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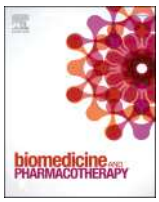

Prenatal THC exposure drives sex-specific alterations in spatial memory and hippocampal excitatory/inhibitory balance in adolescent rats

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ABSTRACT

The interaction between the main psychotropic ingredient of Cannabis, Δ⁹- tetrahydrocannabinol (THC), with the endogenous cannabinoid system (ECS) is a critical and underrated issue that deserves utmost attention. The ECS, indeed, contributes to the formation and regulation of excitatory and inhibitory (E/I) neuronal networks that in the hippocampus underly spatial memory. This study explored sex-specific consequences of prenatal exposure to THC in hippocampus-dependent memory and the underlying cellular and molecular contributors of synaptic plasticity and E/I homeostasis. Sprague Dawley dams were exposed to THC (2 mg/kg) or vehicle, from gestational day 5–20. The adolescent progeny of both sexes was tested for: spatial memory retrieval and flexibility in the Barnes Maze; mRNA expression of relevant players of hippocampal synaptic plasticity; density of cholecystokinin-positive basket cells (CCK+BCs) – a major subtype of hippocampal inhibitory interneurons; mRNA expression of the excitatory and inhibitory synaptic proteins neuroligins (Nlgns), as a proxy of synaptic efficiency. Our results show a sex-specific disruption in spatial memory retrieval and flexibility, a male-specific decrease in CCK+BCs density and increase in the expression of markers of neuroplasticity, and consistent changes in the expression of Nlgn-1 and 3 isoforms. Despite a delay in memory retrieval, flexibility of memory was spared in prenatally-THC-exposed female offspring as well as most of the markers of neuroplasticity; a sex-specific increase in CCK+BCs density, and a consistent expression of Nlgn-3 was observed. The current results highlight a major vulnerability to prenatal exposure to THC on memory processing in the male progeny, and sex-specific alterations in the E/I balance and synaptic plasticity.

Abbreviations: CB1Rs, Cannabinoid type 1 receptors; CCK+BCs, Cholecystokinin-positive basket cells; CTRL, Prenatal Treatment with vehicle; ECBs, Endocannabinoids; ECS, Endogenous cannabinoid system; E/I, Excitatory/Inhibitory; GD, Gestational day; HINT1, Histidine triad nucleotide-binding protein 1; LTD, Long-term depression; LTP, Long-term potentiation; Nlgns, neuroligins; NR, NMDARs NR subunit; PBS, phosphate-buffered saline; PND, Postnatal day; PNs, Pyramidal neurons; PTHC, Prenatal Treatment with THC; THC, Δ⁹-tetrahydrocannabinol.

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1. Introduction

The endocannabinoid system (ECS) plays a pivotal role in orchestrating various aspects of the nervous system growth such as the proliferation, migration, specification and survival of neural progenitors, the phenotypic differentiation of neurons, the establishment of synaptic communication, and bioenergetics [1]. Once this multimodal modulatory activity has occurred, the ECS contributes to synaptic efficacy and, modulating both excitatory and inhibitory synaptic neurotransmission in the postnatal brain, helps maintain the excitatory and inhibitory (E/I) balance [2–5]. In particular, in the hippocampus, contextual and spatiotemporal information processing requires the maintenance of an optimal E/I balance, which involves the contribution of various types of GABAergic interneurons at a staggering number of synapses. Among them, cholecystokinin-containing (CCK) basket cells (CCK+BCs) are particularly relevant in the generation of the network properties that underlie hippocampal cognitive functions [6,7]. Importantly, the terminals of CCK+BCs are heavily endowed with cannabinoid type 1 receptors (CB1Rs) [8]. When activated by the endocannabinoids (eCBs) released from postsynaptic pyramidal neurons (PNs), CB1Rs mediate a retrograde signalling that controls excitability and plasticity [9–11]. Dysregulation of the ECS tone during the intrauterine life, thus, may challenge the E/I balance, alter the synaptic strength, and affect hippocampal functioning.

Recently this group of research has reported that prenatal exposure to Δ^9-tetrahydrocannabinol (THC), the principal psychoactive component of cannabis, results in disrupted hippocampal information processing and in modifications in markers of neuroplasticity that suggest an abnormal excitatory synaptic arrangement in the male adolescent offspring [12–14]. Moreover, in a mice model of prenatal cannabinoid exposure, the reduction of CCK+BCs inhibitory tone has been associated with hippocampal hyperexcitability and impairment in spatial memory [15]. Importantly, the preclinical evidence is supported by human studies showing that maternal use of THC during pregnancy is associated with abnormalities in information processing, learning and memory, and cognitive flexibility in children and adolescents [16–27].

Recently, considerable attention has been directed to the Neuroligins (Nlgns), as crucial modulators of synaptic transmission [28–30]. These synaptic adhesion molecules are concentrated at hippocampal excitatory and inhibitory synapses [31–36], where they provide a multivalent signalling platform to modulate synaptic strength [36–38]. Notably, dysregulation in Nlgns expression and functionality is associated with an imbalance in the E/I tone, which involves eCBs signalling at CB1R-expressing CCK+ synapses [33,39,40]. However, as far as we know, a proper link between neurodevelopmental perturbations of the ECS, abnormality in synaptic plasticity and Nlgns expression has not been established yet [41–43].

Based on these premises and our previous findings, we hypothesize that an early and repeated exposure to a mild THC dosage during intrauterine life can jeopardize the ECS-led developmental trajectories and, thus, affect the normative E/I hippocampal networks, the expression of the hippocampal effectors of neuroplasticity and hippocampus-dependent memory processing in the adolescent progeny. Adolescence, indeed, is a period of extensive neurodevelopmental refinement, during which early challenges that affect developmental trajectories are thought to accumulate across a lifetime and unmask psychopathology [44–46].

Therefore, the current research focuses on: testing spatial memory retrieval and flexibility in the Barnes maze as a measure of hippocampal functioning; investigating the mRNA expression pattern of N-methyl-D-aspartate receptor (NMDAR) subunits, metabotropic glutamate receptor subtype 5 (mGluR5) and Homer1 protein, postsynaptic density protein 95 (PSD-95), CB1R, and Histidine triad nucleotide-binding protein 1 (HINT1), as markers of hippocampal neuroplasticity; evaluating the density of CCK+BCs and Nlgn-1,2,3 mRNA expression as gauges of E/I balance. We expanded the investigation to the female adolescent offspring since the existing evidence on sex biases in response to prenatal THC exposure is characterized by ambiguity and paucity [47]. The current results contribute to the intricate puzzle of prenatal cannabis-induced detrimental consequences on memory in the offspring, highlighting a sex- and function-specific vulnerability to prenatal THC exposure that is associated with sex-specific alterations in hippocampal synaptic plasticity and neuronal networks.

2. Materials and methods

2.1. Animals

Sixteen pregnant Wistar rats (Charles River, Italy), each weighing between 200 and 220 g, were individually housed in standard cages measuring 40 cm × 60 cm × 20 cm, with bedding. They had unrestricted access to water and food in a room maintained at a controlled temperature (22 ± 2 °C) and humidity (55 ± 5 %), with a 12-hour light/dark cycle.

After weaning, male and female rat offspring were housed in pairs, with each experimental group consisting of one or two independent rats per sex from each litter of dams, resulting in a total of 57 rats. Maternal body weight was monitored daily for the duration of the gestation to assess pregnancy weight gain. Dams were allowed to deliver normally. Litter size and the number of male and female pups were recorded for each dam [48]. All experiments were ethically approved by the Italian Ministry of Health (819/2021-PR to Carla Cannizzaro) and conducted following animal protocols sanctioned by the Committee for the Protection and Use of Animals at the University of Palermo. The procedures adhered to the current Italian legislation on animal experimentation (D. L. 26/2014) and the European directives (2010/63/EU) governing the care and utilization of laboratory animals. Rigorous efforts were made to minimize the number of animals used and to alleviate their suffering.

2.2. Prenatal THC treatment

The THC resin, obtained from the Forensic Laboratory of Biologically Active Substances at the University of Chemistry and Technology in Prague, Czech Republic, with a purity exceeding 97 % (verified by HPCL) [49], was dissolved in ethanol to achieve a final concentration of 20 %. Subsequently, the solution underwent sonication for 30 minutes. THC was then emulsified in 2 % Tween 80 and dissolved in sterile physiological saline. THC (2 mg/kg) or the vehicle, consisting of 2 % Tween 80 and saline was prepared in a volume of 1 mL/kg. Starting from gestational day (GD) 5 and continuing until GD20, the rats received daily subcutaneous injections of either a vehicle or THC at a dosage of 2 mg/kg. The THC concentration used in this study, as well as in previous research, corresponds to a level of mild THC consumption observed in humans [12].

2.3. Barnes maze test

To evaluate spatial memory, adolescent male and female rat offspring prenatally exposed to either the vehicle (CTRL) or THC (pTHC), were tested in the Barnes maze test, a mild aversive, dry-land behavioural task [50] from postnatal day (PND) 35–46, during the light phase of the light/dark cycle.

2.3.1. Apparatus

The maze apparatus was a circular, grey Plexiglas platform with a diameter of 122 cm and a height of 90 cm. Positioned around the perimeter were twenty holes with a diameter of 10 cm, with only one hole serving as the target hole leading to an under-platform chamber measuring 12 cm × 12 cm × 35 cm – the escape box. The remaining holes were covered with flat boxes and appeared indistinguishable from one another. During the task, the rat was put in the middle of the platform, and had to locate the target hole, whose position changed in the

reversal phases of the task. The task included an aversive element in the form of bright lighting – two 500 W light spots placed 1.5 m above the platform that, coupled with the open elevated spaces, served as motivational factors for the escape behaviour. To aid spatial orientation, visual cues in the form of large colorful geometric figures were placed on the walls of the laboratory room. Objects and apparatus were meticulously cleaned with 70 % isopropanol, dried with tissue paper, and rinsed with water after each rat's experimental session. The behaviour was recorded and scored by an automatic video-tracking system (Any-Maze, Stoelting Europe, Dublin, Ireland), controlled by an experimenter unaware of the experimental groups.

2.3.2. Experimental design

The Barnes maze test comprised habituation, acquisition, probe, and reversal phases [50]. The experimental design was structured as follows.

Habituation – A day before the acquisition phase, the rats underwent a habituation session to familiarize themselves with the platform and escape box. The animals were placed in the middle of the platform and allowed to explore the apparatus for 180 s.

Acquisition phase – After the habituation period, the same rats entered the acquisition phase, which spanned three consecutive days with one training session per day. Each session comprised three 180-s trials, and the location of the escape box remained constant throughout all acquisition trials. Rats were placed in the middle of the maze, covered with an opaque bucket, and, after a brief delay, the bucket was lifted to vary the initial orientation randomly from trial to trial. The trial concluded either after 180 s or when the rat entered the escape box. The hole was covered for 30 s upon entry, and the light was turned off. If a rat failed to enter the escape box within 180 s, it was gently guided there by the experimenter.

Probe task – Twenty-four h after the last day of acquisition, the target hole was closed, and memory retention of the escape box location was assessed for 90 s during the probe task. Rat's behaviour was assessed for primary latency – i.e., the time spent to make initial contact with the target hole and pre-location errors – namely, the number of incorrect holes checked before reaching the target hole. In addition, total distance travelled and mean speed were measured as indexes of exploratory behaviour.

Reversal task – Conducted twenty-four h after the probe task, the reversal task involved rotating the escape box's position 180° from the original. Three 180-s trials were conducted in a single day, and the following parameters were measured (modified from [51]): latency to escape – the time taken to enter the escape box; and location errors – the number of incorrect holes visited. The maze was ideally divided into four zones: the target zone was the one where the escape box was re-located, and the opposite zone was the one where the escape box was formerly located. The preference for the target- or the opposite zone, in percentage, was calculated as the time spent in the target or opposite zone divided by the time spent in the other zones, multiplied *100. Total distance travelled and mean speed were measured as indexes of exploratory behaviour.

2.4. Tissue collection

Within 1 h following the completion of the behavioural assessments, adolescent rats were sacrificed for tissue collection. Their brains were swiftly extracted on ice and processed for the subsequent analysis. The first subset was immediately immersed in cold 4 % paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) for 24 h fixation at 4 °C. Afterwards, the brains were stored in 0.02 % sodium azide in PBS at 4 °C until the sectioning for immunofluorescence. The other brain subset was immediately dissected for collecting the hippocampus, which was flash frozen in liquid nitrogen, and stored at −80 °C, until subsequent gene expression analysis. Brain samples were counter-balanced among experiments.

2.5. CCK+BCs immunofluorescence evaluation

CCK+BCs are a major subtype of hippocampal inhibitory interneurons [52–54]. As the name implies, the defining molecular characteristic of CCK+BCs is the expression of the octapeptide form of the CCK neuropeptide (CCK-8) throughout the somatodendritic and axonal compartments of CCK+BCs [55–58]. However, not all CCK immunoreactive neurons are CCK+BCs, as CCK can also be detected in glutamatergic principal cell subsets [59,60] and additional subpopulations of dendrite targeting interneurons [55,61].

In this study, we performed immunofluorescence to study CCK+ cells in the hippocampus (Fig. 1a). Specifically, we counted immunofluorescence-positive cells outside the stratum pyramidale (Fig. 1b arrows), since they can be safely considered CCK+BCs. Positive cells inside stratum pyramidale (Fig. 1b arrowheads) were not included in the analysis as may be either basket or pyramidal cells, needing additional interneuronal markers to be fully identified [15].

The procedure was performed as previously described, with minor modifications [62,63]. Briefly, brains were coronally sectioned at a thickness of 30 μm using a microtome (Campden Instruments, Loughborough, UK). Serial sections were collected through the rostral-caudal dimension (every sixth slice) and stored at 4°C in 0.02 % sodium azide in PBS until the immunofluorescence staining.

Sections including the hippocampus (five per rat) [15,64] were collected, washed in PBS for 30 minutes, and incubated in blocking solution (3 % Normal Donkey Serum (NDS), 0.3 % Triton X-100 in PBS) for two hours at 37°C under gentle shaking. Afterwards, sections were incubated in primary antibody solution (3 % NDS, 0.3 % Tween-20 in PBS) with rabbit polyclonal antibody against CCK8 (1:300, orb156288, Biorbyt, USA) for 72 hours under gentle shaking. Subsequently, sections were washed in PBS solution for one hour and incubated in secondary antibody solution (Cy2 AffiniPure Donkey Anti-Rabbit IgG; 1:400; Jackson ImmunoResearch, West Grove, PA) for two hours under gentle shaking. After washing for one hour, slices were briefly incubated with DAPI (1 μg/mL). Sections were mounted onto Superfrost Plus slides (Thermo Fisher Scientific, Waltham, MA) and coverslipped using Vectashield HardSetTM Antifade mounting medium. Images of CA1, CA2, CA3 and DG subregions were acquired at 20x using an epifluorescence microscope (Meji Techno, Saitama, Japan) and Deltapix Insight software (Denmark). CCK+ cells outside stratum pyramidale were considered CCK+BCs and quantified by employing Image J by a trained experimenter blind to the treatment. Data were expressed as the percentage of the respective control group.

2.6. Gene expression analysis

RNA extraction was carried out by homogenizing the tissue in Trizol (Invitrogen), followed by chloroform layer separation and isopropanol precipitation. Additionally, 70 % ethanol washes were performed to eliminate any residual salts from the isopropanol RNA precipitation step [65]. The isolated RNA was resuspended in water and quantified using a NanoDrop (ND-1000 Spectrophotometer, Thermo Scientific, Wilmington, DE, USA). Subsequently, the RNA was reverse-transcribed to cDNA using SuperScript IV Reverse Transcriptase from Invitrogen. The resulting cDNA was diluted, mixed with PowerUp SYBR Green Master Mix (Applied Biosystems), and combined with primers. The samples underwent a heating cycle to 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds, 60 °C for 1 minute, 95 °C for 15 seconds, 60 °C for 30 seconds, and 95 °C for 15 seconds. Gene expression analysis was conducted utilizing the delta–delta C(t) method, and the specific primers used are listed in Table 1.

2.7. Statistical analysis

When the data demonstrated normal distribution and equal variance, the two-tailed Student's t-test was employed to analyse the measures of

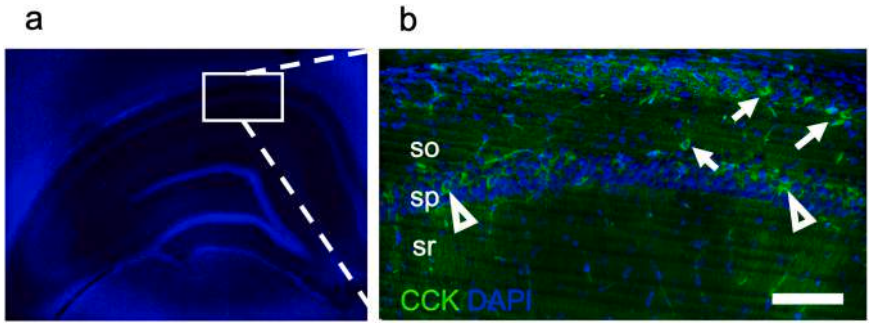

Fig. 1. Representative photomicrographs of immunofluorescent staining for the evaluation of CCK+BCs in the hippocampus. (a) Sections including the hippocampus were stained for CCK; images of CA1, CA2, CA3, and DG hippocampal subregions were acquired at 20x. (b) The analysis included CCK+BCs out of the stratum pyramidale (arrows). Positive cells inside the stratum pyramidale (arrowheads) were excluded from the analysis.20× magnification, scale bar = 100 μm. sp: stratum pyramidale; so: stratum oriens; sr stratum radiatum.

Table 1
Primers employed in qRT-PCR experiments.

Gene Name	Primer Sequence	Product
Gapdh	GTTTGTGATGGGTGTGAACC (Forward) CTTCTGAGTGGCAGTGATG (Reverse)	
NMDAR NR1 subunit-Grin1		Rn_Grin1_1_SG QuantiTect Primer Assay (QT00182287)
NMDAR NR2 subunit-Grin2A		Rn_Grin2a_1_SG QuantiTect Primer Assay (QT00379281)
PSD−95-Dlg4		Rn_Dlg4_1_SG QuantiTect Primer Assay (QT00183414)
mGluR5-Grm5		Rn_Grm5_1_SG QuantiTect Primer Assay (QT01081549)
Homer1-HOM1	CTTCACAGGAATCAGCAGGAG (Forward) GTCCCATTGATACTTTCTGGTG (Reverse)	
CB1R-Cnr1		Rn_Cnr1_1_SG QuantiTect Primer Assay (QT00191737)
Histine triad nucleotide-binding protein 1 (HINT1)		Rn_Hint1_1_SG QuantiTect Primer Assay (QT01602713)
Nlgn−1-NLGN1		Rn_Nlgn1_2_SG QuantiTect Primer Assay (QT01567097)
Nlgn−2-NLGN2		Rn_Nlgn2_1_SG QuantiTect Primer Assay (QT00191100)
Nlgn−3-NLGN3		Rn_Nlgn3_1_SG QuantiTect Primer Assay (QT00178304)

pregnancy outcomes (i.e. maternal weight gain, litter size, and male-to-female ratio of the offspring), behavioural parameters in the probe - (i.e. primary latency; pre-location errors; total distance travelled; mean speed) and the reversal- (i.e. escape latency; location errors; zone preference; total distance travelled; mean speed) tasks of the Barnes Maze test; CCK+BCs density in the immunofluorescence experiments; gene expression results; the Mann-Whitney test was employed in the other cases. The Grubbs's test was used to detect outliers, which were removed from the analysis. Data are presented as mean ± SEM and individual values. Statistical analyses were carried out using the software GraphPad Prism (Version 10.2.0; GraphPad Software Inc.; San Diego CA, USA), and statistical significance was set at alpha = 0.05. A trend was indicated when $p<0.06$.

3. Results

3.1. Maternal weight gain, litter size, and male-to-female ratio of the offspring were not affected by prenatal THC exposure

Pregnant rat dams received either daily doses of vehicle or THC (2 mg/kg s.c.) from GD5 through GD20. Pregnancy weight gain, litter size, and male-to-female ratio were measured to evaluate maternal and neonatal outcomes. Daily administration of THC to pregnant dams did not affect maternal weight gain during pregnancy ($t=0.04773$, $df=10$, $p=0.9629$). In addition, prenatal THC exposure did not alter gestational litter size ($t=0.8796$, $df=10$, $p=0.3997$) or male-to-female ratio ($t=0.4881$, $df=10$, $p=0.6360$). The descriptive statistics is shown in Table 2.

3.2. Prenatal THC exposure affects spatial memory and cognitive flexibility in adolescent male offspring

Rats underwent the probe and reversal sessions in the Barnes maze to test spatial memory retrieval and cognitive flexibility, after the previous acquisition of the escape box location (Fig. 2a).

Spatial memory retrieval was assessed in the absence of the escape box in the probe task; the analysis of the primary latency showed that adolescent male pTHC rats displayed a significant increase in the time spent to locate the target hole, compared to CTRL counterparts (Mann Whitney test, $U=50$, $p=0.0151$) (Fig. 2b). In addition, male pTHC rats showed an increase in the number of pre-location errors, i.e. errors before locating the target hole (Mann Whitney test, $U=54$, $p=0.0217$) (Fig. 2c). No difference was observed in total distance travelled (Student's t test, $t=0.2827$, $df=28$, $p=0.7795$) and mean speed ($t=0.2192$, $df=28$, $p=0.8281$).

The flexibility of memory was evaluated in the reversal task, when the location of the escape box was moved 180° from its previous position (Fig. 2a). Overall, male rats were able to locate and enter the escape box in its novel location. However, male pTHC offspring displayed a significant increase in escape latency (Mann-Whitney test, $U=51$, $p=0.0172$) (Fig. 2d) and a higher number of location errors (Mann-Whitney test, $U=60.50$, $p=0.0302$) (Fig. 2e) indicating a decreased flexibility of memory compared with CTRL counterpart. In addition, male pTHC rats displayed a lower preference for the target zone

Table 2
Maternal and neonatal outcomes.

group	Parameter	N	mean	SEM	p
CTRL dams	Pregnancy weight gain (g)	8	76.95	9.44	
THC-exposed dams		8	77.62	10.34	n.s.
CTRL dams	Pups per litter (n)	8	6.2	1.35	
THC-exposed dams		8	7.6	0.84	n.s.
CTRL dams	male-to-female ratio	8	1.28	0.34	
THC-exposed dams		8	1.52	0.34	n.s.

n.s. = non-significant

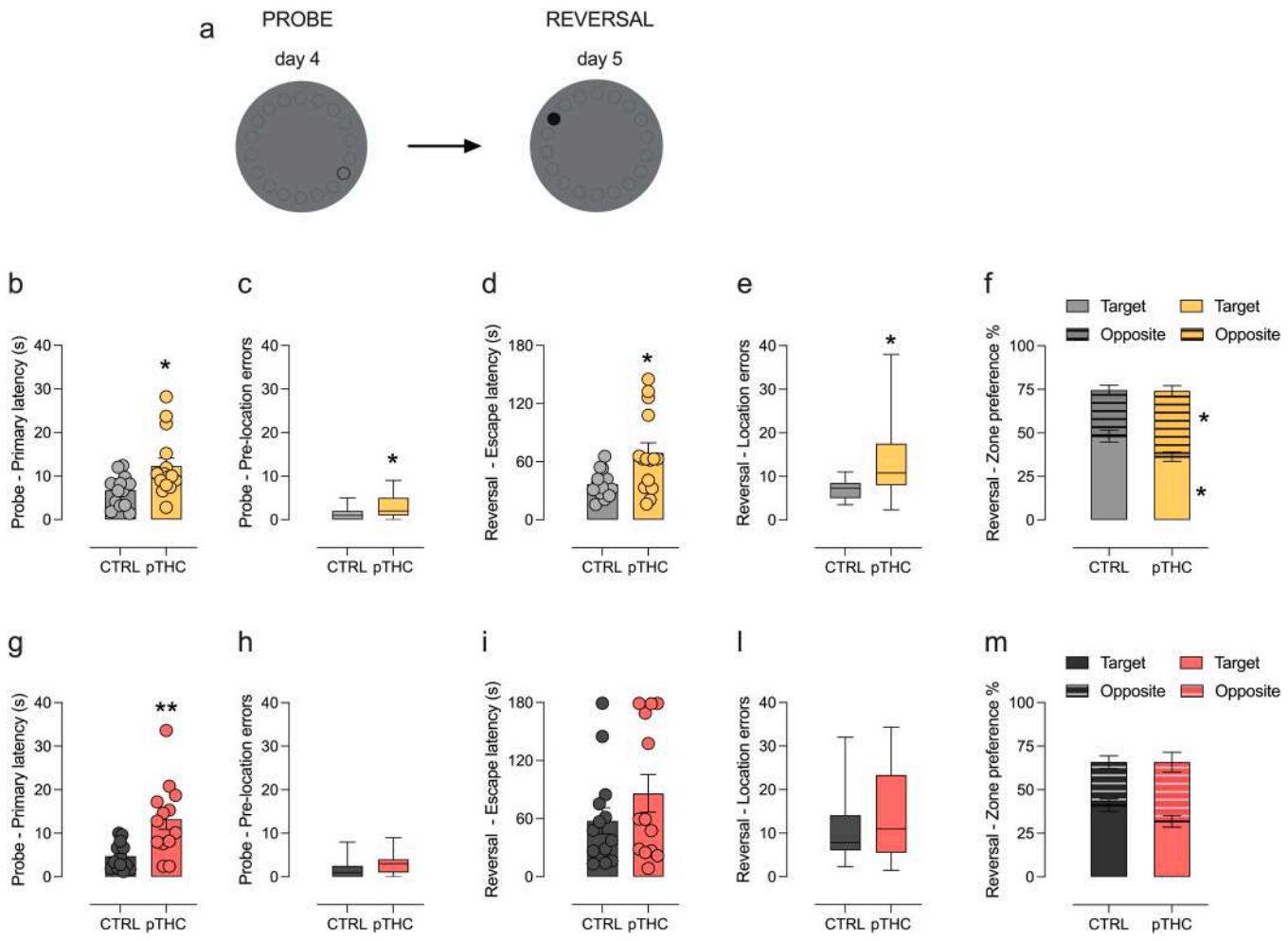

Fig. 2. Prenatal THC exposure induces sex-specific alteration of spatial memory retrieval and flexibility of memory in adolescent offspring. (a) Adolescent rat offspring underwent probe and reversal tasks in the Barnes maze test to assess the effects of prenatal THC exposure on spatial memory retrieval and flexibility of memory. In utero THC-exposed male offspring displayed (b) increased time spent to locate the target hole and (c) increased pre-location errors when compared with CTRL rats in the probe task; (d) increased escape latency, (e) a higher number of location errors, and (f) a higher perseverance in searching the target hole in its previous position than CTRL rats, in the reversal task. Prenatal THC-exposed female offspring showed: (g) increased time spent to locate the target hole and (h) no difference in pre-location errors when compared to CTRL counterparts in the probe task; no difference in (i) escape latency, (l) number of location errors, and (m) zone preference than CTRL rats, in the reversal task. Each bar represents the mean ± SEM of 13–15 rats. *p<0.05; **p<0.01. CTRL=rat offspring prenatally exposed to Veh; pTHC=rat offspring prenatally exposed to THC.

(Student's t test, t=2.743, df=28, p=0.0105) and a higher preference for the opposite zone (t=2.732, df=28, p=0.0108) compared to CTRL counterparts, indicating a higher perseverance of the former memory of the escape box's previous position (Fig. 2f). No difference was observed in total distance travelled (Mann-Whitney test, U=108.5, p=8787) and mean speed (t=0.1688, df=28, p=0.8672).

3.3. Prenatal THC exposure affects spatial memory, but not cognitive flexibility in adolescent female offspring

After the acquisition of the escape box location during the acquisition phase, we assessed spatial memory retrieval in the probe task. Overall, pTHC-exposed female offspring displayed a significant increase in the primary latency in comparison with CTRL counterparts (Mann Whitney test, U=52, p=0.0945) (Fig. 2g); however, no significant

differences were observed in pre-location errors (Mann Whitney test, U=40, p=0.0356) (Fig. 2h). In addition, no difference was observed in total distance travelled (Student's t test, t=0.3593, df=24, p=0.9346) and mean speed (t=0.9269, df=24, p=0.3632).

When female offspring were evaluated for flexibility of memory, no significant group difference occurred either in escape latency (Student's t test, t=1.223, p=0.2327) (Fig. 2i) or in location errors (Mann Whitney test, U=80, p=0.6074) (Fig. 2l). Consistently, no group difference in zone preference was recorded (target zone: t=1.859, df=24, p=0.0753; opposite zone: t=1.386, df=25, p=0.1779) (Fig. 2m). In addition, no differences were observed in total distance travelled (t=0.1401, df=25, p=8897) and mean speed (t=0.5628, df=25, p=0.5786).

3.4. Prenatal THC exposure affects the pattern of mRNA expression of key effectors of synaptic plasticity and ECS signalling in the hippocampus of adolescent male offspring

We first examined the mRNA relative expression levels of key effectors of excitatory synaptic plasticity and downstream signalling of the ECS.

Changes in the ease of plasticity induction can be determined by the type of NMDAR subunit activated, with the mandatory NR1 subunit governing the magnitude of excitatory signalling in the hippocampus and NR2A supporting the efficacy of activity-dependent excitatory signalling during learning and memory formation [66]. In pTHC-exposed male offspring, we observed an increase in the relative expression levels of the obligatory NR1 subunit expression, (Mann-Whitney U=0, p=0.0003), and conversely decreased mRNA levels of hippocampal NR2A subunit (t=4.224, df=13, p=0.0012) and PSD-95, a scaffolding protein which provides structural stability in the synapse and is intimately involved with LTP induction and maintenance (t=3.993, df=13, p=0.0015). Additionally, our data indicated a pTHC effect on the expression of molecular effectors triggering ECS-mediated long-term control of synaptic strength. Specifically, the mRNA expression levels of mGluR5 were shown to be increased by prenatal THC exposure (Mann-Whitney U=0, p=0.0003), as well as of its scaffolding Homer1 (Mann-Whitney U=0, p=0.0003) in the hippocampus of adolescent male offspring. Additionally, results from the Mann-Whitney test revealed that pTHC-exposed male offspring displayed increased levels of CB1R mRNA (Mann-Whitney U=0, p=0.0003) in the hippocampus of adolescent male offspring. HINT1 bridges NR1 NMDARs subunit and CB1Rs, thus maintaining neuronal homeostasis by downgrading NMDAR excitatory signalling. Prenatal THC exposure increased the mRNA expression levels of HINT1 (Mann-Whitney U=3, p=0.0020) (Fig. 3).

3.5. Prenatal THC exposure reduces CCK+BCs interneuron population across hippocampal subregions and accordingly modulates Nlgns expression in adolescent male offspring

CCK+ cells outside the stratum pyramidale, safely considered as CCK+BCs, were quantified in the four hippocampal subregions of the adolescent rat offspring (Fig. 1a, b).

Mann-Whitney test on data from the male offspring revealed that pTHC rats displayed a decreased number of CCK+BCs in CA1 (U=0, p=0.004), CA2 (U=1, p=0.009), CA3 (U=1, p=0.009) and DG (U=0, p=0.004), and in the overall hippocampus (U=0, p=0.004) when compared with CTRL counterpart (Fig. 4a, b). Concurrently, the Student's t test detected a potentiating effect of pTHC exposure on the relative expression levels of the cell adhesion molecules Nlgn-1 in the hippocampus of adolescent male offspring (t=2.188, df=13, p=0.0475); conversely, prenatal THC exposure did not affect the mRNA levels of hippocampal Nlgn-2 (t=1.031, df=13, p=0.3213); lastly, a decrease in Nlgn-3 was found in the hippocampus of adolescent male offspring (Mann-Whitney U = 9, p = 0.0263) (Fig. 5).

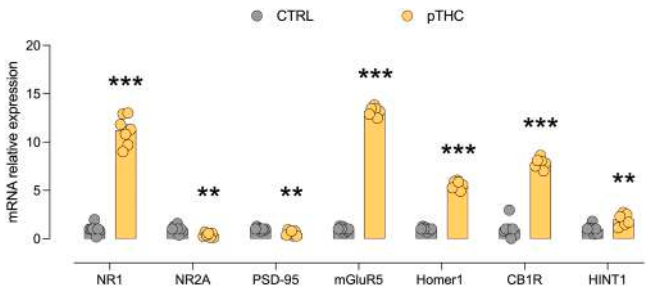

Fig. 3. Exposure to THC during prenatal development at a dosage of 2 mg/kg results in changes in the mRNA relative expression levels of markers and modulators of hippocampal synaptic plasticity in the hippocampus of male adolescent rats. Hippocampal expression levels of the NMDAR NR1 subunit are increased and those of NR2A and PSD-95 are decreased by prenatal THC exposure. Additionally, it induces an increase in mGluR5 expression, along with its scaffolding partner Homer1 isoform, and increases CB1R and HINT1 expression levels in the hippocampus of male adolescent rat offspring. Each bar represents the mean of n=8 rats for the CTRL group and n=7 for the pTHC group; error bars indicate SEM. **p<0.01; ***p<0.001. CTRL=rat offspring prenatally exposed to Veh; pTHC=rat offspring prenatally exposed to THC; NR1=NMDARs NR1 subunit; NR2A=NMDARs NR2A subunit; PSD-95=scaffolding protein post-synaptic density-95; mGluR5=group I metabotropic glutamate receptor 5; CB1R=cannabinoid receptor type 1; HINT1=histidine triad nucleotide-binding protein 1.

3.6. Prenatal THC exposure does not significantly modify the effectors of neuroplasticity but NR2A subunit in the hippocampus of adolescent female offspring

The expression levels of the NMDAR NR1 subunit in the female adolescent offspring were not modified by prenatal THC exposure (Mann-Whitney U=18, p=0.2723). Conversely, results from the Mann-Whitney test revealed a significantly decreasing effect of prenatal THC exposure on the relative expression levels of the NR2A subunit in the hippocampus of adolescent female offspring (Mann-Whitney U=5, p=0.0051), while no effect was shown on PSD-95 expression levels (Mann-Whitney U=24, p=0.6894). When assessing the hippocampal expression levels of molecular effectors responsible for the ECS's long-term regulation of synaptic strength, the analysis found a decreasing trend on mGluR5 (Mann-Whitney U=11.50, p=0.0541) and Homer1 (Mann-Whitney U=11.50, p=0.0531) in pTHC exposed female offspring. On the other hand, no impact of pTHC was detected on CB1R- (Mann-Whitney U=24, p=0.6894) or HINT1 (Mann-Whitney U=17, p=0.2233) level expression in the hippocampus of the adolescent female offspring (Fig. 6).

3.7. Prenatal THC exposure increases CCK+BCs in CA1 and Nlgn-3 expression in the hippocampus of adolescent female offspring

The number of CCK+BCs, which are critical for adjusting the strength and timing of excitatory signals were found to be increased in CA1 (U= 2, p=0.017) of adolescent female pTHC rats in comparison with controls; no significant differences were observed in CA2 (U=5, p=0.082), CA3 (U=8.5, p=0.268) and DG (U=11, p=0.537). When data from the overall hippocampus were considered, a significant increase was observed in pTHC rats in comparison with control counterparts (U=3, p=0.030) (Fig. 7a, b).

Additionally, the Student's t-test revealed that prenatal THC exposure did not affect the expression of Nlgn-1, which modulates excitatory synaptic strength and connectivity (t=0.1182, df=13, p=0.9077), or Nlgn-2 of inhibitory synapses (t=0.3498, df=13, p=0.7321). On the contrary, an increasing effect of pTHC was found in Nlgn-3 mRNA expression levels (t = 2.161, df=13, p=0.049) (Fig. 8).

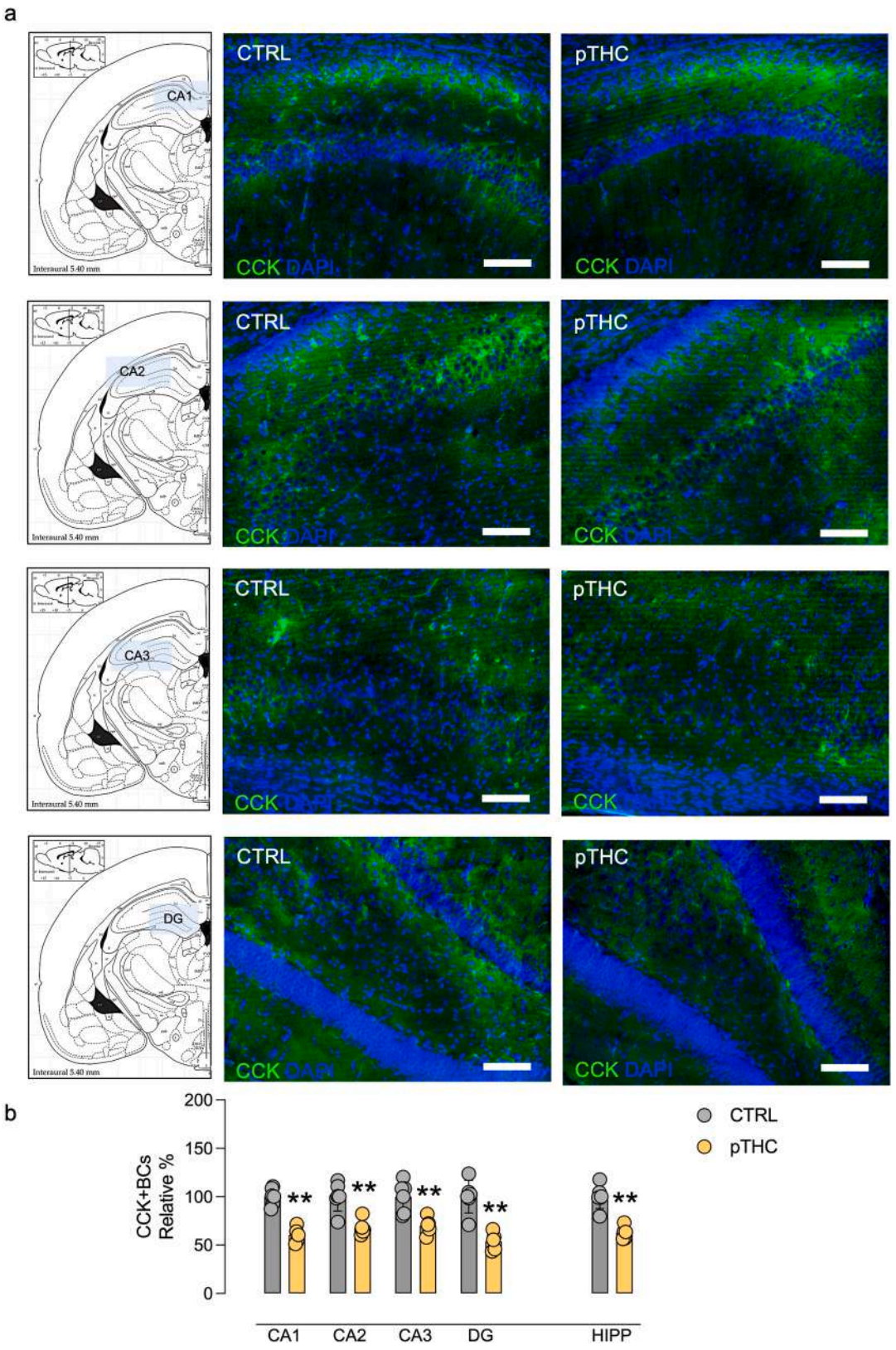

Fig. 4. Prenatal THC exposure decreased CCK+BCs in the hippocampus of adolescent male rat offspring. When we evaluated (a) CCK+BCs in the four hippocampal subregions of CTRL and pTHC-exposed male rat offspring, (b) the quantification analysis showed that adolescent rats exposed to pTHC displayed a significant decrease in CCK+BCs in the CA1, CA2, CA3, DG and overall hippocampus with respect to control rats. Each bar represents the mean of n=6–5 rats; error bars indicate SEM. **p<0.01. CTRL=rat offspring prenatally exposed to Veh; pTHC=rat offspring prenatally exposed to THC. CCK: cholecystokinin; DAPI: nuclear staining with 4′,6-diamidino-2- phenylindole. Images were acquired at 20× magnification, scale bar = 100 μm.

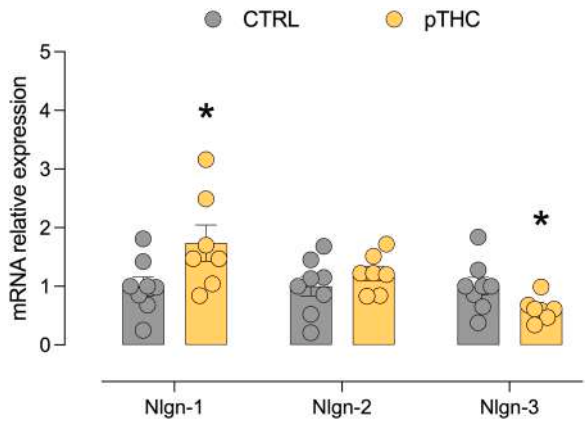

Fig. 5. Effects of prenatal THC exposure on the expression levels of neuroligins in the hippocampus of adolescent male rats. An increase in Nlgn-1 and a decrease in Nlgn-3 were detected in pTHC male adolescent rats. No significant effect was observed for Nlgn-2. Each bar represents the mean of n=8 rats for the CTRL group and n=7 for the pTHC group; error bars indicate SEM. *p<0.05. CTRL=rat offspring prenatally exposed to Veh; pTHC=rat offspring prenatally exposed to THC; Nlgn = neuroligin.

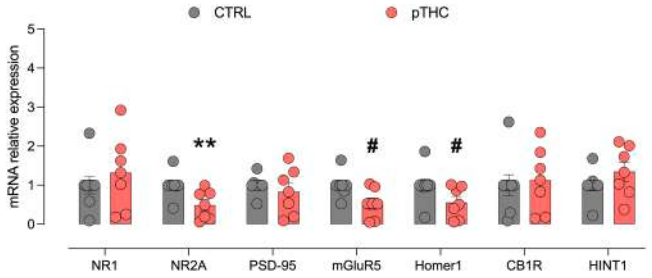

Fig. 6. Exposure to THC during prenatal development at a dose of 2 mg/kg leads to specific alteration in the mRNA relative expression level of effectors of excitatory and inhibitory signalling pathways in the hippocampus of adolescent female rats. Prenatal THC exposure does not induce alteration in NMDAR NR1 subunit expression, while a significant decrease is shown in the NMDAR NR2A subunit expression in the hippocampus. PSD-95 expression levels remain unchanged. pTHC prompts a decreasing trend in hippocampal mRNA expression levels of mGluR5 and Homer1, while no significant changes are observed in the CB1R- or HINT1 expression levels. Each bar represents the mean of n=8 rats for the control (CTRL) group and n=7 rats for the prenatal THC (pTHC) group; error bars indicate SEM. **p<0.01; #p<0.06. Abbreviations: CTRL, rat offspring prenatally exposed to Veh; pTHC, rat offspring prenatally exposed to THC; NR1, NMDAR NR1 subunit; NR2A, NMDAR NR2A subunit; PSD-95, scaffolding protein post-synaptic density-95; mGluR5, group I metabotropic glutamate receptor 5; CB1R, cannabinoid receptor type 1; HINT1, histidine triad nucleotide-binding protein 1.

4. Discussion

The early repeated interference by THC and other exogenous cannabinoids with the normative neurodevelopmental trajectories is currently emerging as a health issue due to recent evidence of behavioural abnormalities in the exposed offspring. Here we show that THC exposure during pregnancy affects the adolescent offspring in a sex-specific manner. Specifically, we show: a deficit in spatial memory retrieval efficacy in both sexes; an impairment in memory flexibility in the male offspring; opposite sex-dependent modifications in CCK+BCs in the hippocampus; a sex-specific disarrangement in Nlgns expression, and in the effectors of excitatory synaptic plasticity.

In their theory, O'Keefe and Nadel [67] designated the hippocampus as the hub where spatial information is built, stored, and flexibly used for navigation. When the animals find themselves in the Barnes maze [51] their behavioural output is: searching for and finding the hidden escape box; remembering its location during the probe task (memory retrieval); navigating to the new escape box location when it has been moved from the previous one, in the reversal learning phase (flexibility of memory).

Here we show that in the probe test, pTHC-exposed adolescent rats of both sexes displayed a longer latency to reach the target hole, suggesting an impaired memory retrieval, compared to controls. Interestingly, we recorded a higher number of incorrect hole checks just in the male progeny before locating the target hole, suggesting a poor memory storage of the target location. On the other hand, in the female offspring, we did not observe increased errors before reaching the target hole, suggesting the occurrence of a delayed retrieval of the memory traces [68]. A sex-related dimorphism has been previously reported in different behavioural outcomes following prenatal exposure to THC and cannabinoid agonists, which also depended on the time of exposure, doses, and rodent model [69–71]. The present findings are consistent with the impaired spatial memory in the probe trial of the Morris water maze detected in females exposed to a higher dose of prenatal THC (100 mg/mL) via a vapor inhalation system [72]. On the other hand, our findings differ from prior data indicating that pTHC exposure – 3 mg/kg, from embryonic day 10.5–17.5 – impairs hippocampal-dependent spatial memory in a non-aversive spatial memory task only in male mice [15]. It is worth noting that our study employed a longer prenatal exposure regimen, in rats, and assessed spatial memory in a mild-aversive context, where stress reactivity may play a role [50]. Overall, in our experimental conditions, the impact of pTHC exposure on memory retrieval shows a partial overlapping in the two sexes, although the errors during the search strategy suggest a greater vulnerability in the male offspring.

When the animals had to locate the new position of the escape box in the reversal task of the Barnes maze [50], sex-specific variations in the cognitive response occurred. In particular, pTHC-exposed male offspring displayed a longer escape latency and more errors throughout the task than the respective controls. During reversal learning, memories of the previously learned task are updated as new memories are acquired [73], making reversal learning a measure of flexibility of memory and adaptability to a changing environment. Therefore, the greater number of errors and the longer time spent searching the target hole in its previous position in pTHC male offspring suggest higher perseverance and decreased flexibility of memory [51]. In most tests of allocentric navigation in rodents, male animals show an advantage and a homogeneous performance compared with female animals [135]. Indeed, when assessed for flexibility of memory, female rat progeny displayed a high degree of variability. Nevertheless, pTHC-exposed female adolescent rats were able to search the escape box's new position as efficiently as the controls, indicating that pTHC exposure did not jeopardize the flexibility of memory in the female offspring.

A selective function-related resilience to low doses of prenatal THC (0.15 mg/kg) has been already reported in the female progeny whereas male rats displayed weakened reverse learning in an avoidance task during the juvenile epoch [74]. On the other hand, our results differ from Weimar et al., 2020 [75] who showed that female offspring prenatally exposed to a higher dose of THC (vapor THC 99.2 mg/mL) suffer from deficits in set-shifting in adulthood, suggesting that female resilience to pTHC exposure may represent a dose- and age-dependent phenomenon. However, the analysis of reports on pTHC effects shows that variations in strains, doses, routes of administration, and experimental setting can reasonably justify most of the discrepancies in the findings and suggests comparing the data according to the consistency of the experimental conditions.

Learning and memory processing in the hippocampus is supported by synaptic plasticity, i.e. the activity-dependent modification of the strength or efficacy of synaptic transmission at preexisting synapses. The eCBs play a role in transiently suppressing inhibitory and excitatory synapses in several brain regions, thus modulating synaptic plasticity.

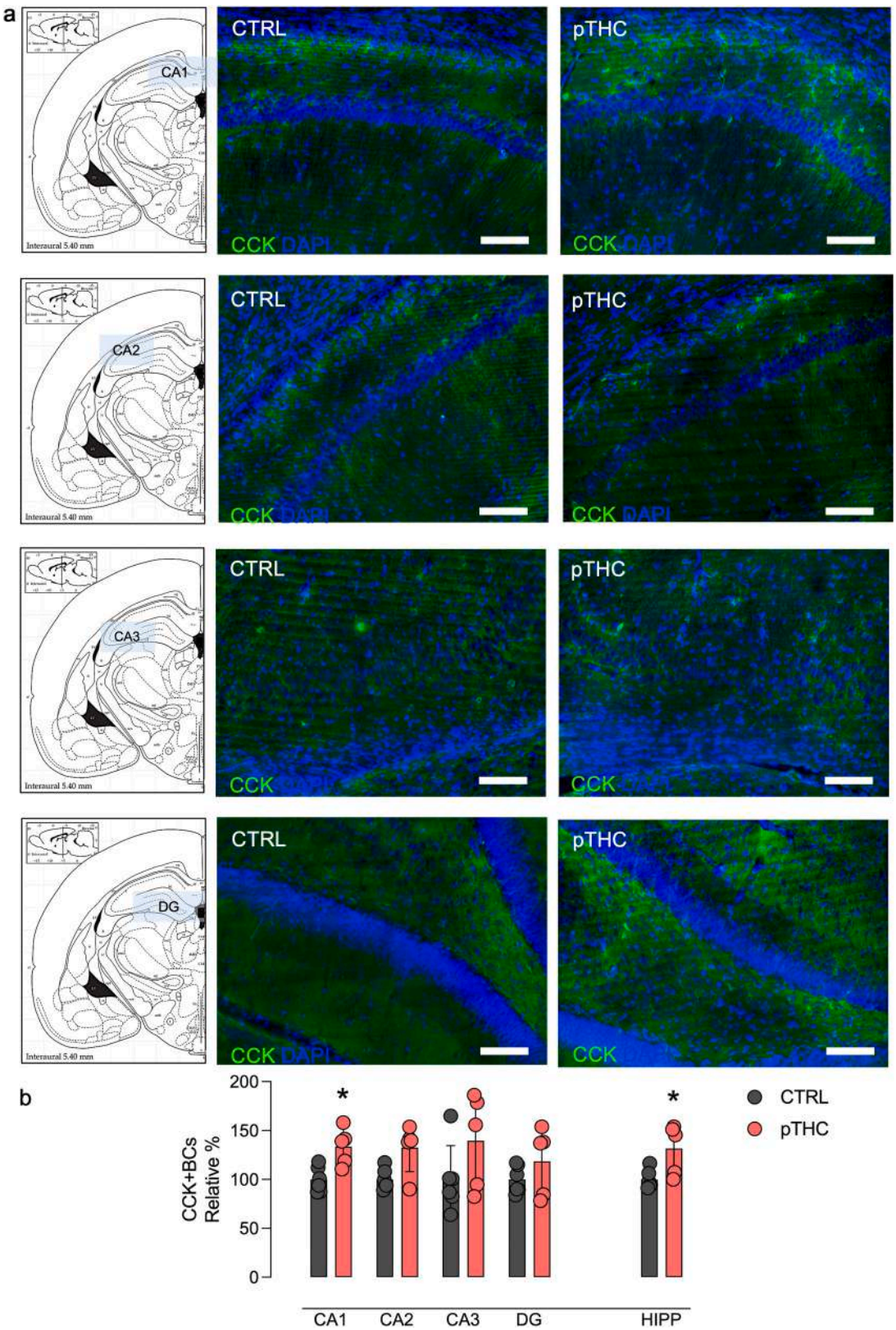

Fig. 7. Prenatal THC exposure increases CCK+BCs in the hippocampus of adolescent female rat offspring. When we evaluated (a) CCK+BCs in the four hippocampal subregions of CTRL and pTHC-exposed female rat offspring, (b) the quantification analysis showed that adolescent rats exposed to pTHC displayed increased CCK+BCs in CA1, and in the overall hippocampus, with respect to control counterparts. Each bar represents the mean of n=5–6 rats; error bars indicate SEM. *p<0.05; CTRL=rat offspring prenatally exposed to Veh; pTHC=rat offspring prenatally exposed to THC. CCK: cholecystokinin; DAPI: nuclear staining with 4′,6-diamidino-2- phenylindole. Images were acquired at 20× magnification, scale bar = 100 μm.

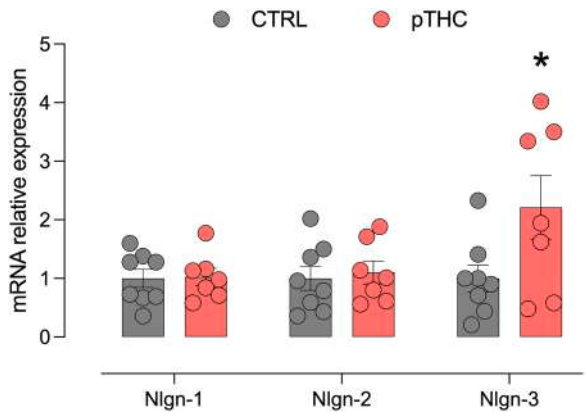

Fig. 8. Effects of prenatal THC exposure on neuroligin expression levels in the hippocampus of adolescent female rats. Prenatal THC exposure did not significantly affect mRNA expression levels of Nlgn-1 or Nlgn-2. However, an increasing effect was observed in Nlgn-3 expression levels. Each bar represents the mean of n=8 rats for the control (CTRL) group and n=7 rats for the prenatal THC (pTHC) group; error bars indicate SEM. Abbreviations: CTRL, rat offspring prenatally exposed to Veh; pTHC, rat offspring prenatally exposed to THC; Nlgn, neuroligin.

According to reports on early disturbances in ECS signalling resulting in impaired synaptic plasticity [76–78], here we show a complex rearrangement in the relevant players of the excitatory synapse that altogether reflects a sexual dimorphism in the effects of pTHC exposure in the adolescent offspring. In the male progeny, specifically, we observed an increase in the mRNA expression of NR1 subunit of the NMDAR, whereas NR2A and PSD-95 mRNA were significantly reduced. Although we did not measure the protein levels of NMDAR subunits, our findings suggest the occurrence of an abnormal NMDAR/PSD-95 combination following pTHC exposure. Moreover, an elevation in the mRNA levels of CB1R and HINT1 was detected: this finding is of particular interest, being HINT1 a scaffold protein that may physically link CB1R to the NR1 to remove NR1 excess from the membrane and prevent excessive activation of NMDARs [79]. Interestingly, we also report an increased expression of mGluR5 and Homer1 in the hippocampus of pTHC-exposed male offspring. It is recognized that mGluR5 is crucial for the expression of hippocampal long-lasting modulation of synaptic strength, and for the formation of spatial representations and memories, both in humans and rodents [80–88]. In particular, by coupling to Homer1 proteins, mGluR5 stimulates eCBs-driven downstream signalling to elicit long-lasting depression of excitatory synaptic transmission. i.e., mGluR5-dependent long-term depression (LTD). Neural representation of memory updating requires a precise balance between synaptic depression and potentiation [89]. If the amplitude of the depression is too high, this might delay or prevent the acquisition of new memories [89,90]. Conversely, if LTD is impaired, this could prevent the updating of memories acquired previously, thus interfering with memory retrieval [91]. The increased gene expression of mGluR5 and Homer1 observed in our study could therefore suggest the occurrence of an abnormal dynamic of mGluR5-LTD that according to Wall and colleagues has been associated with impaired reversal learning [90,92]. Overall, both the overexpression of mGluR5/Homer1 complex, in the presence of an imbalanced NMDA/PSD combination, and higher levels of CB1Rs could be functionally associated with a disruption of synaptic efficiency resulting in the spatial memory deficits promoted by pTHC exposure in the male offspring [93–95].

The ECS is involved in the regulation of neural progenitor proliferation and specification as well as the migration and differentiation of CB1R-expressing CCK-containing GABAergic interneurons in the hippocampus [96–99]. The rationale of our investigation was that pTHC exposure can affect the precise sequelae of neuronal positioning and morphogenesis by occluding eCBs signalling at CB1Rs in the hippocampus during development [100–102]. And indeed, here we show that pTHC exposure is associated with a decrease in the number of CCK+BCs in all the 4 hippocampal subregions evaluated, in the male rat offspring. CB1R expressing CCK+BCs participate in sculpting synaptic plasticity by fine-tuning the strength and timing of the excitatory signalling, thus providing an inhibitory tone on PNs in synergy with other GABAergic sources [103,104]. On this basis, we propose that pTHC exposure, by a curbing effect on CCK+BCs inhibitory signalling in the rat adolescent male offspring, can lead to an imbalance in the hippocampal E/I ratio and contribute to the abnormal neuroplasticity and the deficit in memory retrieval and flexibility here observed [105].

Recent reports point to Nlgns as indispensable factors for the optimal operation of neural circuits and the precise establishment of connections between distinct populations of excitatory and inhibitory neurons [31,33,35,36,106,107]. Shifts in Nlgns expression in specific excitatory and inhibitory neuronal subpopulations in response to experience regulate the dynamic processes of memory consolidation and retrieval [108–110]. Notably, pTHC-exposed male offspring displayed increased expression in Nlgn-1 and decreased levels of Nlgn-3 in the hippocampus.

Nlgn-1 localizes predominantly at excitatory synapses and is considered a crucial player in the regulation of NMDAR-dependent transmission in mature neurons [111], and in synaptic plasticity [31,112]. The deletion of Nlgn-1 in adult neurons causes both a loss of NMDAR-dependent long-term potentiation (LTP) and a decrease in NMDAR-mediated synaptic responses. On the other hand, overexpression of Nlgn-1 leads to abnormal synaptic plasticity and learning deficits by promoting the NR1 subunit recruitment thus shifting the E/I balance in the hippocampus [113]. Indeed, the increase in the mRNA levels of Nlgn-1 observed in the pTHC-exposed male offspring viewed together with the molecular setup of NMDAR abnormal expression, indicates the occurrence of a postsynaptic restructuring suggestive of a strengthened excitatory signalling [114–117]. However, the binding of Nlgn-1 to PDZ domain-containing proteins such as PSD-95, whose gene expression is downregulated in our experimental conditions, is dispensable for both functional and structural synaptic strengthening whereas Nlgn-1 binding to presynaptic neurexins is critical and necessary [118]. Investigation on the neurexin pre-synaptic expression in pTHC-exposed hippocampi is currently undertaken.

Interestingly, Nlgn-3 is preferentially recruited to CB1R-expressing CCK+ inhibitory terminals where it regulates inhibitory signalling but not at other major inhibitory synapses expressing parvalbumin (Pv) or somatostatin (Sst) in the hippocampal areas [119,120]. Indeed CB1R+CCK+BCs display clear potentiation of inhibitory output onto CA1 pyramidal neurons when Nlgn-3 in CA1 pyramidal neurons was overexpressed [120]. Nlgn-3 overexpression was also shown to reduce paired-pulse depression[120–122] suggesting a facilitation of presynaptic GABA release. On the other hand, Nlgn-3 knockdown specifically reduced CCK+BCs inhibitory synaptic transmission in the hippocampal CA1 region while neither Pv+ nor Sst+ neurons were affected [120]. Overall, the reduction in Nlgn-3 gene expression, consistently with the decrease in CCK+BCs here reported, represents further evidence of pTHC-mediated interference in the normative integration of synaptic signal molecules with endocannabinoid components via CB1R during neurodevelopment [33,123].

Intriguingly, pTHC-exposed female adolescent counterparts exhibited a distinct set-up in the expression of cellular and molecular effectors of hippocampal E/I tone. We report a reduction in the expression levels of the NR2A subunit in the absence of substantial changes in the different markers of neuroplasticity. NR2A subunits provide a peculiar contribution to the retrieval of spatial memory, supporting NMDAR's role in spatial information processing [124–126]. Evidence reports that NR2A subunit KO in mice reduces LTP and induces impairment in the water maze task. Our findings, therefore, suggest a role in NMDAR signal depotentiation in the occurrence of the delayed memory retrieval of pTHC female offspring [125] in the Barnes maze.

On the other hand, despite a reduction trend in the mGluR5/Homer1

complex, neither the gene expression of CB1R nor HINT1 were modified, suggesting that, in the female offspring, pTHC exposure was not associated with other signs of aberrant synaptic plasticity. Whether the partial resilience of the markers of neuroplasticity to pTHC exposure may be causally associated with the "spared" normative reversal learning displayed by the pTHC-exposed female offspring is an intriguing open question. Interestingly, we added another piece to the puzzle of pTHC-induced consequences in the female offspring by demonstrating an augmentation in the population of CCK+BCs within the CA1 region and in the overall hippocampus.

CCK+BCs respond to a myriad of sex-specific genetic, hormonal, environmental, and neurotransmitter-related factors, including the eCBs themselves. Specifically, eCB signalling is particularly active at perisomatic CCK+ GABAergic synapses in females suggesting a stronger eCB-mediated inhibition of GABA release from CCK+BCs. In males, such signalling is less pronounced or absent, indicating a significant sex-related difference in how eCBs regulate CCK+BCs [127,128]. This implies that even moderate in utero THC exposure can trigger aberrant patterning of CB1R-expressing CCK+BCs in the postnatal hippocampus in a sex-related fashion, interfering with the delicate balance of eCBs signalling during embryogenesis [129].

In the female offspring, we did not observe changes in the excitatory synapse-related Nlgn-1, whereas we measured elevated levels of Nlgn-3 mRNA in the hippocampus. Therefore, the overexpression of Nlgn-3 could represent a modality to strengthen the CCK+BCs/PNs synapse. E/I imbalances accompany hippocampal cognitive processing in which strength and flexibility of representations play important roles [110]. Thus, based on our observation, it is reasonable to hypothesize that in pTHC-exposed female offspring, the elevated CCK+BCs population and Nlgn-3 expression promote a shift of the hippocampal E/I balance downwards.

Notably, the pattern of expression of neuronal and synaptic markers in the hippocampus of the progeny of both sexes may represent an interaction between the effects of pTHC exposure and task-dependent learning experience; however, the possibility that the neurobehavioural phenotypes may originate from pTHC-induced consequences on the developmental trajectory of other neural sources beyond the hippocampal network system observed herein, cannot be ruled out.

5. Conclusions

We show that pTHC exposure induces sex- and function-specific alterations in spatial memory associated with different patterns of neuronal and synaptic markers in the hippocampus. The male offspring display a high vulnerability to pTHC effects, with detrimental consequences on memory retrieval and memory flexibility associated with pronounced modifications in the excitatory synapse and in the neurons-interneurons connectivity that suggest an upward trend in the E/I balance. The female offspring shows a function-specific resilience to pTHC exposure that is associated with mild changes in the excitatory synapse and modifications of the neurons-interneurons connectivity which indicate a shift of the E/I balance downwards [34,105,130].

Many factors can contribute to sex-specific vulnerability to pTHC effects. Male and female embryos may experience the same external conditions, but their genetic predispositions, sex chromosomes, and variations in sex hormone levels during critical developmental windows (e.i. different pubertal onset) may lead to differential programming of the brain's response to these exposures. The impact of psychoactive substances could, therefore, manifest in the distinct behavioural and neurobiological outcomes observed in male and female offspring [131,132]. For instance, research by Farquhar and colleagues [133] explored the differential vulnerability of male and female fetuses to prenatal alcohol exposure, noting that hormonal fluctuations during early development can modulate the effects of such exposures, potentially accounting for sex-specific outcomes in offspring behaviour and neurodevelopment. This is particularly relevant in the context of the ECS; indeed, the ECS develops in a sex dimorphic manner, with varying expression and functioning of CB1R in male and female brain, which can determine a different susceptibility to exogenous cannabinoids [134].

The further investigation of the intricate role of the ECS, alongside the complex interplay between the excitatory and inhibitory signalling, the neurotrophic factors and hormones is mandatory in order to identify the the sex-specific mechanisms underlying the neurobehavioural alterations induced by gestational THC exposure.

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CRediT authorship contribution statement

Salvatore Feo: Resources, Methodology. **Cesare D'Amico:** Investigation, Formal analysis. **Giuseppe Tringali:** Formal analysis. **Gianluca Lavanco:** Writing – review & editing, Investigation, Funding acquisition. **Valentina Castelli:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Carla Cannizzaro:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization. **Anna Brancato:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Martin Kuchar:** Resources. **Claudio D'Addario:** Methodology. **Martina Di Bartolomeo:** Methodology.

Declaration of Competing Interest

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Data availability

Data will be made available on request.

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