Cannabis Users show Enhanced Expression of CB_1-$5HT_{2A}$ Receptor Heteromers in Olfactory Neuroepithelium Cells

Liliana Galindo[3,9], Estefanía Moreno[4], Fernando López Armenta[1], Daniel Guinart[3,6,7], Aida Cuenca-Royo[1], Mercè Izquierdo-Serra[5], Laura Xicota[1], Cristina Fernandez[1], Esther Menoyo[1], José M. Fernández-Fernández[5], Gloria Benítez-King[8], Enric I. Canela[4], Vicent Casadó[4], Víctor Pérez[3], Rafael de la Torre[1,2*] and Patricia Robledo[1*]

[1]Integrative Pharmacology and Systems Neuroscience, IMIM-Hospital del Mar Research Institute, and Department of Experimental and Health Sciences, University Pompeu Fabra, Barcelona, Spain.
[2]Centro de Investigación Biomédica en Red-Fisiopatología de la Obesidad y Nutrición, Madrid, Spain
[3]Neuropsychiatry and Addictions Institute (INAD) of Parc de Salut Mar, Centro de Investigación Biomédica En Red de Salud Mental G21. Mental Health research group, IMIM-Hospital del Mar Research Institute, Barcelona, Spain
[4]Department of Biochemistry and Molecular Biomedicine, Faculty of Biology, Institute of Biomedicine of the University of Barcelona, University of Barcelona, Barcelona, Spain. Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas, Instituto de Salud Carlos III, Madrid, Spain.
[5]Laboratory of Molecular Physiology, Department of Experimental and Health Sciences, University Pompeu Fabra, Barcelona, Spain
[6]Department of Psychiatry and Legal Medicine, Universitat Autònoma de Barcelona, Barcelona, Spain.
[7]Zucker Hillside Hospital, Feinstein Institute for Medical Research, Northwell Health, New York, USA.
[8]Laboratorio de Neurofarmacología, Subdirección de Investigaciones Clínicas, Instituto Nacional de Psiquiatría Ramón de la Fuente Muñiz, Calzada México-Xochimilco 101 San Lorenzo Huipulco, Tlalpan, Mexico City 14370, Mexico.
[9]Department of Psychiatry, University of Cambridge, Cambridge, UK

* These authors contributed equally to this work

Corresponding Author:
Patricia Robledo, PhD
Integrative Pharmacology and Systems Neuroscience
IMIM-Hospital del Mar Research Institute
PRBB
Calle Dr. Aiguader 88
Barcelona 08003
SPAIN
probledo@imim.es

Running title: CB_1R-$5HT_{2A}$R Heteromers in Human Olfactory Neuroepithelium

ABSTRACT

Cannabinoid CB1 receptors (CB_1R) and serotonergic 2A receptors ($5HT_{2A}R$) form heteromers in the brain of mice where they mediate the cognitive deficits produced by delta9-tetrahydrocannabinol. However, it is still unknown whether the expression of this heterodimer is modulated by chronic cannabis use in humans. In this study, we investigated the expression levels and functionality of CB_1R-$5HT_{2A}R$ heteromers in human olfactory neuroepithelium (ON) cells of cannabis users and control subjects and determined their molecular characteristics through adenylate cyclase and the ERK 1/2 pathway signaling studies. We also assessed whether heteromer expression levels correlated with cannabis consumption and cognitive performance in neuropsychological tests. ON cells from controls and cannabis users expressed neuronal markers such as βIII-tubulin and nestin, displayed similar expression levels of genes related to cellular self-renewal, stem cell differentiation, and generation of neural crest cells, and showed comparable Na^+ currents in patch clamp recordings. Interestingly, CB_1R-$5HT_{2A}R$ heteromer expression was significantly increased in cannabis users and positively correlated with the amount of cannabis consumed, and negatively with age of onset of cannabis use. In addition, a negative correlation was found between heteromer expression levels and attention and working memory performance in cannabis users and control subjects. Our findings suggest that cannabis consumption regulates the formation of CB_1R-$5HT_{2A}R$ heteromers, and may have a key role in cognitive processing. These heterodimers could be potential new targets to develop treatment alternatives for cognitive impairments.

Key Words: CB_1R-$5HT_{2A}R$ heteromers; cannabis; cognitive; progenitor cells; human olfactory neuroepithelium

INTRODUCTION

Cannabis sativa is a recreational drug widely consumed in Europe [1] and in the United States, where its prevalence of use has increased in the last decade [2]. In addition, cannabis use during adolescence raises important concerns regarding its possible detrimental effects on cognitive processing in this vulnerable population, and on the increased risk for developing substance use disorders [3, 4]. Consequently, characterizing the molecular effects of cannabis use and unveiling new and more reliable biomarkers to identify populations at risk, for prevention and treatment, has become a challenge of upmost importance.

Cannabinoid receptors type 1 (CB_1R) are G protein–linked receptors located in the central nervous system (CNS) primarily in cortex, basal ganglia, hippocampus, amygdala and cerebellum [5]. These receptors are modulators of several neurotransmitters including serotonin (5-HT) and dopamine (DA). Delta-9-Tetrahydrocannabinol (THC) is the primary psychoactive component of cannabis producing rewarding effects, changes in sensory perception, psychomotor impairments and cognitive deficits [6, 7]. Recently, we demonstrated that heteromers formed between the 5-HT receptor 2A ($5HT_{2A}R$) and CB_1R are expressed and functionally active in the brain of mice, where they specifically mediate the memory impairments induced by THC [8]. However, it is still unknown whether the expression of this heterodimer is modulated by chronic cannabis use in humans.

The olfactory mucosa has great potential as a tool to examine neurophysiological processes in psychiatric disorders [9, 10]. Olfactory sensory neurons are replaced by neurogenesis continuously throughout adult life from neuronal precursor/progenitors located in the apical and basal membranes [11]. Thus, the olfactory neuroepithelium (ON) contains pluripotent cells that can proliferate in vitro and differentiate into multiple cell types including neurons and glia [12]. Studies in ON cells of patients suffering from several different types of neuropsychiatric disorders show specific alterations in cellular function [13, 14]. Thus, this peripheral tissue can be used as a surrogate of central nervous system (CNS) function [9, 15], and

may provide relevant information related to the neuropathology observed in different mental illnesses including substance use disorders.

Previous studies have shown that human ON cells exhibit a neuronal phenotype comprising several types of receptors including 5-HT 2C receptors and signaling pathways related to olfaction and other functional aspects of the CNS [16]. Likewise, expression of CB_1R and the endocannabinoid, 2-arachidonylglycerol has been found in the ON of experimental animals [17, 18]. Although alterations in CB_1R levels have been described in the brain of cannabis users [19–22], and changes in endocannabinoid levels are detected in plasma following the administration of THC to healthy volunteers [23, 24], no data has been provided yet regarding changes in $5HT_{2A}R$ at central or peripheral level in this population. Nonetheless, adaptations in these receptors have been reported in neuropsychiatric disorders that present cognitive deficits like Alzheimer's disease [25] and schizophrenia [26, 27].

Therefore, in this study we focused on the ON, a peripheral tissue closely related to the CNS, to investigate whether CB_1R-$5HT_{2A}R$ heteromer expression and functionality would be modulated by chronic cannabis use, and to evaluate their role in cognitive processes. Thus, we first determined the functional interaction of this heteromeric complex in ON cells of controls subjects and cannabis users by proximity ligation assays (PLA), and cAMP and pERK analysis. Second, we correlated the expression levels of the heterodimer in ON cells of cannabis users with plasma concentrations of THC metabolites, and with neuropsychological test scores in cannabis users and control subjects.

METHODS AND MATERIALS

Subjects and Clinical Evaluation

17 cannabis users and 16 control subjects matched by age and sex were recruited at IMIM-Hospital del Mar Research Institute. The study was reviewed and approved by the local institutional ethics committee (CEIC-PSMAR). All subjects provided a written informed consent after a complete description about the study and procedures. **Testing was carried out in the morning, and all participants were instructed not to fast on that day in order to avoid any potential interference of different nutritional conditions on heteromer expression profile or cognitive performance.** All subjects fulfilled the following inclusion criteria: subjects of both sexes and age between 18 and 40 years old, and in the case of cannabis users, consumption of more than 5 cannabis cigarettes per week. **Moreover, to avoid the potential confound of acute cannabis intoxication in both neuropsychological and biochemical assessments, subjects we instructed to refrain from cannabis use for at least 12 hours before testing.** The exclusion criteria for all subjects were: (1) meeting criteria for any severe mental disorder according to DSM-V [28]; (2) history of severe mental illness among first-degree relatives; (3) history of severe congenital, medical or neurological illness; (4) present medical condition with nasal repercussions (rhinitis or bleeding) and (5) consumption of other drugs of abuse. (6) Lidocaine allergy. Cannabis consumption was verified by testing for THC metabolites in urine (Instant-View, Multipanel 10 Test Drug Screen, Alfa Scientific Designs, Poway, CA, USA). The exclusion criteria were confirmed through clinical history, the Hamilton Depression Rating Scale (HDRS-17) [29] and the Semi-structured diagnostic interview according to DSM-V criteria (SCID) [30]. Socio-economic status (SES) was measured by the Hollingshead Redlich Scale [31] based on educational attainment and occupational prestige, in this scale lower scores reflect higher SES (SES = [occupation score × 5] + [Education score × 3]). Clinical evaluation also included a complete medical exploration, physical examination, body mass index (BMI = weight in kg/ height in m2) and laboratory tests (drugs detection in urine). Substance-related disorders were assessed with the Spanish version of the Psychiatric Research Interview for Substance and Mental Disorders (PRISM) [32]. Neurological soft signs were evaluated using the Neurological Evaluation Scale (NES), the most commonly used scale in

international literature [33]. The Global Assessment of Functioning Scale (GAF) [34] was administered to measure the functionality level in each subject.

Neuropsychological Assessment

Attention performance was evaluated using the spatial span direct recall with the Cambridge Neuropsychological Test Automated Battery (CANTAB) [35], and the digit span direct recall (WAIS-III) [36] tests. Measures of span length were used for the analysis. Working memory performance was assessed with the spatial span inverse recall (CANTAB), and the digit span inverse recall (WAIS-III) tests. Social and emotional cognition was determined with the emotion recognition task (ERT, CANTAB), executive functions with the semantic verbal fluency test (SVFT) [37], and premorbid intelligence estimation with the vocabulary test (WAIS-III).

Quantification of cannabis consumption in plasma

To estimate the amount of cannabis consumed in cannabis users, we quantified the plasma concentrations of Δ-9-tetrahydrocannabinol (THC), its initial psychoactive metabolite, 11-hydroxy-THC (11-OH-THC), and the main non-psychoactive metabolite of THC, namely 11-nor-9-carboxy-Δ-9-THC (THC-COOH). An extraction protocol from Waters Corporation was applied with some modifications [38]. Briefly, 1 ml of plasma was transferred into a glass tube and spiked with d3-Δ-9-THC (10 µl of 1 µg/ml MeOH solution) as internal standard. A protein precipitation with 2 ml of 0.1% formic acid in acetonitrile was performed prior to a solid phase extraction with Oasis Prime HLB 3 cc, 60 mg column (Waters Co., Milford, MA). After 10 min of centrifugation at 3500 rpm, the supernatant was diluted with 4 ml of MilliQ water and loaded to each cartridge. Subsequently, 2 ml of 25% of methanol was added to wash each column twice. The elution step was carried out with 2 ml of 90:10 acetonitrile:methanol (ACN:MeOH) twofold. The organic phase was evaporated to dryness under a nitrogen stream at <39 °C and <15 psi pressure. Analytes were reconstituted in 50 µl of 90:10 ACN:MeOH and 50 µl of MilliQ water. Quantification of THC, 11-OH-THC and THC-COOH in plasma was performed using an Agilent 1200 series HPLC system (Agilent Technologies) coupled to a 6410 Triple Quadrupole LC-MS (Agilent

Technologies) mass spectrometer with an electrospray interface. Nitrogen was employed as a drying and nebulizing gas.

Nasal Exfoliation and Cell Culture

Samples of the ON were obtained from control subjects and cannabis users during the morning, as previously described [39]. After humidification of the nasal cavity, two separate sterile interdental brushes was used to obtain samples from the lower and middle turbinate, and were placed inside Eppendorf tubes with 250 µl of supplemented medium. This procedure was performed separately for both nostrils. The nasal exfoliates were initially placed in cold Dulbecco's Modified Eagle Medium/Ham F-12 (DMEM/F12) containing 10% FBS, 2% glutamine and 1% streptomycin-penicillin (GibcoBRL). ON cell suspensions were dissociated by mechanical trituration. The primary cultures were grown for 3 weeks in DMEM/F12 supplemented with 10% FBS before passaging into flasks (Thermo Scientific, Madrid, Spain). These were dissociated with 0.25 % trypsin (GibcoBRL), replated at 4000 cells/cm2 into 75 cm^2 flasks and cultured in DMEM/F12 with 10% FBS. Cells were then expanded by passage and banked down in aliquots after harvest by storage in liquid nitrogen with 20% FBS and 10% dimethyl sulfoxide (Sigma-Aldrich, Madrid, Spain). Frozen aliquots of ON cells at passage 3 were used as the starting point for all the experiments described: phenotyping, gene and protein expression, and functional assays. All cultures were grown under standard conditions on tissue culture plastic in DMEM/F12 supplemented with 10% FBS at 37°C and 5% CO2.

Immunofluorescence

ON cells were grown on glass coverslips, fixed in 4% paraformaldehyde for 15 min, and washed with phosphate-buffered saline (PBS) containing 20 mM glycine to quench the aldehyde groups. After permeabilisation with PBS-glycine containing 0.05% Triton X-100 for 5 min, cells were incubated 1 h at room temperature with PBS containing 1% bovine serum albumin and were labelled overnight with the corresponding primary antibody (mouse anti-βIII-tubulin, clone 2G10, neuronal antibody from Millipore (1:500), and rabbit anti-nestin from Abcam (1:200). Cells were washed with PBS, and stained for 2 h with

the secondary antibodies: (Donkey Anti-Mouse IgG Alexa Fluor 488; Jackson ImmunoResearch – 1:500, and Donkey Anti-Rabbit IgG Alexa Fluor 594; Jackson ImmunoResearch – 1:500), and with DAPI (Invitrogen; 300 nM). Samples were rinsed several times in PBS, mounted with Fluoromount-G (SouthernBiotech) medium. Images were acquired with an Eclipse Ni-E microscope (Nikon Instruments, Barcelona, Spain) at 60X for N=6 cannabis users and N=5 for control subjects. **For each subject (cannabis users: n=6 and control subjects: n=5), 10 photographs were captured, and 40-60 cells that could be viewed in their entirety were selected to quantify the intensity levels of βIII-tubulin and nestin in the entire cell using ImageJ software. We excluded those cells that were superimposed with other cells or that appeared only partially in the visual field. The immunostaining was also used to classify ON cells into various differentiation stages according to their morphological characteristics, as previously described [40]. Briefly, in stage 1, cells are spread-out, in stage 2, cells become polarized, in stage 3, neurites are formed, in stage 4, axons start to form and in stage 5, cells are polarized and the axon elongates.**

Western Blot Assays

The extraction of total protein was performed by lysing the cells with Lysis buffer (50 mM Tris pH 7.4, 300 mM NaCl, 1% Triton X-100, 1 mM EDTA, protease inhibitors). Subsequently, electrophoresis was performed with equal amounts of protein (40 µg per well) (DC-micro plate assay (Bio-Rad, Madrid, Spain) in a 7% Acrylamide gel transferred to an Immobilon PVDF membrane (Millipore, Darmstadt, Germany) using antibodies against βIII-tubulin (1:500 in TBS-Tween) (Millipore, Darmstadt, Germany), nestin (1:200), and actin (1:500 in TBS-Tween) (Santa Cruz Biotechnology, Santa Cruz, California) as a loading control. Secondary antibodies were HRP-conjugated. The detection was achieved by chemiluminescent methodology (Chemi-Doc XRS System, Bio-Rad), and protein expression levels were quantified with the quantity one program (Bio-Rad). Each sample from cannabis users (n=6) and controls subjects (n=7) was measured in two independent gels, and the values for each protein were normalized with the detection of actin in the same samples, and expressed as a percentage of the control (relative intensity).

Gene Expression Studies

Harvested cells were trypsinized and pelleted to isolate total RNA using GenElute Mammalian total RNA Miniprep Kit (Sigma-Aldrich, Madrid, Spain). The amount of the purified RNA was determined with NanoDrop 1000 Spectophotometer (Thermo Fisher Scientific, USA). Total RNA was reverse transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's protocol. Resulting cDNA was used for quantitative real-time PCR (qRT-PCR), using SYBR Green PCR master mix on the LC480 Real-Time PCR System (Roche, Madrid, Spain). The primer sequences used were the following: Forward 5'-3' (*GAPDH*: ACAGTTGCCATGTAGACC; *NeuroD1*: GGCGTCAGGCGCATAGACCT; *PAX6*: CACCTACAGCGCTCTGCCGC; *RET*: GGCATCAACGTCCAGTACAAG; *P63*: GGTTGGCAAAATCCTGGAG; *TWIST*: CCGGAGACCTAGATGTCATTGTT; *P75*: CTGCAAGCAGAACAAGCAAG; NANOG: ACAACTGGCCGAAGAATAGCA; SOX2: ACGGTGCCTTGGATGAAGGA. Reverse 5'-3' (*GAPDH*: TTTTTGGTTGAGCACAGG; *NeuroD1*: CGCCCATCAGCCCACTCTCG; *PAX6*: CCCGAGGTGCCCATTGGCTG; *RET*: TGAGGTGACCACCCCTAGC; *P63*: TCACTAAATTGAGTCTGGGCATT; *TWIST*: TTTTAGTTATCCAGCTCCAGAGTCTCT; *P75*: GGCCTCATGGGTAAAGGAGT; *NANOG*: GGTTCCCAGTCGGGTTCAC; *SOX2*: ATGGCTGTTGCCTGGCTTCT). Analysis was performed using LightCycler® 480 Software (Roche, Madrid, Spain). The data for each target gene was normalized to the endogenous reference gene, and the fold change in target gene mRNA abundance was determined using the $2^{-\Delta\Delta Ct}$ method.

Electrophysiological Studies

To examine differences between controls and cannabis users in terms of the functionality of voltage-gated Na^+ channels in ON cell cultures, we performed whole-cell, patch-clamp electrophysiological recordings in **ON cells (Fig. 2D) at stage 3 of differentiation (see Fig. 2C),** as previously described [41]. ON cells were used after 4-5 days of being unfrozen. Pipettes had a resistance of 2-4 MΩ when filled with a solution containing (in mM): 140 KCl, 0.5 EGTA, 4 Na_2ATP, 0.3 Na_3GTP, and 10 HEPES (pH 7.2-7.3 and 290-300

mOsmol/l). The external bath solution contained (in mM): 140 NaCl, 3 KCl, 1 $MgCl_2$, 10 HEPES and 10 D-glucose (pH 7.4 and 300-310 mOsmol/l). When indicated we added tetrodotoxin (Tocris, United Kingdom) to the batch to a final concentration of 5 μM. Current recordings were obtained at a sampling rate of 33 kHz and filtered at 1 kHz with a D-6100 Darmstadt amplifier (List Medical, Germany) and the pClamp8 software (Molecular Devices, USA) was used for pulse generation, data acquisition and subsequent analysis. Current was corrected for leak and capacitive currents using the leak subtraction procedure P/8. Cell capacitance was in average 29 pF, ranging from 18 to 40 pF, no differences in size where found between cells from different conditions. Cells were voltage clamped at -80 mV and to evaluate the current voltage-dependence we run a 20 ms steps protocol at different voltages, from -60 to +70 mV in 5 mV steps. Afterwards, normalized current-voltage (I-V) relationships were individually fitted with the modified Boltzmann equation, as previously reported [42]: $I = (G_{max}(V-V_{rev}))/(1+e^{((-(V-V_{1/2\ act}))/k_{act})})$, where I is the peak current, G_{max} is the maximal conductance of the cell, V is the test membrane potential, V_{rev} is the extrapolated reversal potential of inward Na^+, $V_{1/2\ act}$ is the voltage for half-maximal current activation, and k_{act} is the slope factor of the Boltzmann term.

Biochemical and molecular Assays

The expression of CB_1R-$5HT_{2A}R$ heteromers in ON cells of control subjects and cannabis users was determined using the proximity ligation assay (PLA). In addition, we assessed cAMP production and ERK-1/2 phosphorylation in these heteromers, and evaluated cross-talk and cross antagonism following treatment with CB_1R and $5HT_{2A}R$ agonists and antagonists, as previously described [8]. For proximity ligation assays (PLA), ON cells were grown on glass coverslips and were fixed in 4% paraformaldehyde for 15 min, washed with PBS containing 20 mM glycine, permeabilized with the same buffer containing 0.05% Triton X-100, and successively washed with PBS. Heteromers were detected using the Duolink II in situ PLA detection Kit (OLink; Bioscience, Uppsala, Sweden) as previously reported [8, 43]. A mixture of equal amounts (1:100) of guinea pig anti-CB_1R antibody (Frontier Science, Ishikari, Japan) and rabbit anti-$5HT_{2A}R$ antibody (Neuromics, Edina, MN) was used together with PLA probes detecting guinea pig or rabbit antibodies. Then, cells were processed for ligation and amplification with a Detection Reagent Red

and were mounted using the mounting medium with DAPI. The samples were analyzed in a Leica SP2 confocal microscope (Leica Microsystems, Mannheim, Germany) 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, Bethesda, MD). Nuclei and red spots were counted on the maximum projections of each image stack. The percentage of cells containing one or more red spot versus the total number of cells (blue nucleus), and the mean number of red spots per cell, quantified as the quotient between the number of red spots and the number of cells containing spots were determined considering a total of 200–350 cells from 7–12 different fields for each subject.

To determine cAMP production, homogeneous time-resolved fluorescence energy transfer (HTRF) assays were performed using the Lance Ultra cAMP kit (PerkinElmer, Waltham, Massachusetts, US). The assay is based on competitive displacement of a europium chelate-labelled cAMP tracer bound to a specific antibody conjugated to acceptor beads. We first established the optimal human ON cell density for an appropriate fluorescent signal. This was done by measuring the TR-FRET signal determined as a function of forskolin concentration using different cell densities. The forskolin dose-response curves were related to the cAMP standard curve in order to establish which cell density provides a response that covers most of the dynamic range of cAMP standard curve. Cells (500 cells/well) growing in medium containing 50 µM zardeverine were pre-treated with the CB_1R antagonist, rimonabant and the 5-$HT_{2A}R$ antagonist, MDL 100,907 or the corresponding vehicle in white ProxiPlate 384-well microplates (PerkinElmer, Waltham, Massachusetts, US) at 25°C for 20 min and stimulated with the CB1 agonist, WIN 55,212–2 and the 5HT2A agonist, DOI for 15 min before adding 0.5 µM forskolin or vehicle and incubating for an additional 15-min period. Fluorescence at 665 nm was analyzed on a PHERAstar Flagship microplate reader equipped with an HTRF optical module (BMG Lab technologies, Offenburg, Germany).

To determine ERK-1/2 phosphorylation, ON cells (25.000 cells per well) seeded in 96-well poly-D-lysine-coated plates (Sigma-Aldrich, Madrid, Spain) were pre-treated at 25 °C for 20 min with rimonabant and MDL 100,907 or the corresponding vehicle and stimulated for an additional 7 min with the WIN 55,212–2

and DOI. Phosphorylation was determined in white ProxiPlate 384-well microplates (PerkinElmer Life Sciences) by α-screen bead-based technology using the amplified luminescent proximity homogeneous assay kit (PerkinElmer Life Sciences) following the instructions of the supplier, and using the Enspire multimode plate reader (PerkinElmer Life Sciences). Phosphorylation is expressed in arbitrary units, ALPHAcounts, as measured by light emission at 520–620 nm of the acceptor beads.

Statistical Analysis

For the demographic and clinical data, differences between controls and cannabis users were assessed using two-tailed Chi-square tests for categorical variables (gender and tobacco use). **To determine whether the different data showed a normal distribution, we first applied the Kolmogorov-Smirnov test.** A two-tailed nonparametric Mann-Whitney U test was used to compare the variables which did not meet the assumption of normal distribution, **and differences in variables with normal distributions were assessed with analysis of variance (ANOVA) or two-tailed t-tests**. For neuropsychological assessment all variables were transformed into standardized t-scores. Since a normal distribution was found in both groups, multiple two-tailed t-tests were used to compare all the cognitive results. **The protein levels of βIII-tubulin and nestin in ON cells were analyzed using a two-way ANOVA with protein as within and group as between subject factors.** The percentage of cells with heteromers and the mean number of heteromers per cell were compared with a two-tailed t-test, and a two-tailed non-parametric Mann-Whitney U test, respectively. The cAMP and pERK data were analyzed with a two-way analysis of variance (ANOVA) with group and treatment as between subject factors, followed by a Bonferroni post-hoc test when appropriate. A two-way factorial ANOVA was used to analyze whether group differences in neuropsychological assessment and heteromer expression remained significant after adjusting for tobacco use. Partial correlations were performed to evaluate the association between every neuropsychological test score and heteromer expression levels in the entire sample, and to calculate the correlation between cannabis use (age of onset and THC-COOH plasma levels), and heteromers expression levels in cannabis users only. For significant correlations, multiple linear regressions were performed to evaluate the ability of heteromers expression levels to predict the neuropsychological

performance, and the ability of THC-COOH plasma levels to predict the heteromer expression level. The data were managed and analyzed with the PASW Statistics v.18. P-values lower than 0.05 were considered significant.

RESULTS

Demographic and Clinical Data

Demographic and clinical data from all subjects are shown in Table 1. Cannabis users and controls did not differ in age, sex, body mass index, socio-economic status or clinical assessment (premorbid IQ, depressive symptoms). However, a significantly higher percentage of tobacco smokers was found in cannabis users group ($p<0.05$).

Cannabis consumption

To correlate the amount of cannabis consumption with the expression of heteromers in ON cells and with cognitive performance, we determined the plasma concentrations of THC, 11-OH-THC and THC-COOH in cannabis users and control subjects. Plasma concentrations of THC could not be detected in control subjects (n=16) or in cannabis users (n=17). In the group of control subjects, plasma concentrations of THC metabolites were undetectable. In cannabis users, plasma concentrations of 11-OH-THC were detected in 12 out of 17 subjects (mean concentration: 1.6 ± 0.3 ng/ml), and THC-COOH concentrations were detected in 16 out of 17 subjects (mean concentration: 20.9 ± 3.6 ng/ml). **Cannabis users with non-detectable plasma levels of metabolites (1 subject) or with outlying concentrations (2 standard deviations away from the mean; 1 subject) were excluded from the subsequent correlation analyses. In addition, 4 subjects were further excluded due to cell culture contamination.**

Cannabis users exhibit attention and working memory impairments

Cannabis users showed significantly worse attentional performance assessed in the spatial span direct recall test (Fig. 1A), and worse working memory evaluated in the spatial span inverse recall test (Fig. 1B) than control subjects ($p<0.05$). Since more cannabis users were tobacco smokers compared to controls, we performed a two-way factorial ANOVA considering tobacco use as a fixed factor. Comparisons between groups for inverse spatial recall remained significant after adjusting for tobacco use, but not for direct spatial recall (Supp. Table 1). No significant differences between groups were found for digit span

direct or inverse recall (Fig. 1C and D), semantic verbal fluency (Fig. 1E), or emotional recognition (Fig. 1F).

ON cells derived from control subjects and cannabis users express markers of neural lineage

All ON cells were positive for two specific neuronal markers, namely βIII-tubulin and nestin (Fig. 2 A-C). Most cells studied showed morphologies corresponding to stages 1, 2, 3 of cell differentiation, and very few were observed at stage 4. To analyze differences between groups in terms of βIII-tubulin and nestin protein levels, we first calculated the intensity of fluorescence for these two markers. The statistical analysis revealed significant lower intensity for βIII-tubulin with respect to nestin [$F(1,9)=45.99$, $p<0.001$], but no significant effects of group (controls vs cannabis users), or interaction between factors (Supp. Fig. 1A). To confirm these results with a more quantitative technique, we determined the relative protein intensities in immunoblot experiments. In accordance with immunofluorescence studies, the statistical analysis showed a significant lower intensity for βIII-tubulin with respect to nestin [$F(1,11)=47.76$, $p<0.001$], but no significant effects of group or interaction between factors (Supp. Fig. 1B-C).

We next evaluated whether ON cells of cannabis users would show changes in the expression of genes related to cell differentiation and cell function. In both groups, we found expression of genes associated with stem cell differentiation, self-renewal, and generation of neural crest cell (*NANOG, TWIST, RET* and *P63*), corroborating the neural lineage of the ON cells in culture. However, non-significant changes in the relative expression of these genes were revealed (fold change in cannabis users with respect to control subjects: *NANOG*: 0.996; *TWIST*: 0.673; *P63*: 1.108, and *RET*: 0.768). On the other hand, we did not find expression of characteristic molecular markers of neuronal precursors, globose or horizontal basal cells (*PAX6*, *SOX2*, and *NeuroD1*), or markers of olfactory ensheathing cells (*P75*) in either group[15][15].

Electrophysiological recordings performed **in ON cells (Fig. 2D)** from cannabis users and control subjects revealed the presence of voltage-gated Na^+ channels. In response to membrane depolarizing pulses, we

recorded fast inactivating inward currents **(Fig. 2E),** which were inhibited by tetrodotoxin (5 μM), a toxin that specifically binds to voltage-gated Na^+ channels (**Fig. 2F**). Current density and voltage dependence activation of Na^+ currents showed no differences between cells derived from cannabis users or control subjects (**Fig. 2G),** with a peak current density of -3.3 ± 0.5 pA/pF and -3.0 ± 0.6 pA/pF and potential for half-maximal channel activation ($V_{1/2\ act}$) of -5 ± 2 mV and -8 ± 1 mV, respectively (n=11 cells from 5 cannabis users and n=8 cells from 3 control subjects). Together, these data indicate that the ON cell cultures from cannabis users and controls are viable comprising cells at various stages of neuronal differentiation.

Increased expression of CB_1R-$5HT_{2A}R$ heteromers in ON cells from cannabis users

CB_1R-$5HT_{2A}R$ heteromers were observed in ON cells of both control subjects (Fig. 2H) and cannabis users (Fig. 2I), but not in samples used as negative controls (Suppl. Fig. 1D-E). In both groups, heteromers were observed in all ON cells regardless of their stage of differentiation (Fig. 2J-K). Several cell cultures became contaminated during the incubation process (2 out of 16 in control subjects, and 4 out of 17 in cannabis users). Thus, CB_1R-$5HT_{2A}R$ heteromers expression analysis was performed in a smaller sample (Control group = 14; Cannabis users group = 13). Interestingly, the percentage of cells containing CB_1R-$5HT_{2A}R$ heteromers (**Fig. 2L),** and the mean number of heteromers per positive cell **(Fig. 2M)** were significantly higher in cannabis users with respect control subjects ($p<0.001$ and $p<0.01$, respectively). The differences found in CB_1R-$5HT_{2A}R$ heteromers expression between groups remained significant after being adjusted for tobacco use (percentage of cells $F= 13.995$, $p<0.001$; number of heteromers per cell $F= 5.842$, $p<0.01$). These results clearly show that CB_1R-$5HT_{2A}R$ heteromers are expressed in ON cells, and that cannabis consumption up-regulates their expression.

Functional characteristics of the CB_1R-$5HT_{2A}R$ heterodimer in ON cells

To investigate the biochemical properties of CB_1R-$5HT_{2A}R$ heteromers, we determined signaling through adenylate cyclase and the ERK 1/2 phosphorylation pathway in ON cells. In cells stimulated with forskolin

and treated with WIN 55,212–2, DOI, or both, we found reduced cAMP production to a similar extent in both controls and cannabis users (Fig. 3A). Likewise, both WIN 55,212–2 and DOI alone induced the activation of ERK 1/2 in controls and cannabis users equally (Fig. 3B). However, when cells from both groups were co-stimulated with WIN 55,212–2 and DOI, ERK 1/2 phosphorylation levels were significantly lower than those observed with WIN 55,212–2 alone. This effect was not due to a change in the optimum response time for ERK 1/2 (data not shown). These data indicate that co-stimulation of CB_1R-$5HT_{2A}R$ heteromers in ON cells leads to a negative cross-talk between both receptors. More importantly, compared to controls, the negative cross-talk was more intense in cannabis users, where co-stimulation did not induce ERK 1/2 phosphorylation at all. Some GPCR heteromers have been found to display cross antagonism, i.e. the ability of an antagonist of one receptor to antagonize the signaling of the partner receptor [43, 44]. Cross antagonism requires direct protein-protein interaction since antagonists do not signal on their own, and thus, it is a specific biochemical characteristic of the corresponding heteromer. We found that both rimonabant and MDL 100,907 blocked the reduced cAMP activity produced by WIN 55,212–2 and by DOI (Fig. 3A). Analogously, rimonabant and MDL 100,907 were able to revert the ERK 1/2 phosphorylation induced by both WIN 55,212–2 and DOI (Fig. 3B). In cannabis users, a tendency for a stronger cross antagonism was observed in the group treated with WIN and MDL. These results demonstrate that CB_1R-$5HT_{2A}R$ heteromers in ON cells display bidirectional cross antagonism.

Cannabis use predicts the expression levels of CB_1R-$5HT_{2A}R$ heteromers in ON cells.

Since we found a greater expression of CB_1-$5HT_{2A}R$ heteromers in cannabis users with respect to control subjects, we investigated whether the quantity of cannabis consumed correlated with heteromer expression levels. For this analysis, we selected the more stable metabolite of THC, namely THC-COOH as an estimate of cannabis use. Out of the 17 cannabis users tested, 11 subjects were used for the correlation analysis since 4 subjects did not have measures of heteromer expression, one did not show plasma levels of THC-COOH, and one was an outlier. A significant positive linear correlation was observed between the percentage of cells expressing heteromers and THC-COOH plasma levels, which remained significant after considering tobacco use ($r = 0.747$; $p<0.05$) (Fig. 4A), and a significant negative

correlation was found between the mean number of heteromers per positive cell and the age of onset of cannabis use (r= -0.589; p<0.05) (Fig. 4B). Subsequently, a regression analysis (Supp. Table 3) was used to investigate whether cannabis consumption could predict the expression levels of CB1R-5HT2AR heteromers in the group of cannabis users (n= 11). We found that THC-COOH plasma levels explained up to 40.8% of the total variance in the percentage of cells expressing heteromers, a value that remained significant when tobacco use was included in a further analysis. Age of onset of cannabis use explained up to 29.4% of the total variance in the number of heteromers expressed per cell, but the statistical significance of this value disappeared in a posterior analysis adjusting for tobacco use (Supp. Table 3).

The expression levels of CB_1R-$5HT_{2A}R$ heteromers in ON cells predict neuropsychological performance

A correlation analysis was also performed between the neuropsychological tests scores and heteromer expression considering the entire population (n=27). Inverse spatial span recall values negatively correlated with both the percentage of cells expressing heteromers (Fig. 4C), and with the mean number of heteromers per positive cell (Fig. 4D), and both correlations remained significant after adjusting for tobacco use (Supp. Table 2). A significant negative correlation was also found between direct spatial span scores and both the percentage of cells with heteromers, and the mean number of heteromers expressed per cell. However, only the latter correlation remained significant after adjusting for tobacco use (Fig. 4E) (Supp. Table 2). Direct digit span scores significantly correlated with the percentage of heteromer expression, but lost significance when tobacco use was included in the analysis. For all other neuropsychological tests, no significant correlations with heteromer expression were observed (Supp. Table 2). Multiple regressions were performed to investigate whether expression levels of CB_1R-$5HT_{2A}R$ heteromers could predict the neuropsychological performance in control and cannabis users (n=27). Thus, the percent of cells with heteromers explained up to 25.9% of the total variance in the reverse spatial span test, and 12.5% of the variance in the direct spatial span test (Supp. Table 3). The mean number of heteromers per cell explained up to 14.8% of the total variance in the inverse spatial span test, and 15.2%

of the variance in the direct spatial span test. All of these statistical predictions were still significant when tobacco use was included in a posterior analysis (Supp. Table 3).

DISCUSSION

In this study we report three major findings. First, we revealed the presence of functional CB_1R-$5HT_{2A}R$ heteromers in ON cells of cannabis users and control subjects. Second, we found a significant positive correlation between the expression of these heteromers and the amount of cannabis consumption in cannabis users. Third, we observed a significant negative correlation between the expression levels of this heterodimer and attention and working memory performance in cannabis users and control subjects.

The ON constitutes an ideal substrate to investigate the neuropathophysiology of psychiatric diseases since it is rich in precursor/progenitor cells [11], and it is more closely associated with the central nervous system than other peripheral tissues such as blood or epidermal fibroblasts [15]. **Using immunofluorescence and immunoblot studies we established that the ON cells of control subjects and cannabis users expressed the neuronal markers βIII-tubulin and nestin, as previously reported [39, 45]. The data showed that control subjects and cannabis users do not differ in terms of the expression levels of these proteins, indicating an equivalent stage of cell differentiation in both groups. In addition,** ON cells of both groups showed similar expression levels of genes related to cellular self-renewal, stem cell differentiation, and generation of neural crest cells. The expression of these markers corroborates their neural lineage stage. On the other hand, we did not find expression of molecular markers of neuronal precursors, i.e. basal globose or horizontal cells, or markers of olfactory ensheathing cells. Moreover, in monolayer cell culture conditions we also observed the presence of immature olfactory sensory neurons, which exhibited electrophysiological properties consistent with previous data [39]. The amplitude of these currents, and the potential for half-maximal current activation however, were not significantly different in neurons from control subjects and cannabis users. Together, these data indicate that cannabis use does not induce mayor perturbations in ON cell viability, which guarantees the effective analysis of functional biomarkers of cannabis use disorder in this cell model.

More importantly, in this study we revealed a disparate expression of CB_1R-$5HT_{2A}R$ heteromers in ON cells of cannabis users and controls. Indeed, we found that both the percentage of cells expressing

heteromers and the number of heteromers per positive cell were increased in cannabis users with respect to controls. Notably, this effect was not due to a differential use of tobacco in these two groups. **The morphological analysis shows that under the culture conditions of this study, most ON cells expressing CB_1R-$5HT_{2A}R$ heteromers in both control subjects and cannabis users are in stages 1-3 of differentiation. These findings indicate that the increase in heteromer expression in ON cells from cannabis users is probably related to the use of cannabis, and not to a disparate stage of cell differentiation.**

To further investigate whether changes in expression levels of CB_1R-$5HT_{2A}R$ were associated with alterations in heterodimer function, we determined their biochemical properties. A common consequence of GPCR heteromerization is a specific integrated signaling upon co-activation of the molecularly different protomers as compared to their separate activation [46, 47]. Thus, in line with previous data in mice brain tissue [8], when ON cells were co-stimulated with WIN 55,212–2 and DOI, we found that it led to a lower activation of ERK 1/2 signaling, indicating negative cross-talk between both receptors. Markedly, pERK signaling was blunted in cannabis users during co-stimulation of the heterodimer. In addition, administration of the CB1 antagonist, rimonabant blocked the effects induced by both WIN 55,212-2 and DOI, and reciprocally, the 5-HT_{2A} antagonist MDL blocked the actions of both receptor agonists, indicating that CB_1R-$5HT_{2A}R$ heteromers in ON cells of control subjects and cannabis users display bidirectional cross antagonism. **These data also suggest that a main population of both CB_1R and $5HT_{2A}R$ in these cells are in fact forming functional CB_1R-$5HT_{2A}R$ heteromers.**

THC is the most abundant psychoactive ingredient in *Cannabis sativa* preparations producing psychomotor and mood altering effects, as well as, cognitive impairments [22, 48]. In this study, plasma concentrations of THC could not be detected in cannabis users, while varying levels of two of its metabolites, namely 11-OH-THC and THC-COOH were found. The concentrations of 11-OH-THC and THC-COOH observed are consistent with those previously reported in subjects after smoking one cannabis cigarette more than 6 h before testing, [49, 50]. Importantly, the percentage of cells expressing CB_1R-$5HT_{2A}R$ heteromers was correlated with higher plasma THC-COOH concentrations. Moreover,

plasma levels of THC-COOH significantly predicted the levels of heteromer expression in ON cells of cannabis users, even following correction for tobacco use, suggesting that cannabis consumption may be an important variable regulating the formation of CB_1R-$5HT_{2A}R$ heteromers in the ON. **On the other hand, our results also indicated that subjects who started to use cannabis sooner in life displayed more heteromers per positive cell in adulthood, and vice versa. However, we did not observe a significant correlation between age of onset of cannabis use and cognitive deficits (both in direct and inverse spatial span tests). In addition, the significant predictive value of age of onset on heteromer number was not maintained after a regression analysis adjusting for tobacco use. In this regard, we also found that the significant decrease in spatial attention found in cannabis users with respect to controls disappeared after adjusting for tobacco use. Therefore, in these two instances tobacco use may have influenced the results obtained. Indeed, nicotine and cannabis produce multiple common pharmacological actions in animals and humans including rewarding and affective responses, and alterations in cognitive processing (see** [51] **for review), and bidirectional interactions have been reported between the endocannabinoid and nicotine systems at the cellular and neuronal network levels (reviewed in** [52] **and** [53]**).**

Interestingly, in the entire population studied we found that a high level of CB_1R-$5HT_{2A}R$ heteromer expression in ON cells was associated with worse cognitive performance. Thus, negative correlations were observed between both the percentage of heteromers per cell and the mean number of heteromers per positive cell, and attention and working test scores. These correlations were still significant even after correcting for tobacco use in the analysis. Moreover, we also found that a higher percentage of heteromer expression in ON cells significantly predicted worse performance in attention and working memory tests in controls and cannabis users. Consistent with these findings is our data showing a reduction in p-ERK 1/2 via the heteromer since ERK signaling has been implicated in synaptic plasticity, learning and memory processes [54]. Both CB_1R and $5HT_{2A}R$ modulate cognitive processing in brain structures such as the pre-frontal cortex and hippocampus [55, 56], and CB_1R-$5HT_{2A}R$ heteromers in the rodent brain play a key role in the memory impairments induced by THC [8]. Therefore, our findings showing an association between

the expression of this heterodimer complex in ON cells and cognitive function may be reflecting similar processes in the human brain [9].

Moreover, recent evidence shows that endocannabinoids and exogenous cannabinoids increase odor detection and food intake by acting on CB_1R located in the olfactory bulb in mice [57]**. CB_1R have also been found in the ON of mice, where they do not seem to mediate olfaction** [58]**. On the other hand, there is a dense serotonergic innervation of the olfactory bulb in mammals, where 5-$HT_{2A}R$ could participate in olfactory learning** [59]**, and previous studies have reported the expression of serotonergic receptors in human ON cells** [16]**. Our present molecular data revealed specific biochemical characteristics of the CB_1R-$5HT_{2A}R$ heteromer in ON cells, consistent with previous studies in mouse brain tissue** [8]**. Thus, it is possible that these heterodimers could be mediating certain aspects of olfaction different from those exerted by each receptor alone. Although we did not evaluate olfactory performance in our subjects, further research is warranted to understand the role of CB_1R-$5HT_{2A}R$ heterodimers in ON cells in olfactory processes.**

Our results clearly establish the presence of functional CB_1R-$5HT_{2A}R$ heteromers in the ON, and provide a link between cannabis use and the formation of this heterodimer. Importantly, our study identifies a correlation between the expression of CB_1R-$5HT_{2A}R$ heteromers in the ON and cognitive alterations. These findings contribute to the increasing evidence that the ON is a relevant model to study neuropsychiatric disorders. Finally, the CB_1R-$5HT_{2A}R$ heterodimer could be potential new target to develop treatment alternatives for cognitive impairments in a large spectrum of diseases in which neuropsychological functions are compromised.

FUNDING AND DISCLOSURE

All of the authors declared no financial interests or potential conflicts of interest.

ACKNOWLEDGMENTS

This work was supported by grants from DIUE de la Generalitat de Catalunya (2014-SGR-680 and 2014-SGR-1236 to RTF), Instituto de Salud Carlos III, (P14/00210 to P.R.) FIS-FEDER Funds, Spanish Ministry of Economy and Competitiveness (MINECO/FEDER; grant SAF-2014-54840-R to E.I.C. and V.C., grant SAF-2015-69762-R to J.M.F-F., grant MDM-2014-0370 through the "María de Maeztu" Programme for Units of Excellence in R&D to Department of Experimental and Health Sciences), and the following networks of Instituto de Salud Carlos III: Red de Trastornos Adictivos, CIBER de Salud Mental, CIBER de Fisiopatología de la Obesidad y Nutrición and CIBER de Enfermedades Neurodegenerativas. M.I.-S. holds a "Juan de la Cierva-Formación" Fellowship funded by the Spanish Ministry of Economy and Competitiveness. We would like to thank Dr. María Inmaculada Hernández Muñoz for providing the primers in our gene expression studies and for her invaluable comments and suggestions, Klaus Langohr for his help with the statistical analyses, and Jordi García and Mitona Pujadas for excellent technical assistance. **Laura Xicota is currently at ICM Institut du Cerveau et de la Moelle épinière (CNRS UMR7225, INSERM U1127, UPMC) Hôpital de la Pitié-Salpêtrière, Paris, France"**

REFERENCES

1. Mounteney J, Griffiths P, Sedefov R, et al (2016) The drug situation in Europe: an overview of data available on illicit drugs and new psychoactive substances from European monitoring in 2015. Addiction 111:34–48. doi: 10.1111/add.13056
2. Azofeifa A, Mattson ME, Schauer G, et al (2016) National Estimates of Marijuana Use and Related Indicators - National Survey on Drug Use and Health, United States, 2002-2014. MMWR Surveill Summ 65:1–28. doi: 10.15585/mmwr.ss6511a1
3. Malone DT, Hill MN, Rubino T (2010) Adolescent cannabis use and psychosis: epidemiology and neurodevelopmental models. Br J Pharmacol 160:511–522. doi: 10.1111/j.1476-5381.2010.00721.x
4. Chen K, Sheth AJ, Elliott DK, Yeager A (2004) Prevalence and correlates of past-year substance use, abuse, and dependence in a suburban community sample of high-school students. Addict Behav 29:413–23.
5. Mackie K (2005) Distribution of cannabinoid receptors in the central and peripheral nervous system. Handb Exp Pharmacol 299–325.
6. Ameri A (1999) The effects of cannabinoids on the brain. Prog Neurobiol 58:315–48.
7. Curran HV, Brignell C, Fletcher S, et al (2002) Cognitive and subjective dose-response effects of acute oral Delta 9-tetrahydrocannabinol (THC) in infrequent cannabis users. Psychopharmacology (Berl) 164:61–70. doi: 10.1007/s00213-002-1169-0
8. Viñals X, Moreno E, Lanfumey L, et al (2015) Cognitive Impairment Induced by Delta9-tetrahydrocannabinol Occurs through Heteromers between Cannabinoid CB1 and Serotonin 5-HT2A Receptors. PLoS Biol 13:e1002194. doi: 10.1371/journal.pbio.1002194
9. Lavoie J, Sawa A, Ishizuka K (2017) Application of olfactory tissue and its neural progenitors to schizophrenia and psychiatric research. Curr Opin Psychiatry 30:176–183. doi: 10.1097/YCO.0000000000000327
10. Horiuchi Y, Kano S-I, Ishizuka K, et al (2013) Olfactory cells via nasal biopsy reflect the developing

brain in gene expression profiles: utility and limitation of the surrogate tissues in research for brain disorders. Neurosci Res 77:247–50. doi: 10.1016/j.neures.2013.09.010

11. Leung CT, Coulombe PA, Reed RR (2007) Contribution of olfactory neural stem cells to tissue maintenance and regeneration. Nat Neurosci 10:720–726. doi: 10.1038/nn1882
12. Matigian N, Abrahamsen G, Sutharsan R, et al (2010) Disease-specific, neurosphere-derived cells as models for brain disorders. Dis Model Mech 3:785–798. doi: 10.1242/dmm.005447
13. Mackay-Sim A (2012) Concise review: Patient-derived olfactory stem cells: new models for brain diseases. Stem Cells 30:2361–5. doi: 10.1002/stem.1220
14. Benítez-King G, Valdés-Tovar M, Trueta C, et al (2016) The microtubular cytoskeleton of olfactory neurons derived from patients with schizophrenia or with bipolar disorder: Implications for biomarker characterization, neuronal physiology and pharmacological screening. Mol Cell Neurosci 73:84–95. doi: 10.1016/j.mcn.2016.01.013
15. Borgmann-Winter K, Willard SL, Sinclair D, et al (2015) Translational potential of olfactory mucosa for the study of neuropsychiatric illness. Transl Psychiatry 5:e527. doi: 10.1038/tp.2014.141
16. Borgmann-Winter KE, Rawson NE, Wang H-Y, et al (2009) Human olfactory epithelial cells generated in vitro express diverse neuronal characteristics. Neuroscience 158:642–53. doi: 10.1016/j.neuroscience.2008.09.059
17. Breunig E, Manzini I, Piscitelli F, et al (2010) The endocannabinoid 2-arachidonoyl-glycerol controls odor sensitivity in larvae of Xenopus laevis. J Neurosci 30:8965–73. doi: 10.1523/JNEUROSCI.4030-09.2010
18. Hutch CR, Hillard CJ, Jia C, Hegg CC (2015) An endocannabinoid system is present in the mouse olfactory epithelium but does not modulate olfaction. Neuroscience 300:539–53. doi: 10.1016/j.neuroscience.2015.05.056
19. D'Souza DC, Cortes-Briones JA, Ranganathan M, et al (2016) Rapid Changes in Cannabinoid 1 Receptor Availability in Cannabis-Dependent Male Subjects After Abstinence From Cannabis. Biol Psychiatry Cogn Neurosci Neuroimaging 1:60–67. doi: 10.1016/j.bpsc.2015.09.008
20. Hirvonen J, Goodwin RS, Li C-T, et al (2012) Reversible and regionally selective downregulation

of brain cannabinoid CB1 receptors in chronic daily cannabis smokers. Mol Psychiatry 17:642–9. doi: 10.1038/mp.2011.82

21. Ceccarini J, Kuepper R, Kemels D, et al (2015) [18F]MK-9470 PET measurement of cannabinoid CB1 receptor availability in chronic cannabis users. Addict Biol 20:357–67. doi: 10.1111/adb.12116
22. Mizrahi R, Watts JJ, Tseng KY (2017) Mechanisms contributing to cognitive deficits in cannabis users. Neuropharmacology. doi: 10.1016/j.neuropharm.2017.04.018
23. Walter C, Ferreirós N, Bishay P, et al (2013) Exogenous delta^{9}-tetrahydrocannabinol influences circulating endogenous cannabinoids in humans. J Clin Psychopharmacol 33:699–705. doi: 10.1097/JCP.0b013e3182984015
24. Thieme U, Schelling G, Hauer D, et al (2014) Quantification of anandamide and 2-arachidonoylglycerol plasma levels to examine potential influences of tetrahydrocannabinol application on the endocannabinoid system in humans. Drug Test Anal 6:17–23. doi: 10.1002/dta.1561
25. Salmon E (2007) A review of the literature on neuroimaging of serotoninergic function in Alzheimer's disease and related disorders. J Neural Transm 114:1179–85. doi: 10.1007/s00702-007-0636-5
26. Muguruza C, Moreno JL, Umali A, et al (2013) Dysregulated 5-HT(2A) receptor binding in postmortem frontal cortex of schizophrenic subjects. Eur Neuropsychopharmacol 23:852–64. doi: 10.1016/j.euroneuro.2012.10.006
27. Zavitsanou K, Garrick T, Huang XF (2004) Selective antagonist [3H]SR141716A binding to cannabinoid CB1 receptors is increased in the anterior cingulate cortex in schizophrenia. Prog Neuropsychopharmacol Biol Psychiatry 28:355–60. doi: 10.1016/j.pnpbp.2003.11.005
28. American Psychiatric Association (2013) DSM-5: Diagnostic and Statistical Manual of Mental Disorders, (5th Ed.). Washington, DC
29. Hamilton M (1960) A rating scale for depression. J Neurol Neurosurg Psychiatry 23:56–62.
30. First M, Williams J, Karg R, et al (2015) SCID: Structured clinical interview for DSM-5 (research

version). Arlington

31. Hollingshead AB, Redlich FC (1958) Social class and mental illness. Am J Psychiatry 112:179–185. doi: 10.1176/ajp.112.3.179
32. Torrens M, Serrano D, Astals M, et al (2004) Diagnosing comorbid psychiatric disorders in substance abusers: validity of the Spanish versions of the Psychiatric Research Interview for Substance and Mental Disorders and the Structured Clinical Interview for DSM-IV. Am J Psychiatry 161:1231–7. doi: 10.1176/appi.ajp.161.7.1231
33. Buchanan RW, Heinrichs DW (1989) The Neurological Evaluation Scale (NES): a structured instrument for the assessment of neurological signs in schizophrenia. Psychiatry Res 27:335–50.
34. Endicott J, Spitzer RL, Fleiss JL, Cohen J (1976) The global assessment scale. A procedure for measuring overall severity of psychiatric disturbance. Arch Gen Psychiatry 33:766–71.
35. Robbins TW, Sahakian B (2017) Cambridge Neuropsychological Test Automated Battery.
36. Wechsler D (1997) WAIS-III: Wechsler adult intelligence scale. Spanish Version.
37. Benton A, Hamsher K (1983) Multilingual Aphasia Examination.
38. Zhang X, Danaceau J, Chambers E (2016) Quantitative Analysis of THC and its Metabolites in Whole Blood Using LC-MS/MS for Toxicology and Forensic Laboratories. 1–3.
39. Benítez-King G, Riquelme A, Ortíz-López L, et al (2011) A non-invasive method to isolate the neuronal linage from the nasal epithelium from schizophrenic and bipolar diseases. J Neurosci Methods 201:35–45. doi: 10.1016/j.jneumeth.2011.07.009
40. Galván-Arrieta T, Trueta C, Cercós MG, et al (2017) The role of melatonin in the neurodevelopmental etiology of schizophrenia: A study in human olfactory neuronal precursors. J Pineal Res 63:e12421. doi: 10.1111/jpi.12421
41. Serra SA, Fernàndez-Castillo N, Macaya A, et al (2009) The hemiplegic migraine-associated Y1245C mutation in CACNA1A results in a gain of channel function due to its effect on the voltage sensor and G-protein-mediated inhibition. Pflugers Arch 458:489–502. doi: 10.1007/s00424-009-0637-3
42. Serra M, Brito C, Costa EM, et al (2009) Integrating human stem cell expansion and neuronal

differentiation in bioreactors. BMC Biotechnol 9:82. doi: 10.1186/1472-6750-9-82

43. Moreno E, Moreno-Delgado D, Navarro G, et al (2014) Cocaine Disrupts Histamine H3 Receptor Modulation of Dopamine D1 Receptor Signaling: 1-D1-H3 Receptor Complexes as Key Targets for Reducing Cocaine's Effects. J Neurosci 34:3545–3558. doi: 10.1523/JNEUROSCI.4147-13.2014

44. Moreno E, Vaz SH, Cai N-S, et al (2011) Dopamine-Galanin Receptor Heteromers Modulate Cholinergic Neurotransmission in the Rat Ventral Hippocampus. J Neurosci 31:7412–7423. doi: 10.1523/JNEUROSCI.0191-11.2011

45. Ortiz-López L, González-Olvera JJ, Vega-Rivera NM, et al (2017) Human neural stem/progenitor cells derived from the olfactory epithelium express the TrkB receptor and migrate in response to BDNF. Neuroscience 355:84–100. doi: 10.1016/j.neuroscience.2017.04.047

46. Ferré S, Casadó V, Devi LA, et al (2014) G protein-coupled receptor oligomerization revisited: functional and pharmacological perspectives. Pharmacol Rev 66:413–34. doi: 10.1124/pr.113.008052

47. Gomes I, Sierra S, Devi LA (2016) Detection of Receptor Heteromerization Using In Situ Proximity Ligation Assay. Curr Protoc Pharmacol 75:2.16.1-2.16.31. doi: 10.1002/cpph.15

48. D'Souza DC, Perry E, MacDougall L, et al (2004) The psychotomimetic effects of intravenous delta-9-tetrahydrocannabinol in healthy individuals: implications for psychosis. Neuropsychopharmacology 29:1558–72. doi: 10.1038/sj.npp.1300496

49. Huestis MA, Boyd SJ, Heishman SJ, et al (2007) Single and multiple doses of rimonabant antagonize acute effects of smoked cannabis in male cannabis users. Psychopharmacology (Berl) 194:505–515. doi: 10.1007/s00213-007-0861-5

50. Huestis MA, Henningfield JE, Cone EJ (1992) Blood cannabinoids. I. Absorption of THC and formation of 11-OH-THC and THCCOOH during and after smoking marijuana. J Anal Toxicol 16:276–82.

51. Viveros M-P, Marco EM, File SE (2006) Nicotine and cannabinoids: parallels, contrasts and interactions. Neurosci Biobehav Rev 30:1161–81. doi: 10.1016/j.neubiorev.2006.08.002

52. Castane A, Berrendero F, Maldonado R (2005) The role of the cannabinoid system in nicotine addiction. Pharmacol Biochem Behav 81:381–386. doi: 10.1016/j.pbb.2005.01.025

53. Oz M, Al Kury L, Keun-Hang SY, et al (2014) Cellular approaches to the interaction between cannabinoid receptor ligands and nicotinic acetylcholine receptors. Eur J Pharmacol 731:100–5. doi: 10.1016/j.ejphar.2014.03.010

54. Sweatt JD (2004) Mitogen-activated protein kinases in synaptic plasticity and memory. Curr Opin Neurobiol 14:311–317. doi: 10.1016/j.conb.2004.04.001

55. Bhattacharyya S, Schoeler T (2013) The effect of cannabis use on memory function: an update. Subst Abuse Rehabil 4:11. doi: 10.2147/SAR.S25869

56. Harvey JA (2003) Role of the Serotonin 5-HT2A Receptor in Learning. Learn Mem 10:355–362. doi: 10.1101/lm.60803

57. Soria-Gómez E, Bellocchio L, Reguero L, et al (2014) The endocannabinoid system controls food intake via olfactory processes. Nat Neurosci 17:407–15. doi: 10.1038/nn.3647

58. Hutch CR, Hillard CJ, Jia C, Hegg CC (2015) An endocannabinoid system is present in the mouse olfactory epithelium but does not modulate olfaction. Neuroscience 300:539–53. doi: 10.1016/j.neuroscience.2015.05.056

59. Hardy A, Palouzier-Paulignan B, Duchamp A, et al (2005) 5-Hydroxytryptamine action in the rat olfactory bulb: in vitro electrophysiological patch-clamp recordings of juxtaglomerular and mitral cells. Neuroscience 131:717–31. doi: 10.1016/j.neuroscience.2004.10.034

Figure Legends

Fig. 1 Neuropsychological assessment in control subjects and cannabis users. Significant differences between groups were observed for attention assessed with the direct spatial span test (A), but not the direct digit span test (C). Significant differences between groups were observed for working memory assessed with the inverse spatial span test (B), but not the inverse digit span test (D). No significant differences between groups were observed in the verbal fluency test (E) or the emotion recognition test (F). *p<0.05

Fig. 2 Characterization of olfactory neuroepithelium (ON) cells and quantification of CB_1R-$5HT_{2A}R$ heteromer expression in control subjects and cannabis users. **(A) Representative ON cells stained with anti-βIII-tubulin and with anti-nestin (B), and overlapping of both anti-βIII-tubulin and anti-nestin staining (C). The numbers depict the different stages of cells differentiation from 1 to 4 observed in ON cells (see methods for details). DAPI was used to stain the nuclei (blue). Scale bars at 20 µm. (D) Bright field image of a patched ON cell (scale bar 10 µm).** (E) Whole-cell current recordings from a control (CS, top, grey) and cannabis user (CC, bottom, red) neuron in response to 20 ms steps at different voltages, as indicated. (F) The responses from the same cell in the presence of tetrodotoxin (TTX, 5 µM). The inhibition of the inward peak current with TTX indicates the presence of voltage-gated Na^+ channels on the cells. (G) Normalized current-voltage dependence for CS and CC condition are not different, each point corresponds to the mean ± S.E.M. from 3 CS subjects with 8 cells analyzed and 5 CC subjects with 11 cells analyzed. Each cell was fitted to Boltzmann equation to obtain the parameters $V_{½\ act}$ (mean ± S.E.M.) to compare voltage response from control and cannabis consumer neurons. **In H-I, representative confocal phase contrast microscopy images of ON cells in proximity ligation assays showing heteromers appearing as red spots of a control subject (H), and a cannabis user (I). In J-K, representative confocal microscopy images of ON cells stained with anti βIII-tubulin and processed in proximity ligation assays showing the different cell morphologies containing heteromers (red dots). In all cases, cell nuclei were stained with DAPI (blue) Scale bars = 20 µm. In**

L and M, the percentage of cells containing CB_1R-$5HT_{2A}R$ heteromers (L), and the mean number of heteromers per positive cell (M) were increased in cannabis users (n=13) with respect to controls (n=14). **p<0.01, *** p<0.001

Fig. 3 Quantification of CB_1R-$5HT_{2A}R$ heteromer signaling in olfactory neuroepithelium cells of control subjects and cannabis users. (A) cAMP production was determined in cells pre-incubated either with vehicle or with the CB_1R antagonist rimonabant (1 μM, RIM) or the $5HT_{2A}R$ antagonist MDL 100,907 (300 nM) for 20 min prior to being stimulated with medium, the $5HT_{2A}R$ agonist DOI (100 nM), the CB_1R agonist WIN 55,212–2 (100 nM, WIN), or both in the absence or in the presence of 0.5 μM forskolin. **Values represent mean ± SEM of 12-18 determinations from 5 controls and 6 cannabis users**, and are expressed as the percentage of the forskolin-treated cells in each subject (120–150 pmols cAMP/10^6 cells). **Two-way ANOVA revealed a significant treatment effect [F(10,99)=47.01, p<0.001], but no significant group or interaction effects.** (B) pERK activation was determined in cells pre-incubated or not for 15 min with rimonabant (1 μM, RIM) or MDL 100,907 (300 nM, MDL), and stimulated for 5 min with WIN 55,212–2 (100 nM, WIN), DOI (100 nM) or both. **Quantification of phosphorylated ERK 1/2 was determined by α-screen bead-based technology. Phosphorylation was expressed in arbitrary units (ALPHA counts, light emission at 520–620 nm). Values expressed as percentage of basal (non-treated cells), were mean ± SEM of 10-20 determinations from 5 controls and 5 cannabis users. Two-way ANOVA revealed significant effects of treatment [F(7,64)=17.42, p<0.001], group [F(1,64)=25.60, p<0.001], and interaction between factors [F(7,64)=2.18, p<0.05]. Bonferroni post-hoc tests showed a significant increase in pERK in cells from both groups treated with WIN and DOI with respect to basal activity (***p<0.001). Co-stimulation with WIN and DOI increased pERK in cells to a lower extent than WIN alone in control subjects ($^{\#}$p<0.05), and in cannabis users ($^{\#\#\#}$p<0.001). Compared to controls, in cells from cannabis users, pERK was not activated at all by co-stimulation with WIN and DOI ($^{\&\&}$p<0.01)**

Fig. 4 Correlation analyses in cannabis users (A and B) and in the entire population (C-E). (A) Cannabis users (n=11) showed a positive correlation between the percentage of cells expressing CB_1R-$5HT_{2A}R$ heteromers in olfactory neuroepithelium (ON) cells and the plasma levels of THC-COOH (r=0.747; p<0.05). (B) Cannabis users (n=11) showed a negative correlation between the mean number of CB_1R-$5HT_{2A}R$ heteromers in ON positive cells and the age of onset of cannabis use (r=-0.589; p<0.05). In the entire population (n=27), a significant negative correlation between the percentage of cells expressing CB_1R-$5HT_{2A}R$ heteromers in ON cells and working memory performance assessed in the inverse spatial span test (r=-0.566, p<0.01) was revealed (C). Also, significant negative correlations were observed between the mean number of CB_1R-$5HT_{2A}R$ heteromers per positive cell and attention performance evaluated in the direct spatial span test (D) (r=-0.388, p<0.05), and working memory performance in the inverse spatial span test (E) (r=-0.471, p<0.01). All the correlation analyses were adjusted for tobacco use

Supp. Fig. 1. Biochemical experiments in ON cells. (A) Quantification of relative fluorescence of βIII-tubulin and nestin in ON cells from control subjects and cannabis users. (B) Representative immunoblots for βIII-tubulin, nestin, and actin in controls and cannabis users. (C) Quantification of relative protein intensity of β-III tubulin and nestin in ON cells from control subjects (n=7) and cannabis users (n=6). (D-E) Representative confocal microscopy images of ON cells in proximity ligation assays for of the control condition in the absence of anti-$5HT_{2A}R$ primary antibody of a control subject (D), and a cannabis user (E).

Figure 1

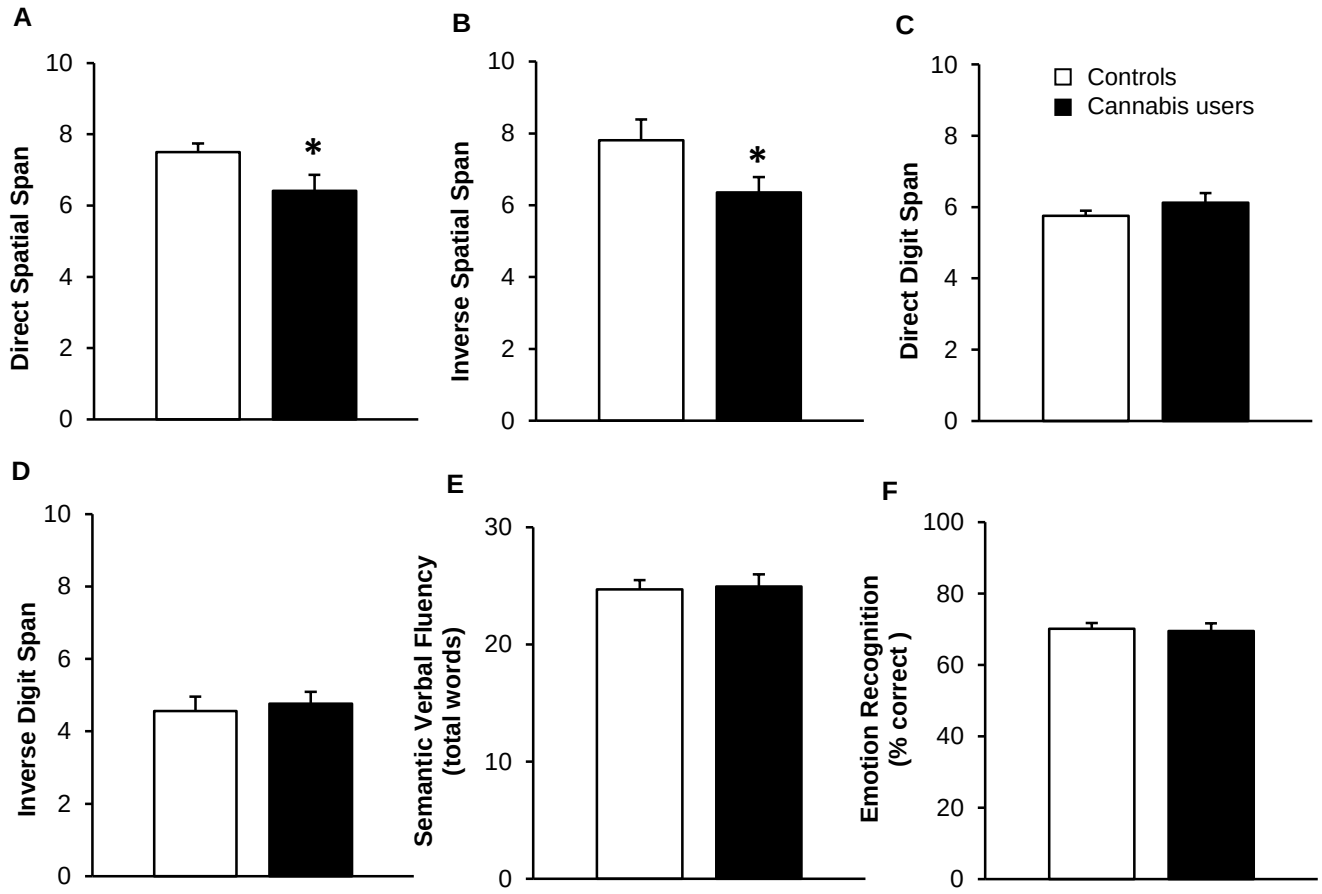

A
Direct Spatial Span
10
8
6
4
2
0
*
B
Inverse Spatial Span
10
8
6
4
2
0
*
C
Controls
Cannabis users
Direct Digit Span
10
8
6
4
2
0
D
Inverse Digit Span
10
8
6
4
2
0
E
Semantic Verbal Fluency (total words)
30
20
10
0
F
Emotion Recognition (% correct)
100
80
60
40
20
0

Figure 2

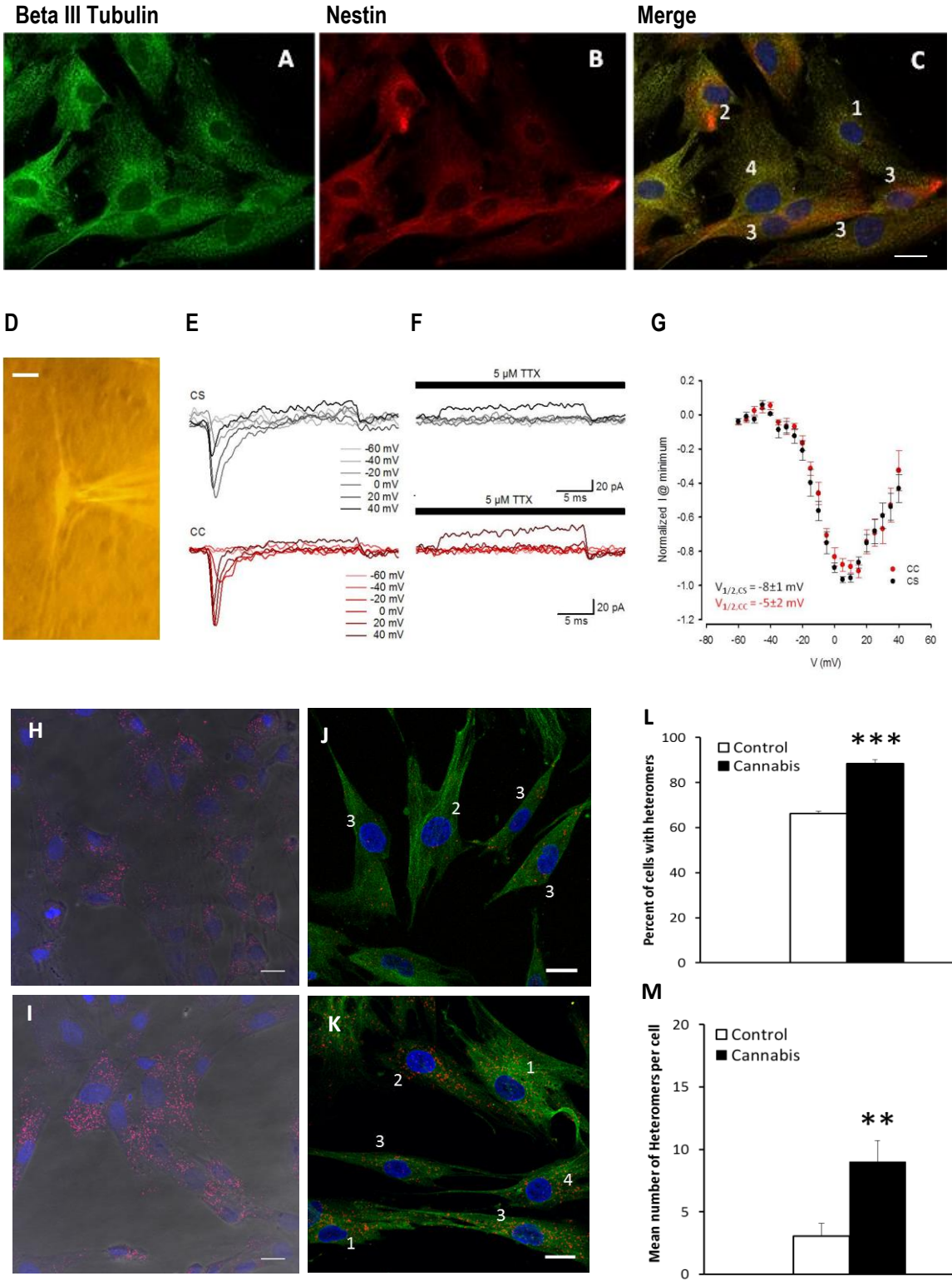
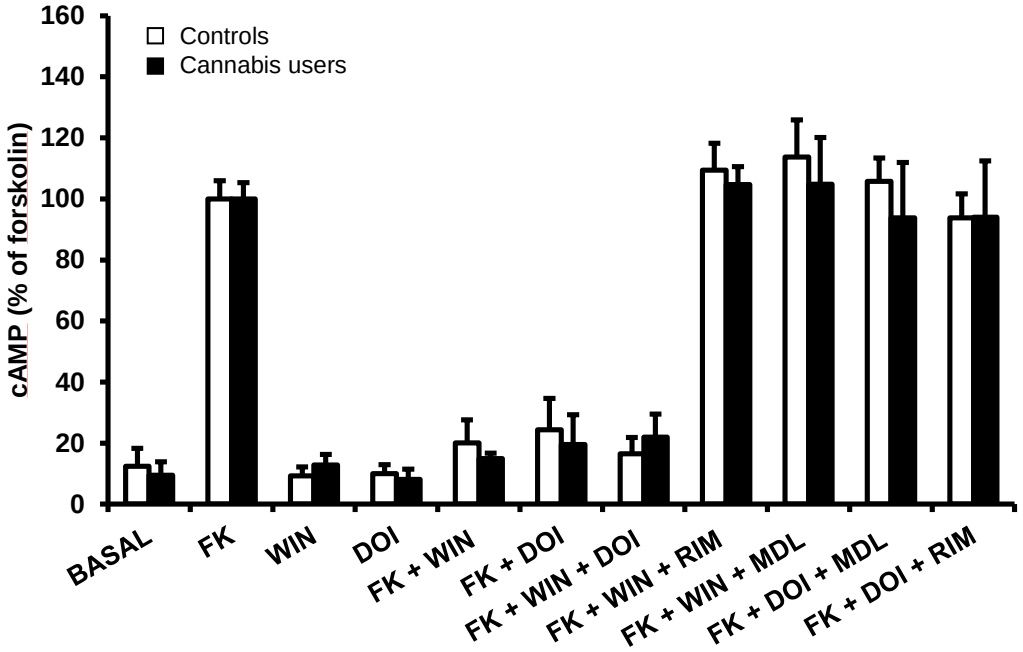

Beta III Tubulin
Nestin
Merge
A
B
C
1
2
3
4
D
E
F
G
CS
CC
-60 mV
-40 mV
-20 mV
0 mV
20 mV
40 mV
5 µM TTX
20 pA
5 ms
Normalized I @ minimum
V (mV)
$V_{1/2,CS}$ = -8±1 mV
$V_{1/2,CC}$ = -5±2 mV
H
I
J
K
L
M
Control
Cannabis
Percent of cells with heteromers
Mean number of Heteromers per cell
***
**

Figure 3

A


Controls
Cannabis users
cAMP (% of forskolin)
160
140
120
100
80
60
40
20
0
BASAL
FK
WIN
DOI
FK + WIN
FK + DOI
FK + WIN + DOI
FK + WIN + RIM
FK + WIN + MDL
FK + DOI + MDL
FK + DOI + RIM

B

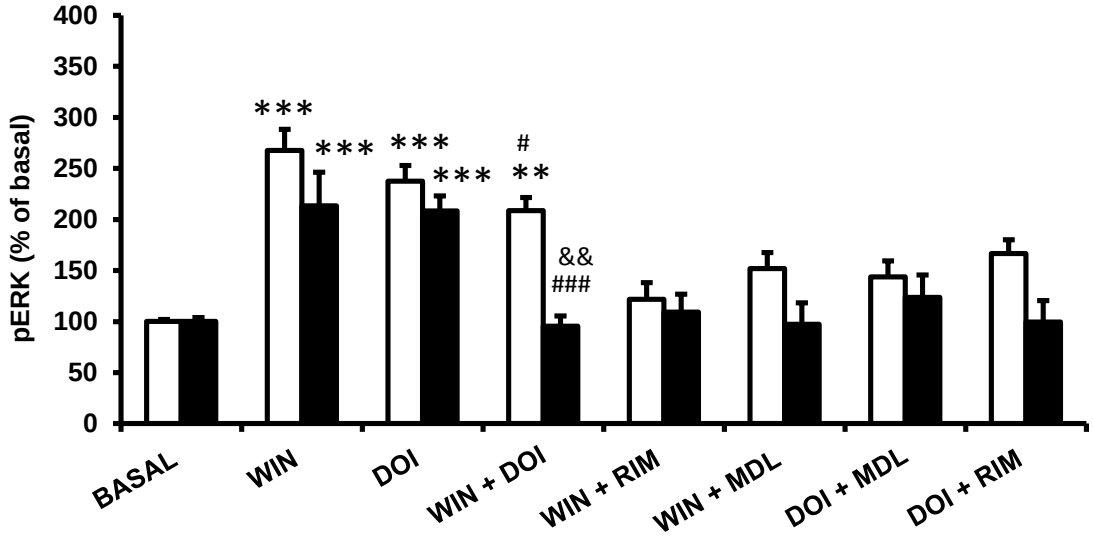
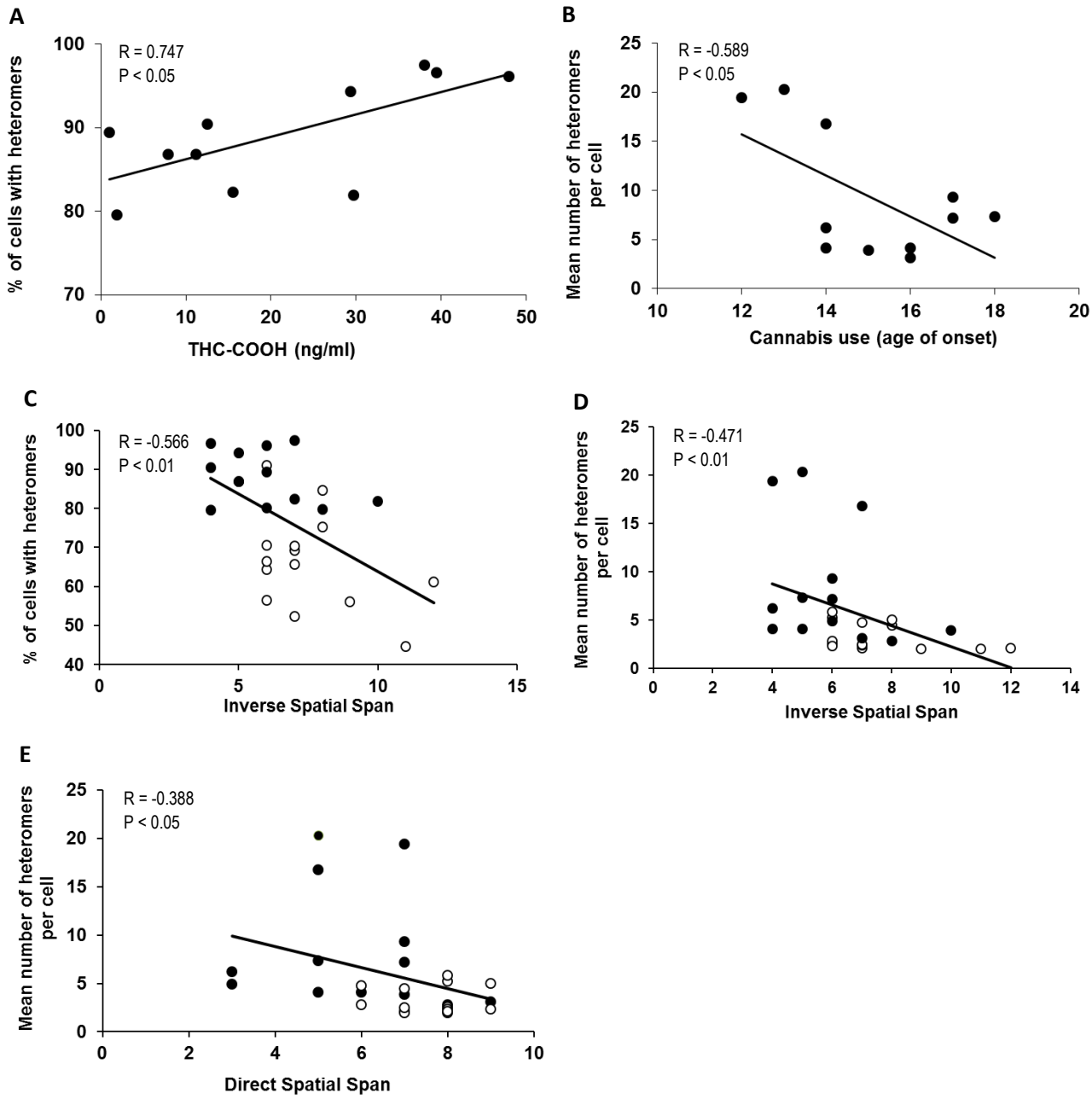

pERK (% of basal)
400
350
300
250
200
150
100
50
0
***
***
***
***
#
**
&&
###
BASAL
WIN
DOI
WIN + DOI
WIN + RIM
WIN + MDL
DOI + MDL
DOI + RIM

Figure 4


A
R = 0.747
P < 0.05
% of cells with heteromers
THC-COOH (ng/ml)
B
R = -0.589
P < 0.05
Mean number of heteromers per cell
Cannabis use (age of onset)
C
R = -0.566
P < 0.01
% of cells with heteromers
Inverse Spatial Span
D
R = -0.471
P < 0.01
Mean number of heteromers per cell
Inverse Spatial Span
E
R = -0.388
P < 0.05
Mean number of heteromers per cell
Direct Spatial Span

Supp. Fig 1

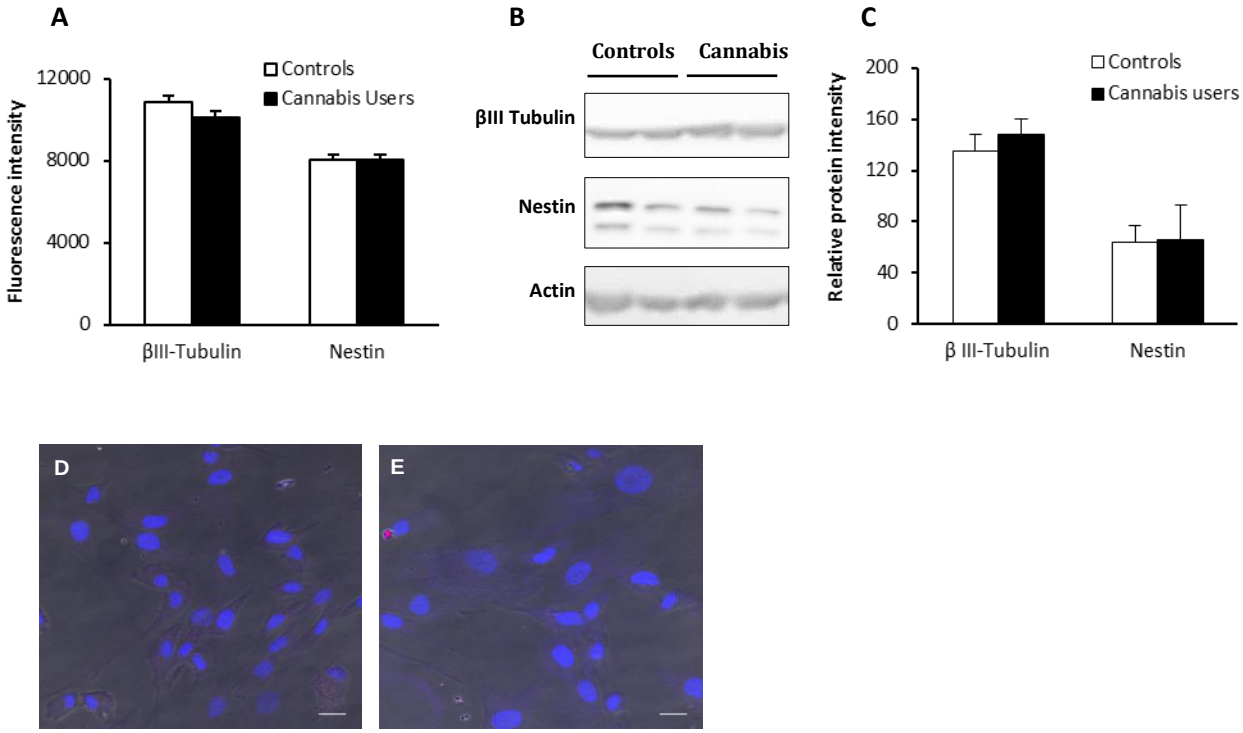

A
Fluorescence intensity
12000
8000
4000
0
Controls
Cannabis Users
βIII-Tubulin
Nestin
B
Controls
Cannabis
βIII Tubulin
Nestin
Actin
C
Relative protein intensity
200
160
120
80
40
0
Controls
Cannabis users
β III-Tubulin
Nestin
D
E

Table 1: Demographic and clinical characteristics of the sample.

	Controls (N = 16)	Cannabis Users (N = 17)
Age – years	29.38 ± 3.38	27.65 ± 7.13
Sex – no. (%)		
Male	10 (62.5)	13 (76.5)
Female	6 (37.5)	4 (23.5)
Socio-economic Status	41.94 ± 15.08	46.76 ± 11.71
Body Mass Index	23.29 ± 3.95	22.48 ± 4.79
Tobacco use		
Users – no. (%)	4 (25)	13 (76.5)*
Cigarettes per week	26.88 ± 51.63	31.29 ± 31.70
Use duration - years	3 ± 5.56	6.06 ± 7.58
Cannabis use		
Onset age – years	-	15.47 ± 1.97
Use duration – years	-	8.36 ± 6.45
Hamilton Depression Rating Scale	0.38 ± 0.71	0.82 ± 1.13
Global Assessment of Functioning (GAF)	99.38 ± 1.70	97.65 ± 4.37
Premorbid IQ	125.43 ± 18.85	123.54 ± 29
Neurological Soft Sings (NSS)	3.31 ± 1.85	4.24 ± 2.38

For continuous variables, results are shown as mean ± standard deviation and for categorical data results are represented as absolute frequency (percentage). *P<0.05

Supp. Table 1: Statistical Analysis comparing performance in neuropsychological tests between cannabis users and controls subjects.

Cognitive domain	Cognitive test	Mean Difference	95% C.I.	T-test (p)	Factorial ANOVA F(p)
Attention	Direct Digit Span	-0.368	-0.99 – 0.26	0.24	-
	Direct Spatial Span	1.088	0.04 – 2.13	0.042*	1.69 (0.192)
Working memory	Inverse Digit Span	-0.202	-1.24 – 0.84	0.696	-
	Inverse Spatial Span	1.46	0.01 – 2.90	0.048*	3.45 (0.029)*
Social cognition	Emotion Recognition Task	0.563	-4.97 – 6.10	0.837	-
Executive functions	Semantic Verbal Fluency	-0.254	-2.98 – 2.47	0.851	-

Initial T-test not including tobacco use in the analysis, followed by a two-way factorial analysis of variance (ANOVA) considering tobacco use as a fixed factor when significant differences were observed in the T-tests. Scores for the cognitive tests are standardized t-scores. * $P<0.05$

Supp. Table 2: Correlation coefficients between the standardized scores in the neuropsychological tests and CB1R-5-HT2AR heteromer expression in the ON.

Cognitive domain	Cognitive test	Model 1 (N=27)		Model 2 (N=27)	
		% of cells with heteromers	Mean number of heteromers per cell	% of cells with heteromers	Mean number of heteromers per cell
Attention	Direct Digit Span	0.398*	0.366	0.332	-
	Direct Spatial Span	-0.399*	-0.450*	-0.356	-0.388*
Working memory	Inverse Digit Span	-0.033	0.024	-	-
	Inverse Spatial Span	-0.536**	-0.425*	-0.566**	-0.471**
Social cognition	Emotion Recognition Task	0.128	-0.015	-	-
Executive functions	Semantic Verbal Fluency	-0.116	-0.095	-	-

In Model 1 the statistical values are for a simple correlation analysis, and in Model 2 the statistical values are for a partial correlation adjusted for tobacco use. * P<0.05; ** P<0.01

Supp. Table 3: Linear regression analyses between heteromer expression, cognitive performance and cannabis

Predictor	Dependent Variable	% of the total variance explained	
		Model 1 R square change (F)	Model 2 R square change (F)
THC-COOH plasma level [(a)]	% of cells with heteromers	40.8% (7.90)*	45.2% (5.123)*
	Mean number of heteromers per cell	27.0% (4.705)	21.7% (2.382)
Age of onset [(a)]	Mean number of heteromers per cell	29.4% (5.160)*	31.9% (3.345)
% of cells with heteromers [(b)]	Inverse Spatial Span	25.9% (10.103)**	26.9%(5.785)**
	Direct Spatial Span	12.5% (4.722)*	18.1% (3.871)*
Mean number of heteromers per cell [(b)]	Inverse Spatial Span	14.8% (5.50)*	16.3% (3.528)*
	Direct Spatial Span	15.2% (5.667)*	20.3% (4.316)*

Model 1 refers to a simple linear regression and Model 2 is a multiple linear regression taking into account tobacco use as a co-factor. [(a)] N=11; [(b)] N=27; * P<0.05; ** P<0.01.