RESEARCH PAPER

Detrimental effects of adolescent escalating low-dose Δ9-tetrahydrocannabinol leads to a specific bio-behavioural profile in adult male rats

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Background and Purpose: Adolescent cannabis use is associated with adult psycho-pathology. When Δ9-tetrahydrocannabinol (THC), mainly in high doses, is administered to adolescence rats there are also long lasting effects in adults. This study aims to determine the specific adult bio-behavioural profile after adolescent low-dose THC, which better mirrors adolescent recreational cannabis use.

Experimental Approach: Adolescent male Sprague–Dawley rats were treated with escalating low-dose of THC. In adulthood, they were evaluated for their spontaneous locomotion, sensorimotor gating, higher order and spatial cognitive functions. Dopaminergic activity and cannabinoid receptor expression were measured in distinct brain regions. Hippocampal neurogenic activity of neural stem cells was determined and protein levels of neuroplasticity-related biomarkers were quantified. Adolescent low-dose THC exposure increased spontaneous open-field activity, without affecting prepulse inhibition and attentional set-shifting performance. Region-specific dopaminergic alterations and CB_1 receptor up-regulation in the prefrontal cortex were observed. Impaired spatial memory, as assessed with the object location task and Morris water maze test, was associated with significantly decreased proliferative activity (SOX2-positive cells), neurogenic potential (decreased doublecortin-positive cells) in the adult hippocampus and defective neuroplasticity, including reduced BDNF expression in the hippocampus and prefrontal cortex.

Key Results: Our findings reveal the adverse impact of adolescent low-dose THC on the psychomotor profile, dopaminergic neurotransmission, compensatory cannabinoid receptor response, cognition-related neurobiological and behavioural functions.

Conclusion and Implications: Our adolescent low-dose THC animal model does not induce tangible psychotic-like effects, such as those reported in high-dose THC studies, but it impairs cognitive functions and points to hippocampal vulnerability and disrupted neurogenesis.

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1 | INTRODUCTION

Cannabis use during adolescence has been associated with psychopathology in adulthood, including addiction vulnerability, psychosis/schizophrenia and affective disorders (Casadio et al., 2011; Silins et al., 2014). Moreover, experimental studies have demonstrated the long-lasting effects of Δ^9-tetrahydrocannabinol (THC), the primary psychoactive constituent of cannabis, during adolescence on the adult bio-behavioural profile (Abela et al., 2019; Renard et al., 2017). However, observed behavioural effects are inconsistent, while the neurobiological underpinnings remain elusive.

Specifically, there is evidence that adolescent THC induces long-lasting motor dysfunction and cognitive abnormalities mostly linked to spatial aspects, but these findings present notable heterogeneity (Abboussi et al., 2014; Abush & Akirav, 2013; Llorente-Berzal et al., 2013; Renard, Rushlow, & Laviolette, 2016; Rubino et al., 2008; Rubino et al., 2009; Shen et al., 2011; Zamberletti et al., 2014). Limited experimental research has also reported either impaired prepulse inhibition (PPI) (Abboussi et al., 2014; Abela et al., 2019; Renard, Rosen, et al., 2016), associated with schizophrenia (Geyer et al., 2001) or no effect on sensorimotor gating after adolescent THC administration (Llorente-Berzal et al., 2013; Silva et al., 2016). However, fewer studies investigate the long-lasting consequences of cannabinoids on executive functions (Gomes et al., 2015).

Converging evidence suggests that adolescent THC exposure affects the development of the endocannabinoid system and neurotransmitter function, including dopamine. However, the enduring effects on adult neurotransmitter function largely remain unknown (Rubino et al., 2008; Silva et al., 2016).

Remarkably, long-term effects of adolescent cannabinoid exposure have been related to hippocampal neurogenesis and neuroplasticity alterations in adulthood (Abboussi et al., 2014; Lee et al., 2014; Silva-Peña et al., 2019; Steel et al., 2014). These alterations have been linked with addiction vulnerability, stress response, depression-like phenotype and cognitive dysfunction. However, the association between adolescent THC exposure and psychosis-like symptomatology warrants an in-depth investigation, especially considering the future consequences of recreational cannabis legalization.

Perhaps, it is most important to note that the majority of behavioural and neurobiological studies use relatively high doses of THC or cannabinoid agonists, with the most commonly used doses being 2.5 –5 –10 mg·kg^{-1} (Moore et al., 2010; Renard et al., 2017; Rubino et al., 2008), although the biphasic effects of THC are well documented in preclinical research (Katsidoni et al., 2013; Polissidis et al., 2010). Clinical findings, which demonstrate the regular cannabis use during early adolescence, escalating to patterns of heavier use during late adolescence and young adulthood, must be back-

translated in experimental studies (Coffey & Patton, 2016; Taylor et al., 2017).

On the basis of our earlier findings (Poulia et al., 2020), the present study aims to shed light on the long-term psychopathological impact of escalating low-dose adolescent THC exposure, in an attempt to accurately model the most common pattern of use in humans (Bagot et al., 2015; Calabrese & Rubio-Casillas, 2018). To this end, psychosis-related behavioural processes related to motor and cognitive functions, including sensorimotor gating, attentional set shifting and spatial learning and memory, were investigated in adulthood. Behavioural outcomes were linked to the neurobiological substrate, including analyses of cannabinoid CB$_1$ and CB$_2$ receptor status, *ex vivo* dopaminergic activity (dopamine and metabolite measurements and dopamine transporter [DAT] expression), adult hippocampal neurogenesis (SRY-box transcription factor 2 [SOX2] and doublecortin) and neuroplasticity indices (BDNF, trkB and p75) in psychosis-linked brain regions (prefrontal cortex, hippocampus, dorsal striatum and nucleus accumbens).

What is already known

- Adolescent cannabinoid exposure affects behavioural and neurobiological indices in adulthood.
- The higher THC doses have been more extensively studied so far.

What this study adds

- Adolescent escalating low-dose THC mainly disrupts spontaneous locomotion, memory-related functions and neurochemicals indices in adulthood.
- Our low-dose THC protocol affects adult neuroplasticity and neurogenesis processes, signifying a profound hippocampal vulnerability.

What is the clinical significance

- At a translational level, adolescent low-dose THC has detrimental effects on adult bio-behavioural profile.
- Low-dose THC induces hippocampus vulnerability. Unlike pro-psychotic high doses, it lacks a tangible psychosis-like profile.

2 | METHODS

2.1 | Animals

Male Sprague–Dawley rats raised in the Animal Facility of the University of Ioannina (license no "EL33-BIObr01") were housed in plastic cages (47.5 cm length × 20.5 cm height × 27 cm width), with natural soft sawdust as bedding, two per cage. The animals were maintained under controlled temperature of 22–24°C, humidity at 55 ± 5% and alternating 12-h light/dark cycles (lights were on between 7:00 a.m. and 7:00 p.m.), with free access to food and water. All experiments took place during the light phase of the cycle. Random allocation of animals to treatment groups and to behavioural tasks and blinding of investigators assessing outcomes were adopted to reduce performance bias in our trials (Curtis et al., 2018). Sample size is indicated in the figure legends and/or represented in the figures as scatter dot plot. In total, 147 rats were studied:- 93 rats for the behavioural tests, 24 rats for the HPLC studies, 20 rats for the western blot analysis and 10 rats for immunohistochemistry studies. The animals were subjected to only a single testing and no retesting of any animal was performed between different experimental procedures. More specifically, a separate set of animals was used for the neurochemical analysis. A separate set of animals was used for the neurobiological analysis (western blots) and a separate set of animals was used for the immunohistochemistry. For the behavioural tests, a separate set of animals was also used. This cohort was further subdivided to the different behavioural experiments; thus, the resulting n per group was between eight and 12 rats. The unequal treatment group size in the behavioural tests is attributed to the exclusion criteria for each behavioural task. Credible justifications have been made in the respective section for each behavioural test. In total, nine rats were excluded. The current experiment is based on the rule of the replacement, refinement or reduction. Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020). All experiments were approved by the Institutional Animal Facility Committee of the University of Ioannina and comply with the guidelines of the European Directive 2010/63/EU.

2.2 | Drugs

THC ethanol solution (25 mg·ml^{-1}) was sublimated and ethanol-free THC was dissolved in vehicle solution (5% DMSO, 5% cremophor EL, 90% NaCl). THC and vehicle were injected in volumes equal to 1 ml·kg^{-1} of body weight.

2.3 | Adolescent THC treatment

THC treatment started on post-natal day 35 (PND 35) and lasted until PND 45 (mid-adolescence). Rats received escalating i.p. THC doses twice daily (0.3 mg·kg^{-1} PND 35–37; 1 mg·kg^{-1} PND 38–41; 3 mg·kg^{-1} PND 42–45) or vehicle (Figure 1a). Body weight was measured daily during treatment. During the 30-day interval between THC administration and behavioural testing, rats were left undisturbed and their body weight was monitored weekly.

2.4 | Behavioural analysis

Behavioural testing started at PND 75 and animals were handled daily for 5 days prior to commencement of testing. All animals were accustomed to the experimental room for 40 min prior to the experiments. In total, 93 rats (43 vehicle and 50 THC-treated rats) were tested in all the behavioural experiments. Predefined exclusion criteria were set before the initiation of any behavioural test. Respective group sizes are included below for each specific test. Rats were killed at PND = 90.

2.4.1 | Motor activity-reaction to novelty

Motor behaviour was recorded for 60 min with a computerized activity monitoring system (ENV515, Activity Monitor, version 5; Med Associates Inc., USA) in a transparent, cubic open field apparatus (40 cm × 40 cm × 40 cm). Analyses were performed in four 15-min intervals. Ambulatory distance, as a measure of animal's overall motor activity and number of vertical counts (rearings), as a measure of animal's reactivity to a novel environment, were used for the evaluation of spontaneous motor activity, as previously described (Polissidis et al., 2013; Poulia et al., 2020). One rat was excluded from the analysis due to lack of reaction to the novel environment (expressed as less than 10 vertical counts).

2.4.2 | Prepulse inhibition

Prepulse inhibition (PPI) was conducted according to previously established protocols (Silva et al., 2016). Each animal was placed in a plexiglass restrainer and habituated to a startle chamber for 5 min with 70-dB white noise (Startle and Fear Interface LE118-8, Panlab, Harvard Apparatus). The rats were presented a series of sound pulses of varying amplitude (70, 80, 90, 100, 110 and 120 dB, in pseudorandom order, 1 s apart; 20 s intertrial interval; five times each) and average startle response of the five trials was determined (acoustic startle response). The following day, a PPI protocol was administered consisting of 5-min habituation (background white noise 70 dB), 10 pulse-alone (115 dB) trials, 10 prepulse (each of 75, 80, 85 and 90 dB) plus pulse trials in pseudorandom order (1 s apart; 20 s intertrial interval) and 10 no stimulus (white noise) trials. PPI was calculated as a percentage score: % PPI = [(S − PreS)/S] * 100, where "S" is the mean startle response for the startle pulse-alone trials and "PreS" is the mean startle response for prepulse plus pulse trials. No rat was excluded from this test due to poor performance.
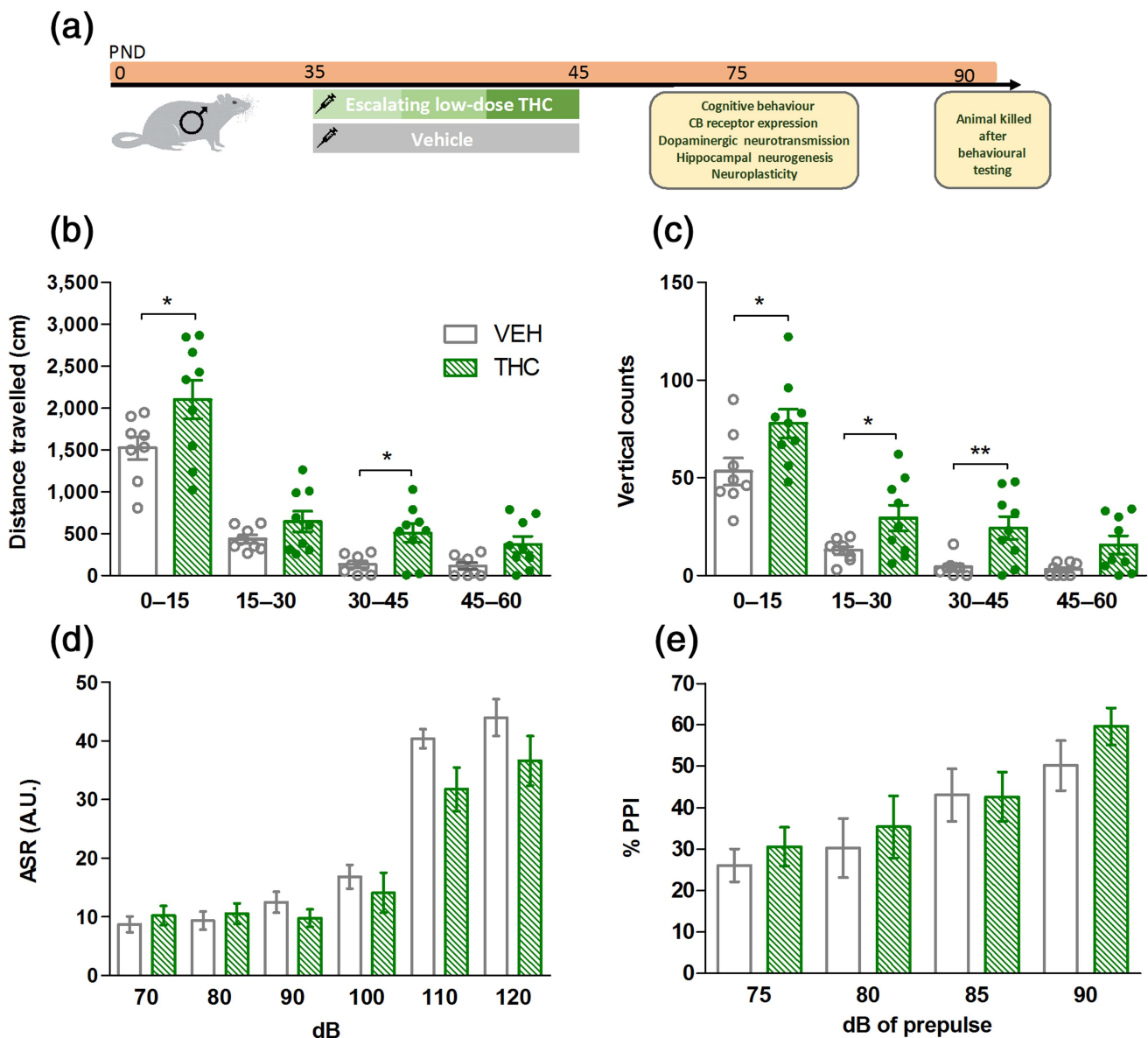
FIGURE 1 (a) Timeline of the experimental protocol: schematic of study design. Spontaneous motor activity in the open-field apparatus: (b) ambulatory distance (cm); (c) vertical counts (VEH n = 8, THC n = 9). One vehicle rat was excluded from the test due to unmet criterion described in Section 2. Prepulse inhibition (PPI): (d) acoustic startle response (ASR) to varying acoustic stimuli (70–120 dB); (e) percent PPI after four combinations of different prepulse (75, 80, 85 and 90 dB)-pulse trials (115 dB) (VEH n = 10, THC n = 10). Values are mean ± SEM. *$P \leq .05$

2.4.3 | Attentional set-shifting test

The attentional set-shifting test was conducted according to a modified version of a previously described protocol (Birrell & Brown, 2000). Food-restricted rats, having access to 80% of their normal food consumption, were trained to dig in small bowls, in order to retrieve food reward, covered with digging media, which was placed in one bowl per trial. Four rats were excluded from the analysis due to poor performance during the training phase (defined as rat's failure to dig and retrieve the food reward). Once rats learned to associate digging into the medium with finding the food reward beneath the bowl's medium, they were trained on a simple discrimination (SD) task, that is, to discriminate between the two bowls that varied along one of two dimensions (odour or digging medium), to a criterion of six consecutive correct trials. On test day, rats performed a series of discriminations: simple discrimination (SD); compound (CD) in which digging media differed according to both odour and texture, but with correct and incorrect exemplars remaining similar to the preceding SD; CD reversal (CDR) in which the reward contingency of the CD exemplars is reversed; intra-dimensional shifting (IDS), in which a novel discrimination is learned with new stimuli, the new correct exemplar being of the same dimension as before; ID reversal (IDR); extra-dimensional shifting (EDS) in which another discrimination with new stimuli is learned, but the correct exemplar is now from the other previously irrelevant dimension and ED reversal (EDR); each with a criterion of six consecutive correct trials.

During each discrimination, the number of trials to reach criterion and errors within each session were counted.

2.4.4 | Object location test (OLT)

This non-rewarded paradigm, based on the spontaneous exploratory behaviour of rodents, assesses short-term spatial recognition memory (Ennaceur, 1998). The task was performed as previously described (Pitsikas & Boultadakis, 2009; Poulia et al., 2020). Rats were allowed to explore the apparatus, an opaque open box (50 × 50 × 50 cm), for 3 min on three consecutive days. On the fourth day, a session that consisted of two 3-min trials, a sample (T1) and a choice (T2) trial, was conducted. During T1, two identical objects were positioned in two opposite corners of the apparatus. Each rat was placed in the middle of the apparatus, facing away from the objects and was allowed to explore them. Two rats were excluded from the analysis due to poor performance during the training phase T1, expressed as lack of motivation to spend time exploring the objects ($t < 10$ s). Immediately after T1, rats were returned to their home cages. The choice trial (T2) was performed following a 1-h intertrial interval. During T2, one of the two identical objects was placed in a novel location (N), while the other remained in the same-familiar position, as presented in T1 (F). All combinations of object locations were counterbalanced to reduce potential bias caused by preference for particular locations. Time spent exploring each object during T1 and T2 was recorded.

Discrimination between familiar (F) and novel (N) location of the object was assessed and a discrimination index (DI) was calculated: $DI = (N - F)/(N + F)$ (Cavoy & Delacour, 1993).

2.4.5 | Morris water maze

The Morris water maze test was conducted according to previously established experimental protocols (Vorhees & Williams, 2006). In brief, spatial cues were placed around the pool and surrounding walls and a hidden platform was placed in a pool filled with opaque water. During the 4-day acquisition phase, the platform is hidden in a specific quadrant of the pool. During each day, four sessions are executed. During each session, the rat is semi-randomly introduced into the water from a different start-point. During all four sessions within each of the 4 days, every rat has to locate the platform before the 90-s cut-off. If a rat did not manage to reach and mount the hidden platform within 90 s, during any session of any acquisition day, the experimenter would assist the rat in locating the platform by gently guiding it to the submerged platform. If a rat failed to meet the criterion of 90 s for more than two sessions in two consecutive acquisition days (not including the first day), then it was excluded from the analysis. Two rats were excluded from the testing due to poor performance. On the fifth day, the rats were subjected to a probe trial where the platform was removed and time spent in the target quadrant was measured as an index of spatial memory. Finally, the platform was relocated to the opposite quadrant of the pool and rats were re-trained in the reversal task, using the same criteria. For the evaluation of visuomotor coordination, latency to reach a visible platform emerging 1 cm from the surface of the water (semi-random placement in the four quadrants over four trials) was measured following the reversal task. Trials were video-recorded and measured automatically using Noldus Ethovision XT 9.0 (RRID:SCR_000441).

2.5 | Neurochemical-neurobiological analyses

2.5.1 | Neurochemical analysis

Adult rats were used for the neurochemical analysis. For the evaluation of dopaminergic activity, brain region homogenates (hippocampus, prefrontal cortex [, dorsal striatum and nucleus accumbens were analysed for dopamine levels and its metabolites DOPAC and homovanillic acid, using reversed phase HPLC on a GBC LC-1150 pump coupled with BAS LC4C electrochemical detector, as previously described (Kokras et al., 2018; Poulia et al., 2020). Dopamine turnover ratios DOPAC/dopamine and homovanillic acid/dopamine were also calculated, as indices of dopamine activity, that is, release, re-uptake and metabolism to DOPAC and homovanillic acid.

2.5.2 | Western blot analysis

The antibody-based procedures used comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018). Adult rats were killed by decapitation under isoflurane anaesthesia and their brains were immediately removed for western blot analysis. Dissected brain areas of the hippocampus, the prefrontal cortex, the nucleus accumbens and the dorsal striatum were homogenized as already described (Polissidis et al., 2010). Equal protein samples (50 μg) were diluted in SDS sample buffer and boiled (95°C) for 5 min. After electrophoresis, gels were semi-dry transferred onto a nitrocellulose membrane. Membranes were blocked in 5% non-fat milk/TBS for 2 h and incubated overnight at 4°C with anti-BDNF antibody (1:300, rabbit polyclonal antibody, Santa Cruz Biotechnology Cat# sc-546, RRID:AB_630940), anti-trkB antibody (Cell Signaling Technology Cat# 4607, RRID:AB_2155128), anti-p75 antibody (Santa Cruz Biotechnology, Cat# sc-6,188-R, RRID:AB_2267254), anti-dopamine transporter (DAT) antibody (Millipore Cat# AB2231, RRID: AB_1586991), anti-CB$_1$ antibody (Sigma-Aldrich Cat# C1108, RRID: AB_476819) and anti-CB$_2$ antibody (Cayman Chemical Cat# 101550, RRID:AB_10079370) following 1-h incubation with an HRP-linked secondary antibody (goat anti-rabbit IgG, 1:1500, Millipore Cat# 12-348, RRID:AB_11214240). All samples were standardized with b-actin (1:1500, rabbit monoclonal antibody, Thermo Fisher Scientific Cat# MA5-16410, RRID:AB_2537929 or GAPDH (1:6000, mouse monoclonal antibody, Millipore Cat# MAB374, RRID:AB_2107445). Immunoreactive bands were visualized with enhanced chemiluminescence detection solutions (Biorad, Clarity™ Western ECL Blotting Substrates, RRID:SCR_008426) using an XRS charge coupled device

camera (Bio-Rad Laboratories) and Quantity One software (RRID: SCR_014280). The optical density of each band was divided by the corresponding b-actin or GAPDH band to yield the corrected band intensity. Optical densities were quantified with ImageJ software (RRID:SCR_003070).

2.5.3 | Immunohistochemistry

The immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018). Adult rats were deeply anaesthetized and perfused transcardially with 0.9% NaCl followed by 4% PFA. Brains were removed and post-fixed in 4% PFA for 4 h at 4°C. Tissues were cryoprotected in 30% sucrose for 2 days, frozen and cut in 40-μm-thick sagittal sections on a cryostat. Sections were collected and

incubated overnight at 4°C with the primary antibody diluted in blocking solution of 10% normal goat serum, 0.1% Triton X-100 in PBS. Sections were washed in PBS and incubated at room temperature for 1 h with the corresponding secondary antibodies in blocking solution. For doublecortin detection, antigen was recovered at 80°C for 20 min in sodium citrate solution (10 mM, pH 6.0). Stained sections were mounted on Superfrost glass slides (Thermo Scientific) and embedded in mounting medium (VectaShield; VECTOR Labs) as an anti-fading agent. Evaluation of hippocampal neurogenesis was conducted using commercially available primary antibodies: anti-SOX2 rabbit antibody (Abcam, Cat# ab97959; RRID:AB_2341193) and anti-doublecortin goat antibody (Santa Cruz Biotechnology, Cat# sc-8,066; RRID: AB_2088494). Fluorescent microscopy was performed using either a confocal microscope Leica TCS SP5 equipped with the LAS AF Software Version I2.1.0 or a Leica DM RXA2 microscope equipped with a Leica DFC500 camera and the LAS AF Lite software. For

FIGURE 2 Attentional set-shifting test: (a) number of trials to reach the criterion of six consecutive correct choices of each individual test; (b) number of errors made until reaching each criterion. SD, simple discrimination; CD, compound discrimination; CB receptors (CBR), CD reversal; IDS, intra-dimensional shifting; IDR, ID reversal; EDS, extra-dimensional shifting (EDS); EDR, ED reversal. Values are mean ± SEM (VEH *n* = 8, THC *n* = 12). Four vehicle rats were excluded from the test due to unmet criterion described in methods. Object location test: (c) decreased discrimination index (DI) in THC-treated rats compared to vehicle (VEH *n* = 10, THC *n* = 12). Two vehicle rats were excluded from the test due to unmet criterion described in Section 2. Morris water maze test: (d) probe trial: time spent in the target quadrant(s) (platform absent) as a measure of spatial memory following acquisition; (e) probe reversal trial: Time spent in the target quadrant(s) (platform absent) following reversal learning; (f) latency to reach the visible platform(s) during the final visual platform trial (VEH *n* = 7, THC *n* = 7). Two rats (one vehicle and one THC-treated rat) were excluded from the test due to unmet criterion described in methods. Data represent the mean ± SEM. *$P \leq .05$
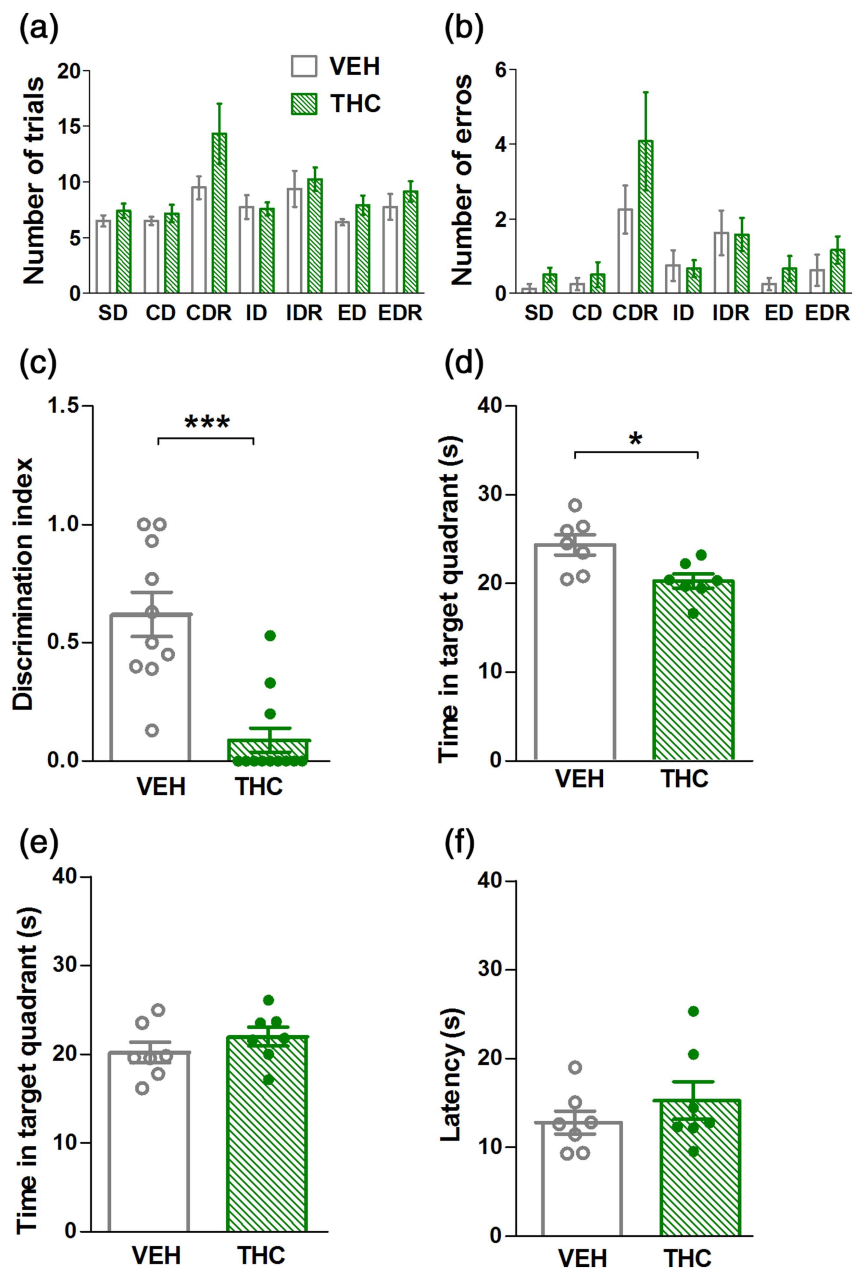
quantification of SOX2- and doublecortin-positive cells, series of every sixth brain section were stained and positive cells were counted throughout the rostro-caudal extent of the granule cell layer (Koutmani et al., 2019).

2.5.4 | Materials

THC was purchased from Sigma–Aldrich Chemie GmbH, Germany (T2386, CAS Number 1972-08-3).

2.5.5 | Data and statistical analysis

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis (Curtis et al., 2018). Randomization was used to assign subjects to the experimental groups and treatment conditions for all *in vivo* studies. Data collection and acquisition of all *in vivo* and

in vitro experimental paradigms were performed in a blinded manner. Statistical analysis was undertaken only for studies where each group size was at least $n = 5$. Data were checked for normality using the Shapiro–Wilk test. Effects of THC treatment on behavioural, neurochemical and neurobiological parameters were analysed using Student's *t*-test. Effects of THC treatment on motor activity and PPI were analysed using two-way repeated measures ANOVA. Post hoc tests were conducted only if F was significant and there was no variance inhomogeneity. When required, the Bonferroni post hoc multiple comparisons procedure was used for between-group individual comparisons. For western blot analysis, the average value of the control group was set to "100," and experimental group measures were reported relative to control values. Group sizes indicate independent animals and any exclusion of outliers in the behavioural tests was based on a predefined and defensible set of exclusion criteria, as already described. Analysis was performed with SPSS v.21 (IBM Corp, NY, USA, RRID:SCR_002865). Data are expressed as mean ± SEM. Overall level of significance was set at $P \leq .05$.
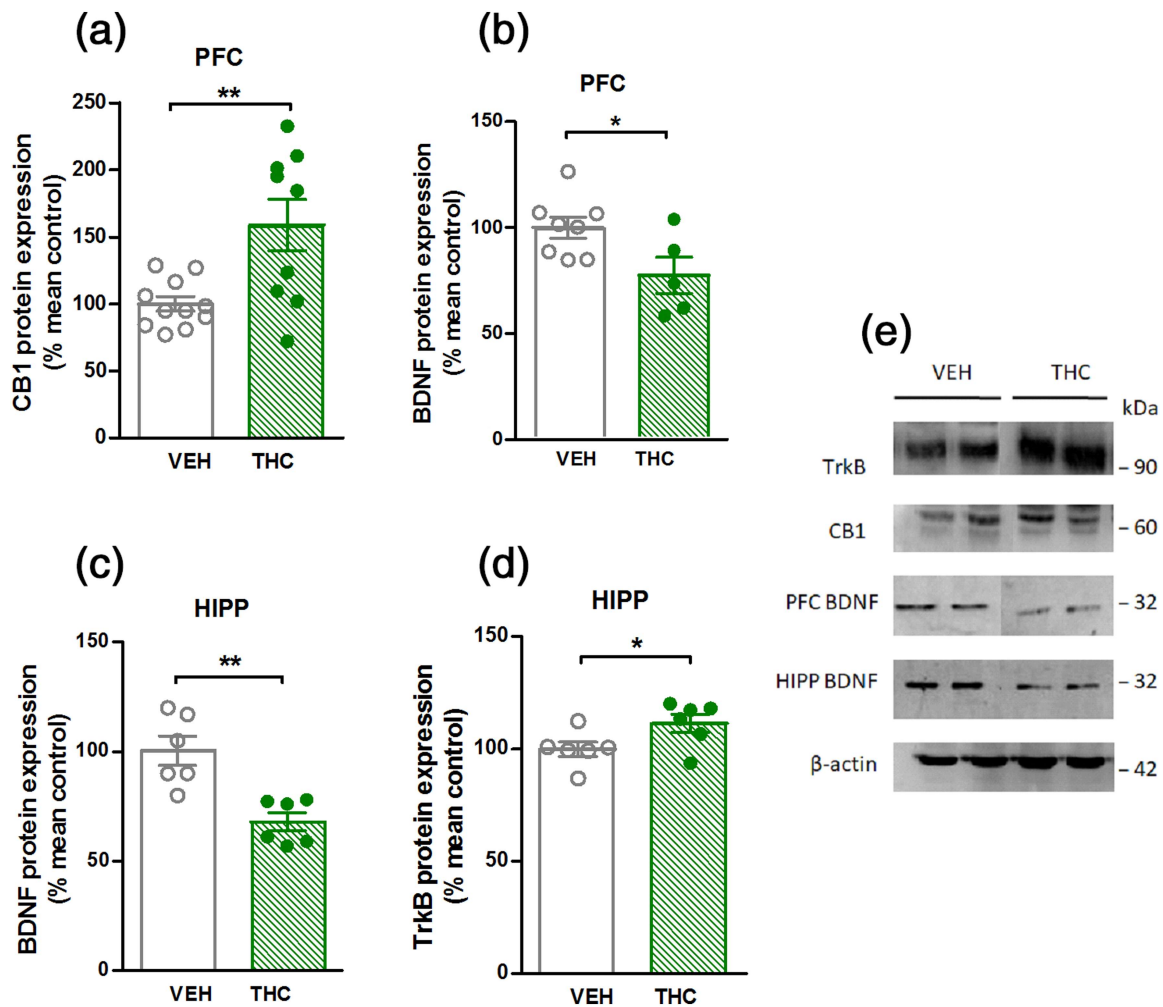

FIGURE 3 Effects of adolescent low-dose THC treatment on protein expression levels in adult male rats: (a) CB1 receptor levels in the prefrontal cortex (PFC); (b) BDNF levels in the prefrontal cortex (PFC) and (c) in the hippocampus; (d) neurotrophic receptor tyrosine kinase 2 (trkB) receptor levels in the hippocampus. The optical density (OD) of each band was normalized to corresponding β-actin bands. Graphical data represent quantification of (e) immunoblot bands. THC groups are expressed as percent change of the VEH group. *$P \leq .05$

2.5.6 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019, 2019a, 2019b, 2019).

3 | RESULTS

3.1 | Body weight

THC versus vehicle treatment during adolescence had no effect on body weight either during treatment or when rats reached adulthood (data not shown).

3.2 | Behavioural analysis

3.2.1 | Adolescent THC treatment increased spontaneous motor activity in adulthood

The 1-h total registration time was analysed in four 15-min intervals.

Two-way repeated measures ANOVA, with treatment and time as factors, revealed a main effect of time both on horizontal and vertical activity. Specifically, both groups showed a significant reduction in ambulatory distance and in vertical counts, over time.

Subsequent post hoc comparisons (Bonferroni test) for each 15-min interval revealed increased ambulatory distance in THC-versus vehicle-treated rats. This was significant between 0 and 15 min and between 30 and 45 min (Figure 1b). Increased significant vertical counts were also observed in THC-treated rats between 0 and 15 min; between 15 and 30 min and between 30 and 45 min (Figure 1c) ($n = 8$ vehicle, $n = 9$ THC-treated rats).

3.2.2 | Adolescent THC treatment did not affect prepulse inhibition in adulthood

Two-way repeated measures ANOVA with time as within-subject factor and treatment as between-subjects factor did not reveal any statistically significant effect on acoustic startle response or PPI between the two groups (Figure 1d,e).

3.2.3 | Adolescent THC treatment did not affect attentional set-shifting in adulthood

Student's t-test showed that adolescent THC administration did not significantly affect attentional set-shifting test execution during adulthood. Specifically, there were no statistically significant differences between the two groups either in the number of trials to reach criterion or errors to reach criterion (Figure 2a,b). It should be noted that the THC-treated group showed a trend towards a deficit in the execution of all trials, compared with vehicle, especially during the CDR, which is the first time that the rule was reversed.

3.2.4 | Adolescent THC treatment impaired special recognition memory in adulthood

Student's t-test demonstrated that adolescent THC administration led to deficits in spatial memory, since THC-treated rats revealed a significantly decreased discrimination index (DI), compared with vehicle (Figure 2c). Total time spent exploring both objects during the habituation phase (T1 SUM) did not show any statistically significant difference between the two groups, indicating no potential confounding effect of treatment on exploration levels (data not shown) ($n = 10$ vehicle, $n = 12$ THC-treated rats).

TABLE 1 Dopaminergic activity status in adult male rats after adolescent low-dose THC or vehicle treatment: Dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) tissue levels and turnover rate values expressed as DOPAC/DA and HVA/DA (mean ± SEM): (A) hippocampus, (B) prefrontal cortex, (C) dorsal striatum and (D) nucleus accumbens

	Vehicle	THC
(A) Hippocampus		
DA	0.019 ± 0.00	0.016 ± 0.00
DOPAC	0.016 ± 0.00	0.018 ± 0.00
HVA	0.002 ± 0.00	0.001 ± 0.00
Turnover rate DOPAC/DA	0.830 ± 0.25	1.330 ± 0.31*
Turnover rate HVA/DA	0.10 ± 0.02	0.18 ± 0.01
(B) Prefrontal cortex		
DA	0.051 ± 0.00	0.072 ± 0.00*
DOPAC	0.038 ± 0.00	0.044 ± 0.00
HVA	0.010 ± 0.00	0.012 ± 0.00
Turnover rate DOPAC/DA	0.740 ± 0.14	0.580 ± 0.15*
Turnover rate HVA/DA	0.20 ± 0.00	0.16 ± 0.07
(C) Dorsal striatum		
DA	24.80 ± 1.92	19.02 ± 0.93*
DOPAC	2.61 ± 0.24	1.90 ± 0.13
HVA	0.94 ± 0.06	0.74 ± 0.03*
Turnover rate DOPAC/DA	0.10 ± 0.01	0.09 ± 0.00
Turnover rate HVA/DA	0.038 ± 0.00	0.039 ± 0.00
(D) Nucleus accumbens		
DA	4.45 ± 1.43	5.68 ± 1.20
DOPAC	1.29 ± 0.30	2.22 ± 0.28*
HVA	0.44 ± 0.07	0.45 ± 0.07
Turnover rate DOPAC/DA	0.36 ± 0.05	0.32 ± 0.04
Turnover rate HVA/DA	0.11 ± 0.08	0.07 ± 0.02

$*P \leq .05$ (THC- versus vehicle-treated rats).

3.2.5 | Adolescent THC treatment impaired spatial memory but not learning in adulthood

Student's t-test showed that adolescent THC administration led to deficits in spatial memory in the Morris water maze test since THC-treated rats spent significant less time in the target quadrant during the memory probe trial, compared with vehicle (Figure 2d). In contrast, in the probe reversal phase, no significant difference in time spent in the new target quadrant was detected, indicating that cognitive flexibility was unaffected (Figure 2e). Finally, there was no significant difference between treatments concerning the latency to find the visible platform, indicating normal visuomotor coordination (Figure 2f).

3.3 | Neurochemical-neurobiological analyses

3.3.1 | CB$_1$ receptor up-regulation in the prefrontal cortex after adolescent THC treatment

First, we sought to identify potential direct changes on cannabinoid receptor expression induced by adolescent low-dose THC treatment. Indeed, we observed significant enhancement of CB$_1$ receptor protein expression levels in the adult prefrontal cortex (Figure 3a), with no

statistically significant changes in other brain regions. CB$_2$ receptor protein levels remained unchanged in all areas examined after adolescent THC treatment (data not shown).

3.3.2 | Altered dopaminergic function after adolescent THC treatment

Next, we assessed potential long-term alterations in dopaminergic neurotransmission in psychosis-linked brain regions following adolescent THC exposure. In adulthood, a significant increased DOPAC/dopamine turnover rate was observed in the hippocampus after adolescent THC exposure, indicating enhanced dopaminergic activity. In parallel, a trend for decreased dopamine levels was observed in THC-treated rats compared to vehicle.

Significant decreased DOPAC/dopamine turnover rate was found in the prefrontal cortex of THC-treated rats, accompanied by significant increased tissue dopamine levels.

Significant decreased tissue dopamine and homovanillic acid levels were determined in the dorsal striatum, following adolescent THC treatment.

Significant increased tissue DOPAC levels were observed in the nucleus accumbens, after adolescent THC treatment (Table 1; Figure S1).
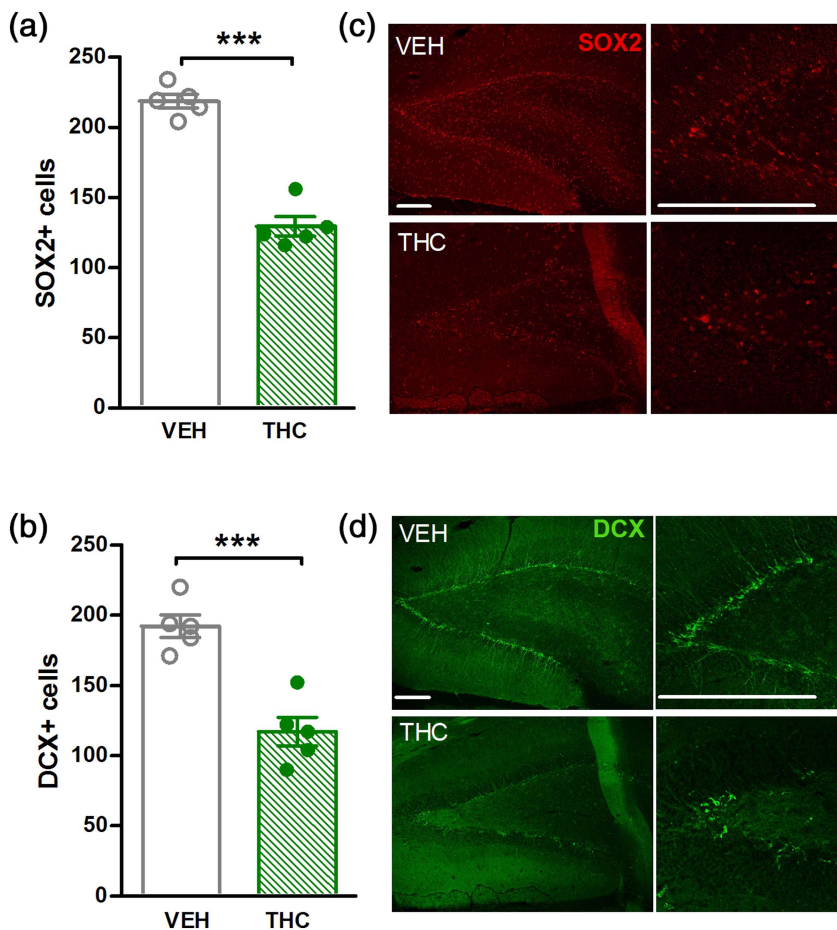

FIGURE 4 Effects of adolescent low-dose THC treatment on adult hippocampal neurogenesis: (a) quantification of the sex determining region (SRY)-box transcription factor 2+ (SOX2+) cells and (b) doublecortin+ (DCX+) cells in the dentate gyrus (DG) of adult vehicle and THC-treated (VEH n = 5, THC n = 5); (c, d) representative images of sagittal DG sections from adult vehicle and THC-treated rats. Sections are stained for SOX2 (c) or DCX (d). Scale bar: 100 μm, values are mean ± SEM. *$P \leq .05$

Adolescent THC exposure did not affect dopamine transporter (DAT) protein levels in any brain area examined (data not shown).

3.3.3 | Reduced BDNF expression in the prefrontal cortex and hippocampus after adolescent THC treatment

BDNF, trkB and p75 levels were assessed as markers of neuroplasticity to further gauge the neurobiological status reflecting the cognitive deficits observed following adolescent THC treatment. Student's t-test revealed a significant reduction in pro-BDNF protein expression levels of THC-treated rats in the prefrontal cortex and the hippocampus after adolescent THC exposure compared to vehicle (Figure 3b,c).

Additionally, our experimental protocol led to significant increased trkB protein levels in the hippocampus compared to vehicle (Figure 3d) (n = 6 vehicle, n = 6 THC-treated rats), while p75 protein expression levels remained unaffected in the hippocampus and the prefrontal cortex (data not shown).

3.3.4 | Impaired hippocampal neurogenesis following adolescent THC exposure

Spatial memory is tightly linked to hippocampal neurogenesis. Since adolescent THC administration led to deficits in spatial memory, as shown both by object location test and Morris water maze test, we reasoned that adult neurogenesis in THC-treated rats might be affected. To assess this hypothesis, both the proliferative and the neurogenic activity of adult neural stem cells were evaluated by detecting the cell-type specific markers SOX2 and doublecortin, respectively. A significantly reduced number of SOX2+ and doublecortin+ cells was identified in the hippocampus of THC-treated rats compared to vehicle (Figure 4).

4 | DISCUSSION AND CONCLUSIONS

It is demonstrated herein that escalating low-dose THC treatment of male rats during adolescence exerts detrimental and enduring effects in adulthood on psychomotor function and spatial memory, along with dopaminergic alterations and hippocampal vulnerability demonstrated by neurogenesis and neuroplasticity aberrations.

Specifically, the spontaneous motor profile was enhanced in THC-treated rats, as reflected by persistently elevated horizontal and vertical activity compared to vehicle. A stimulated motor response, among other indices, has been proposed to model the positive symptomatology of psychosis-like animal models (Powell & Miyakawa, 2006; Santos et al., 2016). Present results are in accordance with our previous work showing hyperlocomotion in a novel environment in adulthood after adolescent THC exposure (Poulia et al., 2020). These findings are also in line with previous reports of enhanced reaction to novelty after adolescent cannabinoid administration (Abush & Akirav, 2013; Llorente-Berzal et al., 2013), yet others have reported either decreased motor response (Renard, Rosen, et al., 2016) or no effects (Rubino et al., 2008; Shen et al., 2011; Zamberletti et al., 2014).

Altered prepulse inhibition (PPI) is present in schizophrenia, indicating sensorimotor gating deficits (Jones et al., 2019; Markou et al., 2009). The PPI of the startle reflex, as an index of the ability to filter out insignificant sensory information from the external environment, is also disrupted in animal models of schizophrenia (Swerdlow et al., 1990; Van den Buuse, 2010). The current study reports that adolescent THC administration did not induce any PPI deficits in adult rats, in line with other studies using this behavioural paradigm (Llorente-Berzal et al., 2013; Silva et al., 2016). Thus, the present findings suggest that escalating low-dose THC during adolescence does not result in perceptual abnormalities and information processing deficits, which are commonly observed in schizophrenia.

Our low-dose THC protocol did not disrupt the execution of complex discriminations and reversals, albeit other studies using high cannabinoid dosages have shown impaired adult attentional set-shifting test (Gomes et al., 2015; Harte & Dow-Edwards, 2010). Attentional set-shifting test has been used as a translational approach for screening higher order cognitive deficits observed in schizophrenia (Tait et al., 2014). This complex task is used as an index of executive function and cognitive flexibility in humans and contributes to the assessment of cognitive deficits in brain disorders, including schizophrenia. Our current attentional set-shifting test and PPI findings converge to form a specific adolescent THC phenotype, devoid of sensorimotor gating deficits and impairments in attention and cognitive flexibility.

Mild effects on dopaminergic activity, as assessed by tissue levels of dopamine and its metabolites, DOPAC and homovanillic acid, the turnover ratio, and dopamine transporter protein expression levels, were observed in the nucleus accumbens and the dorsal striatum. These findings suggest that our treatment protocol did not induce major alterations in dopamine synthesis and release in divisions of the striatum in contrast to what is observed in schizophrenia and its animal models. The aforementioned behavioural data along with these mild striatal dopaminergic alterations suggest that adolescent escalating low-dose THC is not sufficient to promote a psychosis-related phenotype.

Dopaminergic activity, as deduced by the turnover ratio, was decreased in the prefrontal cortex of THC-treated rats. Dopaminergic activity in the prefrontal cortex is involved in the encoding and use of working memory information (Seamans & Yang, 2004), while a low dopamine tone in the prefrontal cortex has been linked to schizophrenia and schizophrenia-related cognitive dysfunction. In this context, the lack of robust effects on striatal function could be related to unaffected performance in attentional set-shifting test and PPI, to the degree that cortico-striatal loops play key roles in behavioural flexibility (Bissonette & Roesch, 2017; Ragozzino, 2007) and sensorimotor gating (Wan & Swerdlow, 1996).

Interestingly, adolescent THC exposure led to CB_1 receptor up-regulation in the prefrontal cortex, while there was no observed alterations in CB_2 receptor expression levels. CB_1 receptors in the prefrontal cortex are mostly located on CCK-positive GABAergic interneurons that contribute substantially to the inhibition of pyramidal neurons (Marsicano & Lutz, 1999). Our current findings would predict increased disinhibition of pyramidal neurons, potentially leading to disruption of cortical functions. Previous studies examining higher doses of adolescent THC showed no effects on CB_1 receptor level expression either in the prefrontal cortex or the hippocampus of adult rats (Steel et al., 2014; Zamberletti et al., 2015), while other studies have shown that chronic high-dose THC treatment in adults or adolescents leads to CB_1 receptor down-regulation and desensitization (Sim-Selley et al., 2006). Present findings of adolescent THC limited impact on CB receptor expression levels may be attributed to the low doses used and possible compensatory developmental mechanisms at play.

Unlike the higher order cognitive tasks, spatial-oriented tasks were impaired in adulthood by adolescent low-dose THC administration. Indeed, THC-treated rats demonstrated memory impairment in the object location test and Morris water maze test. These findings are in line with other studies reporting spatial memory deficits in the object location test and radial maze (Abush & Akirav, 2013; Rubino et al., 2009), along with long-term negative effects on spatial and recognition memories (Abush & Akirav, 2013; Zamberletti et al., 2014) following adolescent cannabinoid treatment. Corroborating previous results, our findings support and further extend the concept that adolescent THC, even at low doses, disrupts spatial aspects of cognitive function. In a broader frame, it could be suggested that cannabinoids during adolescence have a negative enduring impact on memory consolidation, a process tightly related to hippocampal function.

Additionally, adolescent low-dose THC treatment elicited specific dopaminergic changes in the hippocampus. Dopaminergic activity, as deduced by the turnover ratio, was increased in the hippocampus of THC-treated rats. Dopaminergic hippocampal activity contributes to the consolidation of hippocampus-related memories, including spatial (Duszkiewicz et al., 2019) and recognition (Moreno-Castilla et al., 2017) memories. Dysregulation in steady-state dopaminergic activity is expected to interfere with these processes, given that brain functions that depend on dopamine status decline if dopaminergic function is not maintained within a limited range (Bezu et al., 2017; Goldman-Rakic et al., 2000). Thus, the impairments in spatial recognition and memory (object location and Morris water maze tests) may be attributed to the disrupted dopaminergic activity in the hippocampus.

Most interestingly, adolescent THC induced negative effects on the adult neural stem cell pool of the hippocampus, reducing neurogenic potential and, thus, indicating a significant long-term action of THC on adult hippocampal neurogenesis. These findings are in line with other studies showing impaired neurogenesis in adulthood (Abboussi et al., 2014; Lee et al., 2014; Silva-Peña et al., 2019; Steel et al., 2014). Moreover, increased dopaminergic activity in the

hippocampus is related to stress induced effects (Dalla et al., 2008; Perez & Lodge, 2019) also linked to compromised neurogenesis and neuroplasticity (Fenoglio et al., 2006). Detrimental effects on spatial cognitive function, along with disturbances in neurogenesis processes, support the characterization of an impaired bio-behavioural profile in adulthood after adolescent low-dose THC exposure.

Consistent with perturbations in spatial memory, BDNF levels were reduced and trkB increased in the hippocampus. Additionally, decreased cortical BDNF levels were also observed in THC-treated rats. These findings agree with other studies reporting reduced BDNF levels after cannabinoid exposure (López-Gallardo et al., 2012; Maj et al., 2007). Our results suggest that adolescent THC exposure, even at low doses, in adulthood negatively affects spatial aspects of cognitive function and neuroplasticity in relation to BDNF/trkB signalling, in brain regions mediating spatial memory and memory consolidation. Overall, the hippocampus and specific hippocampal-dependent behavioural indices appear to be particularly vulnerable to THC. Importantly, the fact that hippocampal function and related behavioural outputs did not indicate a psychosis-like phenotype, is not surprising since only high THC doses are reported to be pro-psychotic.

In this study, escalating low-dose cannabinoid treatment was chosen as it most closely simulates cannabis use in teenagers, that is, for recreational purposes and not for the sedative actions induced by high doses. The choice of the particular escalating THC doses better mimics casual recreational use in adolescent humans (gradually increased). Our escalating THC dosage (0.3 –1 –3 mg·kg^{-1}) reaches the lower limits of the most widely used escalating scheme reported in the literature (2.5 –5 –10 mg·kg^{-1}) (Renard et al., 2017; Rubino et al., 2008). Our protocol has not been extensively investigated in other studies, thus aims to improve the translational validity of the existing literature towards understanding the long-term consequences of adolescent recreational cannabis use and investigating whether in adolescence even low THC exposure in escalating doses is not devoid of detrimental effects. Our experimental results demonstrate that adolescent low-dose THC leads to a profile in adulthood, characterized by mild psychomotor stimulation, subtle striatal dopaminergic alterations and an aberrant pattern of spatial cognitive function, accompanied by hypodopaminergic cortical and hyperdopaminergic hippocampal status, hippocampal vulnerability, demonstrated by impaired neuroplasticity and neurogenesis. Conversely, lack of alterations in sensorimotor gating and higher order cognitive functions does not support a typical psychosis-like phenotype. However, we could postulate that the detrimental consequences of adolescent low-dose THC in this animal model confers hippocampal vulnerability and may facilitate a premorbid state of psychosis disorder, rather than induce overt psychotic-like behaviour.

In conclusion, our results further our understanding of the impact of early adolescent cannabinoid exposure on CNS function. We described a globally affected bio-behavioural (endo)phenotype, consisting of an enhanced motor output, altered dopaminergic activity, impaired spatial memory function and aberrant neuroplasticity and neurogenesis processes. Our findings provide, at a

translational level, data addressing the key clinical question of whether adolescent low-dose, recreational cannabis consumption could be a risk factor for developing specific aspects of psychopathology later in adulthood.

AUTHOR CONTRIBUTIONS

N.P. conducted the experimental work, analysed the data and contributed to data interpretation and manuscript preparation. F.D. provided input to all the stages of the experimental procedures and contributed to manuscript preparation. C.B. contributed to data interpretation and provided additional insights in the final manuscript. A.P. participated in the execution of the behavioural tests and contributed to the final form of the manuscript. N.K. and C.D. contributed to the HPLC assays and provided additional insights in the final manuscript. Y.K. and P.P. contributed to the immunohistochemistry experiments and provided valuable input in the preparation of the manuscript. K.A. designed and supervised the study and contributed substantially to all the stages of the experimental procedures and the preparation of the manuscript. All authors reviewed, contributed and approved the final manuscript.

CONFLICT OF INTEREST

All authors declare no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design & Analysis, Immunoblotting & Immunohistochemistry and Animal Experimentation and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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