A low repeated dose of Δ9-tetrahydrocannabinol affects memory performance through serotonergic signalling in mice

Lorena Galera-López[1], Victòria Salgado-Mendialdúa[1], Estefanía Moreno[2], Araceli Bergadà-Martínez[1], Alexander Hoffman[3], Irene Manzanares-Sierra[1], Arnau Busquets-Garcia[4], Vicent Casadó[2], Carl Lupica[3], Rafael Maldonado[1,5, #], Andrés Ozaita[1,5,#]

[1]Laboratory of Neuropharmacology, Department of Experimental and Health Sciences, University Pompeu Fabra, 08003 Barcelona, Spain.

[2] Department of Biochemistry and Molecular Biomedicine, University of Barcelona, 08028 Barcelona, Spain.

[3] Electrophysiology Research Section, Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health, Baltimore, United States

[4] Cell-type mechanisms in normal and pathological behaviour Laboratory. IMIM-Hospital del Mar Research Institute, PRBB, Barcelona, Spain.

[5] IMIM-Hospital del Mar Medical Research Institute, Barcelona, Spain

#Corresponding author:

Rafael Maldonado, Laboratori de Neurofarmacologia, Facultat de Ciències de la Salut i de la Vida, Universitat Pompeu Fabra, Parc de Recerca Biomèdica de Barcelona, C/ Doctor Aiguader 88, 08003 Barcelona, Spain. Phone: +34-93-3160824; Fax: + 34-93-3160901; e-mail: rafael.maldonado@upf.edu

Andrés Ozaita, Laboratori de Neurofarmacologia, Facultat de Ciències de la Salut i de la Vida, Universitat Pompeu Fabra, Parc de Recerca Biomèdica de Barcelona, C/ Doctor Aiguader 88, 08003 Barcelona, Spain. Phone: +34-93-3160823; Fax: + 34-93-3160901; e-mail: andres.ozaita@upf.edu

Keywords: Δ9-tetrahydrocannabinol (THC), CB1 cannabinoid receptor, 5-HT2A serotonin receptor, memory

Abstract

Cannabis is the most widely used illicit drug worldwide. Its principal psychoactive component, Δ9-tetrahydrocannabinol (THC), acts as a partial agonist of the main cannabinoid receptor in the brain, the cannabinoid type-1 receptor (CB1R), being the main responsible for the central effects of THC including memory impairment. CB1Rs may form heterodimers with the serotonin 5-HT2A receptor (5-HT2AR) which were found responsible for the memory impairment produced by acute high dose of THC in mice. In this study we investigated whether a repeated low dose of THC (1 mg/kg), with no acute consequence on memory performance, could eventually have deleterious cognitive effects. We found that such a low dose of THC impairs novel object-recognition memory and fear conditioning memory after repeated treatment (7 days). This deficit was also detected 24 h after the last THC administration. At that time, a general enhancement of c-Fos expression was observed in several brain regions of THC-exposed animals. In addition, THC-treated mice showed a decreased spine density at CA1 pyramidal neurons and reduced long-term potentiation at Schaffer collateral-CA1 synapses. Interestingly, an up-regulation in the expression of CB1R/5-HT2AR heterodimers was observed in the hippocampus of THC-exposed mice and a pre-treatment with the 5-HT2AR antagonist MDL 100,907 (0.01 mg/kg) prevented enhanced heterodimerization and the THC-associated memory impairment. Together, these results reveal the significance of serotonergic signalling through 5-HT2ARs in the memory-impairing effects of repeated low doses of THC.

Introduction

Preparations from the *Cannabis sativa* plant have been consumed for medical and recreational purposes over thousands of years (Bonini *et al.*, 2018). In fact, nowadays, cannabis is the most used illicit drug worldwide (Zarei *et al.*, 2020). From the more of 400 compounds, including more than 120 phytocannabinoids, that have been extracted from cannabis, Δ9-tethahydrocannabinol (THC) is the main psychoactive component (dos Santos *et al.*, 2021). THC has been extensively studied due to its potential therapeutic applications including analgesia properties (King *et al.*, 2017; Lötsch *et al.*, 2018; Escudero-Lara *et al.*, 2020), anxiolytic-like effects, and neuroprotective effects (Fraguas-Sánchez and Torres-Suárez, 2018; Maroon and Bost, 2018), among others. However, THC also induces several undesirable effects such as anxiogenic effects (Kasten *et al.*, 2017), dependence (Moreira and Lutz, 2008; Maldonado *et al.*, 2011), and memory impairment (Puighermanal *et al.*, 2012; Volkow *et al.*, 2016). Central effects of THC, including memory impairment, are mostly mediated by the cannabinoid type-1 receptor (CB1R), the main receptor in the endocannabinoid system, a relevant homeostatic system in the organism (Kano *et al.*, 2009). Using transgenic mice lacking CB1R, the memory impairment in object-recognition memory produced by THC is prevented (Puighermanal *et al.*, 2009). Moreover, acute amnesic-like effects of THC in mice are dose dependent, a sub-effective low doses of THC have been described to do not produce memory impairments in object recognition memory in an acute administration (Puighermanal *et al.*, 2009). Additionally, THC also affects synaptic plasticity at a functional and structural level. Repeated treatment with high doses of THC (10 mg/kg) decreases CA1 dendritic spine density in the hippocampus and blocks the ability to produce LTP (Hoffman *et al.*, 2007; Chen *et al.*, 2013). However, 24 h after a unique THC 10 mg/kg administration hippocampal LTP is not affected (Hoffman *et al.*, 2007). Therefore, there are no evidences of whether repeated low doses of THC could affect memory and plasticity.

CB1R is abundantly express in the central nervous system and controls the release of the major neurotransmitters in our brain such as γ-aminobutyric acid (GABA), glutamate, acetylcholine, noradrenaline, dopamine, cholecystokinin and serotonin (Lutz, 2020). Among those, a crosstalk between the endocannabinoid system and the serotonergic system has been described. On the one hand, the activation of serotonin 5-HT2A receptors (5-HT2AR) induces the release of endocannabinoids, the endogenous ligands for cannabinoid receptors (Parrish and Nichols, 2006; Best and Regehr, 2008). On the other hand, the serotonergic system has been involved in several THC-induced effects including hypothermia (Malone

and Taylor, 2001), catalepsy (Egashira *et al.*, 2007), anxiety, sociability and memory (Viñals *et al.*, 2015). Specifically, mice lacking 5-HT2ARs and pre-treatment with the 5-HT2AR antagonist MDL 100,907, prevented the acute effects of a high dose of THC in object recognition memory (Viñals *et al.*, 2015). The interaction between serotonin and cannabinoid receptors is reciprocal since mice lacking CB1R presented a reduced number of DOI-induced head twitches (Mato *et al.*, 2007) and a dysregulation in serotonergic activity in the prefrontal cortex (Aso *et al.*, 2009). Moreover, the formation of heterodimers between CB1R and 5-HT2AR has been described in both humans and mice (Viñals *et al.*, 2015; Galindo *et al.*, 2018). In human studies, enhanced expression of CB1R/5-HT2AR heterodimer has been found in cannabis users (Galindo *et al.*, 2018). In addition, enhanced levels and altered signalling of CB1R/5-HT2AR heterodimer was found in olfactory neuroepithelium cells of schizophrenia patients (Guinart *et al.*, 2020). In mice, the presence of CB1R/5-HT2AR heterodimer has been described in CA3 region of the hippocampus, the cortex, and the striatum. Importantly, CB1R/5-HT2AR heterodimer modulates specific acute effects of THC since its blockade using an interference peptide that avoid heterodimer formation prevent the memory impairment and anxiolytic effects of THC while other classical effects were conserved (Viñals *et al.*, 2015).

In this study, we investigated the effect of repeated exposure to a non-amnesic low dose of THC (1mg/kg, THC 1) in cognitive function in mice and the possible modulation of the 5-HT2AR on those effects. Using well stablished behavioural tasks to assess memory in mice, we found that repeated THC 1 exposure to a low dose with no acute amnesic effect produces specific deficits in non-emotional and emotional hippocampal long-term memory. Moreover, sub-chronic THC 1 exposure produces cellular and molecular alterations in brain regions related with cognitive function such as decreased total spine density in CA1 pyramidal neurons, modified synaptic plasticity in CA3-CA1 synapses of the hippocampus and produced a general enhancement of c-Fos expression compared to control group. A relevant role of 5-HT2AR in the sub-chronic effect of THC 1 exposure in memory performance can be concluded from the protective effect of the simultaneous 5-HT2AR blockade using MDL 100,907. Moreover, enhanced expression of CB1R/5-HT2AR heterodimers in the CA3 region of the hippocampus was found after the sub-chronic treatment THC 1 and MDL pre-treatment prevented those alterations. Altogether, our results indicate that even a sub-effective dose of THC can produce memory impairment and

cellular alterations after a sub-chronic exposure in mice and those effects are mediated by the sensitization of CB1R/5-HT2AR in the hippocampus.

Materials and Methods

Ethics

All animal procedures were conducted following ARRIVE (Animals in Research: Reporting In Vivo Experiments) guidelines (Kilkenny *et al.*, 2010) and standard ethical guidelines (European Union Directive 2010/63/EU) and approved by the local ethical committee (Comitè Ètic d'Experimentació Animal-Parc de Recerca Biomèdica de Barcelona, CEEA-PRBB). All electrophysiological studies were approved by the NIDA IRP Animal Care and Use Committee and carried out in accordance with NIH guidelines.

Animals

Young adult (8-12 weeks old) male Swiss albino (CD-1) mice (Charles River) and C57BL/6J (BL-6) mice (Charles River) were used for pharmacological approaches on behavioural, biochemical, and electrophysiological approaches. The transgenic line Tg(Thy1-EGFP) MJrs/J (Thy1-EGFP mice) in C57BL/6J background (Stock # 007788, The Jackson Laboratories) was used to study the density and morphology of dendritic spines.

Mice were housed in controlled environmental conditions (21 ± 1 °C temperature and 55 ± 10% humidity) and food and water were available *ad libitum*. All the experiments were performed during the light phase of a 12 h light/dark cycle (light on at 8 am; light off at 8 pm). Mice were habituated to the experimental room and handled for 1 week before starting the experiments. All behavioural experiments were conducted by an observer blind to the experimental conditions.

Drug treatment

Delta9-tetrahydrocannabinol (**THC**) (THC-Pharm-GmbH) was diluted in 5 % ethanol, 5 % Cremophor-EL and 90 % saline and injected intraperitoneally (i.p.) at the dose of 1 mg/kg in a volume of 10 mL/kg of body weight. For electrophysiological studies, THC was provided by NIDA Drug Supply (Rockville). MDL 100,907 (**MDL**) (Sigma-Aldrich) was diluted in 0.26 % dimethyl sulfoxide (DMSO; Scharlau Chemie), 4.74 % ethanol, 5 % Cremophor-EL and 90 % saline at the dose of 0.01 mg/kg in a volume of 10 mL/kg of body weight 20 min before Veh/THC administration. The length of the sub-chronic administrations consisted of 6 d or 7 d of treatment, as indicated in the results section.

Behavioural tests

Novel object recognition test

The novel object recognition (NOR) memory test is a behavioural task used to measure non-emotional declarative memory in mice. NOR test was performed as described before (Gomis-González *et al.*, 2021) in a V-shaped maze (V-maze). On day 1, mice were habituated to the empty V-maze for 9 min (habituation phase). On day 2, two identical objects (familiar objects) were presented at the end of each corridor of the V-maze and mice were left to explore for 9 min before they were returned to their home cage (familiarization phase). On day 3 (24 h after the familiarization phase), mice were placed back in the V-maze where one of the familiar objects was replaced by a new object (novel object) to assess memory performance (test phase). The time spent exploring each of the objects (familiar and novel) during the test session was computed to calculate a discrimination index (DI = (Time Novel Object - Time Familiar Object)/(Time Novel Object + Time Familiar Object)), defining exploration as the orientation of the nose toward the object at a distance closer than 1 cm. A higher discrimination index is considered to reflect greater memory retention for the familiar object. Mice that explored <10 sec both objects during the test session or <2 sec one of the objects were excluded from the analysis. For this task, drug treatment was administered after the familiarization phase in acute and sub-chronic treatments. For the measurement of the effects after treatment withdrawal the treatment finished 24 h prior to the familiarization phase. For NOR task CD-1 mice were used (Suppl. Fig. 1a).

Consecutive NOR

The repeated assessment of NOR memory was carried out as previously described (Puighermanal *et al.*, 2013). Briefly, the first memory assessment (T_1) on the consecutive NOR procedure was performed 24 h after the first administration of THC and just before the second drug administration. Memory was tested again 24 h later using the novel object explored the day before (now the familiar object) and a brand-new object (now the novel object, to be the familiar object the day after). In each test session, a discrimination index (DI) was calculated as previously explained. Mice received a total of 6 administrations for this specific task and a total of 10 NOR memory assessments were performed. For consecutive NOR task CD-1 mice were used.

Trace and Context fear conditioning

Fear conditioning (FC) is a behavioural task used to measure emotional memory in mice. **Trace FC** is composed of 2 phases. On day 1, mice were placed in a conditioning chamber with an electrifiable floor. After 2 min of free exploration mice were exposed to an auditory stimulus (conditioned stimulus, CS) for 1 min. Then, 15 sec after the end of the auditory stimulus mice received one foot shock (unconditioned stimulus, US: 2 sec, 0.35 mA intensity) (training phase). On day 2 (Trace FC test phase), 24 h after the training phase, mice were placed in a new environment (transparent cylinder). After 2 min of free exploration, the exact same sound that was presented during training (CS) was played for 1 min; during this time, the freezing behaviour was manually counted. 2 min after the sound end, mice were removed from the cylinder. The time mice showed freezing behaviour during exposure to CS was expressed as a percentage and interpreted as a memory strength index.

In some cases, **Context FC** was also measured. For this purpose, an additional phase was added to the previous protocol. On day 3 (Context FC test phase), 24 h after the Trace FC test phase, mice were placed for 5 min in absence of the shock in the same conditioning chamber used in the training phase on day 1 and the freezing behaviour was manually recorded.

For this task, drug treatment was administered after the training phase for acute and sub-chronic treatment. For the measurement of the effects after treatment withdrawal the treatment finished 24 h prior to the training phase. For FC tasks BL-6 animals were used (Suppl. Fig. 1a).

Spontaneous alternation task

Spontaneous alternation task is a behavioural test to measure spatial working memory. The maze is composed by three identical arms that intersected at 120° (Y- shaped; 6.5-cm width × 30-cm length × 15-cm height, Y-maze). Animals freely explore the Y-maze for 5 min, starting from the end of the same arm in the Y-maze facing the wall. Entries into all arms of the Y-maze were counted (traversing the head and two front paws was considered a valid entry) and the percentage of correct spontaneous alternations was calculated considering the sequential entries in all three arms divided by the total number of possible alternations (triplets) calculated as the total number of entries − 2. For this task, drug treatment was administered 24 h before both the acute and sub-chronic treatments. For the spontaneous alternation task CD-1 animals were used (Suppl. Fig. 1a).

Anxiety-like behaviour (0-maze and elevated plus maze)

Anxiety-like behaviour associated to the acute and chronic administration of THC was measured using the 0-maze and elevated plus maze. The **0-maze** consisted of a black Plexiglas round shape corridor (5 cm wide), where 2 quarters of the maze were closed by walls (20 cm-high) and the other 2 quarters were open. The whole maze is elevated 30 cm above the floor. During 5 min mice freely explored the maze and the percentage of time spent in the open parts of the corridor were counted as a measure of anxiety-like behaviour. Animals that exit the maze during exploration were excluded. The **elevated plus maze** test consisted of a black Plexiglas apparatus with 4 arms (29 cm long x 5 cm wide), 2 closed arms with walls (20 cm high) and 2 open arms, set in cross from a neutral central square (5 x 5 cm) elevated 40 cm above the floor. Light intensity in the open and closed arms was 45 and 5 luxes, respectively. Mice were placed in the central square facing one of the open arms and tested for 5 min. The percentage of time spent in the open arms was determined as a measure of anxiety. Animals that exit the maze during exploration were excluded. The 0-maze task was used to measure the acute effects of THC 30 min after the first administration. The elevated plus maze was performed to measure the effects of repeated THC 24 h after the last administration. For both tasks CD-1 mice were used (Suppl. Fig. 1b).

Barnes maze

Spatial learning and memory was assessed using the Barnes maze, as previously described (Gomis-González *et al.*, 2021). The maze consists of a circular platform (90 cm in diameter) with 20 equally spaced holes through which mice may escape from a bright light (300 lx). Only one hole allows the escape to a dark/target box. Visual cues were placed surrounding the maze for navigational reference. Smart v3.0 software was used to control the video-tracking system. Briefly, mice were first habituated to the maze. In this phase, animals were placed in the centre of the maze for 10 sec covered by an opaque cylinder. After removal of the opaque cylinder, mice were gently guided to the target hole by surrounding them within a transparent cylinder so mice could see where the scape hole was located. Then, they were left inside the target box for 2 min and returned to the home cage. 1 h later, the first training phase was carried out. During training, each mouse performed 2 trials per day on 4 consecutive days. Each training trial started with the mouse placed in the centre of the Barnes maze covered by an opaque cylinder for 10 sec. Then, animals could explore the maze for 3 min. During this period, the time the mice spent to first visit the target hole was

counted (primary latency). Each training trial ended when the mouse entered to the target box or after 3 min of exploration. When mice reached the target box, they stayed there for 1 min before returning to the home cage. When mice did not reach the target box within 3 min, the experimenter guided the mouse gently to the escape box within the transparent cylinder. Primary latency (time to reach the target box) was considered a measure of learning performance. For this task, mice were treated with vehicle or THC for 7 days and the habituation and training started 24h after the last administration. CD-1 animals were used (Suppl. Fig. 1b).

Tissue preparation for immunofluorescence

BL-6 mice were perfused 24 h after the last administration of the sub-chronic treatment (7 days) with vehicle or THC 1 mg/kg (Suppl. Fig. 1c). Mice were deeply anesthetized by intraperitoneal injection (0.2 mL/10 g of body weight) of a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg) prior to intracardiac perfusion with 4 % paraformaldehyde in 0.1 M Na2HPO4/0.1 M NaH2PO4 buffer (phosphate buffer, PB), pH 7.5, delivered with a peristaltic pump at 19 mL/min flow for 3 min. Subsequently, brains were extracted and post-fixed in the same fixative solution for 24 h and transferred to a solution of 30 % sucrose in PB overnight at 4 °C. Coronal frozen sections (30 µm) of the dorsal hippocampus (coordinates relative to Bregma: – 1.22 mm to – 1.82 mm), basolateral amygdala (from Bregma: − 1.22 mm to − 1.82 mm) and the prelimbic prefrontal cortex (from Bregma: 1.98 mm to 1.54 mm) were obtained on a freezing microtome and stored in a solution of 5 % sucrose at 4 °C until used.

Immunofluorescence

Free-floating brain slices were rinsed in PB, blocked in a solution containing 3 % donkey serum (DS) (Sigma-Aldrich) and 0.3 % Triton X-100 (T) in PB (DS-T-PB) at room temperature for 2 h, and incubated overnight in the same solution with the primary antibody to c-Fos (sc-7202, 1:1000, rabbit, Santa Cruz Biotechnology) at 4 °C. The next day, after 3 rinses in PB, sections were incubated at room temperature with the secondary antibody AlexaFluor-555 goat anti-rabbit (1:1000, Life Technologies, Thermo Fisher Scientific) in DS-T-PB for 2 h. After incubation, sections were rinsed and mounted immediately after onto glass slides coated with gelatin in Fluoromont-G with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen, Thermo Fisher Scientific) as counterstaining.

Image analysis

Immunostained brain sections were analysed with a ×10 objective using a Leica DMR microscope (Leica Microsystems) equipped with a digital camera Leica DFC 300FX (Leica Microsystems). Seven different brain subregions were analysed: prelimbic cortex (PL), basal amygdala (BA), central amygdala (CeA), lateral amygdala (LA), dentate gyrus (DG), *Cornu Ammonis* 1 (CA1) and 3 (CA3). For PL analysis, a 430-µm-sided square region of interest (ROI) was delimited for quantification. For the rest of regions, the DAPI signal was used as counterstaining to identify and delimitate each area for quantification. The images were processed using the ImageJ analysis software. c-Fos-positive neurons in each brain area were quantified using the automatic "particle counting" option with a fixed configuration that solely detected c-Fos-positive cell bodies matching common criteria of size and circularity. A fixed threshold interval was set to distinguish the c-Fos-positive nuclei from the background. In addition, all quantifications were individually checked for homogeneity by an expert observer blind to the experimental conditions. 4-6 representative brain sections of each mouse were quantified, and the average number of c-Fos-positive neurons was calculated for each mouse. The data are expressed as density: the mean number of c-Fos-positive cells per squared mm (n = 6 mice per experimental group). For the c-Fos data, the displayed images were flipped for orientation consistency and transformed to inverted grey scale. Circos plot were generated using Pearson r values from c-Fos expression data. Graph network analysis was performed in R (version 4.0.4) using the DescTools (version 0.99) package.

Tissue preparation for in situ proximity ligation assay

CD-1 mice were perfused immediately after the test phase of NOR, 24 h after the last administration of the sub-chronic pre-treatment (7 days) with vehicle/ MDL 0.01mg/kg and treatment with vehicle/THC 1 mg/kg (Suppl. Fig. 1d). Mice were processed for intracardiac perfusion and brain removal as described above. Prior to sectioning, each brain was washed twice for 5 min with NaCl 0.9 % in PB (PBS). After briefly drying, brains were frozen in dry-ice cold methylbutane for 10 sec. Once frozen, coronal frozen sections (15 µm) of the dorsal hippocampus (coordinates relative to Bregma: – 1.22 mm to – 1.82 mm), and the prelimbic prefrontal cortex (from Bregma: 1.98 mm to 1.54 mm) were cut on a cryostat, mounted on super frost slides (Thermo Fisher) and stored at -80 °C until used.

In situ proximity ligation assay

Heterodimers were detected using the Duolink II *in situ* proximity ligation assay (PLA) detection Kit (Sigma) and following the instructions of the supplier. To detect CB1R/5-HT2AR heterodimers, a mixture of equal amounts (1:100) of guinea pig anti-CB1R antibody (Frontier Science) and rabbit anti-5-HT2AR antibody (Neuromics) was used together with PLA probes detecting guinea pig or rabbit antibodies. Then, brain slices were processed for ligation and amplification with a Detection Reagent Red and were mounted using a mounting medium with DAPI. The samples were observed in a Leica SP2 confocal microscope (Leica Microsystems) equipped with an apochromatic 63X oil-immersion objective (1.4 numerical aperture), and a 405 nm and a 561 nm laser line. For each field of view, a stack of two channels (one per staining) and 6 to 10 Z stacks with a step size of 1 µm were acquired. Images were opened and processed with ImageJ confocal software (National Institutes of Health). Nuclei and red spots were counted on the maximum projections of each image stack. After getting the projection, each channel was processed individually. The nuclei were segmented by filtering with a median filter, subtracting the background, enhancing the contrast with the Contrast Limited Adaptive Histogram Equalization (CLAHE) plug-in, and finally applying a threshold to obtain the binary image and the regions of interest (ROIs) around each nucleus. Red spot images were also filtered and thresholded to obtain the binary images. Red spots were counted in each of the ROIs obtained in the nuclei images. The percentage of cells containing one or more red spot (positive cells) versus the total number of cells (blue nucleus) were determined using the Fiji package (http://pacific. mpi-cbg.de/), considering a total of 800–1000 cells from 3 to 7 different fields within each brain region from the different mice per group.

Hippocampal slice preparation for structural plasticity

Thy1-EGFP mice were perfused 24 h after the last administration of the sub-chronic treatment (7 days) with vehicle or THC 1 mg/kg (Suppl. Fig. 1c). Mice were processed for intracardiac perfusion and brain removal as described above 60 µm brain slices were obtained using a freezing microtome (LEICA SM 2000). Finally, slices were washed in 0.1 M PB, and mounted in glass slides with mowiol for subsequent confocal analysis.

Confocal image acquisition and dendritic spine analysis.

Images of dendritic spines from CA1 pyramidal neurons were obtain using a Leica TCS SP5 confocal microscope. Image stacks were of 0.13 µm step thickness. Dendritic spines counting and classification (mushroom, thin, stubby) was assessed through Neuronstudio program (Version 0.9.92; http://research.mssm.edu/cnic/tools-ns.html, CNIC, Mount Sinai School of Medicine) after deconvolution of the images by Huygens Essential software (Huygens compute engine 4.3.1p3 64b, https://svi.nl/HuygensSoftware). We analysed secondary and tertiary dendrites between 20 to 150 µm distance from soma. Spine analysis was carried out along 30 to 80 µm dendrite segment length. The parameters used for the counting and classification were as follow. Voxel dimensions X: 0.08 µm, Y: 0.08 µm, Z: 0.125 µm. For neurite tracing attach ratio: 1.3, Min Length: 3.0 and Discretion Ratio: 1.0. Dynamic and scattered sampling options were selected. For spine detection and classification, the program was trained with 300 examples of spines and analysis was then automatically performed. Clear erroneous detections were manually corrected by an expert observer blind to the experimental conditions.

Hippocampal slice preparation for electrophysiology

BL-6 mice were perfused 24 h after the last administration of the sub-chronic treatment (7 days) with vehicle or THC 1 mg/kg (Suppl. Fig. 1c). Isoflurane was used to anesthetize mice. Then they were euthanized by cervical dislocation and posterior decapitation. Brain was removed and quickly introduced in modified cold and oxygenate artificial cerebrospinal fluid (aCSF) containing NaCl 109 mM, KCl 4.5 mM, MgCl2 1 mM, CaCl2 2.5 mM, NaH2PO4 1.2 mM, NaHCO3 35 mM, Glucose 11 mM, HEPES 20 mM, sodium ascorbate 0.4 mM. Transverse hippocampal slices (280µm) were obtained with a vibratome (Leica VT1200S, Leica Biosystems) while submerged in modified aCSF previously oxygenate. Then slices were transferred to modified and continoulsy oxygenate aCSF at 35ºC for 20min. After this time, they were kept at room temperature for at least 40 minutes. All solutions were oxygenated with 95% O2/5% CO2. Maximum five slices per animal and day were recorded.

Electrophysiological recordings

Electrophysiological recordings consisted of extracellular field EPSPs (fEPSPs) recordings on CA1 from hippocampus. The machinery and the protocol used was the same as previously described (Hoffman *et al.*, 2007). This time, the aCSF used for the recordings was composed

by NaCl 126 mM, KCl 3 mM, MgCl2 1 mM, CaCl2 2.4 mM, NaH2PO4 1.2 mM, NaHCO3 26 mM, glucose 11 mM. Once the baseline was acquired for 15 min theta burst stimulation (TBS) protocol was applied in order to induce long-term potentiation (TBS-LTP). TBS protocol consisted of 4 episodes of 10 bursts of 5 stimuli, at 100Hz. Intervals where 200 msec between bursts and 5 sec between episodes.

RNA extraction and reverse transcription

Brain samples were obtained from CD-1 mice 24 h after the last administration of the sub-chronic treatment (7 days) with vehicle or THC 1 mg/kg (Suppl. Fig. 1c). Hippocampal tissues were rapidly dissected and stored at −80 °C until used. Isolation of total RNA was performed using the RNeasy Mini kit (QIAGEN) according to the manufacturer's instructions. Total RNA concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific). Reverse transcription was performed with 10 ul of total RNA from each animal to produce cDNA in a 20-µl reaction using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's instructions. The cDNAs from brain tissues was diluted to a final concentration of 10 ng/ul and stored at −20 °C until use.

Quantitative real-time PCR analysis

Real-time PCR was carried out in a 10 µl reaction using SYBR Green PCR Master Mix (Roche) using 1ng/ul of cDNA according to the manufacturer's protocol with a QuantStudio 12 K Flex Real-Time PCR System (Applied Biosystems). The following primers specific for mouse were used:

Bdnf (BDNF) 5'-CAGGTGAGAAGAGTGATGACC-3' 5'-ATTCACGCTCTCCAGAGTCCC-3'

Ngf (NGF) 5'-CAAGGACGCAGTTTCTATACTG-3' 5'-CTTCAGGGACAGAGTCTCCTTCT-3'

Actb (β-actin) 5'-CGTGAAAAGATGACCCAGATCA-3' 5'-CACAGCCTGGATGGCTACGT-3'

All primers were used at 10uM. Quantification was performed by using the comparative CT Method (ΔΔCT Method). All the samples were tested in triplicate and the relative expression values were normalized to the expression value of *β-actin*. The fold change was calculated using the eq. $2^{(-\Delta\Delta Ct)}$

Statistical analysis

Comparisons between groups were performed by Student t test or one-way or two-way analysis of variance (ANOVA) for multiple-group comparisons, depending on the factors

involved. *Post hoc* comparisons were performed by Bonferroni test only when significant main effect of one-way ANOVA or significant effect of factors or interaction between factors of two-way ANOVA were revealed or significant repeated-measure ANOVA. For morphology of spine density, *Post hoc* comparisons were performed by Newman-Keuls test. The statistical analysis and the artwork was performed using GraphPad Prism 7. All results were expressed as mean ± s.e.m. Differences were considered statistically significant when $p < 0.05$.

Results

Repeated exposure to a THC dose without acute amnesic-like effects produces deficits in hippocampal long-term memory

A single dose of THC at 10 mg/kg or 3 mg/kg shows significant novel object-recognition (NOR) memory impairment (Busquets-Garcia *et al.*, 2018), with no signs of tolerance development after 7 d of repeated administration (Puighermanal *et al.*, 2013). We wondered whether a dose of THC that does not affect memory performance after an acute administration, could eventually affect memory after repeated dosage (Fig. 1). We used the dose of THC 1 mg/kg (THC 1) that, as previously described, did not affect NOR memory when administered after the familiarization phase (Puighermanal *et al.*, 2009). We administered THC 1 or vehicle to mice after the familiarization phase in a consecutive version of the NOR memory test. In this approach, the memory test phase of one day is equivalent to the familiarization phase for the next day, as previously described (Busquets-Garcia *et al.*, 2011). Such repeated evaluation of NOR memory allowed detecting the deleterious effect of repeated THC 1 on memory consolidation after 5 administrations (Fig. 1a). THC withdrawal gradually improved NOR memory performance reaching control values after two days (Fig. 1a).

Considering this result, we decided to study the effects of an acute vs. a sub-chronic administration of THC 1 mg/kg of 7 days (to secure the instauration of a memory deficit) investigating non-emotional and emotional hippocampal memory and spatial working memory.

In agreement with the observation in Fig. 1a, a single administration of THC 1 after familiarization/training did not affect neither NOR memory (Fig. 1b) nor emotional memory (Trace fear conditioning, Trace FC) performance (Fig. 1c). No effect on the exploratory behaviour was observed in NOR (Suppl. Fig. 2a).

Next, in the cohort previously used for studying NOR memory, mice were injected with THC 1 every day for 6 additional days without behavioural assessment, and the seventh administration of THC 1 was delivered 20 min after the familiarization phase for the NOR memory test (Suppl. Fig. 1a). Then, NOR memory was assessed the next day. Under these experimental conditions, mice exposed to sub-chronic THC 1 showed, as expected, a significant decrease in discrimination index (Fig. 1d) with no effect on the exploratory behaviour (Suppl. Fig. 2b).

Using another cohort of mice, we assessed the effect of 7 doses of THC 1 in the Trace FC paradigm. Emotional hippocampal memory was severely impaired after repeated THC 1 administration, similar to what we observed for non-emotional hippocampal memory in the NOR (Fig. 1e). However, when spatial working memory was tested in another cohort of mice using the Y-maze, no alterations in performance or exploratory behaviour were observed with a single administration or with the seven days of sub-chronic exposure to THC 1 (Suppl. Fig. 3).

We additionally assessed anxiety-like behaviour in the same conditions of acute and sub-chronic administration of THC 1, since the effects of THC are biphasic in this behavioural trait (Puighermanal *et al.*, 2013), but we did not observe any alteration associated to the acute or sub-chronic exposure to THC 1 using the 0-maze (Suppl. Fig. 4a) and the elevated plus maze (Suppl. Fig. 4b), respectively.

Cognitive outcome of repeated THC 1 exposure varies according to the behavioural test

We further assessed the outcome of repeated THC 1 exposure beyond drug withdrawal. In order to study spatial learning, we used the Barnes maze. Mice previous exposed to THC 1 or vehicle were trained for 4 consecutive days after treatment withdrawal. We found that spatial learning was preserved under our experimental conditions, since both groups showed similar primary latency values to find the escape whole in every test (Fig. 2a).

We then analysed the outcome of THC 1 exposure using the NOR and FC tests and performing the training sessions on the next day after treatment withdrawal. To investigate NOR deficits, mice were treated for 7 d and 24 h after the last administration they were subjected to the NOR familiarization phase. The following day, 48 h after the last administration of THC 1, memory was evaluated. Using this protocol, a significant deficit in NOR memory was observed after THC 1(Fig. 2b).

Notably, when Trace FC training was performed the next day after drug withdrawal, the THC 1 exposed group showed a significant memory impairment when mice were exposed to the conditioned stimulus (Fig. 2c), but also when treated mice were exposed to the conditioning box the next day (72 h after the last THC administration) (Fig. 2d) Together, specific alterations in learning and memory performance were derived from THC 1 exposure extending transiently beyond the treatment period.

Repeated THC 1 exposure produces alterations in functional and structural synaptic plasticity.

Alterations in functional and structural synaptic plasticity in the hippocampus have been described after chronic exposure to high doses of THC (10 mg/kg) (Hoffman *et al.*, 2007; Chen *et al.*, 2013). We wanted to study if the sub-chronic treatment with a low THC dose also produced alterations at synaptic plasticity levels. For functional synaptic plasticity assessment, a set of mice was treated for 7 d with THC 1. At the same time-point in which hippocampal-dependent memory deficits were observed, 24 h after the last administration, the ability to generate CA3-CA1 hippocampal LTP was studied. We observed that after theta burst stimulation protocol vehicle group slices presented a standard LTP response, meanwhile THC 1-treated slices presented an LTP that *per se* was not significantly altered, but synaptic potentiation was mildly decrease (Fig. 3a). LTP magnitude was significantly decreased in the last 10 minutes of the session in THC 1-treated hippocampi (Fig. 3b).

To study structural plasticity, Thy1-EGFP transgenic mice were used to study spine density and morphology in the CA1 pyramidal neurons expressing the fluorescent protein EGFP. As above, animals were treated with THC 1 or vehicle for 7 d and 24 h after the last administration histological samples were obtained and CA1 pyramidal dendritic spines were analysed. A significant decrease in total dendritic spine density was observed after THC 1 exposure compared to vehicle-treated mice (Fig. 3c). When the spine structure was analysed, we found a significant decrease thin spine in THC 1 treated mice (Fig. 3d).

These alterations in functional and structural plasticity were not accompanied by changes in RNA levels for two of the most studied neurotrophins BDNF and NGF (Suppl. Fig. 5).

Sub-chronic administration of THC 1 altered overall c-Fos expression and correlation patterns between memory-related brain regions

Acute high and low doses of THC (10, 5 and 0.5 mg/kg) were previously found to induce the expression of the immediate-early gene c-Fos in diverse brain regions (Valjent *et al.*, 2002; Flores *et al.*, 2016; Ruiz *et al.*, 2021). Acute THC exposure also altered functional connectivity as measured by c-Fos expression analysis between brain regions involved in reward and stress responses (Ruiz *et al.*, 2021). We hypothesized that, after the sub-chronic exposure to THC 1, neuronal activity, measured by c-Fos expression analysis, and connectivity, measured by the correlation analysis of c-Fos expression between memory-related brain regions, could be disturbed. We performed the analysis of c-Fos expression the next day

after treatment withdrawal to circumvent any acute effect of THC. Therefore, we treated mice sub-chronically with THC 1 and 24 h after the last administration brain samples were obtained and c-Fos expression was assayed in seven memory-related brain regions including amygdala (BA, CeA and LA), hippocampal (DG, CA3 and CA1) and prefrontal (PL) regions. An overall enhanced c-Fos expression was detected in the regions analysed (Fig. 4a), with a significant increase in hippocampal CA1 region (Fig. 4b).

Subsequent connectivity analysis between control and treated mice revealed an alteration in network connectivity after sub-chronic THC 1 administration. In the vehicle-treated group, the c-Fos expression on most of the brain regions analysed were negatively correlated. Specially, a significant functional de-coupling was observed between DG-CeA and DG-PL (Fig. 4c). After THC 1 treatment, this connectivity map was severely affected and the activity of most of the brain regions analysed passed to be positively correlated. A significant functional coupling was observed between PL-CA1 and PL-CA3 (Fig. 4c).

5-HT2AR blockade prevents THC 1 deficits in non-emotional and emotional hippocampal long-term memory

Since the serotonergic, and specially 5-HT2ARs, have been involved in non-emotional amnesic-like effects of acute administration of THC (Viñals *et al.*, 2015) we hypothesized that the memory impairment that we observed after the sub-chronic exposure with low THC could be also mediated though the serotonergic system. We pre-treated mice using MDL 100,097 (MDL), a 5-HT2A antagonist, before each THC 1 administration and analysed the impact on non-emotional and emotional memory in different cohorts of mice. In agreement with previous results, a single acute administration with THC 1 or with MDL did not alter object-recognition memory (Suppl. Fig. 6a). Notably, memory assessment after repeated THC 1 exposure in mice pre-treated with MDL showed total memory deficit prevention. (Fig. 5a). To investigate whether a similar involvement of 5-HT2ARs was observed in the emotional memory deficit produced by THC 1, two different cohort of mice were used. In the first one, we confirmed that acute THC 1 and MDL did not affect Trace FC memory in the same conditions used for NOR task (Suppl. Fig. 6b). With the second cohort of mice that received repeated THC 1 and MDL as a pre-treatment, we observed that the emotional memory impairment produced after the sub-chronic treatment with THC 1 was totally prevented with 5-HT2AR blockade (Fig. 5b).

Sub-chronic exposure to THC 1 enhances the expression CB1R/5-HT2AR heterodimers

Since we found that 5-HT2ARs were involved in the memory impairment of repeated THC 1, we hypothesized CB1R/5-HT2AR heterodimers could be relevantly involved. Indeed, enhanced levels of CB1R/5-HT2AR heterodimers have been detected in cannabis consumers (Galindo *et al.*, 2018). We therefore studied the expression of these heterodimers after repeated THC 1 exposure. To do so, a cohort of mice received the treatment with THC 1 and the pre-treatment with MDL, as detailed above, and brain tissues were extracted after the performance of the NOR test. We observe that THC 1 treatment produced a significant enhancement in CB1R/5-HT2AR heterodimers in the hippocampus and MDL pre-treatment prevent the heterodimerization (Fig. 6a-b). Notably, the enhanced appearance of the heterodimers was not observed in the prefrontal cortex, another brain region where both receptors are relevantly expressed (Suppl. Fig 7a).

In order to assess whether such enhanced expression of the CB1R/5-HT2AR heterodimers in the hippocampus was responsible for the sensitization to the amnesic-like effects of the THC 1 dose, we pre-treated with MDL acutely. In this case all mice in the experiment were treated with THC 1 and only half of them received MDL 20 min prior the last THC 1 administration, coinciding with the familiarization phase of NOR task. To avoid possible bias between mice in the two groups the acute effect of THC 1 was analysed in all mice of the cohort to discard differences in the NOR task (Suppl. Fig.6c). We observed that a unique administration of MDL on the last day of THC 1 treatment can prevent the amnesic-like effects of THC (Fig. 6c) supporting the relevance of an enhanced expression of CB1R/5-HT2AR heterodimers resulting from THC 1 repeated exposure with relevance for the cognitive performance.

Discussion

In this study we describe the deleterious effect of a low repeated dose of THC on memory performance describing the sensitization of brain region specific serotonergic signalling associated to presence of CB1R/5-HT2AR heterodimers with a potential significant relevance in the appearance of THC memory deficits.

We first assessed memory performance after an acute administration of low THC (1 mg/kg) in different subcategories of memory: declarative non-emotional, emotional, and working memory. In line with previous findings (Puighermanal *et al.*, 2009), in our experimental condition, such a low dose of THC did not affect object-recognition memory, and had no effect in preventing emotional memory measured in Trace FC or working memory assessed on Y-maze. By analysing the effect of THC 1 in the repeated NOR memory paradigm, we observed that THC 1 developed a transient sensitizing effect for memory disruption. This is a somewhat surprising result, since repeated THC exposure produces a rapid tolerance to most of its pharmacological effects such as hypothermia, analgesia, or even anxiety-like effects (Puighermanal *et al.*, 2013) at higher doses, without developing tolerance to the amnesic-like effect. The next step was to study whether at a precise time after a repeated administration with this low dose, THC could produce an effect on different memory types. In this case mice were treated during 7 days with THC and a significant memory impairment was observed in NOR task and in emotional memory. This effect of repeated low THC in hippocampal-dependent long-term memory is comparable to the well-described effect of acute higher doses of THC in NOR memory in mice (Puighermanal *et al.*, 2009, 2013; Viñals *et al.*, 2015), and could be interpreted as caused by the development of sensitization. Regarding the disturbances on emotional memory after THC exposure, we firstly described them in a specific modality of fear conditioning ,Trace FC, but this result is reminiscent to the previous described effect in another hippocampal-dependent emotional task, the Context FC, of acute high dose THC (Puighermanal *et al.*, 2009) and other cannabinoid agonist (Maćkowiak *et al.*, 2009) . However, the repeated exposure to THC 1 did not affect working memory in our experimental conditions. This result is in contrast with the amnesic-like effects observed also in murine models after acute and sub-chronic exposure to high doses of THC in working-memory (Viravathana and Marr, 2001; Han *et al.*, 2012). These results could indicate an independent modulation of low THC on declarative and working memory which could be explored in future studies.

As observed in the repeated NOR paradigm, the deleterious effect of THC 1 was evident when the training phases of the memory tests were performed 24 h after THC withdrawal, probably indicating hippocampal alterations that would go beyond THC presence. This transient effect on memory disruption after THC withdrawal is in concordance with previous finding for object-recognition memory (Puighermanal *et al.*, 2013) where significant amnesic-like effects of sub-chronic administration of THC 10 mg/kg last for 5 d after treatment withdrawal. Taking into account the previous described effects of higher doses of THC and cannabinoids on spatial learning task using Morris water maze (Cha *et al.*, 2006) we decided to assess if our experimental conditions with repeated exposure of low THC could also affect this type of memory on a similar paradigm that also measure spatial learning, the Barnes maze (Sunyer *et al.*, 2007). In this case the learning evaluation was performed once THC 1 treatment was finished and during the following 4 d. However, mice treated with THC showed similar spatial learning to the vehicle-treated group. This result is in accordance with a previous study in which they observed really high doses of THC (100 mg/kg) are needed to produce a spatial learning impairment compared to other pharmacological effects of THC (Viravathana and Marr, 2001).

Biphasic effects of THC have been described regarding its modulation of anxiety in mice. While acute high doses of THC produced anxiogenic-like effects, acute low doses of THC produced anxiolytic-like effects (Puighermanal *et al.*, 2013). We hypothesized that the sub-chronic treatment with THC at the dose of 1 mg/kg would represent a situation in which no anxiogenic nor anxiolytic effects are observed. Indeed, we observed that no effects on anxiety-like behaviours were detected after repeated THC 1 exposure.

At the cellular and molecular level, we focused our analysis on alterations previously described for high amnesic doses of THC. In particular, we investigated the outcome in synaptic plasticity at a functional and structural level in the hippocampus focusing in CA1 pyramidal neurons. We found that THC 1 repeated administration resulted in significant alterations in LTP measured the next day after THC withdrawal. This observation is in agreement with previous findings where repeated treatment with high doses of THC (10 mg/kg) was found to block the ability to produce LTP (Hoffman *et al.*, 2007). However, in the same study, 24 h after a unique THC 10 mg/kg administration hippocampal LTP was not affected (Hoffman et al., 2007). Next, we decided to check synaptic plasticity at a structural level in order to observe if repeated THC 1 also modifies these parameters. We observed a

significant decrease of total spine density at CA1 pyramidal neurons in THC-treated mice. This result is somewhat similar with the previous decreased CA1 dendritic spines density in the hippocampus observed after repeated treatment with high doses of THC (10 mg/kg) (Chen *et al.*, 2013). In further analysis we stratified the structural results based on different dendritic spine morphologies, and we observed that the decrease was most significant in thin spine density, which would indicate a reduction in plasticity potential of brain circuits (Chidambaram *et al.*, 2019). Since neurotrophins are signalling molecules that can modify both synaptic transmission and structure in the hippocampus (Gibon and Barker, 2017) we decided to study the expression of two of the major neurotrophins in brain, BDNF and NGF at the mRNA level. Such levels were not modified in the hippocampus, indicating that the impairment in synaptic plasticity produced by THC would not be associated to deficits in these neurotrophic factors.

We also explored whether brain tissues would be affected with regard to their c-Fos responsiveness. Acute high and low doses of THC (10, 5 and 0.5 mg/kg) were previously found to induce the expression of the immediate-early gene c-Fos in diverse brain regions (Valjent *et al.*, 2002; Flores *et al.*, 2016; Ruiz *et al.*, 2021). However, the effects of THC in c-Fos expression after a sub-chronic low dose administration has not been determined yet. We focused our analysis of c-Fos expression in the next day after THC withdrawal, since there is a clear memory deficit and clear alterations in synaptic plasticity, and to avoid the acute effect of the THC injection. A significant effect of THC exposure was observed in the memory-related brain regions studied, especially in CA1 subregion of the hippocampus. Interestingly, CA1 region is the same brain area in which we observed a decreased in spine density after THC exposure. Since c-Fos expression can also be used to assess the connectivity between regions, and previous findings pointed to altered functional connectivity in reward and stress networks after acute THC exposure (Ruiz *et al.*, 2021), we decided to study the connectivity of the memory-related brain regions. While in vehicle-treated mice the c-Fos expression between brain regions was negatively correlated, mice after THC 1 treatment showed, instead, positively correlated connectivity. Specifically, a significant prelimbic- CA1 and CA3 hippocampal co-activation was found after THC exposure. Importantly, these results are in clear agreement with the previous study of network connectivity after high doses of THC in where using male rats increased overall global connectivity and increased co-activation of PL and the hippocampal CA1–3 subregion was observed (Ruiz *et al.*, 2021). One of the limitations of our study is that all behavioural,

molecular, pharmacological, and biochemical determinations have been performed only with male mice. However, sex-dependent differences in THC effects on all these levels have been described (Ruiz *et al.*, 2021).

Next, we wanted to further understand the mechanism behind the sensitizing effects observed on memory after sub-chronic THC 1 exposure. Considering the crosstalk between the endocannabinoid system and the serotonergic system, including the previously described involvement of the serotonin system in several THC-induced effects such as hypothermia (Malone and Taylor, 2001), catalepsy (Egashira *et al.*, 2007), anxiety, sociability and memory (Viñals *et al.*, 2015), we decided to explore the role of serotonin signalling on the amnesic-like effect observed after sub-chronic THC 1. Of special interest were previous data showing that mice lacking 5-HT2AR, or pre-treatment with the 5-HT2AR antagonist MDL 100,907, prevented the acute effects of a high dose of THC in object recognition memory (Viñals *et al.*, 2015). Using a similar pharmacological approach, we observed that the memory impairment produced by sub-chronic THC 1 mg/kg administration was totally prevented with 5-HT2AR sub-chronic and acute blockade using MDL 100,907 in object-recognition task. In addition, we described for the first time the involvement of 5-HT2AR in emotional memory impairment produced by THC since MDL 100,907 blockade also prevented amnesic-like effects of THC in fear conditioning.

Together, we hypothesize that a significant role of CB1R/5-HT2AR heterodimers could underlie this sustained effect of THC. To prove this hypothesize we decided to study CB1R/5-HT2AR heterodimers in CA3, a hippocampal brain region in which this heterodimers have been previously described in mice (Viñals *et al.*, 2015), and an area directly connected to CA1 region. Interestingly, we observed a significant enhancement of CB1R/5-HT2AR heterodimers after sub-chronic THC 1 exposure and this modification was prevented with MDL 100,907 blockade. This result agrees with previous research done in humans. Specifically, enhanced expression of CB1R/5-HT2AR heterodimers was found in olfactory neuroepithelium cells of cannabis users (Galindo *et al.*, 2018). Additionally, altered signalling in CB1R/5-HT2AR heterodimers also in olfactory neuroepithelium cells was reported in schizophrenia patients (Guinart *et al.*, 2020) were a sensitized serotonergic responses are characteristic. However, further studies addressing the specific relevance of the CB1R/5-HT2AR heterodimers are key to better understand the cognitive deficits associated to repeated THC exposure. Since addressing the enhanced assembly of these heterodimers could prevent some of the cognitive effects of THC, further studies are needed to finally

determine if the memory impairment produced after sub-chronic THC 1 is modulated by CB1R/5-HT2AR heterodimers.

Altogether, our study highlights that even a non-amnesic low dose of THC can produce a deleterious effect on memory function when administered repeatedly at behavioural, molecular, and cellular level and the serotonergic system is involved on this amnesic effects. These evidences pinpoint for the first time 5-HT2AR as a key regulator for repeated low dose THC effects on memory function.

Acknowledgements

We are grateful to Marta Linares, Dulce Real, Raquel Martín, Jolita Jancyte and Francisco Porrón for expert technical assistance and the Laboratori de Neurofarmacologia-NeuroPhar for helpful discussion.

Funding and disclosure

L.G-L. was supported by predoctoral fellowship from Spanish Ministry of Economy, Industry and Competitiveness (BES-2016-077950). V.S-M. was supported by a predoctoral fellowship from Spanish Ministry of Economy and Competitiveness (BES-2013-063545). This work was supported by the Spanish Ministry of Science, Innovation and Universities (RTI2018-099282-B-I00, (AEI/FEDER/UE)) to A.O.

Conflict of interest statement

The authors declare that no conflict of interest exists.

References

Aso, E. *et al.* (2009) Lack of CB1 receptor activity impairs serotonergic negative feedback, *Journal of Neurochemistry*. J Neurochem, 109(3), pp. 935–944.

Best, A. R. and Regehr, W. G. (2008) Serotonin evokes endocannabinoid release and retrogradely suppresses excitatory synapses, *Journal of Neuroscience*. J Neurosci, 28(25), pp. 6508–6515.

Bonini, S. A. *et al.* (2018) Cannabis sativa: A comprehensive ethnopharmacological review of a medicinal plant with a long history, *Journal of Ethnopharmacology*. Elsevier Ireland Ltd, 227, pp. 300–315.

Busquets-Garcia, A. *et al.* (2011) Differential role of anandamide and 2-arachidonoylglycerol in memory and anxiety-like responses, *Biological Psychiatry*. Elsevier Inc., 70(5), pp. 479–486.

Busquets-Garcia, A. *et al.* (2018) Hippocampal Protein Kinase C Signaling Mediates the Short-Term Memory Impairment Induced by Delta9-Tetrahydrocannabinol, *Neuropsychopharmacology*. Nature Publishing Group, 43(5), pp. 1021–1031.

Cha, Y. M. *et al.* (2006) Differential effects of delta9-THC on learning in adolescent and adult rats, *Pharmacology Biochemistry and Behavior*. Pharmacol Biochem Behav, 83(3), pp. 448–455.

Chen, R. *et al.* (2013) Δ9-THC-caused synaptic and memory impairments are mediated through COX-2 signaling, *Cell*. Elsevier B.V., 155(5), p. 1154.

Chidambaram, S. B. *et al.* (2019) Dendritic spines: Revisiting the physiological role, *Progress in Neuro-Psychopharmacology and Biological Psychiatry*. Elsevier Inc., 92, pp. 161–193.

Egashira, N. *et al.* (2007) 2,5-Dimethoxy-4-iodoamphetamine (DOI) inhibits Δ9-tetrahydrocannabinol-induced catalepsy-like immobilization in mice, *Journal of Pharmacological Sciences*. Japanese Pharmacological Society, 105(4), pp. 361–366.

Escudero-Lara, A., Argerich, J., Cabañero, D. and Maldonado, R. (2020) Disease-modifying effects of natural Δ9-tetrahydrocannabinol in endometriosis-associated pain, *eLife*. eLife Sciences Publications Ltd, 9.

Flores, Á., Julià-Hernández, M., Maldonado, R. and Berrendero, F. (2016) Involvement of the orexin/hypocretin system in the pharmacological effects induced by Δ9-tetrahydrocannabinol, *British Journal of Pharmacology*. John Wiley and Sons Inc.,

173(8), pp. 1381–1392.

Fraguas-Sánchez, A. I. and Torres-Suárez, A. I. (2018) Medical Use of Cannabinoids, *Drugs*. Springer International Publishing, 78(16), pp. 1665–1703.

Galindo, L. *et al.* (2018) Cannabis Users Show Enhanced Expression of CB1-5HT2A Receptor Heteromers in Olfactory Neuroepithelium Cells, *Molecular Neurobiology*. Humana Press Inc., 55(8), pp. 6347–6361.

Gibon, J. and Barker, P. A. (2017) Neurotrophins and Proneurotrophins: Focus on Synaptic Activity and Plasticity in the Brain, *Neuroscientist*. SAGE Publications Inc., 23(6), pp. 587–604.

Gomis-González, M. *et al.* (2021) Protein Kinase C-Gamma Knockout Mice Show Impaired Hippocampal Short-Term Memory While Preserved Long-Term Memory, *Molecular Neurobiology*, 58(2), pp. 617–630.

Guinart, D. *et al.* (2020) Altered Signaling in CB1R-5-HT2AR Heteromers in Olfactory Neuroepithelium Cells of Schizophrenia Patients is Modulated by Cannabis Use, *Schizophrenia Bulletin*. Oxford University Press, 46(6), pp. 1547–1557.

Han, J. *et al.* (2012) Acute cannabinoids impair working memory through astroglial CB1 receptor modulation of hippocampal LTD, *Cell*. Elsevier B.V., 148(5), pp. 1039–1050.

Hoffman, A. F. *et al.* (2007) Opposing actions of chronic Δ9-tetrahydrocannabinol and cannabinoid antagonists on hippocampal long-term potentiation, *Learning and Memory*. Learn Mem, 14(1), pp. 63–74.

Kano, M. *et al.* (2009) Endocannabinoid-mediated control of synaptic transmission., *Physiological reviews*, 89(1), pp. 309–80.

Kasten, C. R., Zhang, Y. and Boehm, S. L. (2017) Acute and long-term effects of Δ9-tetrahydrocannabinol on object recognition and anxiety-like activity are age- and strain-dependent in mice, *Pharmacology Biochemistry and Behavior*. Elsevier Inc., 163, pp. 9–19.

Kilkenny, C. *et al.* (2010) Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research, *Journal of Pharmacology and Pharmacotherapeutics*, 1(2), p. 94.

King, K. M. *et al.* (2017) Single and combined effects of Δ9-tetrahydrocannabinol and cannabidiol in a mouse model of chemotherapy-induced neuropathic pain, *British Journal of Pharmacology*. John Wiley and Sons Inc., 174(17), pp. 2832–2841.

Lötsch, J., Weyer-Menkhoff, I. and Tegeder, I. (2018) Current evidence of cannabinoid-based analgesia obtained in preclinical and human experimental settings, *European Journal of Pain (United Kingdom)*. Blackwell Publishing Ltd, 22(3), pp. 471–484.

Lutz, B. (2020) Neurobiology of cannabinoid receptor signaling, *Dialogues in Clinical Neuroscience*, 22(3), pp. 207–222.

Maćkowiak, M., Chocyk, A., Dudys, D. and Wedzony, K. (2009) Activation of CB1 cannabinoid receptors impairs memory consolidation and hippocampal polysialylated neural cell adhesion molecule expression in contextual fear conditioning, *Neuroscience*. Neuroscience, 158(4), pp. 1708–1716.

Maldonado, R., Berrendero, F., Ozaita, A. and Robledo, P. (2011) Neurochemical basis of cannabis addiction, *Neuroscience*. Pergamon, 181, pp. 1–17.

Malone, D. T. and Taylor, D. A. (2001) Involvement of somatodendritic 5-HT1A receptors in Δ9-tetrahydrocannabinol-induced hypothermia in the rat, *Pharmacology Biochemistry and Behavior*. Pharmacol Biochem Behav, 69(3–4), pp. 595–601.

Maroon, J. and Bost, J. (2018) Review of the neurological benefits of phytocannabinoids, *Surgical Neurology International*. Scientific Scholar, 9(1), p. 91.

Mato, S. *et al.* (2007) CB1 knockout mice display impaired functionality of 5-HT 1A and 5-HT2A/C receptors, *Journal of Neurochemistry*. J Neurochem, 103(5), pp. 2111–2120.

Moreira, F. A. and Lutz, B. (2008) The endocannabinoid system: Emotion, learning and addiction, *Addiction Biology*. John Wiley & Sons, Ltd, 13(2), pp. 196–212.

Parrish, J. C. and Nichols, D. E. (2006) Serotonin 5-HT2A receptor activation induces 2-arachidonoylglycerol release through a phospholipase c-dependent mechanism, *Journal of Neurochemistry*. J Neurochem, 99(4), pp. 1164–1175.

Puighermanal, E. *et al.* (2009) Cannabinoid modulation of hippocampal long-term memory is mediated by mTOR signaling, *Nature neuroscience*, 12(9), pp. 1152–8.

Puighermanal, E., Busquets-Garcia, A., Maldonado, R. and Ozaita, A. (2012) Cellular and intracellular mechanisms involved in the cognitive impairment of cannabinoids, *Philosophical Transactions of the Royal Society B: Biological Sciences*, 367(1607), pp. 3254–3263.

Puighermanal, E. *et al.* (2013) Dissociation of the Pharmacological Effects of THC by mTOR Blockade, *Neuropsychopharmacology*, 38, pp. 1334–1343.

Ruiz, C. M. *et al.* (2021) Pharmacokinetic, behavioral, and brain activity effects of ?9-

tetrahydrocannabinol in adolescent male and female rats, *Neuropsychopharmacology*. Springer US, 46(5), pp. 959–969.

dos Santos, R. G., Hallak, J. E. C. and Crippa, J. A. S. (2021) Neuropharmacological Effects of the Main Phytocannabinoids: A Narrative Review, in *Advances in Experimental Medicine and Biology*. Springer, pp. 29–45.

Sunyer, B., Patil, S., Höger, H. and Luber, G. (2007) Barnes maze, a useful task to assess spatial reference memory in the mice, *Protocol Exchange*, pp. 1–12.

Valjent, E. *et al.* (2002) Behavioural and biochemical evidence for interactions between Δ9-tetrahydrocannabinol and nicotine, *British Journal of Pharmacology*. Br J Pharmacol, 135(2), pp. 564–578.

Viñals, X. *et al.* (2015) Cognitive impairment induced by delta9- tetrahydrocannabinol occurs through heteromers between cannabinoid CB1 and serotonin 5-HT2A receptors, *PLoS Biology*. Public Library of Science, 13(7).

Viravathana, P. and Marr, D. W. M. (2001) Differential effects of Δ9-THC on spatial reference and working memory in mice, *Psychopharmacology*. Psychopharmacology (Berl), 157(2), pp. 142–150.

Volkow, N. D. *et al.* (2016) Effects of cannabis use on human behavior, including cognition,motivation, and psychosis: A review, *JAMA Psychiatry*. American Medical Association, 73(3), pp. 292–297.

Zarei, S. *et al.* (2020) A global systematic review and meta-analysis on illicit drug consumption rate through wastewater-based epidemiology, *Environmental Science and Pollution Research*. Springer, 27(29), pp. 36037–36051.

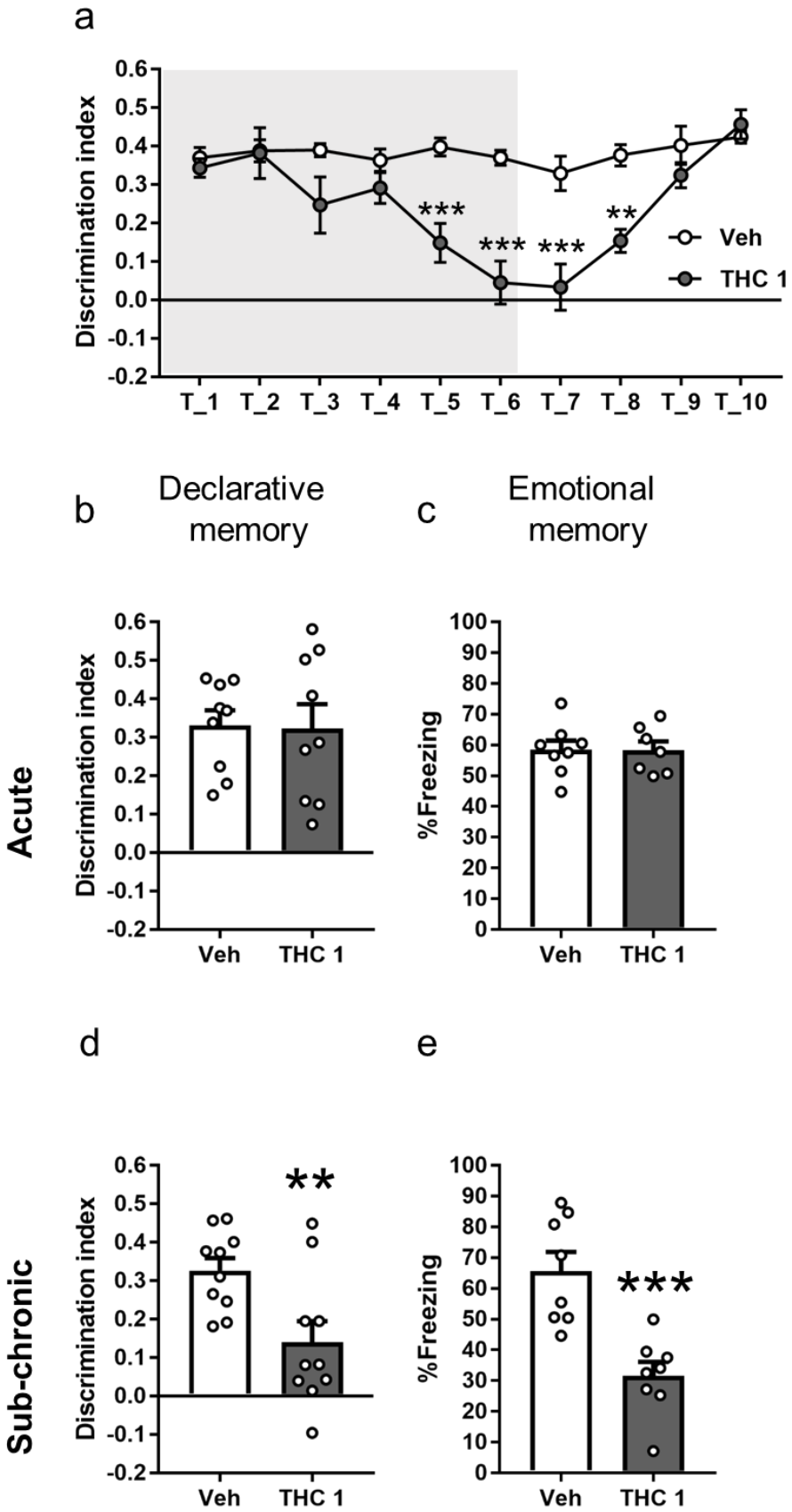

a
Discrimination index
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
T_1
T_2
T_3
T_4
T_5
T_6
T_7
T_8
T_9
T_10
***
***
***
**
Veh
THC 1
b
Declarative memory
c
Emotional memory
Acute
Discrimination index
%Freezing
100
90
80
70
60
50
40
30
20
10
0
Veh
THC 1
d
e
Sub-chronic
**
***

Figure 1.

Figure 1. Repeated exposure to a sub-effective low dose of THC (1 mg/kg, THC 1) produces specific memory impairments in non-emotional and emotional memory in mice.

(a) Consecutive NOR memory test in mice receiving THC 1 mg/kg (THC 1) or vehicle (Veh) on 6 consecutive days (grey area). Memory impairment was detected after 5 administrations, and animals treated with THC totally recovered memory performance two/three days after THC 1 withdrawal (Veh n = 5-10, THC 1 n = 8-10). Statistical significance was calculated by Bonferroni *post hoc* test following two-way ANOVA. ** $p < 0.01$, *** $p < 0.001$ (Veh vs THC 1). A unique THC 1 administration did not produce memory impairment in (b) NOR memory (Veh n = 9, THC 1 n = 9) or in (c) Trace FC (Veh n = 8, THC 1 n = 7). However, after a sub-chronic exposure THC 1 produced significant memory impairments detected in (d) NOR task (Veh n = 10, THC 1 n = 10) and in (e) Trace FC (Veh n = 8, THC 1 n = 8) when these tasks were performed 24 h after the last THC 1 administration. Statistical significance was calculated by Student's t-test. ** $p < 0.01$, *** $p < 0.001$. Data are expressed as mean ± S.E.M.

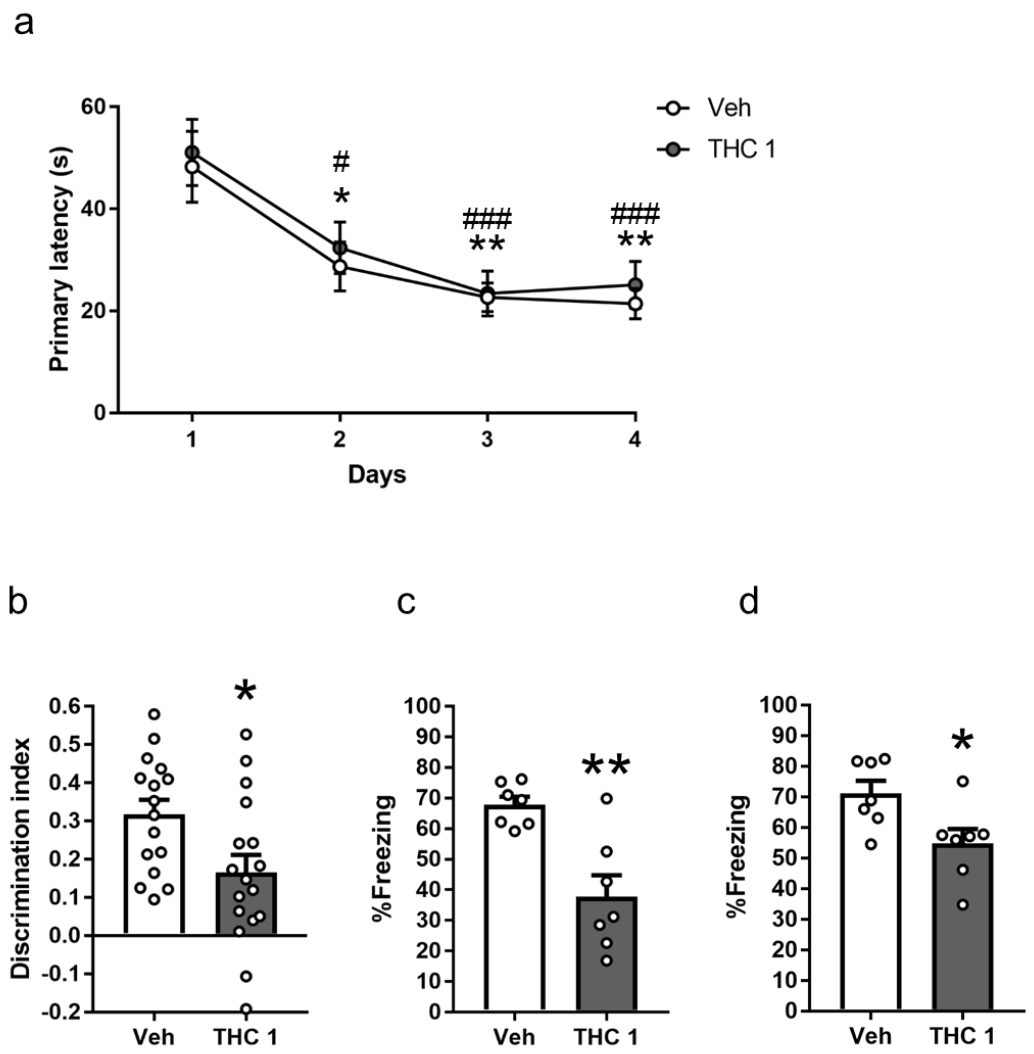

a
Veh
THC 1
Primary latency (s)
Days
#
*
###
**
###
**
b
c
d
Discrimination index
%Freezing
%Freezing
Veh
THC 1
Veh
THC 1
Veh
THC 1

Figure 2.

Figure 2. Cognitive outcome of THC 1 exposure varies according to the behavioural test.

(a) Spatial learning, as assessed in the Barnes maze task, did not show differences between treatment conditions as demonstrated by the similar decrease in primary latency over the training days (Veh n = 18, THC 1 n = 16). Statistical significance was calculated by Bonferroni *post hoc* test following two-way ANOVA repeated-measures. * $p < 0.05$, ** $p < 0.01$ (vs Day 1 Veh). # $p < 0.05$, ### $p < 0.001$ (vs Day 1 THC 1). (b) NOR memory task performed 48 h after the last THC 1 administration showed significant alterations in non-emotional memory (Veh n = 16, THC 1 n = 17). For the emotional memory, sub-chronic exposure with THC 1 mg/kg produced a memory impairment in (c) Trace FC performed 48h after the last administration (Veh n=7, THC 1 n=7) and in (d) Context FC performed 72h after the last administration in the same cohort of mice (Veh n=7, THC 1 n=7). Statistical significance was calculated by Student's t-test. * $p< 0.05$, ** $p< 0.01$. Data are expressed as mean ± S.E.M.

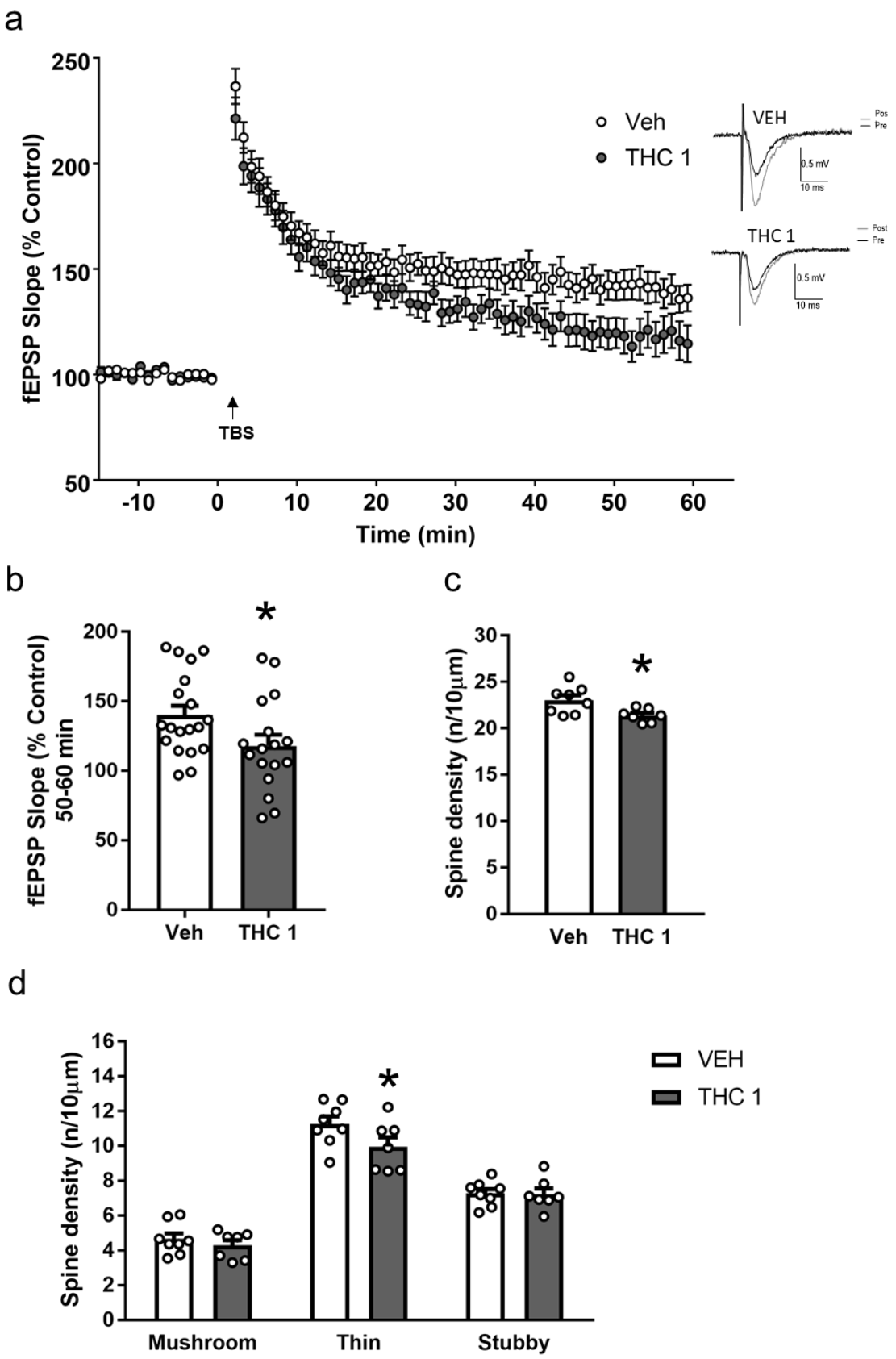

a
fEPSP Slope (% Control)
Time (min)
TBS
Veh
THC 1
VEH
THC 1
0.5 mV
10 ms
b
fEPSP Slope (% Control) 50-60 min
Veh
THC 1
c
Spine density (n/10μm)
Veh
THC 1
d
Spine density (n/10μm)
Mushroom
Thin
Stubby
VEH
THC 1

Figure 3.

Figure 3. Sub-chronic THC 1mg/kg exposure modifies synaptic plasticity in CA1 region of the hippocampus and decreased total spine density.

(a) The average time courses of the change in the slope of the fEPSP in hippocampal slices from mice treated for 7 d with Veh or THC 1. After stimulation, slices from THC 1-treated mice showed a mild decrease in L-LTP (Veh n = 19 slices/4 mice THC 1 n = 17 slices/4 mice). (b) Average fEPSP slope values obtained between 50 min and 60 min after theta burst stimulation (TBS) showing a significant decrease in THC 1 treated slices (Veh n = 19 slices/4 mice THC 1 n= 17 slices/4 mice). Statistical significance was calculated by Student's t-test. * $p< 0.05$. (c) Sub-chronic treatment with THC 1 decreased CA1 pyramidal neuron overall spine density (Veh n = 8, THC 1 n = 7). Statistical significance was calculated by Student's t-test. * $p< 0.05$. (d) Spine morphology analysis showed a significant decrease in thin spine density to (Veh n = 8, THC 1 n = 7). Statistical significance was calculated by Newman-Keuls *post hoc* test following two-way ANOVA. * $p< 0.05$ (Veh vs THC 1). Data are expressed as mean ± S.E.M.

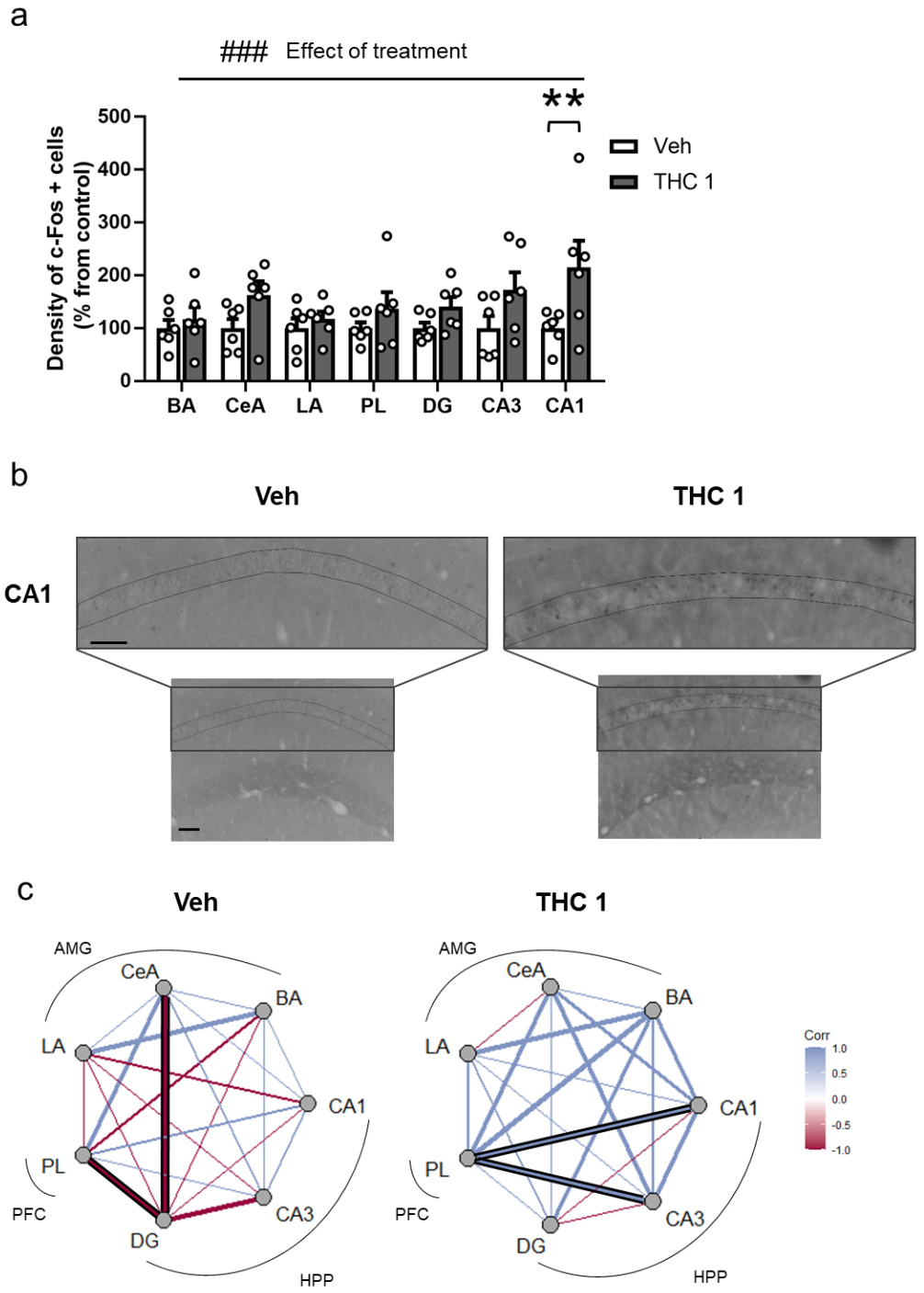

a
Effect of treatment
**
Density of c-Fos + cells (% from control)
500
400
300
200
100
0
BA
CeA
LA
PL
DG
CA3
CA1
Veh
THC 1
b
Veh
THC 1
CA1
c
Veh
THC 1
AMG
CeA
BA
LA
CA1
PL
CA3
DG
PFC
HPP
Corr
1.0
0.5
0.0
-0.5
-1.0

Figure 4.

Figure 4. Sub-chronic THC 1 mg/kg exposure enhances overall c-Fos expression and modifies c-Fos correlation pattern between brain regions.

(a) Quantification of c-Fos immunudetection density in selected brain regions after THC 1 of vehicle (Veh) exposure (Veh n = 6, THC 1 n = 6). Statistical significance was calculated by Bonferroni *post hoc* test following two-way ANOVA. ### $p < 0.001$ (main treatment effect), ** $p < 0.01$ (post-hoc). Data are expressed as mean ± S.E.M. (b) Representative images of c-Fos expression in Veh- and THC 1-treated animals. Scale bar= 75 um. (c) Circos plot analysis of c-Fos expression in different brain regions after sub-chronic THC administration. Network graphs of c-Fos expression based on the Pearson coefficient in Veh and THC 1 between different brain areas, including dentate gyrus (DG), *cornu ammonis* 1 (CA1), *cornu ammonis* 3 (CA3), prelimbic cortex (PL), basal amygdala (BA), lateral amygdala (LA) and central amygdala (CeA). Colours depict correlation indexes. Significant correlations are delimited with black lines.

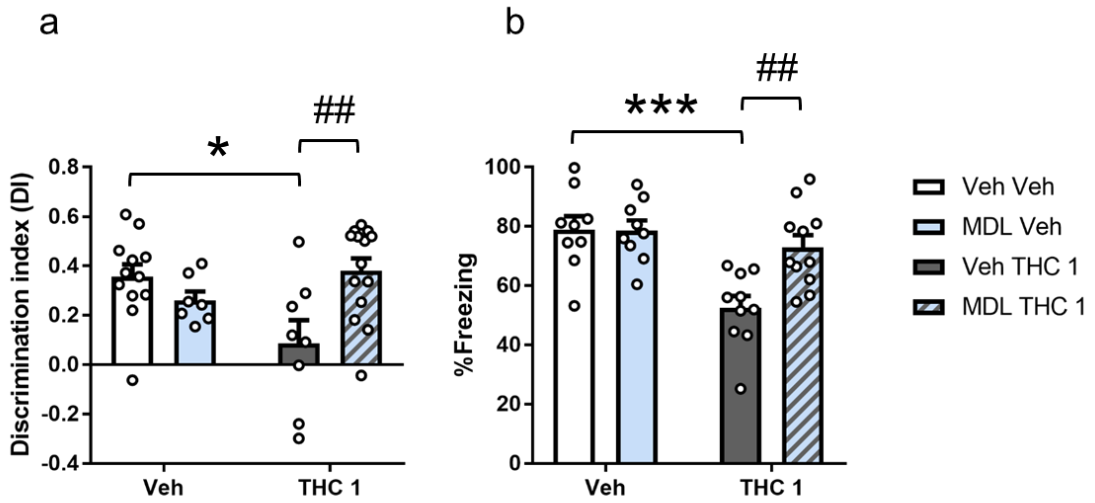
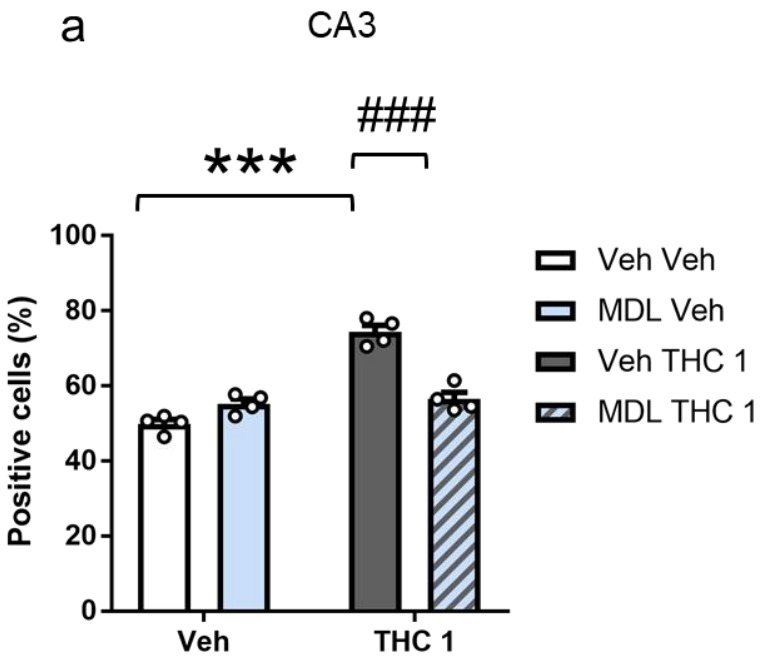
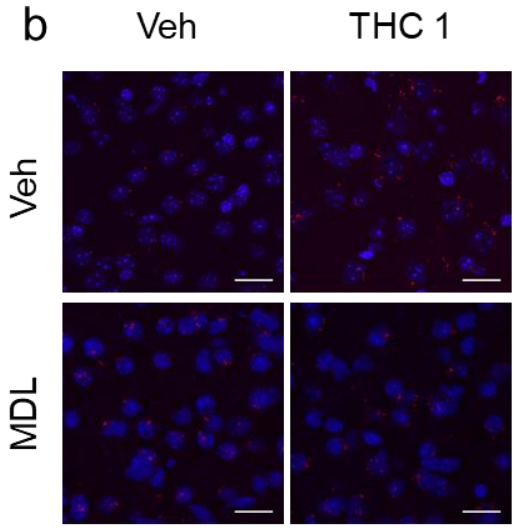


Figure 5.

Figure 5. Memory impairment produced by sub-chronic THC 1 mg/kg administration is prevented with 5-HT2AR blockade.

A sub-chronic pre-treatment with MDL 0.01 mg/kg prevented THC 1 amnesic-like effects in (a) declarative memory measured in NOR task (Veh Veh n = 12, MDL Veh n = 7, Veh THC 1 n = 8, MDL THC 1 n = 14) and (b) emotional memory measured in Trace FC (Veh Veh n = 9, MDL Veh n = 9, Veh THC 1 n = 10, MDL THC 1 n = 11). Statistical significance was calculated by Bonferroni *post hoc* test following two-way ANOVA. * $p < 0.05$, *** $p < 0.001$ (Veh Veh vs Veh THC 1). ## $p < 0.01$ (Veh THC 1 vs MDL THC 1). Data are expressed as mean ± S.E.M.


a
CA3
***
###
Positive cells (%)
100
80
60
40
20
0
Veh
THC 1
Veh Veh
MDL Veh
Veh THC 1
MDL THC 1


b
Veh
THC 1
Veh
MDL

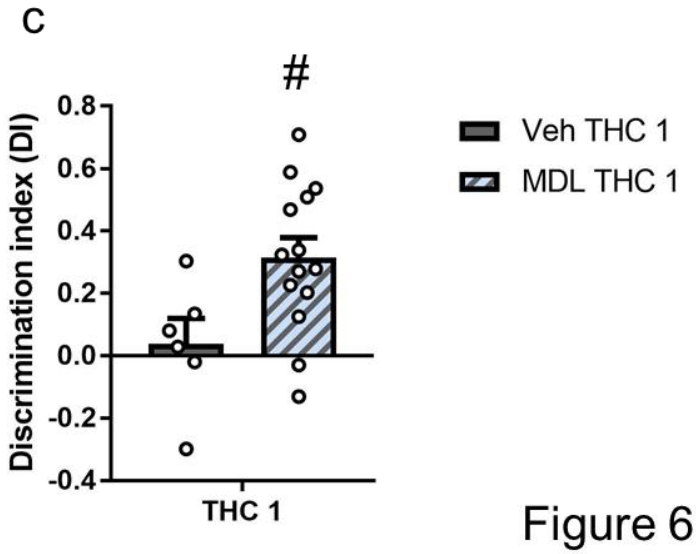
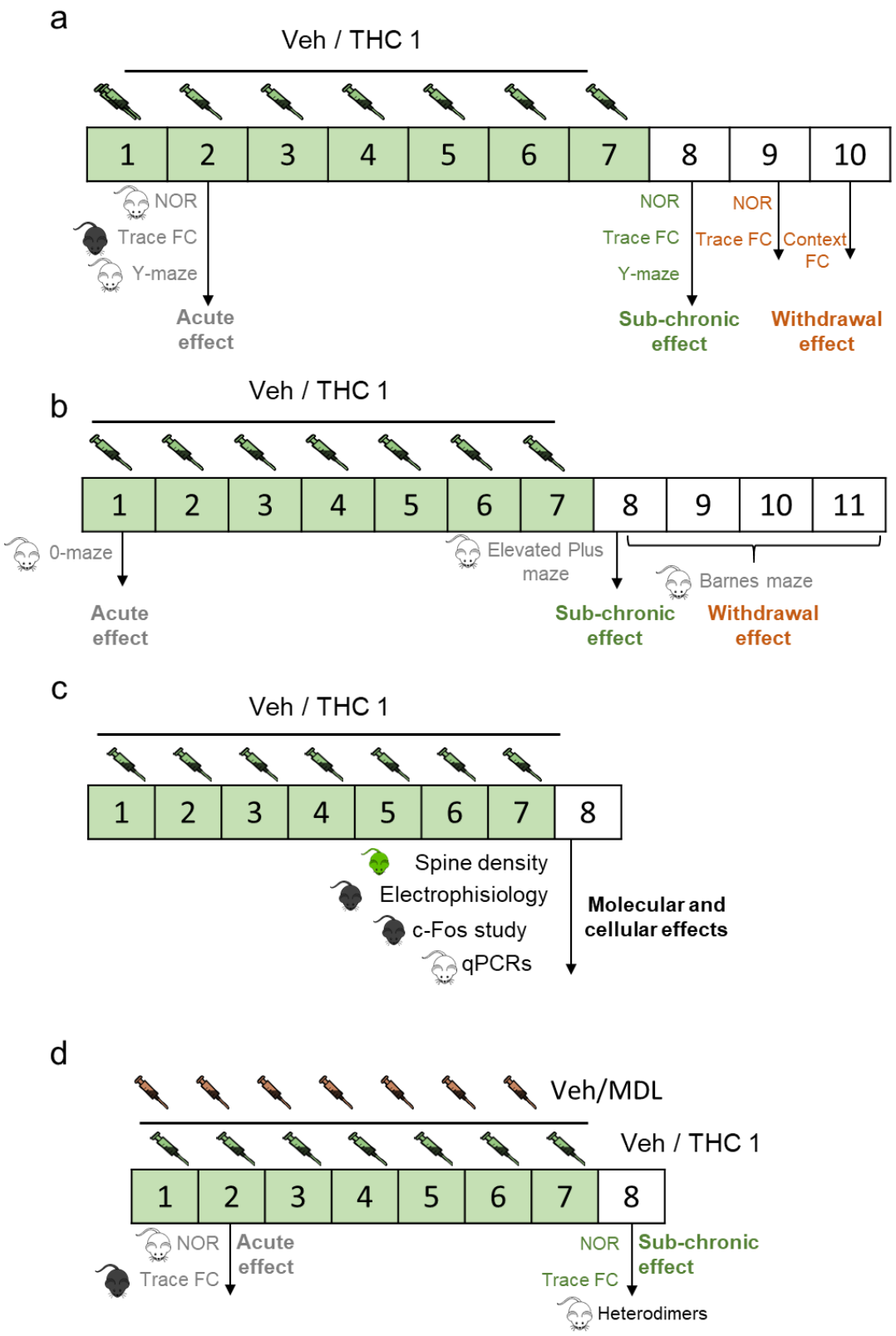

c
#
Discrimination index (DI)
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
THC 1
Veh THC 1
MDL THC 1

Figure 6.

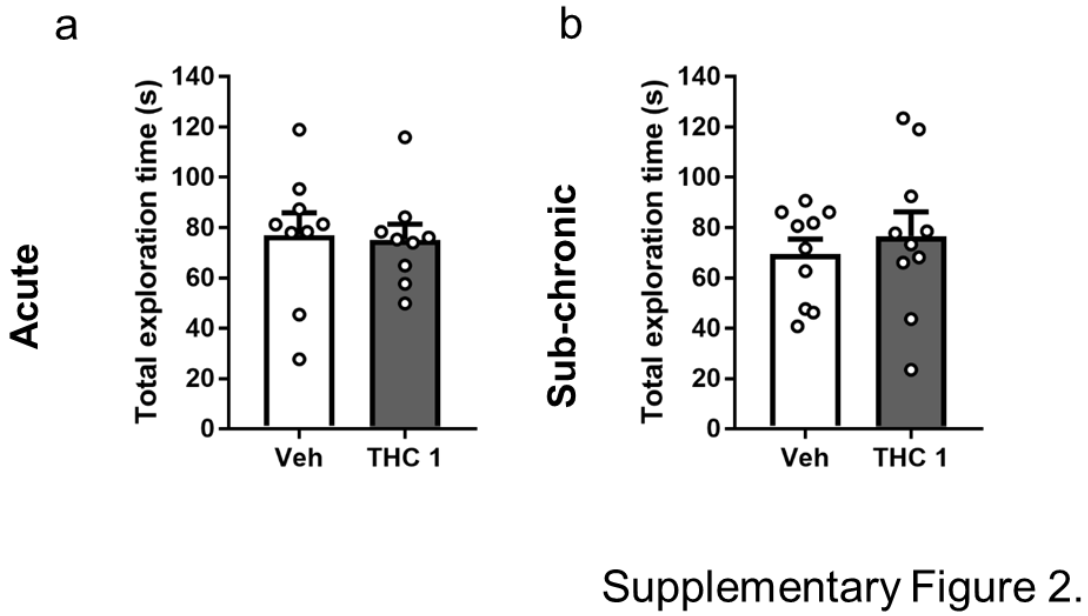
Figure 6. CB1R/5-HT2AR heterodimer detection is enhanced after repeated THC 1 mg/kg exposure.

(a) THC 1 exposure significantly enhanced the detection of cells showing CB1R/5-HT2AR heterodimers, and sub-chronic pre-treatment with MDL prevent the up-regulation of heterodimers formed between CB1 and 5-HT2A receptor after sub-chronic THC 1 treatment in the CA3 region of the hippocampus (Veh Veh n=4, MDL Veh n=4, Veh THC 1 n=4, MDL THC 1 n=4) (see Suppl. Figure 1d for treatment schedule) (b) Representative images of the four groups, scale bar=20 um . Statistical significance was calculated by Bonferroni *post hoc* test following two-way ANOVA. *** $p < 0.001$ (Veh Veh vs Veh THC 1). ### $p < 0.001$ (Veh THC 1 vs MDL THC 1). (c) Acute MDL administration before the seventh administration of THC 1 also prevented THC 1 memory deficits in NOR task (Veh THC 1 n = 6, MDL THC 1 n = 14). Statistical significance was calculated by Student's t-test. # $p < 0.05$ (Veh THC 1 vs MDL THC 1). Data are expressed as mean ± S.E.M.

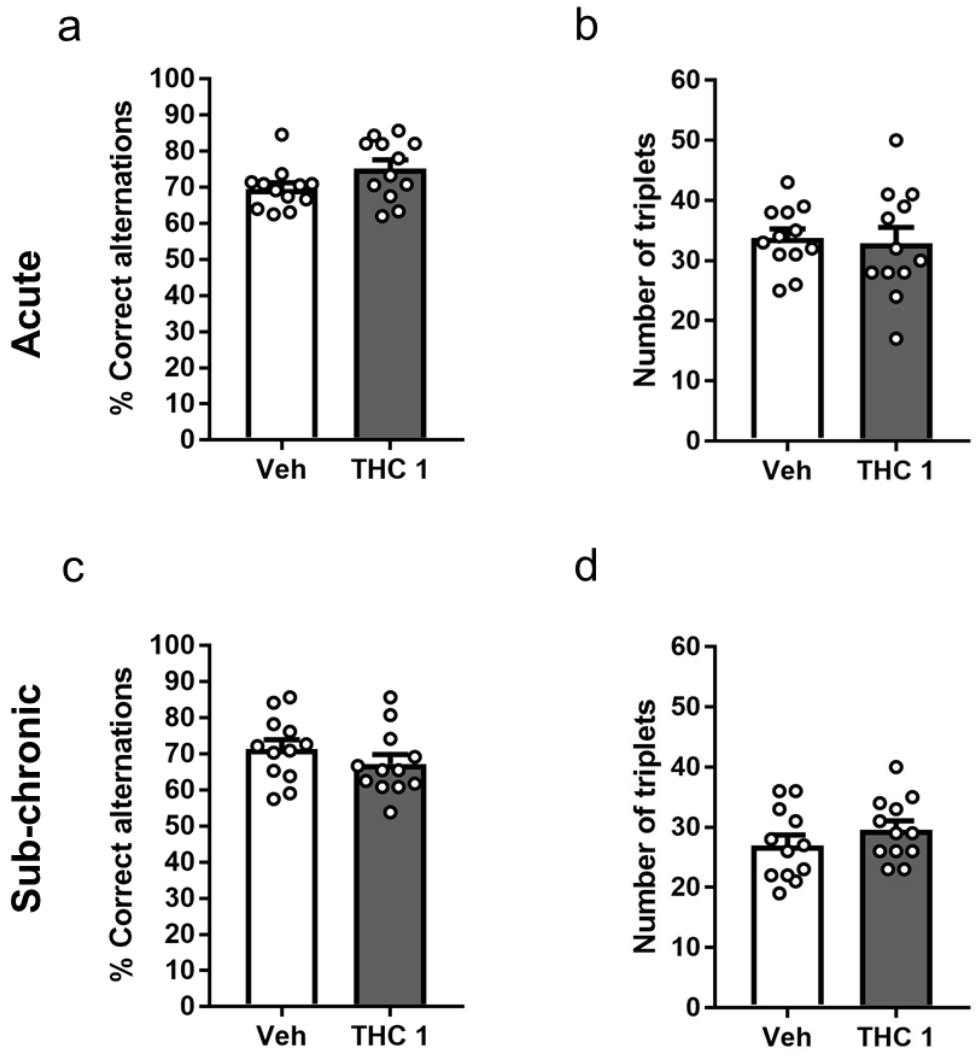


Supplementary Figure 1.

Supplementary Figure 1. Timeline scheme of the behavioral tests, the treatments and animals used in the study. (a) Memory test performed to compare acute with sub-chronic effects of THC 1 were performed in the same way: the acute effect is measured 24h after the first administration and the sub-chronic 24h after the last administration, coinciding with the test phase of NOR task, Trace FC and Y-maze. In the case of treatment withdrawal effects, NOR and Trace FC test were performed 48h after the last administration and Context FC test 72h after last administration. (b) Additional behavioural task was used: anxiety-like behaviour was assessed in 0-maze 30 min after the first administration and in elevated plus maze 24h after the last administration. Spatial learning was measured using Barnes maze during 4 consecutive days starting 24h after the last sub-chronic administration. (c) For molecular and cellular effects studied samples were extracted 24h after the last administration. (d) For the evaluation of 5-HT2AR blockade with MDL, the pre-treatment was administered 20 min prior the THC 1 treatment and acute and sub-chronic affects were studied in NOR and Trace FC in the same way as previously.

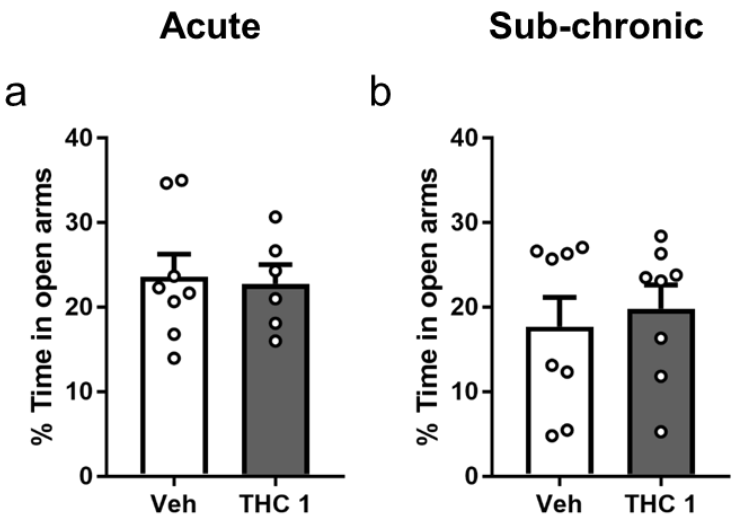


Supplementary Figure 2. Total exploration time measurements in acute and sub-chronic NOR test. After administration of THC 1 no significant differences were observed neither in (a) acute (Veh n=9, THC 1 n=9) or (b) after the sub-chronic treatment (Veh n=10, THC 1 n=10) in total exploration times in NOR task. Statistical significance was calculated by Student's t-test. Data are expressed as mean ± S.E.M.



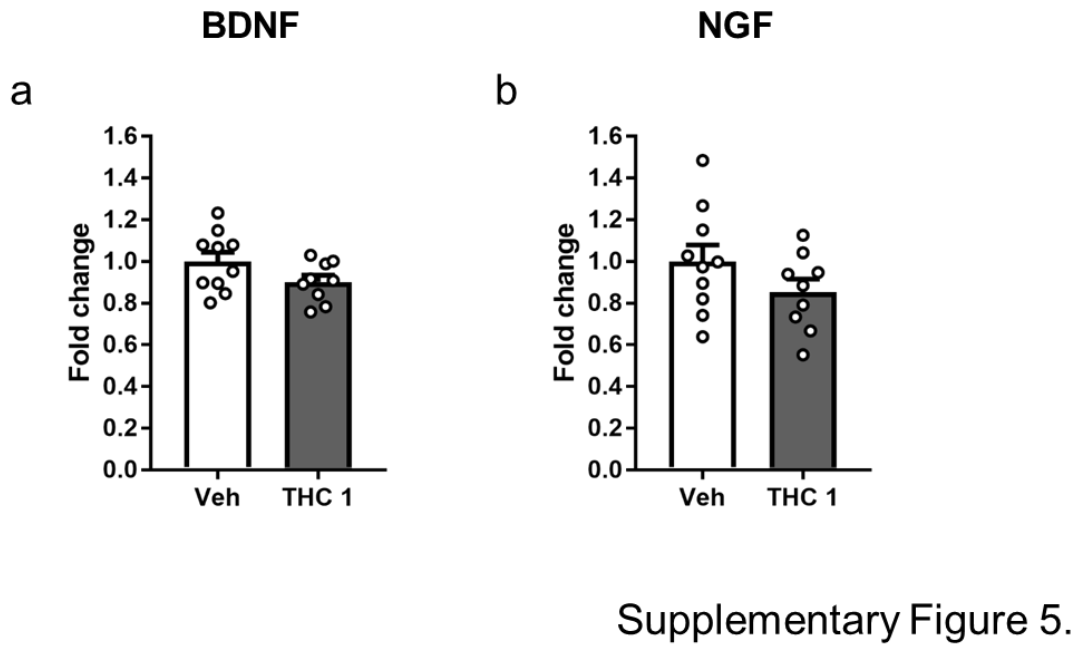
Supplementary Figure 3.

Supplementary Figure 3. No effects of sub-chronic treatment with THC 1 in working-memory. After acute administration of THC 1 no significant differences were observed neither in (a) working-memory assessment Y-maze (Veh n=12, THC 1 n=12) or (b) number of triplets in Y-maze (Veh n=12, THC 1 n=12). After the sub-chronic treatment, no significant differences were observed either in (c) % of correct alternations (Veh n=12, THC 1 n=12) or (d) locomotion in Y-maze (Veh n=12, THC 1 n=12). Statistical significance was calculated by Student's t-test. Data are expressed as mean ± S.E.M.

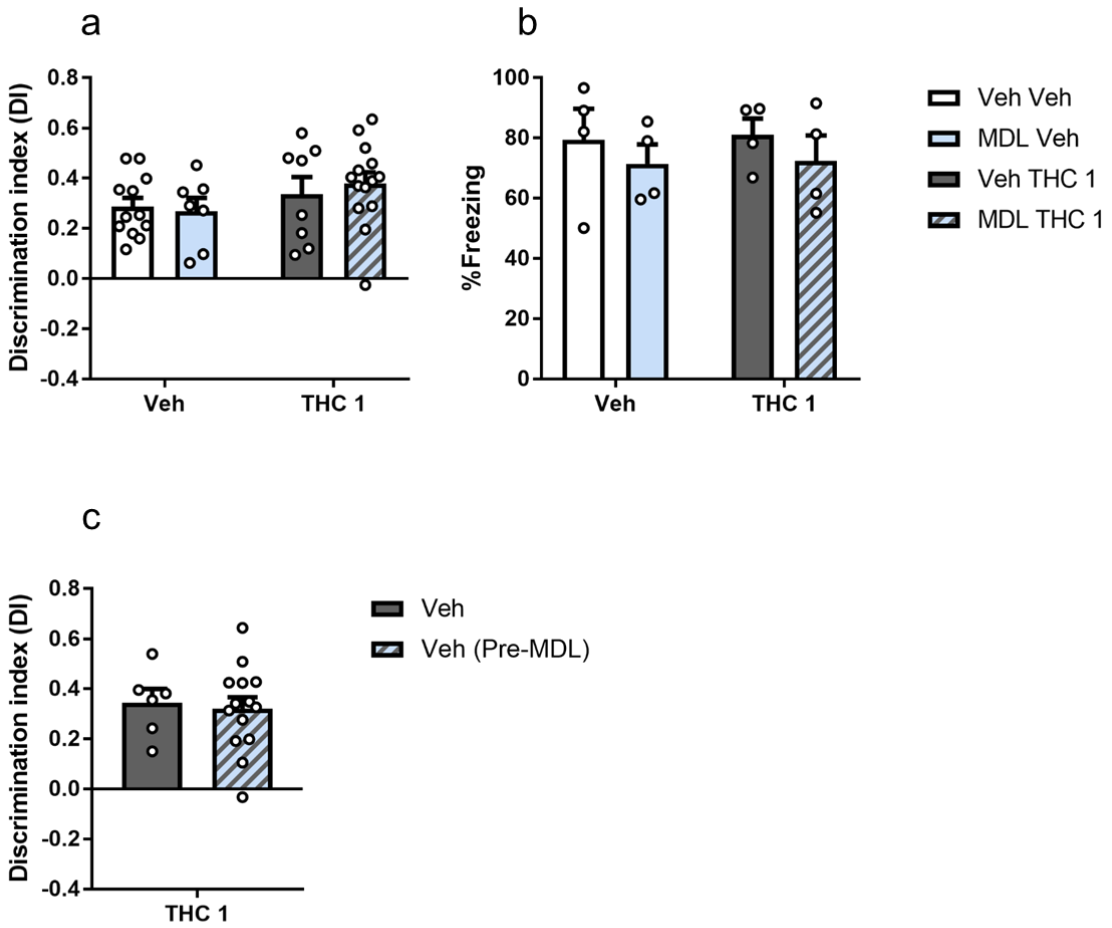


Supplementary Figure 4.

Supplementary Figure 4. Exposure to THC 1 mg/kg do not modify anxiety-like behaviour. (a) 30 min after an acute injection of THC 1 mg/kg no differences in anxiety-like behaviour are observed in 0-maze (Veh n=8, THC 1 n=6). (b) After sub-chronic treatment with THC 1 mg/kg no effects are observed neither in anxiety-like behaviour in elevated plus-maze (Veh n=8, THC 1 n=8). Statistical significance was calculated by Student's t-test. Data are expressed as mean ± S.E.M.

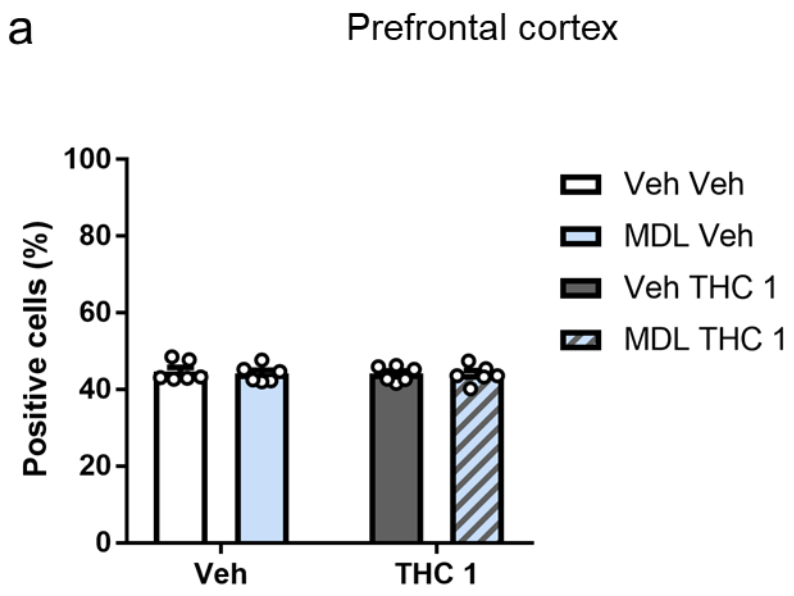


Supplementary Figure 5. Neurotrophic factors after sub-chronic THC 1 exposure showed no changes at RNA level. qPCRs of the two principal neurotrophic factors in the hippocampus of vehicle and THC 1 animals revealed no significant differences neither in (a) BDNF (Veh n=10, THC 1 n=9) or (b) NGF (Veh n=10, THC 1 n=9). Statistical significance was calculated by Student's t-test. Data are expressed as mean ± S.E.M.



Supplementary Figure 6.

Supplementary Figure 6. After an acute administration of THC 1 mg/kg or MDL 0.01 mg/kg no effects on memory are present. (a) Any of the treatments affect memory performance in an acute administration in (a) NOR task (Veh Veh n=12, MDL Veh n=7, Veh THC 1 n=8, MDL THC 1 n=14) and in (b) Trace fear conditioning (Veh Veh n=4, MDL Veh n=4, Veh THC 1 n=4, MDL THC 1 n=4). Statistical significance was calculated by Bonferroni *post hoc* test following two-way ANOVA. (c) No differences are present at memory performance at acute level in the NOR task in the groups in which the acute pre-treatment with MDL was tested. (Veh THC 1 n=6, Veh (Pre-MDL) THC 1 n=14). Statistical significance was calculated by Student's t-test. Data are expressed as mean ± S.E.M.



Supplementary Figure 7.

Supplementary Figure 7. Heterodimers formed between CB1 and 5-HT2A receptor are not altered after THC 1 exposure in prefrontal cortex. (a) In prefrontal cortex no differences are observed in heterodimers levels between groups (Veh Veh n=6, MDL Veh n=6, Veh THC 1 n=6, MDL THC 1 n=6) .Statistical significance was calculated by Bonferroni *post hoc* test following two-way ANOVA. Data are expressed as mean ± S.E.M.