxiety-like behavior.

AdoTHC Exposure Persistently Alters Opioid-Taking Behavior. We have previously demonstrated that AdoTHC exposure alters the acquisition of remifentanil in females, with effects emerging after extended training (5d); however, to prioritize investigation of impact of stressors on opioid-taking behavior, we used a shortened 3d acquisition protocol in the current study.

Acquisition of Remifentanil Self-Administration. After completing behavioral tasks, rats underwent jugular vein catheterization, then were trained to self-administer remifentanil for 3d on an FR1 schedule. Both females and males acquired remifentanil self-administration over the first three FR1 training Days, in that infusions (main effect of Day (1-3): Females: $F_{(1.1, 19.4)}$=5.50, p=0.03; Males: $F_{(1.7, 29)}$=20.10, p<0.0001; both sexes: $F_{(1.5, 51.4)}$=24.68, p<0.0001) and active lever presses increased daily (Females: $F_{(1.3, 23.5)}$=17.44, p=0.0001; Males: $F_{(1.9, 32.3)}$=28.92, p<0.0001; both sexes: $F_{(1.8, 62.5)}$=46.95, p<0.0001), while inactive lever presses remained stable and low (no main effect of Day: Females: $F_{(1.3, 24.2)}$=0.31, p=0.65; Males: $F_{(1.6, 26.6)}$=1.76, p=0.20; both sexes: $F_{(1.7, 58)}$=1.71, p=0.20). Remifentanil acquisition was similar regardless of AdoTX in females (no AdoTX x Day interaction for infusions: $F_{(1.3, 23.5)}$=0.44, p=0.56; active lever: $F_{(1.1, 19.4)}$= 0.48, p=0.51; inactive lever: $F_{(1.3, 24.2)}$= 0.33, p=0.64; data not shown) or males (no AdoTX x Day interaction for infusions: $F_{(1.9, 32.3)}$ =0.75 p=0.47; active lever: $F_{(1.7, 29)}$=3.16, p=0.06; inactive lever: $F_{(1.6, 26.6)}$= 1.80, p=0.19; data not shown). AdoTHC exposure did not affect acquisition overall (no main AdoTX x Day interaction for infusions: $F_{(1.8, 62.5)}$=0.22,

p=0.78; active lever: $F_{(1.5, 51.4)}$= 0.93, *p*=0.38; inactive lever: $F_{(1.7, 58)}$= 1.56, *p*=0.22) and no interactions including Sex were significant for infusions, active lever or inactive lever (no Sex x AdoTX, or Sex x AdoTX x Day interactions: Fs<3.23, *p*s>0.06).

Remifentanil Economic Demand Elasticity. Following acquisition of remifentanil self-administration, rats were trained on a behavioral economic demand elasticity task that allows estimation of both "hedonic setpoint" for remifentanil (represented by Q_0, an estimate of intake at zero cost), and motivation to obtain this desired amount of drug (represented by α, reflecting demand elasticity). In females, hedonic setpoint was unchanged by AdoTX (**Fig. 3.4F**; independent samples t-test: t_{18}=1.11, *p*=0.28), but demand elasticity was decreased (**Fig. 3.4G**; $t_{10.32}$=2.22, *p*=0.05), indicating that AdoTHC selectively increased motivation to pursue remifentanil at high cost without affecting remifentanil intake at low cost conditions. Accordingly, AdoTHC females pressed more in higher-effort than lower-effort Bins (**Fig. 3.4E**; 2-way mixed ANOVA: AdoTX x Cost interaction: $F_{(2.2, 40.3)}$ = 4.56, *p* = 0.01; main effect of AdoTX: $F_{(1, 18)}$=4.89, *p*=0.04), although no individual cost point reached significance (Sidaks: all *ps* > 0.102). P_{max}, or the cost Bin in which the maximum number of infusions was taken, was not altered by AdoTHC in females (t_{18}=0.92, *p*=0.37; **Fig. 3.2H**). In males, AdoTHC did not alter hedonic setpoint, motivation for remifentanil, infusions delivered over cost throughout the session, or P_{max} (**Fig. 3.4A-D**; Q_0: t_{17}=0.23, *p*=0.82; α: t_{17}=0.30, *p*=0.77; Infusions: no AdoTX x Cost interaction $F_{(2, 33.5)}$=2.24, *p*=0.12; no main effect of AdoTX: $F_{(1, 17)}$=1.79, *p*=0.20; P_{max}: t_{17}=0.53, *p*=0.61).

When both sexes were combined, AdoTX did not affect hedonic setpoint (2-way ANOVA: no main effect of AdoTX: $F_{(1, 35)}$=0.69, *p*=0.41) and AdoTHC decrease in α did not reach statistical significance (no main effect of AdoTX: $F_{(1, 35)}$=3.54, *p*=0.07). No significant sex difference in the AdoTX effect on Q_0, α, P_{max}, or infusions delivered over cost throughout the session were seen (no AdoTX x Sex interaction: Q_0: $F_{(1, 35)}$=0.09, *p*=0.77; α: $F_{(1, 35)}$=2.45, *p*=0.13; P_{max}: $F_{(1, 35)}$=1.08, *p*=0.31; Infusions: AdoTX x Sex x Cost: $F_{(2.5, 85.6)}$=1.05, *p*=0.37). However, AdoTHC exposure altered the pattern of remifentanil infusions across increasing cost conditions

(3-way mixed ANOVA: AdoTX x Cost interaction: $F_{(10, 490)}$=3.88, *p*<0.0001; main effect of AdoTX: $F_{(1, 35)}$=6.47, *p*=0.02), with AdoTHC-exposed rats maintaining higher intake at higher cost bins (Sidak: cost point 16.43: $t_{33.5}$=3.52, *p*=0.01).

AdoTHC and Acute Stressors Alter Motivation for Remifentanil in Adulthood. We next sought to determine whether stressors administered prior to opioid access would further modulate AdoTHC-induced opioid-taking. To test this, we administered an acute pharmacological stressor, yohimbine (2.5mg/kg; IP), and an acute physical stressor, three random uncued footshocks (0.5mA), before animals started their behavioral economics session.

Footshock Stressor. In females, remifentanil consumption under low-cost conditions was not altered by either AdoTHC exposure or footshock (Q0; **Fig. 3.5C**; 2-way mixed ANOVA: no main effect of AdoTX: $F_{(1, 18)}$=2.54, p=0.13; no main effect of footshock: $F_{(1, 18)}$=1.32, p=0.27; no AdoTX x footshock interaction: $F_{(1, 18)}$=0.35, p=0.56). However, AdoTHC exposure increased motivation for remifentanil (α; **Fig. 3.5D**; main effect of AdoTX: $F_{(1, 18)}$=7.00, p=0.02), independent of footshock (no main effect of footshock: $F_{(1, 18)}$=0.48, p=0.50; no AdoTX x footshock interaction: $F_{(1, 18)}$=0.21, p=0.65). In males, footshock reduced remifentanil consumption under low-cost conditions (Q0; **Fig. 3.5A**; main effect of footshock: $F_{(1, 17)}$=5.75, p=0.03), independent of AdoTHC history (no main effect of AdoTX: $F_{(1, 17)}$=0.04, p=0.84, no AdoTX x footshock interaction: $F_{(1, 17)}$=0.70, p=0.42), while motivation was not altered (α; **Fig. 3.5B**; no main effect of footshock: $F_{(1, 17)}$=0.07, p=0.79; no main effect of AdoTX: $F_{(1, 17)}$=0.33, p=0.58, no AdoTX x footshock interaction: $F_{(1, 17)}$=2.62, p=0.12).

Group-level analyses confirmed that footshock decreased remifentanil intake under low-cost conditions (Q0; 3-way mixed ANOVA: main effect of Shock: $F_{(1, 35)}$=7.05, p=0.01), regardless of AdoTHC exposure (no main effect of AdoTX: $F_{(1, 35)}$=0.25, p=0.62, no AdoTX x footshock interaction: $F_{(1, 35)}$=1.07, p=0.31; no AdoTX x Sex x footshock interaction: $F_{(1, 35)}$=0.13, p = 0.72). Conversely, AdoTHC exposure increased motivation at higher cost (α; main effect of AdoTX: $F_{(1, 35)}$=4.31, p=0.05), independent of footshock (no main effect of footshock: $F_{(1, 35)}$=0.07, p=0.79; no

AdoTX x footshock interaction: $F_{(1, 35)}$=0.78, p=0.38; no AdoTX x Sex x footshock interaction: $F_{(1, 35)}$=2.27, p=0.14), likely due to the increased motivation in AdoTHC females.

Pharmacological Stressor. We next examined whether yohimbine alters demand for remifentanil. In females, hedonic setpoint was not affected by yohimbine or AdoTHC indicating no change under low-cost consumption (Q_0; **Fig. 3.5G**; 2-way mixed ANOVA: no main effect of yohimbine: $F_{(1, 18)}$=0.19, *p*=0.67; no main effect of AdoTX: $F_{(1, 18)}$=3.27, *p*=0.09; no AdoTX x yohimbine interaction: $F_{(1, 18)}$=0.38, *p*=0.55). However, yohimbine selectively increased motivation for remifentanil in AdoVEH-exposed, but not AdoTHC-exposed animals (α; **Fig. 3.5H**; 2-way mixed ANOVA: AdoTX x yohimbine interaction: $F_{(1, 18)}$=7.94, *p*=0.01; Sidaks: AdoVEH: t_{18}=5.77, *p*<0.0001; AdoTHC: t_{18}=2.18, *p*=0.08). Motivation for remifentanil under yohimbine did not differ between AdoTHC and AdoVEH females (t_{36}=0.76, *p*=0.70), suggesting that yohimbine

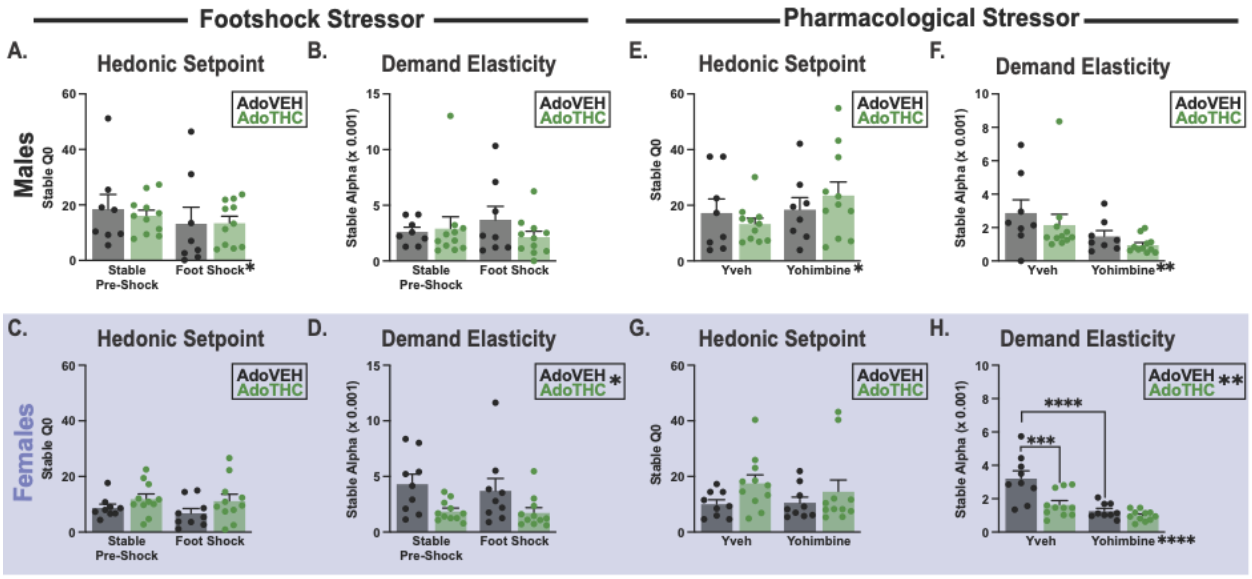


Figure 3.5. AdoTHC exposure persistently alters stressor-induced motivation for remifentanil.
Administration of a footshock stressor, in males **A)** reduced intake, **B)** but not motivation for remifentanil. In females, footshock had no effect on **C)** intake, or **D)** motivation for remifentanil, but AdoTHC-exposure increased motivation for remifentanil overall. Administration of the pharmacological stressor yohimbine, **E)** increased remifentanil consumption, and **F)** motivation in males. In females, yohimbine did not **G)** affect intake, **H)** but increased motivation in AdoVEH (black) animals, mimicking the increased motivation observed in AdoTHC-exposed females at baseline. Data represented as mean + SEM. *p < 0.05, **p< 0.01, ****p < 0.0001.

increased motivation in AdoVEH animals to levels comparable to AdoTHC-exposed females. In males, yohimbine increased consumption (Q_0; **Fig. 3.5E**; main effect of yohimbine: $F_{(1, 17)}$=4.83, *p*=0.04) and motivation for remifentanil (α; **Fig. 3.5F**; main effect of yohimbine: $F_{(1, 17)}$=8.72, *p*=0.009), and AdoTHC did not affect these demand characteristic (no main effect of AdoTX: Q_0: $F_{(1, 17)}$=0.01, *p*=0.92; α: $F_{(1, 17)}$=1.09, *p*=0.31; no AdoTX x yohimbine interaction: Q_0: $F_{(1, 17)}$=3.04, *p*=0.10; α: $F_{(1, 17)}$=0.05, *p*=0.83).

Broader analyses including all groups, confirmed demand elasticity was decreased by both yohimbine and AdoTHC exposure across sex (α; 3-way mixed ANOVA: main effect of yohimbine: $F_{(1, 35)}$ = 28.58, *p* < 0.0001; main effect of AdoTX: $F_{(1, 35)}$=5.60, *p*=0.02; no AdoTX x Sex x yohimbine interaction: $F_{(1, 35)}$=1.24, *p*=0.27), indicating increased motivation for remifentanil under high-cost conditions. However, hedonic setpoint was not altered (Q_0; no main effect of yohimbine: $F_{(1, 35)}$=1.33, *p*=0.26; no main effect of AdoTX: $F_{(1, 35)}$=1.10, *p*=0.30; no AdoTX x Sex x yohimbine interaction: $F_{(1, 35)}$=2.63, *p*=0.11).

AdoTHC Alters Prefrontal Stress Responses Following Yohimbine in Adulthood. Given the behavioral sensitivity to yohimbine in females, we next examined behavioral and neural responses to acute yohimbine administration in a separate cohort of behaviorally naïve AdoTHC/VEH exposed animals.

Locomotion & USVs after Yohimbine. Animals received an acute injection of yohimbine (2.5mg/kg, IP), and locomotion and USVs were then measured for 30min in a novel environment. While 22khz calls, typically associated with negative affect, were not observed, 50khz calls were detected (Lawson et al., 2021). AdoTHC exposure did not alter the number of calls, call length, or sinuosity (complexity of the pitch trajectory; independent samples t-test: Females: all t_8<1.03, all *ps*>0.33; Males: all t_8<1.83, all *ps*>0.10). However, in males, AdoTHC exposure increased delta frequency (difference between end and start frequency) compared to AdoVEH animals (data not shown; t_8=3.05, *p*=0.02), suggesting that AdoTHC alters the pitch trajectory of vocalizations following yohimbine without affecting overall calling behavior, and this

effect was not seen in females (t_8=0.17, p=0.87). Additionally, locomotion was not affected by AdoTHC in either sex (Females: t_{10}=0.84, p=0.42; Males: t_{10}=0.02, p=0.99).

Neural Activity after Yohimbine. To assess stress-related neural activity, cFos expression was quantified in stress- and reward-related brain regions. In females, AdoTHC exposure led to a subregion-specific shift in cFos activation (**Fig. 3.6C**; 2-way mixed ANOVA: AdoTX x Region: $F_{(1, 10)}$=6.95, p=0.03; Sidaks: AdoTHC: t_{10}=4.55, p=0.002), where prelimbic expression was greater than infralimbic cFos expression following yohimbine, and AdoVEH-exposed females showed no difference between subregions (Sidaks: AdoVEH: t_{10}=0.79, p=0.69). In males, AdoTHC exposure increased overall cFos expression within the mPFC after yohimbine (**Fig. 3.6A**; main effect of AdoTX: $F_{(1, 9)}$=7.00, p = 0.03; no AdoTX x Region: $F_{(1, 9)}$=0.12, p=0.74). In contrast, AdoTHC exposure did not alter cFos expression within the amygdala, including basolateral, central, or medial, in females (**Fig. 3.6B**; no main effect of AdoTX: $F_{(1, 9)}$=0.17, p=0.69; no AdoTX x Region: $F_{(1.5, 13.8)}$=1.72, p=0.22) or males (**Fig. 3.6D**; no main effect of AdoTX: $F_{(1, 10)}$=0.01, p=0.93; no AdoTX x Region interaction: $F_{(1.2, 12)}$=1.03, p=0.35).

While AdoTHC had no detectable effect on amygdala activation across sex (3-way mixed ANOVA: no AdoTX x Sex x Region: $F_{(1.4, 26.1)}$=2.26, p=0.14; no main effect of AdoTX: $F_{(1, 19)}$=0.13, p=0.72), the overall ANOVA analyses

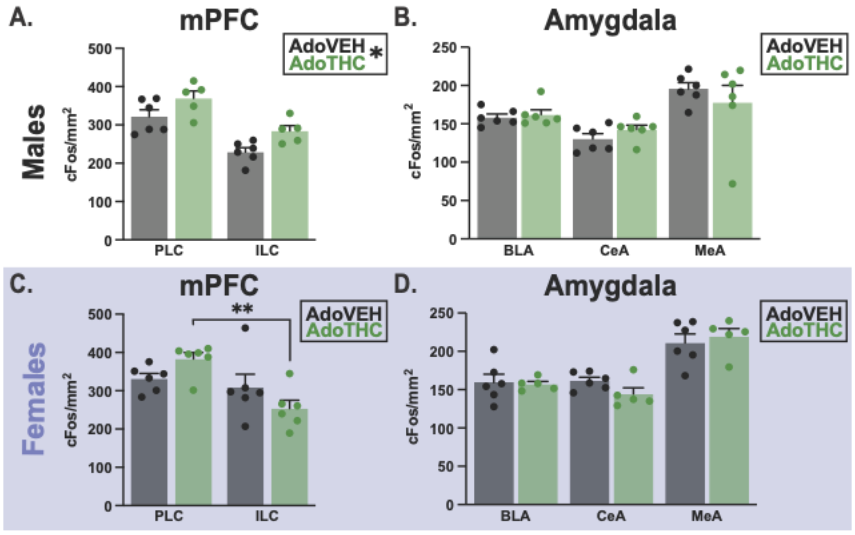


Figure 3.6. AdoTHC exposure sex-specifically alters mPFC neural activity after yohimbine in adulthood.
In mPFC, AdoTHC (green) exposure **A)** decreased cFos expression in males compared to AdoVEH-exposed males. **C)** In females, AdoTHC-exposure reorganized mPFC activation after yohimbine, not seen in AdoVEH-exposed females. There was no effect of AdoTHC exposure on neural activity within the **B)** amygdala in males, or **D)** females. Data represented as mean + SEM. *p < 0.05, **p< 0.01.

confirmed a sex-dependent effect of AdoTHC exposure across the mPFC, with cFos expression differing in a sex- and region-dependent manner following yohimbine (AdoTX x Sex x Region: $F_{(1, 19)}$=5.78, *p*=0.03), despite no main effect of AdoTHC (no main effect of AdoTX: $F_{(1, 19)}$=2.32, *p*=0.14). These findings indicate that following yohimbine, AdoTHC sex-dependently alters mPFC activation, but not amygdala.

Discussion

Here, we show that our AdoTHC exposure protocol (Torrens et al., 2020; Ruiz et al., 2021a; Torrens et al., 2022) produces lasting disruptions in threat responding and opioid motivation. In the shock rod task, AdoTHC-exposed animals exhibited reduced active defensive behavior toward a spatially localized threat, however during fear conditioning displayed sex-dependent responses. AdoTHC-exposed females exhibited heightened reactivity to the first presentation of the novel auditory tone prior to shock during fear acquisition and were more resistant to extinction, whereas AdoTHC-exposed males showed an attenuation in fear responding following cue presentations during acquisition and across extinction. In the remifentanil behavioral economics task, AdoTHC exposure decreased demand elasticity (i.e., increased motivation) in females, without affecting hedonic setpoint, while no changes were observed in males. Acute stressors prior to the session differentially altered opioid-taking behavior. Footshock stress decreased low-cost remifentanil consumption in males but did not affect females. In contrast, yohimbine increased both consumption and motivation for remifentanil in males, while selectively increasing motivation in AdoVEH exposed females. Finally, following an acute administration of yohimbine, AdoTHC exposure sex-dependently altered mPFC activity, but did not alter activity in the amygdala. Together, these findings provide insight into potential mechanisms underlying the association between adolescent cannabis use and contributing risk factors for neuropsychiatric disorders, such as addiction.

AdoTHC Differentially Alters Defensive Responding to Distinct Threats in Adulthood. In the shock rod task, animals confront a localized, spatially defined threat that is paired with shock,

mimicking ethologically relevant danger such as a predator invading a rat's burrow (Pinel and Treit, 1978; Craft et al., 1988; De Boer and Koolhaas, 2003; Fucich and Morilak, 2018). In this situation, when escape is not possible, rats can engage in treading behavior to bury or block the threat with any available material. AdoTHC exposure selectively attenuated active defensive responding, which persisted on the following expression day, although this was more pronounced in females. Notably, during acquisition, AdoTHC-exposed females investigated the shock rod more than counterpart controls, suggesting that AdoTHC-exposed females may have perceived the threat as less aversive or did not learn the association between shock and rod.

In contrast to the localized threat in the shock rod task, fear conditioning involves associating a temporally predictive auditory cue with an impending footshock. During fear acquisition, AdoTHC-exposed females froze more during the first presentation of the auditory cue, prior to receiving any shock, yet both males and females ultimately acquire the cue-shock association. This is consistent with prior work showing no effect of AdoTHC exposure on a single session of cued fear acquisition in either sex (Jouroukhin et al., 2019; Saravia et al., 2019; Smiley et al., 2021), although enhanced freezing has been reported following repeated sessions in males (Smiley et al., 2021). AdoTHC exposure did not alter contextual or cued fear expression; however, during extinction AdoTHC-exposed females were slower to extinguish the learned fear response, while males exhibited a general reduction in freezing. This is in contrast with prior work reporting no AdoTHC effects on cued fear extinction in either sex (Jouroukhin et al., 2019; Saravia et al., 2019), suggesting that this difference may be due to variability in AdoTHC exposure protocol or animal species. Importantly, AdoTHC exposure did not alter anxiety-like behavior in the elevated plus maze, suggesting that the alterations in threat-responding seen here may not be due to differences in anxiety.

Interestingly, the shock rod task heavily implicates mPFC circuitry (Shah and Treit, 2003; Shah et al., 2004), however the BLA also contributes (Roozendaal et al., 1991; Treit et al., 1993; Treit and Menard, 1997; Maren, 2003; Saldívar-González et al., 2003). Given that prior

work has shown that AdoTHC exposure may alter prefrontal top-down control (Renard et al., 2016; Renard et al., 2017a; Renard et al., 2017b), disruption of flexible, context-appropriate defensive responding may be one possibility for the altered threat responding observed. However, we cannot fully disambiguate here whether this alteration reflects a mPFC-dependent failure to integrate threat information and update behavior accordingly or whether threat appraisal is altered at the level of emotion- and motivation-related circuits.

A notable finding across tasks was that AdoTHC-exposed females exhibited divergent responses to two different types of threats. This may suggest that discrete cues predicting imminent, inescapable threat may engage emotional processing differently than physically localized threats that require immediate action in AdoTHC females. Interestingly, recent work has shown that BLA dopamine responses are more robust in females than males during the learning of novel threat and safety cues, prior to stable cue-outcome formation (Brickner et al., 2025). This more broadly raises the possibility that AdoTHC in females may be altering brain circuits important for encoding emotionally salient stimuli such as the amygdala, insula, or paraventricular nucleus of the thalamus (Davis and Whalen, 2001; Menon and Uddin, 2010; Zhu et al., 2018), and warrants direct investigation in future work.

AdoTHC Exposure Produces Enduring Alterations in Stress-Modulated Opioid Motivation. Evidence from our lab and others have shown that AdoTHC exposure enhances later life opioid reward and motivated behaviors (Solinas et al., 2004; Ellgren et al., 2007; Spano et al., 2010; Tomasiewicz et al., 2012; Stopponi et al., 2014; Cadoni et al., 2015; Lecca et al., 2020; Nguyen et al., 2020; Ferland et al., 2023b). Here, we replicated prior findings demonstrating that AdoTHC exposure in females reduced demand elasticity (i.e. increased motivation) without altering hedonic setpoint for remifentanil, an effect not seen in males. Given that perceived threat and stress can shape motivated behavior, alterations to these processes can have downstream consequences for opioid seeking under stress.

Effects of AdoTHC and Footshock Stress on Opioid Demand in Adulthood. In males footshock reduced remifentanil consumption without altering motivation, whereas footshock had no effect in females. The absence of a motivation effect contrasts with footshock reinstatement models that may more directly index motivation, reporting increased opioid-seeking following footshock (Shaham and Stewart, 1994; Shaham et al., 2003; Berridge et al., 2009; Mantsch et al., 2016). Importantly, animals received footshock stress during ongoing opioid self-administration compared to reinstatement which assesses drug seeking after extinction, suggesting that the differences observed here may be due to the animals' behavioral drug state.

Notably, all animals underwent opioid self-administration in operant chambers that they had previously received cued footshocks in during fear conditioning. Here, footshock-induced stress was unpredictable, which in males led to a reduction in remifentanil consumption, possibly suggesting that unpredictable footshock induced a heightened vigilant state that did not allow for increased consumption, despite leaving motivation for remifentanil intact. In contrast, females were unaffected by footshock, despite showing altered defensive responses to predictive footshock cues in fear conditioning. This pattern is consistent with research showing that female rats increase opioid self-administration more following predictable footshock rather than unpredictable (Klein et al., 1997), although we did not directly assess predictable versus unpredictable footshock. This finding does raise an interesting question of whether the predictability of threat, rather than the threat itself, may alter opioid motivated behavior instead. Additionally, our AdoTHC-exposed females exhibited enhanced behavioral demand for remifentanil at baseline, and footshock stress did not potentiate this effect. However, it is unclear given the heightened motivation at baseline, whether AdoTHC-exposed females were unaltered by footshock stress, or whether it failed to further potentiate motivation because animals were already operating at a floor.

AdoTHC and Yohimbine Stressor Alters Opioid Motivation in Females. We used the pharmacological stressor yohimbine, which acts on the noradrenergic system to increase

release of norepinephrine throughout the brain (Ivanov and Aston-Jones, 1995; Jabir et al., 2022; Porrill et al., 2024), especially within the mPFC impairing function, while enhancing amygdala responsivity (Arnsten, 2000, 2009; Arnsten, 2011; Arnsten, 2015). Under stress this shift in control from prefrontal to subcortical motivational and emotional circuits may be necessary to survival, but detrimental when needed to exert cognitively taxing executive functions (Arnsten, 2000, 2009; Arnsten, 2011; Arnsten, 2015). Therefore, given that mPFC may be compromised under yohimbine, we wanted to examine whether yohimbine would potentiate maladaptive opioid seeking in AdoTHC-exposed animals.

Acute administration of yohimbine increased remifentanil consumption in males, and motivation in both sexes. This is consistent with prior work showing yohimbine induces reinstatement of opioid seeking (Banna et al., 2010; Campbell et al., 2012; Zhou et al., 2013), and AdoTHC potentiation of yohimbine-induced heroin reinstatement seen in adult male rats (Stopponi et al., 2014).

Similarly, in females, yohimbine increased motivation for remifentanil in AdoVEH animals to levels comparable to elevated baseline levels of motivation in AdoTHC-exposed females. This may reflect a stress-like motivational state at baseline in AdoTHC-exposed females where the addition of a stressor, like yohimbine or footshock, does not further increase motivation. Indeed, AdoTHC exposure alters the stress system at both physiological and transcriptional levels. Although only examined in males, moderate (5mg/kg) exposure to THC in adolescence produces elevated basal corticosterone levels in adult rats, and recruits gene-networks related to stress signaling in the BLA, that were amplified following adult stress (Ferland et al., 2023b), providing some evidence that AdoTHC exposure may persistently increase stress systems in adulthood. Alternatively, rather than reflecting AdoTHC-induced dysregulation of the stress system, AdoTHC exposure may be persistently altering circuits that the noradrenergic stress system engages, especially the mPFC and amygdala (Arnsten, 2009, 2015), producing a state that resembles stress-circuit engagement. It is worth noting that the absence of enhanced

behavioral responding may again reflect a floor effect, where behavioral demand has reached its lowest detectable point in our task, which limits what we are able to conclude from our data alone. Nevertheless, the convergence of AdoVEH-exposed females under yohimbine and AdoTHC-exposed females at baseline onto a shared motivational state for remifentanil suggests that AdoTHC exposure in females may be producing a 'stress-like' drive for opioids.

Neural Activation and Behavior Following Yohimbine. To better understand the neural mechanisms underlying our observed yohimbine-induced behavioral effects, we assessed cFos expression and behavior following acute yohimbine in behaviorally naïve AdoTHC-exposed animals. While ultrasonic vocalizations were largely unaffected, AdoTHC exposure increased delta frequency in males following yohimbine, suggesting subtle alterations in affective state.

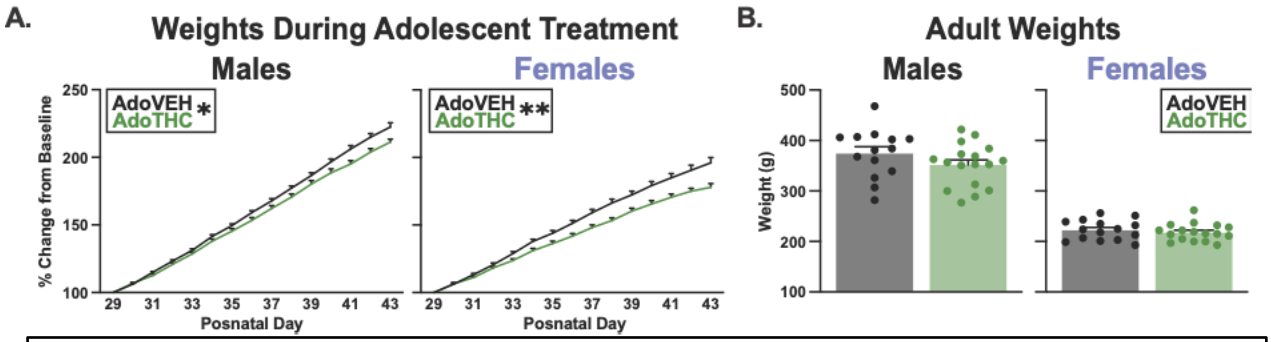
Following yohimbine, AdoTHC exposure in females produced a subregion-specific shift in mPFC activation, with increases in PLC and decreases in ILC activity, whereas in males AdoTHC exposure increased mPFC activation across both PLC and ILC. Yohimbine has been shown to impair PFC executive functions in both humans and rats (Caetano et al., 2012; Chamberlain and Robbins, 2013; Schwager et al., 2014), therefore it may not be surprising that AdoTHC altered mPFC activation. However, yohimbine also engages the amygdala (Buffalari and Grace, 2009; Gozzi et al., 2013; Visocky et al., 2025), but we did not observe any AdoTHC-induced alterations in this region. Together, these findings suggest that AdoTHC exposure may preferentially alter mPFC circuitry; however, what these alterations mean in relation to mPFC function, or how they relate to our opioid motivated behaviors, remains unclear.

Limitations. These studies have several limitations that should be considered. Threat exposure was limited to shock across distinct threat types, and future work would benefit from incorporating non-cued or ethologically relevant threat models (e.g. looming shadow, predator odor). We used yohimbine to induce stress-modulated opioid taking, however, recent work suggests that yohimbine may act as reinforcer, as animals willingly self-administer yohimbine and can elicit conditioned place preference (Renda and Leri, 2025). Here, we used a single,

moderate dose of THC (5 mg/kg, IP), yet the persistent effects of THC are known to be dose-dependent (Amal et al., 2010; Irimia et al., 2015; Freeman-Striegel et al., 2023), and sensitive to the developmental timing of exposure (Gorey et al., 2019; Torrens et al., 2020; Murray et al., 2022; Torrens et al., 2022). Additionally, female rodents exhibit greater blood and brain levels of THC's active metabolites (Tseng et al., 2004; Wiley and Burston, 2014; Craft et al., 2019; Torrens et al., 2020; Baglot et al., 2021; Ruiz et al., 2021a; Ruiz et al., 2021b; Torrens et al., 2022), a pattern that has also been reported in humans (Matheson et al., 2020; Sholler et al., 2021; Arkell et al., 2022), and may contribute to the more robust and persistent effects of AdoTHC seen in females here, and in prior reports (Tseng et al., 2004; Cha et al., 2007; Rubino et al., 2009b; Rubino and Parolaro, 2011; Ruiz et al., 2021a; Ruiz et al., 2021b; Le et al., 2022; Martinez et al., 2025).

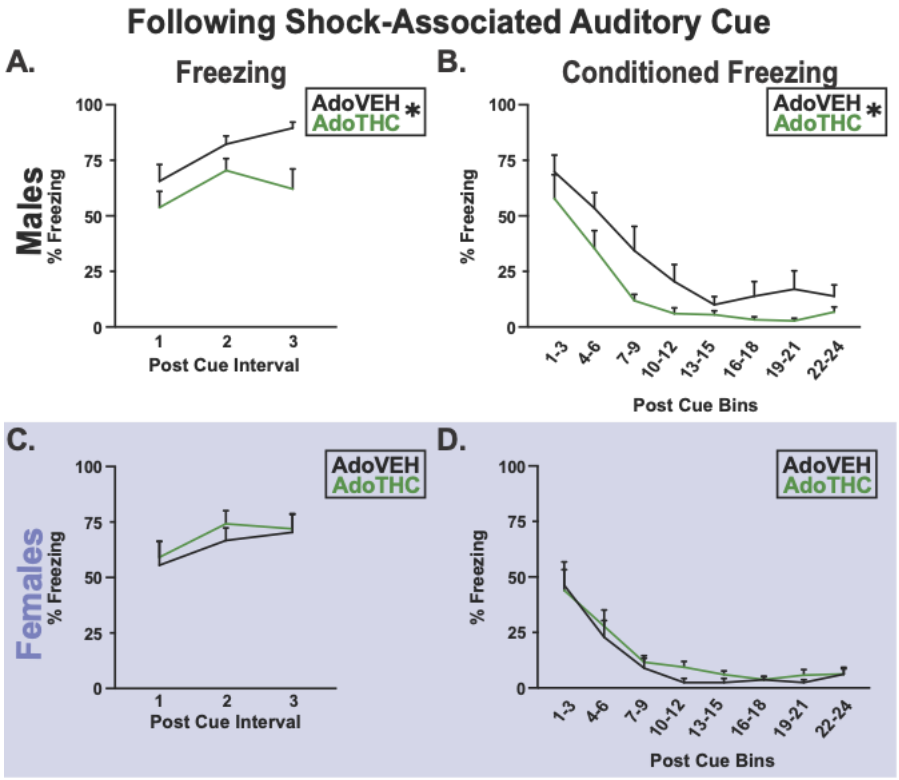
Summary. In sum, these findings demonstrate that AdoTHC may persistently alter sensitivity to different types of stressors faced later in life, and potential new mechanisms that be involved in these effects such as lasting circuit dysfunction that resembles noradrenergic stress system engagement. Additionally, we see that females may be especially vulnerable to these AdoTHC induced effects. Therefore, determining the mechanisms by which AdoTHC exposure persistently alters sensitivity to stressors may provide a greater understanding of the contributing risk factors to neuropsychiatric disorders and guide targeted preventative and intervention strategies to mitigate these risks.

Supplementary Figure 3.1



Supplementary Figure 3.1. AdoTHC transiently attenuates body weight gain. **A**) Adolescent administration of THC from postnatal days 30-43 (green) suppresses weight compared to VEH treatment in the same period (black) in females (left) and males (right). **B**) By adulthood (~PD70), body weight no longer differs between rats that received AdoVEH and AdoTHC. Data represents mean ± SEM. * $p < 0.05$, ** $p < 0.01$.

Supplementary Figure 3.2



Supplementary Figure 3.2. AdoTHC reduces freezing following cue presentations in males, but not females.
A) AdoTHC (green) males exhibit a reduction in freezing following cue presentations during fear acquisition **B**) and fear extinction compared to AdoVEH (black) males. AdoTHC females did not exhibit freezing following cues during **C**) acquisition or **D**) extinction. Data represents mean ± SEM. * $p < 0.05$.

General Discussion

The overall results of this dissertation demonstrate that AdoTHC exposure persistently alters behavioral and neural responses across three domains: cognition, drug reinforcement, and motivation in male and female rats. In the domain of cognition, AdoTHC did not produce any lasting overt baseline deficits in medial prefrontal cortical (mPFC)-dependent cognitive flexibility or on a probability-based decision-making task; instead, impairments in decision-making were only revealed under acute challenge with amphetamine. In the domain of drug reinforcement, AdoTHC exposure altered opioid reward in both sexes, with especially pronounced enhancements in opioid reward and seeking in females. AdoTHC additionally produced distinct patterns of altered region- and network-level cFos expression in males and females, and acutely altered mPFC microglia in both sexes. In the domain of motivation, AdoTHC exposure produced alterations in response to aversive stimuli and the cues predicting them in a sex-dependent manner. Notably, despite sex-dependent brain responses to pharmacological stressors, AdoTHC did not alter physical or pharmacological stress-induced opioid seeking. Across several domains, these behavioral effects were more pronounced in AdoTHC-exposed females than in males, extending the literature that has been predominantly male-biased, and providing greater mechanistic insight into the circuits that may be underlying sex-specific vulnerability.

These results collectively highlight that AdoTHC exposure persistently impairs behavioral and neural responses involved in these three domains and often promotes maladaptive responses when faced with challenges later in life such as drugs of abuse and aversive stimuli. Maladaptive responses within any single one of these domains may not be sufficient to drive addiction vulnerability, but the *collective* impairments across cognition, drug reinforcement, and motivation in adulthood together point toward a brain predisposed to addiction.

Persistent Effects of Adolescent THC Exposure Across Domains.

Persistent Behavioral Effects of Adolescent THC on Cognition. The mPFC promotes goal-directed behavior, and this process relies heavily on executive functions, including our ability to update behavior when rules and contingencies change (cognitive flexibility) and making decisions under uncertainty (Miller and Cohen, 2001; Floresco et al., 2008b; Friedman and Robbins, 2022). Impairments in executive functions can lead to aberrant behavior, as such, both cognitive flexibility and decision-making have been implicated in a wide variety of neuropsychiatric behaviors, such as addiction (Rahman et al., 2001; Volkow et al., 2010) and schizophrenia (Aumer et al., 2024). Additionally, AdoTHC exposure is linked to later life changes in cognition, and animal models suggest this could be a causal effect of THC influencing adolescent development of circuits underlying executive functions, especially in the mPFC.

Along these lines, my work found that AdoTHC exposure produced lasting, distinct cognitive effects in females and males. While AdoTHC exposure did not alter rats' ability to flexibly shift to a new rule, AdoTHC exposure in females showed a specific enhancement in initial learning of a visual cue rule, although all animals learned this rule. When assessing AdoTHC effects on probability-based decision-making, AdoTHC-exposed animals performed comparably to adolescent vehicle (AdoVEH) animals. However, when dopamine circuits were acutely challenged with amphetamine during this task, AdoTHC-exposed animals exhibited greater insensitivity to losses leading to a reduced discounting of improbable reward options. Notably, this effect was more pronounced in females; however, when examining the strategies the animals employed under amphetamine, AdoVEH-exposed females increased sensitivity to negative consequences, shifting toward a safer option following a 'risky' loss, whereas this response was eliminated in AdoTHC-exposed females, instead staying on the 'risky' lever, like males. Therefore, rather than just potentiating an effect, AdoTHC exposure in females appears to wipe out a protective female-specific behavioral response, instead leading AdoTHC females to act more like males under amphetamine.

These results are consistent with the idea that AdoTHC exposure may not produce overt cognitive impairments under baseline conditions but instead produce latent changes that may be compensated for through other systems and only emerge when the brain is sufficiently challenged. Moreover, this suggests that standard cognitive tasks may not capture the full enduring impact of AdoTHC exposure, and may need a “second-hit”, such as with pharmacological or environmental insults, to capture persistent cognitive impairments. This necessity of a second-hit to unmask AdoTHC-induced vulnerability seemingly parallels the “two-hit” hypothesis in humans that suggests that the additive effect of insults in adolescence and adulthood may contribute to the onset of neuropsychiatric disorders, such as schizophrenia (Maynard et al., 2001; Corsi-Zuelli et al., 2022; Guma et al., 2023).

Persistent Effects of Adolescent THC Exposure on Drug Reinforcement. Preclinical studies show that AdoTHC conveys later life susceptibility to opioids (Solinas et al., 2004; Ellgren et al., 2007; Tomasiewicz et al., 2012; Stopponi et al., 2014; Cadoni et al., 2015; Lecca et al., 2020; Nguyen et al., 2020; Ferland et al., 2023b; Hubbard et al., 2024); however this effect has been disproportionately seen in males due to a lack of female inclusion. My work confirms and extends this finding, demonstrating that AdoTHC exposure causes a lasting sex-dependent vulnerability to opioids in rats. AdoTHC exposure did not uniformly enhance all aspects of opioid reward, as we observed that AdoTHC exposure enhanced heroin conditioned place preference overall, an effect that was more prominent in females, and AdoTHC-exposed females exhibited enhanced acquisition of remifentanil self-administration and economic demand for remifentanil. AdoTHC exposure did not induce alterations in heroin locomotor sensitization, consumption of remifentanil in our behavioral economic demand task, or locomotion following acute heroin administration.

Together, this pattern of findings reflects a behavioral profile in which AdoTHC exposure persistently enhances the motivation to seek and obtain opioids, rather than increasing the inherent pleasurable effects of opioids, especially in females. Therefore, AdoTHC exposure may

induce lasting changes in motivational circuits governing opioid reward, such that subsequent opioid insult in adulthood produces an enhanced motivational response, thereby increasing susceptibility to opioid use, and females may be more vulnerable to this effect. Moreover, despite the criticisms of the cannabis 'gateway effect' in humans, our findings and those of other groups show a robust pro-opioid phenotype, across labs and behavioral models, suggesting a biological component underlying this effect.

Persistent Effects of Adolescent THC on Motivation. In humans and animals, aversive stimuli and their associated negative outcomes often redirect future behavior so that they are avoided (Elliot, 1999; Pessoa and Adolphs, 2010; Verharen et al., 2020). These aversive stimuli can range from discrete environmental threats to broader stressors, and disruptions to how they are processed and subsequently responded to may drive maladaptive behavior. Thus, impairments in these processes, such as altered threat responsivity or sensitivity to stressors, may then be particularly relevant to addiction, as failure of adverse consequences to rein in maladaptive behavior can drive sustained drug use despite negative consequences, which is a hallmark feature of addiction (Everitt and Robbins, 2005, 2016; Koob and Volkow, 2016; Verharen et al., 2020).

Along these lines, my work found that AdoTHC exposure produced lasting alterations in responding toward a localized shock-rod, and a sex-dependent difference toward a temporally predictive cue (auditory cue predicting footshock). AdoTHC-exposed males and females exhibited a shared attenuation in active fear responding towards an electrified shock-rod, including in a post shock test in which the rod was deactivated and thus served as a shock cue. However, AdoTHC-exposure sex-dependently altered the behavioral responses during fear conditioning. During fear acquisition, AdoTHC-exposed females exhibited heightened freezing to an auditory stimulus prior to shock delivery, and this was not observed in AdoTHC-exposed males. During fear extinction, AdoTHC-exposed females exhibited resistance to extinguishing

the learned threat cue, whereas AdoTHC-exposed males exhibited reduced fear responding during extinction.

Collectively, these findings demonstrate persistent sex-dependent effects of AdoTHC exposure on responses toward aversive stimuli, yet a standout from these findings is that while AdoTHC exposure altered behavioral responding across the board, in AdoTHC-exposed females the direction of responding was different, whereas AdoTHC-exposed males regardless of aversive stimuli showed an overall attenuation in responding. One possibility for this divergent response in AdoTHC females may be that they are more sensitive to certain types of aversive stimuli, that is, discrete cues versus a spatially localized cue. Alternatively, the type of response elicited matters, as the shock rod induces an active treading response whereas fear conditioning induces a passive freezing response. Future work using different types of aversive stimuli, such as in the looming shadow or predator odor task, would better help triangulate the underlying mechanism driving this divergence.

Beyond threat responding, despite not further altering opioid motivation under pharmacological stress with yohimbine, AdoTHC-exposed females and AdoVEH females converged on a shared elevated motivational state for opioids. AdoTHC exposure did not alter yohimbine-induced consumption or behavioral demand for remifentanil in males, while AdoTHC females exhibited enhanced baseline motivation for opioids that the pharmacological stressor yohimbine in AdoVEH females mimicked. This suggests that noradrenergic stress engagement and AdoTHC exposure may independently converge on the same motivational state for opioids. This raises the possibility that, in females, AdoTHC exposure may cause an enduring shift in the set point at which stress systems are engaged. This type of interpretation is interesting, as while we did not observe any effects of yohimbine in AdoTHC or AdoVEH-exposed males, prior research has shown that AdoTHC-exposed males exhibit elevated basal corticosterone levels in adulthood, and stronger engagement of gene-networks related to stress signaling within the BLA following overnight isolation stress (Ferland et al., 2023b); however, we do not know if this

is also true in females as they were not included in this study. Alternatively, AdoTHC may dysregulate the same circuits that are perturbed under stress, most notably the amygdala and mPFC, such that AdoTHC-exposed females may operate in a state that resembles the effects of stress (Arnsten, 2000, 2009; Arnsten, 2011; Arnsten, 2015). Regardless, these findings suggest possible overlapping circuit mechanisms that may converge on a shared pathway that underlies the AdoTHC enhanced motivational state for opioids seen at baseline in females. Additionally, this possibility, has implications for informing strategies used to mitigate stress precipitated relapse or addiction vulnerability as these strategies may very well look different in females than in males.

Persistent Adolescent THC-induced Brain Changes.

Circuit-Level Contributions to AdoTHC-Induced Cognitive Changes. Circuit-level investigation of AdoTHC-induced cognitive changes implicated mechanisms beyond VTA dopamine neurons and their projections to the mPFC, instead pointing toward the broader catecholamine system. While AdoTHC-exposure potentiated amphetamine 'risky' responding on the probabilistic discounting task, chemogenetic stimulation of VTA dopamine neurons and their projections to mPFC did not recapitulate these effects. This null result suggests that AdoTHC-exposure may alter amphetamine-induced cognitive changes in a manner independent of VTA dopamine neuron signaling, and instead may point to broader circuit-level mechanisms. Therefore, future work should examine other systems that amphetamine can additionally recruit, such as norepinephrine, which has a known role in supporting PFC executive functions (Robbins and Arnsten, 2009).

Adolescent THC-induced Brain Changes in Drug Reinforcement. Sex-specific AdoTHC effects on cFos regional activity and functional connectivity expression following heroin in opioid-experienced rats suggest preferentially altered frontal cortical networks in males, and amygdala-related networks in females. In females, AdoTHC exposure increased engagement of amygdala subregions including basolateral amygdala (BLA) and central amygdala (CEA), and enhanced

BLA connectivity with the broader network, while in males, AdoTHC exposure increased engagement of mPFC subregions, including prelimbic (PLC) and infralimbic cortex (ILC), but decoupling from the broader network. Together, these neural profiles suggest that AdoTHC alters the communication within aversive-, emotion-, and reward-related brain circuits, a disruption that was not fully apparent from regional activity alone. While both sexes show distinct neural profiles under heroin, these findings raise more questions than they answer: What is the relationship between these neural profiles observed following acute heroin and the pro-opioid behavioral phenotype in females, or limited phenotype in males? Under what conditions, if any, would we be able to map the pro-opioid behavioral phenotype to the neural profile? Would these neural patterns be different if instead of a history of opioid exposure in adulthood we examined neural patterns to the first insult of adulthood heroin? Nonetheless, these sex-specific neural patterns identify candidate brain circuits that may be involved in AdoTHC-induced vulnerability to opioid reward in adulthood and warrant targeted future investigation.

Adolescent THC-induced Brain Changes in Motivation. AdoTHC induces lasting, sex-dependent alterations in the pattern of mPFC activation under noradrenergic stress recruitment. cFos activity was assessed within the mPFC and amygdala subregions following pharmacological challenge with yohimbine in behaviorally naïve AdoTHC-exposed adult rats. While AdoTHC did not induce amygdala neural activity in either sex, AdoTHC-exposed females exhibited a subregion-specific shift in cFos activity, increased PLC and decreased ILC, whereas in AdoTHC-exposed males, cFos activity was increased across both mPFC subregions. Together, these findings indicate that AdoTHC-exposure alters mPFC activation under noradrenergic stress system engagement in a sex-dependent manner. While we had observed that AdoVEH females under pharmacological stress mimic the elevated baseline motivation for opioids observed in AdoTHC females, it is unclear what the nature of the relationship is between these findings and altered mPFC activity under pharmacological stress. Additionally, while we

observed alterations in mPFC in AdoTHC males, there was no difference in opioid-motivated behavior seen across treatment groups. Therefore, these findings suggests that AdoTHC exposure alters mPFC activation under pharmacological stress insult; however, how this relates to stress-induced opioid motivated behavior or whether the type of stressor matters is unclear.

Persistent Effects of Adolescent THC on the Brain & Behavior: What Might Drive Them?

Adolescence represents a period of extensive neurodevelopment, marked by profound structural and functional alterations within the brain's architecture and synaptic circuitry, especially in the PFC and its connections with limbic regions such as the amygdala and hippocampus (Caballero et al., 2016). This coordinated maturation supports the emergence of adult executive and emotional functions (Casey et al., 2000; Miller and Cohen, 2001; Casey et al., 2008; Ott and Nieder, 2019); however, it also renders the brain vulnerable to external insults, such as THC. Thus, how might THC exposure during adolescence lead to the behavior and brain changes seen later in adulthood?

The eCB System as a Possible Mechanism Underlying AdoTHC-induced Alterations in the Brain. The endocannabinoid (eCB) system plays an important regulatory role in synaptic plasticity and excitatory/inhibitory balance during circuit refinement (Freund et al., 2003; Kano et al., 2009; Castillo et al., 2012), while also undergoing its own significant refinement during adolescence (de Fonseca et al., 1993; Ellgren et al., 2008; Bara et al., 2021; Peters et al., 2022; Rodrigues et al., 2024). The active maturation of this system and its contributions to greater circuit refinement thus open a critical sensitive period during which AdoTHC can produce its lasting effects. Indeed, chronic AdoTHC exposure leads to an overall downregulation and desensitization in CB_1Rs throughout brain regions, with some effects extending into adulthood (Ellgren et al., 2008; Rubino et al., 2008; Burston et al., 2010; Rubino et al., 2015; Silva et al., 2015; Farquhar et al., 2019; Kruse et al., 2019; Poulia et al., 2021).

Notably, enhancement of eCB signaling can restore behavioral and neural abnormalities persistently produced by AdoTHC exposure. In AdoTHC-exposed females, increasing the

availability of anandamide, an endogenous eCB ligand, in adulthood restores abolished PFC eCB-mediated long term depression and decreases in CB_1Rs (Rubino et al., 2015; Cuccurazzu et al., 2018), functional neurogenesis within hippocampal dentate gyrus newborn neurons (Cuccurazzu et al., 2018), and reverses a “depressive”-like phenotype in adulthood (Realini et al., 2011; Cuccurazzu et al., 2018). Moreover, this restorative effect is blocked by concurrent CB_1R blockade, suggesting the necessity of CB_1Rs in this effect (Cuccurazzu et al., 2018). Together, these findings suggests that AdoTHC-exposure may produce persistent dysregulation in eCB-mediated signaling rather than irreversible developmental impairment, at least in females. Endocannabinoid system manipulations may then be an interesting avenue to further explore and raises several questions: Could enhancing eCB signaling ameliorate the AdoTHC-induced effects seen on cognition, drug reinforcement, or responding to aversive stimuli? Does the type of exogenous insult matters, and how does sex play a role?

Microglia as a Possible Mechanism Underlying AdoTHC-induced Alterations in the Brain. Microglia, the resident immune cells of the brain, aid in shaping neural connectivity, synaptic remodeling, and regulation of neural and synaptic activity, especially in developmental sensitive periods like adolescence (Mallya et al., 2019; Dziabis and Bilbo, 2021; Schalbetter et al., 2022; von Arx et al., 2023; Durán Laforet and Schafer, 2024; Martinez and Mahler, 2025). Microglia are also closely linked with the eCB system, expressing both cannabinoid receptors (Pocock and Kettenmann, 2007) and eCB signaling machinery (Walter et al., 2003; Carrier et al., 2004; Witting et al., 2004; Muccioli et al., 2007; Gabrielli et al., 2015; Mecha et al., 2015). Furthermore, disrupting CB_1R signaling on microglia prevents AdoTHC-induced alterations in microglia, including apoptosis and gene expression (Lee et al., 2022; Hasegawa et al., 2023). Additionally, chronic AdoTHC exposure persistently alters microglia morphology in the PFC (Zamberletti et al., 2015; Gabaglio et al., 2021; Freels et al., 2023) and hippocampus (Lee et al., 2022), in a sex-dependent manner. Similarly, I found that chronic AdoTHC exposure acutely alters microglia morphology within the mPFC of both sexes (Chapter 2).

Together, these findings suggest that AdoTHC exposure induces both functional and structural changes in microglia during adolescence and in the adult brain. Given the role of microglia in synaptic remodeling and circuit refinement in adolescence, this alteration may represent a potential mechanism through which AdoTHC disrupts the maturation of developing circuits, leading to long-term changes in the brain and behavior. However, whether these microglial alterations contribute to the AdoTHC-induced effects on brain function and behavior remains unknown, though chemogenetic manipulation (Coffey et al., 2022; Raymundi et al., 2026) or acute pharmacologic depletion and repopulation (Elmore et al., 2015; Green et al., 2020) methods can allow for investigation of the necessity of microglia in these behaviors and whether 'resetting' microglia may reverse these effects. Given their role in shaping the adolescent brain, microglia may be a promising candidate underlying the lasting effects of AdoTHC on the brain and behavior, warranting future investigation.

Multiple Interacting Mechanisms Underlying Persistent AdoTHC Effects. Although the eCB system and microglia represent two plausible mechanisms through which AdoTHC exposure may produce long-term neural and behavioral alterations, they are not the only systems vulnerable to AdoTHC exposure. AdoTHC likely disrupts other systems as well, such as dopamine, which innervates the PFC during adolescence (Hoops and Flores, 2017; Peters et al., 2022), and GABA, which undergoes maturation during this period (Caballero et al., 2016). Consistent with this, AdoTHC has been shown to persistently alter both GABA expression and VTA dopaminergic activity (Renard et al., 2017a; Renard et al., 2017b), and these alterations are likely interconnected.

Although the studies in this dissertation did not directly manipulate these possible mechanisms, it is likely that AdoTHC disrupts multiple interacting developmental processes. Determining how these processes collectively contribute to the lasting effects of AdoTHC exposure, and whether they play a different role in each sex, remains an important challenge for future work.

Persistent Effects of Adolescent THC on the Brain & Behavior: Where Does This Leave Us? The studies in this dissertation examined distinct behavioral and neural outcomes following AdoTHC exposure in adulthood across three domains: cognition, drug reinforcement, and motivation. However, stepping back and assessing these findings collectively, reveal several overarching themes.

Persistent Effects of AdoTHC Exposure: A 'Two-Hit' Framework. The first major theme to emerge across studies is that AdoTHC exposure may induce latent vulnerabilities in the circuits that govern decision-making and appetitive and aversive motivation, which the adult brain may compensate for through alternate circuits. The result is that AdoTHC and AdoVEH animals appear indistinguishable at baseline. However, when the system was sufficiently challenged, a pattern arose across studies: amphetamine revealed risky decision-making, opioid exposure enhanced motivation for opioid drug-seeking, especially in females, and aversive stimuli revealed impaired adaptive responding. The implications of each of these findings ultimately converge on the same outcome of increased maladaptive responses in AdoTHC-exposed animals following an external challenge in adulthood. These findings map onto the 'two-hit' hypothesis, which suggests that both genetics and early environmental factors increase the risk of neuropsychiatric disorders. One of the strongest associations supporting this framework has been shown with early-life cannabis use and schizophrenia (Maynard et al., 2001; Corsi-Zuelli et al., 2022; Guma et al., 2023), and is further supported by the cannabis "gateway" effect (Yamaguchi and Kandel, 1984; Lynskey et al., 2003; Hall and Lynskey, 2005). Importantly, as the aversive threat findings show, the type of insult does not need to be pharmacological in nature. This invites the question of whether naturally occurring events, such as pregnancy, may be just as capable of unmasking these impairments. Therefore, identifying what types of insults later in life are sufficient to unmask AdoTHC-induced dysfunction has meaningful clinical implications for our understanding of the lasting consequences of adolescent cannabis use.

Preferential AdoTHC-induced Changes in mPFC Circuitry. The second theme to emerge across these studies is that the mPFC repeatedly appears as a site of AdoTHC-induced dysfunction. Direct measures of mPFC activity, via cFos, revealed that yohimbine produced sex-dependent alterations in mPFC activity in behaviorally naïve AdoTHC-exposed animals, and under heroin, AdoTHC-exposed males exhibited recruitment of mPFC activity but decoupling from the broader network. Behaviorally, AdoTHC-exposure increased risky decision-making under amphetamine, attenuated active defensive strategies toward a shock rod, and altered fear extinction, all of which require mPFC engagement. This convergence of persistent AdoTHC-induced alterations in mPFC neural activity and related behaviors is perhaps unsurprising given that the mPFC is actively developing during adolescence (Caballero et al., 2016) rendering it particularly susceptible to THC insult. However, the broader implications of these findings are that AdoTHC exposure may persistently compromise the ability of the mPFC to engage in executive functions that are important for goal-directed behavior and regulatory control over subcortical emotional and motivational circuits. This is of considerable concern as disruptions in these processes have been associated with greater vulnerability to neuropsychiatric disorders such as addiction (Goldstein and Volkow, 2011), post-traumatic stress disorder (Milad and Quirk, 2012), and impulse control disorders (Dalley et al., 2011).

Preferential AdoTHC-Induced Vulnerability in Developing Emotion Circuits. The third theme to arise across studies is that AdoTHC induces preferential changes within emotion-related systems. AdoTHC-exposed males and females exhibited altered threat responding on the shock rod task and fear conditioning, which require coordinated mPFC and amygdala communication. Beyond these shared findings, AdoTHC-exposed females exhibited additional alterations in emotion-related circuits and behaviors including increased BLA and CEA recruitment and broader BLA network connectivity under heroin, heightened reactivity to an auditory tone prior to shock during fear acquisition, and a motivational state for opioids that is recapitulated in AdoVEH females under yohimbine. Collectively, these findings suggest that AdoTHC exposure

may leave enduring alterations on the development of emotion-related circuitry. This notion aligns well with the active innervation process from the amygdala to the mPFC that occurs during adolescence, and is thought to facilitate the maturation of emotional regulation in adulthood (Caballero et al., 2016). Notably, these AdoTHC-induced alterations in emotion-related circuits may render females more vulnerable to emotional dysregulation, which is consistent with the literature suggesting AdoTHC induces a lasting 'emotional' behavioral phenotype in females (Rubino et al., 2008). This may have meaningful implications for the types of psychiatric outcomes associated with adolescent cannabis use as these may manifest differently in females than in males, with females exhibiting greater depression and anxiety (Calakos et al., 2017; Prieto-Arenas et al., 2022; Mostallino and Fattore, 2026). Sex should therefore be taken into consideration when assessing the vulnerability to psychiatric disorders in individuals with a history of adolescent cannabis use.

Sex as a Biological Variable in Enduring AdoTHC Outcomes. One of the most striking patterns across these studies is the pronounced and persistent nature of AdoTHC-induced sex differences, and there are several converging reasons why this may be the case. First, females and males carry different sex chromosomes that differ in gene content, ultimately resulting in brains that are built differently in males and females (Arnold, 2004; Cahill, 2006). Second, females enter puberty earlier than males (Spear, 2000), meaning AdoTHC exposure during an identical adolescent window will capture different pubertal windows in each sex. Additionally, during this window sex hormones are actively aiding in the organization of neural circuits and do so in a sex-specific manner (Sisk and Zehr, 2005; Juraska et al., 2013; Schulz and Sisk, 2016; Peper et al., 2020). Furthermore, these sex hormones also interact with neuromodulatory systems (Barth et al., 2015; McEwen and Milner, 2017), and are bidirectionally linked with the eCB system, as sex hormones can regulate eCB signaling and CB_1R expression, while eCBs can regulate hormone release (López, 2010; Paola Castelli et al., 2014; Struik et al., 2018;

Farquhar et al., 2019). Thus, AdoTHC exposure occurs during a period in which female and male brains are already interacting differently with the eCB system.

Third, females metabolize THC differently than males, resulting in greater levels of THC's active metabolites in the brain and blood that persist longer (Torrens et al., 2020; Ruiz et al., 2021a; Ruiz et al., 2021b; Torrens et al., 2022), suggesting that identical doses of THC may engage CB_1Rs more extensively in females, especially in regions such as the PFC and amygdala, where CB_1Rs are more densely expressed (Fitzgerald et al., 2012; Fitzgerald et al., 2019). Finally, eCB-mediated long-term depression is expressed earlier in females, while appearing at puberty in males (Bernabeu et al., 2023), suggesting that the circuits THC disrupts may be at different maturational stages in each sex.

Collectively, these factors indicate that AdoTHC exposure, despite the same dose and time window, is insulting two inherently different systems in males and females. Thus, appropriately characterizing the lasting effects of AdoTHC exposure requires doing so in both sexes, not only to extend preclinical research that tends to be male-biased but also because understanding how AdoTHC produces these lasting divergent responses can inform targeted preventative and intervention strategies for each sex.

Conclusion. The present dissertation supports the conclusion that AdoTHC exposure produces persistent impairments across cognitive, drug reinforcement, and motivational domains, often in a sex-dependent manner. While alterations in any single domain do not necessarily confer addiction vulnerability, a brain in which decision-making is compromised, motivation for opioids is augmented, and adaptive behavioral responding to aversive stimuli is impaired is a brain predisposed to engage in maladaptive behaviors. Therefore, when taken together, these findings suggest that AdoTHC exposure is enduringly shaping the landscape of the adult brain across multiple domains in a way that increases predisposition to addiction later in life. Thus, as the availability of cannabis and adolescent cannabis use continue to rise, understanding the neurobiological underpinnings of these lasting effects is of great public health concern.

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