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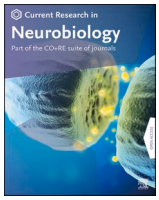

Δ9-Tetrahydrocannabinol does not upregulate an aversive dopamine receptor mechanism in adolescent brain unlike in adults

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ABSTRACT

Earlier age of cannabis usage poses higher risk of Cannabis Use Disorder and adverse consequences, such as addiction, anxiety, dysphoria, psychosis, largely attributed to its principal psychoactive component, Δ9-tetrahydrocannabinol (THC) and altered dopaminergic function. As dopamine D1-D2 receptor heteromer activation causes anxiety and anhedonia, this signaling complex was postulated to contribute to THC-induced affective symptoms. To investigate this, we administered THC repeatedly to adolescent monkeys and adolescent or adult rats. Drug-naïve adolescent rat had lower striatal densities of D1-D2 heteromer compared to adult rat. Repeated administration of THC to adolescent rat or adolescent monkey did not alter D1-D2 heteromer expression in nucleus accumbens or dorsal striatum but upregulated it in adult rat. Behaviourally, THC-treated adult, but not adolescent rat manifested anxiety and anhedonia-like behaviour, with elevated composite negative emotionality scores that correlated with striatal D1-D2 density. THC modified downstream markers of D1-D2 activation in adult, but not adolescent striatum. THC administered with cannabidiol did not alter D1-D2 expression. In adult rat, co-administration of CB1 receptor (CB1R) inverse agonist with THC attenuated D1-D2 upregulation, implicating cannabinoids in the regulation of striatal D1-D2 heteromer expression. THC exposure revealed an adaptable age-specific, anxiogenic, anti-reward mechanism operant in adult striatum but deficient in adolescent rat and monkey striatum that may confer increased sensitivity to THC reward in adolescence while limiting its negative effects, thus promoting continued use and increasing vulnerability to long-term adverse cannabis effects.

1. Introduction

In recent years, North America, Western Europe and Australia have seen rapid growth of cannabis use in parallel with reduced legal restrictions and lower perception of harm (WHO, 2022). In Canada, a nation that legalized cannabis, 25% of the population 16 and older used cannabis in the past year (CCS, 2021), with similar values corresponding for past year use in the United States (17.9% of the population ages 12 and older) (SAMHSA, 2010). With repeated long-term exposure, use of cannabis can lead to cannabis use disorder (CUD), cognitive impairment, anxiety and psychosis in susceptible individuals. The magnitude of neuropsychological consequences is associated with dose, frequency, duration of use and importantly, age of onset of use (Lowe et al., 2019; Rubino et al., 2012; Stark et al., 2021).

Adolescence is a critical period of brain maturation, in which refinements in brain function and behaviour occur, especially in brain regions implicated in cognition, reward, and affect. Of core relevance, endocannabinoids have an integral role in brain development and regulate vital functions throughout the lifespan in rodent and human brain. Particularly during adolescence, dynamic changes in endocannabinoid signaling regulate the maturation and plasticity of the major dopaminergic pathways involved in reward processing in brain, comprising the mesocorticolimbic circuitry, together with the development of associated complex behaviors (Meyer et al., 2018). The introduction of exogenous cannabinoids conceivably could interfere with the trajectory of normal development involving this system, as borne out by accumulating evidence. Adolescent-onset cannabis use confers a higher risk for addiction, cognitive impairment and psychiatric symptoms,

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including psychosis, depression, anxiety, and suicidality, which may persist into adulthood (Bloomfield et al., 2016; Chadwick et al., 2013; Di Forti et al., 2019; Gobbi et al., 2019; Han et al., 2019; Hurd, 2020; Renard et al., 2017; Schaefer et al., 2021). Recent trends among youth emphasize the greatly amplified overall risks: 35.2% of 12th graders used cannabis in the United States in the past year (Han et al., 2019), and 46% usage among 16–19 year olds was recorded in Canada (CCS, 2021). More high school students are using the drug daily (6.9% of 12th graders) and consuming high potency strains of cannabis containing a high percentage of Δ9-tetrahydrocannabinol (THC), the primary psychoactive component of cannabis. These factors in adolescents magnify the risks of developing drug dependence, poor cognitive function, and more severe psychological symptoms (Chan et al., 2021; Gobbi et al., 2019; Han et al., 2019; Kiburi et al., 2021; Walker et al., 2017).

Acting at the cannabinoid type 1 receptor (CB1R) in brain, THC elicits its addictive and psychotomimetic effects, in part by modulating dopamine signaling (Bloomfield et al., 2016; Bossong et al., 2015). Frequent use, especially of high THC concentration cannabis, can engender anxiety and anhedonia, which correlate with reduced dopaminergic function (van de Giessen et al., 2017; Volkow, 2016; Volkow et al., 2014).

In adolescents, increased risk-taking and enhanced reward-value is thought to be related to extensive developmental remodeling of the dopaminergic system (Walker et al., 2017) including robust changes in dopamine D1 and D2 receptor expression in the striatum and prefrontal cortex (Andersen et al., 2000; Walker et al., 2017). D1 and D2 receptors have been shown to oligomerize *in vivo* to form the dopamine D1-D2 receptor heteromer, a complex which is expressed in discrete populations of striatal medium spiny neurons (MSNs) (Perreault et al., 2010) and in other brain regions (Hasbi et al., 2020b). The D1-D2 heteromer couples to $G\alpha_q$, stimulating activation of phospholipase C, intracellular calcium release and phosphorylation of calcium calmodulin kinase IIα (CaMKIIα) (Hasbi et al., 2009; Rashid et al., 2007). D1-D2 heteromer-activated signaling cascades include proteins which are highly implicated in regulation of synaptic plasticity and addiction-related behaviour, including CaMKIIα, brain-derived neurotrophic factor (BDNF) and its receptor TrkB, glycogen synthase kinase 3 (GSK-3), cyclin dependent kinase 5 (Cdk5), dopamine-and cAMP-regulated phosphoprotein of MW 32 kDa (DARPP-32), extracellular regulated kinase (ERK), phosphorylated α-amino-3-hydrooxy-5-methyl-4-isoxazoleproprionic acid (AMPA) receptor subunit GluA1 and transcription factor ΔFosB (Hasbi et al., 2018; Perreault et al., 2016). Activation of D1-D2 produces aversion, anxiety, and anhedonia in rodent models (Hasbi et al., 2018; Shen et al., 2015), not unlike the aversive and anxiety-like symptoms observed after chronic cannabis exposure (van de Giessen et al., 2017; Volkow, 2016; Volkow et al., 2014). It also attenuates the rewarding and sensitizing effects of cocaine (Hasbi et al., 2018). Therefore, D1-D2 conceivably serves as a negative restraint on the dopaminergic reward system. Furthermore, disrupting the D1-D2 heteromer has been shown to ameliorate the anxiety and anhedonia resulting from chronic THC exposure and withdrawal (Hasbi et al., *In press* 2023).

Aversive mechanisms have been postulated to be less active in adolescence, showing greater responsiveness to rewarding experiences with reduced reactions to aversive experiences (Giedd, 2004; Li and Xu, 2008). Greater dopamine release by drugs of abuse in adolescent rat nucleus accumbens has been shown (Jobson et al., 2019; Laviolette, 2021; Shearman et al., 2008), unlike in adults (Shram et al., 2006), likely responsible for greater subjective reward. However, no mechanism that may specifically account for reduced aversion in adolescence has been identified. As D1-D2 heteromer expression has been shown to be lower in juvenile rat striatum compared to adult (Perreault et al., 2012), a potential mechanism could be a lower density of neurons expressing the heteromer in adolescent nucleus accumbens and dorsal striatum compared to adult, with maturation-dependent functional differences in MSNs expressing this complex potentially underlying differences in the regulation of reward and aversion between the age groups.

Importantly, we have previously demonstrated that the dopamine D1-D2 receptor heteromer is upregulated in striatum by THC administered daily to adult nonhuman primate (Hasbi et al., 2020a). However, it is unknown whether THC elicits a similar adaptive response in adolescents. In addition, cannabidiol (CBD), another prominent phytocannabinoid in cannabis does not impair cognition, is not addictive, and has been shown to be anxiolytic, anti-psychotic and neuroprotective, mitigating some of the adverse consequences of THC (Boggs et al., 2018; Casadio et al., 2011; Colizzi and Bhattacharyya, 2017; Englund et al., 2013; Fusar-Poli et al., 2009; Hudson et al., 2019; Schubart et al., 2011). To elucidate the molecular differences governing the age-related differential sensitivity, THC or THC combined with a therapeutic dose of CBD was administered daily to adolescent nonhuman primates (Hasbi et al., 2020a; Withey et al., 2020, 2021), and to adolescent and adult rats. D1-D2 neuron density and downstream signaling system activation were evaluated in striatum of THC-treated animals. Rimonabant, a selective CB1 receptor inverse agonist, was also co-administered with THC to determine whether D1-D2 heteromer regulation occurred downstream of THC activation of the cannabinoid CB1R. By comparing molecular adaptations and behavioural phenotypes in the adolescent and adult subjects, we sought evidence for involvement of the D1-D2 heteromer in the heightened susceptibility of adolescents to the detrimental effects of THC.

2. Materials and methods

2.1. Animals

54 adult (PND 70) and 28 adolescent (PND 35) male Sprague-Dawley rats (Charles River, Canada) were singly housed and maintained in a 12:12 h light:dark cycle with food and water available ad libitum. Animals were acclimatized for one week before the start of experiments. Procedures were carried out in compliance with guidelines described in the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care). The protocol was approved by the University of Toronto Animal Use Committee.

12 adolescent (2–2.5 years) male squirrel monkeys (*Saimiri sciureus*) were housed individually in a temperature and humidity-controlled vivarium in a 12:12 h light:dark cycle with food and water available ad libitum. Animals were maintained in a facility licensed by the U.S. Department of Agriculture. The experimental protocol for the present studies was approved by the Institutional Animal Care and Use Committee at McLean Hospital and were in accord with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research Committee of the Institute of Laboratory Animals Resources, Commission on Life Sciences, National Research Council.

2.2. Drug administration

Chronic treatment in rat consisted of 28 days of daily intraperitoneal (i.p) injections of vehicle, THC (1 mg/kg), CBD (3 mg/kg) or THC + CBD (1 mg/kg, 3 mg/kg). For adolescents, this spanned PND 35–62 and for adults, PND 70–97. On day 28, animals were euthanized 1 h after final drug injection and brains were harvested for biochemical analyses. The rimonabant experiment consisted of 1 week of daily i. p injections of vehicle, THC (1 mg/kg) or THC + RIM (1 mg/kg: 1 mg/kg) in adults, from PND 70–76. The 1 mg/kg dose of THC was based on the percentage of THC in a standard cannabis joint, approximately 20.6% THC, or 60 mg THC, which translates to 0.85 mg/kg for a 70 kg person (Grotenhermen, 2003; Manthey et al., 2021). CBD dose was selected based on available high CBD:THC ratio preparations in which CBD may attenuate the effects of THC (Colizzi and Bhattacharyya, 2017; Englund et al., 2017; Schubart et al., 2011) and in line with a previous study by our group (Hasbi et al., 2020a). The dose of rimonabant 1 mg/kg was

selected to block effects of THC based on previous studies in rats (Jarbe et al., 2010; Ravula et al., 2018) and humans (Huestis et al., 2007).

Monkeys were divided into three treatment groups and received injections of vehicle, THC (1 mg/kg), THC + CBD (1 mg/kg, 3 mg/kg). Doses of THC were progressively introduced over a 3-week period; during week 1, animals received a low dose on a single day (0.1 mg/kg for THC, 0.3 mg/kg for CBD), in week 2, an intermediate dose on a single day (0.3 mg/kg for THC, 1 mg/kg for CBD) and in week 3, a higher dose on a single day (1 mg/kg for THC, 3 mg/kg for CBD), as previously described (Withey et al., 2020). On day 1 of week 4, animals began daily treatment with the final 1 mg/kg dose of THC for 114 or 117 days. Animals from the control group were injected with vehicle for each weekly, and then daily injection, in line with the THC injections. Injections began in adolescent monkeys around 2–2.5 years of age for approximately 3.8–3.9 months until the end of the experiment.

2.3. Drugs

THC and CBD were generously supplied by Cannascribe Inc. And the National Institute of Health (NIH). For chronic THC experiments in rat, drug was prepared in a vehicle of 95% ethanol, Tween80 and saline (1:1:18 ratio). For the CB1R antagonism experiment, rimonabant (AdooQ Bioscience, A11547) and THC were prepared in a vehicle of 61.47% saline, 30% PEG400, 5% propylene glycol, 3.03% 95% ethanol and 0.5% Tween80 as per manufacturer's instructions. For monkeys, THC and CBD were prepared in a 20:20:60 solution of 95% ethanol, Tween80 and saline.

2.4. Behavioural measurements

Light-dark box: Test was performed in a two-chamber apparatus (each compartment 20″ x 20″ x 20"), with one brightly lit and open compartment connected to a covered dark compartment by a door. The rat was placed in the dark compartment to begin, and latency to enter light side was recorded. The rat freely explored the two compartments for a total of 5 min, and total time in each compartment was quantified. This test was performed on day 10 of THC injections (PND 44 for adolescents, PND 79 for adults).

Novelty-induced hypophagia: Rats were exposed to one bottle of 1:3 dilution of commercially available condensed milk and one bottle of water in their home cage for 1 h, on 3 consecutive days for habituation using a 2-bottle, free-choice paradigm. On the fourth day, rats were tested in their home cage for their latency to drink milk and total milk consumption during a 1-h test session. On the fifth day, rats underwent the same test in a novel, anxiety-inducing environment, consisting of a new brightly lit cage with no bedding. This test was performed on days 14–18 of injections (PND 48–52 for adolescents, PND 83–87 for adults).

Open field: Rats were placed in a plexiglass chamber (16″ x 16″ x 16″). The animal's location and movement were tracked by sensors on the bottom and sides of the chamber and collected by the Fusion software (Omnitech Electronics). Variables scored were distance traveled (cm), centre time (whole body), vertical activity (time), stereotypy (episode count) and time in centre zone. This test was performed on day 21 of injections (PND 54 for adolescents, PND 89 for adults).

Emotionality score: A composite emotionality score was calculated for each animal to integrate behavioural measures from the light-dark box, open field, and novelty-induced hypophagia test (adapted from (Guilloux et al., 2011)). Z-normalization was computed for each animal, and for each test. These scores were then combined to generate an emotionality Z-score for each animal, calculated as: $Emotionality\ Score = \frac{Z_{LD\ Box}+Z_{OF}+Z_{NIH}}{Number\ of\ tests}$ (where $Z_{LD\ Box}$ = z-score for latency to enter light box (in the light-dark box test), Z_{OF} = z-score for centre time (in open field test) and Z_{NIH} = z-score for latency to drink condensed milk consumed (in the novelty-induced hypophagia test)).

2.5. D1-D2 dopamine receptor heteromer quantification by in situ proximity ligation assay

D1-D2 dopamine receptor heteromer density and expression in brain regions was determined by *in situ* proximity ligation assay (PLA) of D1 and D2 receptors as previously described (Hasbi et al., 2018), using validated antibodies for D1 and D2 receptors (Lee et al., 2004; Perreault et al., 2010) and for PLA (Hasbi et al., 2018). Antibodies against D1 and D2 receptors were directly conjugated to complementary Plus and Minus oligonucleotides such that receptors in close proximity (<40 nm distance) ligate and induce a fluorescent signal. To generate the PLA probes, rat anti-D1 receptor antibody (Sigma, D2944) was directly conjugated with the PLUS oligonucleotide (In Situ Probemaker PLUS DUO92009, Sigma-Olink) and rabbit anti-D2 antibody (Millipore AB5084P) with the MINUS oligonucleotide (In Situ Probemaker MINUS DUO92010, Sigma-Olink) according to manufacturer's instructions. Rat brain coronal slices of 20 μm thickness were post-fixed with 4% paraformaldehyde (PFA) solution for 20 min. Sections were permeabilized with 0.1% Triton-X-PBS solution for 15 min, then incubated with Duolink Blocking Solution (DUO82007, Sigma) for 30 min at 37 °C. Sections were then incubated at 4 °C overnight with PLA probes diluted at a final concentration of 63 μg/mL. Duolink II *in situ* PLA detection kit (DUO92008, Sigma-Olink) was used for subsequent ligation and amplification steps. Slides were mounted with a DAPI containing mounting medium (Vectashield Antifade Mounting Media, Vector Laboratories, H-1800).

PLA signals appeared as fluorescent puncta and were visualized by confocal microscopy using a Fluoview Olympus microscope (FV 1000) with a 60 × objective. 10 Z-stack images were acquired with a step size of 2 μm. 4–6 images per animal were randomly acquired within a given brain region. Quantification of PLA signals and DAPI cell counts were conducted using the dedicated ImageTool software (Duolink DUO90806, Sigma-Olink). The number of D1-D2 heteromer-positive cells was normalized to the number of DAPI labeled nuclei to calculate the percentage of D1-D2 positive cells.

2.6. In situ hybridization of Drd1 and Drd2

Drd1 and Drd2-expressing cell density was determined by *in situ* hybridization (ISH) of D1 and D2 receptors mRNA using the dedicated RNAScope Multiplex Fluorescnt Detection Kit v2 (323,110, ACD Biotechne) and RNAscope probes Rn-Drd2-C2 *Rattus norvegicus* dopamine receptor 2 (Drd2) mRNA (315,641-C2, ACD Biotechne) and Rn-Drd1a *Rattus norvegicus* dopamine receptor D1 (Drd1) mRNA (317,031, ACD Biotechne). ISH was performed as per manufacturer's kit instructions. Briefly, fresh frozen tissue sections were post-fixed with 4% PFA, then dehydrated with increasing concentrations of ethanol and stored in 100% ethanol overnight at −20 °C. The next day, slides were incubated with hydrogen peroxide solution (RNAScope H202 and Protease reagents kit, 322,381, ACD Biotechne) for 10 min at room temperature, then Protease III (RNAScope H202 and Protease reagents kit, 322,381, ACD Biotechne) for 30 min at 40 °C. A 1:50 ratio of probe Rn-Drd2-C2: Rn-Drd1a-C1 was incubated with tissue for 2 h at 40 °C. RNAScope Multiplex Fluorescent Detection Kit v2 was used for subsequent amplification steps (323,110, ACD Biotechne). Fluorescein fluorophore (TSA-Plus Fluorescein, NEL741001KT, PerkinElmer) was diluted in TSA buffer (1:750) (Cat. #322809, ACD Biotechne) and incubated 30 min at 40 °C. HRP-blocker and HRP-C2 were then each incubated with tissue for 15 min at 40 °C (RNAScope Multiplex Fluorescnt Detection Kit v2, 323,110, ACD Biotechne). Cyanine 3 fluorophore (TSA-Plus Fluorescein, NEL744001KT, PerkinElmer) was diluted in TSA buffer (1:750)

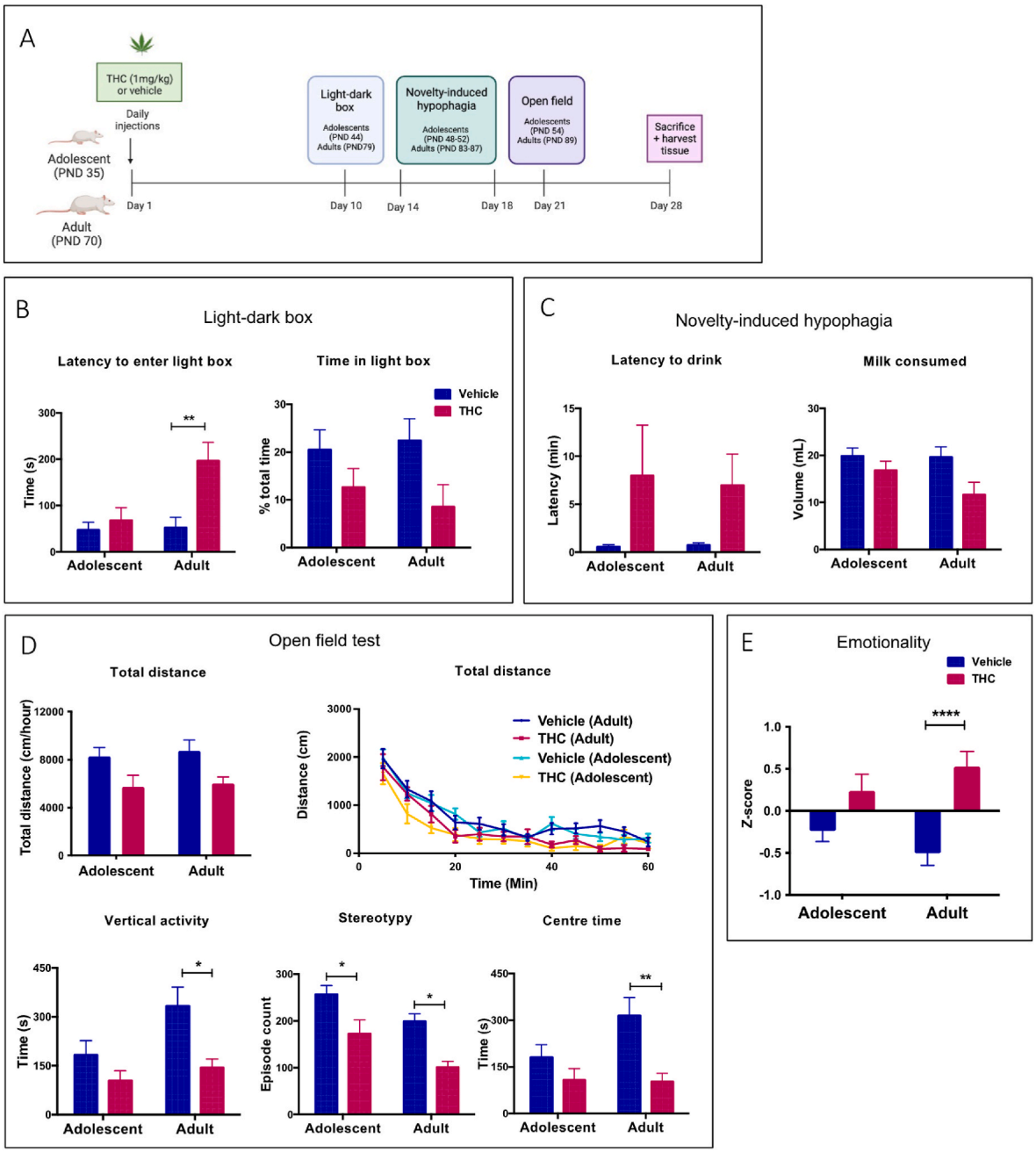


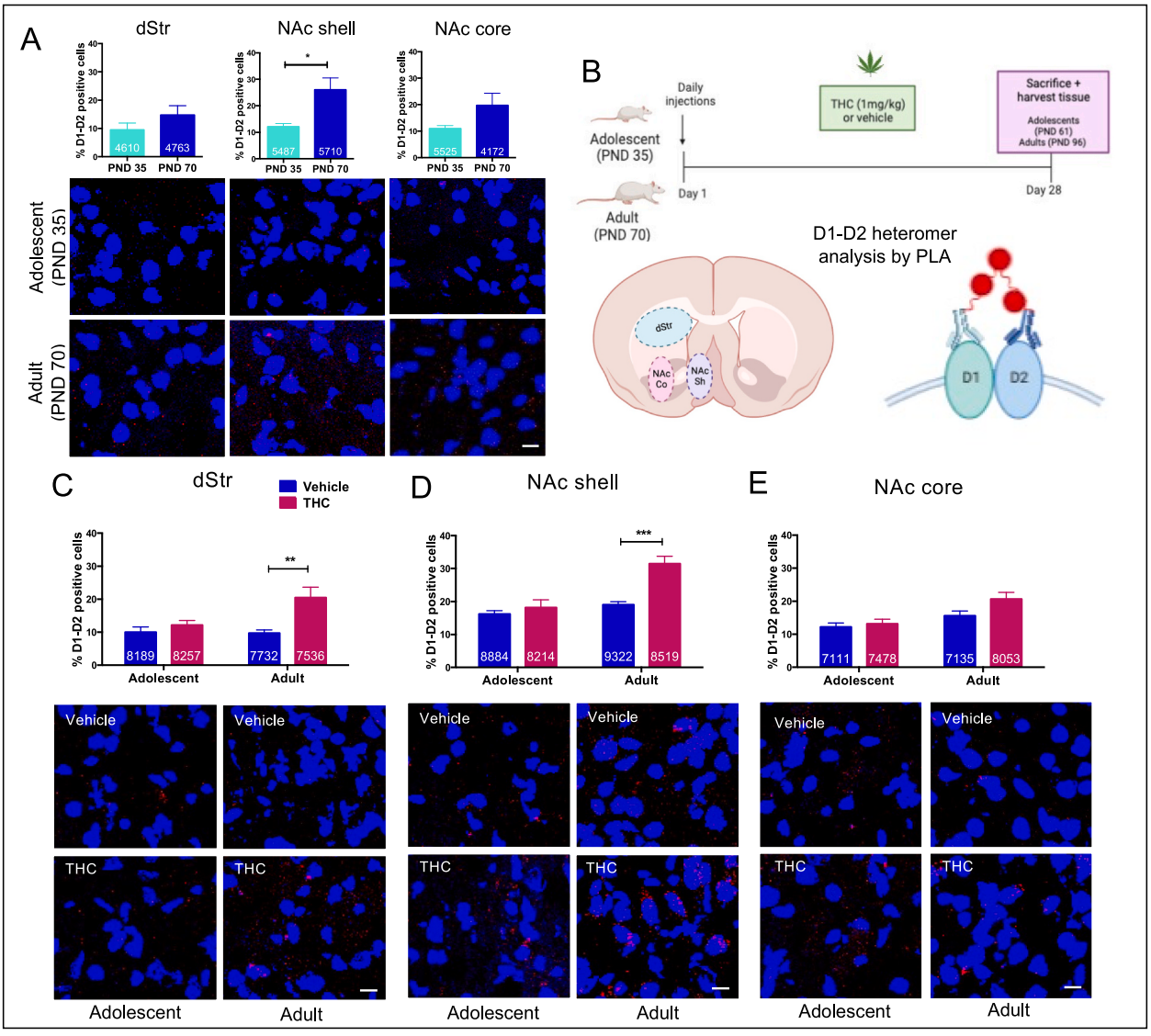
Fig. 1. Effects of repeated daily THC administration on anxiety and depression-like behaviors in adult and adolescent rats. A) Experimental timeline: THC 1 mg/kg or vehicle was administered daily by i. p injection for 28 days total starting at PND 35 for adolescents and PND 70 for adults. Light-dark box test was conducted on day 10 of injections, novelty-induced hypophagia on day 14–18 of injections, open field test on day 21 of injections. B) Light-dark box test: measure of latency to enter light box and total time in light box. C) Novelty-induced hypophagia test: measure of latency to drink milk and total milk consumed. D) Open field test: measure of total distance traveled, vertical activity, stereotypy and time in centre zone of the open field E) Emotionality was calculated as the average Z-score of all three behavioural tests for each animal. Results are expressed as mean ± SEM (two-way ANOVA with Tukey's test for multiple comparisons (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

(322,809, ACD Biotechne) and incubated 30 min at 40 °C. HRP-blocker was incubated with tissue for 15 min at 40 °C, followed by DAPI staining (RNAScope Multiplex Fluorescent Detection Kit v2, 323,110, ACD Biotechne) and slide mounting.

Signal was visualized by confocal microscopy using a Fluoview Olympus microscope (FV 1000) with a 60 × objective. 10 Z-stack images were acquired with a step size of 2 μm. 4–5 images per animal were acquired for each brain region. Quantification of Drd1 and Drd2 signal and DAPI cell counts were conducted using the ImageTool software (Duolink DUO90806, Sigma-Olink). The number of Drd1 or Drd2-positive cells was normalized to the number of DAPI labeled nuclei to calculate the percentage of Drd1-or Drd2-positive cells.

2.7. Western blot

Tissue was punched from brain regions of interest (dorsal striatum and nucleus accumbens) and suspended in lysis buffer containing protease inhibitor (Sigma, P8340) and phosphatase inhibitor (Cell Signaling Technology, 5870 S). Samples were maintained in constant agitation for 30 min then centrifuged at 10 000 g for 10 min and supernatant protein



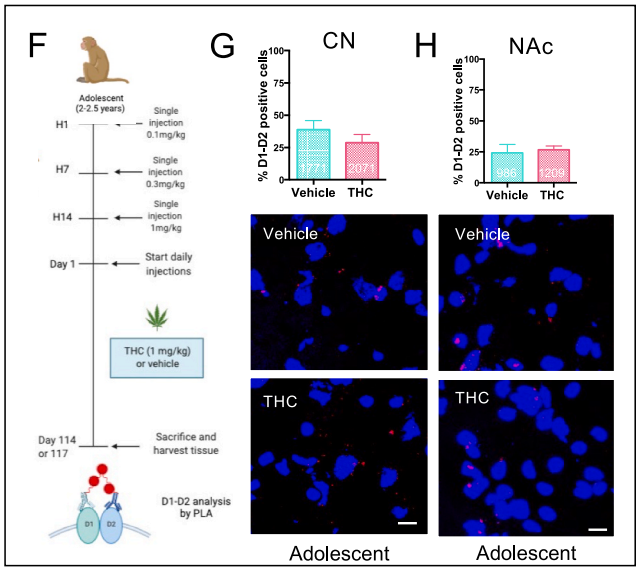


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Fig. 2. D1-D2 dopamine receptor heteromer expression following daily THC or vehicle administration in adult and adolescent rat and monkey striatum. A) Baseline D1-D2 dopamine receptor heteromer expression in striatal subregions of dorsal striatum (dStr), nucleus accumbens (NAc) shell and NAc core in adult (PND 70) and adolescent (PND 35) drug-naïve male Sprague Dawley rats (n = 6/group). B) Adult (PND70) and adolescent (PND35) male rats (n = 10–11/group) received i. p. injections of THC or vehicle daily for 28 days, followed by brain tissue harvesting and PLA analysis of D1-D2 dopamine receptor heteromer in striatal subregions. C) D1-D2 heteromer expression by PLA in the dStr, D) NAc shell, E) NAc core. Results are mean ± SEM, two-way ANOVA with Tukey's multiple comparisons test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). F) Adolescent monkeys (2–2.5 years old) were pre-treated with a single injection of either vehicle or 0.1 mg/kg THC on habituation day 1 (H1), 0.3 mg/kg THC on H7 and 1 mg/kg THC on H14. One week later, they began daily treatment with 1 mg/kg THC or vehicle for 114 or 117 days (n = 3–4/group). G) PLA analysis of D1-D2 heteromer in the caudate nucleus (CN) and H) NAc of monkeys. Results are mean ± SEM and numbers in bar graph denote numbers of neurons evaluated. Student's t-test, (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). Representative images imaged at 60*X*. Scale bar is 10 μM.

was collected. Samples for Western blot contained 30 μg of protein in 2-mercaptoethanol (Sigma, M-6250) and 8% SDS sample buffer and were boiled at 95 °C for 5 min to denature protein. Protein samples were run on 4–20% polyacrylamide gels under denaturing conditions (SDS-PAGE) and transferred onto nitrocellulose membranes (Invitrogen, PB7320) using a semidry transfer system. Membranes were blocked in 5% non-fat milk/TBS-Tween (TBS-T) or 5% bovine serum albumin/(BSA)/TBS-T for 1 h, then incubated in primary antibody diluted in 5% milk/TBS-T or BSA/TBS-T overnight at 4 °C. Membranes were washed in TBS-T, then incubated with secondary antibodies for 1 h at room temperature. Blots were imaged using Li-Cor Odyssey (ODY-2212) and analyzed using the dedicated Image Studio software.

Primary antibodies and dilutions used were: phospho-CaMKIIα (Cell Signaling Technologies, 12,726; 1:1000), CaMKIIα (Cell Signaling Technologies, 3362; 1:1000), phospho-GSK3αβ (Cell Signaling Technologies, 9331; 1:1000), GSK3αβ (Cell Signaling Technologies, 5676; 1:1000), phospho-TrkB (Sigma, ABN1381; 1:500), TrkB (Cell Signaling Technologies, 4607; 1:1000), phospho-Thr75-DARPP-32 (Cell Signaling Technologies, 2301 S; 1:1000), prodynorphin (Neuromics, GP10110; 1:700), kappa opioid receptor (Abcam, ab183825; 1:1000), kappa opioid receptor phospho-Ser369 (SAB Bio; 12,227), phospho-Ser845-GluA1 (Millipore, 1:1000), AB5849; phospho-CB1R (Abcam, ab186428; 1:1000), GAPDH (Sigma, G8795; 1:10,000) and β-actin (Abgent, AM1021B; 1:1000). Secondary antibodies used were IRDye 680RD anti-mouse (Li-Cor, 925–68,072) and IRDye 800RD anti-rabbit (Li-Cor, 926–32,213).

2.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism. Results are reported as mean ± SEM. Two-way ANOVA with Tukey's multiple comparisons was performed on behavioural data, PLA D1-D2 heteromer analysis between vehicle vs THC administration, vehicle vs THC vs THC + CBD administration, and naïve adult vs adolescent. Pearson's correlation and linear regression was calculated between D1-D2 heteromer density and emotionality score. Student's t-test was used to evaluate statistical significance for immunoblot data. One-way ANOVA followed by Tukey post-hoc test was used for CB1R antagonism experiments.

3. Results

3.1. Behavioural effects of daily THC administration in adult and adolescent rats

Male adult (PND 70) or adolescent (PND 35) rats (n = 10–11/group) received daily injections of THC or vehicle for 28 days and anxiety-, depressive-like and anhedonic behaviours were compared in THC-treated adolescent and adult rats (n = 8–11) with testing performed between injection days 10–21 (PND 44–54 in adolescents and PND 79–89 in adults) (Fig. 1A). In the light-dark box test (Fig. 1B), after 10 days of THC exposure, adult male rats, but not adolescent males showed a striking increase in latency to enter the light compartment compared to vehicle control (Two-way ANOVA: main effect of age, F (1,36) = 6.160, p = 0.0179; main effect of treatment, F (1.36) = 9.325, p = 0.0042; age × treatment interaction, F (1,36) = 5.227, p = 0.0282) (Tukey's multiple comparisons test: Adult:Vehicle vs Adult:THC, Mean difference = −144.2, 95% CI of difference = −252.1 to −36.33, p < 0.005), suggestive of THC-induced elevated anxiety in adults but not in adolescents. Moreover, the magnitude of THC effect on light compartment entry latency was significantly higher in adults than adolescents (Tukey's multiple comparisons test Adult:THC vs Adolescent:THC, Mean difference = −128.8, 95% CI of difference = −231.6 to −25.91, p < 0.005). Adult and adolescent rats both exhibited decreased total time spent in the light compartment (Two-way ANOVA: main effect of treatment, F (1,36) = 6.389, p = 0.0160). In the novelty-induced hypophagia test (Fig. 1C), after 18 days of THC exposure, both adult and adolescent rats exhibited an increase in latency to drink milk (Two-way ANOVA: main effect of treatment; F (1,42) = 5.043, p = 0.0301), indicative of anxiety-like behaviour and a reduction in total milk consumption (Two-way ANOVA: main effect of treatment, F (1,42) = 1.567, p = 0.0148), indicative of anhedonia-like behaviour.

The open field test (Fig. 1D), conducted on treatment day 21, showed that total horizontal distance traveled was lower in THC-treated groups compared to vehicle controls (Two-way ANOVA: main effect of treatment, F (1,42) = 10.76, p = 0.0021), with decrease of vertical rearing activity more pronounced in the adult THC group compared to the adolescent group (Two-way ANOVA: main effect of age, F (1,30) = 4.657, p = 0.0391; main effect of treatment, F (1,30) = 9.250, p = 0.0049) (Tukey's multiple comparisons test Adult:Vehicle vs Adult:THC, Mean difference = 189.4, 95% CI of difference = 35.83 to 343, p < 0.05). Stereotypic purposeless behaviours such as repetitive sniffing and head movements were also attenuated in the THC group, affecting the adult group more significantly (Two-way ANOVA: main effect of age, F (1,34) = 10.69, p = 0.002; main effect of treatment, F (1,34) = 21.40, p < 0.0001). THC-treated adult, but not adolescent rats showed decreased time in the centre of the open field compared to control (Two-way ANOVA: main effect of treatment, F (1,41) = 11.13, p = 0.001), suggestive of increased anxiety-like behaviour.

An "emotionality score" (Fig. 1E) was calculated for each rat based on z-scores from the latency to enter light box, latency to drink milk and time in centre of open field, all behavioural readouts of anxiety-like behaviour. Emotionality was significantly elevated by THC exposure in adults, but not in adolescents after 10–21 days of THC treatment (Two-way ANOVA: main effect of treatment, F (1,42) = 21.17, p < 0.0001) (Tukey's multiple comparisons test Adult:Vehicle vs Adult THC, Mean difference = −1.034, 95% CI of difference = −1.641 to −0.4263, p < 0.001) highlighting age-specific behavioural manifestations of repeated THC administration.

3.2. Effects of daily THC administration in adult and adolescent rats and adolescent monkeys on dopamine D1-D2 receptor heteromer

In drug-naïve adult (PND 70) and adolescent (PND 35) male Sprague-Dawley rats (Fig. 2A), D1-D2 heteromer density in striatal subregions was assessed by *in situ* proximity ligation assay (PLA) of D1 and D2 receptors. Numbers in PLA bar graphs denote total number of cells evaluated. In drug-naïve animals, D1-D2 expression was significantly higher in adults than adolescents (Two-way ANOVA: Main effect of age, F (1,30) = 13.08, p = 0.0011) with higher D1-D2 expression in the adult NAc shell compared to adolescent (Tukey's multiple

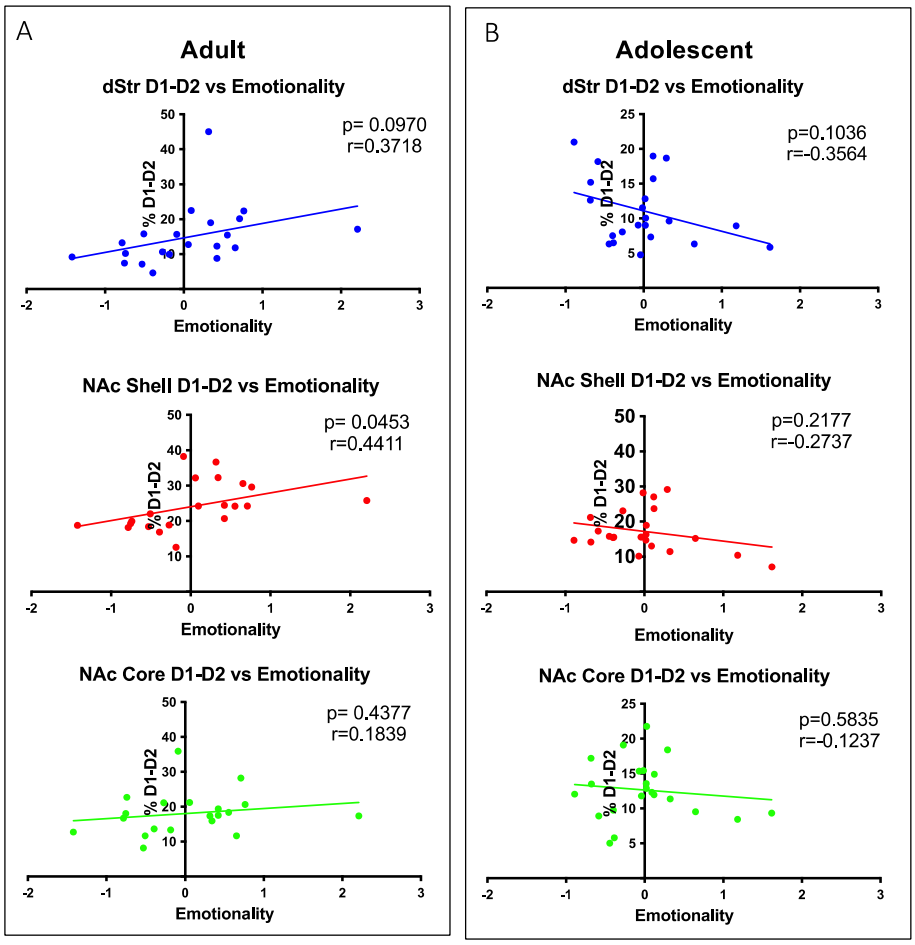


Fig. 3. THC-mediated effects in adolescent and adult rats: association between striatal D1-D2 dopamine receptor heteromer expression and behavioural scores. Regression line for correlation between composite emotionality Z-score and D1-D2 heteromer density in the dorsal striatum (dStr), nucleus accumbens shell (NAc shell) or nucleus accumbens core (NAc core) for A) adult or B) adolescent THC-treated rats. (Pearson correlation, n = 20–21, using data from both vehicle and THC-treated animals).

comparisons test: NAcShell:Adolescent vs NAcShell:Adult, Mean difference = −13.93, 95% CI of difference = −27.47 to −0.3818, $p < 0.05$), demonstrating evidence for developmental maturation of D1-D2 heteromer expression.

After 28 days of chronic injections of either THC or vehicle, animals were euthanized 1 h after the final injection (Fig. 2B) and D1-D2 heteromer expression was evaluated in striatal subregions by PLA (Fig. 2C–E). In the dStr of THC-treated adult rats (Fig. 2C), the proportion of D1-D2 heteromer expressing MSNs was significantly increased 111% compared to vehicle controls, but in the group treated with THC in adolescence, D1-D2 heteromer expressing MSNs remained unchanged (Two-way ANOVA: main effect of age, F (1,39) = 4.199), p = 0.0472; main effect of treatment, F (1,39) = 10.83, p = 0.002; age × treatment interaction, F (1,39) = 4.199, p = 0.0341) (Tukey's multiple comparisons test: Adult:Vehicle vs Adult:THC, Mean difference = −8.351, 95% CI of difference = −15.72 to −0.9779, $p < 0.005$). A parallel effect was observed in the NAc shell (Fig. 2D), as D1-D2 expressing MSNs increased 65% in THC-treated adults rats compared to vehicle, but not in NAc shell of adolescent THC-treated rats (Two-way ANOVA: main effect of age, F (1,40) = 21.22, $p < 0.0001$; main effect of treatment, F (1,40) = 16.98, p = 0.0002; age × treatment interaction, F (1,40) = 8.771, p = 0.0051). In the NAc core (Fig. 2E), the number of neurons expressing D1-D2 heteromer was lower in adolescent than adult-treated rats and was not upregulated following chronic daily THC administration. (Two-way ANOVA: main effect of age, F (1,39) = 13.00, p = 0.0009). It is likely that the repeated stress of injections itself may affect D1-D2 expression as our studies (*unpublished*) have revealed that chronic mild stress decreased D1-D2 heteromer expression in the nucleus accumbens, which may explain why percentage values for vehicle animals are lower in Fig. 2C–E compared to Fig. 2A for treatment naïve animals. This would not affect the observed effect of THC to upregulate D1-D2 as both the vehicle and THC treatment groups receive the same number of repeated injections.

An evaluation of striatal D1-vs D2-expressing cells was performed by *in situ* hybridization of receptor mRNA (Fig. S1). Neither age of treatment or THC treatment altered the proportion of cells expressing Drd1 mRNA in the dStr, NAc shell or NAc core compared to vehicle control (Two-way ANOVA: dStr, Age: F (1,22) = 0.1183, p = 0.7341, Treatment: F (1,22) = 0.1143, p = 0.7385; NAcSh, Age: F (1,22) = 0.3542, p = 0.5578, Treatment: F (1,22) = 0.1900, p = 0.6671; NAcCo, Age: F (1,21) = 0.3821, p = 0.5431, Treatment: F (1,21) = 0.2756, p = 0.6051). Similarly, Drd2-expressing cell numbers were not altered by age of treatment or THC treatment in the dStr, NAc shell or NAc core (Two-way ANOVA: dStr, Age: F (1,22) = 1.198, p = 0.2855, Treatment: F (1,22) = 0.2690, p = 0.6090; NAcSh, Age: F (1,22) = 0.01365, p = 0.9081, Treatment: F (1,22) = 0.1125, p = 0.7405; NAcCo, Age: F (1,21) = 0.2449, p = 0.6258, Treatment: F (1,21) = 0.4181, p = 0.5249). These results suggest that the observed THC exposure-induced increase in D1-D2-expressing cells in the NAcSh and dStr was not driven by increased transcription of D1 or D2 receptor mRNA and instead may be a protein translational mechanism downstream of mRNA generation.

Previously, our group demonstrated that chronic THC administration in adult Rhesus monkey led to a dramatic increase in D1-D2 heteromer density in the NAc and caudate nucleus (CN), the primate homolog of the rodent dStr (Hasbi et al., 2020a). There are clear species differences in D1-D2 heteromer expression in striatal subregions showing increased levels in higher species, therefore with presumed greater importance of this system in higher-order species (Hasbi et al., 2020a). As nonhuman primates showed higher density of D1-D2 heteromer in striatum compared to rodents, it was crucial to validate the lack of D1-D2

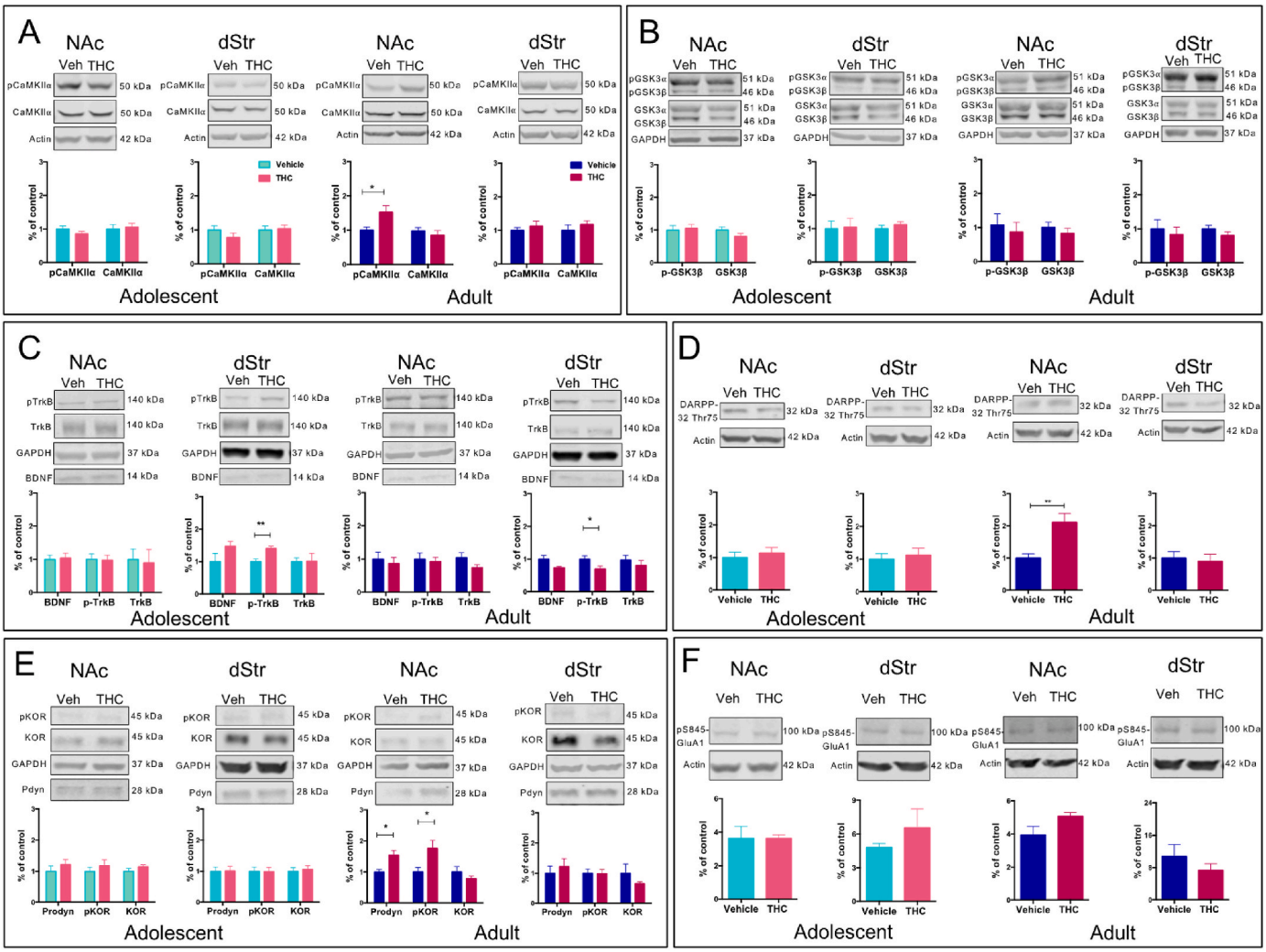


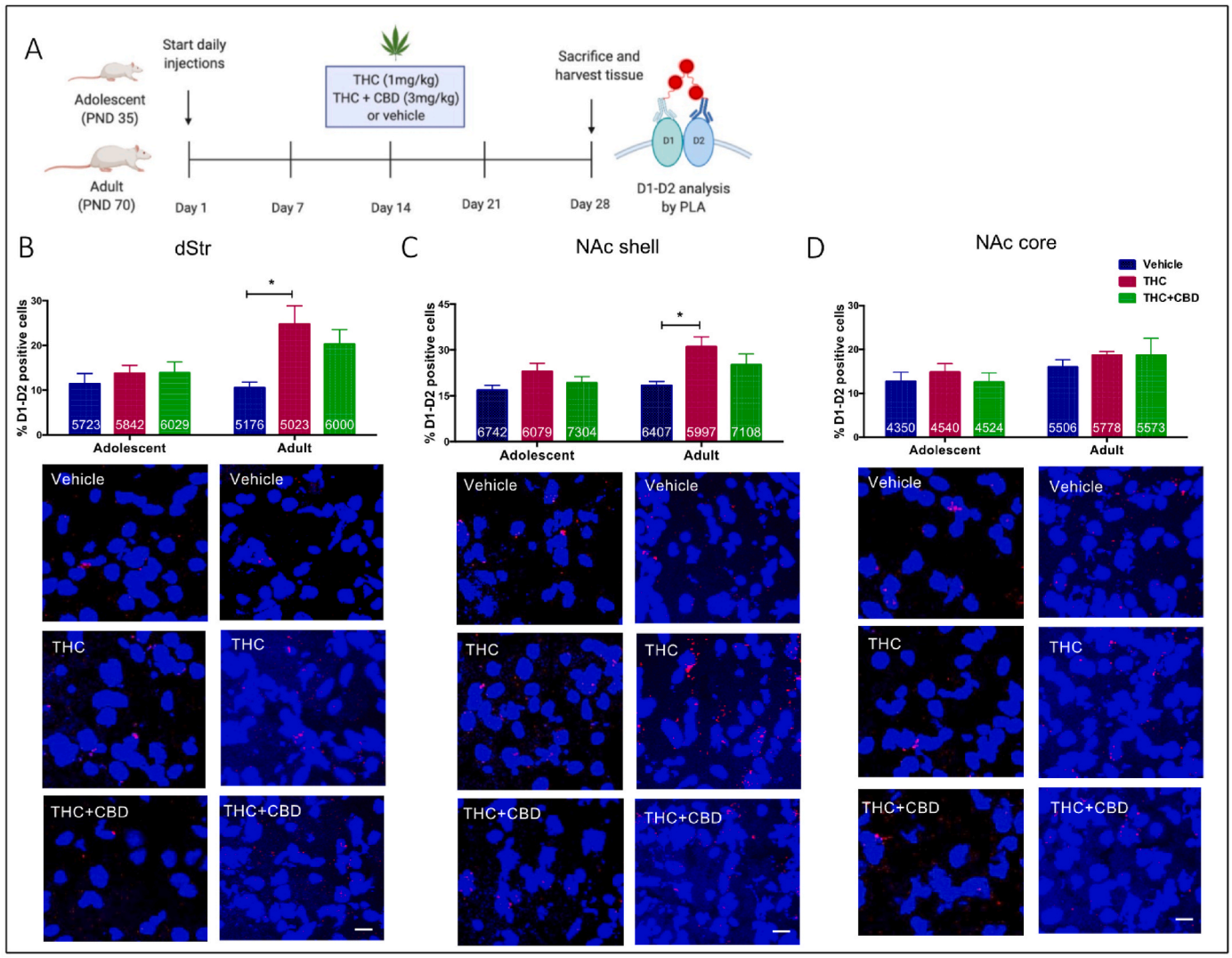
Fig. 4. Downstream effectors of D1-D2 dopamine receptor heteromer after chronic THC administration. Homogenates from dorsal striatum (dStr) and nucleus accumbens (NAc) of adult and adolescent rats chronically treated with vehicle (Veh) or THC were subjected to Western blot using antibodies for A) CaMKIIα and phospho-CaMKIIα (pCamKIIα), B) GSK3αβ and phospho-GSK3αβ (pGSK3αβ), C) BDNF and its receptor TrkB, and phosphorylated TrkB (pTrkB), D) phosphorylated Thr75-DARPP-32 (pThr75) E) and prodynorphin (Pdyn) and its receptor KOR and phosphorylated KOR (pKOR). F) GluA1 phosphorylated at serine845 (pS845-GluA1). GAPDH and β-actin antibodies were used as loading controls. Representative images are shown. Results are expressed as percent of control, mean ± SEM, with statistical significance measured by Student's t-test (*$p < 0.05$, **$p < 0.01$).

upregulation by chronic adolescent THC exposure in higher-order species to provide better translational significance to human cannabis users. Therefore, D1-D2 heteromer expression following THC exposure in adolescent non-human primates was evaluated. In this study, adolescent squirrel monkeys were treated with THC or vehicle for 114 or 117 days, a comparable length of time as were the adult monkeys (Withey et al., 2020) (Fig. 2F). Daily THC administration in adolescent monkey did not increase the proportion of D1-D2 heteromer expressing cells in the CN (Fig. 2G, Student's t-test: Vehicle = 38.95 ± 6.957, THC = 28.71 ± 6.302, t = 1.091 df = 5.942, p = 0.3176) or NAc (Fig. 2H, Student's t-test: Vehicle = 24.26 ± 6.725, THC = 26.68 ± 3.017, t = 0.3295 df = 1.414, p = 0.7840). In parallel with results from THC-treated rats, daily THC exposure in primates increased the proportion of D1-D2 heteromer-expressing cells in dopamine-rich striatal regions of adult (Hasbi et al., 2020a), but not adolescent monkeys.

To determine whether the emotionality score was associated with adaptive changes in D1-D2 receptor heteromer expression, correlation analyses were performed (Fig. 3). In THC-treated adult rats, increased negative emotionality and D1-D2 heteromer expression were significantly and positively correlated in the NAc shell, but not in dStr or NAc core (Fig. 3A, Pearson's correlation: dStr D1-D2 density and emotionality, r = 0.3718, p = 0.0970; NAc shell D1-D2 density and emotionality r = 0.4411, p = 0.0453; NAc core D1-D2 density and emotionality r = 0.1839, p = 0.4377). In contrast, adolescent THC-treated rats did not exhibit any correlation between emotionality and D1-D2 expression in the examined brain regions (Fig. 3B, Pearson's correlation: dStr D1-D2 density and emotionality r = −0.3564, p = 0.1036; NAc shell D1-D2 density and emotionality, r = −0.2737, p = 0.2177; NAc core D1-D2 density and emotionality r = −0.1237, p = 0.5835). In summary, daily THC administration triggered adult-specific anxiety- and anhedonia-like behaviour, characterized by an elevated composite z-score for negative emotionality. Furthermore, exclusively in adult THC-treated rats, the negative emotionality score was positively correlated to D1-D2 heteromer expression in NAc shell, with such a correlation absent in adolescent-treated rat, thus implicating the elevated D1-D2 heteromer density in the NAc shell as contributory to the negative emotionality induced in adult rat by THC.

3.3. *Effects of daily THC administration in adult and adolescent rats on dopamine D1-D2 receptor heteromer signaling cascades*

It was next determined if THC-induced changes in D1-D2 heteromer



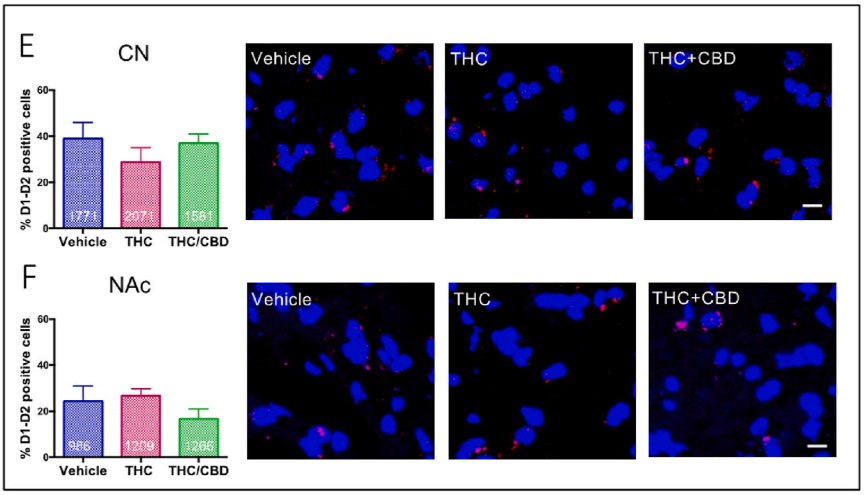


Fig. 5. Effect of CBD co-administration to THC on D1-D2 heteromer expression. A) Adult and adolescent rats (n = 7) received daily injections of THC or THC + CBD or vehicle for 28 days B) PLA analysis of D1-D2 dopamine receptor heteromer in the dorsal striatum (dStr), C) nucleus accumbens shell (NAc shell) D) and nucleus accumbens core (NAc core). Results are mean ± SEM and numbers in bar graph denote numbers of neurons evaluated. Two-way ANOVA (*p < 0.05, **p < 0.01, ***p < 0.001). E) Adolescent monkeys received daily treatment with 1 mg/kg THC, 3 mg/kg CBD + THC or vehicle for 114 or 117 days (n = 3–4/group). PLA analysis of D1-D2 heteromer in the caudate nucleus (CN) and F) NAc of monkeys. Results are mean ± SEM and numbers in bar graph denote numbers of neurons evaluated. One-way ANOVA. Representative images imaged at 60*X*. Scale bar is 10 μM.

expression were reflected in downstream mediators of D1-D2 signaling, such as $G\alpha_q$-mediated calcium release and subsequent increased activation of CaMKIIα, modulation of glycogen synthase kinase 3 (GSK-3), BDNF expression and activation of its receptor TrkB (Hasbi et al., 2009, 2018). In line with our findings that THC increased D1-D2 heteromer in adult rat striatal regions, we observed that activated CaMKII phosphorylated at Thr^{286} (pCAMKIIα) was significantly elevated in the NAc but not in the dStr of the adult THC group compared with vehicle control (Fig. 4A; Student's t-test: NAc, Vehicle = 29.69 ± 2.699, THC = 45.45 ± 5.587, t = 2.539 df = 8.656, p = 0.0327; dStr, Vehicle = 46.94 ± 3.937, THC = 53.14 ± 6.660, t = 0.8014 df = 8.114, p = 0.4457). In adolescent-treated rats, where D1-D2 expression was unaltered following THC exposure, pCaMKIIα in the NAc or dStr was unchanged, reflecting lack of D1-D2 activation (Fig. 4A, Student's t-test: NAc, Vehicle = 34.27 ± 3.126, THC = 29.45 ± 2.597, t = 1.187 df = 13.54; dStr, Vehicle = 40.76 ± 5.141, THC = 31.65 ± 5.465, t = 1.214 df = 13.95, p = 0.2450). THC administration did not alter levels of inactivated GSK3β, phosphorylated at Ser^9 (pGSK3β) in adult-treated dStr or NAc (Fig. 4B, Student's t-test: dStr, Vehicle = 4.354 ± 1.154, THC = 3.613 ± 0.9290, t = 0.5005 df = 9.563, p = 0.6280; NAc Vehicle = 2.078 ± 0.6113, THC = 1.728 ± 0.5180, t = 0.4371 df = 13.63, p = 0.6689) or adolescence-treated dStr or NAc (Fig. 4B, Student's t-test: dStr, Vehicle = 4.587 ± 1.048, THC = 4.789 ± 1.209, t = 0.1258 df = 14, p = 0.9017; NAc, Vehicle = 3.91 ± 0.5455, THC = 4.013 ± 0.4311, p = 0.9415). In dStr of THC-treated adult rats, activated phosphorylated TrkB (pTrkB) was significantly decreased (Fig. 4C, Student's t-test: Vehicle = 27.15 ± 2.592, THC = 19.08 ± 2.317, t = 2.320 df = 11.85, p = 0.0390), along with a trend to decreased expression of BDNF (Student's t-test: Vehicle = 4.308 ± 0.4636, THC = 3.150 ± 0.2100, t = 2.275 df = 6.969, p = 0.0572). In sharp contrast, pTrkB was significantly increased in dStr of adolescent THC-treated rats (Fig. 4C, Student's t-test: Vehicle = 11.32 ± 0.9223, THC = 15.90 ± 0.8798, t = 3.591 df = 11.97, p = 0.0037). These findings were specific to the dStr, as pTrkB was not altered in NAc of adult (Student's t-test: Vehicle = 3.264 ± 0.5930, THC = 3.015 ± 0.4142, t = 0.3449 df = 12.52, p = 0.7359) or adolescent THC-treated rats (Student's t-test: Vehicle = 2.049 ± 0.3364, THC = 1.995 ± 0.3125, t = 0.1173 df = 11.94, p = 0.9086) (Fig. 4C).

D1-D2 activation is also linked to increased Cdk5 (cyclin-dependent kinase)-mediated phosphorylation of DARPP-32 at threonine 75 (Thr75-DARPP-32) (Hasbi et al., 2018). In the NAc but not dStr of adult THC-treated rats, the expression of pThr75-DARPP-32 was significantly elevated (Fig. 4D, Student's t-test: NAc, Vehicle = 6.320 ± 0.9385, THC = 11.91 ± 1.538, t = 3.103 df = 8570, p = 0.0134; dStr, Vehicle = 3.588 ± 0.7244, THC = 3.234 ± 0.7863, t = 0.3309 df = 10.68, p = 0.7471). In adolescent THC-treated rats, pThr75-DARPP-32 expression was unchanged in NAc and dStr (Fig. 4D, Student's t-test: NAc, Vehicle = 6.665 ± 1.105, THC = 7.595 ± 1.121, t = 0.5907 df = 14.00, p = 0.5642; dStr, Vehicle = 3.658 ± 0.6159, THC = 4.125 ± 0.7645, t = 0.4759 df = 13.39, p = 0.6418).

As a major neuropeptide relevant to mediating aversive behaviour, dynorphin is expressed in D1 receptor expressing striatal neurons (Steiner and Gerfen, 1998), as well as in D1-D2 neurons (Perreault et al., 2010) and may be under regulatory control of D1-D2 signaling in the latter. Therefore, we measured parameters of dynorphin signaling after chronic THC exposure. In NAc but not in dStr of adult THC-treated rats, prodynorphin and its activated receptor, phosphorylated kappa opioid receptor (pKOR) were both upregulated, (Fig. 4E, Student's t-test: NAc prodynorphin, Vehicle = 13.17 ± 1.083, THC = 20.26 ± 2.073, t = 3.033 df = 9.047, p = 0.0141; NAc pKOR, Vehicle = 4.398 ± 0.6414, THC = 7.754 ± 1.152, t = 2.546 df = 7.830, p = 0.0350; dStr prodynorphin, Vehicle = 11.51 ± 2.776, THC = 14.08 ± 3.018, t = 0.6265 df = 8.668, p = 0.5471; dStr pKOR, Vehicle = 6.973 ± 1.231, THC+7.286 ± 1.206, t = 0.1817 df = 11.99, p = 0.8589). Once again, in brain regions of adolescent-treated rats, THC administration had no effect on prodynorphin or pKOR in the NAc or dStr (Fig. 4E, Student's t-test: NAc prodynorphin, Vehicle = 13.98 ± 2.464, THC = 16.95 ± 2.239, t = 0.8929 df = 11.89, p = 0.3896; NAc pKOR, Vehicle = 4.715 ± 0.6362, THC = 5.540 ± 0.9171, t = 0.7395 df = 7.405, p = 0.4824; dStr prodynorphin, Vehicle = 14.00 ± 1.889, THC = 14.13 ± 2.106, t = 0.04612 df = 12.57, p = 0.9639; dStr pKOR, Vehicle = 7.928 ± 0.9451, THC = 7.788 ± 1.124, t = 0.09548 df = 12.24, p = 0.9255).

The signaling cascade comprised of D1 receptor/cAMP/protein kinase A (PKA) facilitates reward and is engaged by increases in dopamine release, such as following exposure to drugs of abuse (Wang et al., 2006). PKA enhances glutamatergic AMPA receptor activity through phosphorylation of the glutamate receptor subunit GluA1 at its Ser845 site, important for synaptic plasticity. Thus, Ser845-GluA1 phosphorylation is used as a marker of cAMP/PKA pathway activation. Our previous study (Hasbi et al., 2020a) demonstrated that chronic THC exposure in adult non-human primate inhibited Ser845-GluA1 phosphorylation in the NAc, suggesting inhibition of the cAMP/PKA/MAPK cascade. Here, THC exposure in adult rats did not alter pS845-GluA1 in dStr or NAc (Fig. 4F, Student's t-test: dStr, Vehicle = 10.77 ± 2.920, THC = 7.332 ± 1.600, t = 1.033 df = 6.307, p = 0.3397; NAc, Vehicle = 3.948 ± 5.206, THC = 5.073 ± 0.2141, t = 1.999 df = 5.315, p = 0.0986) and neither did THC exposure in adolescent rats (Fig. 4F, Student's t-test: NAc, Vehicle = 3.628 ± 0.7177, THC = 3.632 ± 0.2117, t = 0.0047 df = 5.871, p = 0.9964; dStr, Vehicle = 4.807 ± 0.3516, THC = 6.559 ± 1.664, t = 1.030 df = 5.445, p = 0.3465) indicating an absence of the expected AMPA receptor activation by the cAMP/PKA cascade in response to D1 receptor activation by THC-induced dopamine release. However, no inhibition of this pathway was observed as documented in adult monkey striatum. Together, these results highlight further differences in neuroadaptive striatal responses induced by daily THC in adult and adolescent animals.

3.4. Effects of CBD co-administered with THC on D1-D2 dopamine receptor heteromer expression

CBD has been reported to reverse or attenuate specific effects of THC (Boggs et al., 2018; Freeman et al., 2019), including our discovery that co-administration of CBD with THC to adult monkeys attenuated the THC-induced upregulation of the D1-D2 receptor heteromer in striatum (Hasbi et al., 2020a). Daily co-administration of CBD with THC for 28 days was performed to determine CBD effect on chronic THC-induced D1-D2 heteromer upregulation in adult rats (Fig. 5A). While daily THC administered to adult rats increased D1-D2 heteromer density in dStr by 134% compared to vehicle control (Fig. 5B, Two-way ANOVA: main effect of age, F (1,36) = 6.306, p = 0.0167; main effect of treatment, F (2,36) = 5.130, p = 0.0110), co-administration of CBD with THC did not show densities of D1-D2 heteromer significantly different from vehicle control (Tukey's multiple comparisons test: Adult:Vehicle vs Adult:THC, Mean difference = −14.18, 95% CI of difference = −25.60 to −2.765, p < 0,005; Adult:Vehicle vs Adult:THC + CBD, Mean difference = −9.767 95% CI of difference = −21.18 to 1.650, p = ns). Similarly, in the NAc shell, there was a significant effect of age and treatment (Fig. 5C, Two-way ANOVA: main effect of age, F (1,36) = 6.465, p = 0.0154; main effect of treatment, F (2,36) = 7.095, p = 0.0025), with chronic THC in adult rats upregulating D1-D2 heteromer expression by 69% compared to control, whereas daily co-administration of CBD and THC was not different from control (Tukey's multiple comparisons test: Adult:Vehicle vs Adult:THC, Mean difference = −12.66, 95% CI of difference = −23.30 to −2.011, p < 0.05; Adult Vehicle vs Adult:THC + CBD, Mean difference = −6.739, 95% CI of difference = −17.39 to 3.908, p = ns). In the NAc core, THC alone or in combination with CBD had no effect on D1-D2 expression (Tukey's multiple comparisons test: Adult:Vehicle vs Adult:THC, Mean difference = −2.705, 95% CI of difference = −1165 to 6.239, p = ns; Adult Vehicle vs Adult:THC + CBD, Mean difference = −2.715, 95% CI of difference = −12.02 to 6.593, p = ns). Furthermore, chronic 28-day administration of CBD alone compared to vehicle did not alter expression of D1-D2 in the adolescent (Fig. S2, Student's t-test: dStr, Vehicle =

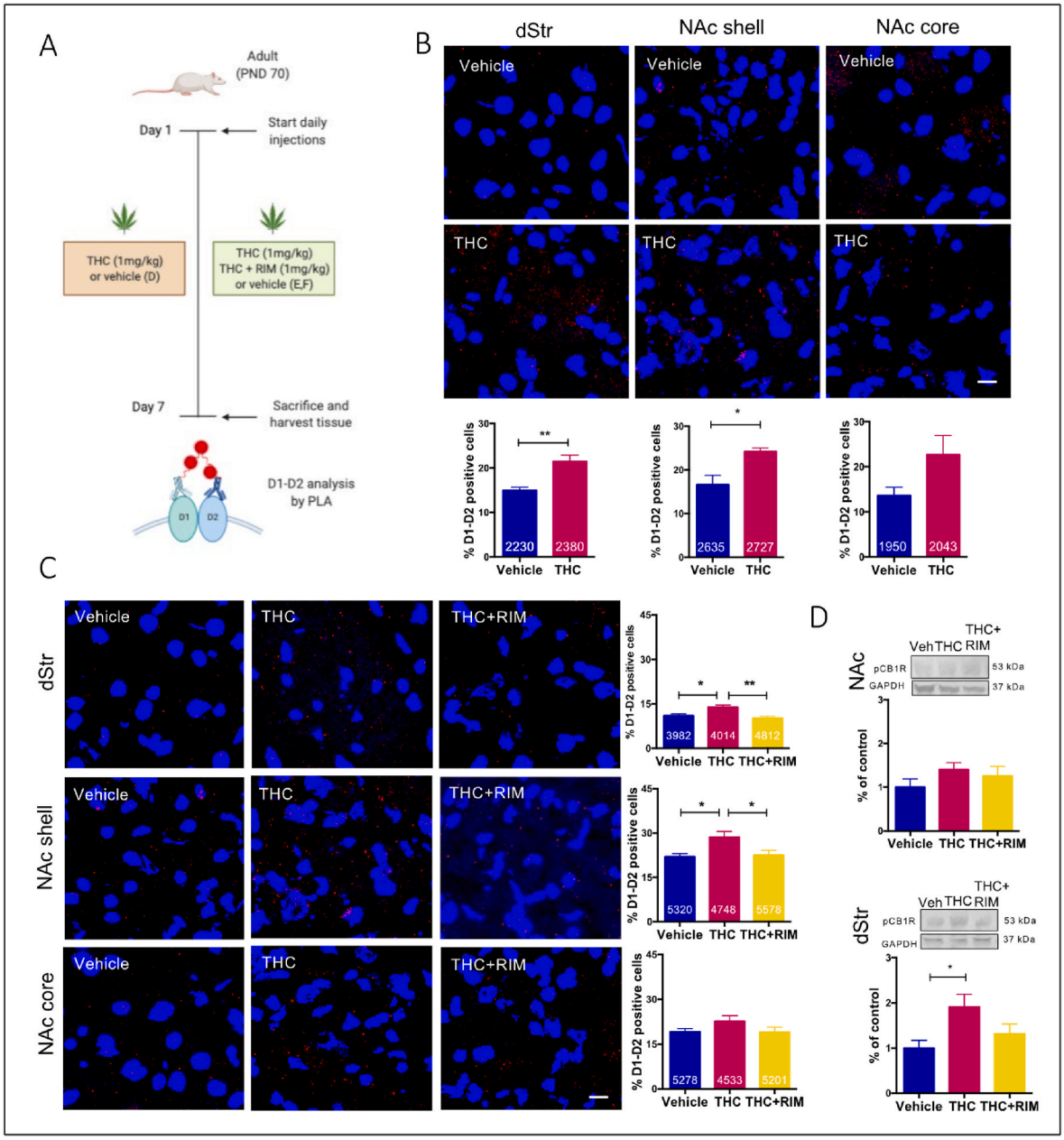


Fig. 6. THC-regulated D1-D2 dopamine receptor heteromer: contribution of cannabinoid receptor-1 (CB1) activity. A) Adult rats (n = 4) received daily injections of vehicle or THC (left, panel D), or THC and the CB1 inverse agonist rimonabant (THC + RIM, right, panel E–F) for 7 days. B) PLA analysis of D1-D2 heteromer in the dStr, NAc shell and NAc core after 7 days THC vs vehicle. Results are mean ± SEM and numbers in bar graph denote numbers of neurons evaluated. Student's t-test, (*p < 0.05, **p < 0.01). C) PLA analysis of D1-D2 heteromer in the dStr, NAc shell and NAc core after 1-week vehicle, THC or THC + RIM administration. One-way ANOVA with Tukey's multiple comparisons (*p < 0.05, **p < 0.01). Representative images imaged at 60*X*. Scale bar is 10 μM. D) Homogenates from NAc and dStr of adult rats treated with vehicle (Veh), THC or THC + RIM were processed by Western blot using an antibody for the phosphorylated CB1 receptor (pCB1R). GAPDH was used as loading control. Results are presented as percentage of control, mean ± SEM, analyzed using one-way ANOVA with Tukey's multiple comparisons. (*p < 0.05).

11.32 ± 2.208, THC = 8.249 ± 0.8775, t = 1.293 df = 7.849, p = 0.2327; NAcSh, Vehicle = 16.89 ± 2.016, THC = 20.32 ± 1.850, t = 1.255 df = 11.91, p = 0.2335; NAcCo, Vehicle = 14.01 ± 2.065, THC = 11.63 ± 1.808, t = 0.8681 df = 11.79, p = 0.4027) or adult rats (Fig. S2, Student's t-test: dStr, Vehicle = 12.56 ± 1.737, THC = 16.30 ± 1.675, t = 1.551 df = 11.98, p = 0.1469; NAcSh, Vehicle = 20.51 ± 2.186, THC = 24.06 ± 3.995, t = 0.7788 df = 9.298, p = 0.4554; NAcCo, Vehicle = 18.79 ± 2.527, THC = 23.14 ± 3.425, t = 1.022 df = 11.04, p = 0.3285), suggesting that striatal modulation of D1-D2 is specific to the cannabinoid THC. Adolescent squirrel monkeys treated with chronic THC, CBD in combination with THC, or vehicle, revealed no change in D1-D2 expression by THC or CBD in the CN (Fig. 5E, One-way ANOVA, F (2,9) = 0.2964, p = 0.4623) or NAc (Fig. 5F, One-way ANOVA, F (2,5) = 0.4478, p = 0.295. Together, these data reveal that when CBD was administered in combination with THC, D1-D2 heteromer expression was not upregulated in adult rodent, just as in adult nonhuman primate striatum.

3.5. *Effects of cannabinoid CB1R inhibition on D1-D2 dopamine receptor heteromer expression*

To determine whether THC-induced striatal D1-D2 heteromer

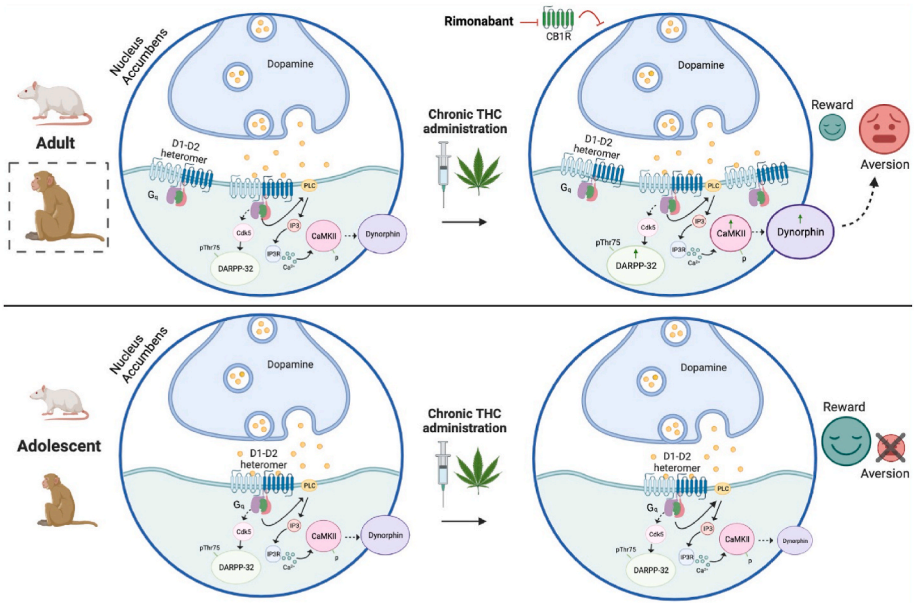


Fig. 7. Proposed mechanism for D1-D2 heteromer-induced negative emotionality in adults, absent in adolescents Chronic THC in adult but not adolescent rat and monkeys upregulates the dopamine D1-D2 heteromer in nucleus accumbens medium spiny neurons (MSNs) with increased expression of downstream markers phospho-CaMKII, Thr75-DARPP-32 and dynorphin leading to anxiety- and depression-like aversive behaviour. In adolescents, lack of D1-D2 heteromer upregulation conceivably increases the aversion threshold and rewarding effects of THC predominate such that there is little anxiety- and depression-like behaviour and potentially greater propensity for drug abuse.

upregulation requires activation of the cannabinoid receptor CB1R by THC, adult rats were administered THC alone or THC combined with a CB1R inverse agonist rimonabant (RIM) for 7 days (Fig. 6A). After 7 days of THC treatment, adult rats showed D1-D2 heteromer upregulation in dStr and NAc shell, but not NAc core (Fig. 6B, Student's t-test: dStr, Vehicle = 15.04 ± 0.6214, THC = 21.49 ± 1.434, t = 4.122 df = 6, p = 0.0062, NAc shell, Vehicle = 16.63 ± 2.145, THC = 24.20 ± 0.8223, t = 3.298 df = 6, p = 0.0165, NAc core, Vehicle = 13.63 ± 1.839, THC = 22.71 ± 4.231, t = 1.968 df = 6, p = 0.0967). Co-administration of RIM blocked the THC-induced upregulation of D1-D2 heteromer in dStr (Fig. 6C, One-way ANOVA: F (2,21) = 0.2050, p = 0.0021; NAc shell, p = 0.0120) (Tukey's multiple comparisons test, THC vs THC + RIM, Mean difference = 3.701, 95% CI = 1.307 to 6.095, $p < 0.005$) and NAc shell (One-way ANOVA: F (2,21) = 0.4588, p = 0.0120) (Tukey's multiple comparisons test, THC vs THC + RIM, Mean difference = 6.043, 95% CI = 0.4442 to 11.64, $p < 0.05$) but did not alter D1-D2 heteromer levels in NAc core (One-way ANOVA: F (2,21) = 1.826, p = 0.1958). CB1R activation was evidenced by increased phosphorylation of the receptor (pCB1R) in dStr of rats treated with 7 daily doses of THC (Fig. 6D, One-way ANOVA: F (2,18) = 0.2970, p = 0.0332) (Tukey's multiple comparisons test, Vehicle vs THC, Mean difference = −160.0, 95% CI = −304.2 to −15.88, $p < 0.05$) but this was not apparent in the NAc (One-way ANOVA, F (2,18) = 0.9863, p = 0.4387). Together, these findings indicate that upregulation of the D1-D2 heteromer in striatum occurred downstream of CB1R activation by THC.

4. Discussion

This study identifies age-specific adaptable differences in the density and regulation of the aversion linked dopamine receptor heteromer in striatum by THC, the principal psychoactive component of a drug of abuse, cannabis. Age-dependent activation of the dopamine D1-D2 receptor heteromer and dynorphin signaling following chronic THC conceivably constitutes a crucial signaling pathway that results in a reduction of dopamine release from nerve terminals and promotes aversive behaviour exhibited by adult rats that was not present in adolescent animals. The dopamine D1-D2 receptor heteromer has been implicated in the regulation of reward, anxiety, depression and addiction-related processes (Hasbi et al., 2018; Shen et al., 2015) and has previously been shown to be upregulated by chronic THC administration in adult nonhuman primate (Hasbi et al., 2020a) and by repeated cocaine exposure in adult rat (Hasbi et al., 2020b). The present study revealed significant differences in the responses of adult and adolescent rat to repeated daily administration of THC, differences supported by parallel, limited experiments in adolescent nonhuman primate. First, daily THC administration to adult rats for several weeks, or adult nonhuman primates for almost 4 months (Hasbi et al., 2020a; Withey et al., 2020, 2021) resulted in significant upregulation of the dopamine D1-D2 receptor heteromer in NAc and dStr. In stark contrast, D1-D2 heteromer expression was unchanged by THC exposure in adolescent rat or adolescent nonhuman primate in comparable experimental paradigms (Fig. 7). Second, D1-D2 heteromer expression in dStr and NAc shell positively and significantly correlated with the negative emotionality scores on behavioural testing performed after 10–21 days of THC exposure, calculated for each individual adult rat, a correlation not detected for adolescent rats. THC-treated adult, but not adolescent rats exhibited elevated negative emotionality scores compared to vehicle-treated controls, obtained from behaviour in light-dark box, novelty-induced hypophagia and open field tests. Third, these stark age-related differences in response to repeated THC exposure were recapitulated in measures of the associated downstream signaling markers of D1-D2 heteromer activation. THC caused upregulation of pCAMKIIα, pThr75-DARPP-32, prodynorphin and pKOR (dynorphin signaling) in NAc after adult-, but not adolescent-onset THC exposure. Opposite adaptive responses elicited by THC administration also were detected, as pTrkB was downregulated in dStr by adult THC treatment and upregulated by adolescent THC treatment. Finally, the increase in dopamine D1-D2 receptor heteromer was attenuated in dStr and NAc shell by a CB1R inverse agonist, implicating involvement of CB1R in mediating the increase in D1-D2 heteromer expression. D1-D2 heteromer expression after CBD treatment in combination with THC was not different from control in the same regions.

The previously demonstrated increase in dopamine D1-D2 receptor heteromer expression by chronic THC in adult nonhuman primate occurred alongside phenotypic reprogramming of striatal neurons (Hasbi et al., 2020a) to coexpress both D1 and D2 receptors, together with evidence of D1-D2 heteromer activation in MSNs that classically were designated to only express one or the other receptor. It highlighted *de novo* D1-D2 heteromer expression in adult monkey striatum, now shown in adult rat striatal MSNs, cell types that in the basal state coexpress both D1 and D2 receptors only in a small subpopulation of striatal neurons in rodents (Gagnon et al., 2017; Gangarossa et al., 2013; Matamales et al., 2009; Perreault et al., 2010). This robust change that occurred in the number of MSNs coexpressing D1-D2 heteromer,

indicating an altered phenotype of adult rat striatal neurons with evidence of D1-D2 heteromer activation is not only unprecedented but was also absent in adolescence. The basal level of coexpressed D1 and D2 dopamine receptors in striatal MSNs reportedly is low in all species, but the previously held dogma of strict segregation of D1 and D2 receptors in MSNs has been challenged by recent advances in single-cell transcriptomics that showed phenotypic diversity in striatal MSNs in mouse (Gokce et al., 2016; Puighermanal et al., 2020; Stanley et al., 2020) and monkey (He et al., 2021) striatum. Interestingly, THC-induced changes in D1-or D2-mRNA transcript expressing cells were not discerned and therefore were not responsible for driving the observed increased in D1-D2 heteromer formation. Indeed, GPCR heteromer formation is not necessarily contingent on increased levels of the individual constituent receptors as observed in the example of cocaine-induced formation of D2-NMDA receptor heteromer, which occurs concurrently with decreased expression of D2 receptor in the striatum (Andrianarivelo et al., 2021). Increased heteromer density may be due to alternative factors promoting the assembly of receptors into heteromers, such as concurrent protein folding of receptors in the endoplasmic reticulum or promotion of co-trafficking to cell surface membranes (Juhasz et al., 2008; Law et al., 2005; Terrillon and Bouvier, 2004).

The use of cannabis and subsequent development of cannabis use disorder conceivably results from an imbalance between the aversive and rewarding effects of the drug. The present study provides a potential mechanism for a higher THC reward:aversion balance in adolescent brain compared with adult that may promote continued cannabis use in adolescents. Accumulating evidence attributes a regulatory role in the brain reward system to the dopamine D1-D2 heteromer, conceivably as a homeostatic feedback response mechanism that can modulate reward from drugs or natural rewards that augment dopamine release and thus have abuse potential. The heteromer is upregulated by the psychostimulants cocaine and amphetamine in adult rat striatum (Hasbi et al., 2020b; Perreault et al., 2010). Its functional significance was revealed, as activating the D1-D2 heteromer with SKF83959 dampened the rewarding effects of cocaine, whereas attenuating D1-D2 heteromer function with a disrupting peptide enhanced psychostimulant rewarding effects (Hasbi et al., 2018; Perreault et al., 2016). With repeated drug use and subsequent upregulation of D1-D2 heteromer, this ceiling would be altered, lowering the threshold for aversive effects of drugs like cannabis and raising the threshold for its rewarding effects. THC administration elicited D1-D2 heteromer upregulation in adult rats and monkeys, such that this “braking system” of drug-induced reward was potently activated. Significantly, THC did not recruit this candidate braking system that was not present in adolescent brain from both species examined.

In addition to lack of D1-D2 upregulation by THC in adolescent striatum, the basal expression of D1-D2 was lower in adolescent compared to adult rat as shown, and associated with dampened aversive behavioural response to pharmacological activation of D1-D2 in juvenile animals (Perreault et al., 2012). The lower D1-D2 heteromer “brake” on the dopaminergic reward system (Hasbi et al., 2018; Shen et al., 2015) in adolescents may contribute to characteristic traits such as enhanced reward sensitivity, emotional impulsivity and risk-taking in adolescence (Burton and Fletcher, 2012; Ernst and Spear, 2009; Kuhnen and Knutson, 2005), which are linked to immaturity of corticolimbic striatal reorganization during this developmental period (Islam et al., 2021; Marinelli and McCutcheon, 2014; Spear, 2014). Both human (Ernst et al., 2005; Volkow and Morales, 2015) and rodent (Sturman and Moghaddam, 2012) neuroimaging studies show hyperactivity of the NAc and dStr of adolescents compared to adults during reward-related tasks. At a molecular level, expression of dopamine D1 and D2 receptors in the striatum and prefrontal cortex (PFC) peak in adolescence, with increased D1 receptor expression on NAc neuron terminals originating from the PFC thought to underlie increased sensitivity to reward cues (Andersen et al., 2000; Brenhouse et al., 2008). This elevated reward reactivity in adolescence may be related to a combination of high “rewarding” D1 receptor and low “aversive” D1-D2 heteromer expression, such that dopamine release and receptor activation in adolescents favors activation of the rewarding D1 receptor pathway with lesser contribution from the reward inhibitory D1-D2 pathway activation.

Daily THC activated the aversive dynorphin-KOR signaling pathway in adult rat NAc with resultant anxiety- and anhedonia-like behaviour. Dynorphin-KOR and dopamine systems are functionally interconnected in this area, with activation by dynorphin of KOR on dopaminergic nerve terminals in striatum leading to inhibition of dopamine release (Bruijnzeel, 2009; Tejeda and Bonci, 2019), with a hypodopaminergic state ensuing. Activation of KOR in the mesocorticolimbic dopamine system produces dysphoria, anhedonia, anxiety and depressive-like effects (Hang et al., 2015; Knoll and Carlezon, 2010; Muschamp and Carlezon, 2013) in adult, but not adolescent rodents (Anderson and Becker, 2017), similar to behavioural effects observed in adult rats following chronic THC administration. The aversive effects of THC reportedly are mediated by activation of KOR by dynorphin in the NAc shell (Norris et al., 2019) as in dynorphin-deficient adult mice, THC does not produce dysphoric or aversive effects (Zimmer et al., 2001). Functionally, the resistance of the adolescent D1-D2 heteromer system to THC with failure to augment prodynorphin expression to engage KOR may render this age cohort impervious to the aversive effects of THC, to maintain a lower reward threshold.

Daily THC administration also increased expression of pCaMKIIα and pThr75-DARPP32 in NAc of adult rat as in adult monkeys (Hasbi et al., 2020a). Once again, the adolescent brain responded differently than the adult to THC; expression of these markers was unaltered, reflecting a lower basal level of D1-D2 heteromer that was not increased by THC in this age group. CaMKIIα activation leads to phosphorylation of AMPA receptors and plays a critical role in regulation of synaptic plasticity (Derkach et al., 1999; Zhou et al., 2006). Particularly, CaMKIIα activation and downstream NAc plasticity is crucially involved in the behavioural response to addictive drugs (Liu et al., 2012; Loweth et al., 2010) and CaMKIIα-induced plasticity may play a role in drug-induced emotionality, as anxiety-like behaviour was alleviated by inhibition of CaMKIIα in the NAc (Soltani et al., 2020). Therefore, increased expression of phospho-CaMKIIα in the NAc of adult rats treated with THC may contribute to downstream synaptic plasticity leading to maladaptive and anxiogenic response to addictive drugs.

Thr75-DARPP-32 phosphorylation has been proposed to be a marker of chronic drug exposure (Bibb et al., 2001). The altered dopaminergic state postulated to occur following drug exposure (Volkow et al., 2007, 2009) and activation of the D1-D2 heteromer signaling pathway leads to accumulation of pThr75-DARPP32 (Hasbi et al., 2018), which may contribute to observed THC-induced negative effects, as it has been linked to depressive-like behaviour (Brito et al., 2019; Yger and Girault, 2011). DARPP32 phosphorylation at Thr75 is mediated by protein kinase Cdk5 (Bibb et al., 2001) and inhibition of Cdk5 has been shown to directly block the aversive effects of D1-D2 activation (Hasbi et al., 2018). Therefore, the lack of pThr75-DARPP-32 induction in adolescent NAc by THC would be another important factor resulting in the deficiency of development of aversive or negative emotional responses in this age group.

In adolescent rat dStr, THC stimulated a rise in activated pTrkB, the receptor for BDNF. Neurotrophin signaling of BDNF through TrkB regulates synaptic plasticity-related gene expression (Lu et al., 2008). Increased BDNF and TrkB plasticity plays an important role in enhancing lability to addictive drugs; for instance, elevated BDNF signaling in the NAc is linked to heightened cocaine-induced conditioned place preference and relapse in rats (Bahi et al., 2008). Another example of BDNF-TrkB mediating drug-induced maladaptive behaviour is the finding that adolescent WIN55212-2 (CB1 receptor agonist) exposure-mediated hyperlocomotion is alleviated by inhibition of TrkB in the striatum (Dong et al., 2019). Together, these findings suggest that increased TrkB activation in the dStr following adolescent THC exposure may contribute to enhanced risk of addictive and psychosis-like

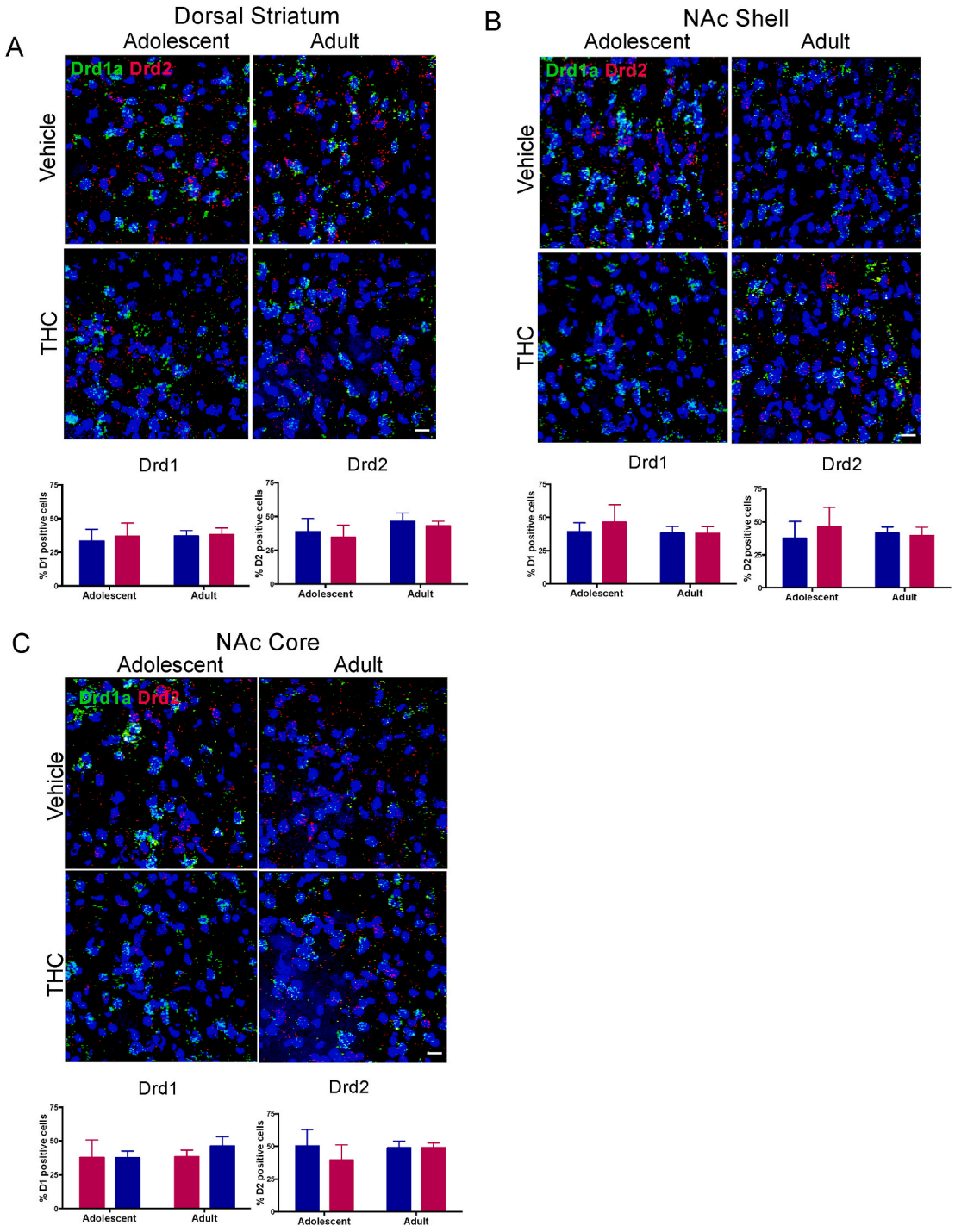


Fig. S1. D1-and D2-expressing MSN density after chronic THC exposure in adolescent and adult rats. *In situ* hybridization of Drd1 and Drd2 in the A) dorsal striatum, B) NAc shell and C) NAc core of rats treated with THC or vehicle in adolescence or adulthood. Bar graphs denote quantification of percentage of Drd1 or Drd2-positive cells, normalized to total DAPI counts. Results are mean ± SEM, analyzed using two-way ANOVA. Representative images imaged at 60*X*. Scale bar is 10 μM.

behaviour in this age group.

In contrast, THC-treated adult rats manifested a decrease in pTrkB in dStr, which differed from previous findings of TrkB activation in adult monkey caudate nucleus (Hasbi et al., 2020a). It is unlikely that decreased pTrkB in adult dStr following chronic THC is the signaling pathway contributing to elevated emotionality in this age group as TrkB knockdown in the dStr of male mice showed anti-anxiety and anti-depressive behavioural outcomes compared to control, suggesting that TrkB in this region is anxiogenic and pro-depressive (Unterwald et al., 2013)

In seeking compounds to mitigate the adverse effects of THC, we investigated CBD, which reportedly attenuates some (cognitive deficits, anxiety and psychosis), but not all detrimental effects of THC in heavy cannabis users (Casadio et al., 2011; Colizzi and Bhattacharyya, 2017; Englund et al., 2013, 2017; Hudson et al., 2019; Karniol et al., 1974; Morgan and Curran, 2008; Morgan et al., 2010, 2012; Schubart et al., 2011). CBD is an allosteric inhibitor of CB1 cannabinoid receptor, the principal target of THC in brain. In this study, while D1-D2 heteromer upregulation in the NAc shell and dStr occurred after THC exposure in adult rat, CBD co-treatment with THC showed similar densities of D1-D2

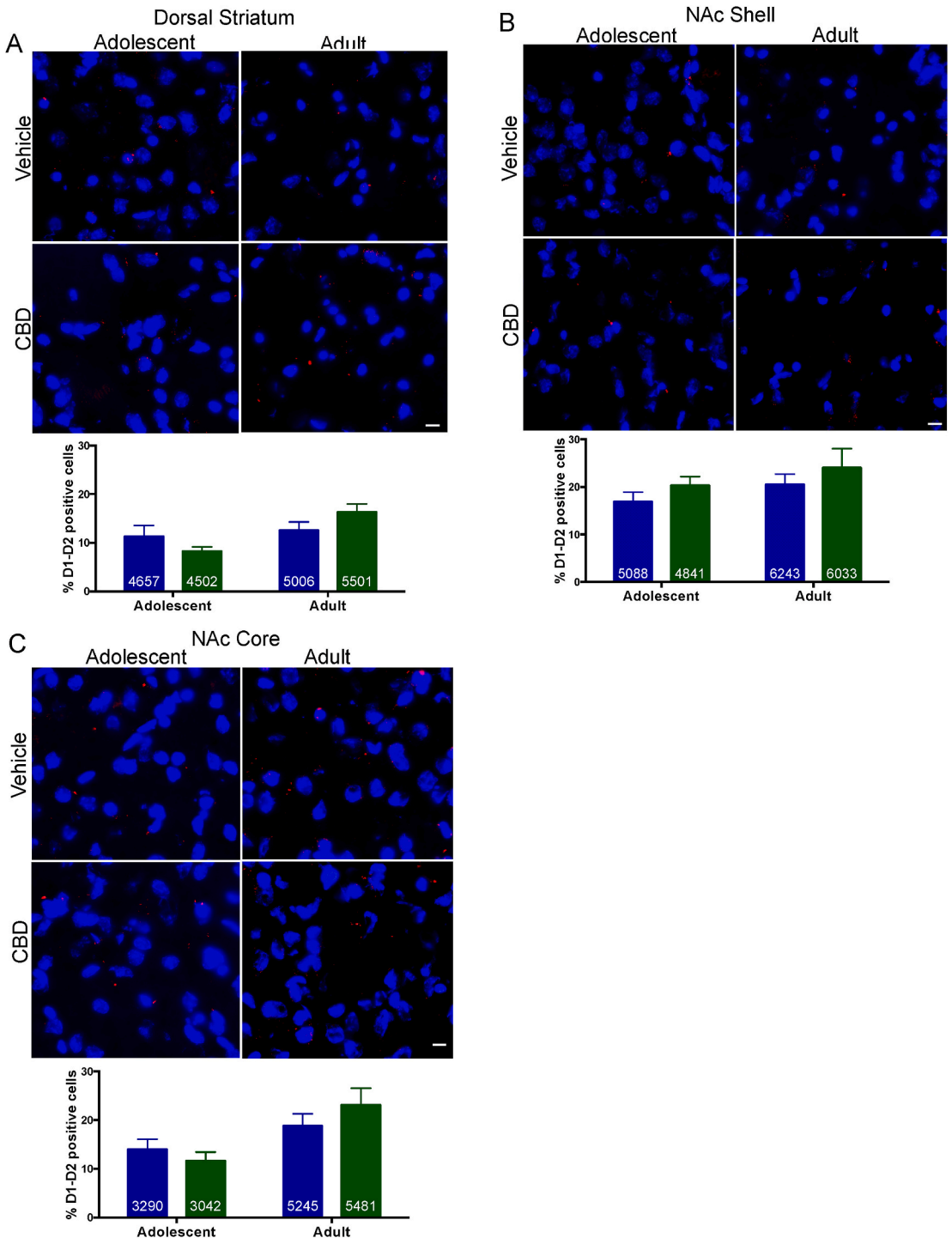


Fig. S2. Dopamine D1-D2 heteromer expression after chronic CBD exposure in adolescent and adult rats. D1-D2 heteromer expression by PLA in the A) dStr, B) NAc shell, C) NAc core. Results are mean ± SEM, analyzed by two-way ANOVA and numbers in bar graph denote numbers of neurons evaluated. Representative images imaged at 60*X*. Scale bar is 20 μM.

heteromer compared to control. Previous studies from our group have shown that CBD administration concurrent with THC in adult monkey reversed THC-induced striatal upregulation of D1-D2 heteromer (Hasbi et al., 2020a). The molecular mechanisms underlying the therapeutic effects of CBD are unresolved, as CBD targets a number of brain receptors, including serotonin, cannabinoid, opioid, dopamine receptors and modulators of endocannabinoid tone (Boggs et al., 2018; Campos et al., 2012; Pertwee, 2008; Seeman, 2016). To determine whether THC exerts its effects on the D1-D2 heteromer through the CB1R, we showed that an inverse agonist of the CB1R prevented THC-induced D1-D2 upregulation in adult rat, indicating that THC activation of CB1R is a necessary initial step in regulating the D1-D2 receptor heteromer in striatum.

5. Conclusions

In sum, we present evidence that striatal dopamine D1-D2 heteromer upregulation in adulthood underlies a physiological homeostatic

mechanism counteracting drug-induced repeated striatal dopamine release to limit the rewarding effects of THC, a process that was deficient in adolescents of two species. The D1-D2 heteromer may mediate the observed THC-induced aversion indicators of anhedonia and anxiety-like behaviour and function in adulthood by attenuating the rewarding effects of cannabis consumption and its associated consequences. Conversely, the inability of the adolescent striatum to respond to THC with D1-D2 heteromer upregulation as shown in rat and monkey, may contribute to heightened THC reward in adolescence, with reduced negative emotional and behavioural manifestations, predisposing to continued use and the associated risks for other consequences of chronic cannabis use. Adulthood-specific THC-induced D1-D2 heteromer upregulation was observed both in rat and as previously reported in monkey striatum (Hasbi et al., 2020a), providing translational significance and suggesting that this biological adaptation to THC, or lack thereof, may also be relevant for human adult and adolescent cannabis users, respectively. Since D1-D2 heteromer upregulation in striatum has also been shown in adult rat with cocaine (Hasbi et al., 2020b), this mechanism may generalize to other drugs of abuse. As the consequences of heavy cannabis use on psychiatric, behavioural and cognitive symptoms in adolescents are increasingly documented, ongoing investigation of the molecular mechanisms underlying maturation of the striatal D1-D2 heteromer system during adolescent transition to adulthood will help define an important factor mediating adolescent susceptibility to CUD and other manifestations and may reveal targets for candidate medications. Future studies will investigate whether D1-D2 disruption can alleviate THC-induced emotionality in adult rat and whether activation of D1-D2 neurons in adolescents can engender an adult-like behavioural phenotype to temper and reduce the rewarding effects of THC in adolescent rats.

Author contributions:

MED, MM, MS, Formal analysis. MED, SRG, BKM interpreted results. AH contributed to reagents. SRG and BKM conceived the study and Funding acquisition. MED, SW, JB designed the experiments. MED, SRG, BKM wrote the paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crneur.2023.100107.

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