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**IN-UTERO  $\Delta^9$ -TETRAHYDROCANNABINOL EXPOSURE  
IMPINGES ON BRAIN EXECUTIVE PROCESSES TOWARDS  
EMOTIONAL, COGNITIVE IMPAIRMENT, AND ALCOHOL  
VULNERABILITY IN THE RAT OFFSPRING:  
BEHAVIORAL AND NEUROCHEMICAL EVIDENCE**

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## Abstract

Cannabis use during pregnancy is on the rise on a global scene, mainly for its antiemetic and analgesic properties. As so, concern raises over the foster impingement of  $\Delta^9$ -tetrahydrocannabinol – THC, the main psychoactive ingredient – on neonatal outcomes, since it interacts with the endogenous cannabinoid system (ECS) widely detected in the central nervous system (CNS) from the earliest stage of embryonic development. The abnormal activation of the ECS plays de facto a role in cognitive and emotional dysregulation, and excessive alcohol intake observed in heavy cannabis users at the peculiar, vulnerable epoch of adolescence, by targeting the underpinning neural signaling in discrete brain regions, including the ventral tegmental area (VTA), the nucleus accumbens (NAc), the prefrontal cortex (PFC), and the hippocampus. On these bases, the working hypothesis is that prenatal THC exposure may lead to aberrant plasticity in the mesocorticolimbic pathway, therefore leading to defective processing of emotionally salient information, and making adolescents more prone to engage in health-risk behaviors such as alcohol use. As in the first part of the present research, *in-utero* THC exposure impaired the formation of memory traces when integration between environmental encoding and emotional processing was required and promoted the development of alcohol-addictive behaviors in the adolescent progeny. The occurrence of this phenotype is associated with a reduction in Neuropeptide Y positive- (NPY+) neurons and disarrangement in the post-synaptic density (PSD) make-up in the mesocorticolimbic regions. Furthermore, in the second part of this work, prenatal THC was associated with defective core executive functions, namely spatial learning and memory processing, and cognitive flexibility triggered by both reward- and aversive settings in the adolescent and adult progeny, along with alterations in the expression levels of components of hippocampal glutamatergic signaling and the scaffold PSD partners. Lastly, as recognized as the THC counterpart, cannabidiol (CBD) administration induced an improvement in reward-induced memory consolidation and retrieval, behavioral flexibility, as well as in neurochemical correlates of the adolescent offspring.

## PART 1

### Introduction

In the last decade, cannabis has been catapulted into the medical arena on a global scene, by an explosion of new evidence affirming the ancient use of cannabis for its antiemetic, analgesic, and anti-inflammatory properties, which is fueling the global trend and prompting the wave of cannabis programs worldwide (Weisman and Rodríguez, 2021). Notably, drug production and trafficking appear to have adapted rapidly to pandemic-related restrictions, with the adoption of new technologies to facilitate drug distribution. Thus, cannabis still is the most commonly used drug: the prevalence of use is about five times that of other substances (EMCDDA, 2021). The combination of easy access to cannabis and enhanced potency poses an important public health and clinical practice challenge. Since the functional effects of the ECS involve sensory pathways, appetite and food intake, affective responses, and mood states, cannabis is commonly perceived as an efficient, harmful self-medicating strategy for a vast array of medical conditions (Fitzcharles and Eisenberg, 2018; Klumpers and Thacker, 2018). This matter becomes even thornier when cannabis use concerns pregnant women with morning sickness and vomiting during pregnancy (Chang et al., 2019; Metz and Borgelt, 2018). The impact of *in-utero* cannabis exposure has been inconsistent and difficult to interpret so far, due to a lack of consistent measures, as well as multiple confounding variables. Although there is no universal agreement regarding the implications for pregnant women using cannabis, accumulating pieces of evidence suggest that cannabis exposure can have a detrimental impact on pregnancy and neonatal outcomes (Crume et al., 2018). Further investigations of molecular and behavioral features of *in-utero* THC exposure will address the need for clarifications on the neuro-teratogenic effects of THC.

### The dynamic expression of the endocannabinoidergic system during brain development

The endocannabinoid signaling pathway is expressed and functional from early developmental stages, when it starts regulating neural stem-cell proliferation, differentiation, migration and survival, neuronal connectivity, and synaptogenesis, until complete maturation of the CNS (Fernández-Ruiz et al., 2000; Fride et al., 2009; Grant et al., 2018). Since that, the ECS controls many of the critical aspects of mammal physiological, behavioral, immunological, and metabolic functions, such as learning and memory, emotional processing, temperature and control, inflammatory and immune responses, and eating (Pacher et al., 2006). The ubiquitous ECS and its complex role are fulfilled by ECS components located in both excitatory and inhibitory synapses – mainly glutamatergic and

GABAergic interneurons – of all the classical neurotransmissions throughout the central and peripheral nervous systems: endocannabinoids (eCBs) and their synthesizing and degrading enzymes; specific transmembrane eCBs transporters, and cannabinoid receptors (CBR; Joshi and Onaivi, 2019). A major function of the ECS in the CNS is the inhibitory, retrograde messenger-mediated synaptic plasticity: eCBs are produced from postsynaptic terminals upon neuronal activation, readily cross the membrane, and travel in a retrograde fashion to activate cannabinoid receptor type 1 (CB1R) located in the presynaptic terminals. The “made-on-demand” feature of eCBs defines the ECS signaling in a temporally and spatially strict fashion (Alger and Kim, 2011). This contrasts strongly with the administration of exogenous cannabinoid ligands, such as THC, which engage receptors indiscriminately and sustainably.

The developmental pattern of CBR – mRNA expression, binding, and activation of signal transduction mechanisms in the fetal rat brain – has been extensively characterized in the developing brain of rats. CB1R binding and mRNA expression are measurable at gestational day (GD) 14, but only located in discrete regions at GD 16. Among these, are the hippocampus, the cerebellum, and the caudate-putamen area, namely the classic areas that contain these receptors in adulthood. The signal for CBR mRNA in these areas progressively increased from GD 16 up to GD 21, until reaching the classical adult values in 70-day-old animals (Berrendero et al., 1998, 1999; Romero et al., 1997; Belue et al., 1995).

Ontogenetic development of cannabinoid receptor expression and signal transduction functionality in the human brain has been analyzed, as well. A significant density of CB1R at 19-week-gestation in the same areas that contain these receptors in the adult human brain has been detected. In parallel, brain CB1R is functionally coupled to signal transduction mechanisms from early prenatal stages. Further, an intense expression of CB1R mRNA has been found in human 20-week fetal brain, preferentially in the limbic regions, specifically in the hippocampal CA2 and CA3 regions and basal amygdala (Wang et al., 2003; Mato et al., 2003). The high CBR mRNA expression in the fetal hippocampus and Amy indicates that these limbic structures might be most vulnerable to prenatal cannabis exposure.

The early pattern of expression of functionally active CBR, along with the transient and atypical localization of these proteins in brain areas during the prenatal stages, suggest a specific role of the ECS in the events related to rodent and human neural development. This poses an open concern about how THC or synthetic cannabinoids consumed by the mother affect CB1R and the long-term consequences of their engagement by THC. Aberrant stimulation of the ECS by early exposure to exogenous cannabinoids, such as THC, during neurodevelopment, may interfere with the homeostatic

role of eCBs and might potentially confer increased vulnerability to adverse neuropsychiatric outcomes (Schonhofen et al., 2018).

### Endocannabinoid signaling and emotional processing: implication for limbic memory and alcohol vulnerability

The ability to differentiate and record stimuli associated with positive or negative outcomes, which is critical for survival, requires the combination of both emotional processes and cognitive functions for the formation of reward- or fear-associated memory traces (Mineka et al., 1998; Haselton and Nettle, 2006) – namely *limbic memory*, i.e. explicit memory traces of an emotionally salient experience (Brancato et al., 2016). In recent years, endogenous cannabinoid players have emerged as key modulators of affect and motivation (Campolongo and Trezza, 2012). As their “on-demand” functioning, eCBs are released in response to both physically harmful stimuli and psychological threats. Generally, their release activates protective mechanisms and counteracts aversive responses by CB1R-mediated processes, aiming at disrupting aversive limbic memory formation and leading to fear extinction. Disturbances in processing external stimuli represent a core endophenotype across various neuropsychiatric disorders, including addiction, mood, and anxiety disorders.

ECS interacts with the brain’s intrinsic “reward circuitry” (Wise, 1996; Gardner, 2002; Lupica et al., 2004), inducing synaptic plasticity in the VTA: CB1R activation triggers long-term depression (LTD) of excitatory inputs to GABA interneurons (Friend et al., 2017), and consequently promotes an enhancement in VTA dopaminergic (DA) signaling. Nonetheless, a complex interaction between ECS and NPY signaling is crucial for emotional control and resilience to stress, by its wide expression in key regions for neuro-excitability and plasticity (Plescia et al., 2014; Reichmann and Holzer, 2016; Robinson and Thiele, 2017). NPY can modulate synaptic strength and plasticity by regulating presynaptic calcium entry and the rearrangement of the architecture of the PSD, including the trafficking of ion channel receptors and the recruitment of scaffold proteins functional to synaptic plasticity, such as the Homer isoforms (Bacci et al., 2002; Goyal and Chaudhury, 2013; Molosh et al., 2013). Indeed, neurochemical analyses have revealed that its overexpression in both the hypothalamus and Amy drives cognitive resiliency and ameliorates the negative effects of stress on memory (Sweis et al., 2013). A potential role for NPY in cannabinoid effects on neurotransmission and synaptic functioning was shown by the association of cannabis use with increased NPY receptor type Y1 (Y1) mRNA expression levels in the PFC (Caberlotto and Hurd, 2001), where cannabinoid receptors are highly localized (Glass et al., 1997). A complex interaction between ECS and NPY signaling has been suggested to contribute to the emotional imbalance induced by chronic stress in

mice (Lomazzo et al., 2017), and to anxiety-like behavior and cognitive deficits observed in adulthood following adolescent exposure to intermittent alcohol (Sanchez-Marin et al., 2017). Given that CB1R signaling in the mesocorticolimbic system, as well as the interaction with relevant neurotransmissions, are fundamental prerequisites for the expression of motivation to seek rewarding stimuli, the involvement of ECS in alcohol rewarding properties is established, with evidence for its role in the regulation of both the reinforcing properties and the mechanisms responsible for cell injury and inflammation in the hepatic tissue (Lavanco et al., 2018). This is confirmed by studies showing that the administration of CB1R agonists increases alcohol consumption in animal models of addiction (Hungund et al., 2004; Agoglia et al., 2016).

Besides the involvement of classical neurotransmitters, the ECS modulation of the response to alcohol involves the recruitment of reward/stress-related neuropeptides – such as corticotropin-releasing hormone (CRH) and NPY – which play a critical role in the development of addiction (Vengeliene et al., 2008; Olling et al., 2009). Indeed, rat exposure to a high concentration of acetaldehyde – ACD, the alcohol's first oxidative metabolite – increases eCBs transmission, resulting in a downregulation of NPY expression and the occurrence of withdrawal symptoms following ACD suspension. Moreover, the study showed that CB1R blockade by the antagonist/inverse agonist AM281 is able to increase NPY expression, decrease ACD seeking behavior, and boost homeostatic functional recovery (Plescia et al., 2013, 2014). Notably, NPY promotes robust reductions in alcohol- craving and taking through the activation of Y1 in the extended Amy and medial PFC (mPFC; Cippitelli et al., 2010; Heilig and Thorsell, 2002; Pandey et al., 2003; Robinson et al., 2019; Robinson and Thiele, 2017).



## Aim

Since the ECS governs synaptic plasticity processes from the early stages of life in the interactive network with other excitability endogenous modulators, such as NPY and PSD machinery, its aberrant manipulation might contribute to the occurrence of a vulnerable phenotype later in life.

The aim of part 1 of the current research was to investigate whether prenatal exposure to THC may induce deficits in emotional/cognitive processes and alcohol vulnerability in adolescent offspring. To assess this, following *in-utero* THC exposure (2 mg/kg, delivered subcutaneously), preadolescent male rat offspring were assessed for different behavioral patterns: behavioral reactivity in the Open Field Test, neutral declarative and aversive limbic memory in the Novel Object, and Emotional Object Recognition tests; given that the manipulation of ECS modulatory signaling by THC during critical periods of neurodevelopment, such as pregnancy, may lead to aberrant plasticity, immunofluorescence for NPY neurons and excitatory PSD proteins Homer-1, 1b/c and 2 were measured in PFC, NAc and Amy at adolescence (cohort 1), where alterations in NPY signal together with modifications in PSD protein make-up can represent the background of a vulnerable phenotype for emotional/cognitive dysregulation. Further, instrumental learning and alcohol vulnerability in terms of discrete alcohol-related behaviors, such as alcohol-taking, alcohol-seeking following a forced abstinence – relapse – and alcohol use despite negative consequences – conflict behavior – were assessed in the operant chamber throughout adolescence until early adulthood (cohort 2).

## Methods

### Animals and treatment

Twelve adult female nulliparous Wistar rats (200-220 g; Envigo, Milan, Italy) were housed in pairs in standard rat cages (40 cm × 60 cm × 20 cm), with ad libitum access to water and food in a temperature- and humidity-controlled room ( $22 \pm 2^\circ\text{C}$  and  $55 \pm 5\%$ , respectively) on a 12-h light/dark cycle. Timed pregnancy was performed by housing each female rat with a single breeding male rat. The day on which sperm was detected in the vaginal smear was designed as the GD 0 (Cannizzaro et al., 2008). Pregnant females were then given daily subcutaneous (s.c.) injections with THC (2 mg/kg) or vehicle (Veh) from GD 5 to GD 20. This dose was chosen because it does not elicit substantial behavioral responses or tolerance after repeated administration and fails to significantly affect maternal or litter outcomes (Traccis et al., 2021). Notably, this dose of THC is equivalent to the current estimates of moderate cannabis consumption in humans since it is similar to the THC content in mild joints (5%; Frau et al., 2019). The route of administration results in the transmission of THC and its metabolites in placenta and fetal brain and mimics the pharmacodynamics of human THC inhalation, allowing fast high concentration in maternal blood (Baglot et al., 2021). After weaning, male rats were housed in pairs, and each experimental group for the behavioral and operant drinking paradigms included one or two independent male rats per each litter of Veh- or THC-treated dams. All experiments were conducted in accordance with animal protocols approved by the Committee for the Protection and Use of Animals of the University of Palermo in accordance with the current Italian legislation on animal experimentation (D.L. 26/2014) and the European directives (2010/63/EU) on care and use of laboratory animals. Every effort was made to minimize the number of animals used and their sorrow.

### Drugs

THC (THC Pharm GmbH, Frankfurt, Germany) was suspended in a Veh of 1% ethanol + 1% Tween 80 in saline or Veh. Rat dams were subcutaneously administered THC (2 mg kg/L) or Veh (1-2% Tween 80, saline) in a volume of 2 ml kg/L. Alcohol (96%; Carlo Erba Reagenti, Milan, Italy) was dissolved in tap water at 10% v/v.

### Behavioral procedures

Male rat offspring, prenatally exposed to either Veh (CTRL) or THC (pTHC) were tested during pre-adolescence and adolescence, starting from postnatal day (PND) 25 onwards, during the light phase of the light/dark cycle. Offspring underwent a behavioral battery tailored to explore behavioral

reactivity, neutral and aversive limbic memory, and alcohol vulnerability (figure 1). In detail, behavioral reactivity in a novel environment was assessed by the Open Field test (OFT; Plescia et al., 2015); neutral declarative and aversive limbic learning and memory were explored with the Novel Object Recognition test (NORT) and the Emotional Object Recognition (EOR) test, respectively (Brancato et al., 2016). In a different cohort of offspring, instrumental learning, that is, the ability to acquire a simple instrumental response in order to gain access to water, was investigated by using an operant-conditioning chamber as previously described, in water-restricted conditions (modified from Oakeshott et al., 2011). The offspring's vulnerability to alcohol motivational properties was then tested in an operant paradigm designed to explore discrete alcohol-related behaviors, such as alcohol-taking in the acquisition phase, alcohol-seeking following forced abstinence in the relapse phase, and alcohol use despite negative consequences, that is, resistance to punishment, in the conflict phase (Brancato et al., 2014; Cacace et al., 2012; Deroche-Gamonet et al., 2004). At the end of the conflict session, rats were tested for nociception in the tail-flick test (Cannizzaro et al., 2016). The objects and the apparatus used were thoroughly cleaned at the end of each experimental session. The behavior of the rats was monitored and quantified by the experimenter and an automatic video-tracking system, AnyMaze (Stoelting Europe, Dublin, Ireland).

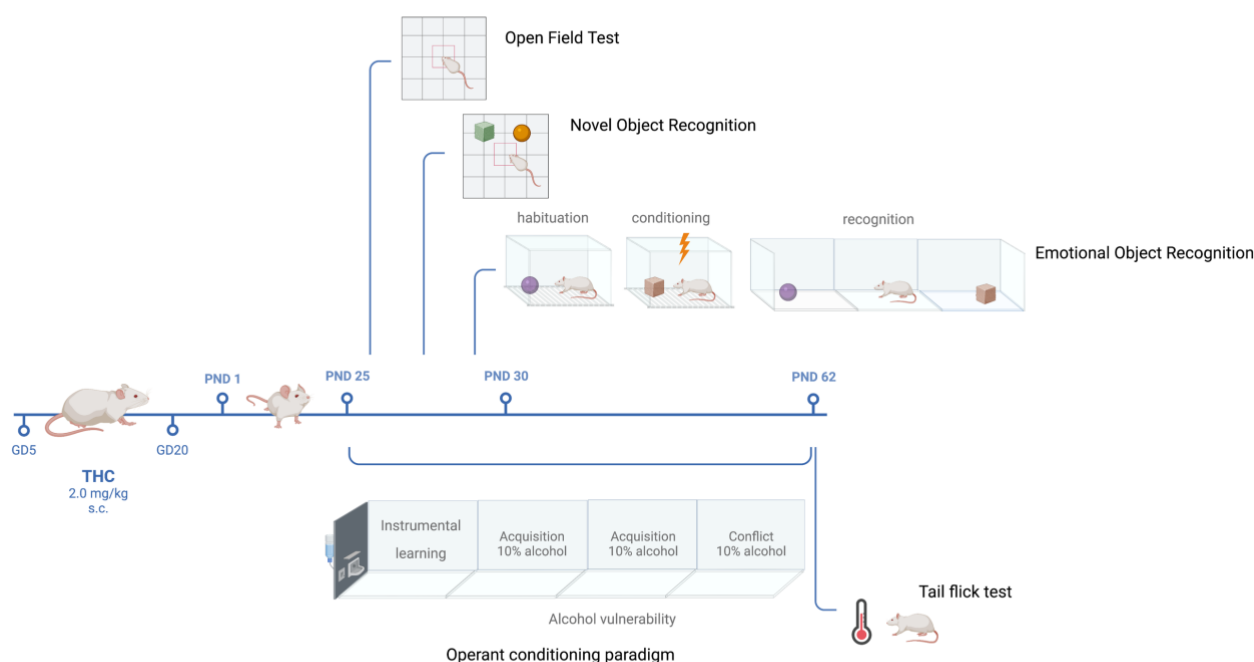


Figure 1. Behavioral procedures. Male rat offspring, prenatally exposed to either vehicle (CTRL) or THC (2 mg/kg; pTHC) were tested from PND 25 onwards for behavioral reactivity in a novel environment by the open field test; for declarative and limbic learning and memory by the Novel Object Recognition test and the Emotional Object Recognition test, respectively; for instrumental learning, by operant conditioning; for vulnerability to alcohol in an operant paradigm by alcohol taking in the acquisition phase, alcohol-seeking following forced abstinence in the relapse phase and alcohol use despite negative consequences (i.e. resistance to punishment) in the conflict phase; for nociception by the tail-flick test. GD = gestational day; PND = postnatal day; s.c. = subcutaneous; THC =  $\Delta^9$ -tetrahydrocannabinol.

## OFT

Locomotor activity and behavioral reactivity in a novel environment were measured in an open field. The apparatus was a Plexiglas square box, 44 cm long, 44 cm wide, and 20 cm high located in a mean light intensity (100 lx) illuminated chamber. The test provides a quali-quantitative mapping of the motor pattern, measuring total distance traveled – TDT, as a measure of locomotor activity – and the number of central transitions from the peripheral to the central area of the arena – NCT, as measures of explorative behavior (Cacace et al., 2011). Each experimental session lasted 5 m.

## NORT

The offspring were tested for declarative learning and memory employing the NORT, as previously described (Cannizzaro et al., 2016). On day 1, a 5-m habituation session was performed at 10:00 am in order to let the animals freely explore the arena, which was the same as in the OFT. Twenty-four h after the habituation session, rats underwent a 5-m training session when they were presented with two identical, non-toxic objects (i.e. two red metal cans) which were placed against a wall in the arena. To prevent coercion to explore the objects, rats were released against the center of the opposite

wall with their backs to the objects. A 2 cm<sup>2</sup> area surrounding the objects was defined such that nose entries were recorded as time for object exploration. After the training session, animals were placed in their home cage for a 24-h retention interval. Then, the animals were returned to the arena where they found two objects: one was identical to the familiar one but previously unused (to prevent olfactory cues and the necessity to wash objects during experimentation), and the other was a novel object (a yellow hard plastic cup). Time spent exploring the novel (TN) and familiar (TF) objects was recorded during a 5-min session. Objects were randomized and counterbalanced across animals. The recognition index (RI %), that is, the percentage of time spent investigating the novel object out of the total object investigation time ( $RI \% = TN/(TN + TF)\%$ ), is a measure of novel object recognition and the main index of retention (Mumby et al., 2002). A RI % of > 50% indicated that the rat spent more time exploring the novel object, thus recalling the memory of the familiar object; a RI % of < 50% indicated that time was mainly spent exploring the familiar object, as it was a novel one.

## EOR

The EOR test was used for the assessment of aversive limbic memory (Brancato et al., 2016). It employed two distinct contexts (A and B) placed in different rooms. The context A chamber was a rectangular arena with a white floor (100 cm long × 30 cm wide × 43 cm high). A left and a right zone (40 cm × 30 cm) on both ends of the context A chamber, as well as a neutral zone in the center of the box (20 cm), represented the arena settings (modified from Ramirez et al., 2015). The context A chamber was customized with two different non-toxic objects (i.e. a plastic ball, 3.5 cm diameter, and a plastic pepper, 3 cm × 3 cm × 4 cm) that were placed against the end walls of the left and the right zones of the arena, according to the procedure described below. The objects and their position were counterbalanced within the experimental groups. The context B chamber was a rectangular arena (45 cm × 22 cm × 22 cm) equipped with a grid floor, opaque ceiling, and dark walls. Rats were allowed to explore the context B chamber for habituation and conditioned/cued learning; they were then tested in the context A chamber for emotional recognition. Rats were transported to and from the experimental room in their home cages using a wheeled cart. The cart and cages remained in an anteroom to the experimental rooms during all behavioral experiments (figure 2).

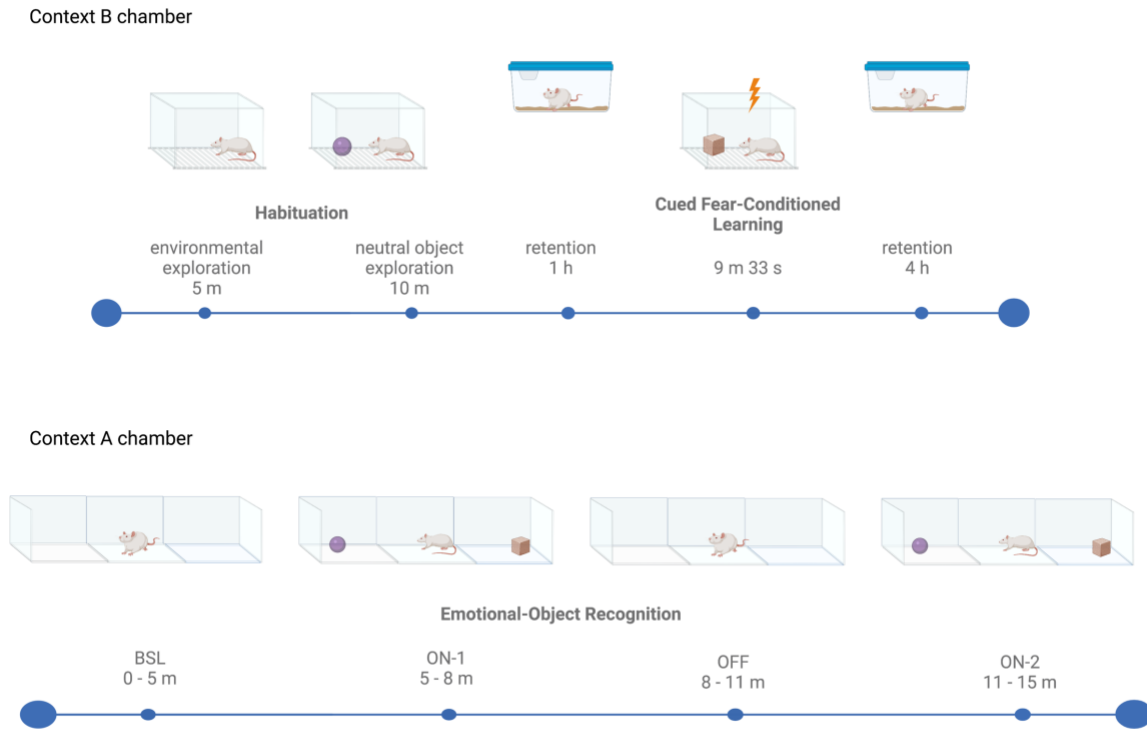


Figure 2. Schematic summary of the Emotional-Object Recognition Test. During the habituation phase in the context B chamber, rats were left undisturbed to explore the chamber and then presented to a neutral object (i.e. plastic ball or pepper) placed in the opposite corner to the rat's entry. In the Cued fear-conditioned learning, rats were re-placed in the context B chamber, exposed to a novel object (emotional object), and trained for fear conditioning. The Emotional object recognition phase took place in the context A chamber where rats were tested for emotional object discrimination and object place aversion. They were allowed to explore the new context with or without either the neutral- or emotional objects throughout the epochs baseline (BSL), ON-1, OFF, and ON-2.

### Experimental design

**Habituation.** Habituation took place in the context B chamber and consisted of two separate sessions: environmental exploration, during which rats were put in the arena and left undisturbed to explore the chamber for 5 m; neutral-object exploration, in which an object (i.e. plastic ball or pepper) was placed in the opposite corner to the rat's entry and presented to the animals for 10 m. Between the two sessions, rats were taken and returned to their home cages for 10 m.

**Cued fear-conditioned learning.** One h after neutral-object exploration, rats were re-placed in the context B chamber, exposed to a novel object (emotional object), and trained for fear conditioning. The session lasted 560 s, and six 2-s 0.3 mA shocks were delivered at seconds 120, 200, 280, 360, 440, and 520. At the end of the session, animals were returned to their home cages for a 4-h retention interval.

*Emotional object recognition: context A chamber.* Four h after cued fear-conditioned learning, rats were put into the context A chamber and tested for emotional object discrimination and object place aversion in the neutral context A chamber. They were allowed to explore the new context freely for 5 m. For each rat, the favorite zone, between the left and right ones, was recorded at epoch baseline (BSL). Afterward, the object experienced during fear conditioning (the emotional object) was placed in the favorite zone; the object experienced during habituation (the neutral object) was placed in the less preferred zone. Rats explored objects and zones during minutes 5 - 8 (epoch ON-1). During minutes 8 - 11, objects were removed from the arena (epoch OFF). During minutes 11 - 14, the objects were reintroduced in the same positions as during minutes 5 - 8 (epoch ON-2). Finally, rats were placed in their home cages and returned to the holding room. At the end of each experimental session, both the objects and the arena were cleaned with a 70% solution of isopropanol. Time spent exploring the objects and zones was recorded along the epochs. Emotional object discrimination was measured by the percentage of emotional object avoidance, which was calculated as “ $100 - (\text{time spent on the emotional object} / \text{time spent on neutral} + \text{emotional object})\%$ ”. Object place conditioning was measured by the percentage of target zone preference, which was calculated as the percentage of time spent in the target zone during BSL, ON, and OFF epochs.

## Operant tasks

### *Apparatus*

The experimental sessions were carried out in custom-built operant-conditioning chambers (30 cm × 28 cm × 37 cm) located within a dimly lit, sound-attenuating shell, with a fan mounted at one end of the cubicle that was active throughout the session for white noise. Each chamber was equipped with two levers located on opposite walls of the chamber, 3.5 cm above the grid floor, controlling the delivery of 0.05 mL of liquid reinforcement for each lever press into two separate dippers and a light stimulus above the lever. The grid floor was connected to an electric-shock generator to deliver a footshock (0.2 mA) during the punished period (see conflict experiment described below). The hardware was controlled by an Arduino-based control unit (patent pending, application n. 102019000022341), which allowed to record all the events during the experimental sessions.

### *Experimental design*

*Instrumental learning.* During the instrumental learning experiment, rats were trained to press a lever via a simple free operant procedure where each lever press was reinforced with 0.05 mL of tap water throughout a 20-m session. No reinforcement was delivered without a lever press. The instrumental learning experiment was carried out once daily along 5 days. Rats' access to water was restricted

since they were allowed to drink for 1 h per day at the end of the experimental session. The number of lever presses and water intake were recorded. Animals were trained to a learning criterion, requiring them to obtain 20 reinforcements across two consecutive experimental sessions (modified from Oakeshott et al., 2011).

*Alcohol vulnerability.* The offspring that reached the learning criterion within 5 days underwent an alcohol vulnerability assessment in terms of motivation for alcohol in the operant chamber. In order to match the sample size of the CTRL and pTHC groups, and to control for litter effect, six rats per group were used, composed of one pup per litter. The operant paradigm included acquisition, relapse following forced abstinence, and conflict experiments. During the acquisition experiment, the rats would lever press via a Fixed Ratio 1, so each lever press was reinforced with 0.05 mL of alcohol 10% (lever 1) or water (lever 2) throughout a 20-m session. The acquisition experiment was carried out daily for 21 sessions. Rats were not water-restricted. The number of lever presses and fluid intake were recorded. Weekly and daily mean numbers of lever presses and alcohol intake (g/kg) were calculated. Following the 21-day acquisition experiment, alcohol operant administration was suspended to achieve forced abstinence. Thus, rats were left undisturbed in their home cages for one week and received water and food ad libitum. Afterward, in the relapse experiment, rats were exposed to alcohol following the forced abstinence period and allowed to lever press for either 10% alcohol or water in the operant chamber, in the same experimental conditions as for acquisition, for 5 days. The number of lever presses and fluid intake were recorded. Daily mean numbers of lever presses and alcohol intake (g/kg) were calculated. The difference between the mean daily alcohol intake, in terms of g/kg, during the relapse session and the mean alcohol intake during the last 5 days of the acquisition session was calculated as a measure of the alcohol deprivation effect, that is, the marked increase in alcohol intake that follows periods of withdrawal. In the conflict experiment, the delivery of the reinforcement (both water and alcohol) was paired with the delivery of a mild footshock, according to a within-session schedule. In detail, each session started with a non-punished interval (3 m in duration), when each lever press was reinforced as described above (Fixed Ratio 1, water or alcohol 10% according to the lever). During the punished response interval (1 m long), the light stimulus was on, and each lever press controlled the delivery of the reinforcement (either alcohol or water, according to the lever), along with a mild footshock (0.2 mA; Cacace et al., 2012). Non-punished and punished periods alternated according to the 3-m – 1-m schedule for one 20-m session. The number of unpunished and punished lever presses was automatically recorded. The percentage of punished responses, over the total number of non-punished and punished operant responses, emitted in each of the 5 time-bins of the conflict session was calculated as a measure of punishment resistance.



### Tail-flick test

At the end of the conflict session, nociception was explored by measuring tail-flick latency in the hot-water immersion tail-flick test (Cannizzaro et al., 2016). Briefly, 2 cm of the rat tail was immersed in a water-bath apparatus (MPM Instruments Srl, Bernareggio, Italy) maintained at  $52 \pm 0.5^{\circ}\text{C}$ . Latency to response was determined by a vigorous tail-flick. A cut-off time of 10 s was imposed to minimize tissue damage.

### Immunofluorescence experiments

At PND 35, a subset of offspring from the first cohort of rats was anesthetized (chloral hydrate, 300 mg/kg) and trans-cardially perfused with cold phosphate-buffered saline (PBS; pH 7.4), followed by 4% paraformaldehyde in PBS. Brains were dissected and post-fixed overnight in the same fixative used for perfusion. Fixed brains were coronally sectioned at a thickness of 40  $\mu\text{m}$  using a microtome (Campden Instruments, Loughborough, UK). Serial sections were collected through the rostral-caudal dimensions (every sixth slice) and stored at  $4^{\circ}\text{C}$  in 0.02% sodium azide in PBS until the immunofluorescence staining (Brancato et al., 2017). Sections containing mPFC (from the bregma 3.20 mm to 2.20 mm), NAc (from the bregma 2.20 mm to 1.60 mm), and Amy (from the bregma  $-1.80$  mm to  $-2.30$  mm) were selected (Paxinos and Watson, 1998), washed in PBS for 30 min and incubated in blocking solution (3% Normal Goat Serum (NGS), 0.3% Triton X-100 in PBS) for 2 h at room temperature under gentle shaking. Afterward, sections were incubated in primary antibody solution (3% NGS, 0.3% Tween-20 in PBS) with either rabbit anti-NPY (T-4070 1:1000; Peninsula Laboratories International, Inc., San Carlos, CA), mouse monoclonal anti-Homer 1 (#sc136358; 1:250; Santa Cruz Biotechnology, Dallas, TX), mouse monoclonal anti-Homer 1b/c (#sc25271; 1:250; Santa Cruz Biotechnology), rabbit polyclonal anti-Homer 2 (#160 203, 1:650; Synaptic Systems, Goettingen, Germany) for 72 h. Subsequently, sections were washed in PBS solution for 1 h, incubated in secondary antibody solution (Alexa Fluor® 488 AffiniPure Goat Anti-Rabbit IgG or Alexa Fluor® 594 AffiniPure Goat Anti-Mouse IgG; 1:200; Jackson ImmunoResearch, West Grove, PA) for 2 h under gentle shaking. After washing for 1 h, slices were briefly incubated with DAPI (1  $\mu\text{g}/\text{mL}$ ). Sections were mounted onto adhesive slides (Superfrost® Plus; Thermo Fisher Scientific, Waltham, MA) and coverslipped using Vectashield® HardSet™ Antifade mounting medium. Images were acquired at 20 $\times$ , 40 $\times$ , and 100 $\times$  magnification using an epifluorescence microscope (Meiji Techno, Saitama, Japan) and Deltapix Insight software (figure 3). NPY-positive (NPY+) neurons (from 20 $\times$  images) and Homer proteins immunofluorescence (from 40 $\times$  images) were quantified using Image J.

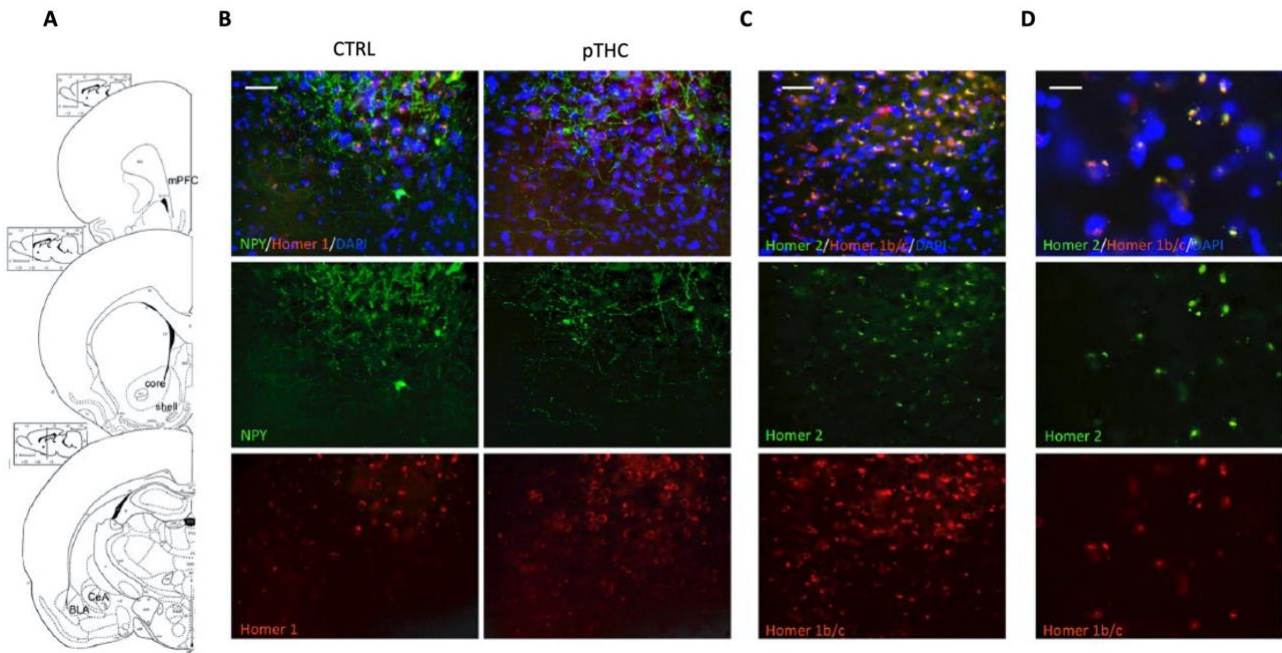


Figure 3. Immunofluorescent experiments. NPY+ neurons and Homer proteins were separately quantified in the mPFC, shell and core subregions of the NAc, BLA, and CeA in the two experimental groups (A). Representative photomicrographs of immunofluorescent staining for NPY and Homer 1 (B) in the two experimental groups (magnification 40 $\times$ , scale bar = 40  $\mu$ m for all images) and for Homer 1b/c and 2 at (C) magnification 40 $\times$ , scale bar = 40  $\mu$ m for all images and (d) magnification 100 $\times$  scale bar = 10  $\mu$ m for all images.

NPY = neuropeptide Y; mPFC = medial prefrontal cortex; NAc = nucleus accumbens; BLA = basolateral amygdala; CeA = central amygdala; CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC.

### Statistical analysis

All data were tested for normality and equal variances. When data exhibited normality and equal variances, differences between groups were determined using either Student's t-test or two-way analysis of variance (ANOVA) either regular or for repeated measures, followed by a Bonferroni post hoc test. Data that did not display equal variances were analyzed using nonparametric tests. The performance of rats to reach the learning criterion was analyzed using Kaplan-Meier event analysis over the instrumental learning period, and the resulting curves were compared by employing the log-rank Mantel-Cox test. Statistical analysis was carried out using GraphPad Prism v. 9 (GraphPad Software, Inc., San Diego, CA). All values represent the mean  $\pm$  standard error of the mean. An alpha level of 0.05 was adopted throughout.

## Results

### Prenatal THC exposure does not alter reproduction parameters

*In-utero* cannabinoid exposure did not significantly affect reproduction parameters such as the number of dams giving birth, length of pregnancy, litter size at birth, postnatal mortality (the number of pups that died before weaning), and male:female ratio (table 1). Unpaired Student's t-test analysis on data from body weight at birth showed a small but significant decrease in pTHC pups compared to CTRL rats, which disappeared at the time of the first behavioral assessment (PND 25; table 1).

Reproduction parameters	CTRL	pTHC	Statistics
Percentage of dams giving birth	100%	100%	
Length of pregnancy (days), median (IQR)	21 (1.8)	21 (1.0)	n.s. (Mann–Whitney U-test, $p = 0.394$ , $U = 11.0$ )
Litter size at birth, median (IQR)	10.5 (1.5)	10 (3.5)	n.s. (Mann–Whitney U-test, $p = 0.632$ , $U = 14.5$ )
Body weight of pups (g), mean $\pm$ SD per litter			
At birth	$6.3 \pm 0.4$	$5.7 \pm 0.4$	* (Student's t-test, $p = 0.028$ , $t = 2.57$ , $df = 10$ )
At weaning time	$31.4 \pm 3.1$	$29.8 \pm 4.4$	n.s. (Student's t-test, $p = 0.480$ , $t = 0.733$ , $df = 10$ )
Postnatal mortality, median (IQR) per litter	0.00 (0.02)	0.06 (0.43)	n.s. (Mann–Whitney U-test, $p = 0.182$ , $U = 10.5$ )
Male:female ratio, median (IQR) per litter	1.3 (0.4)	1.4 (1.6)	n.s. (Mann–Whitney U-test, $p = 0.972$ , $U = 17.5$ )

IQR: interquartile range; SD: standard deviation; n.s.: not significant.\*Statistically significant. CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC

Table 1. Effects of prenatal cannabinoid exposure on reproduction parameters

### Behavioral assessment

#### OFT - Prenatal THC exposure impacts on behavioral reactivity of the preadolescent offspring

At PND 25, male offspring were tested in the open field arena to evaluate the effects of prenatal exposure to THC on locomotor activity and exploratory behavior. Statistical analysis highlights that pTHC rats significantly increased TDT ( $t = 4.925$ ,  $df = 22$ ,  $p < 0.001$ ; figure 4, A) compared to the CTRL group. No significant differences were observed in NCT ( $U = 41.00$ ,  $p = 0.0708$ ; figure 4, B).

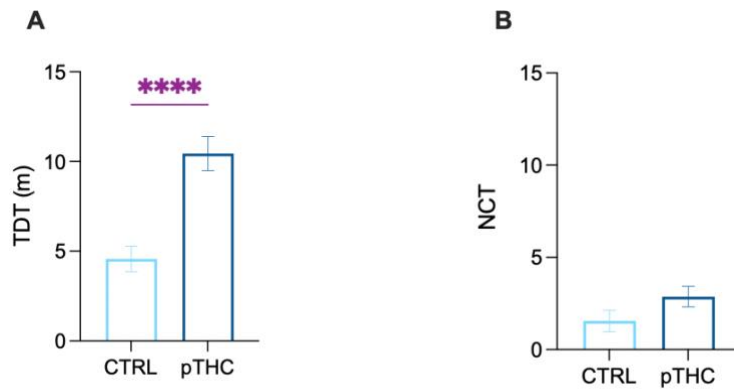


Figure 4. Effects of prenatal THC exposure (2 mg/kg) on behavioral reactivity in preadolescent offspring in the OFT. *In-utero* exposure to THC increased locomotor activity, in terms of TDT (A), while inducing no significant effects in exploratory activity, in terms of NCT (B). Data are shown as the mean  $\pm$  SEM. \*\*\*\* $p < 0.0001$ . CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC; TDT = total distance traveled; NCT = number of central transitions.

#### NORT - Prenatal THC exposure induced no alteration in neutral declarative memory of the offspring

Offspring underwent the NORT in order to evaluate the effect of prenatal exposure to THC on declarative learning and memory. The results of Student's t-test on the RI% show no significant differences in pTHC offspring when compared to CTRL counterparts ( $t = 1.007$ ,  $df = 22$ ,  $p = 0.3249$ ; figure 5).

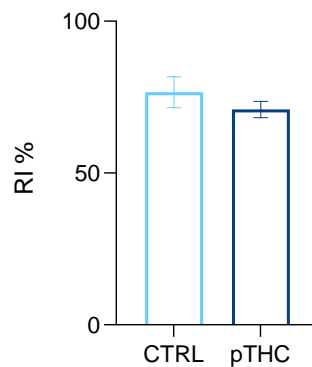


Figure 5. Effects of prenatal THC exposure (2 mg/kg) on declarative learning and memory in the NORT. *In-utero* exposure to THC induced no significant effects in neutral declarative learning and memory, in terms of RI%, compared to the CTRL group. Data are shown as the mean  $\pm$  SEM. CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC; RI = recognition index.

#### EOR - Prenatal THC exposure impaired aversive limbic memory

Offspring were tested in the EOR test for assessing the effects of *in-utero* exposure to THC on the acquisition of fear-associated declarative memory. When data from the emotional object avoidance (%) were considered, CTRL rats clearly avoided the emotional object, previously associated with the

aversive experience in the context B chamber, and displayed  $69 \pm 4\%$  emotional object avoidance, whereas pTHC offspring's emotional object avoidance reached  $54 \pm 3\%$ . The results of the two-tailed Mann-Whitney test highlight that pTHC rats showed a significant decrease in the avoidance of the fear-associated object compared to CTRL ( $U = 28.50$ ;  $p = 0.0105$ ; figure 6, A). In the Emotional Object Recognition test, the preferred zone of the context A chamber is determined during BSL and paired to the emotional object afterwards. When the target zone preference (%) along the test epochs was analyzed, a repeated measures two-way ANOVA showed a significant effect of test epoch ( $F(3, 66) = 7.874$ ;  $p = 0.0001$ ) and pTHC ( $F(1, 22) = 9.367$ ;  $p = 0.0057$ ; figure 6, B).

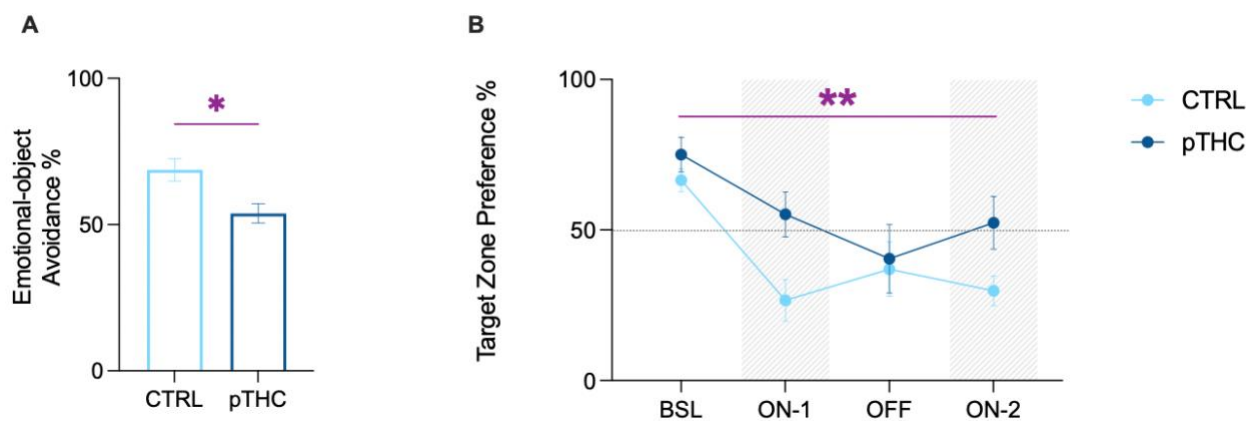


Figure 6. Effects of prenatal THC exposure (2 mg/kg) on aversive limbic memory of the offspring in the EOR. *In-utero* exposure to pTHC decreased limbic learning and memory in the EOR test compared to the CTRL group in terms of emotional object avoidance (A) and target zone preference (B). Data are shown as the mean  $\pm$  SEM. \* $p < 0.05$ ; \*\* $p < 0.01$ . CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC; BSL = baseline .

**Operant task - Prenatal THC exposure decreases instrumental learning and increased alcohol drinking, relapse, and conflict behavior in the operant chamber.**

**Instrumental learning.** The effects of prenatal exposure to THC on the offspring's instrumental learning were assessed by evaluating the animal's ability to acquire a simple instrumental task. The offspring performance was measured in terms of days elapsed to reach the learning criterion, and the Kaplan-Meier analysis showed that median learning times were two days for CTRL rats and five days for pTHC offspring. In addition, 0.0% of CTRL rats and 41.7% of pTHC rats failed to reach the criterion within 5 days, and thus were censored and withdrawn from the alcohol vulnerability experiment. The log-rank Mantel-Cox test for comparison of survival curves indicated that learning performance was significantly decreased in pTHC offspring compared to CTRL rats ( $\chi^2 = 12.10$ ,  $df = 1$ ,  $p = 0.0005$ ; figure 7).

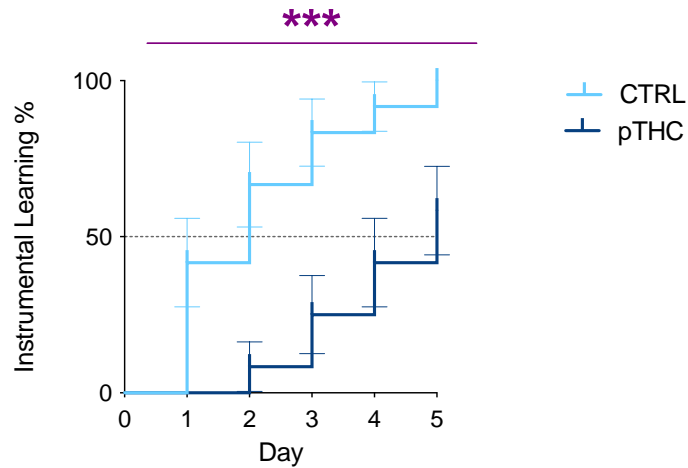


Figure 7. Effects of prenatal THC exposure (2 mg/kg) on instrumental learning in adolescent offspring in the operant paradigm. pTHC decreased instrumental learning in a simple operant task compared to the CTRL group. Data are shown as the mean  $\pm$  SEM. \*\*\* $p < 0.001$ . CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC.

**Alcohol vulnerability.** In order to assess the effects of *in-utero* exposure to THC on the vulnerability to the motivational properties of alcohol, six offspring per experimental group were tested in an operant paradigm that models discrete alcohol-related behaviors, such as alcohol-taking in the acquisition phase, alcohol-seeking following forced abstinence in the relapse phase and alcohol use despite negative consequences, that is, resistance to punishment, in the conflict phase. When rats underwent the acquisition phase, the number of lever presses for 10% alcohol ( $M \pm SD$ ) increased along the three-week paradigm (week 1: CTRL =  $13 \pm 3$ ; pTHC =  $12 \pm 5$ ; week 2: CTRL =  $16 \pm 4$ ; pTHC =  $19 \pm 2$ ; week 3: CTRL =  $15 \pm 3$ ; pTHC =  $20 \pm 2$ ; figure 8, A). pTHC offspring performed a higher number of lever presses (average per week) than CTRL rats. A repeated-measures two-way ANOVA that included pTHC as the between-subject factor and week as the repeated-measure factor showed a significant main effect of pTHC ( $F(1, 10) = 5.102$ ,  $p = 0.0475$ ), weeks ( $F(2, 20) = 11.70$ ,  $p = 0.0004$ ) and their interaction ( $F(2, 20) = 3.535$ ,  $p = 0.0485$ ) on the number of responses emitted throughout the 21 days. In particular, a Bonferroni post hoc test indicated no significant differences between the two groups during the first two weeks of the acquisition period ( $t = 0.6329$ ,  $df = 30$ ,  $p > 0.05$ ;  $t = 1.564$ ,  $df = 30$ ,  $p > 0.05$ ), while pTHC rats displayed a significant increase in the number of lever presses compared to CTRL animals on the last week of the paradigm ( $t = 3.065$ ,  $df = 30$ ,  $p < 0.05$ ; figure 8, A, top panel). No significant effect of pTHC was observed on number of lever presses for water ( $F(1, 10) = 1.145$ ,  $p = 0.3097$ ; figure 8, A, lower panel). Following the acquisition experiment, the adolescent rats were tested for alcohol-seeking behavior after forced abstinence in the relapse paradigm. pTHC offspring emitted a higher number of lever presses for 10% alcohol compared to CTRL rats ( $M \pm SD$ : CTRL =  $19 \pm 4$ ; pTHC =  $24 \pm 4$ ; figure 8, B). In particular, a

repeated-measures two-way ANOVA showed a significant main effect of pTHC ( $F(1, 10) = 5.841$ ,  $p = 0.0363$ ) and days ( $F(4, 40) = 5.096$ ,  $p = 0.0021$ ) on the number of lever presses throughout the 5-day relapse period (figure 8, B, top panel). No significant effects of pTHC were observed on number of lever presses for water ( $F(1, 10) = 1.811$ ,  $p = 0.2081$ ; figure 8, B, lower panel). In addition, in order to assess the effects of prenatal THC exposure on the occurrence of the deprivation effect, the mean alcohol intake during the relapse session was compared with the mean amount of alcohol consumed during the last 5 days of training. The results of a repeated-measures two-way ANOVA, that included pTHC as the between-subject factor and deprivation as the within-subject factor, showed a significant effect of pTHC ( $F(1, 10) = 18.84$ ,  $p = 0.0015$ ), deprivation ( $F(1, 10) = 67.12$ ,  $p < 0.0001$ ) and their interaction ( $F(1, 10) = 9.818$ ,  $p = 0.0106$ ). A Bonferroni post hoc test indicated that CTRL offspring displayed a significant increase in mean alcohol consumption during relapse compared to their own levels during the last 5 acquisition days (CTRL:  $t = 3.577$ ,  $df = 10$ ,  $p = 0.0101$ ). pTHC offspring showed increased alcohol consumption following the deprivation compared to their own levels during the last five acquisition days (pTHC:  $t = 8.009$ ,  $df = 10$ ,  $p < 0.001$ ), and they consumed more alcohol than CTRL rats during the last 5 days of acquisition ( $t = 3.284$ ,  $df = 20$ ,  $p = 0.0074$ ) and during relapse ( $t = 5.046$ ,  $df = 20$ ,  $p = 0.0001$ ; figure 8, C). In particular, pTHC progeny showed a higher deprivation effect than CTRL rats in terms of difference between alcohol intake during relapse and the last 5 days of acquisition ( $t = 3.133$ ,  $df = 10$ ,  $p = 0.0106$ ,  $\eta^2 = 0.4954$ ; figure 8, D). Eventually, when the adolescent offspring were tested for punishment resistance in the conflict session, the repeated-measures two-way ANOVA on the percentages of punished responses for alcohol, over total responses – punished plus non-punished alcohol lever presses – throughout the 5 time-bins, showed that pTHC offspring emitted an increased percentage of punished responses for alcohol compared to CTRL rats (pTHC:  $F(1, 10) = 11.57$ ,  $p = 0.0067$ ; figure 8, E, top panel). No significant differences were observed on the percentage of punished lever presses for water (pTHC:  $F(1, 10) = 0.05257$ ,  $p = 0.8233$ ; figure 8, E, lower panel).



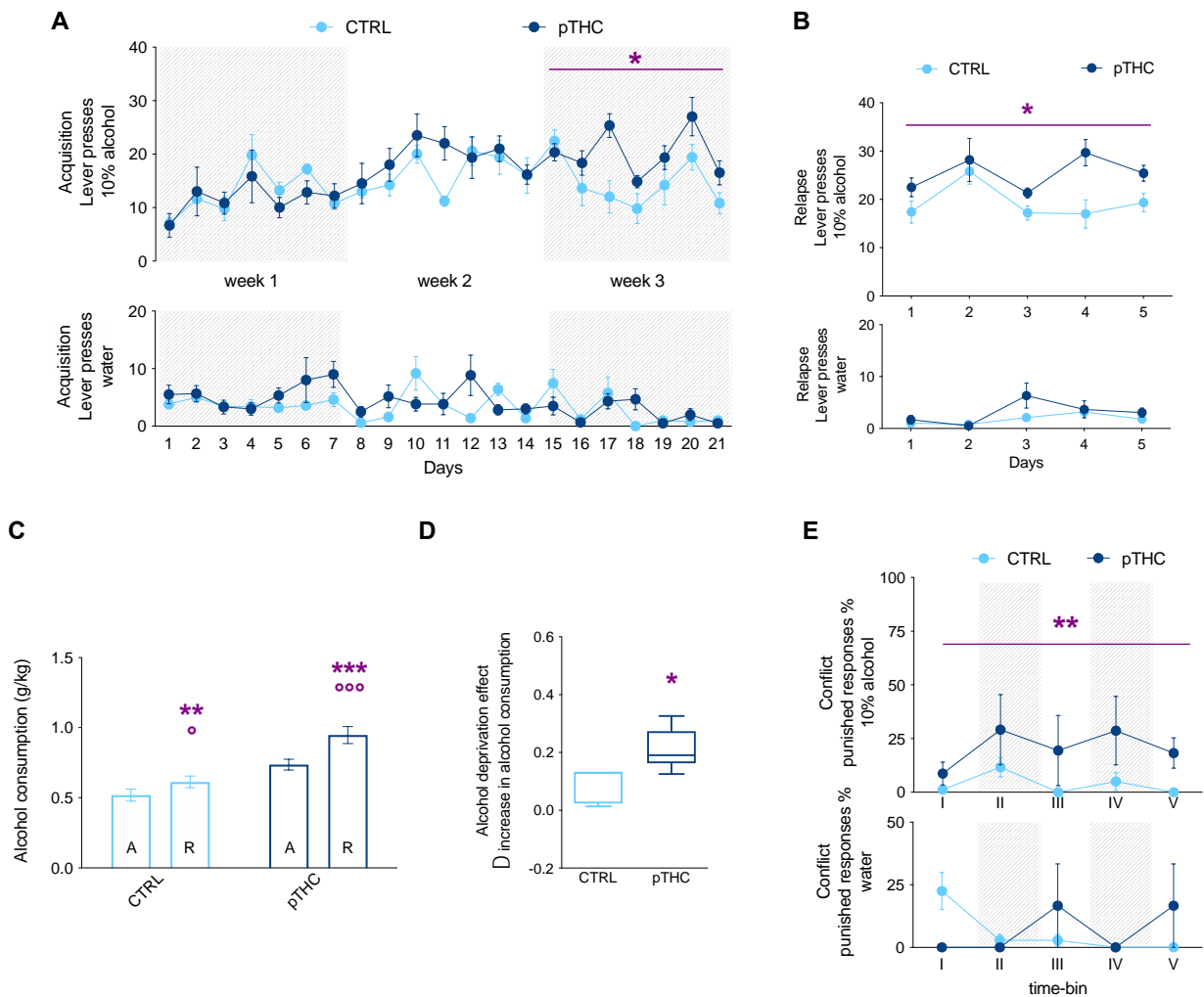


Figure 8. Effects of prenatal THC exposure (2 mg/kg) on motivation for alcohol in adolescent offspring in the operant paradigm. pTHC increased motivation for alcohol in the last week of the acquisition phase of the operant paradigm (A) and the relapse session following forced abstinence compared (B) to the CTRL group. pTHC offspring displayed a higher mean daily alcohol intake (in terms of g/kg) during relapse compared to the CTRL group and when compared to their own mean daily alcohol intake during the previous five days of acquisition (C), showing a higher deprivation effect than the CTRL group in terms of difference ( $\Delta$ ) in alcohol consumption (D). Moreover, pTHC progeny showed higher resistance to punishment for alcohol than the CTRL group along the five time-bins of the conflict session (E). Data are shown as the mean  $\pm$  SEM. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  vs. CTRL group; ° $p < 0.05$ ; °° $p < 0.001$  relapse (R) vs. acquisition (A) phases. CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC.

### Tail Flick - Prenatal THC exposure does not alter nociception

The results of Student's t-test on tail-flick latency showed no significant difference in nociception in pTHC offspring compared to CTRL progeny ( $t = 0.100$ ,  $df = 10$ ,  $p = 0.9223$ ; figure 9).



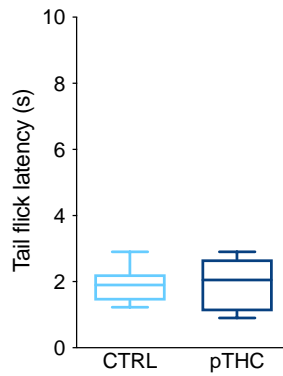


Figure 9. Effect of prenatal THC exposure (2 mg/kg) on nociception of adolescent offspring in the tail-flick test. No differences in nociception were observed. Data are shown as the mean  $\pm$  SEM.  
CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC.

### Exploration of neuroplasticity associated with emotional, cognitive, and alcohol vulnerability

#### Prenatal THC exposure decreases NPY+ neurons in limbic regions

The effects of pTHC on NPY+ neurons were assessed by immunofluorescence and are reported as percentages of the mean values of the CTRL group (figure 10). The analysis of data from mPFC indicates that pTHC significantly decreased the number of NPY+ neurons ( $t = 4.686$ ,  $p < 0.001$ ,  $df = 10$ ) compared to the CTRL group. The results of Student's t-test on the number of NPY+ neurons in pTHC rats in shell and core subregions of the NAc indicate a significant decrease in the number of NPY+ neurons both in shell ( $t = 3.668$ ,  $p = 0.004$ ,  $df = 10$ ) and core ( $t = 2.330$ ,  $p = 0.042$ ,  $df = 10$ ) compared to CTRL rats. NPY+ neurons were also measured in basolateral Amy (BLA) and central Amy (CeA): Student's t-test highlighted a significant decrease in NPY+ neurons in BLA ( $t = 3.529$ ,  $p = 0.0054$ ,  $df = 10$ ) and CeA ( $t = 5.646$ ,  $p < 0.001$ ,  $df = 10$ ) of pTHC rats compared to CTRL rats.

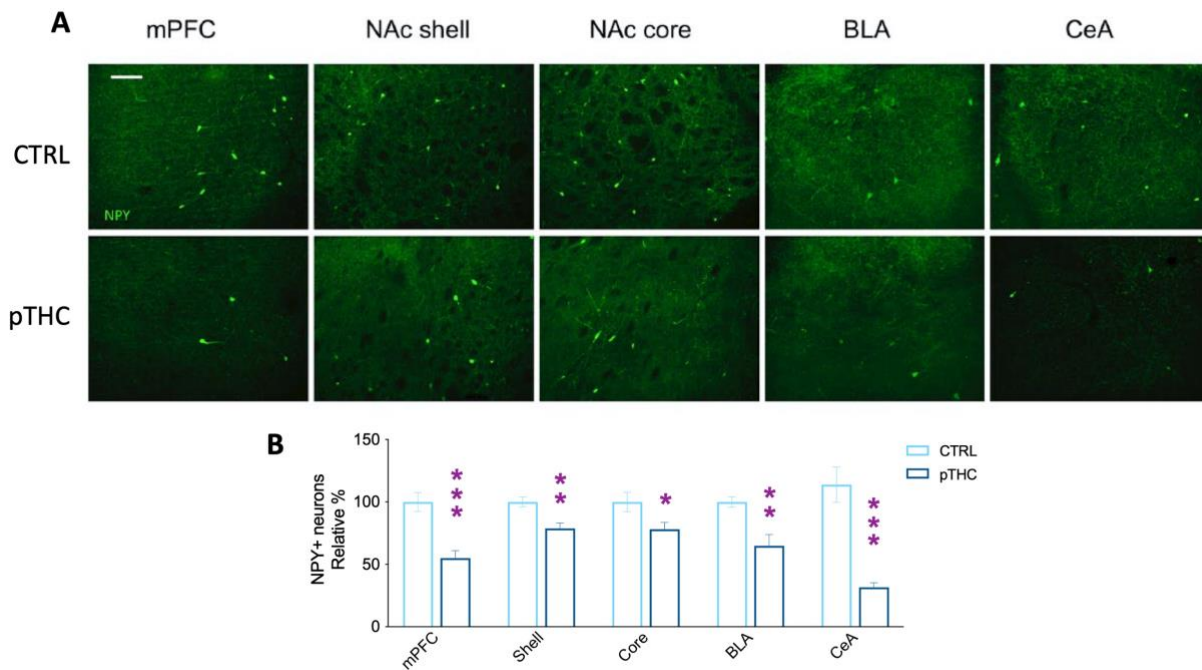


Figure 10. Effects of prenatal THC exposure (2 mg/kg) on NPY+ neurons in the limbic brain regions. (a) Representative photomicrographs of NPY immunofluorescent staining in the mPFC, shell and core subregions of the NAc and BLA, and CeA in the two experimental groups (A). 20 $\times$ , scale bar = 100  $\mu$ m for all images. pTHC significantly decreased the relative % of NPY+ neurons in the mPFC and in the shell and core of the NAc, BLA, and CeA with respect to the CTRL group (B). Data are shown as the mean  $\pm$  SEM. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ . NPY = neuropeptide Y; mPFC = medial prefrontal cortex; NAc = nucleus accumbens; BLA = basolateral amygdala; CeA = central amygdala; CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC.

### Prenatal THC exposure induces a region-specific variation in Homer-1, 1b/c, and 2 immunoreactivity

Expression levels of the PSD proteins Homer 1, 1b/c and 2 in the mPFC, NAc and Amy were assessed by immunofluorescence and are reported as relative immunofluorescence to the mean values of the CTRL group. When the effects of pTHC on Homer 1 protein were analyzed (figure 11, A and B), the results of Student's t-test indicated that pTHC increased the expression of Homer 1 in mPFC ( $t = 2.430$ ,  $p = 0.035$ ,  $df = 10$ ) and in both BLA ( $t = 2.670$ ,  $p = 0.023$ ,  $df = 10$ ) and CeA ( $t = 4.659$ ,  $p = 0.0010$ ,  $df = 10$ ) compared to the CTRL group. Moreover, when the expression of the C–C isoform Homer 1b/c was assessed (figure 11, C and D), the results of Student's t-test indicated that pTHC rats showed a significant decrease in Homer 1b/c in mPFC ( $t = 5.255$ ,  $p < 0.001$ ,  $df = 10$ ) and an increase in BLA ( $t = 2.829$ ,  $p = 0.018$ ,  $df = 10$ ) compared to CTRL rats. In addition, in the limbic regions of pTHC and CTRL rats (figure 11, E and F), the results of Student's t-test indicated that pTHC rats displayed a significant increase in Homer 2 immunofluorescence in NAc shell ( $t = 5.963$ ,  $p < 0.001$ ,  $df = 10$ ) and core ( $t = 2.967$ ,  $p = 0.014$ ,  $df = 10$ ) and BLA ( $t = 4.949$ ,  $p < 0.001$ ,  $df = 10$ ) compared to CTRL rats.

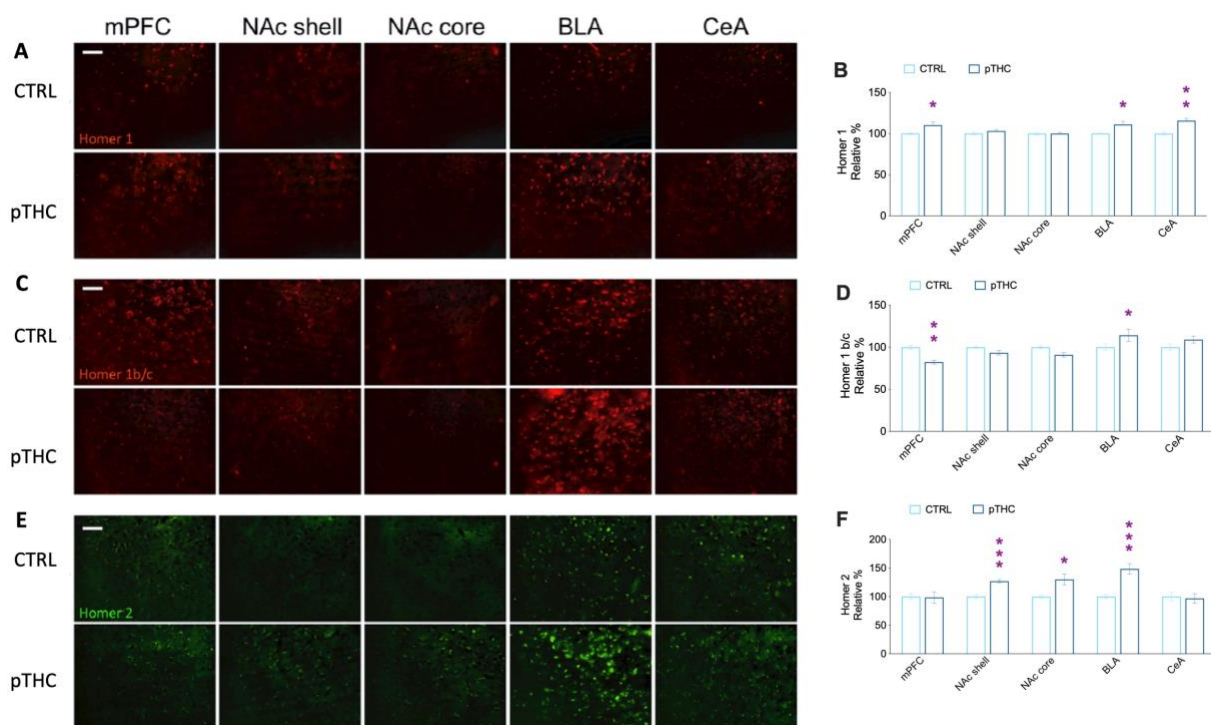


Figure 11. Effects of prenatal exposure to THC (2 mg/kg) on Homer protein expression in the limbic brain regions. Representative photomicrographs of Homer 1 immunofluorescent staining in the mPFC, NAc, and Amy of the two experimental groups (A). pTHC increased Homer 1 in the mPFC and BLA and CeA compared to the CTRL group (B). Representative photomicrographs of Homer 1b/c immunofluorescent staining in the mPFC, NAc, and Amy of the two experimental groups (C). In pTHC rats, we observed decreased Homer 1b/c expression in the mPFC and increased Homer 1b/c expression in the BLA compared to the CTRL group (D). Representative photomicrographs of Homer 2 immunofluorescent staining in the mPFC, NAc, and Amy of the two experimental groups (E). pTHC increased Homer 2 immunofluorescence in the shell and core of the NAc and BLA compared to the CTRL group (F). Magnification 40 $\times$ , scale bar = 40  $\mu$ m for all images. Data are shown as the mean  $\pm$  SEM. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ . mPFC = medial prefrontal cortex; NAc = nucleus accumbens; BLA = basolateral amygdala; CeA = central amygdala; CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC.

## Discussion

The present research investigates the effects of *in-utero* THC exposure on behavioral reactivity, neutral and limbic memory, instrumental learning, and alcohol vulnerability in adolescent offspring. The results highlight how the manipulation of the endocannabinoid signal at critical developmental stages impacts cognitive functions when these are strongly dependent on emotional processing, such as limbic memory. Moreover, for the first time, prenatal exposure to THC is shown to confer vulnerability to alcohol motivational properties and contributes to the development of alcohol addictive-like behaviors in adolescent offspring. The occurrence of this multifaceted phenotype is associated with a reduction in NPY+ neurons and disarrangement in the PSD Homer make-up in relevant function-related brain regions.

Pregnant women use cannabis as a natural substitute for prescribed medications to manage mood, stress, and morning sickness, as well as the well-known recreational effects (Metz and Borgelt, 2018), due to the fact that data on cannabis use is not always consistent and persuasive. Unfortunately, clinical assessment of developmental outcomes of prenatal cannabis exposure is limited by poor control of timing and dosage, disclosure, underreporting, and underestimation. Therefore, although mimicking human developmental cannabis exposure presents some limitations, the animal model of prenatal THC exposure is essential in systematically exploring specific neurobiological mechanisms that underlie putative abnormalities in adolescent brain and behavior, and allows the intervening confounding variables to be controlled (DiNieri and Hurd, 2012). Under these experimental conditions, adolescent offspring that were exposed *in-utero* to THC displayed increased locomotor activity, but no differences in the exploratory pattern of the arena compared to CTRL rats. Preclinical data on the developmental effects of cannabinoid exposure on the ontogeny of motor behavior have shown different and sometimes conflicting results (Brake et al., 1987; Campolongo et al., 2011; Fride and Mechoulam, 1996; Fried, 1976; Navarro et al., 1995; Trezza et al., 2008). However, the present findings are in line with human data showing that children and adolescents prenatally exposed to cannabis are hyperactive as well as impulsive (Fried and Smith, 2001; Fried and Watkinson, 1988; Goldschmidt et al., 2004). Under the neutral conditions of the open field arena, prenatal THC exposure did not induce signs of anxiety-like behavior. Thus, the increase in locomotion could be the result of a decreased habituation to a novel environment compared to the CTRL group. Since habituation per se represents a learning process of the environmental setting, one could speculate that the hyper-locomotor effect observed in this study may underlie a delay in simple learning (Breit et al., 2019) rather than a prominent effect on emotionality. Further investigation of spatial learning abilities, induced by prenatal THC exposure in the present research project, will clarify this point. Interestingly, when adolescent offspring were tested for aversive limbic memory, that is, explicit

memory traces of an emotionally salient experience (Brancato et al., 2016), while CTRL animals recognized and avoided the emotional object paired to the aversive emotional experience and the area of the maze where it was placed, pTHC offspring decreased both avoidance of the emotional object and conditioned place aversion, indicating that prenatal exposure to THC exerted a detrimental effect on the formation of emotionally aversive memory traces. The recognition of the emotional object as fearful – and its avoidance – makes the Emotional Object Recognition task a discriminative paradigm based on active responses of rats – rather than on implicit nonspecific freezing behavior – that enables the researcher to correlate animal cognitive performance with critical aspects of human emotional memory. This comes as no surprise. Indeed, the ECS has emerged as an important regulator of stress response and emotional behavior (Hill et al., 2011; Lutz et al., 2015). Consistently, prenatal exposure to cannabinoids induces subtle yet persisting changes in cognitive functions that emerge when cognitive abilities require emotional integration (Bara et al., 2018; Mereu et al., 2003; Morena et al., 2015). Indeed, whereas activation of CB1R within the mPFC strongly potentiates normally non-salient emotional memory formation (Laviolette and Grace, 2006; Tan et al., 2014), THC administration alters facial recognition (Hindocha et al., 2015), decreases, in the Amy, blood oxygen level-dependent response to threatening faces (Phan et al., 2008) and preferentially impairs memory for emotional events in humans (Ballard et al., 2013). The mechanisms by which CB1R transmission regulates emotional memory formation are not entirely understood, but a single systemic stimulation of the cannabinoid transmission by the CB1R agonist WIN 55, 212-2 decreases limbic memory in the same EOR task in adult rats (Brancato et al., 2016). Besides directly modulating excitatory/inhibitory signaling, the ECS can biphasically modulate both rewarding and aversive emotional information by functionally interacting with the DA system (Laviolette and Grace, 2006; Rey et al., 2012), which is of exceptional importance for gating attention and facilitating conditioned stimulus associations during fear conditioning (Bromberg-Martin et al., 2010; Pezze and Feldon, 2004). Therefore, one could hypothesize that prenatal THC exposure may disrupt the integration between the abilities to form explicit memories and the emotional valence paired with that memory by dysregulating mesocorticolimbic transmission and the processing of emotionally salient information (Cannizzaro et al., 2019). Prenatal THC-induced perturbation of neurodevelopmental trajectories in the mesocorticolimbic system may also pose a risk for vulnerability to alcohol and drug consumption early in adolescence. Strikingly, no data on adolescents' vulnerability to alcohol – the most abused drug among teenagers (UNODC, 2018) – as a result of early exposure to THC are currently available. To test that, an operant paradigm was designed and recorded alcohol-taking, alcohol relapse, and alcohol use despite negative consequences in both pTHC and CTRL adolescent offspring. The first result on the rate of acquisition of the operant paradigm before the alcohol

vulnerability assessment had started: while all CTRL rats acquired the instrumental responding for water in five days, only about half of the preadolescent pTHC rats learned the same operant task in the same time interval and joined the CTRL rats in the operant task for alcohol; the remaining offspring were then excluded from the experiment. The delay in the acquisition of the operant task observed in pTHC rats is consistent with data showing an acquisition deficit in heroin self-administration in THC-exposed rats (Spano et al., 2007), and since a motor deficit can be ruled out by the open field outcome, it might result from impaired effort-related decision making as a consequence of the prenatal THC exposure. On the other hand, the present data show that interference in the cannabinoid system during gestation promoted alcohol vulnerability in adolescent rats. Indeed, whether during the first weeks of alcohol presentation no differences in the operant behavior appeared between the two groups, during the last week of the acquisition window, pTHC rats increased the number of lever presses compared to CTRL animals, likely as a result of increased motivation for alcohol. Moreover, after a week of forced abstinence, both pTHC and CTRL rats elevated their responses to alcohol compared to the acquisition phase, but prenatally THC-treated rats displayed a greater increase than CTRL animals. Indeed, re-presentation of alcohol after a period of forced abstinence usually leads to a robust but temporary increase in alcohol intake over baseline drinking – a relapse-like behavior referred to as the alcohol deprivation effect (Vengeliene et al., 2014), which reflects increased craving or increased reinforcing value of alcohol in humans (Söderpalm et al., 2019). Preclinical and clinical findings have consistently reported how endocannabinoid transmission modulates alcohol- taking and craving (De Vries and Schoffelmeer, 2005; Serra et al., 2002). Indeed, alcohol-rewarding properties implicate ECS-mediated reduction of GABA inhibition onto VTA DA neurons (Barrot et al., 2012; Lupica and Riegel, 2005). The voluntary administration of alcohol in the presence of response-contingent shock punishment reliably models compulsive drug use despite adverse consequences (Cacace et al., 2012; Deroche-Gamonet et al., 2004; Plescia et al., 2013), which mimics a core feature of the vulnerable phenotype to addiction (Vanderschuren and Ahmed, 2013). In the operant conflict procedure used here, responses were alternatively paired to a footshock, signaled by a light cue, which led to a suppression of conditioned responses for the reinforcement in CTRL rats. Instead, the punishment was less effective in inhibiting the operant responding to alcohol in pTHC adolescent rats, which displayed a higher number of punished responses than CTRL animals, indicating either an increase in the motivation for alcohol or lesser sensitivity to the punishment. However, pTHC rats did not show differences in tail-flick latency compared to CTRL rats, ruling out a non-specific effect on nociception. To our knowledge, this is the first report showing that prenatal THC exposure may induce a susceptible phenotype to alcohol addictive properties in adolescent offspring; the inconsistency with previous research that did not highlight a facilitatory effect of

perinatal THC on alcohol operant behavior (Economidou et al., 2007) shows that doses, time of exposure and experimental design may represent fundamental variables in the identification of behavioral read-outs of neurodevelopmental vulnerability. Indeed, in the present experimental conditions, THC exposure from the early gestational period to delivery (a time window equivalent to the first and second trimesters in humans) was able to induce alcohol vulnerability in adolescent offspring, whereas in the study by Economidou et al. (2007), higher THC doses (5 mg/kg) administered within the GD 15 - PND 9 period (corresponding to the second and third trimester in humans) did not exert such an effect. Previous studies have demonstrated that developmental exposure to cannabinoids enhances sensitivity to heroin and morphine self-administration and heroin-seeking following a mild food deprivation stress (Spano et al., 2007; Vela et al., 2008) likely through alterations in DA receptor 2 gene expression in the NAc of the offspring exposed *in-utero* to cannabis (DiNieri et al., 2011). Previous results showed that the systemic administration of a D2 autoreceptor agonist and a specific CB1R antagonist was able to decrease drug-seeking, relapse after forced abstinence, and resistance to punishment for alcohol's neuroactive metabolite ACD (Brancato et al., 2014; Plescia et al., 2013). Altogether, abnormal DA and ECS signaling may result in modifications in the motivational properties of alcohol, thus highlighting that THC *in-utero* exposure may pose a risk for increased vulnerability to alcohol-addictive behaviors. On the other hand, one could parallel the data from the conflict session with the results of the conflict session of the Emotional Object Recognition test, interpreting the abnormal resistance to the punishment in the operant chamber as weaker learning of the pairing between the punishment and the lever pressing. Furthermore, since dysfunctions of the synaptic triad amongst glutamate, GABA, and DA in the mesocorticolimbic regions have long been implicated in the underlying pathogenesis of alcohol use disorders (Cannizzaro et al., 2019; Spiga et al., 2014), it is reasonable to suggest that the increased operant behavior for alcohol observed in this study can result from prenatal THC-induced aberrant plasticity in specific areas of the brain that are functionally associated with reward, motivation and salience attribution (Brancato et al., 2014, 2018; Cannizzaro et al., 2019). Overall, from this first set of data, it appears that aversive and conflicting environmental challenges can be particularly helpful in unveiling the early phenotypic consequences of pTHC. Moreover, it can be hypothesized that prenatal THC exposure can exert a detrimental effect on coping abilities when integration between environmental stimuli's encoding and emotional control is required, making male offspring more vulnerable towards dysfunction in emotionally salient learning and memory and sensitivity to alcohol-addictive properties. Whether female offspring display the same characteristics is not known at the moment. However, male offspring seem to be particularly vulnerable to detrimental effects of *in-utero* THC, since prenatal THC exposure altered sensorimotor gating functions in a THC challenge

in preadolescent male offspring, whereas female offspring were resilient (Frau et al., 2019). Among the players that contribute to adaptive coping with stress, NPY plays a major role. A considerable amount of literature on NPY supports an anxiolytic and antidepressant-like activity (Bowers et al., 2012; Heilig, 2004; Wu et al., 2011) and its association with decreased stress responses and the expression of resilience in rodents and humans (Morgan et al., 2000; Sajdyk et al., 2008). Furthermore, NPY signaling controls alcohol-related vulnerability, since the activation of Y1 and Y2 receptors in the extended Amy and mPFC decreased alcohol consumption and self-administration (Robinson et al., 2019; Robinson and Thiele, 2017). In the current study, NPY+ neurons were measured in brain regions crucial to cognitive functions, motivation, and emotional regulation, such as mPFC, NAc, and Amy. Despite limited evidence on the interplay between ECS and NPY signaling, previous work from this group observed an inverse relation between the two systems (Plescia et al., 2014), since the administration of AM281, a selective CB1R antagonist, significantly increased NPY+ neurons in both the hippocampus and in the NAc of control and ACD-withdrawn rats. Now, it is reported here that THC interference in the endocannabinoid signaling during pregnancy is associated with a decreased number of NPY+ cells in the mPFC. NPY is abundantly expressed in cortical areas where it exerts a potent inhibitory effect on the neuronal excitability of projection neurons (Bacci et al., 2002). As a matter of fact, the decrease in the NPY-ergic tone observed in the PFC of pTHC adolescent offspring was associated with dampened limbic memory and instrumental learning, both functions dependent on the contribution of the mPFC (Caballero et al., 2019). Therefore, based on the existing evidence, it is reasonable to speculate that the decrease in NPY signaling would significantly alter the functional activity of the projecting neurons (Vollmer et al., 2016), contributing to the cognitive impairment observed in pTHC offspring. In addition, NPY is reported to enhance memory retention in T-maze footshock avoidance and step-down passive avoidance training in mice (Flood et al., 1987). Recently, blunted DA signaling and altered glutamate connectivity in the NAc have been proven to be a major neural underpinning of impaired aversive limbic memory (Cannizzaro et al., 2019). In this regard, a significant decrease in NPY+ neurons was observed in the shell and core subregions of NAc of pTHC rats compared to controls. Clinical and preclinical reports indicate that NPY in the NAc may modulate salience attribution, regardless of the stimulus valence (Brown et al., 2000; Josselyn and Beninger, 1993; Warthen et al., 2019), suggesting that the observed reduction in NPY levels in NAc GABAergic interneurons might contribute to the dampened cognitive functions displayed by prenatally THC-exposed rats in this study. In addition, previous evidence reports that perinatal THC exposure induces presynaptic disarrangement of discrete components of glutamatergic transmission, well-known involved in synaptic plasticity during learning and memory (Campolongo et al., 2007; Suárez et al., 2004). Thus, in order to verify the



occurrence of abnormal neuroplasticity at the excitatory postsynaptic site, the expression of different isoforms of the PSD proteins of the Homer family – Homer-1, 1 b/c, and 2 – following prenatal THC exposure was investigated. As a matter of fact, along with decreased NPY expression, a general increase in different Homer isoforms in the corticolimbic brain regions was found. In more detail, in the mPFC of pTHC rats, the increased expression of Homer 1 was paralleled by a decrease in the constitutively expressed long isoform Homer 1 b/c, which normally provides the structural scaffold of the excitatory signalosome (Castelli et al., 2017). This suggests that a Homer 1 increase is related to the overexpression of the activity-induced, short isoform Homer 1a that serves as a transient disruptor of optimal postsynaptic glutamate signaling (Clifton et al., 2019). Again, besides the decrease in the NPY-ergic tone, increased recruitment of the long isoform Homer 2 was measured in both the shell and core subregions of the NAc, suggesting an abnormal excitatory postsynaptic plasticity. Enhanced activity of the mPFC could in turn suppress aversive limbic memory by activating inhibitory Amy microcircuits (Maren and Quirk, 2004; Paré et al., 2004). At this level, prenatally THC-exposed offspring displayed decreased NPY+ cells in the BLA and CeA, and increased Homer expression – namely, Homer 1 in both the BLA and CeA and the long functional isoforms Homer 1b/c and 2 in the BLA compared to controls. This abnormal PSD make-up might contribute to alterations in the local modulatory mechanism of negative limbic memory (Quirk and Mueller, 2008). Intriguingly, alterations of discrete functional components of the PSD at the glutamatergic synapse within the NAc and Amy underlie the development of acute and chronic alcohol-induced behavioral plasticity (Castelli et al., 2017). In particular, preclinical and clinical data suggest that Homer 2 is an essential and active player in the expression of alcohol-induced behavioral and cellular plasticity and, in turn, is crucial in promoting alcohol consumption. Virus-mediated Homer 2 overexpression in the NAc enhanced alcohol motivational properties in an operant self-administration paradigm and facilitated the expression of alcohol-conditioned place preference (Haider et al., 2015; Szumlinski et al., 2008). Therefore, here, it can be suggested that the reduced NPY expression – and the spread disarrangement of the PSD make-up – in the limbic brain regions observed during adolescence, may represent pieces of the intricate puzzle – the neurobiological substrate – that underpins the escalation in alcohol consumption of pTHC male rats. Accordingly, several reports indicate that reduced NPY levels in the NAc contribute to increased sensitivity to alcohol (Barkley-Levenson et al., 2016; Borkar et al., 2016), whereas infusions of NPY into the CeA normalize alcohol intake in alcohol-preferring rats (Zhang et al., 2010). However, an interplay between the disarrangement in NPY and PSD proteins and aberrant plasticity due to the higher alcohol intake during the last week of acquisition in pTHC male rats cannot be ruled out, thus contributing to the occurrence of the alcohol-prone phenotype observed during the relapse and

conflict sessions. Overall, these findings provide the first evidence of deficient NPY signaling and Homer-mediated dysfunctional synaptic plasticity as a common background of limbic memory dysfunction, impaired instrumental learning, and the onset of vulnerability to alcohol in prenatally THC-treated adolescent male offspring.

In conclusion, *in-utero* exposure to THC can involve enduring consequences on the neurodevelopmental trajectories towards adolescence, resulting in a vulnerable phenotype for impaired emotional/cognitive functions and alcohol addictive-like behavior in male rat offspring. This is associated with a dysregulation in NPY expression and signaling and PSD make-up in mesocorticolimbic regions.

## PART 2

### Introduction

The general evidence links cannabis to cognitive decline (Shrivastava et al., 2011). De facto, specific neuropsychological parameters, which demand higher cognitive commitment – i.e. response time, prolongation of word viewing time, residual verbal memory, and executive functioning – have been found to be affected by cannabis consumption (Korver et al., 2010). Pieces of evidence from both animal and human studies suggest that the severity of the effects of cannabis consumption on cognitive development is dependent on the age when this begins (Dragt et al., 2010). Indeed, the use of cannabis during critical developmental periods may induce persistent alterations in the still-maturing brain structures and functions (Welch et al., 2011). Notably, it appears that potentially compensatory mechanisms, that might occur during neurodevelopment, are insufficient to delete vulnerability to neuropsychiatric disorders. However, despite representing an issue increasingly recognized in the field of cannabis research, the impact of cannabis on the developing brain is still not entirely defined (Shrivastava et al., 2011).

### ECS-dependent long- and short-term synaptic plasticity

The role of the ECS in synaptic plasticity is the key to understanding the link between cannabis and cognitive performance (Shrivastava et al., 2011). Both transient and long-lasting fashion of ECS-mediated synaptic plasticity occurs in the CNS. eCBs are the retrograde messenger for depolarization-induced suppression of inhibition (DSI)/depolarization-induced suppression of excitation (DSE) in the hippocampus, thus, the major contributor to short-term synaptic plasticity. The first demonstration that in situ eCBs release could short-term modulate synaptic transmission at hippocampal GABAergic synapses is the blocked DSI in hippocampal pyramidal neurons by the CB1R antagonist SR 141716A (rimonabant, Acomplia; Wilson and Nicoll, 2001), and its absence in mutant mice lacking the CB1R (CB1R<sup>-/-</sup>; Wilson et al., 2001; Ohno-Shosaku et al., 2001). Later, DSE identified at several central glutamate synapses was also found to require the intervention of eCBs (Kreitzer and Regehr, 2001; Melis et al., 2004). Moreover, metabotropic induced suppression of inhibition or excitation (MSI/MSE) is a similarly ubiquitous form of ECS-mediated short-term synaptic plasticity: elicited by the engagement of post-synaptic receptors, including metabotropic glutamate receptor 5 (mGluR5), it leads to the release of 2-arachidonoylglycerol – 2-AG, the most prevalent endogenous cannabinoid ligand in the brain (Sugiura et al., 1995) – which diffuses presynaptically to activate CB1R and suppress synaptic transmission. Nonetheless, the short-term regulation of synaptic

transmission interacts with and facilitates conventional forms of long-term synaptic plasticity (Heifets and Castillo, 2009), such as the facilitation of glutamatergic long-term potentiation (LTP) by DSI. When a weak stimulus that is insufficient to alone trigger LTP is paired with the transient, 2-AG-dependent inhibition of GABA release via DSI, LTP can be observed in hippocampal pyramidal cells (Carlson et al., 2002). On the other hand, ECS signaling is also known to play a direct role in the modification of long-term synaptic plasticity, in both excitatory and inhibitory synapses (Heifets and Castillo, 2009). ECS-long-term depression (LTD) induction typically begins with a transient increase in activity at glutamatergic afferents and a concomitant release of eCBs from a target, postsynaptic neuron. eCBs then travel backward across the synapse, activating CB1R on the presynaptic terminals of either the original afferent (homosynaptic eCBs-LTD) or nearby afferents (heterosynaptic eCBs-LTD). Notably, in the hippocampus, eCBs-LTD also modulates excitability and the induction of LTP at excitatory synapses, a process widely thought to underlie memory acquisition (Heifets and Castillo, 2009).

### Hippocampal CB1R and related functions

The hippocampus is a forebrain structure that participates in cognitive functions such as learning, memory, and sensory integration (Scarante et al., 2017; Galve-Roperh et al., 2013; Mackie, 2005). Here, CB1R is enriched on GABAergic interneurons and widely localized on glutamatergic axon terminals of most excitatory pathways (Katona et al., 1999; Marsicano et al., 1999; Nyiri et al., 2005; Gutierrez-Rodriguez et al., 2018). The peculiar wide localization of CB1R on pre-synaptic terminals reflects its major role in modulating synaptic transmission, by inhibiting inhibitory and excitatory signals (Sugaya et al., 2022). Along with the above-discussed ability of the ECS to affect short- and long-term changes in synaptic strengthening, this evidence points to CB1R a role in cognitive processing (Varvel et al., 2009; Busquets-Garcia et al., 2016; Hampson and Deadwyler, 1999). The inhibition produced by endocannabinoid signaling in hippocampal GABAergic neurons is necessary for complex learning processes such as incidental associations (Busquets-Garcia et al., 2018). In contrast, NMDAR-mediated excessive excitatory signaling, likely facilitated by the suppression of GABAergic inhibition, was shown to be the substrate for the long-term amnesic effects induced by cannabinoids (Puighermanal et al., 2009; Han et al., 2012). Although the THC-induced impairment in hippocampal LTP could result from its actions at CB1R located on either glutamatergic or GABAergic terminals, the genetic deletion of these receptors from only GABAergic neurons reduces LTP, whereas their deletion on glutamatergic neurons enhances LTP (Monory et al., 2015). Using a similar selective CB1R deletion strategy, it was also shown that the memory impairment caused by acute THC was absent when CB1R was deleted on GABAergic terminals, where these receptors are

more abundantly expressed (Puighermanal et al., 2009, 2013). Consistent with these results, long-term THC exposure has a larger impact on CB1R on GABAergic axon terminals than on glutamatergic terminals, and THC-induced alterations contribute to deficits in LTP generated at glutamate synapses (Carlson et al., 2002; Chevalleyre and Castillo, 2004).

### Spatial and object memory: motivated and fear-induced cognitive processing

Spatial discrimination processes are supported by the activation of the hippocampus, the core of the neural memory system. In particular, at the cellular level, the hippocampus encodes and retrieves spatial memories through the firing dynamics of the place cells (Dupret et al., 2010; Kentros et al., 2004; Smith and Mizumori, 2006). Intriguingly, reward expectation, plus their specific locations within an environment, supports and strengthens the encoding and retrieval of spatial information by firing place cells at the location that leads to reward, with high trial-by-trial and day-by-day reliability (Danielson et al., 2016; Hollup et al., 2001; Lee et al., 2006). This mechanism, which is crucial for survival, guides future decisions about which locations lead to reward, likely through a DA-hippocampal circuit. Specifically, DA could promote glutamatergic synaptic transmission of specific sets of inputs that drives place cell firing in real-time (Li et al., 2017). Therefore, disruption of the glutamate network would trigger defective mapping by making place cells unreliable for the reward location (Gill and Mizumori, 2006; Mamad et al., 2017). Nonetheless, since functional interactions between the hippocampus, VTA, and NAc have been shown to be critical for controlling the contextual and emotional salience of various cognitive behaviors, dysregulation of DA transmission within this circuitry has been proposed as a potential underlying mechanism for cognitive deficits, which include distortions in affective salience processing, and memory-related impairments (Caragea and Manahan-Vaughan, 2022; Lee et al., 2014). A critical role for CB1R transmission directly in the hippocampus has been highlighted both in the regulation of the mesolimbic activity pathway and during the processing of memory phenomena. Indeed, activation of hippocampus CB1R transmission was shown to alter the salience of fear-related conditioning cues, causing significant disruptions in recognition memory formation (Tan et al., 2014). These effects were dependent upon the ability of intra-hippocampal CB1R stimulation to induce an overdrive of the mesolimbic DA system (Kramar et al., 2017).

Noteworthy, these reward-place hippocampal neurons encode associations between places- but not objects-and-rewards, rather than object-reward pairing as shown by employing visual discrimination task, in which orbitofrontal cortex neurons are differentially fired (Rolls et al., 1996). Indeed, studies on the hippocampal role in the non-spatial, visual recognition memory and retention of object discrimination reported inconsistent effects of a hippocampal lesion on this ability (Mumby et al.,

1999, Vnek and Rothblat, 1996; Lehmann et al., 2007; Sanderson et al., 2006; Basile et al., 2020). On the other hand, some studies showed impaired retention of visual object discrimination after hippocampal lesion (Epp et al., 2008; Driscoll et al., 2005). It may be that rats use two types of cognitive strategies for discriminating objects – one hippocampal-dependent and other hippocampal-independent: if the objects have no other features except geometric shapes, then the rats need the hippocampus in order to discriminate them (Poulter et al., 2020). However, the fasciola cinereum (FC), an anatomically discrete and functionally critical subregion of the hippocampus, has recently received attention for its role in hippocampal learning and memory. The FC exhibits afferent projections from perirhinal and lateral entorhinal cortex (Hjorth-Simonsen, 1972), and sends its output only to the dentate gyrus (DG) in the rat hippocampus. Place cells found in the FC modify their firing rates in response to visual contexts to a greater extent than CA1 place cells. Moreover, rats with selective lesions in the FC were impaired with respect to the acquisition of novel visual contextual memory compared with controls, but not in retrieving familiar visual contextual memories (Park et al., 2022).

Processing and discriminating (visual) stimuli is crucial for encoding, consolidation, and retrieval of memory traces, thus for attention, inhibitory control abilities (Alain and Woods, 1999; Plescia et al., 2013, 2014). However, concern raises about the equal-opportunity incentive that balances motivation among groups of animals and does not apply to appetitive tasks (Vorhees et al., 2014). Besides, fear-eliciting contexts influence the performance of executive tasks, including attention, working memory, inhibition, and flexibility in the cognitive processes (Chen et al., 2022; Heyser et al., 2000) and physiologically prompt instinctive defensive behaviors. As aversive stimuli are linked with particular places, animals need to learn the association between a space and an aversive event and decide whether to initiate avoidance behavior against aversive locations. In some circumstances, they accept aversive situations to obtain a reward to ensure their survival. Besides the canonical brain regions associated with negative emotions in mammalian systems (i.e. Amy), evidence points to a role of the flexible reorganization and firing of hippocampal neurons in learning processing, in a novel, aversive environment, and the subsequent decision-making (Okada et al., 2017). Indeed, the introduction of an aversive cue into a place has been demonstrated to alter the spatial representation and firing rates of place cells that encode the locations tied to the aversive events (Moita et al., 2004; Wang et al., 2012; Wu et al., 2017). These results demonstrate that hippocampal neurons incorporate information regarding aversive events into an existing spatial framework waiting to undertake approach/avoidance behavior against hostile environments. Different neuronal mechanisms have been involved in the plastic changes of hippocampal neurons. CB1R and eCBs mediate short- and long-term modulation of synaptic transmission and have been shown to be involved in neural

plasticity mechanisms related to the processing of fear memories in the hippocampus (Akirav, 2011; Segev et al., 2018). Consolidation specifically is enhanced by intra-hippocampal administration of anandamide and impaired by intra-hippocampal injection of AM251, a cannabinoid antagonist, in an inhibitory avoidance paradigm (de Oliveira Alvares et al., 2005). Therefore, CB1R in the hippocampus is involved in memory consolidation, and failure to consolidate may suggest a relative decrease in the effectiveness of the endogenous cannabinoid system.

### Cannabidiol as the antithesis of THC in the modulation of cognitive processing

THC, the most prevalent cannabinoid in the cannabis plant, is responsible for the widely known psychoactive effects of the plant. In contrast, CBD, another abundant compound of cannabis, might have an impact on cognitive functioning which is opposite to the effect of THC, so their widely-explored pharmacology (Kowal et al., 2013).

The therapeutic value of CBD in clinical contexts is currently being investigated (Crippa et al., 2018). CBD has received attention mainly due to its anxiolytic (Crippa et al., 2011; Bergamaschi et al., 2011) and antipsychotic effects (Davies and Bhattacharyya et al., 2019; Schubart et al., 2014). Notably, given their different, even antagonistic properties, it is likely that THC and CBD also exert distinct effects on cognitive functioning (Martin-Santos et al., 2012; Bhattacharyya et al., 2010). Recent evidence suggests that CBD has no effect on cognition in healthy individuals, but can improve cognitive processes including attention, executive function, working memory, and episodic memory in various pathological conditions including acute THC intoxication (Osborne et al., 2017; Curran et al., 2020). Plus, CBD has been shown to differ from THC in terms of activation of brain regions during tasks involving response inhibition (Borgwardt et al., 2008), emotional processing (Fusar-Poli et al., 2009), and verbal memory (Bhattacharyya et al., 2010). Despite, the effect of CBD on cognitive performance has been largely unexplored and deceiving (Curran et al., 2020). In contrast to research showing no effect of nabiximol (a compound with a  $\Delta 9$ -THC:CBD ratio of 1:1) on working memory performance impaired by a high dose of synthetic  $\Delta 9$ -THC (dronabinol), studies have also reported that while individuals who used cannabis strains with lower CBD content had marked impairment on various memory tasks, those smoking cannabis high in CBD concentrations showed no performance deficits relative to the placebo condition. Nonetheless, the combination of THC and CBD exposure spared healthy individuals from impaired emotional recognition. Altogether, CBD seems to contrast the deleterious cognitive effects of acute THC exposure, for cognitive processing. While encouraging, these findings need further supporting information for the identification of neurochemical substrates.

## Aim

Given the thrilling results of part 1 of the present research, the next working hypothesis was that, besides or beyond motivational features induced by alcohol vulnerability in the adolescent offspring, the observed lower learning ability may be due to direct cognitive deficits induced by *in-utero* exposure to THC. This may have a huge impact on *in-utero* THC research since the effective demonstration of cognitive processes is required for normal functioning in everyday life (Logue and Gould, 2014). Moreover, in order to provide the solution to the issue, it was tempting to explore the potential recovery approach of CBD – as the non-psychoactive component of marijuana – in rectifying learning paucity.

Thus, part 2 of the present research aimed at evaluating (1) whether prenatal THC exposure induces alteration in the cognitive phenotype resulting in the core of executive functions, which linger to adolescence and adulthood. Specifically, following *in-utero* THC exposure (2 mg/kg delivered subcutaneously), adolescent and adult male rat offspring were assessed for cognitive processes needed at the core of executive functions, namely consolidation and retrieval of reference- and working memory in spatial- and visual discrimination tasks, as well as cognitive flexibility, triggered by both reward – Can Test – and aversive – Barnes Maze Test – prompting settings. To exclude confounding factors, namely altered maternal caregiving or an anxiety-like phenotype of the progeny, maternal behavior and behavioral reactivity of the offspring were assessed. In addition, (2) the ability of CBD in rescuing impaired cognitive functions in adolescent and adult progeny prenatally exposed to THC was explored. Notably, the specific involvement of the CB1R was assessed by the pharmacological pre-treatment with the CB1R antagonist AM281, before CBD administration. Lastly, (3) to propose a cognitive endophenotype, the expression of markers of ECS – CB1R – post-synaptic density – Homer 1 isoform – and glutamate – NMDAR NR1 subunit and mGluR5 – were evaluated in the hippocampus of the adolescent and adult progeny.



## Methods

### Animals and treatment

Twelve adult female pregnant Wistar rats at GD 4 (200–220 g; Envigo, Milan, Italy) were housed singly in standard rat cages (40 cm × 60 cm × 20 cm), with ad libitum access to water and food in a temperature- and humidity-controlled room ( $22 \pm 2^\circ\text{C}$  and  $55 \pm 5\%$ , respectively) on a 12-h light/dark cycle. They were given daily s.c. injections with Veh (Veh dams) or THC (2 mg/kg; THC dams) from GD 5 to GD 20. THC dose was chosen based on previous studies (Brancato et al., 2020; Frau et al., 2019). After weaning, male rats were housed in pairs, and each experimental group for the behavioral paradigms included one or two independent male rats per each litter of Veh- or THC-treated dams. All experiments were conducted in accordance with animal protocols approved by the Committee for the Protection and Use of Animals of the University of Palermo in accordance with the current Italian legislation on animal experimentation (D.L. 26/2014) and the European directives (2010/63/EU) on care and use of laboratory animals. Every effort was made to minimize the number of animals used and their sorrow.

### Drugs

THC resin, extracted by the Forensic Laboratory of Biologically Active Substances of the University of Chemistry and Technology of Prague, Czech Republic, (purity (HPCL) > 97%; Drazanova et al., 2019), was dissolved in ethanol at 20% final concentration, and then sonicated for 30 min. THC was emulsified in 2% Tween 80 and then dissolved in sterile physiological saline. Rat dams were subcutaneously administered THC (2 mg kg/L) or Veh (1-2% Tween 80, saline) in a volume of 2 mL kg/1 from GD 5 until GD 20. CBD, extracted by the Forensic Laboratory of Biologically Active Substances of the University of Chemistry and Technology of Prague, Czech Republic (purity (NMR) > 99%; Hložek et al., 2017), was dissolved in a Veh of ethanol (1%), Tween 80 (1%), saline and, immediately afterward, administered intraperitoneally (i.p.) at the dose of 40mg/kg, 24 h prior to testing (Gasparyan et al., 2020). AM281 (Sigma, Italy) was initially dissolved in 3% ethanol, then 3% Tween 20 and 94% saline (Cannizzaro et al., 2016). AM281 was administered i.p. at a dose of 1 mg/kg, 30 m prior to CBD administration. Control rats received the same volume of Veh at the same time points (1 mL/kg).

### Behavioral procedures

Maternal behavior was observed after delivery from PND 1 to 12. Male rat offspring, prenatally exposed to either Veh (CTRL) or THC (prenatal THC exposure, pTHC) were tested during

adolescence – starting from PND 35 to 55 – and adulthood – from PND 63 to 83 – during the light phase of the light/dark cycle. Firstly, the offspring performed the OFT, to assess locomotor activity and behavioral reactivity in a novel environment. Afterward, the progeny underwent a behavioral battery tailored to explore cognitive processes needed at the core of executive functions (Diamond, 2013), by employing the Can Test – a reinforce-motivated task that allows the assessment of both spatial memory acquisition and visual discrimination (Plescia et al., 2014; Popovic et al., 2001) – and the Barnes Maze Test – a mild aversive, dry-land based behavioral test developed to study spatial learning and memory retrieval, as well as cognitive flexibility in rodents (Barnes, 1979). In detail, the spatial acquisition was explored in the spatial task, and object recognition and memory were assessed by the simple visual task of the Can Test; spatial orientation and acquisition were also measured in the acquisition task of the Barnes Maze Test, while spatial memory retrieval was assessed in the probe task, and the reversal task of the Barnes Maze Test was employed to assess cognitive flexibility. Twenty-four h before the Can Test, the offspring were exposed to either Veh or CBD (CTRL- or pTHC- CBD rats), to assess the potential pro-cognitive impact of CBD. Moreover, in order to likely dissect out the mechanism of CBD, either Veh or CB1R antagonist AM281 (CTRL- or pTHC-AM281 + CBD rats) was administered 30 m before the administration of CBD. Lastly, working memory was assessed in the spontaneous alternation T-Maze test (figure 12). The objects and the apparatus used were cleaned thoroughly with 70% isopropanol, dried with tissue paper, and rinsed again with water at the end of each experimental session. The behavior of the rats was monitored and quantified by the experimenter and an automatic video-tracking system, AnyMaze (Stoelting Europe, Dublin, Ireland).

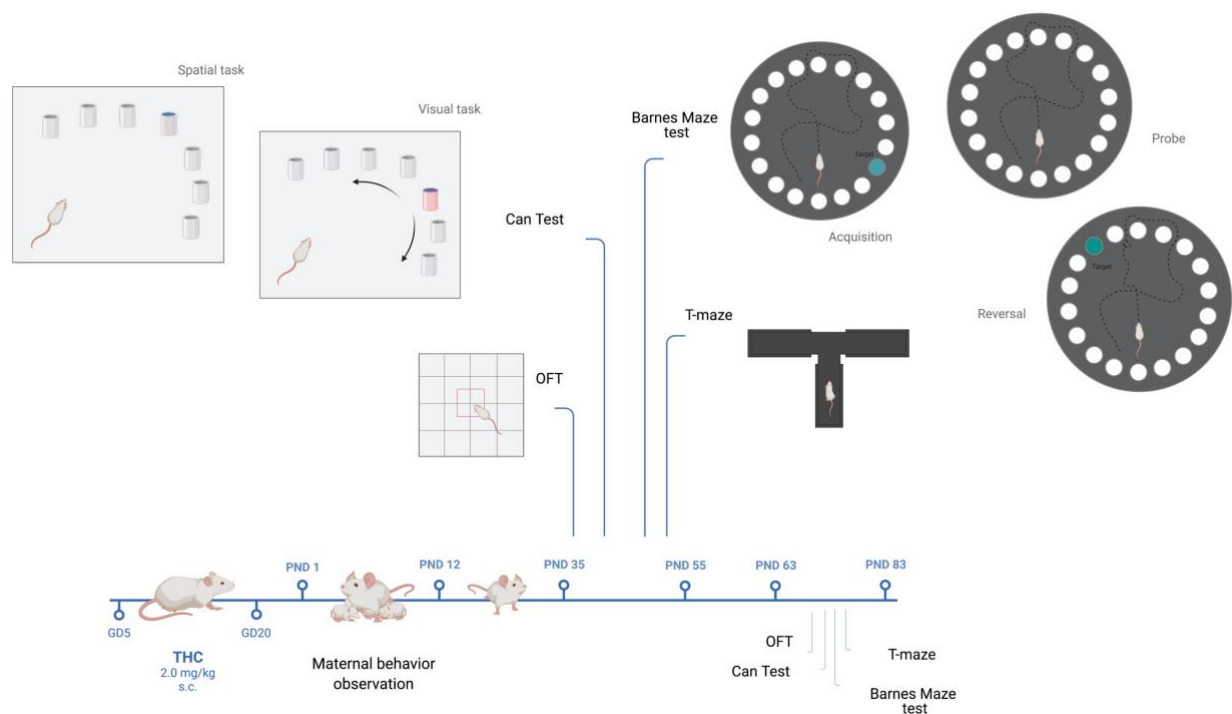


Figure 12. Behavioral procedures. Maternal behavior was observed from PND 1 to 12. Male rat offspring prenatally exposed to either vehicle (CTRL) or THC (2 mg/kg; pTHC) were tested in adolescence, starting from PND 35 to 55, and adulthood, from PND 63 to 83 in the behavioral battery. GD = gestational day; PND = postnatal day; s.c. = subcutaneous; THC =  $\Delta^9$ -tetrahydrocannabinol; OFT = open field test.

### Assessment of maternal behavior

Drugs of abuse may affect a mother's ability to provide optimal care for the progeny (Brancato and Cannizzaro, 2018). Furthermore, it is well known that the duration of specific maternal behaviors can modulate spatial reference and working memory in offspring tested in Morris Water Maze and a spatial/object paradigm (Cannizzaro et al., 2005; Liu et al., 2000). Thus, to assess whether the prenatal THC exposure altered the mother-infant dyad, maternal behavior was observed. The behavior of the dams was analyzed in five daily sessions, conducted at 8:00 AM, 10:00 AM, 2:00 PM, 5:00 PM, and 8:00 PM in the home cage under undisturbed conditions (Pires et al., 2020). The explored phenotypic correlates of maternal behavior are included in two discrete dimensions: pups- and non-pups-oriented behavioral repertoire (table 2; Brancato and Cannizzaro, 2018).

Behavior	Description
Pups-oriented behaviors	
Nursing	The first and most characteristic pup-oriented maternal behavior to occur. Once nursing is initiated, the dam becomes quiescent
Arched-back	The dam is immobile and in a high upright dorsal arch posture; most to all of the pups are attached to the nipples.
Blanket	The dam is over the pups, relatively immobile, in a low dorsal arch posture, or lies flat on top of the pups.
Passive	The dam lies down on her side with more than one pup usually attached to the nipples
Pups-retrieval	The dam picks up the pup gently by its dorsal skin with the incisors and carries it to the nest site
Pups-licking	The dam licks the pup's body, including the anogenital region
Nest-building	The dam shreds cylindrical wooden dowels by chewing or stripping the wood, and takes the shredded material to make a crude nest.
In the nest	The dam spends time in the nest
Pups-sniffing	The dam sniffs the pup's body, including the anogenital region
Non-pups-oriented behaviors	
Digging	The dam digs in the sawdust, moving it around, using the snout and both the forepaws and hind paws, and mostly moves around the cage
Rearing	The dam stands on its hind limbs, often sniffing
Walking	The dam moves around the cage, actively exploring the environment
Eating	The dam nibbles on food pellets held in its forepaws or held in the food containing compartment of the cage
Drinking	The dam drinks from the water bottle
Self-grooming	The dam licks, combs or scratches any part of its own body
Resting / Out of nest	The dam is out of the nest, lying still in a sleeping mode or generally not caring about the pups

Table 2. Items of pups- and non-pups-oriented behaviors scored during the undisturbed observation of dams

## OFT

Behavioral reactivity in a novel environment was measured in an open field. The apparatus was a Plexiglas square box, 44 cm long, 44 cm wide, and 20 cm high. The behavior of the rats was monitored in a mean light intensity (100 lx) illuminated chamber. The test provides a qualitative mapping of the motor pattern, measuring total distance traveled – TDT, as a measure of locomotor activity – and number of central transitions from the peripheral to the central area of the arena – NCT, as measures of explorative behavior (Cacace et al., 2011). Each experimental session lasted 5 m.

## Can Test

For the evaluation of learning and memory processing, rats were tested in the Can Test, a reinforcement-motivated task (Popović et al., 2001). In the task, rats were trained to identify a single rewarded can

among a set of seven cans. The reinforce consisted in a 0.3 mL tap water – 23 h water deprivation schedule was used for motivation. The behavioral protocol consisted of four consecutive steps, as described below. Cans were painted in white or left in their imprinted colors, according to the task administered, and put upside down in a square Plexiglas apparatus (100 cm × 100 cm × 43 cm). This allowed their indented bottoms to hold water. Cans were placed in a fan-shaped pattern, in which the distance from each can to a start point was 70 cm and the distance between the cans was 7 cm. When the rat stood on its hind paws and brought its nose up to the level of the top edge of the can, this was considered a “visit”. The parameters measured were: activity, the number of trials on which rats visited at least one can (up to 10 during each experimental session); correct responses, the number of trials in which the rat visited the rewarded can first, divided by activity score (up to 1 per each experimental session); reference memory errors, the first visits to a non-rewarded can on each trial, divided by activity score (up to 6 per each experimental session); working memory errors, repeated visits to the same non-rewarded can on the same trial divided by the number of activity. Rats were allowed to drink freely for 20 m at the end of the experimental sessions.

### *Experimental design*

*Shaping period.* This period took two days. During this session, rats were drug-free, and they started habituation by familiarizing themselves with the environment. On the first day, rats were put in the apparatus with seven cans, whose bottom was filled with tap water. Each can was painted in white except for one on-centered relative to the others, which was wrapped with white tape. Rats were allowed to explore the apparatus and take water from the cans for 20 m. Animals were then removed and placed in their home cages. On the second day, two cans randomly plus the white-taped one on the center were rewarded with water. The rats had up to 10 m to visit and drink water. After a 15-s interval, the procedure was repeated.

*Spatial task.* Forty-eight h after the shaping period and 24 h following either Veh, CBD or AM281 and CBD administration, on 3 consecutive days and along 10 trials per day, rats were placed in the same environment as the shaping period. The single rewarded can was the white-taped, on-centered one. Rats could spend up to 3 m per trial in order to visit cans and obtain water; once the reward was received, the rat was immediately removed from the apparatus. During the 15-s interval between trials, rats were placed in a small Plexiglas box (50 cm × 30 cm × 30 cm).

*Simple visual task.* Forty-eight h after the spatial task, and 24 h following either Veh, CBD or AM281 and CBD administration, on 3 consecutive days along 10 trials per day, rats were placed in the compartment where the rewarded can was of a different color (i.e. a Pepsi can) than the other identical six cans, and randomly located on each trial. As in the previous step, rats could spend up to 3 m per

trial in order to visit cans and obtain water. During the 15-s interval between trials, rats were placed in the small Plexiglas box.

### Barnes Maze Test

To assess spatial learning and memory retrieval, and cognitive flexibility, adolescent and adult offspring underwent the Barnes Maze Test (Gibula-Tarlowska et al., 2020; Gawel et al., 2019). The apparatus consisted of a circular, gray platform made in Plexiglas with a diameter of 122 cm and a height of 90 cm. On the perimeter of the platform, 20 holes were placed with a diameter of 10 cm each, where only one was the entrance to an under-platform chamber with dimensions of 12 cm × 12 cm × 35 cm – referred to as an *escape box*. The other holes were covered underneath with a flat box, likewise of the same material and color. So that the rats could not discriminate the escape hole from other holes until situated adjacent to it, from the center of the maze, all holes looked identical. In the task, the animal was placed in the middle of the platform and was initially unable to locate the escape box, the location of which varied according to the phase of the task. The additional stimulus was provided during the task in the form of intense lighting – two points of light placed 1.5 m above the platform with a power of 500 W each. Additionally, on the walls of the laboratory room, visual cues were provided in the form of large colorful geometric figures and signs placed to facilitate the location of the escape box by the animal. The Barnes Maze Test consisted of the following phases: habituation; acquisition phase; probe task; reversal task. The experimental design was developed as follows.

### Experimental design

**Habituation.** In order to reduce the levels of anxiety that may affect behavior (i.e., locomotor activity), 24 h before the acquisition phase, the rats were habituated to the platform and the escape box. The animals were placed in the middle of the platform and were allowed to freely explore the apparatus for 180 s.

**Acquisition phase.** Twenty-four h after habituation the same rats were entered into the acquisition phase, to analyze their ability to acquire a simple task such as escape from a hostile environment. The acquisition phase included 1 training session per day for 3 consecutive days. Each training session consisted of three 180-s trials. The location of the escape box remained constant over all the acquisition trials. Rats were placed in the middle of the maze covered with an opaque tube or a bucket. After a delay of a few seconds, the bucket was lifted and the experimental animal was allowed to explore the platform. This approach intends to ensure that the initial orientation of the animal in the maze varies randomly from trial to trial. The trial was completed after 180 s or when the animal entered the escape box. Immediately after the animal entered the escape box, the hole was covered for 30 s, and the light was switched off. If the animal did not enter the escape box within 180 s, it was

gently guided there by the experimenter. The latency to escape – latency to find the escape box – was recorded in each trial to assess spatial learning.

*Probe task.* Twenty-four h later, the probe task was conducted similarly to the acquisition phase, except for the tunnel leading to the escape box was closed. The memory of the location of the escape box was assessed for 90 s. Primary latency – the time required for the rat to make initial contact with the escape box – and total distance traveled were measured to assess for retention of spatial learning and memory retrieval, and locomotor activity in the maze, respectively.

*Reversal task.* The reversal task was performed 24 h after the end of the probe task. At that time, the position of the escape box was rotated 180° to the original, and three 180-s trials were run in 1 day. The reversal task took shorter times than the acquisition phase since the animals have already familiarized themselves with the environment, and they know the rules of how to solve the maze. The latency to escape – latency to find the escape box – was recorded in each trial.

### T-Maze

The T-Maze consisted of a T-shaped platform positioned in a 70-cm high pedestal with three 30 × 10 cm arms joined by a 10 × 10 cm center all closed. The test was carried out with soft light in a soundproofed room. The animal was placed on the middle arm (starting arm) facing away from the closed center. The test consisted of two trials (one set) when the rats had up to 120 s to choose which arm to go on. Once the rat entered one of the arms, a physical barrier was placed to restrain the rat in the chosen arm for 30 s before continuing with the next trial. If the animal failed to make a choice within 120 s, the test is marked as “failed” and the animal is placed in the starting arm to begin the next test which has the same experimental conditions as in trial 1. The results were expressed as % alternation, calculated according to the formula in figure 13 (Wu et al., 2018).

$$\frac{\text{The number of correct choices}}{\text{Total sets performed}} \times 100\% = \% \text{ Alternation}$$

Fig. 13 Calculation of the % alternation

### Tissue Collection and qRT-PCR procedure

After the behavioral battery, a subset of the adolescent and adult male rats were killed and brains were rapidly removed, sliced into 1 mm-thick coronal sections, and hippocampus samples were rapidly dissected. Tissue samples were flash-frozen in dry ice and stored at 80° C until subsequent

analysis. RNA was isolated using homogenization in Trizol (Invitrogen) followed by chloroform layer separation, and precipitation with isopropanol, plus 70% ethanol washes intended to remove any residual salts from the isopropanol RNA precipitation step (Chomczynski and Sacchi, 2006). RNA was resuspended with 50 µl water and then analyzed with NanoDrop (ND-1000 Spectrophotometer, Thermo Scientific, Wilmington, DE, USA). RNA samples were reverse-transcribed to cDNA, using oligo(dT) primers and dNTPs, buffer, DTT, and SuperScript IV Reverse Transcriptase (Invitrogen). cDNA was then diluted and mixed with PowerUp SYBR Green Master Mix (Applied Biosystems), and forward and reverse primers. Samples were then heated to 95° C for 10 m, followed by 40 cycles of 95° C for 15 s, 60° C for 1 m, 95° C for 15 s, 60° C for 30 s, and 95° C for 15 s. Analysis was performed using the delta-delta C(t) method. Primers employed are indicated in table 3.

Gene Name	Primer Sequence	Product
Gapdh	GTTTGTGATGGGTGTGAACC (Forward) CTTCTGAGTGGCAGTGATG (Reverse)	
CB1R - Cnr1		Rn_Cnr1_1_SG QuantiTect Primer Assay (QT00191737)
Histidine triad nucleotide-binding protein 1 (HINT1)		Rn_Hint1_1_SG QuantiTect Primer Assay (QT01602713)
NMDAR NR1 subunit- Grin1		Rn_Grin1_1_SG QuantiTect Primer Assay (QT00182287)
Homer 1 - HOM1	CTTCACAGGAATCAGCAGGAG (Forward) GTCCCATTGATACTTTCTGGTG (Reverse)	
mGluR5 - Grm5		Rn_Grm5_1_SG QuantiTect Primer Assay (QT01081549)

Table 3. Primers employed in qRT-PCR experiments

## Statistical Analysis

When data exhibited normality and equal variance, the difference between groups was determined by employing either Unpaired parametric Student's t-test, one-way-, two-way- or three-way analysis of variance (ANOVA) followed by a Bonferroni post hoc test, when necessary. If data did not show normal distribution or equal variance, nonparametric tests were performed. The performance of rats to reach the learning criterion was analyzed using Kaplan-Meier event analysis over the acquisition period in the Barnes Maze, and the resulting curves were compared by employing the log-rank Mantel-Cox test. Data are reported as mean  $\pm$  SEM. Statistical analysis was performed using Prism v. 9 (Graphpad) and statistical significance was set at  $\alpha = 0.05$ . Trends toward statistical significance are indicated at  $\alpha < 0.1$ .



## Results

### Prenatal stimulation of the endocannabinoid system does not affect the mother-infant dyad

The prenatal treatments might interfere with the neurodevelopment of the offspring, thus the maternal behavior was analyzed in order to dissect out possible hinder which may be reflected on offspring neurodevelopment. No significant differences were detected for % of each observed pups-oriented behavior between Veh- and THC-treated dams, as reported previously (Sagheddu et al., 2021) – nest-building ( $U = 8.00$ ,  $p = 0.1494$ ); pups-licking ( $U = 12.00$ ,  $p = 0.4307$ ); passive nursing ( $U = 12.00$ ,  $p = 0.4307$ ); blanket nursing ( $t = 0.4670$ ,  $df = 10$ ,  $p = 0.6505$ ); arched-back nursing ( $U = 12.00$ ,  $p = 0.4307$ ). As so, for non-pups-oriented behaviors including resting / out of nest ( $t = 1.220$ ,  $df = 10$ ,  $p = 0.2505$ ), self-grooming ( $U = 10.00$ ,  $p = 0.2100$ ), walking ( $U = 16.00$ ,  $p = 0.8009$ ), rearing ( $U = 10.00$ ,  $p = 0.2749$ ), except for a trend detected in drinking ( $t = 2.132$ ,  $df = 10$ ,  $p = 0.0588$ ), and a significant increase in eating behavior of THC dams ( $t = 3.518$ ,  $df = 10$ ,  $p = 0.0056$ ). The results are disclosed in figure 14.

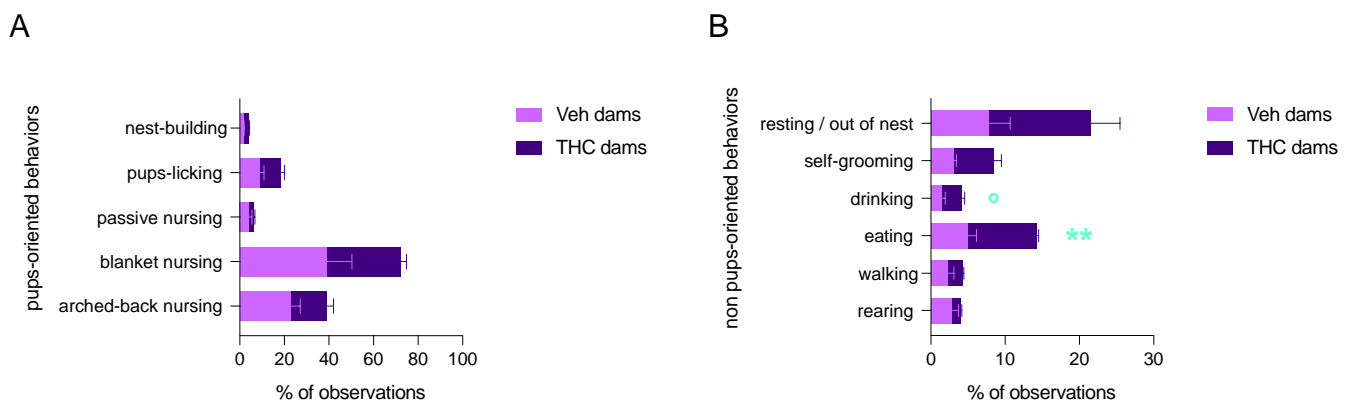


Figure 14. Main pups- (A) and non-pups- (B) oriented behaviors observed. Data are presented as mean  $\pm$  SEM.  $^{\circ}p = 0.0588$ ;  $^{**}p < 0.01$ .

### Behavioral assessment of the offspring

#### OFT - Prenatal THC exposure impacts behavioral reactivity in the adolescent, but not in adult offspring

The offspring were tested in the open field arena to evaluate the effects of prenatal THC exposure on locomotor activity, exploratory- and anxiety-like behaviors. Statistical analysis highlights that prenatal exposure to THC induced a significant increase in TDT ( $t = 3.272$ ,  $df = 48$ ,  $p = 0.0020$ ; figure

15, A) of adolescent offspring compared to the CTRL group. No significant differences were observed in NCT ( $t = 1.901$ ,  $df = 48$ ,  $p = 0.0633$ ; figure X0, B).

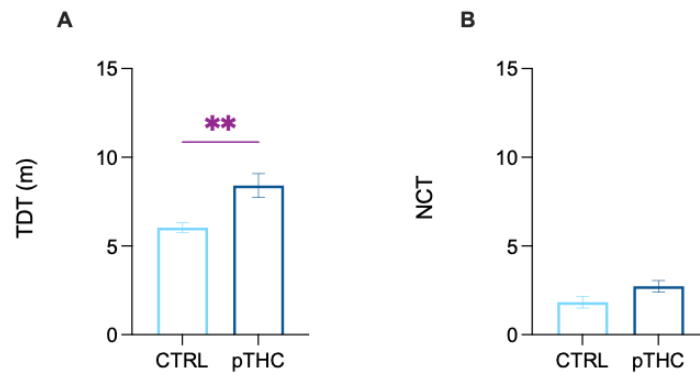


Figure 15. Effects of prenatal TCH exposure (2 mg/kg) on behavioral reactivity of adolescent offspring in the OFT. *In-utero* exposure to THC increased locomotor activity, in terms of TDT (A), while inducing no significant effects in exploratory activity, in terms of NCT (B). Data are shown as the mean  $\pm$  SEM. \*\* $p < 0.01$ . CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC; TDT = total distance traveled; NCT = number of central transitions.

On the other hand, adult pTHC rats showed no differences in TDT ( $t = 1.722$ ,  $df = 14$ ,  $p = 0.1071$ ; figure 16, A) or NCT ( $t = 1.181$ ,  $df = 14$ ,  $p = 0.2571$ ; figure 16, B) when compared to the control group.

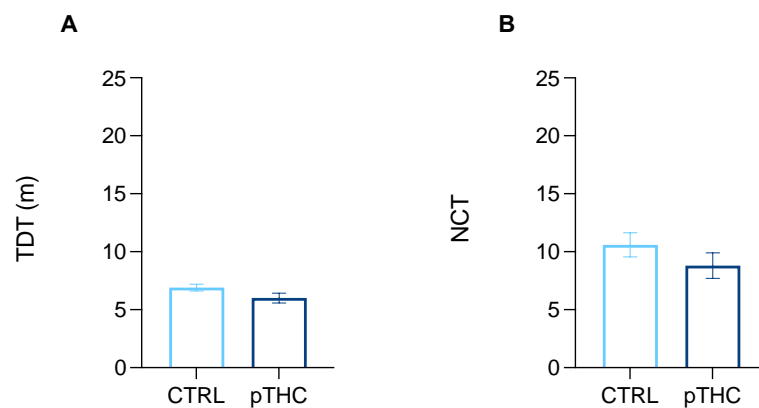


Figure 16. Effects of prenatal TCH exposure (2 mg/kg) on behavioral reactivity of adult offspring in the OFT. *In-utero* exposure to THC induced no significant effects in locomotor activity, in terms of TDT (A) and exploratory activity, in terms of NCT (B). Data are shown as the mean  $\pm$  SEM. CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC; TDT = total distance traveled; NCT = number of central transitions.

## Can Test - Prenatal THC exposure alters spatial memory acquisition and visual discrimination memory of the adolescent offspring in the spatial- and visual tasks

The effect of prenatal THC exposure on spatial learning was assessed by measuring the behavioral strategy of rats during the acquisition phase of the Can Test, where the can containing the reward was centrally positioned among other cans through the sessions. The results of a two-way ANOVA for repeated measures on correct responses of adolescent male rats showed significant effects of days ( $F(2, 26) = 36.18, p < 0.0001$ ) and a main decreasing effect of pTHC ( $F(1, 13) = 7.485, p = 0.0170$ ), but not their interaction ( $F(2, 26) = 2.028, p = 0.1519$ ; figure 17, A). The main effects of days ( $F(2, 26) = 70.12, p < 0.0001$ ), pTHC ( $F(1, 13) = 5.023, p = 0.0431$ ), and their interaction ( $F(2, 26) = 11.53, p = 0.0003$ ) were observed on the reference memory errors. In particular, the post hoc analysis showed an increase in reference memory errors ( $DF = 39, t = 4.749, p < 0.0001$ ) on day 2 of adolescent pTHC rats compared to controls (figure 17, B). No statistically significant difference was detected in working memory errors (days:  $F(2, 26) = 3.351, p = 0.0507$ ; pTHC:  $F(1, 13) = 0.4478, p = 0.5151$ ; days x pTHC:  $F(2, 26) = 1.233, p = 0.3080$ ; figure 17, C) and activity score (days:  $F(2, 26) = 0.3278, p = 0.7234$ ; pTHC:  $F(1, 13) = 0.01453, p = 0.9059$ ; days x pTHC:  $F(2, 26) = 1.837, p = 0.1794$ ; data not shown).

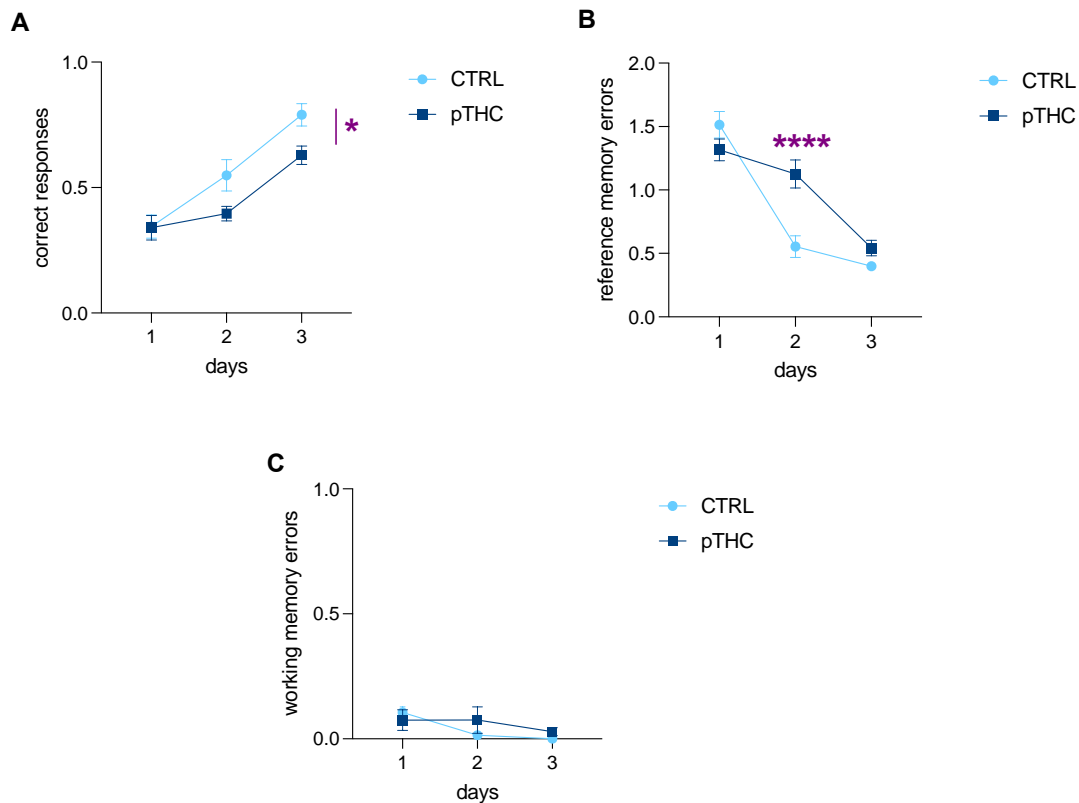


Figure 17. Effect of prenatal THC exposure (2 mg/kg) on the acquisition of spatial memory of the adolescent offspring in the spatial task of the Can Test. pTHC decreased correct responses of adolescent male rats (A) and induced an increase in reference memory errors on day 2 of adolescent male rats compared to controls (B). No effect of prenatal THC treatment was detected in working memory errors (C). Data are shown as the mean  $\pm$  SEM. \* $p < 0.05$ ; \*\*\*\* $p < 0.0001$ . CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC.

To evaluate the ability of the offspring to discriminate a different object among identical ones in a known environment, the simple visual task was performed. The results of a two-way ANOVA for repeated measures on the correct responses of male rats showed main effects of days ( $F(2, 26) = 12.78$ ,  $p = 0.0001$ ) and a significant decreasing effects of pTHC ( $F(1, 13) = 8.905$ ,  $p = 0.0106$ ) but not their interaction ( $F(2, 26) = 1.392$ ;  $p = 0.2665$ ) on correct responses (figure 18, A), as so on reference memory errors, in an increasing fashion (days:  $F(2, 26) = 11.42$ ,  $p = 0.0003$ ; pTHC:  $F(1, 13) = 13.15$ ,  $p = 0.0031$ ; days x pTHC:  $F(2, 26) = 1.095$ ,  $p = 0.3494$ ; figure 18, B). No statistically significant effect of prenatal THC exposure was detected in working memory errors (days:  $F(2, 26) = 8.054$ ,  $p = 0.0019$ ; pTHC:  $F(1, 13) = 0.1132$ ,  $p = 0.7419$ ; days x pTHC:  $F(2, 26) = 0.03543$ ,  $p = 0.9652$ ; figure 18, C), and activity score (days:  $F(2, 26) = 0.000$ ,  $p > 0.9999$ ; pTHC:  $F(1, 13) = 0.000$ ,  $p > 0.9999$ ; days x pTHC:  $F(2, 26) = 0.000$ ,  $p > 0.9999$ ; data not shown).

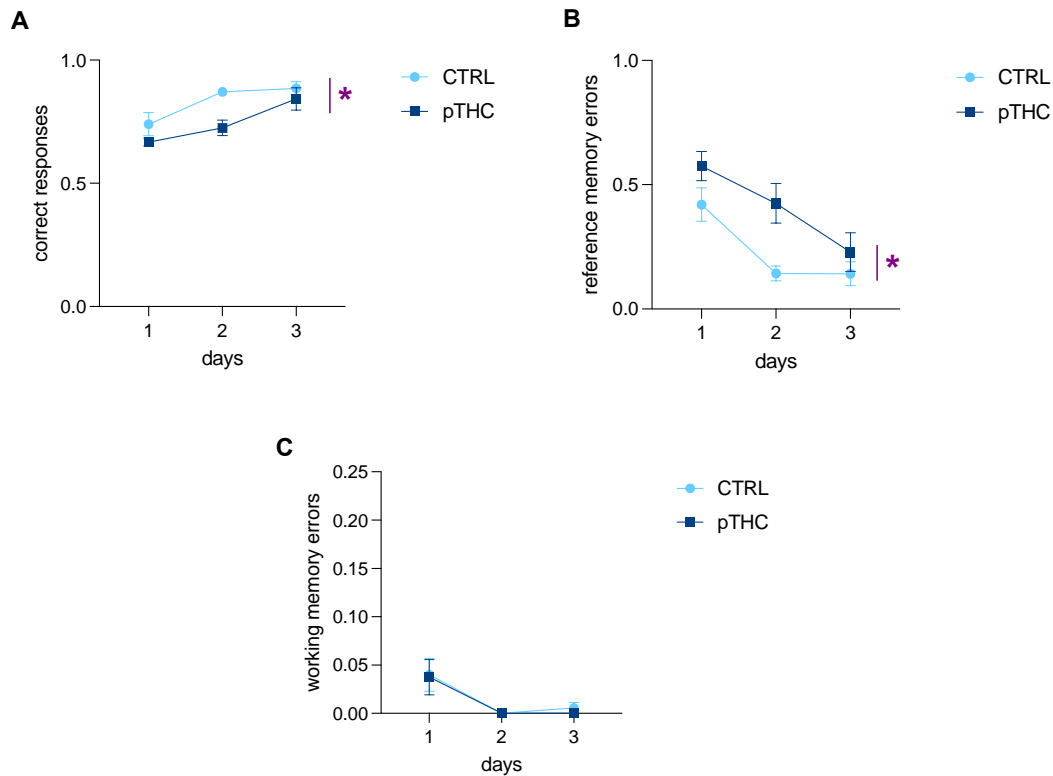


Figure 18. Effect of prenatal THC exposure (2 mg/kg) on the visual discrimination memory of the adolescent offspring in the visual task of the Can Test. *In-utero* exposure to THC decreased correct responses (A) and increased reference error memory (B) of adolescent male rats. No significant effect of prenatal THC treatment was detected in working memory errors (C). Data are shown as the mean  $\pm$  SEM. \* $p < 0.05$ . CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC.

### Can Test - Prenatal THC exposure impairs the acquisition of spatial- and visual discrimination memory of the adult offspring in the spatial- and visual tasks

The results of a two-way ANOVA for repeated measures on correct responses in the spatial task of adult male rats showed a significant effect of days ( $F(2, 20) = 160.2$ ,  $p < 0.0001$ ), pTHC ( $F(1, 10) = 8.425$ ,  $p = 0.0158$ ) and their interaction ( $F(2, 20) = 7.070$ ,  $p = 0.0048$ ). In particular, post hoc analysis showed a lower number of correct responses on day 1 ( $t = 3.502$ ,  $DF = 30$ ,  $p = 0.0044$ ) and day 2 ( $t = 3.413$ ,  $DF = 30.00$ ,  $p = 0.0056$ ) of adult male pTHC rats compared to controls (figure 19, A). A main effect of days ( $F(2, 20) = 159.3$ ,  $p < 0.0001$ ), and its interaction with pTHC ( $F(2, 20) = 10.31$ ,  $p = 0.0008$ ) on the reference error memory was observed. In particular, the post hoc analysis showed an increase in reference memory errors on day 1 ( $t = 4.339$ ,  $DF = 30.00$ ,  $p = 0.0004$ ) of male pTHC rats compared to controls (figure 19, B). No statistically significant effect of prenatal THC exposure was detected either on working memory errors (days:  $F(2, 20) = 22.57$ ,  $p < 0.0001$ ; pTHC:  $F(1, 10) = 4.774$ ,  $p = 0.0538$ ; days  $\times$  pTHC:  $F(2, 20) = 0.8889$ ,  $p = 0.4267$ ; figure 19, C), nor on

activity score (days:  $F(2, 20) = 2.179$ ,  $p = 0.1393$ ; pTHC:  $F(1, 10) = 0.01037$ ,  $p = 0.9209$ ; days x pTHC:  $F(2, 20) = 0.2540$ ,  $p = 0.7781$ ; data not shown).

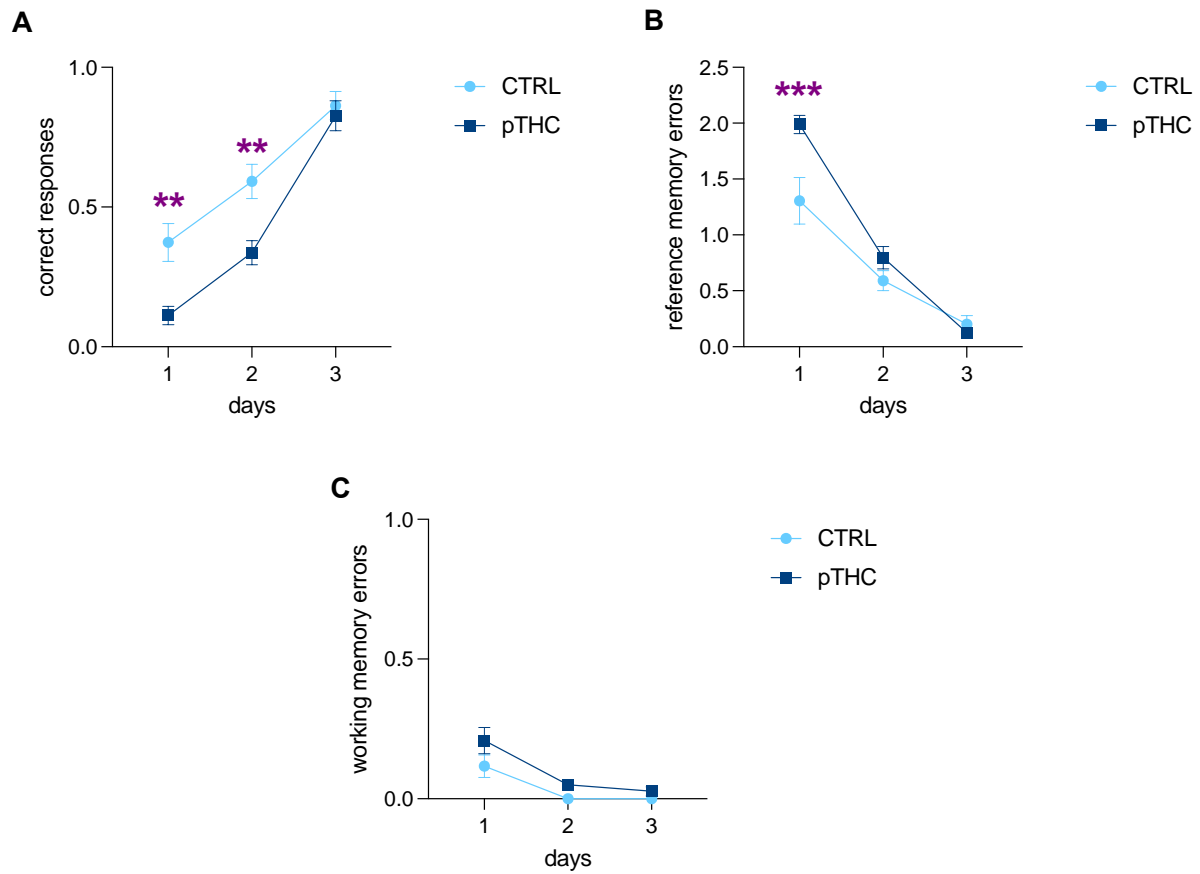


Figure 19. Effect of *in-utero* exposure to THC (2 mg/kg) on the acquisition of spatial memory of the adult offspring in the spatial task of the Can Test. Prenatal THC exposure induced a decrease in correct responses on days 1 and 2 (A), and an increase in reference memory errors on day 1 (B) of adult male rats compared to controls. No effect of prenatal THC treatment was detected in working memory errors (C). Data are shown as the mean  $\pm$  SEM. \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ . CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC.

In the visual task, the results of a two-way ANOVA for repeated measures showed main effects of days ( $F(2, 20) = 6.691$ ,  $p = 0.0060$ ), pTHC ( $F(1, 10) = 15.46$ ,  $p = 0.0028$ ), and their interaction ( $F(2, 20) = 13.33$ ,  $p = 0.0002$ ) on reference memory errors. In particular, post hoc analysis showed an increase in reference memory errors on day 2 of pTHC rats compared to controls ( $t = 6.416$ ;  $DF = 30.00$ ,  $p < 0.0001$ ; figure 20, B). No statistically significant effect of prenatal THC exposure was detected on correct responses (days:  $F(2, 20) = 3.564$ ,  $p = 0.0474$ ; pTHC:  $F(1, 10) = 3.192$ ,  $p = 0.1043$ ; days x pTHC:  $F(2, 20) = 0.9938$ ,  $p = 0.3877$ ; figure 20, A), working memory errors (days:  $F(2, 20) = 6.018$ ,  $p = 0.0090$ ; pTHC:  $F(1, 10) = 1.659$ ,  $p = 0.2268$ ; days x pTHC:  $F(2, 20) = 0.7823$ ,  $p = 0.4708$ ; figure 20, C), or activity score (days:  $F(2, 20) = 2.500$ ,  $p = 0.1074$ ; pTHC:  $F(1, 10) = 2.500$ ,  $p = 0.1449$ ; days x pTHC:  $F(2, 20) = 2.500$ ,  $p = 0.1074$ ; data not shown).

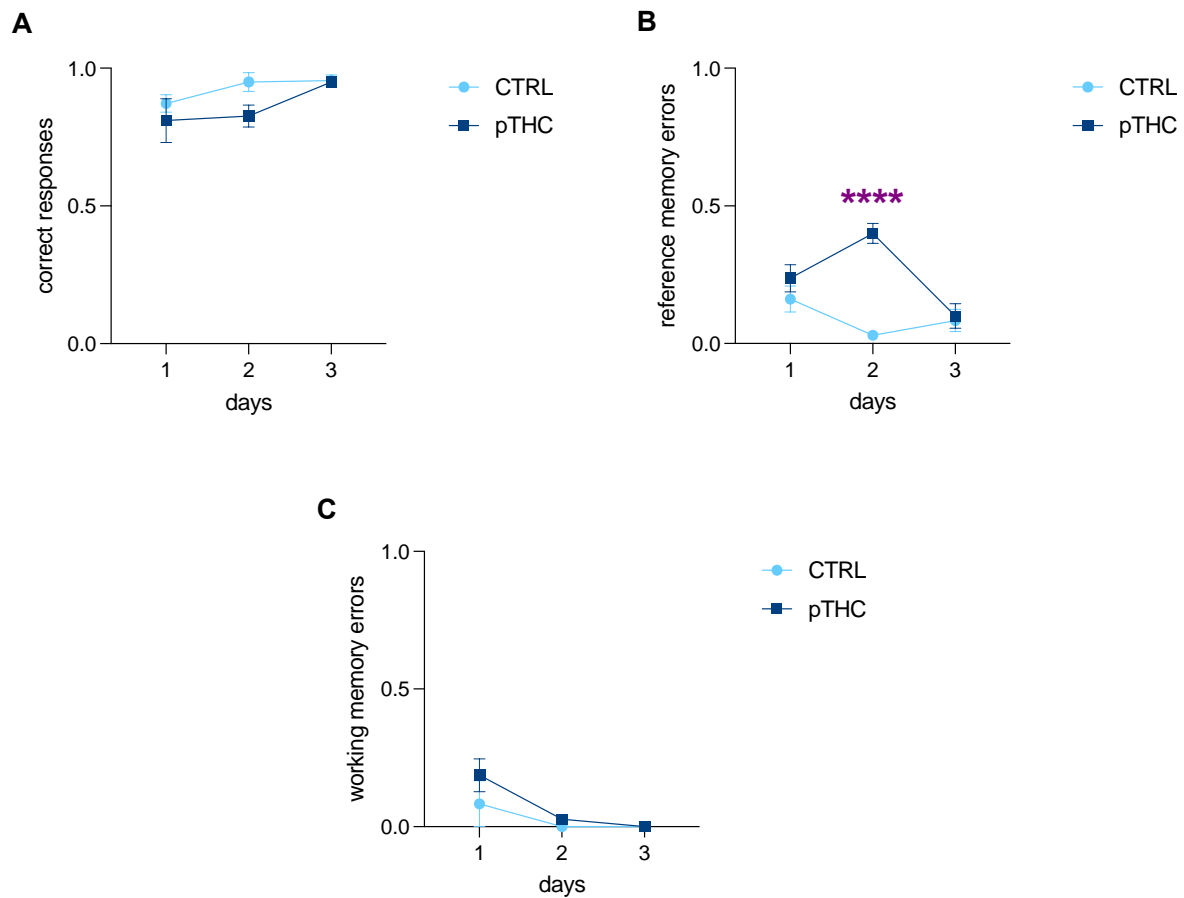


Figure 20. Effect of prenatal THC exposure (2 mg/kg) on the visual discrimination memory of the adult offspring in the visual task of the Can Test. *In-utero* exposure to THC induced an increase in reference memory errors on day 2 of male adult rats when compared to controls (B), while no significant effect was detected in correct responses (A) and working memory errors (C) of adult male offspring. Data are shown as the mean  $\pm$  SEM. \*\*\*\* $p < 0.0001$ . CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC.

### Can Test - Recovery effect of CBD on pTHC-induced spatial memory acquisition and visual discrimination memory impairments in the adolescent offspring during the spatial- and visual tasks

When considering the effect of CBD administration 24 h prior to testing on spatial memory acquisition task, the results of a three-way ANOVA for repeated measures on male rat correct responses showed the main effect of days ( $F(1.869, 42.98) = 59.41, p < 0.0001$ ), CBD ( $F(1, 23) = 11.35, p = 0.0027$ ), and pTHC  $\times$  CBD ( $F(1, 23) = 7.775, p = 0.0104$ ) and days  $\times$  pTHC  $\times$  CBD ( $F(2, 46) = 3.729, p = 0.0316$ ) interactions. Post hoc analysis detected an increase in correct responses on day 3 of male rats prenatally exposed to THC and received CBD 24 h prior to testing compared to pTHC rats ( $t = 5.730, DF = 13.00, p = 0.0046$ ; figure 21, A). The significant effect of days ( $F(1.906, 43.84) = 122.3, p < 0.0001$ ), CBD ( $F(1, 23) = 36.99, p < 0.0001$ ), days  $\times$  pTHC ( $F(2, 46) = 4.864, p = 0.0121$ ), pTHC  $\times$  CBD ( $F(1, 23) = 6.825, p = 0.0156$ ) and days  $\times$  pTHC  $\times$  CBD ( $F(2.46) = 9.111,$

$p = 0.0005$ ) interactions were highlighted in the reference memory errors. A decrease in reference memory errors of prenatal rats exposed to THC who received CBD treatment 24 h prior to testing versus pTHC rats was shown on days 2 ( $t = 9.978$ ,  $DF = 5.937$ ,  $p = 0.0096$ ) and 3 ( $t = 7.068$ ,  $DF = 9.900$ ,  $p < 0.0024$ ; figure 21, B). There was no significant effect of CBD treatment on working memory errors (days:  $F(1.513, 34.81) = 14.76$ ,  $p < 0.0001$ ; pTHC:  $F(1, 23) = 0.04240$ ,  $p = 0.8387$ ; CBD:  $F(1, 23) = 0.1993$ ,  $p = 0.6595$ ; days x pTHC:  $F(2, 46) = 1.535$ ,  $p = 0.2263$ ; days x CBD:  $F(2, 46) = 0.9898$ ,  $p = 0.3794$ ; pTHC x CBD:  $F(1, 23) = 0.0491$ ,  $p = 0.8760$ ; days x pTHC x CBD:  $F(2, 46) = 0.7405$ ,  $p = 0.4825$ ; figure 21, C), or activity score (days:  $F(1.370, 32.88) = 1.613$ ,  $p = 0.2167$ ; pTHC:  $F(1, 24) = 0.9149$ ,  $p = 0.3484$ ; CBD:  $F(1, 24) = 0.3365$ ,  $p = 0.5673$ ; days x pTHC:  $F(2, 48) = 3.679$ ,  $p = 0.0236$ ; days x CBD:  $F(2, 48) = 0.2087$ ,  $p = 0.8124$ ; pTHC x CBD:  $F(1, 24) = 0.5844$ ,  $p = 0.4521$ ; days x pTHC x CBD:  $F(2, 48) = 0.09170$ ,  $p = 0.9125$ ; data not shown).

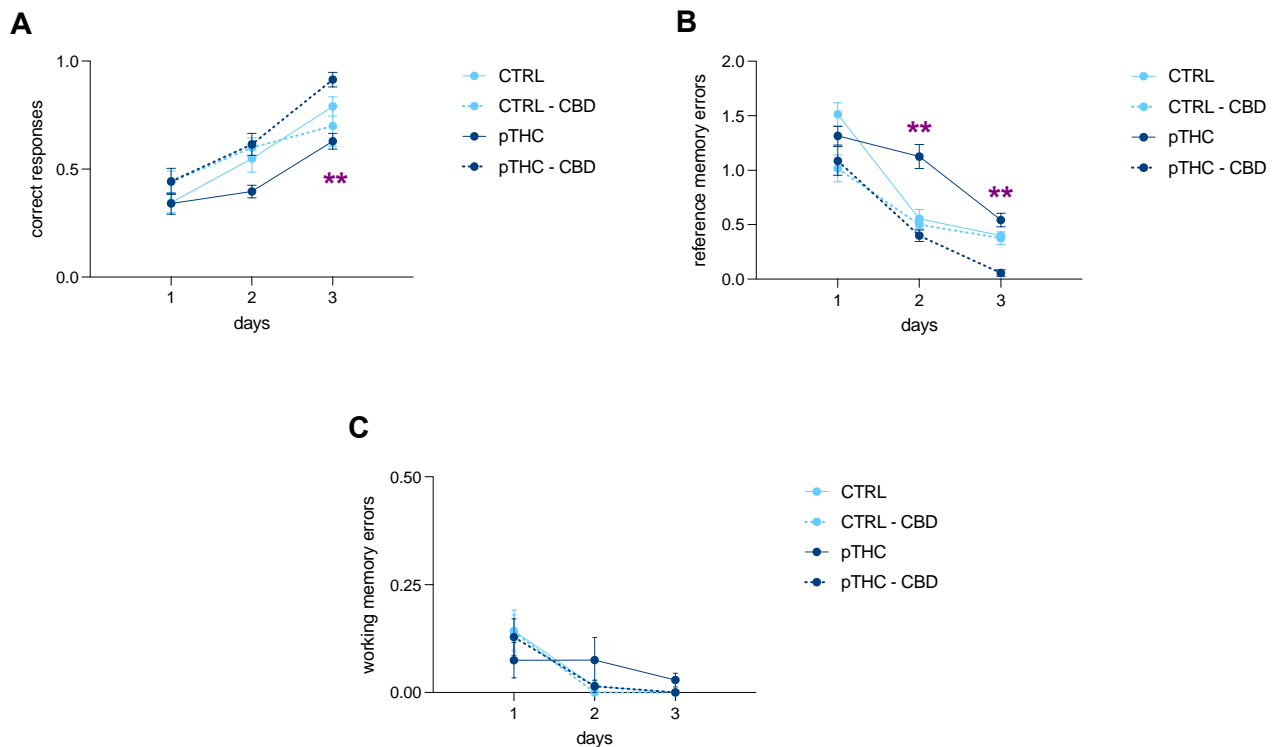


Figure 21. Recovery effect of CBD (40 mg/kg) on spatial memory acquisition of the adolescent offspring in the spatial task of the Can Test. CBD administration increased corrected responses on day 3 (A) and decreased reference memory errors on days 2 and 3 (B) of male rats prenatally exposed to THC compared to non-CBD treated pTHC rats. No effect of CBD treatment was detected in working memory errors (C). Data are shown as the mean  $\pm$  SEM. \*\* $p < 0.01$  pTHC vs. pTHC - CBD. CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC.

When considering the behavioral effect of CBD administered 24 h prior to the simple visual task, the results of a three-way ANOVA for repeated measurements on the correct responses of adolescent



male rats showed a main effect of days ( $F(2, 48) = 32.41, p < 0.0001$ ), CBD ( $F(1, 24) = 10.09, p = 0.0041$ ), days x pTHC ( $F(2, 48) = 5.395, p = 0.0077$ ), pTHC x CBD ( $F(1, 24) = 19.61, p = 0.0002$ ) and days x pTHC x CBD ( $F(2, 48) = 6.789, p = 0.0025$ ) interactions. Post hoc analysis showed an increase in correct responses of adolescent CBD-treated pTHC rats when compared to CBD-treated CTRL rats ( $t = 5.082, DF = 72.00, p = 0.0002$ ) on day 1, and to pTHC rats on days 1 ( $t = 5.134, DF = 72.00, p = 0.0002$ ) and 2 ( $t = 4.143, DF = 72.00, p = 0.0061$ ; figure 22, A). Significant effect of days ( $F(1.919, 46.06) = 28.47, p < 0.0001$ ), CBD ( $F(1, 24) = 16.02, p = 0.0005$ ), days x pTHC ( $F(2, 48) = 3.928, p = 0.0263$ ), pTHC x CBD ( $F(1, 24) = 22.01, p < 0.0001$ ), days x pTHC x CBD ( $F(2, 48) = 4.682, p = 0.0139$ ) interactions were detected on reference memory errors. CBD treatment induced a significant decrease in reference memory errors of adolescent CBD-treated pTHC rats when compared to CBD-treated CTRL rats ( $t = 5.915, DF = 11.00, p = 0.0067$ ) on day 1 and to non-CBD-treated pTHC rats ( $t = 5.343, DF = 13.61, p = 0.0075$ ) on day 1 (figure 22, B). Lastly, significant effects of days ( $F(1.045, 25.08) = 7.261, p = 0.0116$ ), CBD ( $F(1, 24) = 5.374, p = 0.0364$ ) and their interaction ( $F(2, 48) = 3.704, p = 0.0319$ ) were detected on working memory errors. However, post-hoc analysis failed to highlight significant differences among groups (figure 22, C). No significant difference was detected on activity score (pTHC:  $F(1, 24) = 0.000, p > 0.9999$ ; CBD:  $F(1, 24) = 0.000, p > 0.9999$ ; days x pTHC:  $F(2, 48) = 0.000, p > 0.9999$ ; days x CBD:  $F(2, 48) = 0.000, p > 0.9999$ ; pTHC x CBD:  $F(1, 24) = 0.000, p > 0.9999$ ; days x pTHC x CBD:  $F(2, 48) = 0.000, p > 0.9999$ ; data not shown).

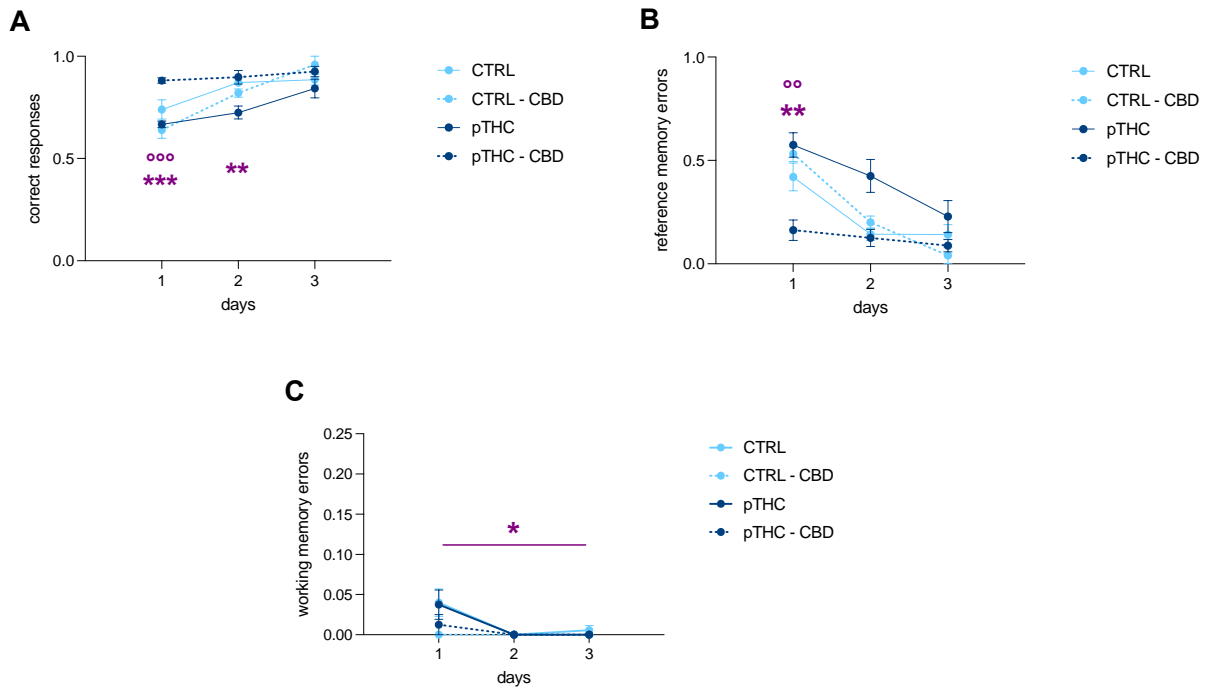


Figure 22. Recovery effect of CBD (40 mg/kg) on simple visual discrimination memory of the adolescent offspring in the simple visual task of the Can Test. CBD treatment increased correct responses of adolescent pTHC rats when compared to CBD-treated CTRL rats (DF = 69.00,  $t = 4.977$ ,  $p = 0.0003$ ) on day 1, and to prenatally THC-exposed adolescent rats on days 1 and 2 (A), and decreased reference memory errors of adolescent pTHC- compared to CTRL- and pTHC rats on day 1 (B). Overall, CBD decreased working memory errors (C). Data are shown as the mean  $\pm$  SEM.  $^{\circ\circ} p < 0.01$ ,  $^{\circ\circ\circ} p < 0.001$  CTRL - CBD vs. pTHC - CBD;  $*p < 0.05$ ,  $**p < 0.01$  pTHC vs. pTHC - CBD. CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC.

### Can Test - Recovery effect of CBD on spatial memory acquisition and simple visual discrimination memory of the adult offspring in the spatial- and visual tasks

When considering the effect of CBD administration 24 h prior to testing on spatial task, the results of a three-way ANOVA for repeated measures on adult male rat correct responses, showed the main effect of days ( $F(1.529, 27.53) = 135.2$ ,  $p < 0.0001$ ), pTHC ( $F(1, 18) = 5.170$ ,  $p = 0.0355$ ), a significant increasing CBD effect ( $F(1, 18) = 8.660$ ,  $p = 0.0087$ ) and days x CBD interaction ( $F(2, 36) = 6.794$ ,  $p = 0.0031$ ). Indeed, post hoc analysis highlighted a CBD treatment-induced increase in correct responses of adult pTHC rats when compared to non-CBD-treated pTHC rats on days 1 ( $t = 5.152$ ,  $DF = 8.561$ ,  $p = 0.0465$ ) and 2 ( $t = 6.797$ ,  $DF = 8.686$ ,  $p = 0.0062$ ; figure 23, A). A main effect of days ( $F(1.815, 32.67) = 462.4$ ,  $p < 0.0001$ ), a significant decreasing CBD effect ( $F(1, 18) = 23.27$ ,  $p = 0.0001$ ), and days x pTHC ( $F(2, 36) = 3.527$ ,  $p = 0.0399$ ), days x CBD ( $F(2, 36) = 30.32$ ,  $p < 0.0001$ ), pTHC x CBD ( $F(1, 18) = 7.272$ ,  $p = 0.0148$ ), days x pTHC x CBD interactions ( $F(2, 36) = 9.047$ ,  $p = 0.0007$ ) on reference memory errors were highlighted. Post hoc analysis detected a significant decrease in reference memory errors of CBD-treated pTHC rats when compared to their

non-CBD-treated counterpart ( $t = 8.510$ ,  $DF = 8.802$ ,  $p = 0.0010$ ; figure 23, B). Significant main effects of days ( $F(1.077, 19.39) = 29.97$ ,  $p < 0.0001$ ) and pTHC x CBD interaction were detected on working memory errors ( $F(1, 18) = 4.512$ ,  $p = 0.0478$ ; figure 23, C). No significant effect of CBD was detected on activity score (days:  $F(1.294, 23.29) = 0.9009$ ,  $p = 0.3784$ ; pTHC:  $F(1, 18) = 0.1673$ ,  $p = 0.6874$ ; CBD:  $F(1, 18) = 0.9326$ ,  $p = 0.3470$ ; days x pTHC:  $F(2, 36) = 0.5312$ ,  $p = 0.5924$ ; days x CBD:  $F(2, 36) = 2.696$ ,  $p = 0.0811$ ; pTHC x CBD:  $F(1, 18) = 0.3373$ ,  $p = 0.5686$ ; days x pTHC x CBD:  $F(2, 36) = 0.4256$ ,  $p = 0.6566$ ; data not shown).

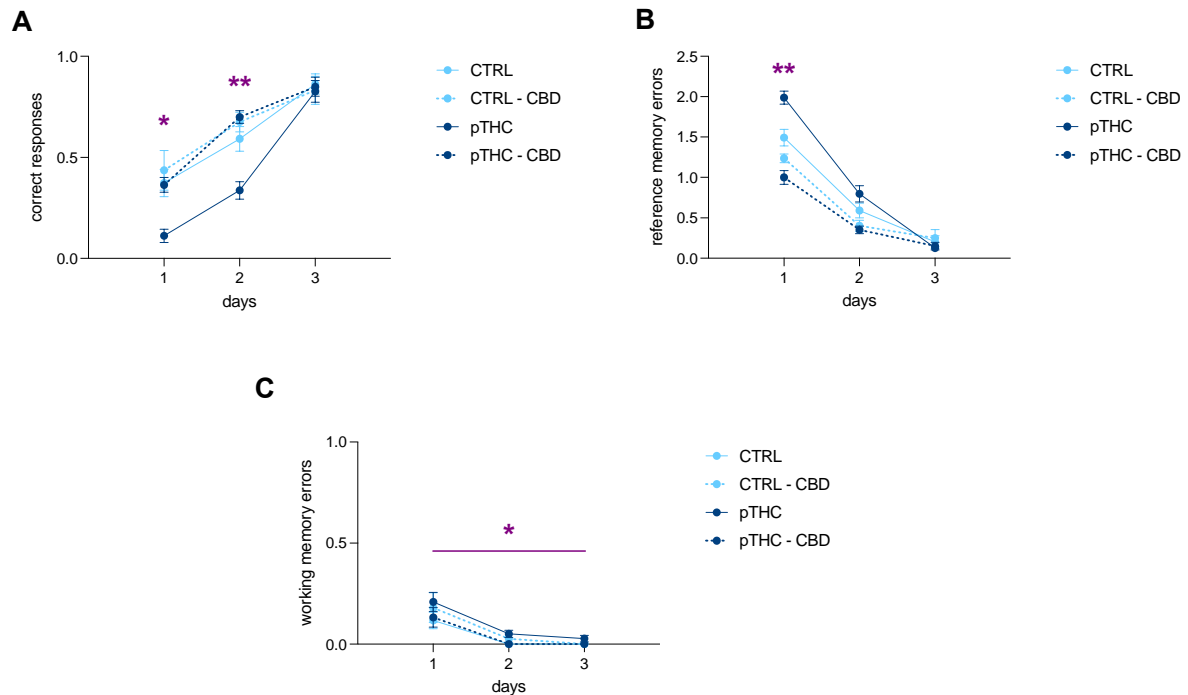


Figure 23. Recovery effect of CBD (40 mg/kg) on spatial memory acquisition of the adult offspring in the spatial task of the Can Test. CBD administration increased corrected responses on days 1 and 2 (A) and decreased reference memory errors on day 1(B) of male adult pTHC rats. A decreasing effect of CBD was detected in working memory errors (C). Data are shown as the mean  $\pm$  SEM. \* $p < 0.05$ ; \*\* $p < 0.01$ .

CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC.

When considering the behavioral effect of CBD administered 24 h prior to the simple visual task, the results of a three-way ANOVA for repeated measurements on the correct responses of male adult rats showed main effects of days ( $F(2, 36) = 26.85$ ,  $p < 0.0001$ ), CBD ( $F(1, 18) = 5.528$ ,  $p = 0.0303$ ) and their interaction ( $F(2, 36) = 8.878$ ,  $p = 0.0007$ ) on correct responses. Post hoc analysis did not detected any significant differences among groups (figure 24, A). Significant effects of days ( $F(1.286, 23.15) = 49.04$ ,  $p < 0.0001$ ), pTHC ( $F(1, 18) = 14.83$ ,  $p = 0.0012$ ), CBD ( $F(1, 18) = 6.537$ ,  $p = 0.0198$ ), and days x CBD ( $F(2, 36) = 34.38$ ,  $p < 0.0001$ ), days x pTHC x CBD ( $F(2, 36) = 7.500$ ,  $p = 0.0019$ ) interactions were detected on reference memory errors. Indeed, post hoc analysis showed

a significant higher score of reference memory errors in pTHC adult offspring when compared to CTRL on day 2 ( $t = 12.69$ ,  $DF = 7.557$ ,  $p = 0.0007$ ) and in CBD-treated pTHC adult offspring with respect to non-CBD-treated pTHC rats on day 1 ( $t = 8.422$ ,  $DF = 8.421$ ,  $p = 0.0071$ ), while lower score on day 2 ( $t = 8.783$ ,  $DF = 8.991$ ,  $p = 0.0044$ ; figure 24, B). CBD treatment had no significant effect on working memory errors (days:  $F(1.012, 15.18) = 6.497$ ,  $p = 0.0218$ ; pTHC:  $F(1, 15) = 0.09434$ ,  $p = 0.7630$ ; CBD:  $F(1, 15) = 0.02818$ ,  $p = 0.8689$ ; days x pTHC:  $F(2, 30) = 0.2255$ ,  $p = 0.7995$ ; days x CBD:  $F(2, 30) = 0.1165$ ,  $p = 0.8904$ ; pTHC x CBD:  $F(1, 14) = 2.020$ ,  $p = 0.1757$ ; days x pTHC x CBD:  $F(2, 30) = 1.544$ ,  $p = 0.2301$ ; figure 24, C). No significant differences were detected on activity score (days:  $F(1.012, 18.22) = 9.257$ ,  $p = 0.0068$ ; pTHC:  $F(1, 18) = 0.1195$ ,  $p = 0.7336$ ; CBD:  $F(1, 18) = 0.03424$ ,  $p = 0.8553$ ; days x pTHC:  $F(2, 36) = 0.3139$ ,  $p = 0.7326$ ; days x CBD:  $F(2, 36) = 0.1656$ ,  $p = 0.8480$ ; pTHC x CBD:  $F(1, 18) = 2.915$ ,  $p = 0.1049$ ; days x pTHC x CBD:  $F(2, 36) = 2.196$ ,  $p = 0.1259$ ; data not shown).

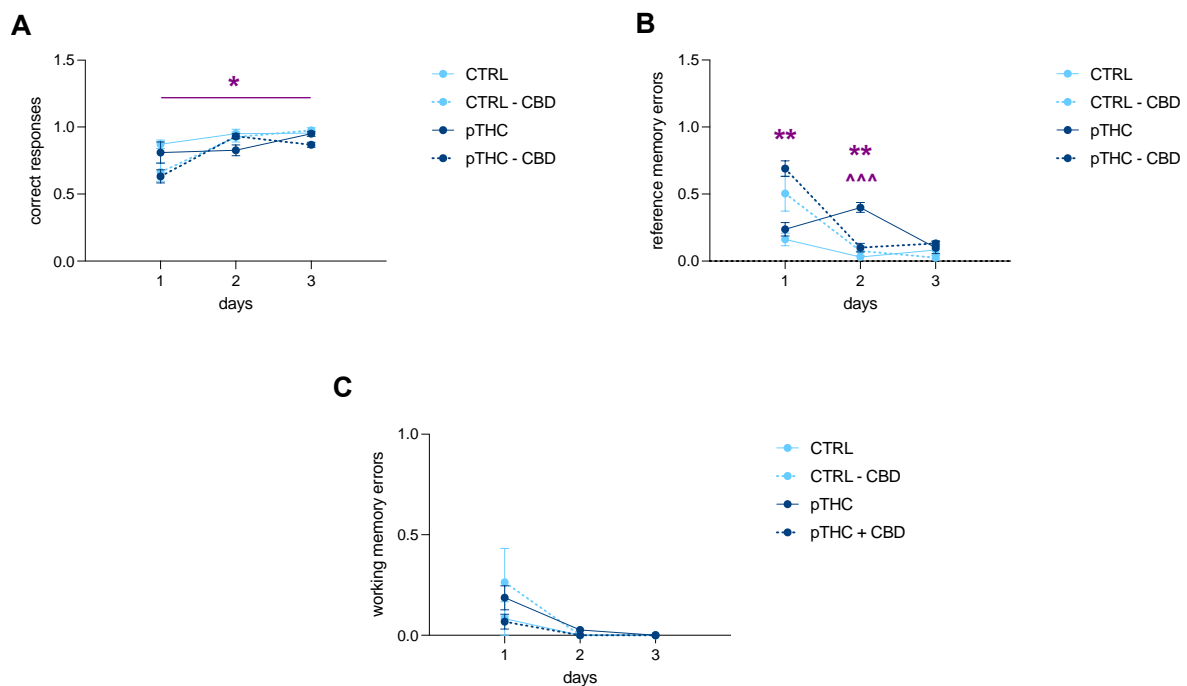


Figure 24. Recovery effect of CBD (40 mg/kg) on visual discrimination memory of the adult offspring in the simple visual task of the Can Test. An increasing effect of CBD was shown on correct responses (A), while a general decreasing effect of CBD was detected on reference memory errors (B) of adult rat offspring. No effect of CBD was detected in working memory errors (C). Data are shown as the mean  $\pm$  SEM. \*\* $p < 0.01$  pTHC vs. pTHC – CBD; ^^^ $p < 0.001$  CTRL vs. pTHC. CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC.

## Can Test – CB1R is not involved in the CBD recovery effect on spatial memory acquisition of the adolescent and adult offspring

In order to find a reading key for CBD mechanism on ECS signaling, AM281, a selective CB1R antagonist, was administered just before CBD to a subset of male adolescent and adult offspring, when tested in the spatial task of the Can Test. The results of the one-way ANOVA showed a significant P value on the average of correct responses ( $F(2, 16) = 18.63$ ,  $p < 0.0001$ ) of adolescent offspring in the spatial task. In particular, pTHC rats displayed a lower score of correct responses when compared to pTHC offspring treated with CBD ( $t = 5.237$ ,  $DF = 16$ ,  $p = 0.0002$ ) which stays when compared to pTHC rats treated with AM281 before the administration of CBD ( $t = 4.949$ ,  $DF = 16$ ,  $p = 0.0004$ ). No significant difference was detected between correct responses of pTHC - CBD- and pTHC - AM281 + CBD rats ( $t = 51.05$ ,  $DF = 16$ ,  $p > 0.9999$ ; figure 25, A). A significant result was detected also in the average of reference memory errors ( $F(2, 16) = 24.24$ ,  $p < 0.0001$ ). In details, multiple comparisons showed a higher number of reference memory errors of pTHC rats when compared to pTHC offspring treated with CBD ( $t = 6.444$ ,  $DF = 16$ ,  $p < 0.0001$ ) which stays when compared to pTHC rats treated with AM281 before the administration of CBD ( $t = 4.966$ ,  $DF = 16$ ,  $p = 0.0004$ ). No significant difference was detected between reference memory errors of pTHC - CBD- and pTHC - AM281 + CBD rats ( $t = 46.96$ ,  $DF = 16$ ,  $p > 0.9999$ ; figure 25, B).

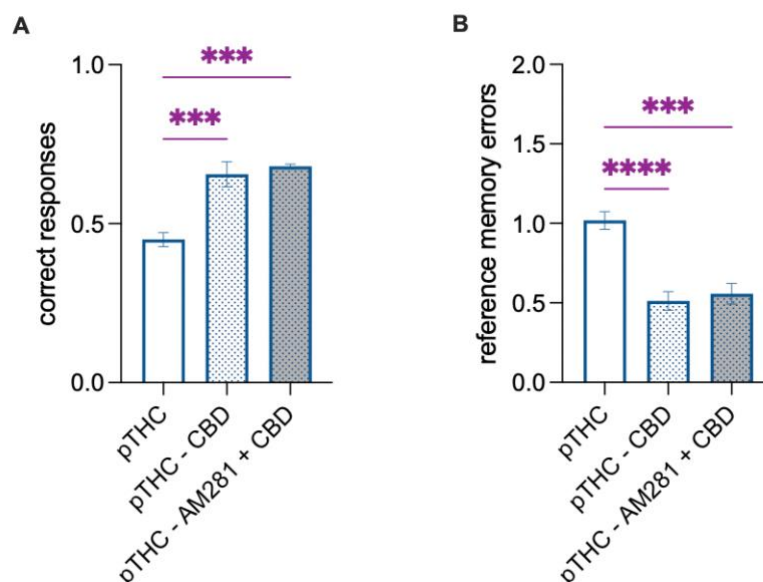


Figure 25. Evaluation of CB1R as a target of the CBD (40 mg/kg) recovery effect on spatial memory acquisition of the adolescent offspring in the spatial task of the Can Test. Adolescent prenatally THC-treated rats showed lower scores of correct response (A) and higher reference memory errors (B) when compared to both pTHC rats treated with CBD and pTHC rats treated with AM281 (1 mg/kg) just before CBD. Data are shown as the mean  $\pm$  SEM. \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ . CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC.

The results of the one-way ANOVA showed a significant P value on the average of correct responses ( $F(2, 9) = 8.11$ ,  $p = 0.0097$ ) of adult offspring in the spatial task. In particular, pTHC rats displayed a lower score of correct responses when compared to pTHC offspring treated with CBD ( $t = 3.469$ ,  $DF = 9$ ,  $p = 0.0212$ ) which is maintained when compared to pTHC rats treated with AM281 before the administration of CBD ( $t = 3.509$ ,  $DF = 9$ ,  $p = 0.0199$ ; figure 26, A). A significant result was detected also in the average of reference memory errors ( $F(2, 9) = 10.50$ ,  $p = 0.0044$ ). In details, multiple comparisons showed a higher number of reference memory errors of pTHC rats when compared to pTHC offspring treated with CBD ( $t = 4.491$ ,  $DF = 9$ ,  $p = 0.0045$ ) which stays when compared to pTHC rats treated with AM281 before the administration of CBD ( $t = 1.032$ ,  $DF = 9$ ,  $p = 0.0426$ ). No significant difference was detected between reference memory errors of pTHC - CBD- and pTHC - AM281 + CBD rats ( $t = 1.459$ ,  $DF = 9$ ,  $p = 0.5357$ ; figure 26, B).

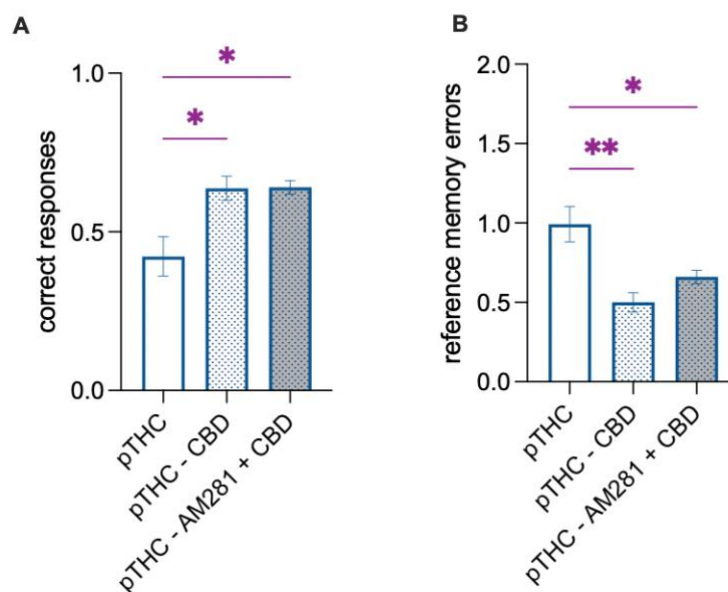


Figure 26. Evaluation of CB1R as a target of the CBD (40 mg/kg) recovery effect on spatial memory acquisition of the adult offspring in the spatial task of the Can Test. Adult prenatally THC-treated rats showed lower scores of correct response (A) and higher reference memory errors (B) when compared to both pTHC rats treated with CBD and pTHC rats treated with AM281 (1 mg/kg) just before CBD. Data are shown as the mean  $\pm$  SEM. \* $p < 0.01$ ; \*\* $p < 0.01$ .

CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC.

## Barnes Maze Test - Prenatal THC exposure impinges on the acquisition of spatial orientation of the adolescent offspring in the acquisition and probe steps, and on cognitive flexibility in the reversal task

Spatial memory impairment was assessed using the Barnes Maze using a mild aversion stimulus. 2-way ANOVA revealed main effects of days ( $F(1.849, 94.28) = 78.43$ ,  $p < 0.0001$ ) and a significant decreasing effect of pTHC ( $F(1, 51) = 4.878$ ,  $p = 0.0317$ ), but not their interaction, on latency to reach the escape box along the acquisition phase of adolescent male offspring (figure 27, A). The performance of the offspring was measured in terms of days elapsed to reach the learning criterion, or to enter the escape box: Kaplan-Meier analysis showed that the mean learning times were 1 day for male CTRL rats and 2 days for THC-treated prenatal rats. However, the Log-rank (Mantel-Cox) test for comparing survival curves indicated no significant differences in learning performance ( $\chi^2 = 2.608$ ,  $df = 1$ ,  $p = 0.1064$ ; figure 27, B).

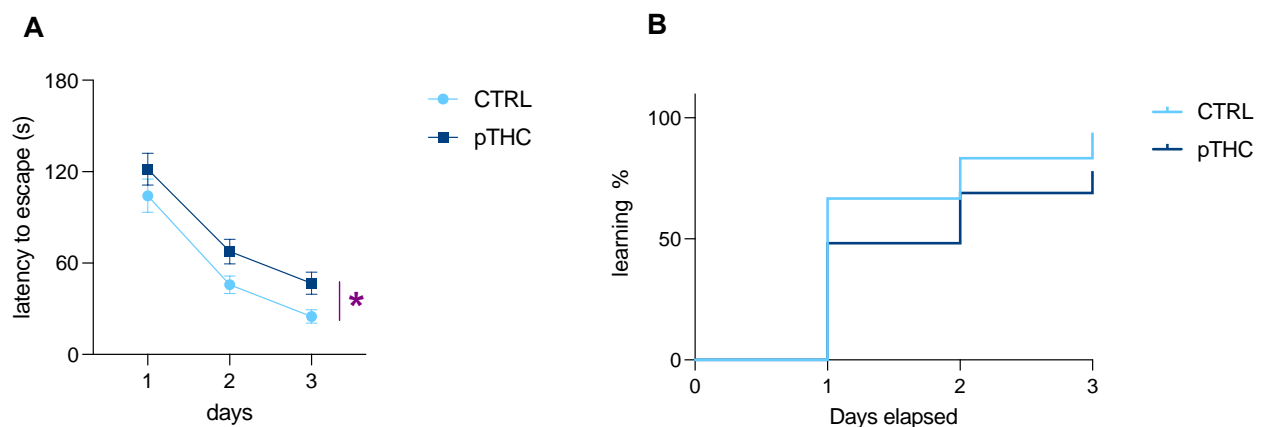


Figure 27. Effect of prenatal THC exposure (2 mg/kg) on the acquisition of spatial orientation of the adolescent offspring in the acquisition phase of the Barnes Maze Test. Prenatal exposure to THC increased latency to reach the escape box throughout the acquisition phase (A). The mean learning time was 1 day for male CTRL rats and 2 days for THC-treated prenatal rats, although the difference in the learning curves was not significant (B). Data are shown as the mean  $\pm$  SEM. \* $p < 0.05$ . CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC.

Next, the impact of prenatal THC exposure was evaluated in the probe task. The results of Student's t-test on primary latency showed significant increase in the primary latency of pTHC adolescent rats when compared to CTRL progeny ( $t = 2.235$ ,  $df = 28$ ,  $p = 0.0336$ ; figure 28, A). No significant difference was detected on the total distance traveled of the adolescent offspring in the maze during

the probe task ( $t = 0.5924$ ,  $df = 13$ ,  $p = 0.5637$ ; data not shown). Further, the effect of prenatal exposure to THC on learning flexibility was assessed in the reversal task. pTHC had a significant increasing effect in the latency to escape of the adolescent offspring ( $t = 2.213$ ,  $df = 28$ ,  $p = 0.0352$ ; figure 28, B).

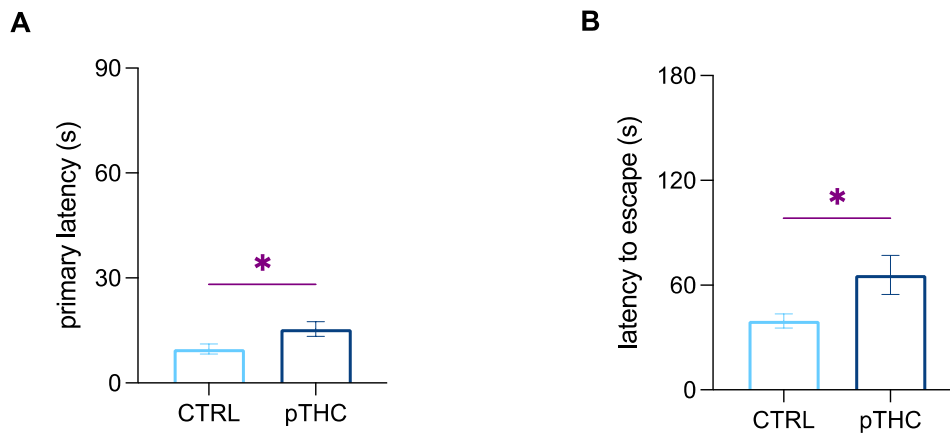


Figure 28. Effect of prenatal THC exposure (2 mg/kg) on the acquisition of spatial orientation of the adolescent offspring in the probe task, and on the cognitive flexibility in the reversal task of the Barnes Maze Test. pTHC increased primary latency (A) and latency to escape (B) of the adolescent progeny, in the probe- and reversal tasks, respectively. Data are shown as the mean  $\pm$  SEM. \* $p < 0.05$ . CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC.

#### Barnes Maze Test - Prenatal THC exposure has no effect on the acquisition of spatial orientation of the adult progeny in the acquisition phase and probe steps, and on cognitive flexibility in the reversal task

The result of a 2-way ANOVA showed no impact of prenatal exposure to THC on latency to reach the escape box of the adult offspring along the acquisition phase, although it revealed a main effect of days ( $F(1.255, 33.87) = 7.556$ ,  $p = 0.0061$ ; figure 29, A). Furthermore, the learning functioning of the adult offspring was evaluated by analyzing their ability to acquire the task to reach a safe place. The curve comparison did not show any significant effect of pTHC ( $\chi^2 = 0.8535$ ,  $df = 1$ ,  $p = 0.3556$ ). Indeed, Kaplan-Meier analysis showed that the mean learning times were 1 day for both male prenatally CTRL- and THC-treated rats (figure 29, B).



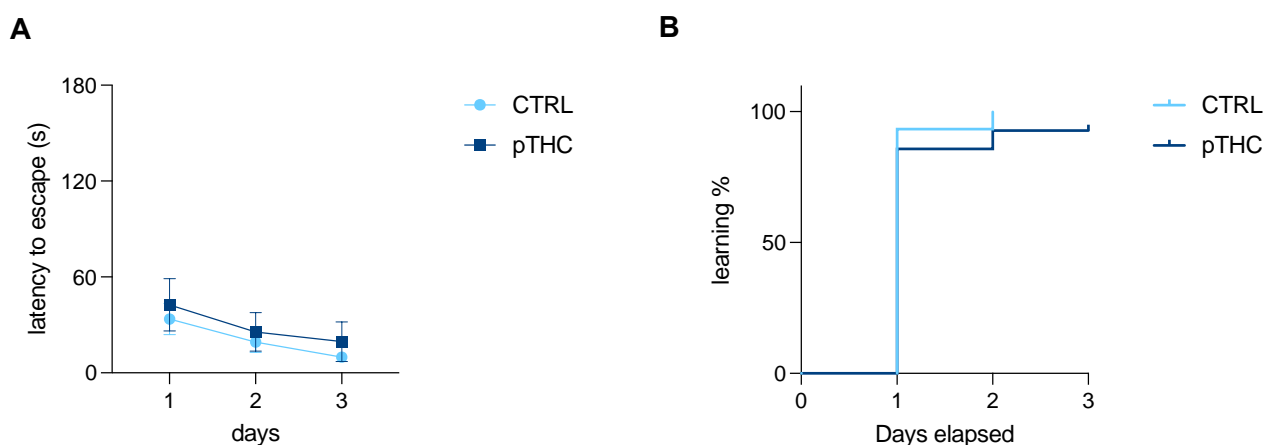


Figure 29. Effect of prenatal THC exposure (2 mg/kg) on the acquisition of spatial orientation of the adult offspring in the acquisition phase of the Barnes Maze Test. pTHC had effect neither on latency to reach the escape box (A), nor in the learning performance, as days elapsed to reach the criterion in the acquisition phase (B). Data are shown as the mean  $\pm$  SEM. CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC.

Next, the impact of prenatal THC exposure was evaluated in the probe task. The results of Student's t-test on primary latency showed no significant effect of pTHC ( $t = 1.255$ ,  $df = 10$ ,  $p = 0.2380$ ; figure 30, A). No significant difference was detected in the total distance traveled of the adult offspring in the maze during the probe task ( $t = 0.7458$ ,  $df = 10$ ,  $p = 0.4729$ ; data not shown). Further, the effect of pTHC on learning flexibility was assessed in the reversal task. Prenatal THC exposure had no significant effect in the latency to escape of the adult offspring ( $t = 0.005076$ ,  $df = 10$ ,  $p = 0.9960$ ; figure 30, B).

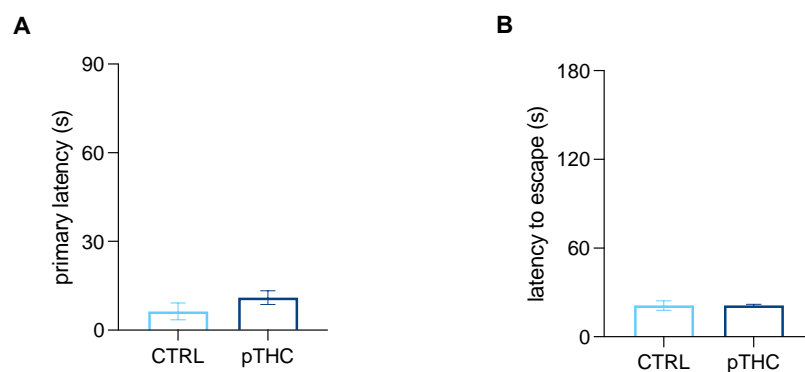


Figure 30. Effect of prenatal THC exposure (2 mg/kg) on the acquisition of spatial orientation of the adolescent offspring in the probe task, and on the cognitive flexibility in the reversal task of the Barnes Maze Test. pTHC had no effect on primary latency (A) and latency to escape (B) of the adult progeny, in the probe- and reversal tasks, respectively. Data are shown as the mean  $\pm$  SEM. CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC.

### T-maze - Prenatal THC exposure has no effect on working memory of adolescent and adult progeny in the spontaneous alternation test

For the evaluation of spatial working memory, the spontaneous alternation T-maze test was used to assess working memory. Both prenatally THC treated- and CTRL offspring showed an alternation rate above 50%, which expresses a normal processing of the working spatial memory. No significant differences were found between the groups, either in adolescence ( $U = 84.00$ ,  $p > 0.9999$ ; figure 31, A) or in adulthood ( $U = 12.50$ ,  $p = 0.4242$ ; figure 31, B).

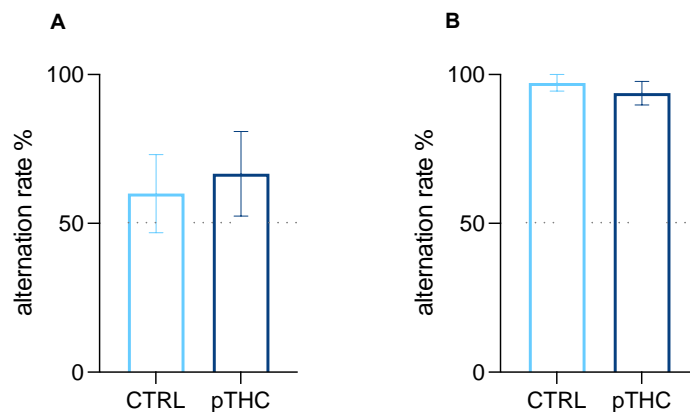


Figure 31. Prenatal THC exposure (2 mg/kg) did not affect working memory in the spontaneous alternation T-Maze test either in adolescence (A) nor in adulthood (B). Data are shown as the mean  $\pm$  SEM.  
CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC.

### Exploration of hippocampal neuroplasticity associated with cognitive processing

#### Prenatal THC exposure impinges on neuroplasticity in the hippocampus of adolescent- and adult offspring

When the effect of prenatal exposure to THC on the hippocampal neuroplasticity of adolescent offspring was explored, data showed significant effect of prenatal THC exposure on CB1R ( $t = 3.458$ ,  $df = 8$ ,  $p = 0.0086$ ; figure 32, A), HINT1 ( $t = 3.171$ ,  $df = 8$ ,  $p = 0.0132$ ; figure 32, B), NR1 ( $t = 8.247$ ,  $df = 8$ ,  $p < 0.0001$ ; figure 32, C), mGluR5 ( $t = 3.261$ ,  $DF = 8$ ,  $p = 0.0115$ ; figure 32, D), and Homer 1 ( $U = 0.00$ ,  $p = 0.0095$ ; figure 32, E) relative expression levels.

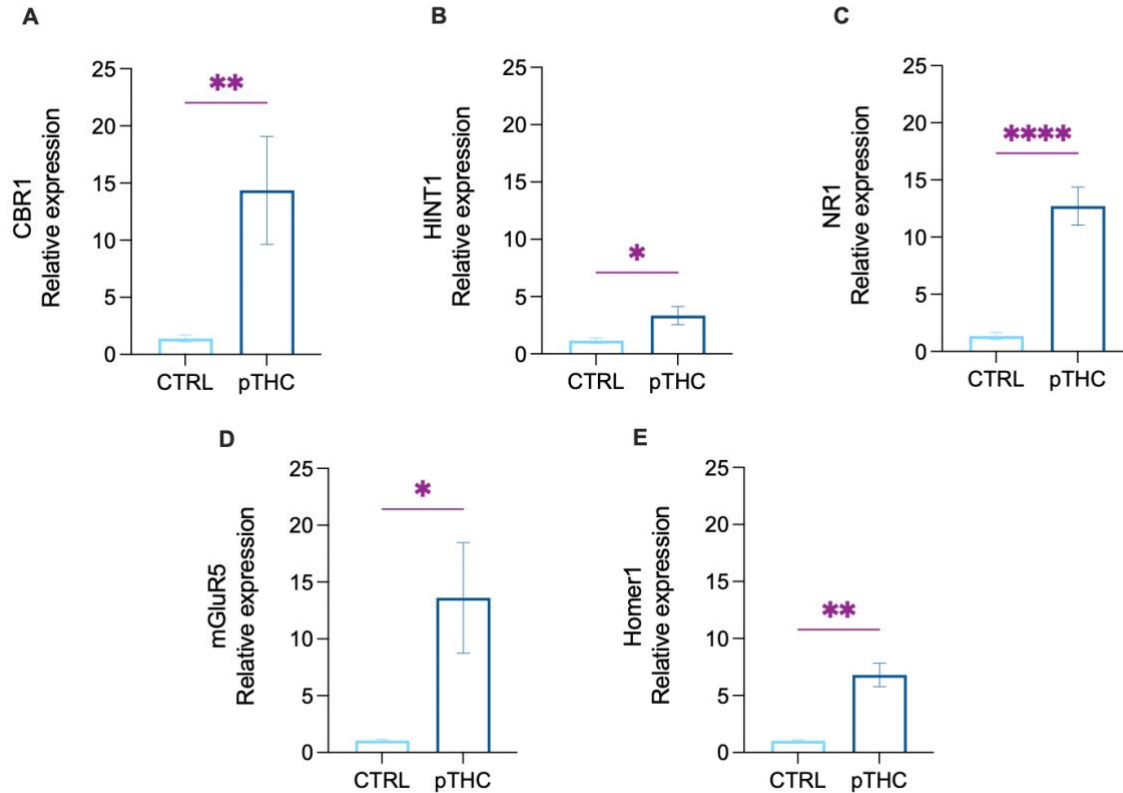


Figure 32. Prenatal THC exposure (2 mg/kg) impacts adolescent hippocampal neuroplasticity. mRNA expression of CB1R (A), NR1 (C), and mGluR5 (D), together with scaffold partners – Homer 1 (E) and HINT1 (B), that are involved in the cascade leading to hippocampal synaptic plasticity – were increased in the hippocampus of adolescent progeny by prenatal THC exposure. Data are shown as the mean  $\pm$  SEM. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\*\* $p < 0.0001$ . CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC.

Investigation on adult hippocampal neuroplasticity of adult progeny, showed a significant impact of *in-utero* exposure to THC, with decreased NR1 ( $U = 0.00$ ,  $p = 0.0286$ ; figure 33, C) and mGluR5 ( $U = 4.00$ ,  $p = 0.0397$ ; figure 33, D) expression levels. On the other hand, pTHC increased HINT1 hippocampal mRNA expression ( $t = 2.343$ ,  $df = 8$ ,  $p = 0.0472$ ; figure 33, B), while had no effect on hippocampal expression of CB1R ( $t = 1.547$ ,  $df = 6$ ,  $p = 0.1729$ ; figure 33, A) and Homer 1 ( $t = 0.9217$ ,  $df = 6$ ,  $p = 0.3922$ ; figure 33, E) in adult offspring.

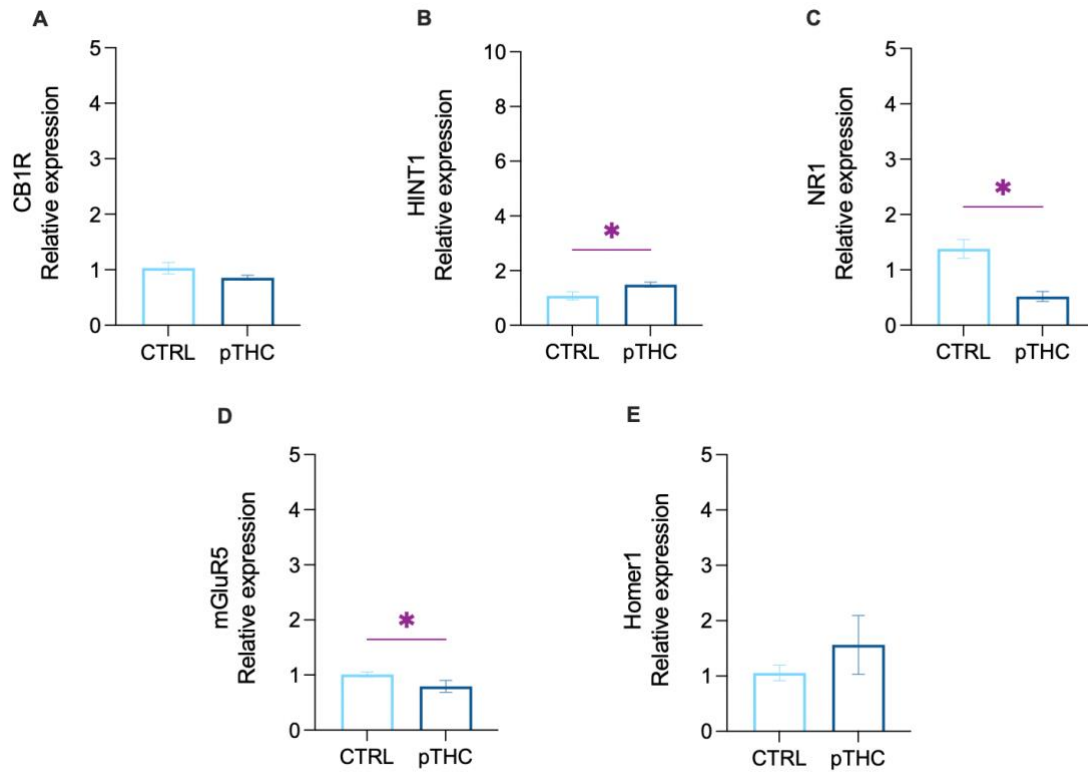


Figure 33. Impact of prenatal THC exposure (2 mg/kg) on adult hippocampal neuroplasticity. Prenatal THC treatment was found to decrease NR1 (C) and mGluR5 (D) in the hippocampus of adult progeny. On the other hand, prenatal exposure to THC increased HINT1 (B), while did not affect the expression level in the hippocampus of CB1R (A) and Homer 1 (E). Data are shown as the mean  $\pm$  SEM. \* $p < 0.05$ . CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC.

### CBD differentially restores hippocampus maladaptive neuroplasticity in prenatal THC-exposed adolescent and adult rats

When administered 24 h prior to testing, CBD ameliorated the hippocampal neurochemical alterations occurred in adolescent rats exposed to THC during prenatal epoch. Data analysis showed a main effect of pTHC ( $F(1, 16) = 18.54$ ,  $p = 0.0005$ ) and its interaction with CBD ( $F(1, 16) = 5.083$ ,  $p = 0.0385$ ) on the relative expression of CB1R (figure 34, A), while no effect of CBD was found in hippocampal mRNA expression (pTHC:  $F(1, 16) = 14.05$ ,  $p = 0.0018$ ; CBD:  $F(1, 16) = 3.375$ ,  $p = 0.0848$ ; interaction:  $F(1, 16) = 0.01741$ ,  $p = 0.8967$ ; figure 34, B). A main effect of CBD ( $F(1, 16) = 9.289$ ,  $p = 0.0077$ ), pTHC ( $F(1, 16) = 47.44$ ,  $p < 0.0001$ ), and their interaction ( $F(1, 16) = 37.92$ ,  $p < 0.0001$ ) was found in NR1 hippocampal expression levels. In particular, whilst the increasing effect of pTHC was confirmed ( $t = 9.132$ ,  $DF = 16.00$ ,  $p < 0.0001$ ), CBD treatment induced a decrease in prenatally THC-exposed rats ( $t = 6.201$ ,  $DF = 16.00$ ,  $p < 0.0001$ ; figure 34, C). The same output was detected for the hippocampal expression level of mGluR5, with a main effect of CBD ( $F(1, 15) = 20.87$ ,  $p = 0.0004$ ), pTHC ( $F(1, 15) = 77.88$ ,  $p < 0.0001$ ), and their interaction ( $F(1, 15) = 32.62$ ,

$p < 0.0001$ ). In detail, as opposed to the increase induced by pTHC ( $t = 10.48$ ,  $DF = 15.00$ ,  $p < 0.0001$ ), CBD treatment induced a decrease in mGluR5 in pTHC rats ( $t = 7.133$ ,  $DF = 15.00$ ,  $p < 0.0001$ ; figure 34, D). Lastly, a main effect of CBD ( $F(1, 16) = 12.14$ ,  $p = 0.0031$ ), pTHC ( $F(1, 16) = 68.94$ ,  $p < 0.0001$ ), and their interaction ( $F(1, 16) = 16.84$ ,  $p = 0.0008$ ) were detected in the hippocampal Homer 1 expression. In particular, while pTHC induced an increase of Homer 1 levels ( $t = 8.685$ ,  $DF = 16.00$ ,  $p < 0.0001$ ), a decreasing effect of CBD was detected ( $t = 5.111$ ,  $DF = 16.00$ ,  $p = 0.0006$ ; figure 34, E).

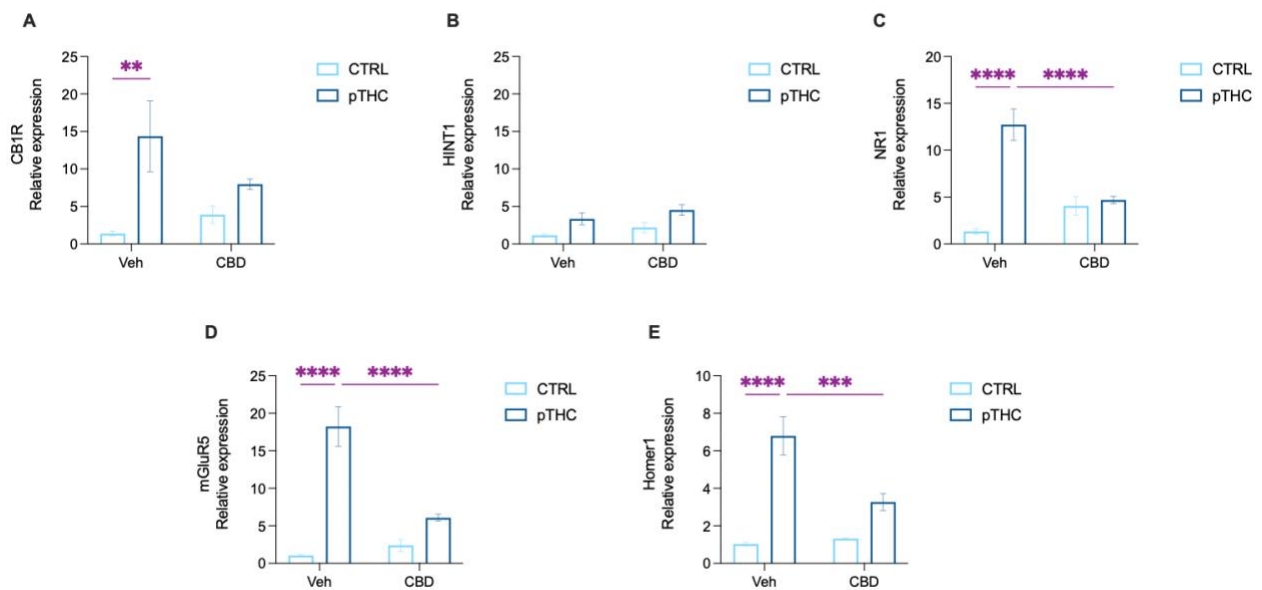


Figure 34. CBD (40 mg/kg) ameliorative effect on adolescent hippocampal maladaptive neuroplasticity induced by *in-utero* THC exposure (2 mg/kg). Whilst no effect was found on CB1R (A) or HINT1 (B), CBD decreased NR1 (C), mGluR5 (D), and Homer 1 (E) hippocampal mRNA expression levels. Data are shown as the mean  $\pm$  SEM. \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ . CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC.

Notably, CBD administration rescued the effects of prenatal THC exposure on adult hippocampal gene expression. Data analysis revealed a main increasing effect of CBD treatment on the relative expression of CB1R ( $F(1, 12) = 16.11$ ,  $p = 0.0017$ ; figure 35, A), HINT1 ( $F(1, 12) = 71.91$ ,  $p < 0.0001$ ; figure 35, B), NR1 ( $F(1, 14) = 26.99$ ,  $p = 0.0001$ ; figure 35, C), and Homer 1 ( $F(1, 12) = 17.43$ ; figure 35, E). A main effect of CBD ( $F(1, 14) = 59.46$ ,  $p < 0.0001$ ), pTHC ( $F(1, 14) = 47.56$ ,  $p < 0.0001$ ) and their interaction ( $F(1, 14) = 38.92$ ,  $p < 0.0001$ ) were detected in the expression levels of mGluR5. In particular, mGluR5 expression levels were higher in CBD-treated CTRL offspring

with respect to both CTRL ( $t = 9.864$ ,  $DF = 14.00$ ,  $p < 0.0001$ ) and lower in CBD-treated pTHC offspring ( $t = 8.812$ ,  $DF = 14.00$ ,  $p < 0.0001$ ; figure 35, D).

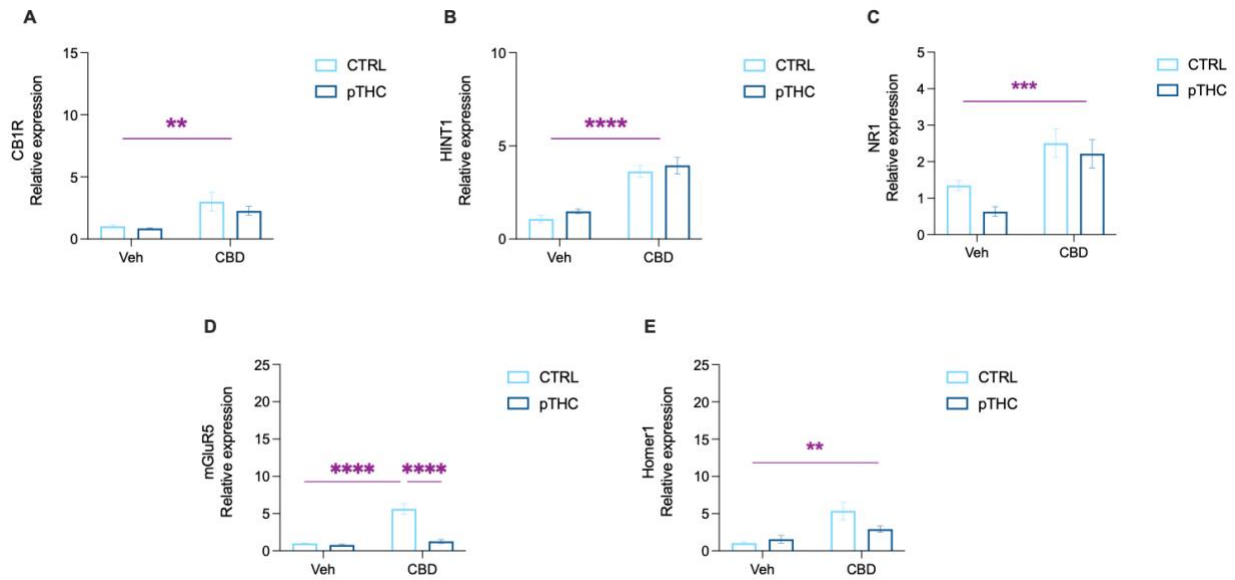


Figure 35. CBD (40 mg/kg) ameliorative effect on hippocampus maladaptive neuroplasticity induced by *in-utero* THC exposure (2 mg/kg) in the adult progeny. CBD increased the expression of CB1R (A), HINT1 (B), NR1 (C), mGluR5 (D), and Homer1 (E) in the hippocampus of adult male rats. Data are shown as the mean  $\pm$  SEM. \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ . CTRL = male rat offspring prenatally exposed to Veh; pTHC = male rat offspring prenatally exposed to THC.

## Discussion

The expanding cannabis legalization results in a pro attitude about cannabis consumption, as it is identified as a harmless and excellent self-medicating strategy for a vast array of medical conditions, including morning sickness and vomiting of pregnancy (Mauro et al., 2019; Metz and Stickrath, 2015; Metz and Borgelt, 2018; Hasin et al., 2017; Martins et al., 2016; Fitzcharles and Eisenberg, 2018; Klumpers and Thacker, 2018). Despite recent findings reporting a negative association between aspects of executive function and *in-utero* cannabis exposure in children beyond the age of 3 (Fried and Smith, 2001; Fried, 2002), clinical reports on the issue are controversial and far from definitive. In this scenario, animal models provide a useful tool for examining the long-term effects of *in-utero* exposure to cannabinoids. *In vivo* research is, indeed, of unquestionable value since it allows for examination, while strictly controlling confounding variables, parameters such as dosage, the timing of exposure, genetic factors, and possible underlying neurological causes for behavioral alterations (Fried, 2002). To date, few data are currently available concerning the long-lasting cognitive consequences of early exposure to THC at doses equivalent to the current estimates of moderate cannabis consumption in humans (Nashed et al., 2021). Thus, the second part of the present research assessed the outcome of *in-utero* exposure to THC on the executive cognitive processing in the adolescent and adult progeny. Peculiarly, defective memory acquisition and retrieval, as well as cognitive flexibility, triggered by both reward – Can Test – and aversive – Barnes Maze Test – settings were detected, along with memory-related glutamatergic synaptic plasticity within the hippocampus. Furthermore, the CBD's potential to rescue cognitive deficits and neurochemical alterations was highlighted.

The aberrant activation of the ECS plays a role in the cognitive impairment observed in cannabis users, primarily during developmental epochs for higher-order reasoning (Gogtay et al., 2004; Maij et al., 2017). More specifically, manipulation of eCBs affects reward-related explicit memory and the efficacy of fear extinction brain circuits, thus altering the ability to successfully recall the emotional salience of normally subthreshold conditioning cues and fear memory (Brancato et al., 2019; Loureiro et al., 2016; Draycott et al., 2014; Hudson et al., 2018). Moreover, the association between cannabis use and reduced complex executive functioning essential for adaptive behavior in many species – as the ability to solve problems, make decisions, control emotions and behavior, as well as sustained attention – is observed in longitudinal studies of cannabis users, suggesting that these are enduring vulnerabilities (Camchong et al., 2017; Crean et al., 2011; Scott et al., 2018; Sagar and Gruber, 2018). So far, this is the first research investigating the core executive functions of *in-utero* THC-exposed rat progeny by employing the Can Test. In the task, rats were trained to identify a single water-

rewarded can among a set of seven cans, using a water-restricted deprivation schedule for motivation. The Can Test, a reward-motivated task, allows the assessment of reference- and working memory in the spatial acquisition, visual discrimination, and cognitive flexibility. This test has a higher translational value since it mimics the creation of a map of the environment based on how far, at what speed, and in which direction they have traveled in order to obtain the reward, based on spatial, tactile, and visual cues, as well as and proprioceptive hints (Jacobs and Schenk, 2003). Indeed, spatial cognition includes all the processes that allow animals to acquire, process, memorize and use spatial cues to perform goal-directed movements towards a reward (Retaillie et al., 2016). In addition, it enables to investigate the ability of the rats to adapt behavioral strategies to the demands of the assignment (Jacobs and Schenk, 2003). Indeed, in the distinct, serial tasks, rats learn to select the can with the reward based on its position and its surface, or its visual appearance (Popovic et al., 2001). This mechanism, which is crucial for survival, requires intact hippocampus functionality (Tsien et al., 1996; Seese et al., 2012; Seese et al., 2014).

Adolescent prenatal THC-exposed rats displayed a lower number of correct responses and a higher score of reference memory errors than controls in the spatial task of the Can Test. Since it has been previously demonstrated, in the present research, that prenatal THC treatment does not induce either an effect on water consumption in the acquisition phase of the operant paradigm or a difference in the locomotor activity among experimental groups, measured by the activity score in the Can Test, these findings indicate that prenatal THC exposure impacts storage and recall of reward-induced spatial memory traces. These results are in line with preclinical data showing that improper excitation of ECS, resultant from exposure to THC at prenatal epoch, is correlated to altered spatial, contextual memory and processing of adolescent rats, modeled in the active place avoidance test (Silva et al., 2012). Importantly, cognitive deficits were associated with decreased hippocampal glutamate outflow and uptake, and impaired hippocampal LTP (Antonelli et al., 2004, Castaldo et al., 2007, Mereu et al., 2003). Nonetheless, to date, the present findings represent the first evidence of the impact of prenatal THC exposure on the spatial orientation acquisition of adolescent offspring in a non-aversive test. Preclinical evidence turns sparser and results conflicting when the formation and recall of memory traces are explored in a behavioral task that includes a set-shifting associated with a visual discrimination strategy. Even more so, when one looks at a pure test – such as the Can Test – rather than sophisticated, operant tasks (Zoccolan et al., 2009). Discrimination processing is crucial for attention, inhibitory control, and sensory memory (Alain and Woods, 1999). Rodent paradigms based upon set-shifting and reversal tasks that assess spatial/visual discrimination memory are crucial in distinguishing disruption in executive functions and the underlying neuroplasticity (Talpos and Shoaib, 2015). Thus, prenatal THC-exposed and non-exposed offspring underwent the second task



of the Can Test, which aims at the exploration of rat visual discrimination, investigating its ability to recognize a different object which changes position among similar others, in a familiar environment (Plescia et al., 2014). *In-utero* THC exposure was shown to decrease correct responses and increase reference memory errors of adolescent offspring. These findings indicate that prenatal THC worsens the skill to solve the simple object-discrimination task in adolescence, which exploits the natural exploratory activity of rodents and assesses the detection of spatial relocations of a known object (Plescia et al., 2014). Likewise, switches between tasks and increases in task complexity of the Can Test accentuated the performance deficits and highlighted the inability of pTHC rats to adapt behavioral strategies to the demands of the tasks. Accordingly, adolescent offspring from mothers exposed to THC exhibited deficits in object recognition memory (Lallai et al., 2022). However, so far, no evidence comes from fine, albeit simple, tests for recognition memory associated with a positive prompt, as the visual task of the Can Test. THC has been shown to cause a residual impairment in object recognition memory of rats when administered during adolescence, along with profound lasting alteration on protein expression profiles in the hippocampus (Quinn et al., 2008). Indeed, prenatal THC-induced deficits in cognitive tasks, including novel object recognition test, have been already characterized via intrahippocampal recordings, showing alterations in main hippocampal oscillations in adult males (de Salas-Quiroga et al., 2020).

Concern raises about the equal incentive that balances motivation among groups of animals and does not apply to appetitive tasks (Vorhess et al., 2014). Besides, fear-eliciting contexts influence the performance of executive tasks, including attention, working memory, inhibition, and flexibility in the cognitive processes (Chen et al., 2022; Heyser et al., 2000) and physiologically prompt instinctive defensive behaviors. Given that, Barnes Maze Test – a rodent dry-land-based, hippocampal-dependent behavioral paradigm – was employed to evaluate cognitive strategies implemented by mammals to allocate attentional resources towards the goal of safety (Pitts, 2018; Gawel et al., 2019; Harrison et al., 2009). It represents a well-established alternative to the more popular Morris Water Maze and offers the advantage of being free from the potentially confounding influence of swimming behavior (Pitts, 2018). Bright light and open spaces are aversive to rodents, thus serve as motivating factors to induce escape behavior (Gawel et al., 2019). Indeed, rodents find open, well-lit spaces aversive and they will search around the platform trying to find the escape box (Sweatt, 2010). Prenatal THC exposure was shown to harm the learning performance of adolescent offspring during the criterion acquisition phase: pTHC rats displayed higher latency to reach the escape box. Although non-significantly different, the control adolescent rats reached the learning criterion 1 day before the prenatally THC-exposed rats. Subsequently, *in-utero* THC-exposed adolescent offspring showed a decrease in primary latency in the probe task, that is, the time to localize the former entrance to the

escape box. Since the Barnes Maze Test assumes that rats learn to solve a maze by using negative environmental factors – open space, as well as bright illumination (Gawel et al., 2019; Locklear and Kritzer 2014; McLay et al., 1998) – it is necessary to bear in mind that the performance of rodents in the Barnes Maze Test may be influenced by numerous non-cognitive factors – anxiety, low exploratory activity (McHail et al., 1998). For instance, if the animals show a high level of anxiety, they may spend the time on the surface of the maze not actively looking for the escape box, but just grooming or freezing. In contrast, when the rodents show low levels of anxiety, they may fast explore the maze rather than look for a safe shelter (Gawel et al., 2019). In both cases, the time to reach the escape box may be prolonged and not truly reflect memory impairments. These factors, however, do not affect the behavior of the animal if properly controlled. The habituation phase, preceded by the handling of the animals for a few days prior to the Barnes Maze Test, is considered enough for the rats to familiarize themselves with both the platform and escape box, to reduce anxiety (Gawel et al., 2016; Marszalek-Grabska et al., 2018). In addition, prenatal THC exposure did not affect the measure of explorative behavior in the OFT – the number of central transitions from the peripheral to the central area of the arena. Furthermore, although pTHC rats displayed an increased locomotor activity when tested in the open field, they displayed no differences in the total distance traveled when compared to controls under the experimental conditions of the probe task. On the other hand, in the first part of the present research, the abnormal resistance to the punishment in the operant chamber was linked to weaker learning of the pairing between the punishment and the lever pressing. Thus, once potential confounding factors are excluded, one could interpret the higher latency to escape of the pTHC offspring as a deficit in the context of memory acquisition and retrieval. These findings are in line with a previous study on prenatal WIN 55,212-2 – a potent cannabinoid receptor agonist – exposure inducing a disruption of memory retention in late adolescent offspring subjected to a passive avoidance task, correlated with alterations of hippocampal LTP and glutamate release (Mereu et al., 2003). De facto, the hippocampus is a key structure in navigation and spatial learning and memory (Morris et al., 1982), while it is also essential for remembering aversive contexts (Phillips and LeDoux, 1992; Curzon et al., 2009). When aversive stimuli are linked with spatial representation, animals learn this association and decide whether to initiate avoidance behavior against aversive locations. In some circumstances, they accept aversive contexts to obtain a reward to ensure their survival. In addition, late adolescent offspring prenatally exposed to THC displayed a significant treatment effect in the reversal performance tested in an active place avoidance task (Silva et al., 2012). Accordingly, in the present research, prenatal THC exposure was shown to exert a negative impact also in the latency to escape of adolescent progeny in the reversal phase, when the position of the escape box is moved to a location directly opposite the initial target site to assess learning

flexibility.

Spatial learning, along with contextual conditioning, is one of the most studied forms of place learning, since remembering the location and context of feeding resources and escape routes is likely one of the most essential forms of higher cognitive processing in mammals (Silva et al., 1998). Thus, the detection of impaired encoding and retrieval of spatial and recognition memory traces, and cognitive flexibility in this research naturally raises the question of whether there is a difference in their neural correlates. Accordingly, in the present study, the hippocampal neuroplasticity in the expression levels of specific markers was explored (Rubino et al., 2009). The ability of synapses to express plasticity is a key component of the learning machinery, which is tightly regulated by various intercellular signaling molecules (Abraham, 2008). Notably, NMDAR, mGluR5, and CB1R, together with scaffold partners – Homer 1 and HINT1 protein – that are involved in the cascade leading to hippocampal synaptic plasticity (Izumi and Zorumski, 2017; Doherty and Dingledine, 2003) were explored. In the adolescent progeny, prenatal THC exposure altered developmental programs relating to the levels of the endocannabinoidergic and glutamatergic markers, in an overexpressing fashion. eCBs have been shown to signal at glutamatergic synapses on dendritic spines throughout the hippocampus: diacylglycerol lipase  $\alpha$ , the precursor to 2-AG, is highly concentrated on the heads of dendritic spines indicating involvement with retrograde signaling from glutamatergic synapses to presynaptic CB1R (Katona et al., 2006). Within the DG specifically, mossy cells contain abundant presynaptic CB1R, and granule cell spines release 2-AG forming a bridge for retrograde signaling to adjust network activity following excitation (Uchigashima et al., 2011). Since the CB1R are located at the end of mossy cells synapses releasing glutamate onto granule cell spines, following exogenous cannabinoid agonist stimulation, the Gi/o coupled receptor would prevent glutamate release and the postsynaptic spine may retract due to loss of excitation, leading to synaptic weakening (Gipson and Olive, 2017). Concurrently, the reduction in hippocampal glutamatergic synaptic transmission due to the CB1R agonism (Misner and Sullivan, 1999; Domenici et al., 2006) would lead to ECS signaling empowerment and glutamatergic receptors' compensatory upregulation, as has been shown in the present study (Candelaria-Cook et al., 2012; Shen et al., 1996; Mereu et al., 2003). On the other hand, eCBs may exploit the indirect control over excitatory glutamate transmission via the direct modulation of inhibitory interneuron signaling. A rich variety of inhibitory interneurons limits excitatory activity via a well-tuned excitatory-inhibitory balance, which appears to be essential in enabling memory-related synaptic plasticity and neuronal network development (Froemke, 2015; Jazmati et al., 2018; Letzkus and Wolff, 2015). Cholecystokinin (CCK)-expressing basket cells (CCK<sup>+</sup> BC) encompass a subclass of inhibitory GABAergic interneurons that regulate memory-forming oscillatory network activity of the hippocampal formation (Ledri, 2011). Similarly, NPY has

the potential to modulate excitatory synaptic transmission within the hippocampus, by inhibiting presynaptic glutamate release via NPY receptor type 2 (Y2; Klapstein and Colmers, 1993; Sørensen et al., 2008). In the hippocampus, CB1R is localized primarily on CCK+ BC GABAergic interneurons (Tsou et al., 1999; Monoroy et al., 2006). Notably, CB1R signaling in developing GABAergic interneurons is required for the adequate maturation of CCK-dependent hippocampal function in male mice (de Salas-Quiroga et al., 2020). In addition, it has been demonstrated that CB1R located on hippocampal GABAergic interneurons is the main target for embryonic THC exposure. Indeed, prenatal THC exposure interferes with the development of CCK+ BC interneurons, thereby leading to altered feedback inhibition (Vargish et al., 2016). Importantly, further interference with inhibitory projecting neurons has been shown in the first part of the present research, with a decrease in the NPY signaling in limbic areas induced by prenatal THC exposure. The persistent alteration in excitatory and inhibitory activity thus appears to be the reason for impaired hippocampal information coding, as has been shown in the present study. Besides, prenatal THC exposure induced hippocampal overexpression of HINT1, a potential marker of appropriate neuroplasticity, in the adolescent offspring. HINT1 is highly related to many neuropsychiatric diseases including schizophrenia, mood disorder, and drug addiction, although the specific mechanism is yet to be determined (Liu et al., 2017). What is established is that HINT1 interacts with the NR1 subunit of NMDAR and GPCRs, such as CB1R, to ensure an adequate inhibitory control of NMDAR activity (Dracheva et al., 2001). In the cell membrane, the physical association of CB1-NMDAR and its functional relevance are dependent upon the HINT1 protein (Sánchez-Blázquez et al., 2013; Vicente-Sánchez et al., 2013). Following agonist challenge, CB1R is co-internalized with HINT1 and NR1 subunits, and whereas the HINT1 protein conserves its association with the CB1R, it dissociates from the NR1 subunits. When the CB1-HINT1 complexes are recycled back to the cell surface, they rapidly re-associate with new NR1 subunits for their co-internalization (Sánchez-Blázquez et al., 2014). Thus, in a context with overexpression of NMDAR subunits and control-like expression levels of CB1R, HINT1 is called to regulate the higher excitability and prevent excitotoxicity (Vicente-Sánchez et al., 2013). The overexpression of NR1 mRNA, which exceeds the physiological increase following hippocampal LTP (Cammarota et al., 2000), and the concomitant increase in HINT1 and CB1R expression levels, might result in an inappropriate assembly of a neuronal network that could represent the substrate for the observed cognitive dysfunctions displayed by the adolescent THC-exposed offspring.

When the cognitive phenotype of adult pTHC and control progeny was examined, prenatal THC exposure turned out to differentially impact core executive functions. In the Can Test, although no statistically significant differences were detected in the number of correct responses to the visual task, adult prenatally THC-treated rats showed spatial impairment detected during the spatial task of the

paradigm, together with an increase in reference memory errors in the visual task. This notion is in line with previous findings showing impairment in spatial memory acquisition of rodents exposed to either THC or WIN 55,212-2 during embryonic development, while no or slight alteration in the performance in the Novel Object Recognition test (de Salas-Quiroga et al., 2020; Bara et al., 2018). Nonetheless, a significant reduction in the hippocampal glutamatergic marker, as the mGluR5 and NR1, and an increase in HINT1 expression levels, as the inhibitory control of glutamatergic neurotransmission, were detected in adult prenatally THC-exposed rats, suggesting a dampened glutamatergic signaling strength. Similarly, impairment in spatial memory performance was found linked to decreased levels of mGlu5R mRNA, plus an ablated ECS-mediated LTD in the PFC of adult male offspring prenatally treated with synthetic cannabinoid WIN55,212-2 (Bara et al., 2018). One can hypothesize that the decrease in hippocampal glutamatergic by prenatal THC exposure is due to the selective aberrant actions of persistent long-term forms of synaptic plasticity over time (Massey et al., 2007). No differences in CB1R expression levels were expected, since previous evidence indicates no impact of prenatal THC exposure on adult PFC's ECS mRNAs, including CB1R, CB2R, and synthesizing/metabolizing enzymes of the eCBs (Bara et al., 2018). Furthermore, no significant effect of prenatal THC exposure was found when adult rats were tested for spatial memory acquisition and reversal learning in the Barnes Maze Test. Since no evidence exists on the outcome of prenatal THC exposure on adult offspring behavior in aversive contexts, a comparison may be done with findings from THC exposure during the peculiar, vulnerable adolescence epoch. In particular, adult rats subjected to THC during adolescence showed less potent deficits in spatial learning and memory, and cognitive flexibility, than did adolescents when tested in the Barnes Maze Test, and only in combination with exposure to alcohol in adolescence (Gibula-Tarlowska et al., 2020). Allegedly, differences in the type of reinforcement – appetitive in the Can Test vs. aversive in the Barnes Maze – may relate to differences in performance across the two conditions. Somewhat different brain processes may be engaged during memory tasks that employ appetitive versus aversive reinforcement. For example, long-term memory recall is facilitated when memorizing material with a strong emotional component, compared with more neutral items (Mickley et al., 2016; Tyng et al., 2017). Indeed, in the precise context of prenatal THC exposure and the observed adolescent aberrant synaptic plasticity, a different asset in neurochemical circuits required to perform the Barnes Maze-peculiar tasks may occur in the adult epoch, likely related to resilience-involved NPYergic neurotransmission. Functionally, NPY release is associated with appetite, anxiety, and stress regulation. Furthermore, Y2 are significantly involved in hippocampus-dependent spatial memory and their expression is dynamically regulated by the learning experience. NPY acting on Y2 facilitates fear extinction suggesting a role in associative memory and resilience (Mendez-Couz et

al., 2021; Cohen et al., 2012). Notably, NPY is released as a co-transmitter with plasticity-related neurotransmitters such as glutamate in the hippocampus, in order to depress hyperexcitable activity. On this basis, and given the observed depleted glutamatergic asset of adult pTHC offspring, it is reasonable to assume that NPY may have taken over excitatory neurotransmission and settled a robust resilience strategy (Golob et al., 2002).

Remarkably, when the potential rescue of CBD was explored in adolescent and adult progeny before the Can Test, the observed correlates of prenatal THC exposure were prevented in a peculiar fashion. CBD administration in adolescents restored spatial memory consolidation and accessibility, as well as facilitation in the rat ability to draw up a successful response of the adolescent rats exposed to THC *in-utero*. This is shown by an increased number of correct responses, and a decrease in reference memory errors in the spatial and visual tasks of the Can Test. Therefore, it seems that this phytocannabinoid interferes positively not only with the acquisition of spatial information, but also when adolescent rats are requested to switch to the increasingly complex version of the task in its visual discrimination component. This evidence is in agreement with the pro-cognitive effect of CBD described in the clinical setting. Studies in healthy subjects showed that CBD modulates brain activity and has opposite effects when compared to THC in resting state and during several cognitive paradigms – emotional processing, memory, response inhibition – following task-specific activation patterns (Batalla et al., 2021). CBD discretely rectified indices of glutamatergic neuroplasticity in the hippocampus of adolescent rats, with expression levels of NMDAR NR1 subunit, mGluR5, and Homer 1 of pTHC offspring being decreased to control levels. Notably, the effect of CBD treatment on changes in the expression of the NMDAR subunit has been already assessed in the rat hippocampus in a model of chronic epilepsy. Similarly, the results revealed a marked decrease in the quantity of NR1 mRNA in the CBD treatment groups (40 and 80 mg/kg) compared to the model control group, thus protecting from hippocampal neuronal damage (Mao et al., 2015). The answer to how CBD might exert its recovery effect in the present study is to be found in the increasingly explored poly-pharmacodynamics of CBD (Pretzsch et al., 2019). In this context, the relationship between CBD and glutamatergic receptors expression is apparently indirect and likely resides in its influence on the ECS signaling. CBD arguably inhibits the activity of fatty acid amide hydrolase (FAAH), a major enzyme involved in anandamide (AEA) hydrolysis (Bisogno et al., 2013). The ability of CBD to inhibit AEA hydrolysis and reuptake causes an increase in the concentration of available endogenous cannabinoids to bind their respective receptors (de Almeida et al., 2020) and exploit the appropriate inhibitory control on excitatory, glutamatergic signaling in the hippocampus. An additional strategy to reverse prenatal THC exposure-induced deficits based on our findings may be that Transient Receptor Potential Vanilloid 1 (TRPV1R) mediates LTD as well. AEA is an

endogenous ligand of TRPV1R (Di Marzo et al., 2002), thus the inhibition of anandamide's main degrading enzyme FAAH would increase anandamide levels to the threshold for LTD induction in the prenatal THC-exposed males (Rubino et al., 2015). Indeed, incubation of mPFC slices prepared from prenatally THC-treated male rats with the FAAH inhibitor URB597 was shown to effectively restore eCBs-LTD (Bara et al., 2018). Since CBD enhanced the release of eCBs, the indirect effect of down-regulation of CB1R in adolescent and adult progeny was expected. Nonetheless, CBD may operate throughout components of the serotonin (5-HT) pathways, likely acting as a 5-HT<sub>1A</sub> receptor positive allosteric modulator (Mori et al., 2021). The 5-HT systems have been depicted as neuromodulators on the operations of the CNS that require a high cognitive demand (Buho, 1997). Indeed, 5-HT neurotransmission modulates appropriate learned behavioral responses, by adjusting the timing of responses to ensure more adapted behavior (Bari and Robbins, 2013; Bacqué-Cazenave et al., 2020). The regulatory role of 5-HT in cognitive processing is linked to its simultaneous effects on a multiplicity of neural targets underlying these functions, including glutamatergic signaling. Indeed, the release of glutamate from the DG has been reported to be under 5-HT inhibitory regulation via the postsynaptic 5-HT<sub>1A</sub> receptor (Matsuyama et al., 1996).

Accordingly, the recovery effect of CBD herein observed appears not to be mediated by direct interaction with CB1R, since it is not significantly different from the effect of CBD when administered together with the selective antagonist of CB1R – AM281 – in the spatial task of the Can Test, neither in adolescence nor in adulthood. The use of selective antagonism on CB1R has been previously used to disentangle CBD pharmacodynamics (Zuardi et al., 2017) providing conflicting results. Although AM251 – a CB1R antagonist – was able to prevent CBD effects on processes related to aversive memories, such as extinction and reconsolidation (Bitencourt et al., 2008; Do Monte et al., 2013; Stern et al., 2012), it failed to interfere with the anxiolytic effects of locally injected CBD. Lastly, it is important to highlight that in adult offspring, CBD was slightly able to restore the impaired cognitive functioning in the spatial and visual tasks of the Can Test. Indeed, CBD moderately recovers the prenatal THC exposure-induced defective glutamatergic signaling, by generally increasing the expression of ECS- and glutamatergic transmission markers. This highlights rigid synaptic plasticity in the adult epoch that makes the prenatally THC-exposed adult offspring less sensitive to CBD rescue. Therefore, it is crucial to identify behavioral damage at its early onset, before the consolidation of an aberrant metaplasticity. Additional research will be needed to characterize the interactive effects of this drug in hippocampal circuits underlying cognitive processing.

## Conclusion

Clinical and experimental evidence indicates that ECS governs many ontogenetic stages of the CNS. The manipulation of such regulators by the exposure to phytocannabinoids during brain developmental, susceptible epoch is an important determinant in the long-term cognitive and behavioral deficits found in the progeny.

The present findings indicate that *in-utero* THC exposure differentially impinges on prominent excitatory and inhibitory signaling of adolescent and adult offspring, which contributes to deficits in limbic memory, alcohol vulnerability and core executive functioning. Our results turns out to be unique in the use of specific protocols that allow a high refined analysis of long-term behavioral outcomes in the progeny exposed *in-utero* to cannabis, to grasp the traits necessary for the evaluation of higher-order functions. Even more, a distinction is made between the behavior induced by a reward- and an aversive stimulus.

The modulation of the ECS during the vulnerable, prenatal epoch allow to add useful elements to the drawing-up of recovery targets. Worthy of note is the evaluation of the preventive therapeutic potential of long-chain  $\omega 3$  polyunsaturated fatty acids (LC  $\omega 3$ -PUFA), which are paramount for brain development and function (Martinat et al., 2021). Nonetheless, relevant evidence of the efficacy of CBD to improve cognitive deficits and restore hippocampal neuroplasticity associated with prenatal THC exposure are reported here. Hence, the difference in the outcome and the potential recovery strategy of CBD in adolescent and adult progeny. Besides crucial a priori strategies, as prevention and awareness campaigns, the attempts to evaluate a pharmacological post-approach for THC-induced impairment are a research priority. Indeed, the legalization, resulting production, and increasing commercialization of cannabis fuel the plague and leave no other option but to study potential recovery strategies.

The diversity of findings and the lack of clear-cut results make the extent and nature of this modulation unclear, which may be explained by various factors, including differential activation of the cognitive and affective subdivisions of the hippocampus and the interactive neuronal network (Bush et al., 2000). The present research will be extended with further analysis of the neurochemical substrate in other brain areas related to executive functions, including PFC, and, hopefully, with the in-depth investigation of the dendritic spines asset underlying the memory and learning mechanism. Lastly, evidence for the impact of sex-dimorphic prenatal cannabinoid exposure on human brain development is scarce and its interpretation is extremely complex due to a wide array of confounding factors (de Salas-Quiroga et al., 2020). Considering the existence of sex-dependent differences in



intensity of phytocannabinoid-induced interference, neurodevelopmental outcomes of prenatal THC exposure have been assessed, and the results are depicted in the supplementary section below.

## Supplementary results

Although gender differences in neurobehavioral consequences of *in-utero* exposure to drugs of abuse have not been fully investigated, some longitudinal studies indicate that male and female progeny exposed *in-utero* to drugs of abuse show different vulnerabilities to deleterious effects of these substances in cognitive, executive, and behavioral domains. Males and females possess different expression levels of ECS elements: males seem to have higher CB1R binding sites than females, but females seem to possess more efficient CB1R (Rubino and Parolaro, 2011). Hence there is a gender-different impact on various cannabinoid-related outputs (Cuttler et al., 2016). On this basis, in the current study, the importance of addressing sex differences when investigating the neurodevelopmental changes induced by cannabis exposure and the neurodevelopmental consequences in the offspring has been taken into account.

To the same extent as male offspring, prenatal exposure to THC decreased the number of correct responses and increased the reference memory errors score of both adolescent and adult female progeny in the spatial- and visual- tasks of the Can Test. So far, there is no evidence on the impact of *in-utero* THC exposure on the spatial- and visual discrimination performance of adolescent female offspring. However, unlike our findings, developmental THC exposure has been shown to impact hippocampal oscillations and spatial memory impairment in a sexual dimorphism fashion, with the adult females being unaffected (de Salas-Quiroga et al., 2020). Likewise, prenatal THC exposure turned out not to alter adolescent female offspring's ability to cope with acute stress, experience pleasure, and learn to avoid an unpleasant stimulus (Traccis et al., 2021). This is in contrast with the present findings on adolescent female rats tested in an aversive context, showing that prenatal THC exposure altered the cognitive performance in the acquisition phase of the Barnes Maze Test, increased primary latency and the latency to reach the escape box in the probe- and reversal tasks, respectively. Notably, as for adult male offspring, pTHC had no impact on the behavioral strategy of adult female rats in the Barnes Maze, except for the reversal task, indicating a vigorous interference of developmental THC exposure to cognitive flexibility, even on potential compensatory neurosignaling in the aversive environment.

Although female offspring, so far, appear to be spared to some extent from prenatal exposure to THC (Traccis et al., 2021), the present preliminary findings show that pTHC induced impaired behavioral strategy in adolescent and adult female offspring hardly to the same extent of male progeny. Rather, by comparing male and female behavioral strategies, adolescent and adult female pTHC offspring performed worse in the spatial acquisition and in cognitive flexibility tasks than their male counterparts.

The controversial existence of sex- and age-dependent bias in prenatal THC exposure interference with cognitive processing, and its neurochemical correlate, remains needy for further investigation. Indeed, in an attempt to get further insights, the impact of pTHC on hippocampal neuroplasticity will be examined as an extension of the present research.

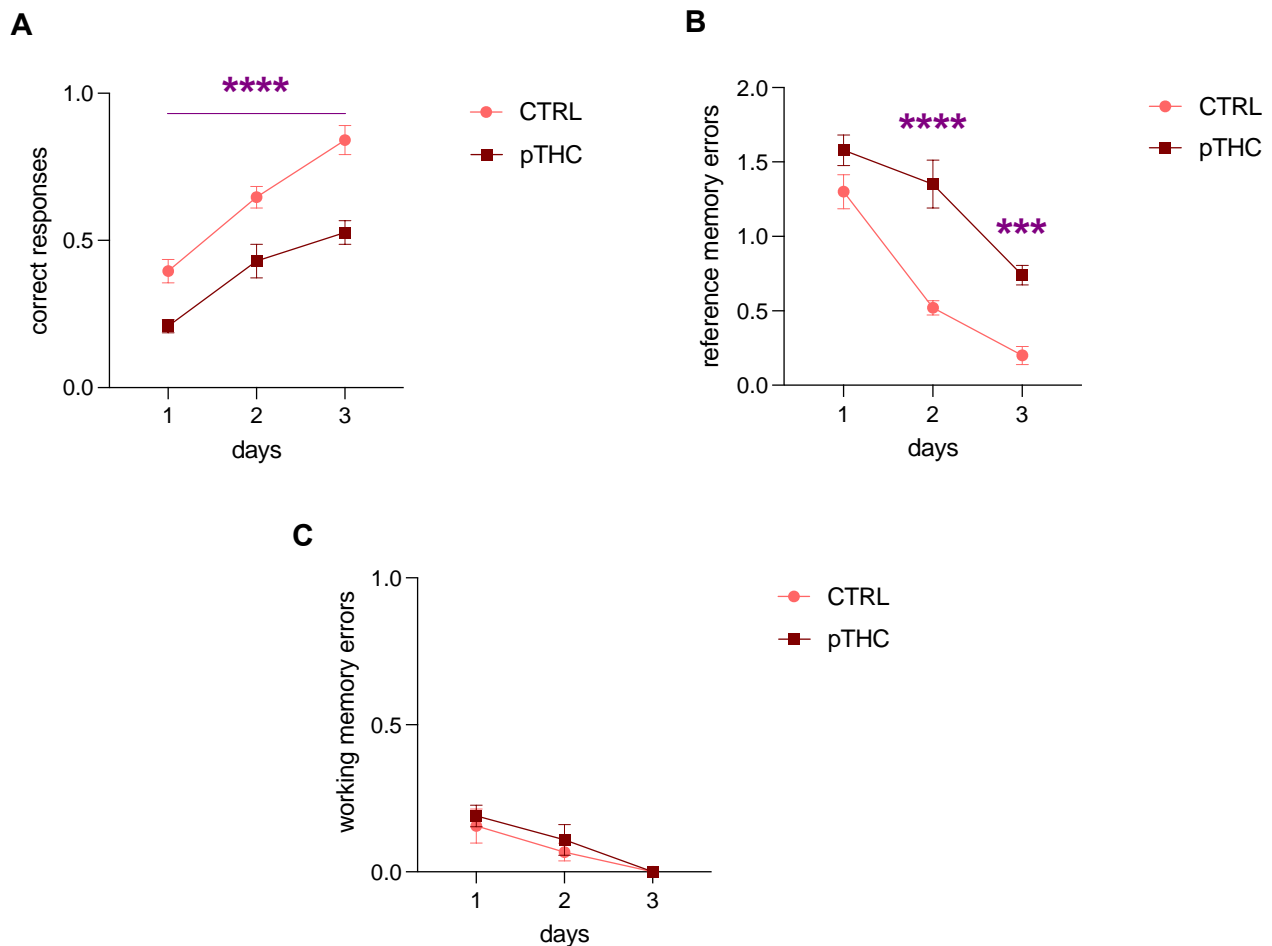


Figure X1. Prenatal THC exposure (2 mg/kg) impinges on the acquisition of spatial orientation of the adolescent female offspring in the spatial task of the Can Test. Statistical analysis of the behavioral strategy of females showed the main effects of days ( $F(2, 30) = 51.50, p < 0.0001$ ) and pTHC ( $F(1, 15) = 30.68, p < 0.0001$ ), but not their interaction, on correct responses (A). The significant effect of days ( $F(2, 30) = 69.92, p < 0.0001$ ), pTHC ( $F(1, 15) = 32.24, p < 0.0001$ ), and their interaction ( $F(2, 30) = 5.689, p = 0.0080$ ) are highlighted on reference memory errors. Post hoc analysis showed an increase in reference memory errors on days 2 ( $t = 6.138, DF = 45.00, p < 0.0001$ ) and 3 ( $t = 3.987, DF = 45.00, p = 0.0007$ ) of the female pTHC rats compared to controls (B). No significant differences were detected in working memory errors (C). Data are shown as the mean  $\pm$  SEM. \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ . CTRL = female rat offspring prenatally exposed to Veh; pTHC = female rat offspring prenatally exposed to THC.

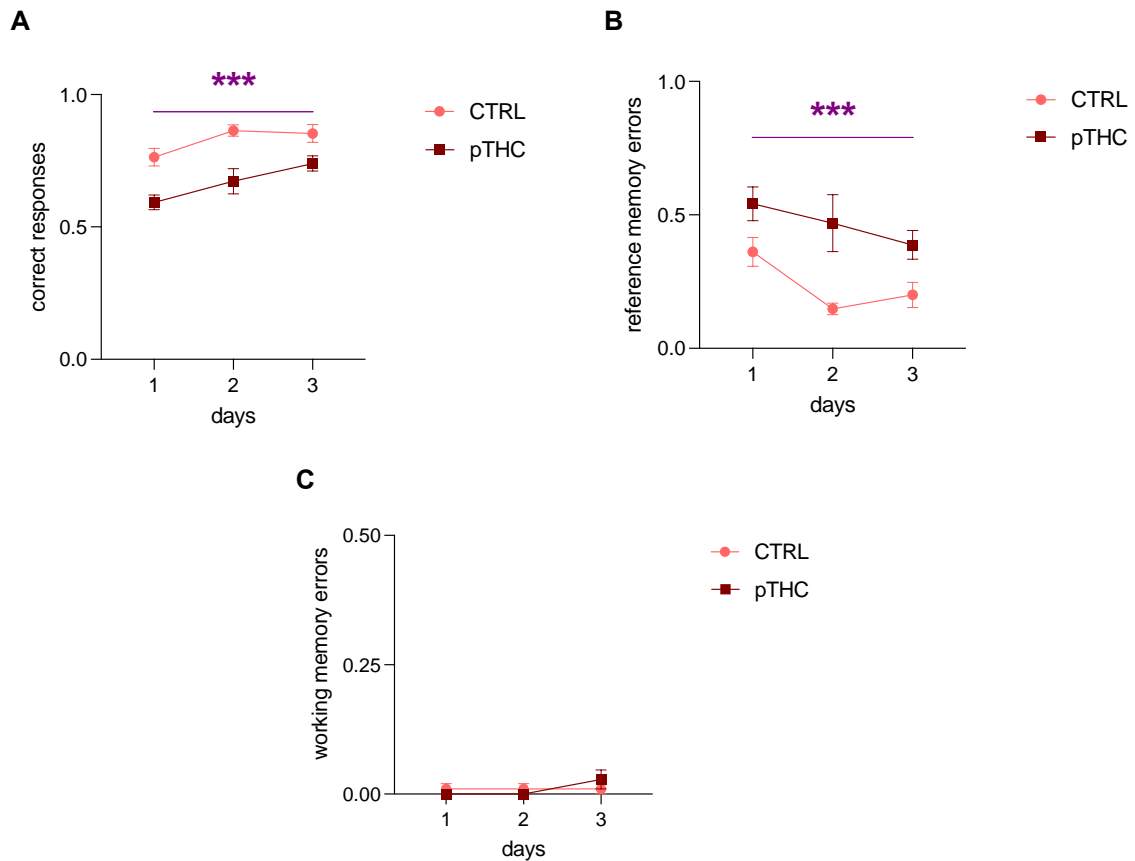


Figure X2. Prenatal exposure to THC (2 mg/kg) alters visual discrimination memory of the adolescent female offspring in the simple visual task of the Can Test. Statistical analysis of female behavioral strategy showed the main effects of days and pTHC but not their interaction on correct responses (A;  $F(2, 30) = 8.105$ ,  $p = 0.0015$ ;  $F(1, 15) = 26.63$ ,  $p = 0.0001$ ) and reference memory errors (B;  $F(2, 30) = 4.264$ ,  $p = 0.0235$ ;  $F(1, 15) = 24.54$ ,  $p = 0.0002$ ). No significant differences were detected in working memory errors (C). Data are shown as the mean  $\pm$  SEM. \*\*\* $p < 0.001$ . CTRL = female rat offspring prenatally exposed to Veh; pTHC = female rat offspring prenatally exposed to THC.

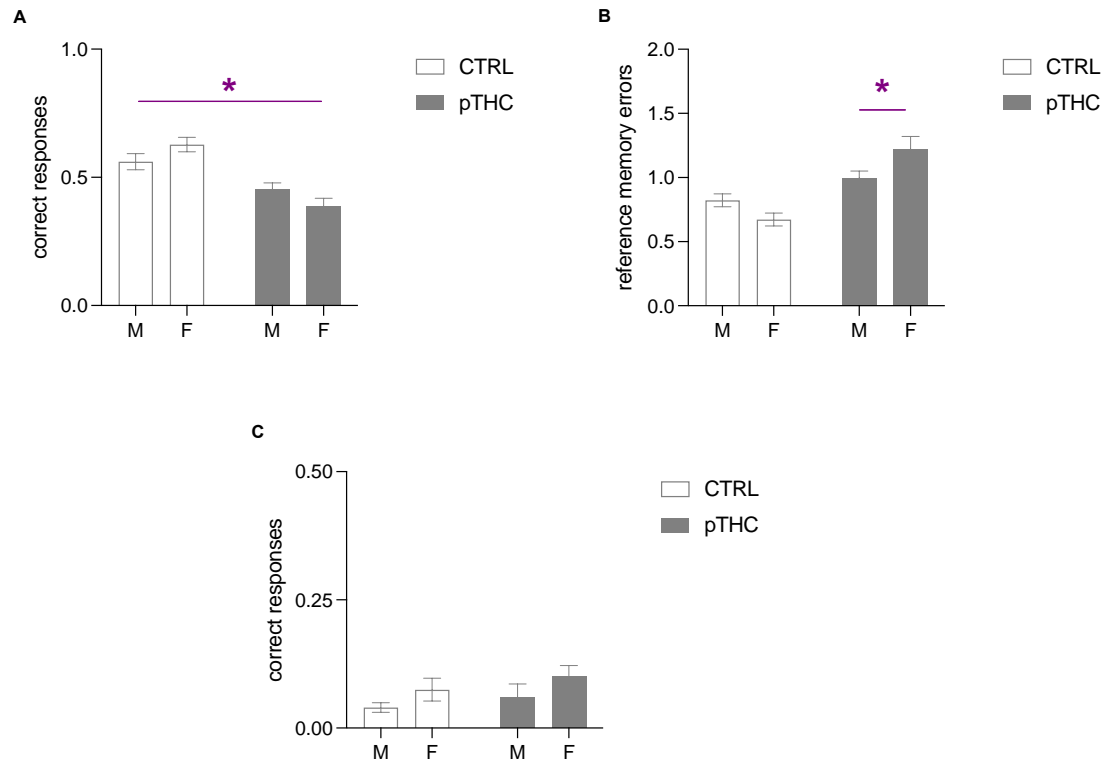


Figure X3. Gender interacts with prenatal exposure to THC (2 mg/kg) on the acquisition of spatial orientation of the adolescent offspring in the spatial task of the Can Test. Statistical analysis showed a significant P value for pTHC ( $F(1, 28) = 34.73$ ,  $p < 0.0001$ ) and its interaction with gender ( $F(1, 28) = 5.204$ ,  $p = 0.0303$ ) on the average of correct responses (A). Furthermore, a main effect of pTHC ( $F(1, 27) = 31.28$ ,  $p < 0.0001$ ) and its interaction with gender ( $F(1, 27) = 8.579$ ,  $p = 0.0068$ ) was found on the average of reference memory errors, with post hoc test showing a higher score of pTHC female offspring with respect to their male counterpart (B;  $t = 2.461$ ,  $DF = 27.00$ ,  $p = 0.0411$ ). No significant differences were detected on working memory errors (C). Data are shown as the mean  $\pm$  SEM. \* $p < 0.05$ . CTRL = rat offspring prenatally exposed to Veh; pTHC = rat offspring prenatally exposed to THC; M = male; F = female.

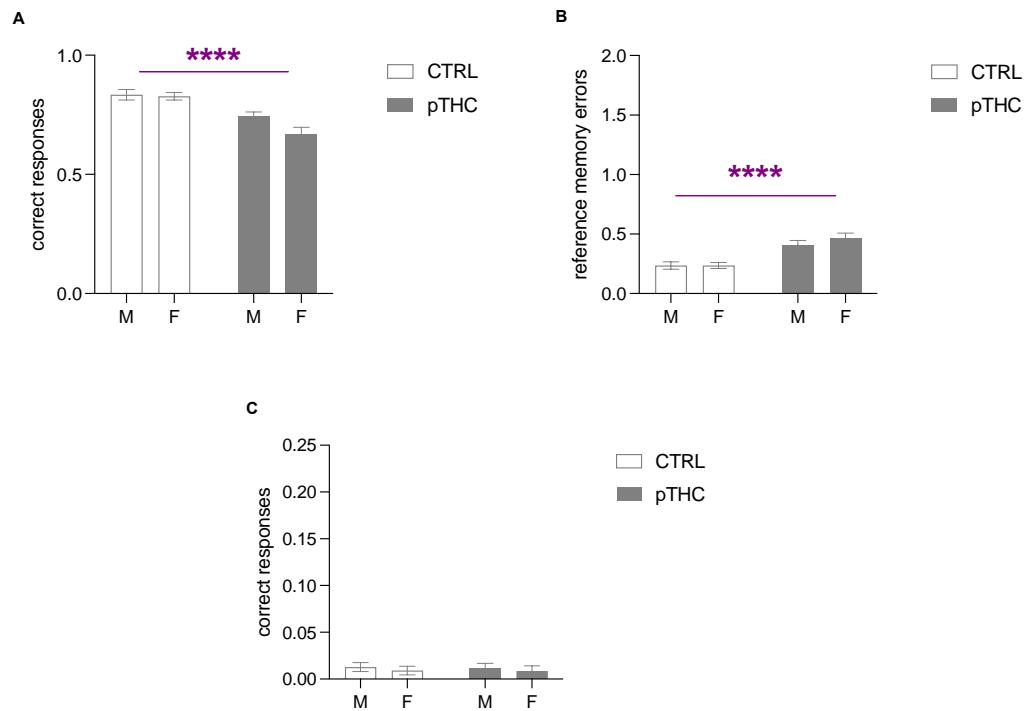


Figure X4. Gender does not interact with prenatal exposure to THC (2 mg/kg) on visual discrimination of the adolescent offspring in the simple visual task of the Can Test. Statistical analysis showed the main effect of pTHC on the average of correct responses (A;  $F(1, 28) = 34.53$ ,  $p < 0.0001$ ) and reference memory errors (B;  $F(1, 28) = 36.56$ ,  $p < 0.0001$ ). No significant differences were detected on working memory errors (C).

Data are shown as the mean  $\pm$  SEM. \*\*\*\* $p < 0.0001$ . CTRL = rat offspring prenatally exposed to Veh; pTHC = rat offspring prenatally exposed to THC; M = male; F = female.

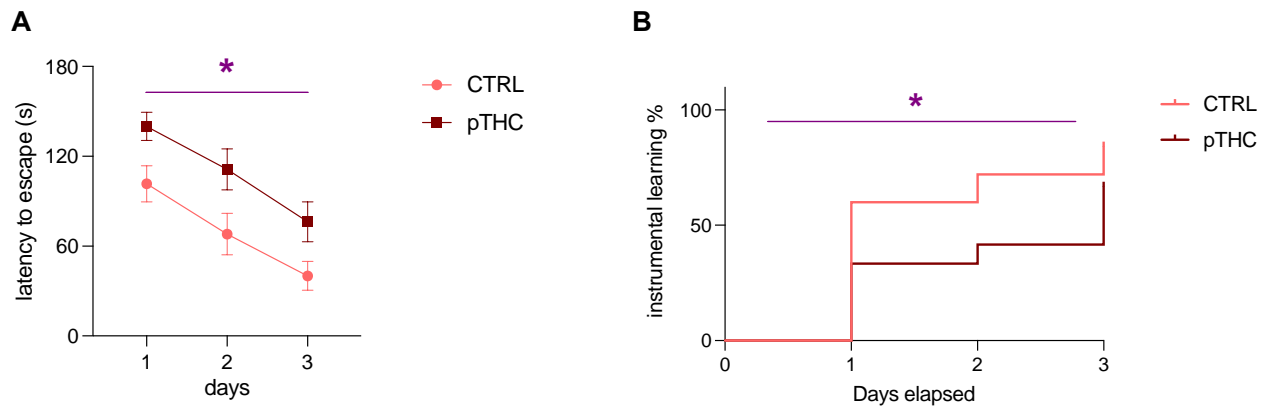


Figure X5. Prenatal THC exposure (2 mg/kg) impinges on the acquisition of spatial orientation of the adolescent female offspring in the acquisition phase of the Barnes Maze Test. 2-way ANOVA revealed main effects of pTHC ( $F(1.47) = 6.553$ ,  $p = 0.0137$ ) and days ( $F(1.887, 88.71) = 43.36$ ,  $p < 0.0001$ ), but not their interaction, on latency to reach escape box along the acquisition phase (A). Furthermore, the performance of the offspring was measured in terms of days elapsed to reach the learning criterion: the mean learning times were 1 for control and 3 days for prenatal THC-treated female adolescent offspring. The Gehan-Breslow-Wilcoxon test for comparing survival curves indicated that learning performance was significantly reduced in female offspring treated with prenatal THC compared to their respective controls (B;  $\chi^2 = 4.074$ ,  $df = 1$ ,  $p = 0.0436$ ). Data are shown as the mean  $\pm$  SEM. \* $p < 0.05$ . CTRL = female rat offspring prenatally exposed to Veh; pTHC = female rat offspring prenatally exposed to THC.

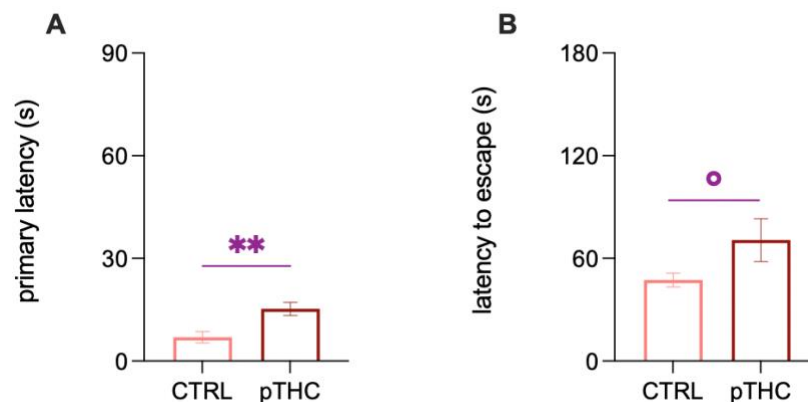


Figure X6. Prenatal THC exposure (2 mg/kg) impinges on the spatial memory retrieval and cognitive flexibility of the adolescent female offspring in the probe- and reversal tasks of the Barnes Maze Test. Student's t-test analysis detected an increase in the primary latency of pTHC rats when compared to control (A;  $t = 3.324$ ;  $df = 25.00$ ,  $p = 0.0027$ ) in the probe task. An increasing trend in the latency to escape of pTHC offspring was detected in the reversal task (B;  $t = 1.829$ ,  $df = 25$ ,  $p = 0.0793$ ). Data are shown as the mean  $\pm$  SEM. \*\* $p < 0.01$ ; °  $p = 0.0793$ . CTRL = female rat offspring prenatally exposed to Veh; pTHC = female rat offspring prenatally exposed to THC.

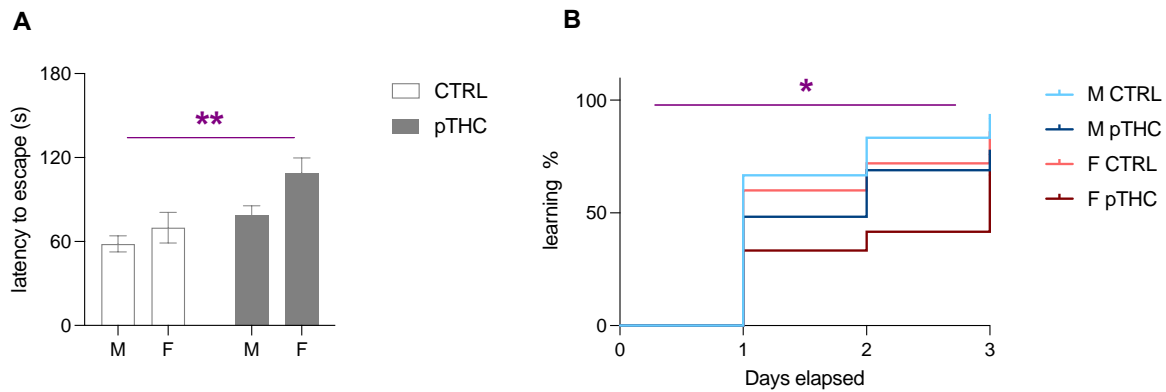


Figure X7. Gender affects the acquisition of spatial orientation of the adolescent offspring in the acquisition phase of the Barnes Maze Test. Statistical analysis revealed main effects of pTHC ( $F(1, 98) = 11.48, p = 0.0010$ ) and gender ( $F(1, 98) = 5.750, p = 0.0184$ ), but not their interaction, on latency to reach the escape box along the acquisition phase (A). Furthermore, the performance of the offspring was measured in terms of days elapsed to reach the learning criterion. The Gehan-Breslow-Wilcoxon test for comparing survival curves shows a significant P value (B;  $\chi^2 = 8.203, df = 3, p = 0.0420$ ). Data are shown as the mean  $\pm$  SEM. \* $p < 0.05$ ; \*\* $p < 0.01$ . CTRL = rat offspring prenatally exposed to Veh; pTHC = rat offspring prenatally exposed to THC; M = male; F = female.

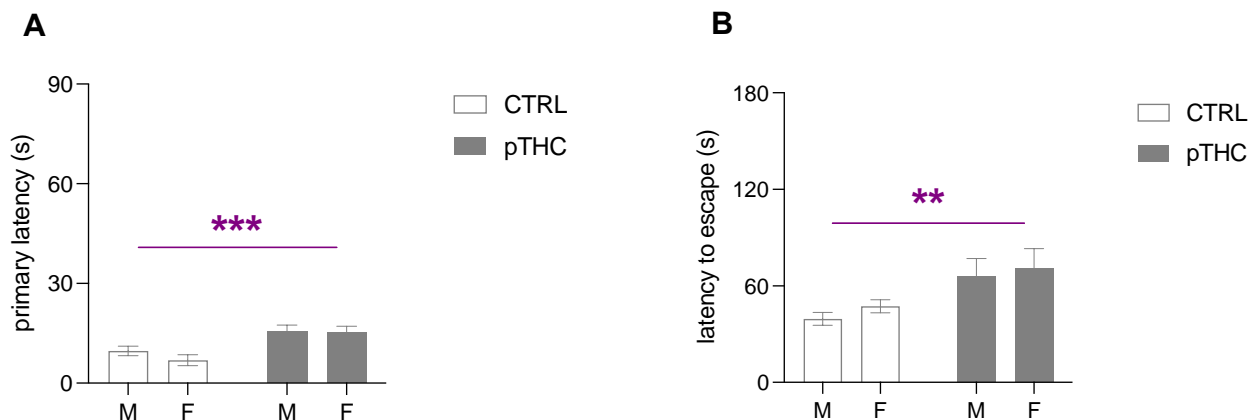


Figure X8. Gender does not interact with prenatal THC exposure (2 mg/kg) on the spatial memory retrieval and cognitive flexibility of the adolescent offspring in the probe- and reversal tasks of the Barnes Maze Test. The statistical analysis detected the main effect of pTHC on the primary latency in the probe- (A;  $F(1, 53) = 15.24, p = 0.0003$ ) and reversal- (B;  $F(1, 53) = 8.120, p = 0.0062$ ) tasks.

Data are shown as the mean  $\pm$  SEM. \*\* $p < 0.01$ ; \*\*\* $p = 0.001$ . CTRL = rat offspring prenatally exposed to Veh; pTHC = rat offspring prenatally exposed to THC; M = male; F = female.



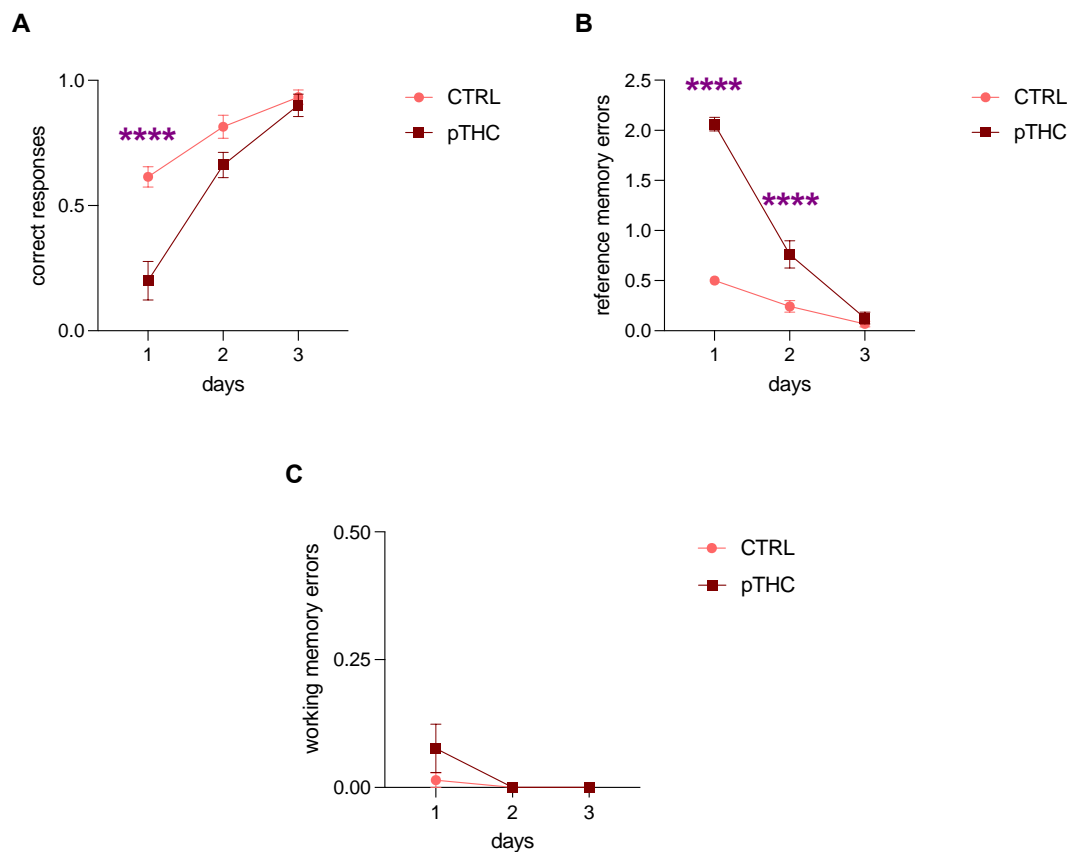


Figure X9. Prenatal THC exposure (2 mg/kg) impinges on the acquisition of spatial orientation of the adult female offspring in the spatial task of the Can Test. Statistical analysis of the behavioral strategy of females showed main effects of days ( $F(2, 22) = 92.65$ ,  $p < 0.0001$ ), pTHC ( $F(1, 11) = 14.09$ ,  $p = 0.0032$ ), and their interaction ( $F(2, 22) = 13.20$ ,  $p = 0.0002$ ) on correct responses. Post hoc analysis revealed a decrease in the correct responses on day 1 of the pTHC group with respect to prenatal vehicle group (A;  $t = 6.005$ ;  $DF = 33.00$ ,  $p < 0.0001$ ). The significant effects of days ( $F(2, 22) = 203.8$ ,  $p < 0.0001$ ), pTHC ( $F(1, 11) = 103.0$ ,  $p < 0.0001$ ), and their interaction ( $F(2, 22) = 83.38$ ,  $p < 0.0001$ ) were highlighted on reference memory errors. Post hoc analysis showed an increase in reference memory errors on days 1 ( $t = 15.87$ ,  $DF = 33.00$ ,  $p < 0.0001$ ) and 2 ( $t = 5.274$ ,  $DF = 33.00$ ,  $p < 0.0001$ ) of adult female pTHC rats compared to controls (B). No significant differences were detected on working memory errors (C). Data are shown as the mean  $\pm$  SEM. \*\*\*\* $p < 0.0001$ . CTRL = female rat offspring prenatally exposed to Veh; pTHC = female rat offspring prenatally exposed to THC.

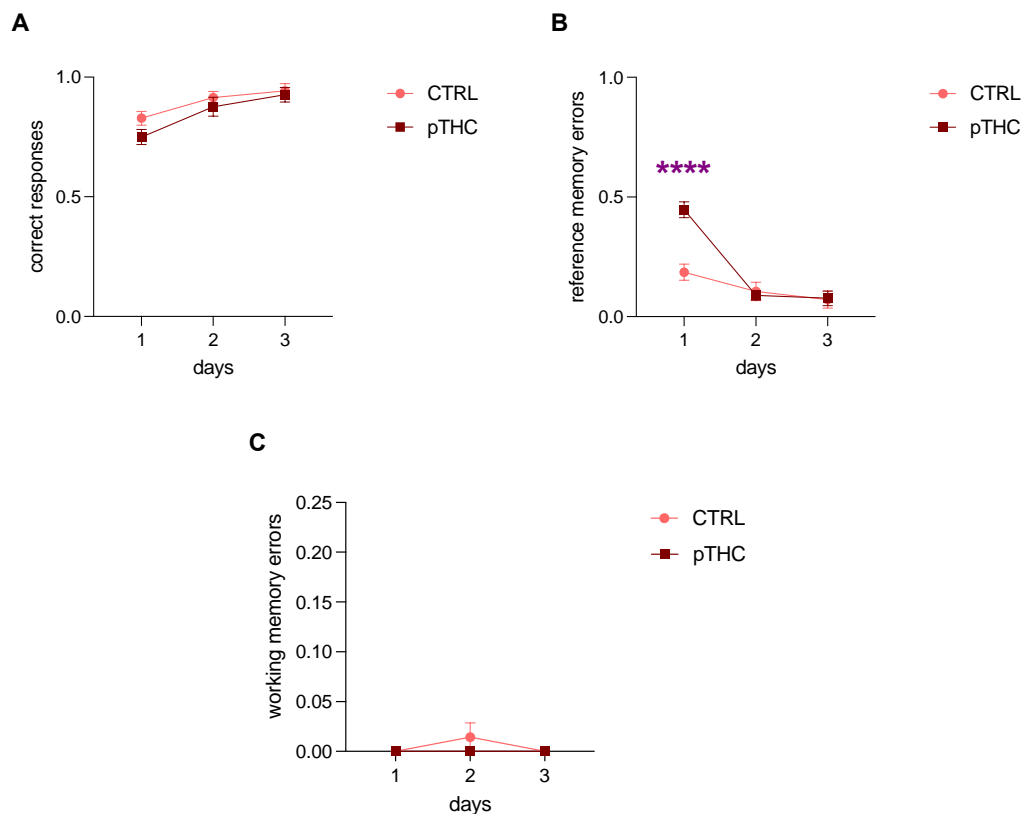


Figure X10. Prenatal exposure to THC (2 mg/kg) alters visual discrimination of the adult female offspring in the simple visual task of the Can Test. The main effects of days ( $F(2, 22) = 33.48$ ,  $p < 0.0001$ ), pTHC ( $F(1, 11) = 8.702$ ,  $p = 0.0132$ ), and their interaction ( $F(2, 22) = 11.20$ ,  $p = 0.0004$ ) were detected on reference memory errors. Post hoc analysis showed an increase in reference memory errors of pTHC offspring with respect to control group on day 1 (B;  $t = 5.547$ ,  $DF = 33.00$ ;  $p < 0.0001$ ). No significant differences on correct responses (A) and working memory errors (C) were found. Data are shown as the mean  $\pm$  SEM. \*\*\*\* $p < 0.0001$ . CTRL = female rat offspring prenatally exposed to Veh; pTHC = female rat offspring prenatally exposed to THC.

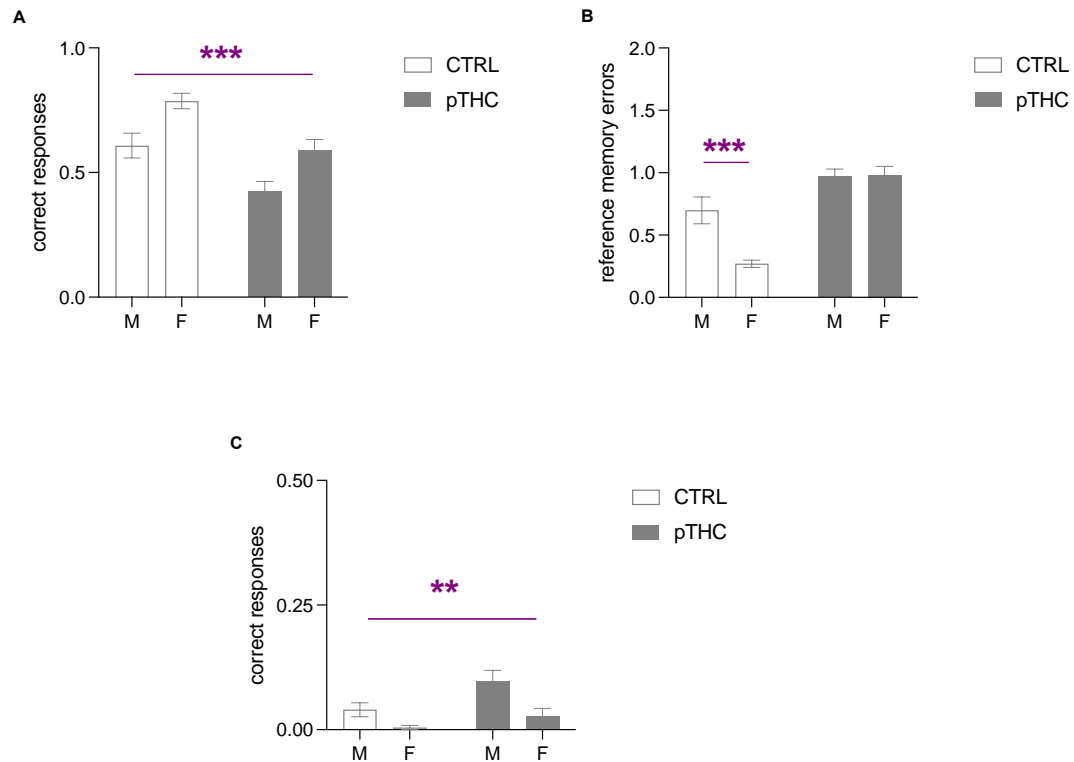


Figure X11. Gender significantly interacts with prenatal exposure to THC (2 mg/kg) on the acquisition of spatial orientation of the adult offspring in the spatial task of the Can Test. Statistical analysis showed a significant P value for pTHC ( $F(1, 21) = 21.46, p = 0.0001$ ) and gender ( $F(1, 21) = 17.21, p = 0.0005$ ), but not their interaction, on the average of correct responses (A). Furthermore, main effects of pTHC ( $F(1, 21) = 49.62, p < 0.0001$ ), gender ( $F(1, 21) = 8.868, p = 0.0072$ ), and their interaction ( $F(1, 21) = 10.04, p = 0.0046$ ) was found on the average of reference memory errors, with post hoc test showing a higher score of CTRL male offspring with respect to their female counterpart (B;  $t = 4.429, DF = 21.00, p = 0.0005$ ). The main effects of pTHC ( $F(1, 21) = 6.981, p = 0.0152$ ) and gender ( $F(1, 21) = 12.49, p = 0.0020$ ), but not their interaction, were detected on working memory errors (C). Data are shown as the mean  $\pm$  SEM. \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ . CTRL = rat offspring prenatally exposed to Veh; pTHC = rat offspring prenatally exposed to THC; M = male; F = female.

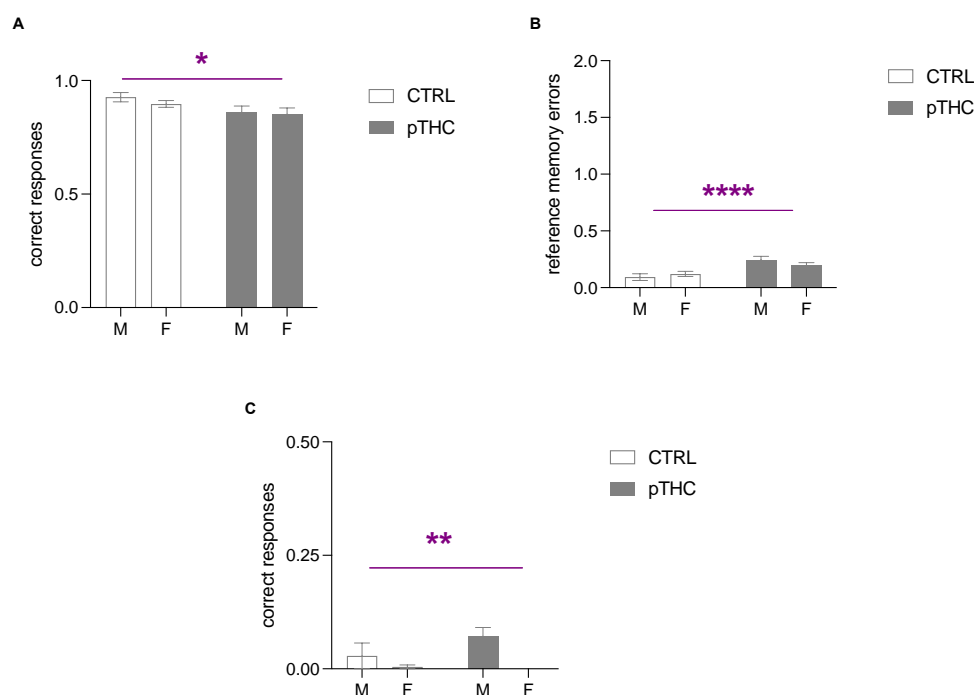


Figure X12. Gender does not interact with prenatal exposure to THC (2 mg/kg) on visual discrimination of the adult offspring in the simple visual task of the Can Test. Statistical analysis showed the main effect of pTHC on the average of correct responses (A;  $F(1, 21) = 5.975$ ,  $p = 0.0234$ ) and reference memory errors (B;  $F(1, 21) = 24.64$ ,  $p < 0.0001$ ). On the other hand, a significant effect of gender ( $F(1, 21) = 8.281$ ,  $p = 0.0090$ ) was detected on working memory errors (C). Data are shown as the mean  $\pm$  SEM. \*\* $p < 0.01$ ; \*\*\*\* $p < 0.0001$ . CTRL = rat offspring prenatally exposed to Veh; pTHC = rat offspring prenatally exposed to THC; M = male; F = female.

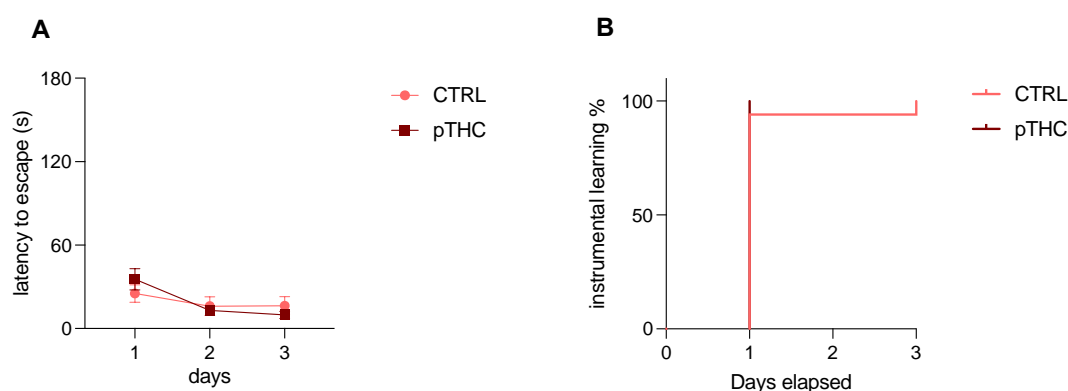


Figure X13. Prenatal THC exposure (2 mg/kg) does not alter the acquisition of spatial orientation of the adult female offspring in the acquisition phase of the Barnes Maze Test. 2-way ANOVA did not reveal significant effect of pTHC or days (A;  $F(1, 29) = 0.0005996$ ,  $p = 0.9806$ ;  $F(1.283, 37.21) = 7.174$ ,  $p = 0.0068$ ). Furthermore, the curve comparison did not show any significant effect of pTHC (B;  $\chi^2 = 0.8235$ ,  $df = 1$ ,  $p = 0.3642$ ). Data are shown as the mean  $\pm$  SEM. CTRL = female rat offspring prenatally exposed to Veh; pTHC = female rat offspring prenatally exposed to THC.

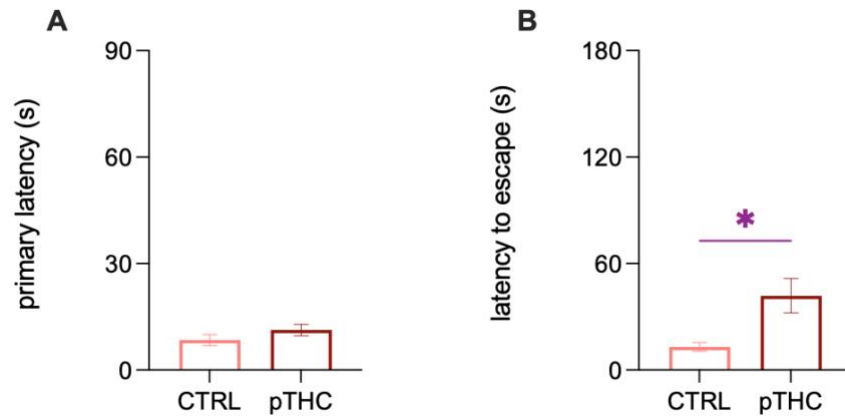


Figure X14. Prenatal THC exposure (2 mg/kg) effects on the spatial memory retrieval and cognitive flexibility of the adult female offspring in the probe- and reversal tasks of the Barnes Maze Test. No significant impact of pTHC was detected in the probe task (A;  $U = 15$ ,  $p = 0.4225$ ). On the other hand, Student's t-test analysis detected an increase in the latency to escape of pTHC rats when compared to control ( $t = 3.089$ ,  $df = 11$ ,  $p = 0.0103$ ) in the reversal task (B). Data are shown as the mean  $\pm$  SEM. \* $p < 0.05$ . CTRL = female rat offspring prenatally exposed to Veh; pTHC = female rat offspring prenatally exposed to THC.

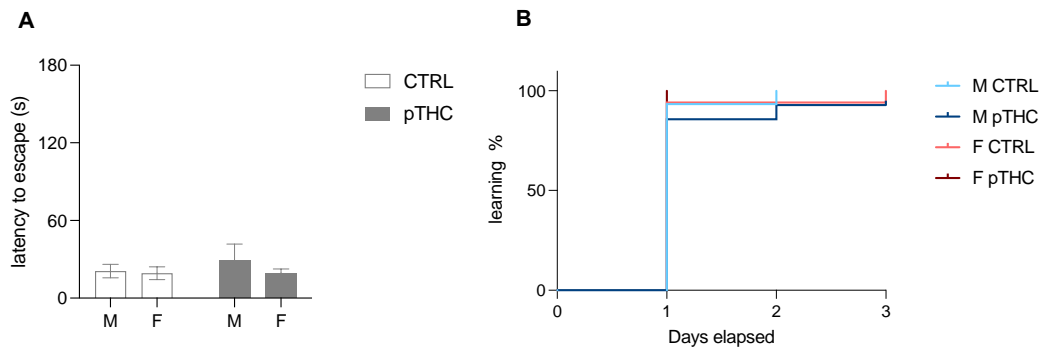


Figure X15. Gender does not interact with prenatal THC exposure (2 mg/kg) on the acquisition phase of the adult offspring in Barnes Maze Test (A; pTHC  $\times$  gender:  $F(1, 56) = 32.84$ ,  $p = 0.5689$ ). Furthermore, the performance of the offspring was measured in terms of days elapsed to reach the learning criterion. The Gehan-Breslow-Wilcoxon test for comparing survival curves shows no significant difference (B;  $\chi^2 = 2.319$ ,  $df = 3$ ,  $p = 0.5089$ ). Data are shown as the mean  $\pm$  SEM. CTRL = rat offspring prenatally exposed to Veh; pTHC = rat offspring prenatally exposed to THC; M = male; F = female.

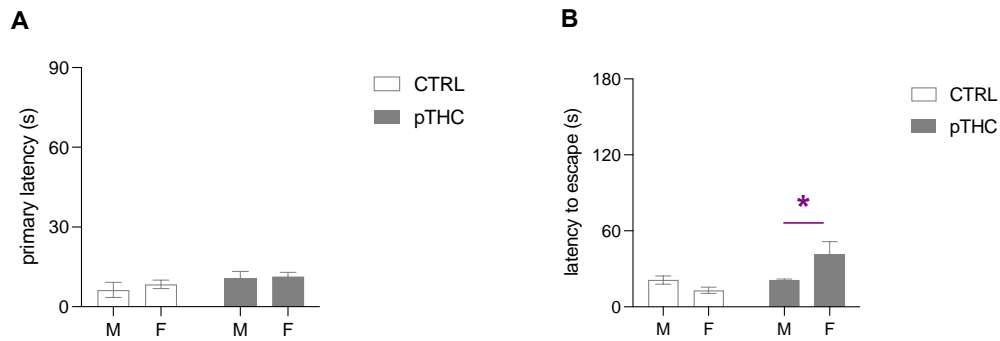


Figure X16. Gender interacts with prenatal THC exposure (2 mg/kg) on cognitive flexibility, but not on the spatial memory retrieval, of the adult offspring in the Barnes Maze Test. The statistical analysis failed to detect significant pTHC x gender interaction ( $F(1, 21) = 0.1778$ ,  $p = 0.6775$ ) on the primary latency in the probe task (A). On the other hand, the main effect of pTHC ( $F(1, 21) = 7.929$ ,  $p = 0.0104$ ) and its interaction with gender ( $F(1, 21) = 7.948$ ,  $p = 0.0103$ ) were detected on the latency to escape in the reversal task.

Indeed, the post hoc analysis revealed a higher score in female pTHC rats when compared to their male counterpart (B;  $t = 2.807$ ,  $DF = 21.00$ ,  $p = 0.0211$ ). Data are shown as the mean  $\pm$  SEM. \* $p < 0.05$ . CTRL = rat offspring prenatally exposed to Veh; pTHC = rat offspring prenatally exposed to THC; M = male; F = female.

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