The Tea on THC: How Exposure in Early Adolescent Male and Female Mice Leads to Divergent Outcomes on Dopamine Development and Adult Cognition

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Abstract

Exposure to delta 9 - tetrahydrocannabinol (THC) – the principle psychoactive constituent of cannabis – is steadily increasing among youth. This raises concerns because core neurodevelopmental processes are occurring during adolescence, including long-distance axonal pathfinding. Specifically, dopamine axons continue to grow from the striatum towards the prefrontal cortex across adolescence, promoting the refinement of inhibitory control. This growth is controlled by the Netrin-1 guidance receptor, DCC (deleted in colorectal cancer), whose expression in dopamine neurons is regulated by the microRNA, miR-218. In addition, cortical refinement is highly influenced by microglial action. I treated adolescent male and female C57/Bl6 mice or Cx3Cr1$^{GFP/+}$ (postnatal day 22) with 5 intraperitoneal injections of vehicle or THC 5 mg/kg once every other day and brains were processed to quantify the extent and organization of dopamine connectivity within frontal cortical regions. Results show that repeated exposure to THC in early adolescence leads to sex-specific effects in adult cortical dopamine connectivity. Were females show no changes, but males show a robust reduction in the extent of the dopamine input to the prefrontal cortex and in the number of dopamine axon presynaptic sites in this region. In contrast, within the OFC – a region involved in action inhibition – there is an increase in the density of dopamine presynaptic sites. These male-specific and dichotomous effects raise the intriguing possibility that THC in adolescence triggers dopamine axon mistargeting, deviating the growth of prefrontal cortex dopamine axons, to orbitofrontal regions. Next, I investigated if these adult dopaminergic changes, or lack thereof in females, would lead to changes in adult impulse control. In adulthood separate mouse cohorts were treated in adolescence and tested in the Go/No-go task to measure impulse control. Compared to their vehicle-treated counterparts, adult males exposed to THC in adolescence show (i) increased premature responding in the Go/No-go task, which is

reflective of impaired waiting impulsivity, but (ii) reduced number of commission errors, indicating improved action inhibition. None of these effects were observed in females. In addition, molecular changes associate with differential THC effects on mesocortical dopamine and cognitive maturation in male but not female mice. Males show increased *Dcc* and decreased miR-218, suggesting that in males, adolescent THC exposure induces changes via DCC/miR-218. Females have decreased *Dcc* but show no changes in miR-218, however, display an altered mPFC microglia profile, possibly buffering against the effects of decreased *Dcc* on dopamine.

These findings show that THC in early adolescence impacts dopamine and impulse control development in males, but not in females, and divergent molecular and epigenetic processes are dysregulated. DCC-mediated events may mediate outcomes in males, but in females, compensatory mechanisms may be recruited.

Résumé

L'exposition au delta 9 – tétrahydrocannabinol (THC) – le principe psychoactif principal du cannabis – est en augmentation constante chez les jeunes. Cela soulève des inquiétudes, car des processus neurodéveloppementaux fondamentaux se produisent pendant l'adolescence, notamment la migration d'axones sur de longues distances. En particulier, les axones dopaminergiques continuent de croître du striatum vers le cortex préfrontal durant cette période, promouvant le renforcement du contrôle inhibiteur. Cette croissance est contrôlée par le récepteur de guidage DCC (supprimé dans le cancer colorectal), dont l'expression dans les neurones dopaminergiques est régulée par le microARN miR-218. J'ai traité des souris adolescentes mâles et femelles C57/Bl6 (jour postnatal 22) avec 5 injections intrapéritonéales de véhicule ou de THC 5 mg/kg une fois tous les deux jours et les cerveaux ont été traités pour quantifier l'étendue et l'organisation de la connectivité dopaminergique au sein d'une analyse corticale frontale ou moléculaire. Les résultats montrent qu'une exposition répétée au THC au début de l'adolescence entraîne des effets spécifiques au sexe sur la connectivité dopaminergique corticale de l'adulte. Les femelles ne montrant aucun changement, mais les mâles présentent une forte réduction de l'étendue de l'apport de dopamine au cortex préfrontal et du nombre de sites présynaptiques des axones dopaminergiques dans cette région. En revanche, au sein de l'OFC – une région impliquée dans l'inhibition de l'action – on observe une augmentation de la densité des sites présynaptiques de la dopamine. Ces effets dichotomiques et spécifiques aux males soulèvent la possibilité intrigante que le THC à l'adolescence déclenche un mauvais ciblage des axones dopaminergiques, déviant la croissance des axones dopaminergiques du cortex préfrontal vers les régions orbitofrontales. Ensuite, j'ai étudié si ces changements dopaminergiques chez l'adulte, ou leur absence dans le cas des femelles, entraîneraient des changements dans le contrôle des impulsions de l'adulte. À l'âge adulte, des cohortes de

souris distinctes ont été traitées à l'adolescence et testées dans le cadre de la tâche Go/No-go pour mesurer le contrôle des impulsions. Comparés à leurs homologues traités par le véhicule, les mâles adultes exposés au THC à l'adolescence présentent (i) une réponse prématurée accrue à la tâche Go/No-go, ce qui reflète une impulsivité d'attente altérée, mais (ii) un nombre réduit d'erreurs de commission, indiquant amélioration de l'inhibition de l'action. Aucun de ces effets n'a été observé chez les femelles. De plus, les changements moléculaires sont associés aux effets différentiels du THC sur la dopamine mésocorticale et la maturation cognitive chez les souris mâles mais pas chez les souris femelles. Les males présentent une augmentation du *Dcc* et une diminution du miR-218, ce qui suggère que chez les mâles, l'exposition au THC chez les adolescents induit des changements via le DCC/miR-218. Les femelles ont diminué le *Dcc* mais ne présentent aucun changement dans miR-218. Cependant, elles affichent un profil de microglies mPFC modifié, protégeant peut-être des effets d'une diminution du *Dcc* sur la dopamine.

Ces résultats montrent que le THC au début de l'adolescence a un impact sur le développement de la dopamine et du contrôle des impulsions chez les mâles, mais pas chez les femelles, et que de différents processus moléculaires et épigénétiques sont dérégulés. Les processus médiés par la protéine DCC peuvent induire des conséquences chez les mâles, mais des mécanismes compensatoires peuvent être recrutés chez les femelles.

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List of Abbreviations

THC	Tetrahydrocannabinol
DCC	Deleted in Colorectal Cancer
OFC	Orbitofrontal Cortex
PFC	Prefrontal Cortex
PrL	Prelimbic
IL	Infralimbic
I.P.	Intraperitoneal Injection
TH	Tyrosine Hydroxylase
daiPFC	dorsal agranular insular OrbitoPrefrontal Cortex
voPFC	ventral OrbitoPrefrontal Cortex
PND	Postnatal Day
VTA	Ventral Tegmental Area
RNA	Ribonucleic Acid
mRNA	Messenger Ribonucleic acid
PCR	Polymerase Chain Reaction
miR	microRNA
VEH	Vehicle
AUC	Area Under the Curve

Author Contributions

I, Tanya Capolicchio oversaw and lead each experiment, molecular experiments, treated animals, statistical analysis, all under the guidance of Dr. Cecilia Flores. The following individuals contributed to the project (in no particular order): Dr. Zdenka Pausova, Giovanni Hernandez, Sammy Shi, Brian J. Nieman, Delaram Shizrad, Emilie Dube, Katherina Estrada, Michel Giroux, Del MacGowan.

Figures were created with Illustrator and BioRender.

Experiment 1 – Dopamine Innervation to the PFC and OFC

T.C. treated both male and female mice with VEH or THC in early adolescence. T.C. and D.M. perfused mice. T.C. performed brain slicing, immunohistochemistry, and E.D. assisted in preparing male brain sections onto microscope slides. T.C. acquired images through microscopy and completed volume quantification and varicosity analysis.

The present study used the services of the Molecular and Cellular Microscopy Platform in the DHRC. Melina Jaramillo Garcia and Bita Khadivjam helped set up the imaging experiments.

Experiment 2 – Male Go/No-go Task

T.C., G.H., K.E., ran the task. T.C. and D.M. perfused and collected brains.

Experiment 3 – Male VTA qPCR and mPFC microglia profiling

T.C., M.G., G.H., treated male mice, collected brains, and performed tissue punching. T.C. and M.G. performed the qPCR.

T.C., G.H., treated male mice, perfused, and collected brains in skull. S.S., imaged, processed and analyzed brains, B.J.N., assisted, Z.P. supervised. S.S., T.C., performed data analysis and statistical analysis.

Experiment 4 – Female Go/No-go Task

T.C., G.H., and D.S., performed the task. T.C. and D.M. perfused and collected brains.

Experiment 5 – Female VTA qPCR mPFC microglia profiling

T.C., M.G., G.H., treated female mice, collected brains, and performed tissue punching. T.C. performed the qPCR.

T.C., G.H treated male mice, perfused, and collected brains in skull. S.S., imaged, processed and analyzed brains, B.J.N., assisted, Z.P. supervised. S.S., T.C., performed data analysis and statistical analysis.

Introduction and Statement of Problem

Adolescent exposure to high levels of the main psychoactive constituent of cannabis, delta-9-tetrahydrocannabinol (THC), poses a unique risk to develop enduring cognitive dysfunction and psychotic-related disorders (Malone et al., 2010; Rice et al., 2023; Trezza et al., 2008). This is concerning considering the surge in cannabis use over recent years within North America and the increasing concentration of THC (Kourgiantakis et al., 2022; Patrick et al., 2022; Wellman et al., 2023). Adolescence is a period of substantial physical, emotional, and social development and of noticeable refinement of neuronal circuits particularly those mediating executive function and goal-directed behavior (Best & Ban, 2021; Goldstein & Volkow, 2011; Naneix et al., 2012; Ordaz et al., 2013; Sturman & Moghaddam, 2011). The prefrontal cortex (PFC) which is one of the last brain regions to reach full maturity, undergoes significant changes in adolescence across species (Blakemore & Robbins, 2012; Caballero et al., 2016; Paquola et al., 2019). These protracted modifications occur in parallel to the stabilization in cognitive control and render PFC and cognitive development quite sensitive to beneficial and detrimental influences of environmental factors (Areal & Blakely, 2020; Gulley & Juraska, 2013; Larsen & Luna, 2018; Ordaz et al., 2013; Paus et al., 2008; Reynolds & Flores, 2021; Tottenham & Galván, 2016), including THC exposure during adolescence (Malone et al., 2010; Renard et al., 2017, 2018). However, the cellular and molecular mechanisms underlying the effects of THC in adolescence on PFC and cognitive maturation remain to be elucidated.

Background Information

Dopamine axons are still growing from the nucleus accumbens towards the PFC throughout adolescence – a phenomenon occurring in rodents, primates, and likely in humans too

(Avramescu & Flores, 2023; Hoops et al., 2018; Padmanabhan & Luna, 2014; Reynolds, Pokinko, et al., 2018; Reynolds et al., 2023; Reynolds & Flores, 2021; Rosenberg & Lewis, 1995). The fate of growing dopamine axons is controlled by the Netrin-1 receptor DCC (Deleted in Colorectal Cancer), which segregates mesolimbic and mesocortical dopamine pathways in adolescence, determining the extent and organization of synaptic dopamine inputs in the PFC (Reynolds, Pokinko, et al., 2018). In mice, gene editing- or experience-induced variations in DCC expression in dopamine axons in adolescence triggers their mistargeting and ectopic growth, inducing enduring alterations in cognitive function, most notably impulse control (Manitt et al., 2011, 2013; Reynolds, Pokinko, et al., 2018; Reynolds et al., 2023; Vassilev et al., 2021). These effects, which involve epigenetic processes, depend on the specific adolescent age of exposure, the type of experience, and differ significantly between males and females (Cuesta et al., 2018; Manitt et al., 2013; Pantoja-Urbán et al., 2023; Reynolds, Yetnikoff, et al., 2018; Reynolds et al., 2023; Vassilev et al., 2021). THC exposure during early adolescence has been shown to disrupt PFC dopamine development (Renard et al., 2016; Rubino et al., 2015). In addition, consequences of THC in adolescence on cognition are seemingly sexually dimorphic (Johnson et al., 2015; Rubino et al., 2015; Savulich et al., 2021; Stringfield & Torregrossa, 2021). However, the enduring effects of THC exposure during adolescence on dopamine development and cognitive control remains unclear.

Summary

Through a combined approach of molecular, behavioral, and neuroanatomical experiments, we sought to investigate here if THC exposure in adolescence dysregulates the Netrin-1/DCC system, impacting the development of the PFC dopamine system and impulse control in adulthood. We assessed if alterations in microRNAs known to regulate DCC receptor expression could serve as a possible indicator of THC-induced molecular changes. Microglia

are integral components of synaptic pruning and refinement processes (Lenz & Nelson, 2018; Schalbetter et al., 2022), including those occurring in the mesocorticolimbic dopamine system during adolescence (Kopec et al., 2018; Mallya et al., 2018). Therefore, we also investigated if adolescent THC alters microglia profiles in the developing PFC. We determined across all aspects of the study, the short and long-term outcomes of THC exposure in adolescence in male and female mice.

Aims

1- *Ascertain if THC in early adolescence alters adult frontal cortical dopamine connectivity in a sex-specific manner.*

2- *If THC in early adolescent males leads to changes in adult performance in the Go/No-Go task.*

3- *Assess if changes in DCC/miR-218 and mPFC microglia are involved in adult dopamine connectivity and Go/No-go task performance.*

4- *To investigate whether THC exposure in early adolescent female alters adult performance in the Go/No-Go task.*

5- *Determine if DCC/miR-218 and mPFC microglia are involved in adult female dopamine connectivity and Go/No-go task performance.*

Methods

Animals

Experimental procedures were all performed in accordance with the guidelines of the Canadian Council of Animal Care and approved by the McGill University and the Douglas Hospital Animal Care Committee. Male and female C57BL/6J and Cx3cr1$^{GFP/+}$ mice (Charles River Laboratories, Saint-Constant, Qc, Canada) were housed in a temperature- and humidity-controlled (21–22°C; 60%) room in the Neurophenotyping center of the Douglas Mental Health University Institute, on a 12/12 h light/dark cycle (light on at 8 A.M.). The mice were given *ad libitum* access to food and water throughout all experiments, except during behavioral testing in adulthood. Drug administration and behavioral testing took place during the light part of the cycle. Mice were housed in groups of 3-4 animals per cage throughout the study and were assigned randomly to each of the experimental groups.

Drug

Delta-9^{o} tetrahydrocannabinol (Cayman Chemical Company, MI, USA) was dissolved in cremophor, 0.9% saline, 100% anhydrous ethanol in a 1:18:1 ratio. All THC injections were administered interperitoneally at a volume of 0.1 ml/10 g and at a dose of THC 2.5 and 5 mg/kg.Vehicle (referred to as VEH) consisted of (cremophor, 0.9% saline, 100% anhydrous ethanol in a 1:18:1 ratio) and was also administered intraperitoneally at a volume of 0.1 ml/10g.

Experimental Design

Early adolescent male and female C57BL/6J and Cx3cr1$^{GFP/+}$ mice were administered VEH or THC injections from postnatal day (PND) 22 ± 1 to PND 31 ± 1. Mice received one injection of THC or VEH every other day, for a total of 10 days. This treatment regimen was based on our

previous studies showing robust and consistent dysregulation of DCC receptor expression in dopamine neurons by exposure to amphetamine in early adolescence (Cuesta et al., 2018; Reynolds et al., 2023; Yetnikoff et al., 2011). Separate mouse cohorts were used in the neuroanatomical, behavioral, and molecular experiments. Experimental timelines and procedures are indicated with diagrams on each figure. We began by assessing via stereological quantifications the expanse of TH+ axons occupied within the prelimbic (PrL) and infralimbic (IL) subregions of the prefrontal cortex in male and female mice. The doses selected for assessment were based on both current and pre-existing research, where 2.5 and 5 mg/kg THC has been reported to result in cognitive consequences (Cha et al., 2007; Ferland et al., 2022; Halbout et al., 2023; Murphy et al., 2017). Although we are aware that our study utilizes a non-contingent administration regiment, mouse intraperitoneal injection (I.P.) of THC 5 mg/kg has been reported to reach peak plasma drug concentrations that mimic that observed in nonmedical human cannabis smokers (Freeman & Lorenzetti, 2020; Guma et al., 2023; Huestis, 2007; Huestis & Cone, 2004; Lin et al., 2023; Torrens et al., 2020; Zamberletti et al., 2014).

Neuroanatomical Analysis

Tissue preparation. Adult male and female mice were anesthetized with an intraperitoneal overdose of a combination of ketamine 50 mg/kg, xylazine 5 mg/kg, acepromazine 1 mg/kg. Mice were perfused intracardially with 50mL of 0.9% saline, followed by 75 mL of chilled 4% PFA in phosphate-buffered saline (pH 7.4). Brains were collected and placed in 4 % PFA overnight at 4^{o}C, they were then transferred to a phosphate-buffered saline and stored for a maximum of 2 days. Coronal sections of the prefrontal cortex were obtained at 35-*u*m using a vibratome.

Immunofluorescence. As previously done, every second coronal brain section was processed for immunoflorescent labeling (1:2 series) (Auger et al., 2013; Manitt et al., 2010, 2011; Reynolds et al., 2022). Sections of tissue were blocked (2% bovine serum albumin, 0.2% Tween-20 in PBS) for 1 hour, incubated for 48 hours in a rabbit polyclonal anti-tyrosine hydroxylase (TH) antibody (1:500 dilution, catalog #AB152; Millipore Bioscience Research Reagents) followed by an Alexa Fluor 594-conjugated secondary antibody raised in donkey (1:500 dilution, 1 hr incubation, Invitrogen). Tissue sections were then mounted onto gelatin-coated slides and using a SlowFade Gold Antifade mounting medium (Invitrogen) were cover slipped.

Stereology. Tissue was stained with a TH+ antibody which labels DA axons specificity in the prefrontal cortex, with negligible labeling of norepinephrine axons (Manitt et al., 2011, 2013; Reynolds et al., 2022). The volume that TH-positive (TH+) fibers occupy and the total number of TH+ axonal varicosities in the prelimbic, and infralimbic subregions of the pregenual medial prefrontal cortex evaluated using a stereological fractionator sampling design(West et al., 1991), with the optical fractionator probe of the Stereoinvestigator software (MicroBrightField), as previously described (Auger et al., 2013; Reynolds et al., 2022; Reynolds, Pokinko, et al., 2018). Regions of interest were delineated according to the mouse brain atlas (Stereological Coordinates - Paxinos and Franklin, 2019), and contours of the TH+ projection within these regions were traced at 5 X magnification with a Leica DM4000B microscope. For each brain region Stereoinvestigator calculates a volume (in cubic micrometers) measurement from the contour area, section thickness, and section periodicity (MicroBrightField). Sections spanning plates 14– 18 of the mouse brain atlas were studied. Stereoinvestigator calculates the total number of TH+ varicosities based on the experimenter's random sampling of a

known fraction of the region. Counting frame and grid size were chosen to consistently sample 33 sites per region.

In addition, we measured the density of TH+ varicosities within sections of the obirtofrontal cortex corresponding to plates 14-20 of the mouse brain atlas (Paxinos and Franklin 2013). We delineated the following subregions of the orbitofrontal cortex: the dorsal agranular insular orbitoprefrontal cortex (daiPFC), the ventral agranular insular orbitofrontal cortex (vaiPFC), and the ventral orbitofrontal cortex (voPFC) using landmarks derived from Paxinos and Franklin (2013). We did not examine the volume of the dopamine input to this region, because the innervation is scattered in comparison to the prefrontal cortex, and there is no distinct, well-defined region of dopamine presence (Hoops & Flores, 2017).

Go/No-go

To assess impulse control, we used the Go/No-go task we modified and implemented for the use of mice (Reynolds, Pokinko, et al., 2018; Reynolds, Yetnikoff, et al., 2018). Mice were food-restricted to approximately 1.5 g of food per day for the duration of the task to maintain 85% of their initial free-feeding body weight. Training phases for this behavioral task began at PND75 ± 15. Chocolate-flavored dustless precision pellets (BioServ, Inc.) were used as a reinforcer, with sessions conducted in operant behavioral boxes equipped with a house light, an adjustable tone generator, two nose-poke holes (right and left) capable of being illuminated, and a pellet dispenser. The behavioral paradigm consisted of 3 stages: discrimination training, reaction time training, and the Go/No-Go behavioral task.

Discrimination Training: These sessions were 20 minutes in duration. Mice were trained for 6 consecutive days, followed by one rest day, until the target performance rate was attained.

In the first stage, mice were trained to nose-poke the correct illuminated hole (left or right, the conditions balanced across mice), constituting a “rewarded” trial. Mice moved to the next phase when the cohort reached approximately a performance rate of 70% rewarded trials.

Reaction time training: The second training stage was divided into two subsections, the five second reaction time and the three second reaction time. In the first stage mice were trained to nose-poke the correct illuminated hole within five seconds of the cue light turning on. A pretrial period, wherein the house light was illuminated for several seconds without the cue light, was designed during this stage to train mice to attend exclusively to the cue light. This continued until the cohort reached a rate of approximately 50% or fewer premature responses. Responding to the house light during this pretrial period constituted a “Premature Response” taken as a measure of waiting impulsivity (Bari & Robbins, 2013; Dalley & Robbins, 2017; Jentsch et al., 2014). Following the pretrial period, mice who nose-poked within five seconds of the cue light being illuminated were rewarded with a reinforcer. In the second stage, mice were trained to nose-poke the correct illuminated hole within three seconds of the cue light turning on. Responding to the house light during this pretrial period constituted a “Premature Response”. This stage continued until the cohort showed less than 25% of premature responses during the pretrial period, whereupon they advanced to the Go/No-go task.

Go/No-Go task: Mice were tested in this task for a total of 14 consecutive days. Mice had to nose-poke in response to an illuminated “Go” cue within a limited amount of time (average of 3 sec) or inhibit their response to this cue when presented together with an auditory “No-go” cue. Nose poke responses to the Go cue were recorded as “Hits” and resulted in the delivery of a food pellet, while the same response to the No-go cue was considered a “Commission

Error" and was taken as a measure of action impulsivity (Dalley & Robbins, 2017; Jentsch et al., 2014).

Progressive Ratio (motivation for food reward)

Following the Go/No-Go task, mice were trained in the same operant chambers to nose poke for a food reward for 2 days. To investigate differences in motivation for food reward, the number of nose pokes required to obtain a reward increased exponentially after each trail. Following training, mice were to a one-day test where breakpoint performance was recorded, which is the maximum number of nose pokes mice are willing to make in exchange for a singular reward (Gourley et al., 2016).

RNA Extraction and RT-qPCR

One week following VEH or THC treatment, mice were rapidly decapitated, and their brains were collected and flash-frozen in 2-methylbutane (Fisher Scientific, Hampton, New Hampshire). Bilateral punches of the ventral tegmental area (VTA) were taken from 1-mm thick coronal slices starting from sections corresponding to plate 55 (−2.92 mm, anterior/posterior relative to Bregma) and 15 (1.94 mm, anterior/posterior relative to Bregma), respectively, of Paxinos and Franklin. Total RNA and microRNA fractions were isolated using the miRNeasy Micro Kit protocol (Qiagen, Toronto, ON, Canada) (Cuesta et al., 2018; Manitt et al., 2013; Torres-Berrío et al., 2017). All RNA samples were determined to have 260/280 and 260/230 values ≥ 1.8, using the Nanodrop 1000 system (Thermo Scientific, Toronto, ON, Canada). Reverse transcription for *Dcc*, and *Glyceraldehyde-3-phosphatedehydrogenase* (GAPDH) mRNA was performed using a high-capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's instructions. Real-time

polymerase chain reaction (PCR), using TaqMan assay (Applied Biosystem) was carried out with an Applied Biosystems QuantStudio 6 Flex Real-Time PCR system. Data for *Dcc* mRNA expression were analyzed by using the relative quantification standard curve method and the level of these transcript was quantified relative to the expression of the reference gene *Gapdh*. Reverse transcription for miR-218 was performed using the TaqMan MicroRNA Reverse Transcription Kit together with the corresponding miRNA TaqMan probes (Applied Biosystems). Expression levels were calculated using the relative quantification standard curve method. The small nucleolar RNA (snoRNA) RNU6B was used as an endogenous control to normalize the expression of miR-218. In all cases, the real- time PCR was run in technical triplicates.

Medial Prefrontal Cortex Microglia Morphology Analysis

Tissue preparation. Adolescent male and female mice were anesthetized with an intraperitoneal overdose of a combination of ketamine 50 mg/kg, xylazine 5 mg/kg, acepromazine 1 mg/kg. Mice were perfused intracardially with 30mL 0.1M phosphate-buffered saline (PBS) solution containing 1 uL/mL heparin and 2 mM ProHance® was used, followed by a 30 mL 4% paraformaldehyde (PFA) solution with 2 mM ProHance®. Both solutions were perfused at a rate of 1.0 mL/min. Following perfusion, the skulls, with the brains intact, were removed and stored in a 4% PFA solution with 2 mM ProHance® at 4°C for 24 hours. Subsequently, the samples were transferred to a 0.1M PBS solution containing 2 mM ProHance® and 0.02% sodium azide, maintained at 4°C for future ex vivo imaging. Following ex vivo MRI, brains were extracted from mouse skulls utilizing a standardized surgical procedure. The brains were washed in PBS and then embedded in 3% agarose prepared with phosphate buffer for stabilization required for serial two-photon tomography.

Ex vivo T2 Weighted MRI: Ex vivo T2-weighted MRI was done on brains retained in skulls. Images were captured using a 7.0 T multi-channel magnet system (Varian Inc. Palo Alto, CA, USA), housed at the Mouse Imaging Centre at the Hospital for Sick Children (Toronto, ON, Canada). Sixteen samples underwent simultaneous scanning through the utilization of a specially engineered solenoid array (Dazai et al., 2011). The anatomical scans were conducted according to the following specifications: T2-weighted, three-dimensional imaging employing a fast spin-echo sequence in conjunction with cylindrical k-space acquisition, set with a TR of 350 ms, TE of 12 ms, echo train length of six, and four averages (Nieman et al., 2005). The scanning parameters included a field-of-view of 20mm × 20mm × 25mm, a matrix configuration of 504 × 504 × 630, and an isotropic image resolution at 40 µm.

Serial two-photon tomography image acquisition: To investigate the distribution of GFP expression across the entire brain, we utilized a serial two-photon tomography system (STPT) (Ragan et al., 2012), configuring the imaging parameters based on established research (Vousden et al., 2015). The individual brains embedded in 3% agarose were positioned in an orientation perpendicular to the blade. The cerebellum was sectioned and imaged initially, followed by the anterior progression towards the olfactory bulbs, which were the final structures to be imaged. The STPT software performed an automated acquisition to record GFP expression in brain samples, taking roughly 8 hours per specimen. Individual image tiles, or raw two-photon microscope captures, were subsequently merged utilizing custom-developed Matlab and Python scripts, following the methodology outlined by Vousden et al., to create all-encompassing coronal sections throughout the full mouse brain (Vousden et al., 2015). Upon completion of the image assembly, the compiled data consisted of an estimated

170 coronal sections per mouse brain, exhibiting 1.37-µm in-plane resolution and 100-µm slice spacing, and were retained as 16-bit (.tiff) image files.

Serial-two photon tomography image processing: The obtained coronal (.tiff) images served as input images for microglia location identification. To normalize signal intensities across samples, we employed OpenCV tools (version 4.6.0, https://docs.opencv.org/4.6.0 (Bradski & Kaebler, 2008) in Python (version 3.8.0, https://www.python.org/downloads/release/python-380) (Heetinger 2019). Specifically, we used Contrast Limited Adaptive Histogram Equalization (CLAHE) to enhance local image contrast by distributing pixel intensities across a wider range of intensity values, thereby improving microglia conspicuity. By leveraging the Scipy "Winsorize" tool (version 1.9.0, https://docs.scipy.org/doc/scipy) (Virtanen et al., 2020) we clipped pixel intensities within a predetermined range to minimize the impact of intensity outliers on microglia label detection and segmentation (lower bound threshold = 85%, upper bound threshold = 0.5%). Subsequently, we normalized contrast intensities across samples to ensure they fell within a consistent range (0-255). These normalized coronal images were then employed as input images for a cell-segmentation pipeline (fastCell) developed at MICe (Wang & Lerch 2021), which relies on an artificial neural network incorporating supervised machine learning to detect and segment individual cells (https://github.com/fastai/fastai). The neural network was trained using a set of manually segmented microglia labels (n ~ 8000) with varying size, shape, density, and signal intensity, derived from distinct samples and slices. The refined neural network was subsequently applied to segment the normalized coronal sections, generating output images that displayed segmented GFP-labelled microglia throughout the brain.

Serial-two photon tomography image registration: The normalized coronal (.tiff) sections were assembled to produce 3D files, saved as mnc files. To align the STPT data with MR images, these images were smoothed and downsampled to a resolution of 1.37 µm, yielding images more akin to anatomical structures observable in MRI. The downsampled STPT images were first registered with one another to form a consensus average image, employing the pydpiper toolkit (Friedel et al., 2014) built upon MNI-autore (Collins et al., 1994) and ANTS (*Advanced Normalization Tools (ANTS)*, n.d.) registration tools. In some images, microglia labels were presented with faint label due to technical difficulties during image acquisition process of the STPT. As a result, we devised STPT sample-specific atlases that masked out areas with weak microglia labels before aligning them with their respective MR images. These "masked" volume estimates were subsequently utilized for more accurate cellular density calculations. In order to obtain MRI-derived volume estimates, the pre-segmented atlas was aligned with the MR images, resulting in 182 segmented brain regions (combining left and right regions as required) (Reddick et al., 2003). Additionally, the STPT sample-specific atlases were matched with their associated MR images to generate masked volume estimates, which were then employed in the creation of density maps for each individual sample.

Statistical Analysis:

Statistical analysis was performed using Graphpad Prism 7.0. A significance threshold of $\alpha<0.05$ was used in all the experiments. All values are represented as mean ± S.E.M. Statistical differences between two groups were analyzed with Student's t-tests with two-tailed analysis. Otherwise, one- way, or two-way ANOVAs (with Repeated Measures) were performed, followed by Holm-Sidak's multiple comparison. Outliers were screened using the

ROUT's 1% method. All data were normally distributed, and variance was similar between groups, supporting the use of parametric statistics.

Results

Aim 1: THC in adolescence alters dopamine connectivity in the adult prefrontal cortex in a sex-specific manner

Alterations in the volume that prefrontal cortex dopamine axons occupy in adulthood often serve as a proxy of changes in mesocortical dopamine axon growth in adolescence (Hoops et al., 2023; Reynolds et al., 2023; Reynolds, Pokinko, et al., 2018). We began by assessing in males and females if THC exposure in early adolescence impacts ongoing prefrontal cortex dopamine development. We quantified the expanse of the dopamine input to prefrontal cortex in adulthood using stereology (Fig. 1A). There is a decrease in the span of prefrontal cortex dopamine axons in adult males administered THC in early adolescence compared to VEH (Fig. 1B). This reduction is observed in the prelimbic and infralimbic subregions. Conversely, there is no significant difference in the span of prefrontal cortex dopamine fibers in adult females administered THC in early adolescence compared to VEH (Fig. 1B: three-way repeated measures ANOVA, subregion x sex x drug $F_{(1, 12)} = 4.962$, $p = 0.045$ main effect of treatment condition $F_{(1, 12)} = 26.27$, $p = 0.0003$; main effect of subregion, $F_{(1,12)} = 421$, $p < 0.0001$; main effect of sex, $F_{(1, 12)} = 12.03$, $p = 0.04$).

The male-specific change in the expanse of the prefrontal cortex dopamine input in the THC group is accompanied by a corresponding reduction in the total number of dopamine axonal varicosities in both the prelimbic and infralimbic subregions (Fig. 1C: two-way repeated measures ANOVA, main effect of treatment condition $F_{(1, 6)} = 9.182$, $p = 0.023$; main effect of subregion, $F_{(1,6)} = 84.80$, $p < 0.0001$).

Since dopamine axons also continue to grow towards the orbitofrontal cortex (OFC) across adolescence (Hoops & Flores, 2017), we next quantified the density of dopamine axon varicosities in the adult orbitofrontal cortex of the same adult males administered THC or vehicle in early adolescence (Fig. 1A). There is an *increase* in the density of TH+ varicosities in THC versus control groups (Fig. 1D, OFC, one- way ANOVA, main effect of treatment $F_{(1, 6)}$ = 10.22, p = 0.018). This change is opposite to the one observed in the prefrontal cortex and raises the intriguing possibility that axons that normally grow to the prefrontal cortex are deviated to the orbitofrontal cortex in THC-exposed adolescent mice.

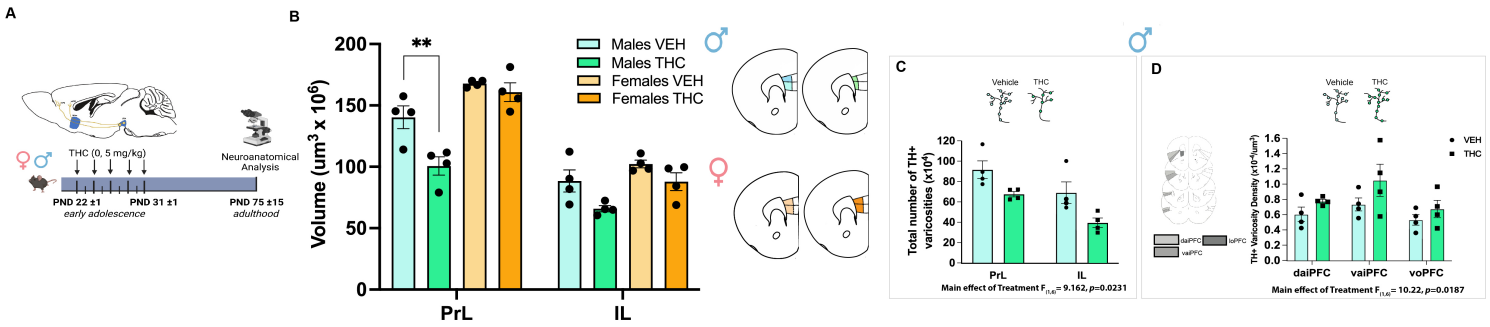

FIGURE 1. THC (5 mg/kg) in adolescence disrupts adult PFC dopamine connectivity in males but not females. **A**, Timeline of treatment and experimental procedures. **B**, Male mice treated with THC (5 mg/kg) during adolescence show decreased expanse of the dopamine input to the PFC, but these differences are not observed in females. **C,** Reduced dopamine input to the PFC in males administered THC in adolescence is associated with a concomitant decrease in total number of TH+ varicosity within the prelimbic (PrL) and infralimbic (IL) subregions of the PFC. **D,** Males treated with THC in adolescence show an *increase* in the density of TH+ varicosities in the orbitofrontal cortex (OFC). ** significantly different, $p < 0.01$; * significantly different, $p < 0.05$. All data are shown as mean ± SEM (n=4 per group).

Aim 2: THC in adolescent males improves action impulsivity in adulthood but impairs waiting impulsivity

To begin examining if changes in dopamine connectivity in the prefrontal cortex and orbitofrontal cortex of males administered THC in adolescence associate with alterations in impulse control in adulthood, we used the Go/No-go task. This task is heavily dependent on dopamine signaling within the frontal cortex (Shiner et al., 2015; Yamamuro et al., 1994). This experiment was performed in a separate cohort of adult male mice exposed to (5 mg/kg) THC (*n* = 9) or VEH (*n* = 9) in early adolescence (Fig. 2A, B). Adult males that received THC in early adolescence show *improved* action impulsivity as in indicated by an overall lower proportion of commission errors, compared to VEH (Fig. 2C, AUC commission errors, $t_{(17)}$=2.192; p = 0.042). The proportion of hits is similar between VEH and THC male groups indicating equal engagement in the task (Fig. 2D, AUC hits, $t_{(17)}$=1.180; p = 0.254).

However, the opposite effect was observed when assessing premature responding, wherein males that received THC in early adolescence show *deficits* in their waiting impulsivity, as indicated by an overall increased proportion of premature responses compared to VEH (Fig. 2E, F, AUC premature responses, $t_{(15)}$=3.287; p = 0.005). These results show that the enduring effects of THC in early adolescence on impulse control in males vary according to impulsivity dimension outcome. The prefrontal cortex and orbitofrontal cortex are preferentially engaged in waiting and action impulsivity (Dalley & Robbins, 2017), respectively. It is intriguing that THC in adolescence increases dopamine innervation to the orbitofrontal cortex but reduces dopamine input to the prefrontal cortex in adult male mice.

Twenty-four hours after the Go/No-go task, mice underwent a progressive ratio test to assess motivation for food reward. The breakpoints are similar between VEH and THC groups indicating similar sensitivity to food reward (Fig. 2G inset, $t_{(17)}$ = 0.2851, p = 0.779).

Males that received THC during early adolescence show altered weight gain compared to mice that received VEH (Fig. 2H left panel inset, $t_{(20)} = 4.814$, $p = 0.0001$). Interestingly, the difference in body weight gain does not persist until adulthood (i.e. their weight on the last day of injection and their weight in adulthood; Fig. 3H right panel inset, $t_{(20)} = 0.7817$, $p = 0.443$), consistent with previous reports in male mice (Lin et al., 2023; Rubino et al., 2008; Silva et al., 2016).

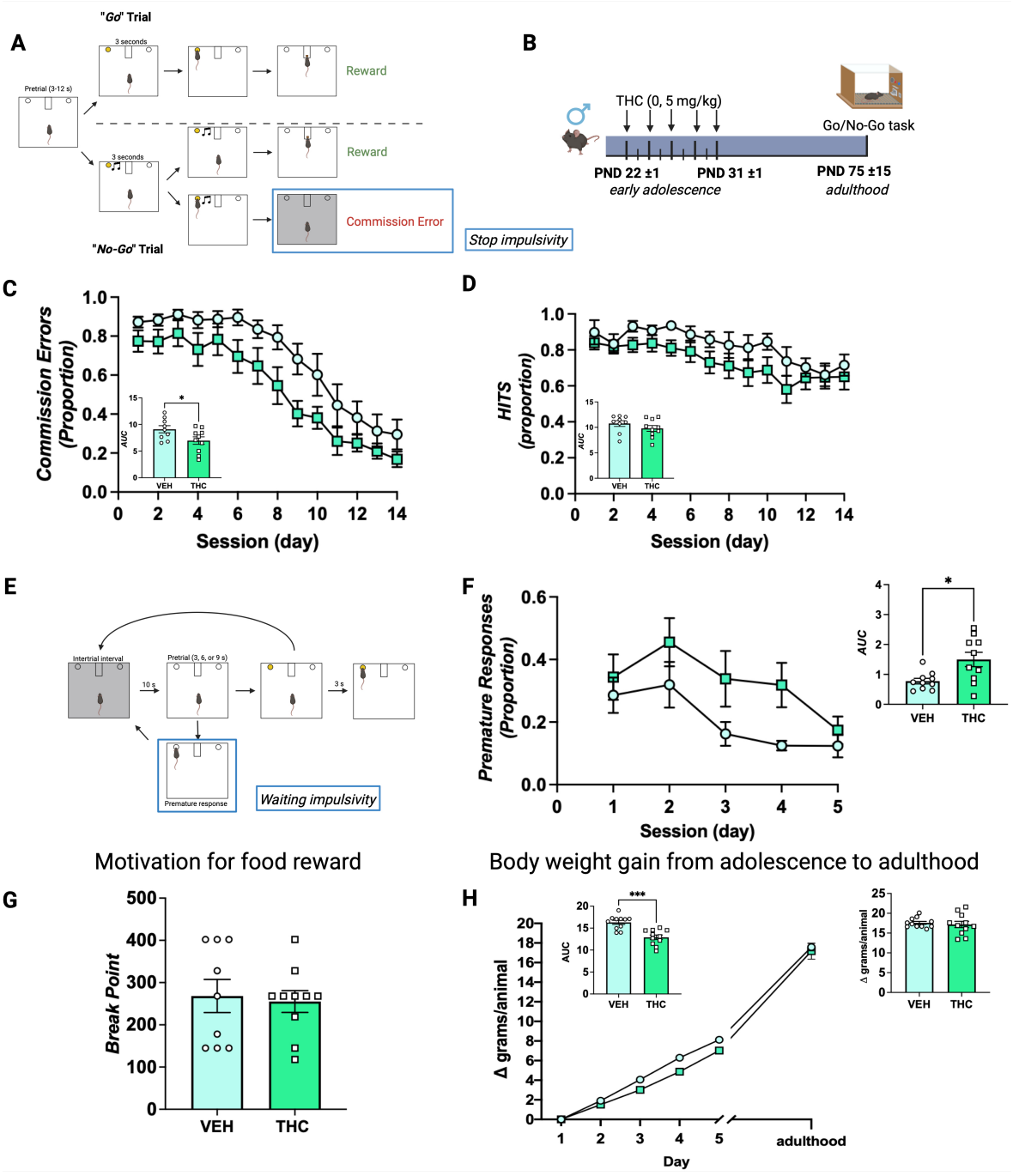

FIGURE 2. THC in early adolescent male mice improves action impulsivity in adulthood but impairs waiting impulsivity. **A**, Go/No-go experimental design. **B**, Experimental timeline, and procedures. **C**, Adult males that received THC in early adolescence have a lower proportion of commission errors compared to VEH. Area under the curve (AUC, inset) illustrates a lower proportion of commission errors overall in the THC compared to the VEH group. **D,** THC (5 mg/kg) does not affect the proportion of hits in Go trials. Area under the curve (AUC, inset) illustrates similar proportion of hits between treatment groups. **E**, Experimental design to capture premature responses. **F**, THC in early adolescence leads to increased proportion of premature responses in adulthood compared to VEH. Area under the curve (AUC, inset) illustrates a larger proportion of premature responses overall for the THC compared to VEH group. **G,** THC and VEH male groups show similar progressive ratio breakpoints in adulthood. **H,** Males that received THC in early adolescence show reduced body weight gain compared to VEH treated males across treatment days. Data are presented as the increment (Δ) in body weight (grams) per animal at each time point with their *Day 1* body weight as the reference point. Area under the curve (AUC, left panel inset) illustrates lower Δ body weight (g)/animal gain in the THC group compared to VEH during treatment. The difference in body weight between treatment conditions does not persist into adulthood. Right panel inset: difference in weight between postnatal day (PND) 75 and PND 30. ** significantly different, $p < 0.01$; * significantly different, $p < 0.05$. All data are shown as mean ± SEM (n= 9-11 per group).

Aim 3: THC in adolescence upregulates *Dcc*, but downregulates miR-218 in the VTA of male mice without altering mPFC microglia

In the VTA, 99% of dopamine neurons express the guidance cue gene DCC, with similar results in male and female rodents (Phillips et al., 2022). We next assessed if THC in early adolescence

would alter *Dcc* mRNA expression in the VTA of male mice one week later (Fig. 3A,B), when dopamine axons are undergoing targeting events (Hoops et al., 2018; Reynolds et al., 2023; Reynolds, Pokinko, et al., 2018). In this experiment we tested 2 doses (either 2.5 or 5 mg/kg) to determine the sensitivity of this guidance cue system to different THC concentrations. THC increases *Dcc* mRNA levels compared to VEH, regardless of dose (Fig. 3C, VTA *Dcc*, one- way ANOVA $F_{(2, 33)} = 4.832$, $p = 0.032$, Holm–Sidak *post hoc* tests: VEH vs THC 2.5, $p = 0.027$, VEH vs THC 5, $p = 0.044$).

In contrast, exposure to either dose of THC decreases miR-218 expression, compared to VEH, suggesting the involvement of microRNAs processes in the regulation of *Dcc* by THC (Fig. 3D, miR-218, one- way ANOVA $F_{(2, 17)} = 8.286$, $p = 0.003$, Holm–Sidak *post hoc* tests: VEH vs THC 2.5, $p = 0.038$, VEH vs THC 5, $p = 0.01$). This finding is consistent with previous findings from studies in male mice administered amphetamine in adolescence (Cuesta et al., 2018; Reynolds et al., 2023).

Treatment with THC (5 mg/kg) during early adolescence in males reduces body weight gain across treatment days compared to VEH (Fig. 3E, AUC (right), one-way ANOVA, $F_{(2, 21)} = 4.935$, $p = 0.017$, Holm–Sidak *post hoc* tests: VEH vs THC 2.5, $p = 0.064$, VEH vs THC 5, $p = 0.019$).

Across adolescent development microglia serve a pivotal role in synaptic refinement, specifically within the PFC (Mallya et al., 2018; Schalbetter et al., 2022). To address potential THC-induced changes in microglia profiles in the developing PFC of males, we assessed the density and soma size of microglia, one week following THC or VEH exposure (Fig. 3F). We chose this time because it coincides with the period when dopamine axons are still undergoing pathfinding decisions (Cuesta et al., 2020; Reynolds et al., 2023). We found no differences in

PFC microglia density between THC and VEH groups (Fig. 3G $t_{(6)}$=0.739; p = 0.478) and no changes in microglia soma size (Fig. 3H $t_{(6)}$=0.865; p = 0.409).

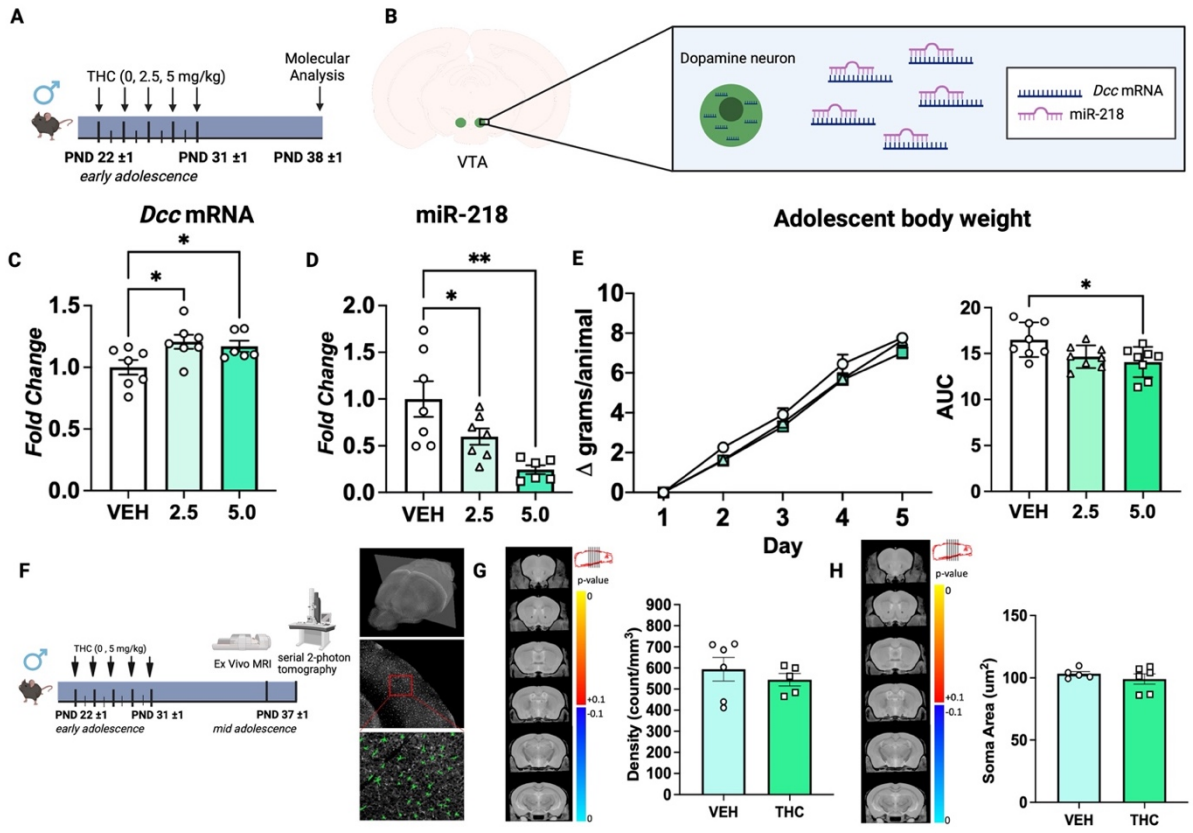

FIGURE 3. THC in early adolescence upregulates *Dcc* and downregulates miR-218 in VTA dopamine neurons of male mice. **A**, Experimental timeline, and procedures. **B**, Majority of dopamine neurons within the VTA express *Dcc* mRNA, which is repressed by the microRNA miR-218. **C**, THC upregulates *Dcc* mRNA expression in the VTA of males one week after last drug exposure in early adolescence. **D**, Male mice treated with THC (2.5 and 5 mg/kg) show a significant decrease in miR-218. **E,** Across treatment days, body weight gain is only altered between THC (5 mg/kg) and VEH groups. Data are presented as the increment (Δ) in body weight (grams) per animal at each time point with their *Day 1* body weight as the reference point. Area under the curve (AUC) illustrates altered Δ body weight (g)/animal gain only

between THC (5 mg/kg) and VEH groups. **F**, Experimental timeline and procedures **G**, Representative images of PFC microglia density in males (left). THC does not alter microglia density in the PFC of males one week after last drug exposure (right). **H,** Representative images of microglia soma size in the male PFC (left). Male mice treated with THC (5 mg/kg) show no significant change in microglia density or average soma size (right). ** significantly different, $p < 0.01$; * significantly different, $p < 0.05$. All data are shown as mean **±** SEM (n= 6-8 per group).

Aim 4: THC during adolescence does not impact impulse control in adulthood in female mice

Despite the lack of changes in PFC dopamine connectivity we still wanted to assess whether THC altered impulse control in adult females. We examined Go/No-go performance in adult female mice exposed to (5 mg/kg) THC ($n = 10$) or VEH ($n = 11$) in early adolescence (Fig. 4A,B). There are no differences in the numbers of commission errors or hits between THC and VEH groups (Fig. 4C AUC commission errors, $t_{(19)}=0.7377$; $p = 0.469$, Fig. 4D AUC hits, $t_{(19)}=1.022$; $p = 0.319$). Furthermore, during the training phase, both groups show comparable number of premature responses (Fig. 4E,F AUC premature responses, $t_{(19)}=0.4614$; $p = 0.649$). One day after the Go/No-go task, females underwent a progressive ratio test to assess their motivation to work for a food reward. Both groups show similar breakpoints, indicating no differences in sensitivity to food reward (Fig. 4G, break point, $t_{(19)}=0.2997$; $p = 0.767$). These results are in line with the lack of changes in females in the extent of the dopamine input to the prefrontal cortex.

Females administered THC or VEH show similar weight across treatment days during early adolescence (Fig. 4H left panel inset, $t_{(19)} = 0.1147$, $p = 0.909$) as well as when they reach

adulthood (i.e. when we compare their weight in the last day of injection and their adult weight; Fig. 4H right panel inset, $t_{(19)} = 1.416$, $p = 0.172$).

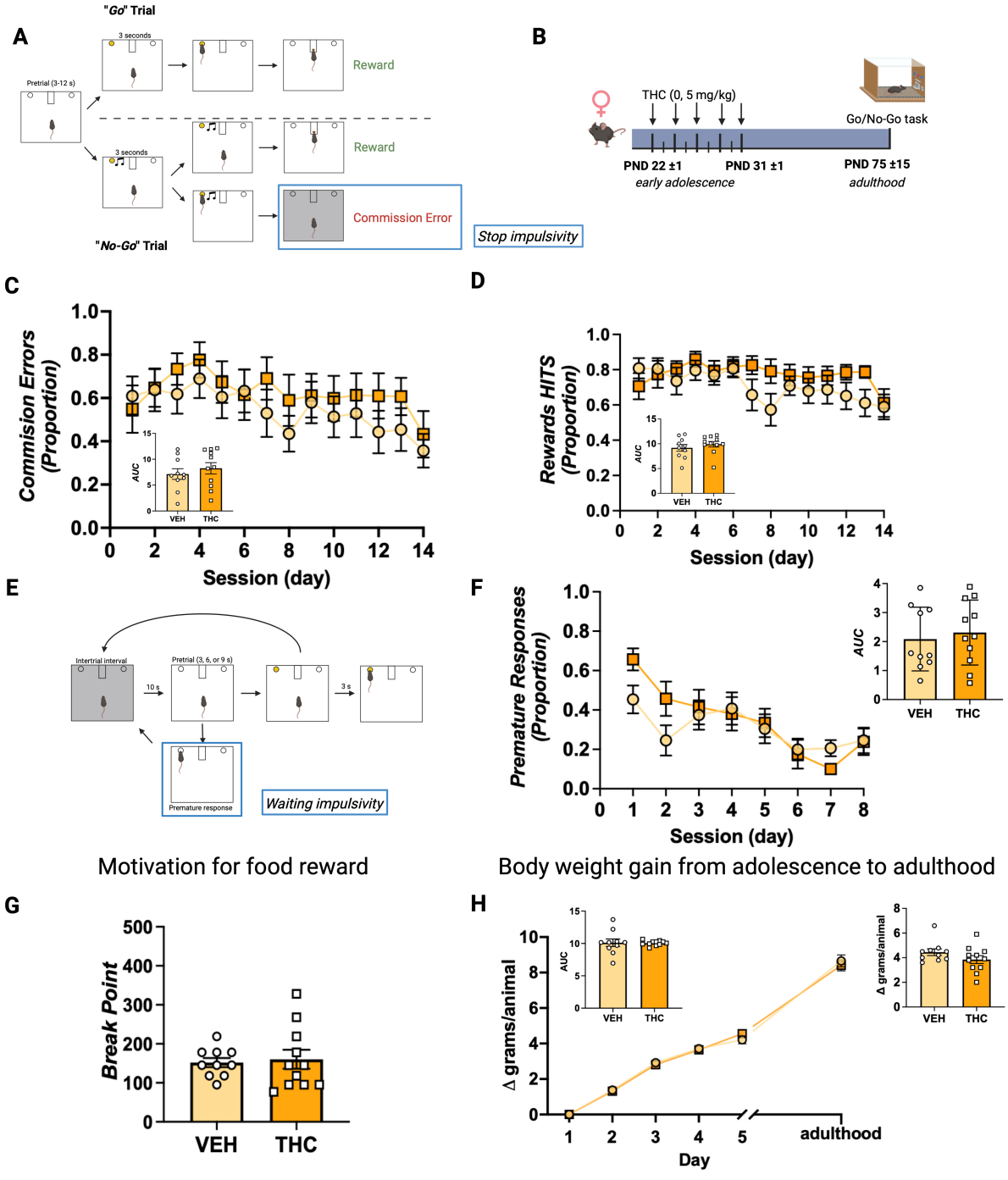

FIGURE 4. THC in early adolescence does not alter impulse control in adulthood in female mice. **A**, Experimental timeline, and procedures. **B**, Go/No-go experimental design. **C**, THC and VEH female groups show similar proportion of commission errors in No-Go trials. Area under

the curve (AUC, inset) illustrates similar proportion of commission errors between THC and VEH groups. **D**, Similar proportion of hits in Go trials between adult female mice administered THC (5 mg/kg) or VEH in early adolescence. Area under the curve (AUC, inset) illustrates similar proportion of hits between THC and VEH groups. **E**, Experimental design to capture premature responses. **F**, THC and VEH female groups show similar proportion of premature responses during the training phase. Area under the curve (AUC, inset) illustrates similar proportion of premature responses between THC and VEH groups. **G**, THC and VEH female groups show similar progressive ratio breakpoints in adulthood. **H**, Body weight gain is similar between THC and VEH treated females across treatment days. Data are presented as the increment (Δ) in body weight (grams) per animal at each time point with their *Day 1* body weight as the reference point. Area under the curve (AUC, left panel inset) illustrates similar Δ body weight (g)/animal gain between THC and VEH groups during treatment. The lack of change in body weight between treatment conditions persists into adulthood. Right panel inset: difference in weight between postnatal day (PND) 75 and PND 30. ** significantly different, $p < 0.01$; * significantly different, $p < 0.05$. All data are shown as mean ± SEM (n= 9-11 per group).

Aim 5: THC in adolescent females downregulates *Dcc* levels in a dose-dependent manner but does not alter miR-218

We quantified *Dcc* mRNA and miR-218 expression in the VTA of female mice one week after exposure to THC (either 2.5 or 5mg/kg) or VEH (Fig.5A). There is a significant decrease in *Dcc* levels following THC treatment with the 5, but not the 2.5 mg/kg dose (Fig. 5B, VTA *Dcc*, one-way ANOVA $F_{(2, 33)} = 4.832$, $p = 0.014$, Holm–Sidak *post hoc* tests: VEH vs THC 2.5 $p = 0.755$, VEH vs THC 5 $p = 0.023$). Regardless of treatment condition, there is no difference in VTA miR-

218 expression across groups (Fig. 5C, VTA miR-218, one- way ANOVA $F_{(2, 31)} = 1.708$, $p = 0.197$). These data indicate that despite downregulating *Dcc* expression in dopamine neurons in early adolescence, exposure to THC (5mg/kg) in females does not alter adult impulse control or dopamine connectivity.

All female groups show similar weight gain across treatment days (Fig. 5D, AUC (right), one-way ANOVA, $F_{(2, 32)} = 1.069$, $p = 0.355$).

Serial two-photon tomography analysis revealed no differences in microglia density in the PFC between THC and VEH female groups (Fig. 5F $t_{(6)}=1.664$; $p =0.147$). However, THC (vs. VEH) leads to a reduction in soma size one week following early adolescent exposure (Fig. 5G $t_{(6)}=3.318$; $p = 0.016$). Although direct evidence is needed, this initial finding raises the intriguing possibility that microglia changes in females may play a protective role against potential THC-induced disruption in dopamine development, for example by affecting the pruning of synaptic dopamine terminals (Kopec et al., 2018; Schalbetter et al., 2022).

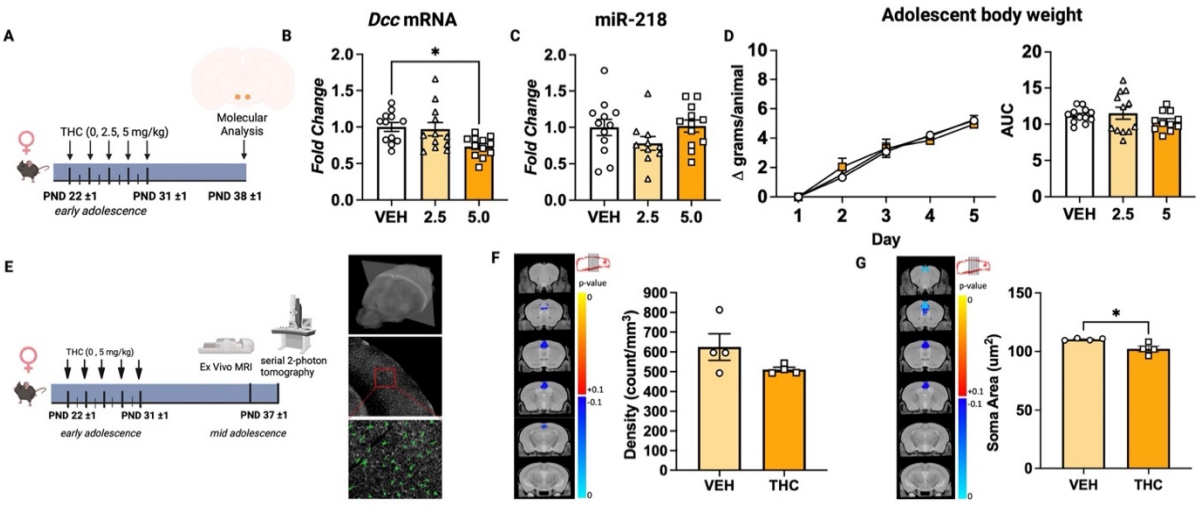

FIGURE 5. In females, THC in early adolescence induces dose-dependent *Dcc* downregulation in dopamine neurons, without altering miR-218 levels. **A**, Experimental timeline and

procedure. **B**, Treatment with 5, but not 2.5 mg/kg THC in early adolescent female mice downregulates *Dcc* mRNA expression in the VTA, one week after the last drug exposure. **C**, THC (either 2.5 or 5 mg/kg) does not alter VTA miR-218 expression in females. **D**, Body weight gain across treatment days is similar between THC and VEH groups – data are presented as the increment (Δ) in body weight (grams) per animal at each time point with their *Day 1* body weight as the reference point. Area under the curve (AUC) illustrates similar Δ body weight (g)/animal gain between THC and VEH groups. **E**, Experimental timeline and procedures. **F**, Representative images of microglia density in the female PFC (left). THC does not alter microglia density in the PFC of females one week after last drug exposure in early adolescence (right). **G**, Representative images of microglia soma size in the female PFC (left). Female mice treated with THC (5 mg/kg) show a significant decrease in average soma size (right). ** significantly different, $p < 0.01$; * significantly different, $p < 0.05$. All data are shown as mean ± SEM (n= 11-12 per group).

Discussion

The use of THC in young individuals is increasing at a unprecedent rate (Kourgiantakis et al., 2022; Patrick et al., 2022; Wellman et al., 2023). In this study we examined the short and long-term effects of non-contingent THC exposure in early adolescence in male and female mice. Our findings indicate that THC in early adolescence reduces dopamine innervation in the adult PFC of males, but not females, and that this change in males is associated with *increased* dopamine connectivity in the orbitofrontal cortex. This suggests that THC may induce axons destined to innervate the PFC to ectopically grow to the orbitofrontal cortex. Indeed, THC in adolescent males alters *Dcc* expression in the VTA – an event tightly linked with errors in dopamine axon targeting (Reynolds et al., 2023; Reynolds, Pokinko, et al., 2018) – and induces dimension-specific changes in impulse control in adulthood. In males, THC in adolescence also leads to changes in cognitive control in adulthood, with opposite changes in waiting versus action impulsivity. In females, THC *decreases* VTA *Dcc* expression, but does not alter adult cognitive control. Interestingly, in females only, THC reduces microglial soma size within the PFC. Since microglia mediate synaptic pruning and refinement during adolescence, including in the developing PFC (Mallya et al., 2018; Schalbetter et al., 2022), these findings suggest that while in males THC alters adolescent dopamine and impulse control development, in females protective processes are recruited.

In males, the decrease in the extent of the dopamine input to the PFC is accompanied by reduced number of dopamine presynaptic sites within this region and is likely to result in aberrant PFC dopamine neurotransmission (Jentsch et al., 1998; Verrico et al., 2003). In the orbitofrontal cortex, however, there is an increase in dopamine connectivity. This PFC versus

orbitofrontal cortex dichotomy may be causally linked to its opposite impact on waiting versus action impulsivity, since dopamine in these two cortical regions has been shown to participate in different impulsivity dimensions as measured in the Go/No-go task (Dalley et al., 2011; Dalley & Robbins, 2017; Donnelly et al., 2015). Wait impulsivity involves, alongside other brain regions, the prelimbic and infralimbic subregions of the PFC, while action impulsivity seems to engage preferentially the orbitofrontal portion of the cortex (Dalley et al., 2011; Dalley & Robbins, 2017; Donnelly et al., 2015). Our results raise the intriguing possibility that THC in adolescence induces ectopic growth of PFC dopamine axons to the orbitofrontal cortex, leading to hypo and hyper dopamine neurotransmission in these cortical regions, respectively, and impacting adult impulse control in a dimension-specific manner. It is interesting to note that in humans chronic, but not acute THC alters presynaptic dopamine function and that this is amplified by earlier onset of cannabis use (Batalla et al., 2013; Bloomfield et al., 2014; Volkow et al., 2014). Our findings are in line with evidence in male mice showing that adolescent THC exposure alters different components of adult impulsivity and that changes in PFC *drd2* expression seem to be involved (Cajiao-Manrique et al., 2023)

DCC receptors in dopamine axons mediate axon targeting in adolescence, controlling the proper segregation of mesolimbic and mesocortical dopamine pathways and development of impulse control (Reynolds et al., 2023; Reynolds, Yetnikoff, et al., 2018). The THC-induced increase in *Dcc* expression in dopamine neurons of early adolescent males may mediate the disruption of PFC and orbitofrontal cortex dopamine development, and in turn the alterations in adult impulse control. Indeed, previous work form our group has shown that exposure to a therapeutic-like dose of amphetamine in early adolescence increases DCC receptor expression in the VTA and induces improved overall cognitive performance in the Go/No-go task in adult (Cuesta et al., 2020). In contrast, early adolescent exposure to a recreational-like

amphetamine dose decreases VTA *Dcc* expression, *causing* targeting errors and ectopic growth of dopamine axons and impairment in impulse control in adulthood (Reynolds et al., 2023). Females are insensitive to these amphetamine effects or engage protective processes (Reynolds et al., 2023). Different substances of abuse in adolescence may induce mistargeting events of dopamine axons, with the ectopic pathfinding trajectory dictated by type of drug and buffered by female sex (Avramescu et al., 2023).

In males, THC-induced dysregulation of *Dcc* expression appear to involve miR-218, which plays a critical role in adolescent brain development (Morgunova & Flores, 2021; Torres-Berrío et al., 2021). This finding is in line with our studies with amphetamine showing that miR-218 mediates drug-induced changes in *Dcc* levels in dopamine neurons in early adolescent male, but not female mice (Cuesta et al., 2020; Reynolds et al., 2023). Epigenetic alterations following adolescent exposure to cannabis have been explored in the context of histone modification (Szutorisz & Hurd, 2016) and more recently regarding the role of microRNAs (Lee et al., 2022; Tomasiewicz et al., 2012). For instance, shifts in microRNA expression profiles in brain and peripheral blood cells have been reported following THC exposure in macaques and mice, respectively (Chandra et al., 2015; Molina et al., 2011; Szutorisz & Hurd, 2016). Furthermore, 7 microRNAs have been shown to be dysregulated in the entorhinal cortex following exposure to a cannabinoid agonist in male rats (Hollins & Cairns, 2016), and RNA sequencing of whole brain showed miR-155hg as enduringly altered after adolescent THC exposure in male mice (Lee et al., 2022).

Females exposed to THC during adolescence show a decrease in *Dcc* expression, but this decrease does not seem to involve miR-218. This raises two intriguing questions: which epigenetic processes may be involved and why are females protected against disruption in dopamine development, despite reduced *Dcc* expression in the VTA. Perhaps, as seen before

in our work with amphetamine (Reynolds et al., 2023), compensatory molecular changes are at play, including microglia-mediated alterations in synaptic pruning (Kopec et al., 2018; Mallya et al., 2018; Schalbetter et al., 2022). In addition, adolescent females may be sensitive to the impact of THC on dopamine and impulse control development at a later adolescent chronological age. For example, a recent report shows that cortical endocannabinoid-mediated long-term depression (eCB-LTD) reaches maturity at a much younger age in female than male mice (Bernabeu et al., 2023) and that female rats treated with THC later in adolescence show in adulthood, disrupted PFC glutamatergic signaling (Rubino et al., 2015). The clear changes occurring within components of the eCB system during distinct windows of adolescence strongly suggests that different periods may incur differential sensitivity to THC exposure (Wiley et al., 2021).

The lack of effects of THC on the developing prefrontal cortex dopamine system and on impulse control in females, does not preclude the possibility of having detrimental consequences in other domains. The endocannabinoid system has been suggested as a buffer of physiological and behavioral responses to stress, and its dysregulation can affect behavioral outcomes (Rubino et al., 2008; Viveros et al., 2005). Female rats administered escalating doses of THC intraperitoneally in adolescence, show increased immobility in the forced swim test and decreased CB1 receptor expression in adulthood in brain regions involved in emotional regulation (Rubino et al., 2008). Further, female-specific brain region activation and behavioral output following THC treatment has been reported by many groups, for example following THC 5mg/kg treatment the hippocampus, the nucleus accumbens core and shell of female rats is activated (Ruiz et al., 2021; Silva et al., 2016; Zuo et al., 2022). It is important to consider that at the time of THC treatment, levels of circulating gonadal hormones in C57/BL6 female mice may be low (Schneider, 2013), and this may confer them with protection (Hill et al., 2007;

Riebe et al., 2010; Rubino & Parolaro, 2011; Schneider, 2008, 2013). Circulating female gonadal hormones have been shown to have interactive effects with cannabis exposure and with the endocannabinoid system (Fonseca et al., 1993; Mize & Alper, 2000), although the mechanistic underpinning of this interplay remains elusive.

Regarding the reduced microglia some size observed in the PFC of females one week after THC exposure, it is important to mention that microglia, alongside the endocannabinoid system (eCBs) play a pivotal role in the extensive synaptic remodeling occurring in the PFC during this period (Berghuis et al., 2007; Mallya et al., 2018; Schalbetter et al., 2022) and that reduced soma size may result in reduced synaptic pruning (Mallya et al., 2018; Schalbetter et al., 2022). Indeed, when microglia are activated in response to external perturbations, their morphology shifts from a rested, surveying state, to a more complex configuration and increased soma size (Cornell et al., 2021). Therefore, reduced synaptic pruning by microglia may be protective against reduced PFC dopamine axonal growth, particularly given their role in the elimination of dopamine receptors (Kopec et al., 2018). Further investigation is needed to dissect the precise role of microglia in THC-induced changes on adolescent neurodevelopment. However, this work contributes to the limited understanding of early adolescent THC exposure on mPFC microglia profile. However, exposure to THC in adolescent female rats has been shown to alter adult PFC microglia morphology (Freels et al., 2023; Zamberletti et al., 2015, 2016). Our findings demonstrate male-specific changes in dopamine connectivity, suggest that THC engages divergent processes in males and females, with modulation by THC dose and adolescent chronological age of exposure. These insights provide valuable information on potential mechanisms through which THC use may lead to lasting behavioral and cognitive changes.

Future Directions

This work adds to the small body of evidence surrounding adolescent THC use and axonal guidance cue systems. Specifically, this project addressed many questions regarding early adolescent THC use on dopamine development and adult impulse control. However, this project represents only the beginning, with many more important aspects to be addressed. Our neuroanatomical data shows that adult males exposed to THC in early adolescence have decreased dopamine connectivity in prelimbic and infralimbic subregions of the mPFC and increased dopamine connectivity in the orbitofrontal cortex, suggesting perhaps axons are being rerouted towards the OFC. Utilizing a dual viral labelling strategy, previously employed in our lab we can address this possibility. By labeling dopamine axons originating from cell bodies in the VTA that are growing to the frontal cortex via the striatum, followed by THC treatment, and perform adult neuroanatomical experiments (Reynolds, Pokinko, et al., 2018). Furthermore, these changes observed in adult male dopamine connectivity may be mediated by changes in *Dcc*, to address this we can apply gene-editing methods. Specifically, constructing CRISPRa constructs to downregulate DCC expression in dopamine neurons and assess whether this buffers against early adolescent-THC induced changes in adult male dopamine connectivity. With regards to adult female dopamine connectivity, we do not observe any changes following early adolescent exposure to THC 5 mg/kg, despite the changes in *Dcc*. We can address the possibility of compensation via microglia by inhibiting microglia activation through a non-selective phosphodiesterase enzyme (PDE) inhibitor while treating females with THC in a similar treatment protocol used in this project and assess microglia profile one week later, and in a separate cohort assess adult frontal cortical dopamine connectivity (Gibson et al., 2006; Liu et al., 2019; Zamberletti et al., 2015). Lastly, given the

change in *Dcc* in THC 5 mg/kg but no change in miR-218, we can begin by assessing other known targets of *Dcc* (Torres-Berrío et al., 2017), if unsuccessful then perform whole miRNA sequencing on brain tissue, following the same treatment protocol utilized in this project to help elucidate the epigenetic mechanism that may be involved in females.

Conclusions and Expected Contributions

This body of work contributes to our knowledge of adolescent THC exposure on frontal cortical dopamine development and impulse control once reached adulthood. THC-treated males show a robust reduction in both the extent of the dopamine input and in the number of dopamine axon presynaptic sites in the PFC in adulthood, however show increased dopamine connectivity within the orbitofrontal cortex. These observed changes suggest a disruption to adolescent male dopamine axon pathfinding whereas females are seemingly protected. THC in early adolescence may impact male and female dopamine and cognitive development differently and by recruiting divergent processes. Our findings show that males exposed to THC show increased premature responding in the Go/No- Go task, reflective of impaired waiting impulsivity, but have fewer commission errors, indicating improved action inhibition, compared to vehicle groups. Females, in line with the lack of changes in dopamine connectivity, show no difference compared to control in adult impulse control. DCC-mediated events may mediate outcomes in males, but in females' changes in microglia profile are observed, perhaps buffering against changes in dopamine connectivity.

This work sheds light on how sex and experiences intersect to shape the developing dopamine system in adolescence. This study aims to further investigate this connection by examining the effects of THC exposure on the development of these conditions. Psychiatric disorders often arise during adolescence, and sex seems to play a crucial role in influencing vulnerability or resilience to their emergence. The research sought to uncover how THC exposure affected neuroanatomical, behavioral and molecular outcomes in adulthood, in males then in females. Ultimately, I hope our discoveries emphasize the importance of (i) continuing research on the developmental consequences following early exposure to THC (ii)

considering potential male and female differences when exploring how any external factors may influence brain development.

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