CANNABINOID THERAPEUTICS: WHAT'S HOT

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Table of Contents

06 ***The Grass Might Be Greener: Medical Marijuana Patients Exhibit Altered Brain Activity and Improved Executive Function After 3 Months of Treatment***
Staci A. Gruber, Kelly A. Sagar, Mary K. Dahlgren, Atilla Gonenc Rosemary T. Smith, Ashley M. Lambros, Korine B. Cabrera and Scott E. Lukas

19 ***Cannabinoid Receptor Type 1 Expression in the Developing Avian Retina: Morphological and Functional Correlation With the Dopaminergic System***
Luzia da Silva Sampaio, Regina C. C. Kubrusly, Yolanda P. Colli, Priscila P. Trindade, Victor T. Ribeiro-Resende, Marcelo Einicker-Lamas, Roberto Paes-de-Carvalho, Patricia F. Gardino, Fernando G. de Mello and Ricardo A. De Melo Reis

29 ***Suppression of Cisplatin-Induced Vomiting by* Cannabis sativa *in Pigeons: Neurochemical Evidences***
Ihsan Ullah, Fazal Subhan, Javaid Alam, Muhammad Shahid and Muhammad Ayaz

39 ***Endocannabinoid System and Migraine Pain: An Update***
Rosaria Greco, Chiara Demartini, Anna M. Zanaboni, Daniele Piomelli and Cristina Tassorelli

46 ***Emerging Role of (Endo)Cannabinoids in Migraine***
Pinja Leimuranta, Leonard Khiroug and Rashid Giniatullin

53 ***Cannabidiol as a Promising Strategy to Treat and Prevent Movement Disorders?***
Fernanda F. Peres, Alvaro C. Lima, Jaime E. C. Hallak, José A. Crippa, Regina H. Silva and Vanessa C. Abílio

65 ***Cannabigerol Action at Cannabinoid CB_1 and CB_2 Receptors and at CB_1–CB_2 Heteroreceptor Complexes***
Gemma Navarro, Katia Varani, Irene Reyes-Resina, Verónica Sánchez de Medina, Rafael Rivas-Santisteban, Carolina Sánchez-Carnerero Callado, Fabrizio Vincenzi, Salvatore Casano, Carlos Ferreiro-Vera, Enric I. Canela, Pier Andrea Borea, Xavier Nadal and Rafael Franco

79 ***Chemical Proteomic Analysis of Serine Hydrolase Activity in Niemann-Pick Type C Mouse Brain***
Eva J. van Rooden, Annelot C. M. van Esbroeck, Marc P. Baggelaar, Hui Deng, Bogdan I. Florea, André R. A. Marques, Roelof Ottenhoff, Rolf G. Boot, Herman S. Overkleeft, Johannes M. F. G. Aerts and Mario van der Stelt

88 ***Long-Term Stress and Concomitant Marijuana Smoke Exposure Affect Physiology, Behavior and Adult Hippocampal Neurogenesis***
Kitti Rusznák, Kata Csekő, Zsófia Varga, Dávid Csabai, Ágnes Bóna, Mátyás Mayer, Zsolt Kozma, Zsuzsanna Helyes and Boldizsár Czéh

108 ***Cannabidiol as a Therapeutic Alternative for Post-traumatic Stress Disorder: From Bench Research to Confirmation in Human Trials***
Rafael M. Bitencourt and Reinaldo N. Takahashi

118 ***Inhibition of Diacylglycerol Lipase Impairs Fear Extinction in Mice***
Victoria S. Cavener, Andrew Gaulden, Dante Pennipede, Puja Jagasia, Jashim Uddin, Lawrence J. Marnett and Sachin Patel

128 ***Cannabidiol (CBD) is a Novel Inhibitor for Exosome and Microvesicle (EMV) Release in Cancer***
Uchini S. Kosgodage, Rhys Mould, Aine B. Henley, Alistair V. Nunn, Geoffrey W. Guy, E. L. Thomas, Jameel M. Inal, Jimmy D. Bell and Sigrun Lange

145 ***Self-Reported Effectiveness and Safety of Trokie® Lozenges: A Standardized Formulation for the Buccal Delivery of Cannabis Extracts***
Kenton Crowley, Sieta T. de Vries and Guillermo Moreno-Sanz

150 ***The Endocannabinoid/Cannabinoid Receptor 2 System Protects Against Cisplatin-Induced Hearing Loss***
Sumana Ghosh, Sandeep Sheth, Kelly Sheehan, Debashree Mukherjea, Asmita Dhukhwa, Vikrant Borse, Leonard P. Rybak and Vickram Ramkumar

165 ***Potential Clinical Benefits of CBD-Rich* Cannabis *Extracts Over Purified CBD in Treatment-Resistant Epilepsy: Observational Data Meta-analysis***
Fabricio A. Pamplona, Lorenzo Rolim da Silva and Ana Carolina Coan

174 ***Anandamide Effects in a Streptozotocin-Induced Alzheimer's Disease-Like Sporadic Dementia in Rats***
Daniel Moreira-Silva, Daniel C. Carrettiero, Adriele S. A. Oliveira, Samanta Rodrigues, Joyce dos Santos-Lopes, Paula M. Canas, Rodrigo A. Cunha, Maria C. Almeida and Tatiana L. Ferreira

188 ***Cannabis Therapeutics and the Future of Neurology***
Ethan B. Russo

199 ***Cannabis and the Anxiety of Fragmentation—A Systems Approach for Finding an Anxiolytic Cannabis Chemotype***
Brishna S. Kamal, Fatima Kamal and Daniel E. Lantela

213 ***Behavioral and Pharmacokinetic Profile of Indole-Derived Synthetic Cannabinoids JWH-073 and JWH-210 as Compared to the Phytocannabinoid Δ^9-THC in Rats***
Libor Uttl, Ewa Szczurowska, Kateřina Hájková, Rachel R. Horsley, Kristýna Štefková, Tomáš Hložek, Klára Šíchová, Marie Balíková, Martin Kuchař, Vincenzo Micale and Tomáš Páleníček

223 ***The Endocannabinoid System and Oligodendrocytes in Health and Disease***
Alexander A. Ilyasov, Carolanne E. Milligan, Emily P. Pharr and Allyn C. Howlett

233 ***Case Report: Clinical Outcome and Image Response of Two Patients With Secondary High-Grade Glioma Treated With Chemoradiation, PCV, and Cannabidiol***
Paula B. Dall'Stella, Marcos F. L. Docema, Marcos V. C. Maldaun, Olavo Feher and Carmen L. P. Lancellotti

240 ***Acute Cannabinoids Produce Robust Anxiety-Like and Locomotor Effects in Mice, but Long-Term Consequences are Age- and Sex-Dependent***
Chelsea R. Kasten, Yanping Zhang and Stephen L. Boehm II

258 ***The Cannabinoid CB1 Antagonist TM38837 With Limited Penetrance to the Brain Shows Reduced Fear-Promoting Effects in Mice***
Vincenzo Micale, Filippo Drago, Pia K. Noerregaard, Christian E. Elling and Carsten T. Wotjak

267 ***Effects of CBD-Enriched* Cannabis sativa *Extract on Autism Spectrum Disorder Symptoms: An Observational Study of 18 Participants Undergoing Compassionate Use***
Paulo Fleury-Teixeira, Fabio Viegas Caixeta, Leandro Cruz Ramires da Silva, Joaquim Pereira Brasil-Neto and Renato Malcher-Lopes

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The Grass Might Be Greener: Medical Marijuana Patients Exhibit Altered Brain Activity and Improved Executive Function after 3 Months of Treatment

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The vast majority of states have enacted full or partial medical marijuana (MMJ) programs, causing the number of patients seeking certification for MMJ use to increase dramatically in recent years. Despite increased use of MMJ across the nation, no studies thus far have examined the specific impact of MMJ on cognitive function and related brain activation. In the present study, MMJ patients seeking treatment for a variety of documented medical conditions were assessed prior to initiating MMJ treatment and after 3 months of treatment as part of a larger longitudinal study. In order to examine the effect of MMJ treatment on task-related brain activation, MMJ patients completed the Multi-Source Interference Test (MSIT) while undergoing functional magnetic resonance imaging (fMRI). We also collected data regarding conventional medication use, clinical state, and health-related measures at each visit. Following 3 months of treatment, MMJ patients demonstrated improved task performance accompanied by changes in brain activation patterns within the cingulate cortex and frontal regions. Interestingly, after MMJ treatment, brain activation patterns appeared more similar to those exhibited by healthy controls from previous studies than at pre-treatment, suggestive of a potential normalization of brain function relative to baseline. These findings suggest that MMJ use may result in different effects relative to recreational marijuana (MJ) use, as recreational consumers have been shown to exhibit decrements in task performance accompanied by altered brain activation. Moreover, patients in the current study also reported improvements in clinical state and health-related measures as well as notable decreases in prescription medication use, particularly opioids and benzodiapezines after 3 months of treatment. Further research is needed to clarify the specific neurobiologic impact, clinical efficacy, and unique effects of MMJ for a range of indications and how it compares to recreational MJ use.

Keywords: medical marijuana, cannabis, neuroimaging, fMRI, cognition, executive function, MSIT

INTRODUCTION

Currently, 30 states and the District of Columbia have medical marijuana (MMJ) programs or pending MMJ legislation, an additional 16 states have passed laws to allow limited access to MMJ, and an estimated 2.6 million individuals in the United States are certified for MMJ use (Procon.org). Since societal attitudes toward marijuana (MJ) have generally warmed, an increasing number of individuals are turning to MMJ to help treat a variety of medical conditions, as patients often do not achieve full symptom alleviation with conventional medications and experience unwanted side effects. Data gathered from several US surveys of MMJ patients indicate that the most common indications for MMJ use included pain-related concerns (i.e., chronic pain, headaches), psychiatric disorders (i.e., anxiety, depression), and insomnia (Nunberg et al., 2011; Reinarman et al., 2011; Bonn-Miller et al., 2014; Park and Wu, 2017). Although considerable research efforts have clarified the impact of recreational MJ use, particularly among adolescent and young adult populations, to date, there is a paucity of research focused on examining the impact of MMJ use on neurobiologic measures, including brain function and structure. As the number of MMJ patients continues to grow, research efforts designed to understand potential changes associated with MMJ use are critically important.

A large body of evidence from the past several decades suggests that recreational MJ use is related to cognitive decrements, including deficits in verbal memory (Tait et al., 2011; Auer et al., 2016; Shuster et al., 2016), processing speed (Fried et al., 2005; Lisdahl and Price, 2012; Jacobus et al., 2015), attention (Ehrenreich et al., 1999; Cousijn et al., 2013; Becker et al., 2014) and executive function (Crean et al., 2011; Gruber et al., 2012b; Solowij et al., 2012; Dougherty et al., 2013; Hanson et al., 2014; Jacobus et al., 2015; Dahlgren et al., 2016). While these deficits have been observed in adult MJ users (Nader and Sanchez, 2017), they are most salient among MJ-using adolescents (Lisdahl et al., 2014) who are in the midst of critical neurodevelopment (Giedd et al., 1999). Furthermore, these decrements have also been linked to alterations in brain structure and function. Although the directionality of structural alterations appears to be dependent on the brain region under investigation (Batalla et al., 2013), studies show that gray and white matter alterations are associated with increased executive dysfunction (Medina et al., 2009, 2010; Churchwell et al., 2010; Clark et al., 2012; Price et al., 2015). In addition, functional magnetic resonance imaging (fMRI) studies have reported altered activation patterns within the prefrontal cortex as well as orbitofrontal, cingulate, and subcortical/limbic regions of recreational MJ users compared to non-using control subjects during tasks of executive function (Lisdahl et al., 2014, for review). Further, similar to studies of cognitive performance and brain structure, fMRI studies have revealed that earlier onset of MJ use is related to altered patterns of brain activation during tasks requiring cognitive control and inhibition (Tapert et al., 2007; Gruber et al., 2012a; Sagar et al., 2015).

Although many have posited that MMJ use would be associated with similar deficits, preliminary studies have suggested that this may not be the case. In our own recent pilot investigation (Gruber et al., 2016), the only study to date to examine the impact of whole plant-derived MMJ products on cognitive performance, we found that MMJ patients did not demonstrate decrements in performance on measures of executive function following 3 months of MMJ treatment. In fact, patients generally demonstrated *improved* performance on a number of measures, particularly those assessing executive function. Improvements were also noted on several measures of quality of life, sleep, and depression relative to pre-MMJ treatment levels. Differences between recreational and MMJ users may be related to a variety of factors, including age of onset of MJ use, duration, magnitude and frequency of use, and choice of actual cannabis products used. Although products used by recreational MJ consumers and MMJ patients are derived from the same plant species, they are generally utilized for different purposes (i.e., to get high/alter one's current state of being vs. symptom alleviation). Accordingly, recreational and medical users often seek different MJ products with various constituent compositions based on the desired effect. Recreational MJ users often seek products high in Δ^9-tetrahydrocannabinol (THC), the main psychoactive constituent of the cannabis plant, and while medical patients may also choose products with high THC levels they often seek products high in other potentially therapeutic cannabinoids. Research has begun to focus on the beneficial effects of cannabidiol (CBD), the primary non-intoxicating constituent of MJ, which has been touted for its antipsychotic, anxiolytic, anti-seizure, and anti-inflammatory properties (Rong et al., 2017). While studies from recreational MJ users have reported a relationship between higher levels of THC and poorer cognitive performance (Ramaekers et al., 2006; Kowal et al., 2015) the acute administration of CBD prior to THC has been shown to *improve* cognitive function (Morgan et al., 2010; Englund et al., 2013), underscoring the need for further study. Moreover, Yücel et al. (2016) recently found that although MJ users exposed to THC exhibit alterations in hippocampal volume and neurochemistry, those who utilized CBD-containing products did not demonstrate differences relative to healthy controls. Similarly, a recent review of the effects of THC and CBD on neuroanatomy concluded that MJ users are prone to brain alterations in regions with high cannabinoid receptor density, and although THC exacerbates these alterations, CBD appears to protect against these deleterious changes (Lorenzetti et al., 2016). In addition, several researchers have administered pure THC or CBD to healthy control participants to investigate the impact of these constituents on brain activation patterns using fMRI. In general, studies suggest that THC and CBD have opposite effects on cognition-related brain activation (Bhattacharyya et al., 2010, 2015; Winton-Brown et al., 2011). This may be related to the fact that THC is a CB1 agonist with strong binding affinity for CB1 receptors, while CBD appears to exert effects through more indirect mechanisms, which include additional receptor types (Zuardi, 2008; Ashton and Moore, 2011). Despite preliminary work investigating the acute effects of pure THC and CBD on neural networks associated with cognitive domains impacted by MJ use, to our knowledge, no studies thus far have examined the impact of treatment with whole-plant-derived MMJ products on brain activation patterns.

In order to investigate whether pilot observations of improved executive function (Gruber et al., 2016) persist with larger sample sizes and to determine whether these changes co-occur with altered brain activation patterns, MMJ patients from an ongoing longitudinal study underwent fMRI while completing the Multi-Source Interference Test (MSIT). The MSIT is a robust measure of cognitive interference, a core facet of executive functioning, which is related to attentional control and inhibitory processing and requires actively shifting attention by inhibiting automatic responses (Lezak et al., 2004). This task reliably activates frontal brain regions associated with executive functioning, particularly the cingulo-frontal-parietal (CFP) network (Bush and Shin, 2006). Given our previous findings (Gruber et al., 2016), we hypothesized that following 3 months of treatment, MMJ patients would demonstrate improved task performance, and that this improvement would coincide with changes in brain activation patterns measured by fMRI. We have previously utilized the MSIT to better characterize patterns of cingulate and frontal brain activation within clinical and non-clinical cohorts (Gruber et al., 2012a, 2017), and although no studies thus far have examined MMJ patients using neuroimaging techniques, we hypothesized that improved MSIT task performance would be associated with increased activation in these regions following 3 months of MMJ treatment. We also posited that these changes would occur in the context of improved mood and quality of life ratings.

MATERIALS AND METHODS

Participants

To date, of 45 consented participants, 41 MMJ patients were enrolled and data from 22 patients' pre-treatment (Visit 1) and 3-month check-in visits (Visit 2) were available for analyses. In addition, patients who were free of MRI contraindications also completed neuroimaging procedures ($n = 15$). In order to qualify for study entry, patients had to be over the age of 18, and have an estimated IQ of 75 or higher as assessed by the Wechsler Abbreviated Scale of Intelligence (WASI; Wechsler, 1999). In order to minimize the effects of previous MJ exposure on study findings, patients were required to be MJ naïve or be abstinent from MJ use for at least 2 years for their pre-treatment visit. Patients were also required to be certified for MMJ use, or describe a plan to use industrial hemp derived products (which do not currently require certification). All subjects received payment for each study visit, and those who completed MRI procedures were compensated additionally in accordance with Partners IRB-approved protocol procedures.

Study Design

Prior to participation, all study procedures were explained, and each participant was required to provide written informed consent in accordance with the Declaration of Helsinki. This document and all study procedures were approved by the Partners Institutional Review Board. Eligible participants were enrolled in a larger longitudinal study designed to assess the impact of MMJ on cognition and brain function over the course of 12–24 months. Patients completed all assessments and imaging *prior* to initiation of MMJ treatment and again after 3 months of treatment.

As part of a larger neuroimaging protocol, participants completed the MSIT (Bush et al., 2003; Bush and Shin, 2006) with concurrent fMRI scanning using identical task parameters as reported in our previous studies of recreational MJ users, patients with bipolar disorder, and healthy controls (Gruber et al., 2012a, 2017). Using aspects from well-established measures of cognitive interference (e.g., Stroop, Simon, and Flanker tasks), the MSIT incorporates both spatial and flanker types of interference to measure cognitive control (Bush et al., 2003; Bush and Shin, 2006). During the task, three-digit stimuli sets comprised of the numbers 0, 1, 2, or 3 are presented briefly on a screen. Each set contains two identical distractor numbers and a target number that differs from the distractors. Using a button box, participants report the *identity* of the target number that differs from the two distractor numbers during two conditions: during the Control condition, distractor numbers are always zeros, and the identity of the target number always corresponds to its position on the button box (i.e., 100, 020, 003). During the Interference condition, patients are required to inhibit a prepotent response in favor of a less automatic response (i.e., indicate the identity of the target number rather its position). Distractor numbers are always numbers other than 0, and the identity of the target number is always incongruent with its position on the button box (e.g., 211, 232, 331, etc.). Performance is measured by reaction time and percent accuracy, which can be further subdivided by error type. Omission errors occur when no response is given and are typically reflective of slower or overloaded cognitive processing while commission errors, or incorrect responses, generally indicate difficulty inhibiting inappropriate responses. The entire task is comprised of four blocks of control trials alternating with four blocks of interference trials; the task begins and ends with a fixation period (30 s), making the total run time 6 min and 36 s (see **Figure 1** for graphic representation of the task design).

Patients also completed a battery of self-report rating scales. Briefly, these included the Profile of Mood States (POMS), Beck Depression Inventory (BDI), Beck Anxiety Inventory (BAI), Pittsburgh Sleep Quality Index (PSQI), Barratt Impulsiveness Scale (BIS-11), and the Short Form-36 Health Survey (SF-36), a measure of functional health and quality of life. During each study visit, participants also provided information regarding dose, frequency, and duration of use for all conventional medications, which were categorized into different classes, including opioids, antidepressants, mood stabilizers, and benzodiazepines. Percent change data was calculated to assess potential changes in medication use from pre- to post-3 months of MMJ treatment.

After completing pre-treatment assessments, patients began MMJ treatment at their discretion. Although patients selected their own products and determined their own treatment regimens, we collected detailed data about MMJ use patterns and products. Between study visits, patients submitted biweekly diaries documenting MMJ use and were contacted by phone on

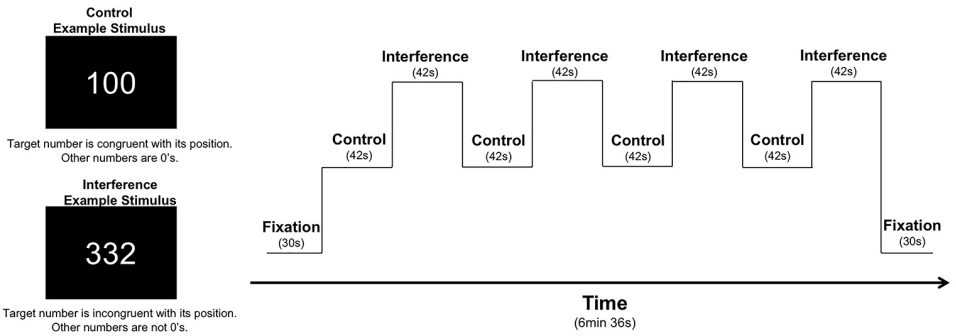


FIGURE 1 | MSIT task design: the MSIT begins and ends with a 30 s fixation. It contains eight total blocks, which alternate between Control (C) and Interference (I) conditions: C, I, C, I, C, I, C, I. Each block is 42 s long, containing 24 trials each, for a total of 384 total trails across the task. For each trial, stimuli are presented for 1.25 s with an interstimulus interval of 0.5 s, which yields a total run time of 6 min and 36 s.

a monthly basis to acquire information regarding MMJ product type, frequency, magnitude, and modes of use using a modified timeline follow-back procedure (TLFB; Sobell et al., 1998). Following a minimum of 3 months of regular MMJ treatment, patients returned for their first of several check in visits (Visit 2) where they repeated all study measures. In addition, participants were asked to provide a sample of their most frequently used MMJ product(s) to an outside laboratory (ProVerde Laboratories, Inc.) for cannabinoid constituent profiling. These analyses, which quantified the levels of 10 major cannabinoids including THC and CBD, will be used to identify the unique effects of specific cannabinoids in future analyses.

Statistical Analyses

Descriptive statistics were calculated for demographic and MMJ use variables. Repeated-measure analyses of variance (ANOVAs) were used to assess changes in clinical state from Visit 1 to Visit 2. The assumption of homogeneity of variance was confirmed using Levene's F; however, Shapiro–Wilk tests indicated that data for the MSIT were not normally distributed. Accordingly, non-parametric, repeated-measures Wilcoxon Signed Rank Tests were used to assess changes from Visit 1 to Visit 2 for MSIT data. It is of note, however, that the non-parametric tests resulted in similar findings as the ANOVAs; all significant results remained. For the MSIT analyses, alpha was set at 0.05 for the response time and percent accuracy variables. In cases where percent accuracy differed significantly between Visits 1 and 2, comparisons of the two different error types (omission, commission) utilized a Bonferroni correction for multiple comparisons ($\alpha/2 = 0.025$).

fMRI Methods and Analyses

All imaging was performed on a Siemens Trio whole body 3T MRI scanner (Siemens Corporation, Erlangen, Germany) using a 12-channel phased array head coil. For the MSIT, 40 contiguous coronal slices were acquired from each participant, ensuring whole brain coverage (5 mm thick, 0 mm skip), and images were collected with TR = 3000, using a single shot, gradient pulse echo sequence (TE = 30 ms, flip angle = 90, with a 20 cm field of view and a 64 × 64 acquisition matrix; in plane resolution 3.125 mm × 3.125 mm × 3.125 mm). A total of 132 images per slice were collected.

fMRI images were analyzed using SPM8 (version 4667, Wellcome Department of Imaging Neuroscience, University College, London, United Kingdom) software package running in MATLAB (version R2010b, MathWorks, Natick, MA, United States). First, blood-oxygen-level dependent (BOLD) fMRI data were corrected for slice timing and for motion in SPM8 using a two-step intra-run realignment algorithm that uses the mean image created after the first realignment as a reference. A criterion of 3 mm of head motion in any direction was used as an exclusionary criterion. The realigned images were then normalized to an EPI template in Montreal Neurological Institute stereotactic space using DARTEL. Normalized images were re-sampled into 3 mm^3 voxels and then spatially smoothed using an isotropic Gaussian kernel with 6 mm full width at half maximum. Global scaling was not used, high-pass temporal filtering with a cut-off of 168 s was applied, and serial autocorrelations were modeled with an AR(1) model in SPM8. Using a general linear model, statistical parametric images were calculated individually for each subject showing Interference > Control. These images were subsequently entered into second level model, subjected to a voxel-wise contrast and *t*-test to assess statistical significance. In addition to the realignment during the preprocessing, effects of motion were further corrected by removing motion related components from the data by including the calculated motion parameters from the realignment as regressors in the GLM (e.g., six nuisance regressors corresponding to three directions of translation and three axes of rotation). Regions of interest (ROI) masks were created using the Wake Forest University Pickatlas utility (Maldjian et al., 2003) and included cingulate and frontal regions. Specifically, the cingulate ROI was comprised of both bilateral anterior and mid cingulate regions (22,302 voxels) while the frontal ROI was comprised of bilateral superior frontal, middle frontal and inferior frontal gyri (6,857 voxels; see Supplementary Figure 1). These regions were selected as the cingulate (CC) and frontal cortices are associated with inhibitory processing and are reliably activated during the completion of the MSIT (Bush and Shin, 2006; Gruber et al., 2012a, 2017). Contrast analyses consisted of the subtraction of one map from

the other; for example, the cingulate activity of Visit 1 was subtracted from cingulate activity of Visit 2 to determine which areas showed increased activity over the course of treatment. As in previous studies (Heckers et al., 2004; Harrison et al., 2007; Yucel et al., 2007; Shin et al., 2011; Harding et al., 2012), the fixation point was not included in planned contrast analyses. The statistical threshold was set at $p < 0.05$ for cluster level family-wise-error (FWE), $p < 0.001$ for voxel level FWE with a minimum cluster extent $k = 15$ contiguous voxels in accordance with previously published manuscripts that have utilized the MSIT and have used a *k*-value of 15 (Gruber et al., 2012a) or lower (Harding et al., 2012; Bush et al., 2013). In addition to utilizing previously published *k*-values, we also conducted Monte Carlo simulations (Ward, 2000) to determine a more rigorous cluster extent for $p < 0.001$ which yielded $k = 91$. As two ROIs were used for analyses, data was also corrected for multiple comparisons, generating a new statistical threshold ($p < 0.0005$). One patient was excluded from MSIT analyses as they requested early termination of scanning procedures.

RESULTS

Demographics and MMJ Use

All patients (11 male, 11 female) were between the ages of 28–74 ($M = 50.64$, $SD = 13.15$) who reported seeking MMJ treatment for a variety of conditions including pain ($n = 13$), anxiety/PTSD ($n = 10$), sleep ($n = 10$), mood ($n = 8$), and "other" conditions ($n = 8$), which included gastrointestinal issues, difficulty with attention, and additional indications not specified by the state of Massachusetts. Patients in the current sample were generally well-educated; all had earned a high school diploma, many completed advanced education ($M = 15.91$ years, $SD = 1.97$), and all were of at least average intelligence as measured by the WASI ($M = 117.23$, $SD = 7.63$). Upon initiation of MMJ treatment, all patients reported at least weekly use, which ranged from 1.5 times per week to multiple times per day. As noted in **Table 1**, patients reported using MMJ products an average of 5.34 days per week and 1.83 times per day for an overall average of 10.26 total episodes of MMJ use per week. Patients also indicated various routes of administration, including smoking and vaporizing flower, as well as use of oil and concentrates (vaporized and oral administration), tinctures, edibles, and topicals.

TABLE 1 | Demographics and MMJ use.

Demographic variable ($n = 22$)	Mean (*SD*)
Age	50.64 (*13.15*)
Education (years)	15.91 (*1.93*)
WASI[a] Full Scale IQ	117.23 (*7.63*)
MMJ use[b]	
Days of MMJ use/week	5.34 (*1.99*)
Times/day used	1.83 (*1.02*)
Total MMJ use episodes/week	10.26 (*7.71*)
Mode of use	**Number of patients**
Smoke (flower)	8
Vaporize (flower)	9
Vaporize (oil/concentrates)	6
Oil/concentrates (non-smoked/vaporized)	5
Tincture	6
Edibles	7
Topicals	2

[a]*WASI, Wechsler Abbreviated Scale of Intelligence;* [b]*reflects average use from the start of regular treatment through Visit 2.*

MSIT Behavioral Performance

Relative to pre-treatment, patients demonstrated improved MSIT performance following 3 months of MMJ treatment (**Table 2**). During the Control condition, patients exhibited improved performance, marked by fewer omission errors; however, qualitative analyses revealed that patients approached near perfect levels of performance pre-and post-treatment for this condition. During the Interference condition, patients performed notably better at Visit 2, demonstrating significantly fewer omission and a trend for fewer commission errors, and thus significantly improved percent accuracy. In addition, MMJ patients also demonstrated faster response times during Visit 2, relative to Visit 1, across both Control and Interference trials.

MSIT fMRI Data

Interestingly, in addition to improved task performance, MMJ patients exhibited notable changes in brain activation patterns in terms of both magnitude and location from Visit 1 to Visit 2. Results are provided in **Table 3** which includes data for both the *a priori* threshold of $k = 15$ and also indicates which values survived the new threshold of $k = 91$ determined by the Monte Carlo simulations. After initiating MMJ treatment, patients generally exhibited increased activation within both the cingulate and frontal ROIs. Specifically, within the CC ROI, single-sample analyses revealed no significant activation at Visit 1, yet at Visit 2 patients exhibited focal activation within the midcingulate cortex ($k = 165$). Within-subjects contrast analyses between Visit 1 and Visit 2 revealed no significant activation differences for Visit 1 > Visit 2, but the Visit 2 > Visit 1 contrast indicated activation differences within the right anterior cingulate ($k = 43$). Within the frontal ROI, single-sample analyses revealed activation at Visit 1 within the left superior ($k = 65$) and the right inferior frontal gyrus ($k = 19$), and at Visit 2, within the right inferior ($k = 575$), left middle frontal gyrus ($k = 217$), and the left precentral gyrus ($k = 19$). Within-subjects contrast analyses between Visit 1 and Visit 2 yielded no significant activation differences for the Visit 1 > Visit 2 contrast; however, the Visit 2 > Visit 1 contrast revealed significant activation differences within the right middle gyrus ($k = 88$) and superior frontal gyrus ($k = 25$). See **Figure 2**.

Clinical Ratings and Conventional Medication Use

Following 3 months of MMJ treatment, patients reported some improvement on measures of mood and quality of life (**Table 4**). Across all rating scales, no significant worsening

of clinical state or quality of life was observed. Moreover, consistent with a previous report (Gruber et al., 2016), patients reported significant *improvements* on measures of depression (BDI), impulsivity (BIS-11), sleep (PSQI), and quality of life (SF-36). Specifically, on the SF-36, patients indicated significantly improved energy/fatigue and fewer role limitations due to physical health, which reflects how often patients' physical health affects their work and other life

TABLE 2 | Repeated measures Wilcoxon signed rank tests assessing Multi-Source Interference Test (MSIT) performance at pre-treatment and after 3 months of MMJ use (post-treatment).

MSIT variable	Visit 1 Pre-treatment Mean (*SD*)	Visit 2 Post-treatment Mean (*SD*)	Wilcoxon	
			Z	*p* (*r*)
Control condition				
Response time (ms)	608.90 (*97.20*)	582.62 (*64.97*)	2.062	**0.020 (0.500)***
Percent accuracy	97.40 (*2.57*)	98.82 (*1.74*)	2.282	**0.011 (0.553)***
Omission errors[a]	1.73 (*2.25*)	0.68 (*1.09*)	1.974	**0.024 (0.479)***
Commission errors[a]	0.77 (*0.97*)	0.46 (*0.86*)	1.461	0.072 (0.354)
Interference condition				
Response time (ms)	914.23 (*76.56*)	886.62 (*82.76*)	2.743	**0.003 (0.665)***
Percent accuracy	79.03 (*18.87*)	86.55 (*11.88*)	2.858	**0.002 (0.693)***
Omission errors[a]	11.96 (*12.01*)	7.27 (*7.92*)	2.750	**0.003 (0.667)***
Commission errors[a]	8.18 (*9.11*)	5.77 (*5.57*)	1.718	*0.043* (*0.417*)^

df = 1,21; [a]*corrected for multiple comparisons using Bonferroni method; *results significant at p ≤ 0.05 when α = 0.05 or, for Bonferroni corrected analyses, at p ≤ 0.025 when α = 0.025; ^results trending toward significance at p ≤ 0.10 when α = 0.05 or, for Bonferroni corrected analyses, at p ≤ 0.05 when α = 0.025.*

TABLE 3 | Multi-Source Interference Task (Interference-Control condition): activation local maxima within cingulate cortex (CC) and frontal cortex regions of interest (ROIs).

ROI **Visit** Region	Cluster size (voxels)	*x*	*y*	*z*	SPM {t}	Voxel *p* (FWE - corrected)
CC						
Visit 1						
No activation $k \geq 15$	–	–	–	–	–	–
Visit 2						
Right middle cingulate cortex	**150**	6	12	45	8.45	<0.0005
Right anterior cingulate cortex	15	12	36	15	5.18	<0.0005
Visit 1 > Visit 2						
No activation $k \geq 15$	–	–	–	–	–	–
Visit 2 > Visit 1						
Right anterior cingulate cortex	20	3	18	24	5.41	<0.0005
Right anterior cingulate cortex	23	9	30	18	5.21	<0.0005
FRONTAL						
Visit 1						
Left superior frontal gyrus	65	−30	−3	69	9.51	<0.0005
Right inferior frontal gyrus (*p. triangularis*)	19	45	12	24	8.48	<0.0005
Visit 2						
Right inferior frontal gyrus (*p. opercularis*)	**575**	42	9	27	10.75	<0.0005
Left middle frontal gyrus	**217**	−24	−6	51	10.08	<0.0005
Left precentral gyrus	19	−45	9	36	7.87	<0.0005
Visit 1 > Visit 2						
No activation $k \geq 15$	–	–	–	–	–	–
Visit 2 > Visit 1						
Right middle frontal gyrus	88	48	9	54	6.94	<0.0005
Right superior frontal gyrus	25	24	12	66	6.58	<0.0005

The statistical threshold was initially set at p < 0.05 for cluster level family-wise-error (FWE), and p < 0.0005 for voxel level FWE (corrected for multiple comparisons) with a minimum cluster extent k = 15 contiguous voxels. Bolded results indicate values that survived the Monte Carlo simulation minimum cluster extent (k = 91).

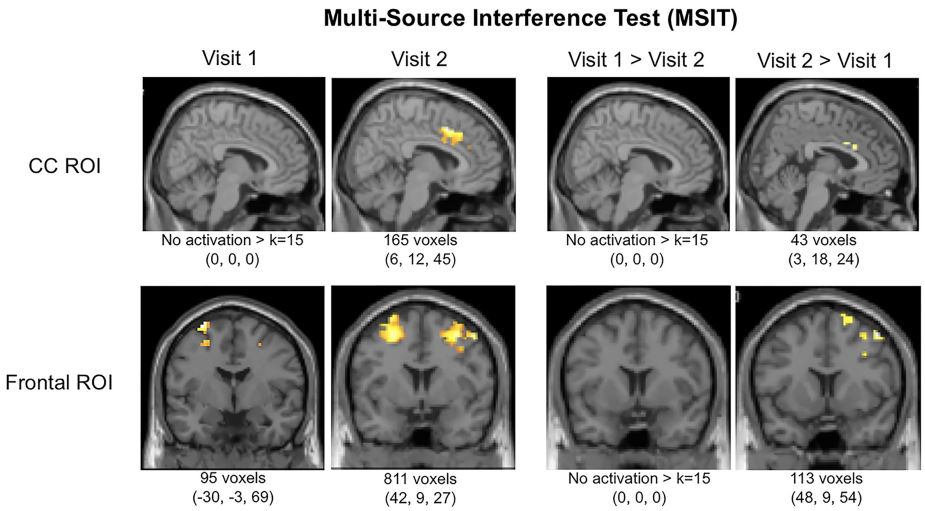


FIGURE 2 | Functional magnetic resonance imaging (fMRI) activation in cingulate cortex (CC) and frontal regions of interest (ROIs) during the MSIT (Interference-Control). Local maxima and total k (voxels activated within ROIs per contrast) are displayed below images.

activities. A trend also emerged suggesting improved social functioning.

In addition to improvements in clinical state and quality of life, following 3 months of MMJ treatment, patients reported reductions in their use of conventional pharmaceutical products across several drug classes. Specifically, patients taking opioids reported a 47.69% reduction in use and those prescribed benzodiazepines reported a 46.91% reduction in use. Antidepressant use decreased by 22.35% while the use of mood stabilizers decreased by 28.57% between Visit 1 and Visit 2.

DISCUSSION

Following 3 months of MMJ treatment, patients exhibited improved task performance and related alterations in frontal brain activation patterns during the completion of the MSIT, a measure of executive function and cognitive control, relative to pre-MMJ treatment. Within the cingulate cortex (CC), patients did not exhibit any significant pre-treatment activation during the Interference condition of the MSIT; however, after 3 months of treatment, robust activation was noted within this region. In fact, the magnitude of activation significantly increased over the course of treatment such that post-treatment activation patterns appeared more similar to that of healthy controls observed in previous studies (Bush and Shin, 2006; Gruber et al., 2012a). Activation within the frontal ROI was also notably increased following 3 months of MMJ treatment relative to pre-MMJ treatment. Taken together, these changes may be reflective of a potential "normalization" of brain function following 3 months of MMJ use.

Further, changes in brain activation patterns were observed in the context of improved task performance and self-reported improvements in mood and quality of life as well as reduced sleep disturbance and lower motor impulsivity, consistent with previously published preliminary data (Gruber et al., 2016). It is possible that improvements in symptomatology (i.e., relief of symptoms, improved mood/sleep) are directly related to observed improvements in cognitive function and alterations in brain activation. Patients in the present study most commonly endorsed pain and anxiety as their reasons for MMJ certification; both of these conditions have previously been associated with reduced cognitive performance (Moriarty et al., 2011; Vytal et al., 2013). Symptom improvement may therefore result in improved cognitive performance, and subsequently impact patterns of brain activation during completion of these tasks.

In addition to reduced symptomatology resulting in improved cognitive performance, it is also possible that several other factors may have also contributed to the observed changes. Patients reported notable decreases in their use of opioids, benzodiazepines, antidepressants, and mood stabilizers, and it is possible that reductions in conventional medications influenced changes in brain activation patterns. In fact, several studies have shown that mood stabilizers, benzodiazepines, and antidepressants generally attenuate activation (Bell et al., 2005; Del-Ben et al., 2005; Paulus et al., 2005; Arce et al., 2008; Murphy et al., 2009). In particular, Bell et al. (2005) reported that use of mood stabilizers is related to hypoactivation of the CFP network, a key region implicated in cognitive interference processing. While we are not aware of fMRI research focused on the effects of short-term prescriptive doses of opioids in humans, one study examining opioid dependence reported that normalization of frontal brain activation patterns was related to days since last drug use (Bunce et al., 2015). Reduction or cessation of use of these medications may therefore alter patterns of brain activation. Accordingly, future studies are needed to disentangle the effects

TABLE 4 | Mood and health ratings at pre-treatment and after 3 months of MMJ use (post-treatment).

Rating scale	Visit 1 Pre-treatment Mean (*SD*)	Visit 2 Post-treatment Mean (*SD*)	ANOVA	
			F	*p* (η^2)
Clinical ratings				
Profile of Mood States (POMS)[a]				
Vigor	16.86 (*6.19*)	16.14 (*6.53*)	0.543	0.235 (0.025)
Anger	8.68 (*10.33*)	8.59 (*9.21*)	0.003	0.477 (<0.001)
Confusion	8.00 (*6.38*)	6.73 (*4.92*)	*2.748*	*0.056 (0.116)^*
Tension	12.59 (*9.96*)	12.05 (*9.97*)	0.153	0.350 (0.007)
Fatigue	9.91 (*7.29*)	8.77 (*7.24*)	*1.946*	*0.089 (0.085)^*
Depression	13.18 (*16.78*))	14.14 (*16.95*)	0.217	0.323 (0.010)
TMD	35.50 (*51.08*)	34.14 (*48.89*)	0.055	0.409 (0.003)
Beck Depression Inventory (BDI)[a]				
Total	13.77 (*12.60*)	9.73 (*11.65*)	***9.559***	***0.003 (0.313)****
Beck Anxiety Inventory (BAI)[a]				
Total	10.55 (*10.32*)	9.73 (*9.93*)	0.227	0.319 (0.011)
Impulsivity				
Barratt Impulsiveness Scale (BIS-11)[a]				
Attention	16.59 (*5.77*)	16.59 (*5.47*)	0.000	0.500 (0.000)
Motor	23.00 (*5.43*)	21.23 (*5.37*)	***12.531***	***0.001 (0.374)****
Non-planning	23.41 (*5.53*)	23.59 (*5.50*)	0.077	0.392 (0.004)
Total	63.00 (*15.08*)	61.41 (*14.64*)	1.626	0.108 (0.072)
Health and quality of life ratings				
Pittsburgh Sleep Quality Index (PSQI)[b]				
Total	8.26 (*4.46*)	6.05 (*3.26*)	***7.167***	***0.008 (0.285)****
SF-36[a]				
Physical functioning	71.59 (*21.40*)	71.82 (*26.03*)	0.002	0.483 (<0.001)
Role limitations (physical)	44.32 (*43.60*)	56.82 (*44.44*)	***3.915***	***0.031 (0.157)****
Role limitations (emotional)	63.64 (*43.53*)	60.61 (*45.58*)	0.096	0.380 (0.005)
Energy/fatigue	42.73 (*25.39*)	51.36 (*21.67*)	***10.738***	***0.002 (0.338)****
Emotional well-being	67.64 (*26.18*)	65.82 (*27.32*)	0.827	0.187 (0.038)
Social functioning	62.50 (*29.12*)	68.75 (*29.06*)	*1.819*	*0.096 (0.080)^*
Pain	52.73 (*2.05*)	56.59 (*26.06*)	0.918	0.174 (0.042)
General health	57.50 (*19.75*)	60.91 (*19.56*)	1.668	0.105 (0.074)

Clinical rating scales (POMS, BDI, BAI), lower scores reflect lower levels of clinical symptoms; BIS-11, lower scores indicate lower levels of self-reported impulsivity; PSQI, lower scores reflect improved sleep quality; SF-36, higher scores indicate higher quality of life. [a]*df = 1,21;* [b]*df = 1,18; *bolded results are significant at p ≤ 0.05; ^italicized results approach significance at p ≤ 0.10.*

of MMJ treatment and conventional medication use on brain activation patterns, and may benefit from limiting clinical samples to only those on a single specific class of conventional medication.

Although findings from this study indicate *improvements* in cognitive task performance and more normalized patterns of brain activation after 3 months of MMJ treatment, previous studies, exclusively focused on recreational MJ users, have reported *decrements* in cognitive performance and accompanying atypical neural alterations. A recent review highlighting neuroimaging findings in recreational MJ users found evidence for altered frontal neural function during completion of executive function tasks (Weinstein et al., 2016), a finding observed in our own previous research (e.g., Gruber and Yurgelun-Todd, 2005; Gruber et al., 2012a; Sagar et al., 2015). A number of critical factors may account for the differences between our current findings in MMJ patients relative to findings from recreational MJ consumers. The majority of studies of recreational MJ use have included adolescent and young adult populations. Overwhelmingly, studies have demonstrated that early/adolescent onset of recreational MJ use is related to poorer task performance and changes in brain structure and function (Lisdahl et al., 2013, 2014; Jacobus and Tapert, 2014; Levine et al., 2017). Given that participants in the current sample are adults (*Mean age* = 50.64) who are well-beyond the critical stages of neurodevelopment (Giedd et al., 1999), they are likely less vulnerable to the adverse neural effects of THC. Interestingly, recent preclinical evidence indicates that THC may have the potential to *improve* cognition in older individuals (Bilkei-Gorzo et al., 2017). Mature and old mice administered low doses of THC demonstrated a reversal of age-related cognitive decline, hypothesized to be related to upregulation of the aging endocannabinoid system via increased signaling secondary to low dose THC exposure. Moreover,

the same exposure resulted in cognitive decrements among young mice. Additional research is needed to more fully understand the mechanisms underlying these improvements and to examine the impact of cannabis and cannabinoids in older adult populations as well as the effects of low doses of THC, as these factors likely influence the impact of MJ use.

It is also important to consider patterns of MJ use, including frequency and duration of use, in order to understand potential reasons for the different outcomes among recreational users and medical patients. In the current study, all patients reported using MMJ at least weekly; on average, they reported using 5 days per week and 1–2 times per day. Traditionally, studies of recreational users have examined chronic, heavy use; although criteria for "heavy use" can vary across investigations, most studies have required participants to use MJ at least 1–4 days per week (Tait et al., 2011; Gruber et al., 2012a; Macher and Earleywine, 2012; Dougherty et al., 2013; Cousijn et al., 2014), similar to the frequency of use among the current sample of medical patients. Given these similarities, it is unlikely that differences between recreational and MMJ patients are solely attributable to frequency of use. Additionally, studies of recreational MJ users typically include consumers with a longer duration of MJ use relative to the current sample of MMJ patients, and differences in cumulative exposure should also be considered. For this reason, our ongoing study is designed to examine MMJ users after increasingly longer periods of use to explore the impact of longer durations of MMJ use on cognitive function.

Further, MMJ patients and recreational MJ users also typically differ in terms of the products they use. Recreational MJ products are often prized for high THC levels, and the goal of the recreational consumer is to change their current state of being or to 'get high.' MMJ patients seek symptom alleviation and tend to choose products with rich and varied cannabinoid profiles including constituents other than THC, which may also impact clinical state, cognitive processing and other domains. For example, CBD, which has been touted for its clinical benefits (Rong et al., 2017), has demonstrated efficacy in mitigating the negative cognitive effects of THC (Yücel et al., 2016) and appears to exert opposite effects on task-related brain activation relative to THC (Colizzi and Bhattacharyya, 2017). In addition, although there is a paucity of research in this area, some studies have examined the direct impact of acute CBD administration on cognitive performance. Englund et al. (2013) reported that administration of CBD prior to the administration of THC resulted in better episodic memory relative to placebo pre-treatment in healthy controls. Morgan et al. (2010) examined verbal memory performance in current recreational MJ users and found that those using products without CBD (confirmed by hair sample analysis) performed more poorly on verbal memory measures than those with detectable levels of CBD. While no studies have examined the impact of whole plant-derived MMJ products or assessed the long-term impact of MMJ treatment, some studies have utilized fMRI techniques to examine the acute effects of individual cannabinoids. Borgwardt et al. (2008) studied the acute impact of THC, CBD, and placebo on executive function in healthy controls using a Go/No go task. Although the authors did not report any performance differences between cannabinoids or placebo, fMRI data demonstrated that THC reduced activation in frontal and anterior cingulate regions, while CBD reduced activation in temporal and insular regions relative to placebo. In addition, Bhattacharyya et al. (2010) found that intravenous administration of THC and CBD resulted in opposite effects on brain activation patterns across multiple regions during the completion of memory, inhibitory function, and affective measures. Given these findings, data from the present study may reflect the direct neurobiologic effects of cannabinoids, as increased endocannabinoid signaling is associated with improved cognition (Egerton et al., 2006), reduced stress response, emotional regulation, and increased endogenous reward signaling (Hill and McEwen, 2010; Befort, 2015), and as previously noted, specific alterations in brain activation patterns (Borgwardt et al., 2008; Bhattacharyya et al., 2010). Results may also reflect the indirect impact of whole plant-derived products, which include an array of cannabinoid constituents, and may exert downstream affects on multiple receptor types and neural systems (i.e., pain and reward circuitry). While THC and CBD are generally the most abundant cannabinoids in patients' products, and as noted, several studies have begun to explore their impact on cognition and brain activation patterns, a number of other cannabinoids including cannabigerol (CBG), cannabinol (CBN), cannabichromene (CBC), and tetrahydrocannabidivarin (THCV), are often present in MMJ products, and may have moderated or indirectly affected the impact typically associated with THC exposure (Englund et al., 2016). Further research is clearly indicated for assessing the specific impact of individual cannabinoids on cognitive and clinical variables in patients using cannabis for medical purposes.

Limitations and Future Directions

Despite the compelling nature of the study findings, several limitations must be noted. First, the current investigation is designed as an observational, longitudinal pre–post study in which patients choose their own products and treatment regimen. The ability to assess the impact of whole plant-derived cannabis-based products is more ecologically valid than studies involving synthetic or non-plant derived products; however, the current legal landscape prohibits the use of dispensary-based products within a clinical trial model and allows only the use of products supplied by the National Institute on Drug Abuse (NIDA). While NIDA's drug supply program has expanded their portfolio of MJ products available for research, their supply does not currently include the range and scope of products (i.e., product type, potency, constituent profiles, etc.) that patients are seeking and obtaining through dispensaries and caregivers across the nation. Accordingly, as a clinical trial model could not be utilized, the present study collected comprehensive data on product source, selection, dose, frequency, and mode of use. Further, as previously noted, patients also provided a sample of their most frequently

used MMJ products for cannabinoid constituent profiling. Interestingly, 13 of the 22 patients (59%) in the current study were identified as taking products high in CBD which may have contributed to study findings given previous data highlighting its clinical benefits (McGuire et al., 2017; Rong et al., 2017). As initial laboratory analyses revealed a range of cannabinoid constituents from patients' products, additional analyses will be conducted to examine the impact of specific cannabinoids and their relationship with cognitive and clinical variables. Further, future studies will assess potential differences between patients who choose products high in THC compared to those using products high in CBD, as these data may provide critical information regarding efficacy of individual constituents and combinations of constituents for specific indications and conditions to better inform selection of MMJ products.

The current study utilized a pre–post, within-subjects design in which all patients are MJ naïve at Visit 1 and are followed over the course of 12–24 months in order to clarify the impact of prolonged duration of exposure to MMJ treatment. As results from the current study represent only data from baseline and patients' first check-in visit after 3 months of MMJ treatment, data must be considered preliminary. Additional analyses, which are planned for the future, are needed to understand the impact of MMJ over longer treatment periods. Further, as a result of the pre–post study design, repeated administration of cognitive measures was required, and thus practice effects cannot be completely ruled out. Although no studies to date have specifically examined practice effects for the MSIT, given the lengthy duration of time between visits (at least 3 months) and the computerized nature of the task, it is highly unlikely that practice effects would persist. In addition, given the longitudinal nature of the design, each subject's baseline assessment serves as their own control from which to assess change after initiation of MMJ treatment. It could, however, prove beneficial for future investigations to also recruit a control group of patients who report similar symptoms (i.e., pain, insomnia, anxiety, etc.) but who do *not* choose to utilize MMJ. Comparing outcomes of MMJ patients and "treatment as usual" patients over time could strengthen findings if MMJ patients display more positive outcomes relative to those who do not use MMJ but suffer from similar symptoms or conditions.

In addition, statistical thresholds for fMRI analyses were set in accordance with previous investigations (Shin et al., 2011; Gruber et al., 2012a; Harding et al., 2012) in order to aid in the interpretation of findings. While more stringent thresholds were also applied and are noted within the results, it is important to recognize that some findings did not survive the more rigorous thresholds, a common issue in fMRI studies with limited sample sizes.

In order to gain a more thorough understanding of how MMJ impacts cognitive functioning, it will be important for future studies to replicate current study findings using other measures of executive function and to examine additional cognitive domains. As executive functioning has been shown to be impacted by recreational MJ use (for review, Crean et al., 2011), this domain was targeted for the current investigation; however, it is crucial to examine additional cognitive variables, including verbal memory, which has also been shown to be sensitive to MJ use (for review, Solowij and Battisti, 2008; Broyd et al., 2016).

Finally, this study included MMJ patients using products for a variety of indications, which resulted in a varied clinical sample. While this approach provides a broad assessment of the impact of MMJ, it is likely that individual conditions and symptoms will have unique patterns of responses associated with MMJ treatment. A number of medical and psychiatric conditions have been shown to negatively impact cognitive processing; accordingly, future studies may derive additional power by limiting inclusion to patients with a single condition or indication (i.e., patients using MMJ exclusively for pain) or including only patients taking medications from a single drug class.

CONCLUSION

To our knowledge, this study represents the first neuroimaging investigation of patients using marijuana for medical purposes. Following 3 months of MMJ treatment, brain activation patterns appear more similar to those exhibited by healthy controls from previous studies than at pre-treatment. This finding provides strong evidence that MMJ treatment may normalize brain activity. Importantly, these changes were accompanied by improved task performance as well as positive changes in ratings of clinical state, impulsivity, sleep, and quality of life. Further, patients reported notable decreases in their use of conventional medications, including opioids. In light of the national opioid epidemic, these data clearly underscore the need to expand and extend this study to determine if a reduction in opioid use persists with continued MMJ treatment. Results from the current study raise the possibility that the observed improvements in cognition and related changes in functional activation patterns may be related to direct and/or indirect effects of cannabinoids, specifically within an adult population beyond the stages of critical neuromaturation. Patients utilizing MMJ appear to use products with different cannabinoid profiles (i.e., high CBD) relative to recreational users, which is also likely to impact cognitive function. Observed changes may also be related to secondary or more indirect effects, including the reduction of clinical symptoms, improved sleep, and decreased use of conventional medications. Additional studies using both observational and clinical trial models to examine the impact of actual MMJ products used by patients are needed to clarify the underlying neural mechanisms associated with clinical and behavioral changes that accompany MMJ treatment.

AUTHOR CONTRIBUTIONS

SG conceptualized and designed the current study in consultation with SL. KS assisted SG in manuscript preparation with

additional help provided by the remaining authors. SG, KS, RS, AL, and KC recruited the patients, carried out the study procedures and administered the neuropsychological and clinical assessments. MD completed the statistical analyses and AG completed the neuroimaging analyses.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2017.00983/full#supplementary-material

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frontiers in Cellular Neuroscience

ORIGINAL RESEARCH
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Cannabinoid Receptor Type 1 Expression in the Developing Avian Retina: Morphological and Functional Correlation With the Dopaminergic System

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The avian retina has been used as a model to study signaling by different neuro- and gliotransmitters. It is unclear how dopaminergic and cannabinoid systems are related in the retina. Here we studied the expression of type 1 and 2 cannabinoid receptors (CB_1 and CB_2), as well as monoacylglycerol lipase (MAGL), the enzyme that degrades 2-arachidonoylglycerol (2-AG), during retina development. Our data show that CB_1 receptor is highly expressed from embryonic day 5 (E5) until post hatched day 7 (PE7), decreasing its levels throughout development. CB_1 is densely found in the ganglion cell layer (GCL) and inner plexiform layer (IPL). CB_2 receptor was also found from E5 until PE7 with a decrease in its contents from E9 afterwards. CB_2 was mainly present in the lamination of the IPL at PE7. MAGL is expressed in all retinal layers, mainly in the IPL and OPL from E9 to PE7 retina. CB_1 and CB_2 were found both in neurons and glia cells, but MAGL was only expressed in Müller glia. Older retinas (PE7) show CB_1 positive cells mainly in the INL and co-expression of CB_1 and tyrosine hydroxylase (TH) are shown in a few cells when both systems are mature. CB_1 co-localized with TH and was heavily associated to D_1 receptor labeling in primary cell cultures. Finally, cyclic AMP (cAMP) was activated by the selective D_1 agonist SKF38393, and inhibited when cultures were treated with WIN55, 212–2 (WIN) in a CB_1 dependent manner. The results suggest a correlation between the endocannabinoid and dopaminergic systems (DSs) during the avian retina development. Activation of CB_1 limits cAMP accumulation via D_1 receptor activation and may influence embryological parameters during avian retina differentiation.

Keywords: cannabinoid, retina, development, dopamine, cAMP

INTRODUCTION

The retina is a unique tissue located in the posterior part of the eye involved with light transduction in visual information. The vertebrate retina, easily accessible to experimental manipulation, formed by seven major cell types, reviewed in Masland (2012), is extensively used as an experimental model to investigate cell interactions. Neurons and glia cells interact bidirectionally, and the avian retina has been used in the last 40 years, as a model to study the role of neuro-glia interactions in cell migration and development (Reis et al., 2007; de Melo Reis et al., 2008).

Dopamine is the main catecholamine found in a subtype of retinal amacrine cells located in the inner nuclear layer (INL; Reis et al., 2007). Emergence of amacrine cells begins at early stages (around embryonic day 3, E3) and is concluded at E9 (Prada et al., 1991; Calaza Kda and Gardino, 2010). Tyrosine hydroxylase (TH) expression is only found around E12, while D_1/D_5 type dopaminergic receptors coupled to cyclic AMP (cAMP) production are fully functional on E7 (de Mello, 1978) as compared to the end of the proliferation period (Gardino et al., 1993). Indeed, analysis of chick retina sections show TH positive dopaminergic amacrine cells located in the dorsal retina at E13/E14, and beginning to be defined at E16, with a rising arborization complexity until hatching, in a way that at E18/2 days post hatching dopaminergic cells are uniformly distributed throughout the retina (Gardino et al., 1993). In addition, specific dopaminergic amacrine cell subpopulation shows a more restrict neurogenesis period than the general amacrine cell population. However, dopaminergic system (DS) development only becomes complete in the post-embryonic phase, when TH expressing in amacrine cells in the INL are found in a highly connected wiring pattern with retinal ganglion cells, located in the ganglion cell layer (GCL), exerting a major inhibitory input. TH expression is modulated and dependent on several extrinsic and intrinsic factors that determine the dopaminergic phenotype, such as the cAMP signaling cascade activated by PACAP (Reis et al., 2007; Fleming et al., 2013). However, a clear picture of dopaminergic amacrine cell development is still not completely understood.

The endocannabinoid system (ECS) is present in almost all classes of vertebrates (Cottone et al., 2013). The modulation of cannabinoid activity is mediated by selective type-1 and type-2 cannabinoid receptors (CB_1 and CB_2), which are members of the G protein-coupled receptor (GPCR) family that inhibit adenylate cyclase leading to a decrease in cAMP levels. Specific endogenous endocannabinoids agonists drive these receptors. These bioactive lipids, such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG), are synthesized and degraded by a limited group of enzymes presented in different cell types (Howlett, 2002). One of these enzymes, monoacylglycerol lipase (MAGL), is a serine hydrolase involved in the metabolism of 2-AG in the brain (Chanda et al., 2010).

In the CNS, CB_1 are found mainly in presynaptic neurons, functioning as a regulatory mechanism for the release of both excitatory and inhibitory neurotransmitters (Katona and Freund, 2008).

The ECS is expressed at very early stages of the avian retinal development, as evidenced by CB_1 expression in neurons (Leonelli et al., 2005). Recently, CB_2 has been described in Müller glia in mammals (Bouskila et al., 2013). Straiker et al. (1999) showed for the first time that CB_1 is expressed in the avian retina in two synaptic retinal layers, the inner plexiform and outer plexiform layers (IPL and OPL, respectively). CB_1 expression was also reported in some amacrine and ganglion cell bodies and axons (Straiker et al., 1999). We recently showed that CB_1 and CB_2 receptors are found in both neurons and glial avian retinal cells in culture. These receptors regulate avian retina signaling (GABA release, calcium mobilization and cAMP levels) at critical embryonic stages during synapse formation (Kubrusly et al., 2018).

Dopaminergic neurons are modulated by ECS in different areas of the CNS. CB_1 and endocannabinoids are abundant in the DS, acting to modulate dopamine release. It has been reported that selective and non-selective CB_1 agonists decrease the dopamine release in the guinea-pig retina (Schlicker et al., 1996). However, the relationship between dopaminergic and ECSs during retinal development is still unclear. Therefore, we characterized the expression of CB_1 and CB_2 receptor, and also MAGL, during the development of avian retina as well as the possible functional correlation between the dopaminergic and ECSs in this process.

MATERIALS AND METHODS

All materials used were of analytical grade. Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12), fetal calf serum (FCS) and gentamycin were obtained from Gibco (USA).

Animals

All experiments involving animals were approved by and carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Federal University of Rio de Janeiro (permit number IBCCF-035), and experimental procedures were carried out in accordance with the guidelines of the Brazilian Society of Neuroscience and Behavior (SBNeC). Fertilized White Leghorn eggs (*Gallus gallus*) were obtained from a local hatchery and kept in an appropriate incubator under 12 h light and 12 h dark cycles until the day of use. The eyes from chick embryos ranging from 5, 7, 9 and 14 days of incubation to 7 days post-hatching stage were used in this study. We started with embryonic day 5 (E5) since amacrine cells begin to differentiate early (E3; Calaza Kda and Gardino, 2010), CB_1 receptors are found around E5 (Leonelli et al., 2005), and the first dopaminergic marker is described around E6 (dopamine transporter, DAT, Kubrusly et al., 2003). D_1 receptors are found around E7 (de Mello, 1978), and amacrine neurons end their migration process (Gardino et al., 1993). A peak on retinal synaptogenesis is found around E14 (Calaza Kda and Gardino, 2010). And at P7, DS in the retina is fully differentiated (Gardino et al., 1993).

Preparation of Tissue and Retinal Radial Sections

After decapitation, the embryos and chicks eyes were enucleated and fixed for 2 h in 4% paraformaldehyde in phosphate buffer 0.16 M, pH 7.4 (PB); after these procedures, the eyes were washed in PB three times. In 24 h, the eyes were cryoprotected in sucrose 15% and 30%, embedded in medium optimal cutting temperature (O.C.T. compound, Sakura Finetek, Torrance, CA, USA), frozen and then cut perpendicularly to the vitreal surface on a Leica CM3050 S cryostat (12 μm).

Mixed Retinal Cells in Culture

Primary cell cultures of chick retinal cells at stage E8 (8-days embryo) were prepared as described (Paes de Carvalho and de Mello, 1982). Briefly, retinas were dissected on a Ca^{2+}- and Mg^{2+}-free salt balanced medium (CMF) and cleared of the pigmented epithelium. The retinas were dissociated using TrypLE® (Thermo Fisher Scientific) and incubated at 37°C for 10 min. After brief centrifugation, the pellet was suspended in 1 ml of DMEM/F-12 medium supplemented with 10% FCS and mechanically dissociated with a Pasteur pipette. Cells were plated on 13 mm coverslips (Marienbad, German; 1.6×10^6 cells), or 33.7 mm plates (TPP, Trasadingen, Switzerland; 20×10^6 cells), previously coated with Poly-L-Lysine. The cultures were maintained in an incubator at 37°C under 5% CO_2 atmosphere for 6 days (E8C6 cells in culture). E8C6 on 13 mm coverslips were fixed for 15 min in 4% paraformaldehyde in PBS.

Immunofluorescence

Retina sections and primary cell cultures of retinal cells were washed three times with PBS and incubated for 30 min with 0.15% Triton X-100 and 3% FCS in PBS. This buffer was removed and the primary antibodies of interest added in an overnight incubation at 4°C. The sections and cultures were washed with PBS and incubated with fluorochrome-conjugated secondary antibody for 2 h at room temperature protecting from the light. After rinsing with PBS, the retinas and cultures were mounted using a PBS containing 40% glycerol and DAPI 0.04 μg/ml solution and were examined in a Zeiss Axio Imager 2 Fluorescence Microscope with Apotome and/or confocal microscope (LSM 510 Meta, Zeiss, Germany).

For the immunofluorescence results, specific antibodies were used according to the recommendation of each manufacturer. For primary antibodies, anti-CB1 (anti-cannabinoid receptor 1 produced in goat—SAB2500190 from Sigma-Aldrich), dilution of 1:100; anti-CB2 (CB2 antibody (M-15) produced in goat—sc-10076 from Santa Cruz Biotechnology) dilution of 1:100; anti-MAGL (anti-MAGL produced in rabbit—ab152002 from abcam) dilution of 1:100; anti-TH (anti-tyrosine hydroxylase produced in rabbit—AB152 from EMD Millipore) dilution of 1:100; anti-MAP2 (anti-MAP2 produced in mouse—ab11267 from abcam) dilution of 1:400; anti-2M6 (monoclonal anti-2M6 (kindly provided by Dr. B. Schlosshauer; Max-Planck-Institute, Tübingen, Germany, Schlosshauer et al., 1991)), dilution of 1:400; anti-D_1 (anti-dopamine D_{1R} produced in rabbit—NBP2–16213 from Novusbio) dilution of 1:100.

For secondary antibodies, Alexa Fluor® 488 donkey anti-goat (A11055 produced from Thermo Fisher Scientific); Alexa Fluor® 488 donkey anti-rabbit (A21206 produced from Thermo Fisher Scientific); Alexa Fluor® 594 donkey anti-rabbit (A21207 produced from Thermo Fisher Scientific); Alexa Fluor® 594 donkey anti-mouse (A21203 produced from Thermo Fisher Scientific), all were used at the 1:500 dilution.

Western Blotting

Fresh retinas from embryos and chicks, and E8C6 cells culture were washed twice with PBS, homogenized with a lysis buffer (EDTA 10 mM, HEPES-Tris 50 mM, Sucrose 1 M, trypsin inhibitor 0.15 mg/ml) and the total protein concentration was quantified (Lowry et al., 1951). Total proteins were separated by polyacrylamide gel electrophoresis (10% SDS-PAGE) and transferred to nitrocellulose membranes (Laemmli, 1970). After 1 h in blocking buffer (5% low fat dried milk in Tris-buffered saline—TBS), the immunodetection was performed by incubating the membrane with a specific primary antibody overnight at 4°C. The membranes were washed with TBS and incubated with secondary horseradish peroxidase (HRP)-conjugated antibody for 2 h at room temperature. The proteins of interest were finally detected using Luminata™ Forte (Merck Millipore) and ChemiDoc MP system (Bio-Rad).

For the western blotting results, we used specific antibodies, according to the recommendation of each manufacturer. For primary antibodies, anti-CB_1 (anti-cannabinoid receptor CB_1 (1–77) produced in rabbit–209550 from Calbiochem) was used at a dilution of 1:1000; anti-CB_2 (anti-CNR2 antibody produced in mouse—WH0001269M1 from Sigma-Aldrich) was used at a dilution of 1:1000; anti-TH (anti-tyrosine hydroxylase produced in rabbit—AB152 from EMD Millipore) was used at a dilution of 1:500; anti-D_1 (anti-dopamine D_{1R} produced in rabbit—NBP2–16213 from Novusbio) was used at a dilution of 1:500; anti-DARP32 (anti-DARPP-32 antibody [EP720Y] produced in rabbit—ab40801 from abcam) was used at a dilution of 1:500; anti-Nurr-1 (anti-Nurr-1 antibody produced in rabbit—ab93332 from abcam) was used at a dilution of 1:500; anti-ERK1/2 (anti p44/42 MAPK (Erk1/2) antibody produced in mouse–4696 from cell signaling) was used at a dilution of 1:1000; anti-β actin (anti-β-actin clone AC-15 produced in mouse—A5441 from Sigma-Aldrich) was used at a dilution of 1:5000. For secondary antibodies, HRP-conjugated goat anti-mouse (A5278 from Sigma-Aldrich) and HRP-conjugated goat anti-rabbit (A0545 from Sigma-Aldrich) were used at a dilution of 1:5000 (for the β actin protocol, we used the dilution 1:25,000). For statistical analysis, densitometry for each protein of interest was performed using Scion Image software followed by ANOVA.

cAMP Accumulation

Accumulation of cAMP was assayed according to the competitive binding assay as described previously (Kubrusly et al., 2005). Mixed retinal cells were washed with PBS and pre-treated with 5×10^{-4} M IBMX, an inhibitor of phosphodiesterases, for 15 min in 1 ml of DMEM/F-12 plus 20 mM HEPES pH 7.4 at 37°C. To measure adenylyl cyclase activity, we used 5×10^{-5} M SKF38393,

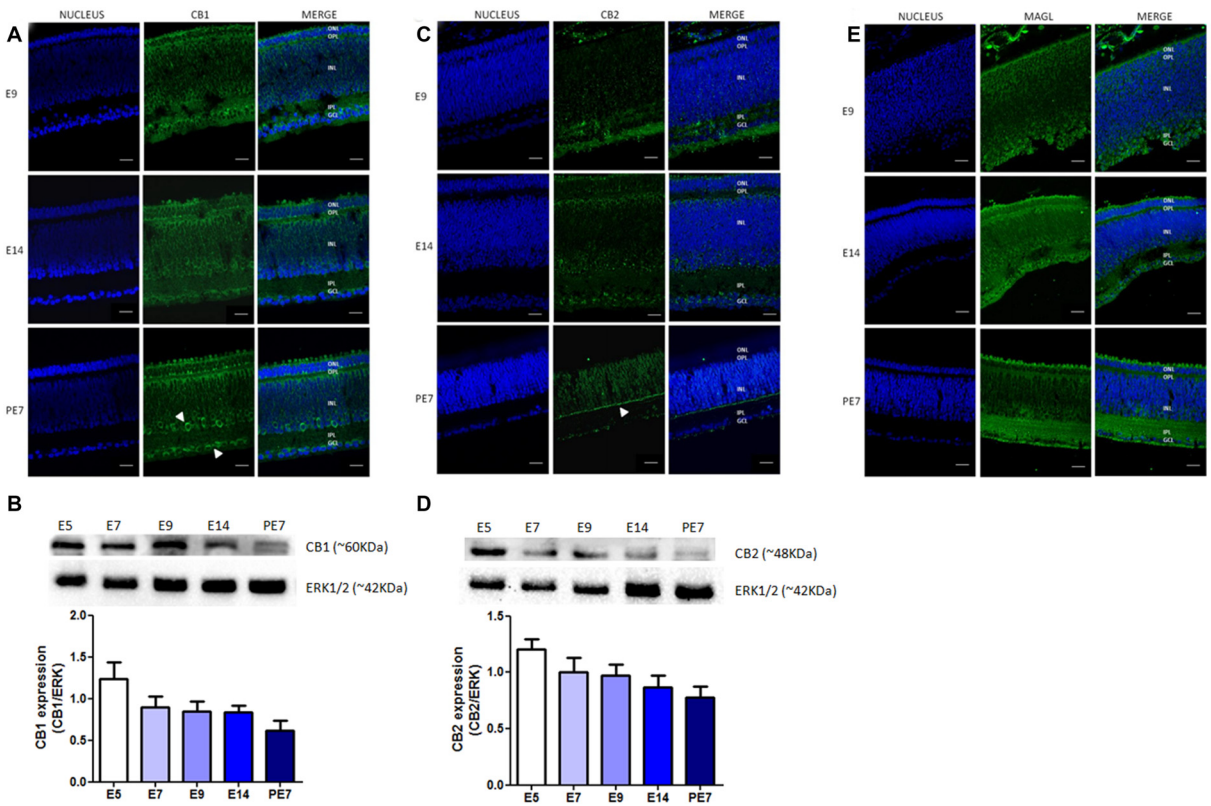


FIGURE 1 | Expression of selective endocannabinoid markers during avian retina development. Expression of CB_1, CB_2 and monoacylglycerol lipase (MAGL) using immunofluorescence and western blotting. In **(A)**, expression of CB_1 (green) at embryonic stages E9 and E14 and post-hatched 7 days (PE7) in chick retina. At PE7, CB_1 is mainly found in cells of the inner nuclear layer (INL) and ganglion cell layer (GCL; white arrows). **(B)** Western blot highlights CB_1 expression from embryonic day 5 (E5) to PE7. Expression of ERK 1/2 was used as loading control. In **(C)**, CB_2 (green) expression from E9 to PE7 primarily in the inner plexiform layer (IPL) lamina (white arrow). CB_2 expression from E5, as shown in **(D)**. In **(E)**, MAGL expression (green) from E9 to PE7 is shown, its distribution is evident across all layers of the retina. Scale bar of 20 μm, all images were obtained with 40× magnification in a Zeiss fluorescence microscope. *N* = 6 for each analysis.

a D_{1R} agonist, for 30 min at 37°C, or 1×10^{-7} M forskolin, a general activator for this enzyme. The treatments performed were 10^{-6} M AM251, a CB_1 antagonist, for 15 min; 10^{-6} M WIN55, 212–2, a non-selective cannabinoid receptor agonist, for 30 min. The reaction was stopped by addition of 100 μl trichloroacetic acid 100% (5% final) acid followed by storage at −20°C for 24 h. After centrifugation (1000 *g* for 10 min), the supernatant was passed through an ion-exchange resin column (Dowex 50) to remove the trichloroacetic acid and other nucleotides. The samples containing cAMP were then incubated in the presence of the regulatory subunit of PKA and a fixed trace amount of [^{3}H]cAMP in 50 mM acetate buffer, pH 4.0, at 4°C for 90 min. The reaction was interrupted by the addition of 200 mM phosphate buffer at pH 6.0. The samples were filtered through Millipore filters and the radioactivity determined in a Tri Carb 2810 TR Perkin Elmer liquid scintillation analyzer.

Statistical Analysis

All the results are shown as means ± SEM. Graphpad Prism5® software was used to analyze the results by one way ANOVA, followed by Tukey post-test. For all the results, $p < 0.05$ was taken as the significance level, and the number of experiments is described in each figure legend.

RESULTS

Our results confirm that CB_1 receptor is highly expressed in the chick retina. Indeed, we analyzed its expression during ontogenesis from embryonic day 9 (E9) for immunofluorescence and E5 for western blotting, until post-hatched day 7 (PE7; **Figures 1A,B**). Western blotting data show a decrease, but not statistically different, in the CB_1 expression during avian retinal development (**Figure 1B**). CB_1 is located in almost all retinal layers from E14 to PE7, and is more densely present in the GCL and IPL (**Figure 1A**).

We also evaluated the expression of other ECS proteins, such as CB_2 receptor and MAGL, the enzyme that degrades the endocannabinoid agonist 2-AG. Our data clearly show the expression of CB_2 receptor throughout development, between E5 and PE7 retinas (**Figure 1D**). We also observed a decrease in CB_2 protein content from E5 afterwards and that these receptors are mainly present in the lamination of the IPL at PE7 retinas

(**Figure 1C**). MAGL is expressed in all retinal layers, but more clearly in the IPL and OPL from E9 to PE7 retina (**Figure 1E**).

To test our hypothesis of a link between the ECS and the DS, we asked whether the CB_1 receptor is co-expressed with TH in retina dopaminergic cells. We performed western blotting for TH at different stages, confirming that TH is highly expressed at PE7 (**Figure 2A**). TH amacrine neurons express CB_1 receptor in the INL and GCL cells around PE7 (**Figure 2B**). Indeed, CB_1 positive cells mainly in the INL are found co-expressed with a few TH positive cells (**Figure 2B**).

Since dopaminergic neurons express CB_1 receptor, we decided to investigate whether this receptor modulate dopaminergic functions in the avian retina. For this purpose, we used primary cell cultures of mixed neurons and Müller glia (**Figure 3**), as these cells express both CB_1 and CB_2 receptors (Kubrusly et al., 2018) and also MAGL. CB_1 and CB_2 are found both in neurons and glia cells, but MAGL is only expressed in Müller glia. CB_1 is highly expressed in neuron bodies (**Figure 3**), and in Müller cells purified culture (**Figure 3C**).

CB_1 was also found to be co-localized with TH and heavily associated with D_1 receptor labeling in primary cell cultures (**Figures 4A,B**). To explore the presence of selective dopaminergic markers in mixed retinal cell cultures, we performed western blot analysis of different proteins associated with the DS. Data revealed the clear expression of the nuclear receptor related 1 protein (Nurr1), D_1 receptor and dopamine- and cAMP-regulated phosphoprotein 32 (Darpp32; **Figure 4C**) in addition to CB_1.

Next, E8C6 cultures were exposed to SKF38393 (SKF), a selective D_1 agonist, which was able to increase the cAMP levels when compared to controls. Our results show that the cAMP accumulation induced by SKF is lost when cultures were pretreated with WIN55, 212–2 (WIN), a non-selective CB agonist (**Figure 5**). When cultures were pre-treated with AM251, a CB_1 selective antagonist, and then co-exposed to SKF+WIN, the SKF response was restored (**Figure 5**), indicating that the repressive effect of WIN on SKF promoted cAMP accumulation was due, mainly, to activation of CB_1 receptors.

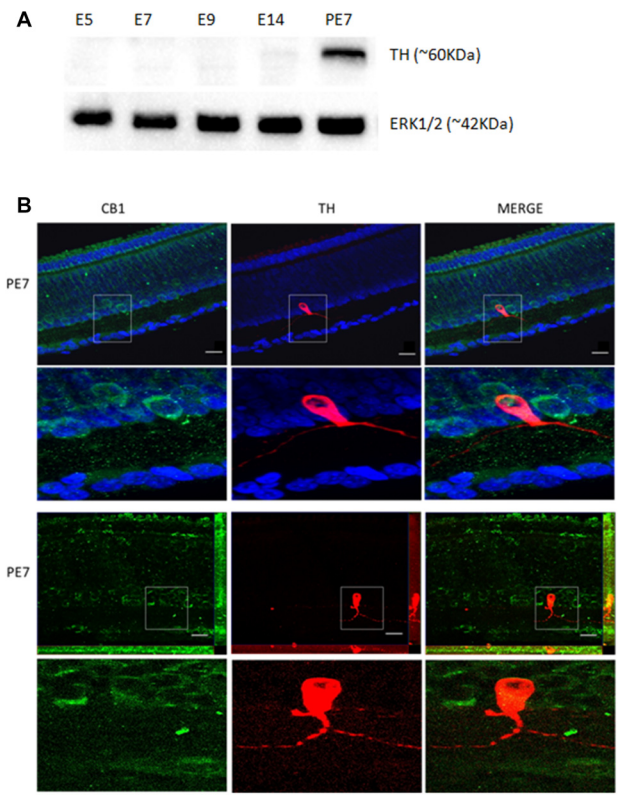


FIGURE 2 | Co-localization of CB_1 receptor and tyrosine hydroxylase (TH) enzyme in the avian retina. We evaluated whether dopaminergic neurons, TH positive cells (red) express CB_1 receptor (green) by immunofluorescence. In **(A)**, western blotting for TH at different stages, confirming that TH is highly expressed at PE7. ERK1/2 is shown as the loading control. In **(B)**, CB_1 and TH co-expression in PE7 avian retina. As shown, a discrete TH co-expression with CB_1 that is mainly present in neighboring cells in the INL. ApoTome images in upper row and confocal images in lower row. Scale bar of 20 μm, all images were obtained with 40× magnification. *N* = 5 for each analysis.

DISCUSSION

Here we show the expression of CB_1 receptors in the avian retina throughout development, being detected since an early embryonic stage (E5) up to a more mature period (PE7). Moreover, we show a decrease in CB_1 protein content during development, but no significance was reached upon ANOVA. CB_1 is mainly located in ganglion as well as amacrine cells, present in the GCL and in the INL, respectively. Our work confirms previous studies that have shown the presence of CBs and endocannabinoids metabolic enzymes in the retina of several species such as goldfish, rat, mouse, chick and monkey (Schwitzer et al., 2016). Some of these previous data have shown CB_1 expression in ganglion, horizontal and amacrine cells, cone pedicles and rod spherules of photoreceptors (Zabouri et al., 2011a). Leonelli et al. (2005) showed for the first time that, in avian retina, CB_1 emerges in E4, in ganglion cells, while at E18 CB_1 is expressed in neurons located in the GCL and INL.

We recently showed that CB_1 and CB_2 receptors are found in both retinal neurons and Müller glia of chick embryos. We found that WIN decreases cAMP production in retinal cells in basal conditions, decreases the number of glial cells that increased Ca^{2+} evoked by ATP, and inhibited [^{3}H]-GABA release induced by KCl or L-Aspartate (Kubrusly et al., 2018). Now we evaluated the expression of CB_1, CB_2 receptor and MAGL enzyme in the developing retina. CB_2 has been reported in rat, mouse and monkey but not in avian retina. CB_2 has been shown to be expressed in photoreceptors, horizontal, amacrine and ganglion cells and also in fibers in the INL in adult rat retina (López et al., 2011). Interestingly, CB_2 expression appears in Müller cells in the monkey retina (Bouskila et al., 2013). However, there is no information regarding CB_2 expression during development of the avian retina. Our data show that CB_2 is expressed beginning at E5 and is clearly observed in the lamination of the IPL between E14 and PE7 (**Figure 1C**). In early retinogenesis, up to E5, retinal cells are essentially in the neuroblastic layer made of multipotent progenitors that generate early-born cells (RGCs, horizontal, some types of

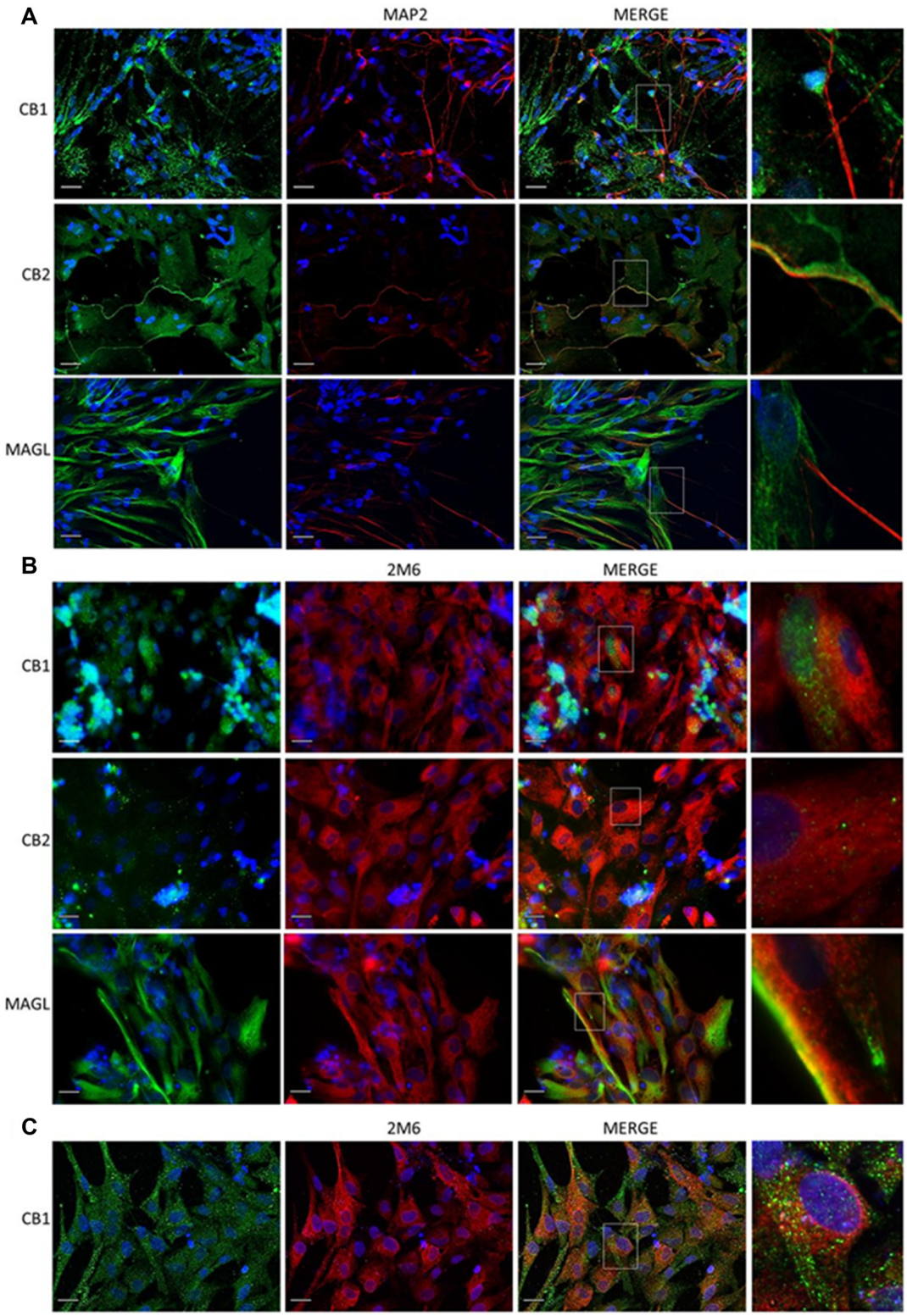


FIGURE 3 | Expression of cannabinoid markers in primary retinal cell in cultures. Expression of CB_1, CB_2 and MAGL in mixed cultures of avian retina cells, displaying neurons and glia prepared from 8-day old embryos (E8), as described. In **(A)**, CB_1, CB_2, MAGL (all in green) and MAP2 (red) as a specific neuronal marker. In **(B)**, expression of CB_1, CB_2, MAGL (green) and 2M6 (red) as a selective glial marker for Müller cells. In **(C)**, purified culture of Müller cells show expression of CB_1 (green). Scale bar of 20 μm, all images were obtained with 40× magnification in a fluorescence microscope with ApoTome software. *N* = 5.

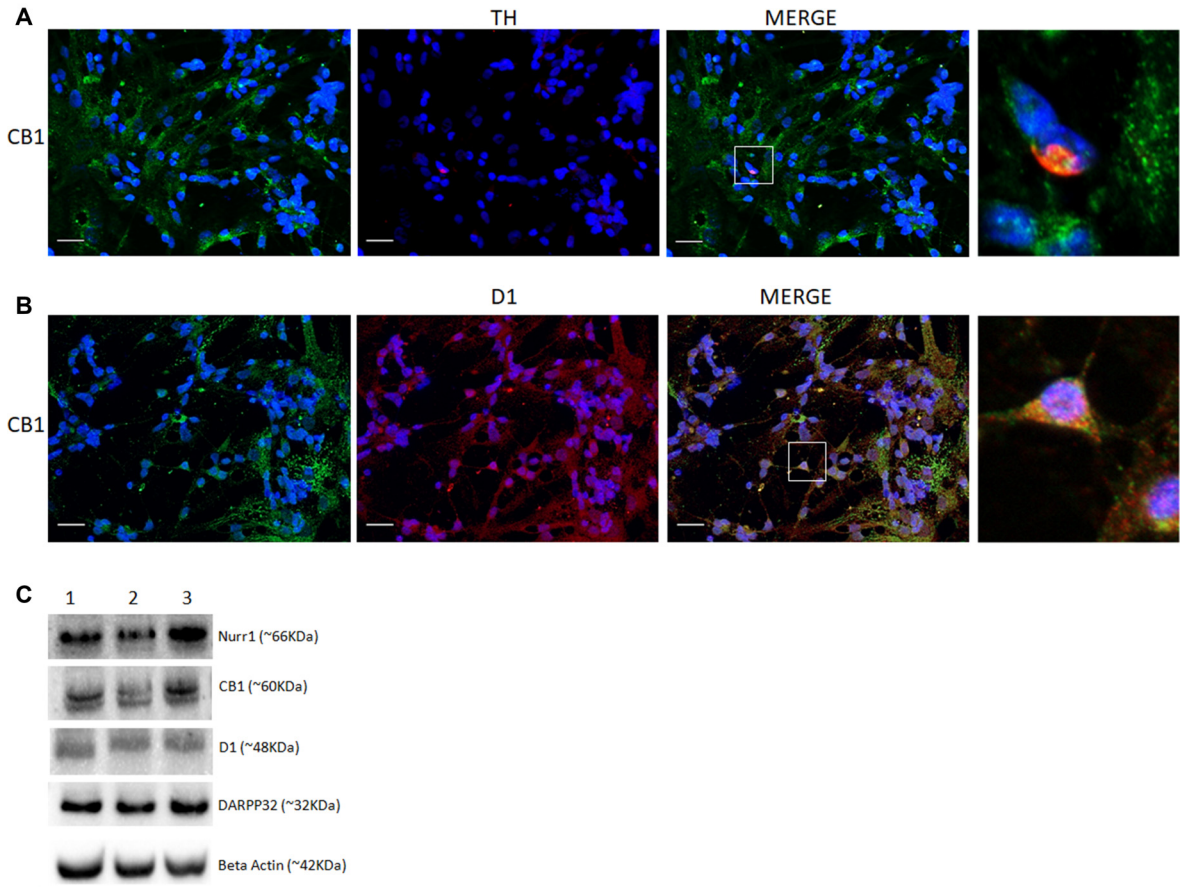


FIGURE 4 | Co-expression of selective cannabinoid and dopaminergic markers in cultured retinal cells. Expression of CB_1 receptor in E8C6 cultures showing the main components of the dopaminergic system (DS). In **(A)**, co-localization of CB_1 (green) in TH positive neurons (red). In **(B)**, co-localization of CB_1 (green) in cells expressing the D_1 receptor (red). In **(C)**, western blotting analyses confirm the expression dopaminergic markers (Nurr1 and Darpp32) and D_1 receptor, in addition to CB_1 expression, β Actin is shown as the loading control (lanes represent triplicate assay as shown for 1, 2, 3). Scale bar of 20 μm, all images were obtained with 40X magnification in a fluorescence microscope with ApoTome software. *N* = 3 for each analysis.

amacrines and cones). Around E14, late-born progenitors give rise to bipolar, rods and Müller glia, so the precise number and proportion of cells into different layers form a fully mature retina (Martins and Pearson, 2008). Since CB_1 (Xapelli et al., 2013) and CB_2 (Bravo-Ferrer et al., 2017) receptors are correlated with proliferation and/or neurogenesis in the central nervous system, their presence early in the embryonic avian retina could modulate the generation of retinal cells. Moreover, activation of these receptors at early stages suggests that the retinal circuitry might be altered (morphologically) or in terms of function (CBs inhibit the release of GABA and aspartate (Kubrusly et al., 2018), and decrease cAMP levels, as shown in **Figure 5**. This could possibly reflect in changes during synapse remodeling during retinal development.

The main endocannabinoids are AEA and 2-AG, described in the retina of several vertebrates along with the main enzymes that degrade them (Schwitzer et al., 2016). MAGL, one of these enzymes, is present in rat retinal layers mainly in amacrine and Müller cells (Cécyre et al., 2014). We found that, in the avian retina, MAGL is expressed beginning at E9 and up to post-hatched PE7, where it is detected in all the five retinal layers, mostly in IPL and GCL (**Figure 1E**). Our data provide that MAGL is located primarily in the glial compartment as the enzyme is co-localized with 2M6 (**Figure 3B**, lower panel), at least *in vitro*. As glial cells are the last to be generated in the retina (Martins and Pearson, 2008), perhaps this explains the appearance of labeling at E9. We still cannot affirm how the developmental profile of CB_1, CB_2, MAGL and endocannabinoids match the interactions between different retinal neurons and Müller glia. However, data on **Figure 5** suggest an important interplay between receptors that decrease the levels of cAMP (CB_1 and CB_2) and those that increase cAMP levels (dopamine, adenosine or PACAP) during retina differentiation.

In addition, CB receptor activation decreases the number of glial cells that increased Ca^{2+} evoked by ATP, and inhibited [^{3}H]-GABA release induced by KCl or L-Aspartate (Kubrusly et al., 2018). The first part of the retina that differentiates is the central part and RGCs are the first to emerge. Synaptogenesis peak in the avian retina around E14, and it is known to have

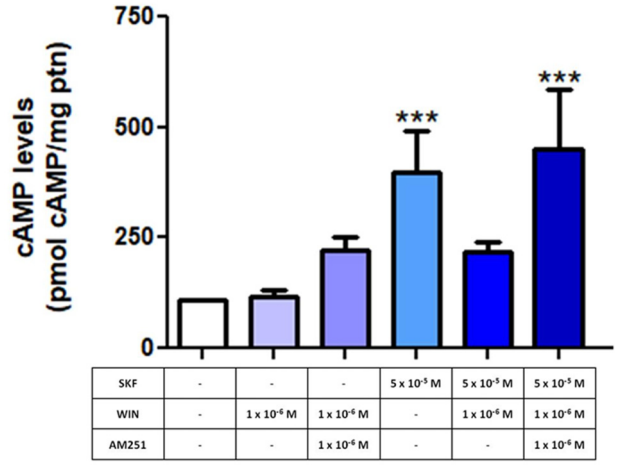


FIGURE 5 | Endocannabinoid system (ECS) modulates the function of dopamine-induced increase in cyclic AMP (cAMP) levels. E8C6 retinal mixed cell culture was subjected to cAMP quantification analysis, as described. When the selective D_1 receptor agonist, SKF38393 (SKF) at 5×10^{-5} M was used, cAMP levels increased significantly. When the culture was treated with WIN after SKF stimulation, the SKF-induced increase in cAMP levels was lost. However, in cultures pretreated with AM251, a selective CB_1 antagonist, cAMP levels were increased (AM251 + SKF + WIN), similar to SKF-only application. AM251 was added 15 min before SKF and WIN, which were added for 30 min, all at 37°C. ***< 0.001. *N* = 6 for each analysis.

two main axis: glutamatergic in the vertical axis (photoreceptors, bipolar and the RGCs) and GABAergic in the horizontal axis (horizontal and amacrine; Barnstable, 1993). Moreover, Müller glia is an active compartment that shapes the circuitry (de Melo Reis et al., 2008). The ECS seems to be a powerful regulator of the efficacy of retinal circuitry.

The retina presents the main neurotransmitters found in the brain such as glutamate (Connaughton, 1995), GABA (Bringmann et al., 2009) and dopamine (Reis et al., 2007). A direct action of the ECS on retinal transmitter release and in general retinal physiology has been described in different vertebrate retinas mainly in retinal bipolar cells (Straiker et al., 1999). Interestingly, dopamine and noradrenaline release is inhibited by CB_1 activation in perfused guinea-pig retina (Schwitzer et al., 2016). In the developing vertebrate retina, the DS is one of the first phenotypes to appear mainly in amacrine and interplexiform cells (Reis et al., 2007). Here, we first evaluated the emergence of CB_1 and TH co-expression in PE7 avian retina. We observed that CB_1 is co-localized with dopaminergic neurons (**Figure 2**) present in the IPL. CB_1 is also localized in neighboring cells in IPL and also GCL, suggesting a more complex role in retina signaling. It is likely that CB_1 positive cells are responsive for the dopamine released by TH amacrine cells and vice versa. Bosier et al. (2007) showed an important relationship between the ECS and DS using a murine neurospheres model, where CB_1 agonists promoted a reduction in TH expression.

It is known since the 70s that dopamine increases cAMP levels in the chick retina and in mixed cultures of embryonic chick retina cells (de Mello, 1978). Since it is described that CB_1 expression occurs in retinal cells in response to dopaminergic stimuli (Fan and Yazulla, 2005), we investigated the correlation between ECS and the DS. Primary cell cultures of mixed neurons and Müller glia express CB_1 and CB_2 receptors as well as MAGL enzyme. CB_1 is predominantly in cell bodies while CB_2 is found mainly in neuronal processes. On the other hand, MAGL is mainly located in Müller cells.

In order to evaluate a functional correlation between CB_1 and D_1 receptors, a mixed neuron-glia culture was used at E8C6, a stage where most of dopaminergic proteins are expressed. The D_1 receptor agonist SKF38393 promotes a significant increase in cAMP accumulation in retina cells (Castro et al., 1999). In order to test the effect of the ECS on this D_1-mediated effect, we added AM251, a CB_1 receptor selective antagonist, and WIN 55, 212–2 (WIN), a non-selective cannabinoid CB_1/CB_2 agonist. Our results show that WIN decreases cAMP accumulation induced by SKF, and this response is blocked when AM251 was used as a pretreatment. Additionally, WIN did not change cAMP accumulation promoted by forskolin (not shown), a general adenylyl cyclase activator, suggesting that WIN is selectively acting on dopaminergic receptor level. Therefore, we provide evidence here that CB_1 receptor can modulate the response of D_1 receptor activation through cAMP accumulation. We conclude that the endocannabinoid and the DS interact in retinal cells.

Emergence of the ECS (CB_1/CB_2 receptors), endocannabinoids and enzymes during embryonic retinal development might influence dopaminergic communication that seems to use two separated systems during development. One operates in early stages of retina formation, and the other, later in development. No TH is detected in the tissue between E5 and E10/12. However, E5 retinas display several cells (neuroblasts) clearly labeled for L-Dopa decarboxylase (DDC; da Silva et al., 2009). At this stage, or even before, the presumptive neuroretina is already associated with the pigmented epithelium, characterized by the presence of melanin. Melanin, as dopamine, use as precursor L-Dopa that in the epithelium comes from tyrosinase activity. Our group has shown that conditioned media from the pigmented epithelium of embryonic eyes fed to embryonic retina tissue prior to TH expression, is capable, of synthesizing dopamine (Kubrusly et al., 2003). This activates cAMP formation prior to TH expression. Therefore, based on the findings that D_1 (increasing cAMP) or D_2 receptors activation (decreasing cAMP levels) in embryonic avian retina can modify the dynamics of neurites outgrowth, interference with these systems via CBs might influence dopaminergic circuitry. D_1 and CB_1 have been shown to exhibit antagonistic effects in goldfish bipolar retinal cells, as D_1 activation increases cAMP level, while CB_1 (WIN) reduces the concentration of cAMP (Fan and Yazulla, 2005). Our data strengthen this point using avian retinal cells to assess the interaction between the endocannabinoid and DSs.

A recent article has revealed in a pioneer way the presence of both cannabinoid receptors (CB_{1R} and CB_{2R}, the synthesizing and degrading enzymes in mice, shrews and monkey retinae (Bouskila et al., 2016). These data, added to ours in chicks (and many others) suggest a strong conservation of the ECS

in the retinal tissue in vertebrates. This opens a possibility to create strategies to ameliorate vision problems with cannabinoid modulators. A recent article has shown that activation of CB_2 receptors with a selective agonist (JWH-133) increases inflammation in human retinal pigment epithelium, decreasing cellular viability through release of pro-inflammatory cytokines and might be a central element in avoiding vision problems (Hytti et al., 2017). Both neuronal and glial CB_1 and CB_2 receptors might have an important role in retinal development through modulation of intracellular pathways (Kubrusly et al., 2018). Indeed, 2-AG is found in early retinal development together with DAGLα, 2-AG degrading enzyme), while MAGL, develops later (Cécyre et al., 2014). FAAH, (AEA, degrading enzyme) emerges transiently at postnatal day 1 in ganglion and cholinergic amacrine cells (Zabouri et al., 2011b).

Moreover, we show that endocannabinoids may act on the developing CNS (here exemplified by the retina), possibly influencing developmental characteristics of CNS systems such as plasticity during synapses formation (Fleming et al., 2013). We also call attention to the possibility that cannabinoid consumption might have a dramatic effect in the developing CNS.

AUTHOR CONTRIBUTIONS

LSS and RCCK: conception and design, provision of study material, collection and assembly of data, data analysis and interpretation, manuscript writing and final approval of manuscript. YPC, PPT, VTR-R, ME-L and PFG: collection and assembly of data, data analysis and interpretation. RP-C, FGM and RAMR: conception and design, provision of study material, assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript, financial support and administrative support.

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Suppression of Cisplatin-Induced Vomiting by *Cannabis sativa* in Pigeons: Neurochemical Evidences

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Cannabis sativa (*CS*, family *Cannabinaceae*) has been reported for its anti-emetic activity against cancer chemotherapy-induced emesis in animal models and in clinics. The current study was designed to investigate *CS* for potential effectiveness to attenuate cisplatin-induced vomiting in healthy pigeons and to study the impact on neurotransmitters involved centrally and peripherally in the act of vomiting. High-performance liquid chromatography system coupled with electrochemical detector was used for the quantification of neurotransmitters 5-hydroxytryptamine (5HT), dopamine (DA) and their metabolites; Di-hydroxy Phenyl Acetic acid (Dopac), Homovanillic acid (HVA), and 5-hydroxy indole acetic acid (5HIAA) centrally in specific brain areas (area postrema and brain stem) while, peripherally in small intestine. Cisplatin (7 mg/kg i.v.) induce emesis without lethality across the 24 h observation period. *CS* hexane fraction (*CS*-HexFr; 10 mg/kg) attenuated cisplatin-induced emesis ~ 65.85% ($P < 0.05$); the reference anti-emetic drug, metoclopramide (MCP; 30 mg/kg), produced ~43.90% reduction ($P < 0.05$). At acute time point (3rd h), CS-HexFr decreased ($P < 0.001$) the concentration of 5HT and 5HIAA in the area postrema, brain stem and intestine, while at 18th h (delayed time point) CS-HexFr attenuated ($P < 0.001$) the upsurge of 5HT caused by cisplatin in the brain stem and intestine and dopamine in the area postrema. *CS*-HexFr treatment alone did not alter the basal neurotransmitters and their metabolites in the brain areas and intestine except 5HIAA and HVA, which were decreased significantly. In conclusion the anti-emetic effect of *CS*-HexFr is mediated by anti-serotonergic and anti-dopaminergic components in a blended manner at the two different time points, i.e., 3rd and 18th h in pigeons.

Keywords: cisplatin, emesis, *Cannabis sativa*, pigeon, neurotransmitters

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INTRODUCTION

Cytotoxic agents like cisplatin and cyclophosphamide have the side effects of nausea and vomiting most feared by patients undergoing chemotherapy (Hesketh et al., 2003a). These stressful side effects often result in poor compliance and even refusal of treatment (Tanihata et al., 2000; Hesketh, 2008). The D_2 receptor blocker "metoclopramide" was found to be effective against chemotherapy induced vomiting (CIV) at higher doses, where the anti-emetic effect is reported to be mediated

through antagonism of 5-hydroxytryptamine type 3 ($5HT_3$) receptors (Coronas et al., 1975; Miner and Sanger, 2012). These findings led to the discovery of $5HT_3$ receptor antagonists (Ondansetron, etc.).

Dopamine (DA), 5-hydroxytryptamine (5HT), and neuropeptide substance P are involved in emetic circuitry. The neurotransmitter 5HT (Serotonin) is primarily responsible for the initiation of the vomiting produced by cisplatin (Grunberg and Koeller, 2003). Up to 95% of 5HT is present in the enterochromaffin (EC) cells in the gastrointestinal mucosa along with substance P (Diemunsch and Grelot, 2000; Minami et al., 2003). The noxious stimulus caused by highly emetogenic chemotherapy (HEC) agents like cisplatin results in the release of 5HT (Wolff and Leander, 1997; Percie du Sert et al., 2011). The released 5HT then activates $5HT_3$ receptors on vagal afferents which stimulate the brain centers to initiate the vomiting response (Hesketh et al., 2003b). Furthermore, in human and animal studies, there is evidence for the increased level of the 5-HT metabolite, 5-Hydroxy Indole Acetic Acid (5HIAA, urine) (Cubeddu et al., 1995; Veyrat-Follet et al., 1997), 5HT in the intestinal mucosa (ileal segment), Tryptophan Hydroxylase (TPH, ileum), Aromatic L-amino Decarboxylase (AADC, ileum) (Endo et al., 1993) and in the brain stem (Minami, 1995), following cisplatin treatment. Furthermore, a decrease in Monoamine Oxidase (MAO, ileum) has also been reported (Endo et al., 1993). This enhancement in 5HT biosynthesis and reduction in degradation ultimately led to the upsurge of serotonin which initiates the vomiting response (Ullah, 2013).

The selective activation of D_2 receptors, localized in the limbic system, hypothalamus, amygdala and in the brain stem emetic circuitry trigger the vomiting response (Le Moine and Bloch, 2004). This involvement of dopamine receptors advocates dopamine as important mediator for vomiting act. Dopaminergic agonists like apomorphine have been reported to be emetic in a variety of species including dogs (Foss et al., 1998), ferrets (Osinski et al., 2003, 2005), least shrew (Darmani et al., 1999), and humans (Schofferman, 1976). The emetic action of apomorphine and loperamide has been suggested to be mediated in the chemoreceptor trigger zone/area postrema through stimulation of dopamine receptors. Where, the ablation of area postrema abolished the vomiting response advocating the involvement of area postrema in the mediation of vomiting by apomorphine and loperamide (Miller and Leslie, 1994; Yoshikawa et al., 1996).

The identification of cannabinoid receptors resulted in the discovery of endocannabinoids (Pacher et al., 2006). Delta-9-Tetrahydrocannabinol (Δ^9-THC) and synthetic cannabinoids exert their cannabimimetic effects via CB_1 receptors (Mackie and Stella, 2006). CB_1 receptors are primarily located centrally and peripherally while CB_2 receptors occur mainly on immune cells (Pertwee, 2006). Furthermore, a family of nuclear hormone receptor PPAR (α, β, and γ) are also been reported to be involved in the mediation of some effects in analgesia, anti-inflammatory, neuroprotection, cardiovascular, gastrointestinal, and anti-tumor properties of some cannabinoids (O'sullivan, 2016). Endocannabinoids like oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) are reported to activate PPARα. Other endocannabinoids including noladin ether, virodhamine, 2-arachidonoyl-glycerol, and Anandamide are also shown to stimulate PPARα and transient receptor potential vanilloid type 1 (TRPV1) cation channel. In continuation, Synthetic cannabinoids like WIN55,212-2 activates the transcriptional activity of PPARα and PPARγ (Brown, 2007; O'sullivan, 2016; Stampanoni Bassi et al., 2017). Activation of the Endocannabinoid system, PPARγ and CB1 receptors are associated with decrease in the dopaminergic activity in the basal ganglia and levodopa induced abnormal involuntary movements (AIMs) which can be extrapolated to the anti-emetic effect of *CS* in the brain stem emetic center (Martinez et al., 2015; Stampanoni Bassi et al., 2017).

Cannabinoids have been shown to affect neuronal circuits that modulate nausea, vomiting, and other gastrointestinal functions. Evidence is emerging regarding the interaction of cannabinoid (CB_1), serotonin ($5HT_3$), neurokinin-1 (NK_1) and dopamine receptors (D_2 and D_3), implicating an important role for cannabinoids in vomiting circuits. The old era of neurotransmitter understanding advocate primarily the involvement of the monoaminergic neurotransmitters especially serotonergic system, while the late phase is associated with monoaminergic system excluding the serotonergic system (Tanihata et al., 2000). The current literature provide evidences for the substantial overlapping of serotonergic, dopaminergic, and neurokininergic mechanisms for the entire time course of cisplatin-induced vomiting (Saito et al., 2003; Darmani et al., 2009; Higgins et al., 2012).

Considering the relevance of DA and 5HT in cisplatin-induced vomiting, this study was designed to evaluate the participation of these monoamine neurotransmitters and their metabolites in cisplatin-induced vomiting, and to examine the impact of *Cannabis sativa* (*CS*) extract on neurotransmitters implicated in the act of vomiting in specific brain areas and intestine in pigeons. *Cannabis sativa* hexane fraction was selected based on our previous studies where it was proved to be anti-emetic against cisplatin-induced vomiting in pigeon model (Ullah et al., 2012).

MATERIALS AND METHODS

Animals

Pigeons of either sex (mixed breed, Department of Pharmacy, University of Peshawar, Peshawar, Pakistan) weighing between 250 and 350 g were used. They were housed in groups of eight (n = 8) at 22–26°C under a 12 h light/dark cycle and had free access to food (locally available food; Millet + Wheat) and water before and during experimentation. All of the experimental procedures were approved by the Ethical Committee of the Department of Pharmacy, University of Peshawar (Ref. No. 5/EC/Pharm) and were in accordance with the UK Animal Scientific Procedure Act, 1986 (Ullah, 2013).

Drugs and Chemicals

High-performance liquid chromatography (HPLC) grade acetonitrile (99.9%), methanol (99.9%), 1-octane sulfonic acid sodium salt (>98%) (Fisher Scientific, United Kingdom), sodium

dihydrogen orthophosphate (99%) and ethylene diamine tetra acetic acid (≥99%) (EDTA) were purchased from the Merck local distributor in Peshawar, Pakistan. Noradrenaline (≥98%), DOPAC (≥98%), dopamine (≥99%), 5HIAA (≥98%), HVA (≥98%), and serotonin (≥99%), were from Acros Organics, Belgium. Cisplatin (≥99.9%) was from Korea United Pharm., Inc. (South Korea). Metoclopramide (MCP; ≥98%) was purchased in solution from GlaxoSmithKline (GSK Pakistan, Ltd.). Commercial grade *n*-Hexane was from Haq Chemicals Peshawar (Pakistan). The plant was collected at a farm, from Malakand Division (Khyber Pukhtoonkhwa, Pakistan) at its bloom season and was authenticated by Prof. Dr. Muhammad Ibrar, Department of Botany, University of Peshawar, a specimen was preserved in the herbarium for future reference (voucher No. 8717) (Ullah, 2013).

Extraction of *Cannabis sativa*

Leaves and flowering tops of *Cannabis sativa* plant were separated, shade dried, coarsely ground and then extracted as shown in the extraction scheme (**Figure 1**) (Ali et al., 2017; Ayaz et al., 2017).

Drug Formulation

Cisplatin was dissolved in normal saline by heating up to 60°C and then cooled up to 40–45°C before administration (Ullah et al., 2014). *Cannabis sativa n*-hexane fraction (CS-HexFr) was dissolved in absolute ethanol, mixed with emulsifier and made the volume with distilled water in such a way that the final mixture consists of ethanol: emulsifier: distilled water in a ratio of 5: 5: 90 (Feigenbaum et al., 1989a; Ullah, 2013).

Drug Administration

Cotton wool and methylated spirit were used to sterilize the skin prior to drug administration. Intravenous (Cisplatin) and intramuscular (Treatment) administrations were done through brachial wing vein and chest muscle, respectively using Neoject 2 ml non-pyrogenic syringes with sharp painless needles (27G × 1/2″ for the i.v. route, and 23G × 1″ for the i.m. route). Immediately, after the last injection, the animals were put back in the specially designed confining/observation cages and the number of Retching plus Vomiting ($R + V$) and latency to first vomit were recorded for 24 h. At the end of experiment, body weight loss was calculated. Subsequently, the animals were decapitated to terminate the experiment (Ullah, 2013).

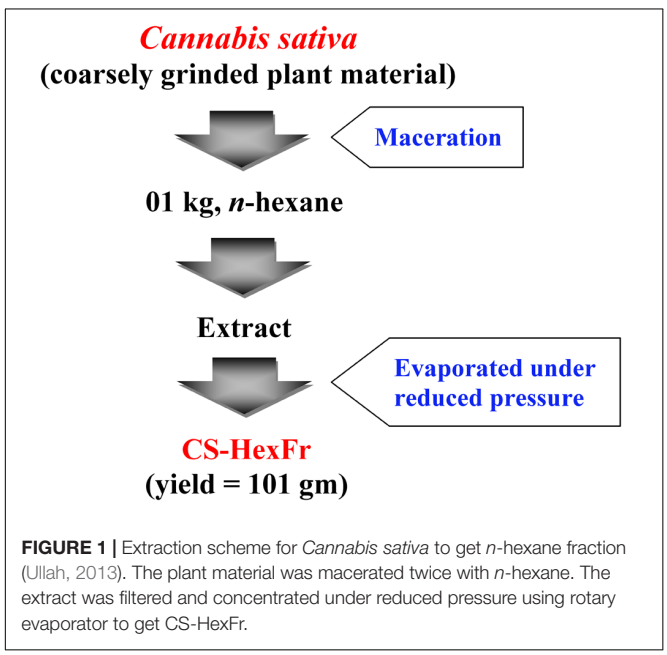


FIGURE 1 | Extraction scheme for *Cannabis sativa* to get *n*-hexane fraction (Ullah, 2013). The plant material was macerated twice with *n*-hexane. The extract was filtered and concentrated under reduced pressure using rotary evaporator to get CS-HexFr.

Anti-emetic Assay

On the day of experiment, the pigeons were placed in individual cages specially designed for video observation. Cisplatin at the dose of 7 mg/kg was administered intravenously via the brachial wing vein at $t = 0$ (Ullah et al., 2014). The behavior of the pigeons was then recorded for 24 h. Food and water were available during the observation period and each animal was used only once. The vomiting response with or without oral expulsion was considered as one vomiting episode (Preziosi et al., 1992). The latency to first vomit and the number of vomiting episodes were recorded. A vomiting episode was considered complete when the pigeon adapted relaxed posture. Jerking episodes, which are indicative of vomiting intensity, were also recorded. In these studies, *CS* fraction and MCP or respective vehicles, were administered 30 min before cisplatin administration, In case of twice (BD) administration of *CS* the second dose was administered intramuscularly at 12th of cisplatin administration (Ullah, 2013).

Tissue Sampling for Neurotransmitters Analysis

Two discrete parts of the brain (brain stem and area postrema) as well as the intestinal samples 5–6 cm from the pylorus (initial segment of Jejunum) were collected for the neurotransmitter analysis and the effects of CS-HexFr and MCP were investigated. The dissection of brain parts was carried out according to the atlas of Karten and Hodos (1967) and Duvernoy and Risold (2007). In brief, after decapitation of experimental animals, the dorsal surface of the skull was exposed by making an incision along the mid line and the temporal muscles were stripped off to expose skull bone. After exposing the skull, bones, and meninges were carefully removed in a way to expose the brain hemispheres and especially to make brain stem prominent from the ventral aspect. The long strip of capillaries stretching from the obex on the median line to the lateral angles of the fourth ventricle (area postrema) was dissected followed by dissection of brain stem. Jejunal samples of about 2 cm were rapidly removed and washed with ice cold saline. The collected samples were rapidly frozen on an ice plate and stored at −80°C until analysis (Ullah, 2013).

Determination of Neurotransmitters and Their Metabolites

Tissue samples were homogenized in cold 0.2% perchloric acid (PCA) at 5000 rpm with the help of Teflon glass homogenizer (Wise stir HS 30 E). After centrifugation

(Centurion, United Kingdom) at 12000 *g*/min (4°C) and filtered through a 0.45 micron filter. Neurotransmitters and their metabolites were analyzed using High-Performance Liquid Chromatography system (HPLC, Shimadzu, Japan) coupled with Electrochemical Detection (ECD, ESA Coulochem III model 5300), a pump (model LC-20AT), and an analytical column (Teknokroma 3 × 150, 3 um). The mobile phase consisted of 94 mM sodium dihydrogen orthophosphate, 40 mM Citric acid, 2.3 mM sodium 1-octane sulfonic acid, 50 uM EDTA, and 10% acetonitrile (pH 3). The flow rate was maintained at 0.6 mL/min. The standards used were noradrenaline hydrochloride (NA), 3, 4-dihydroxyphenylacetic acid (DOPAC), dopamine hydrochloride (DA), 5-hydroxyindole-3-acetic acid (5HIAA), Homovanillic acid (HVA), and serotonin (5HT). The HPLC method already reported by our laboratory (Ullah et al., 2014) was used where all the neurotransmitters and their metabolites were separated within 13 min (Ullah, 2013).

Statistical Analysis

The differences between means were evaluated using a one way analysis of variance (ANOVA) followed by Dunnett or Tukey multiple comparison tests. $P < 0.05$ was considered as statistically significant. The animals which showed complete suppression of Retching Plus Vomiting ($R + V$) were not included in statistical analysis for latency. Data represent the mean ± SEM unless otherwise indicated.

RESULTS

Anti-emetic Effect of *Cannabis sativa* Hexane Fraction (CS-HexFr)

Cisplatin at the dose of 7 mg/kg (Ullah et al., 2014) induced reliable $R + V$ in all the animals tested with intense vomiting occurring in the first 3 h while the treatments attenuated it **Figure 2** and **Table 1**.

In these experiments, cisplatin-induced $R + V$ following a latency of ∼ 67 min that comprised ∼ 41 episodes. *CS* hexane fraction (CS-HexFr; 5, 10, and 15 mg/kg) attenuated cisplatin-induced $R + V$ in non-dose-dependent manner (**Figure 3**), showing significant reduction with 10 mg/kg once (OD) and twice (BD) dosing up to 17 ± 3.4 (58.53% protection) and

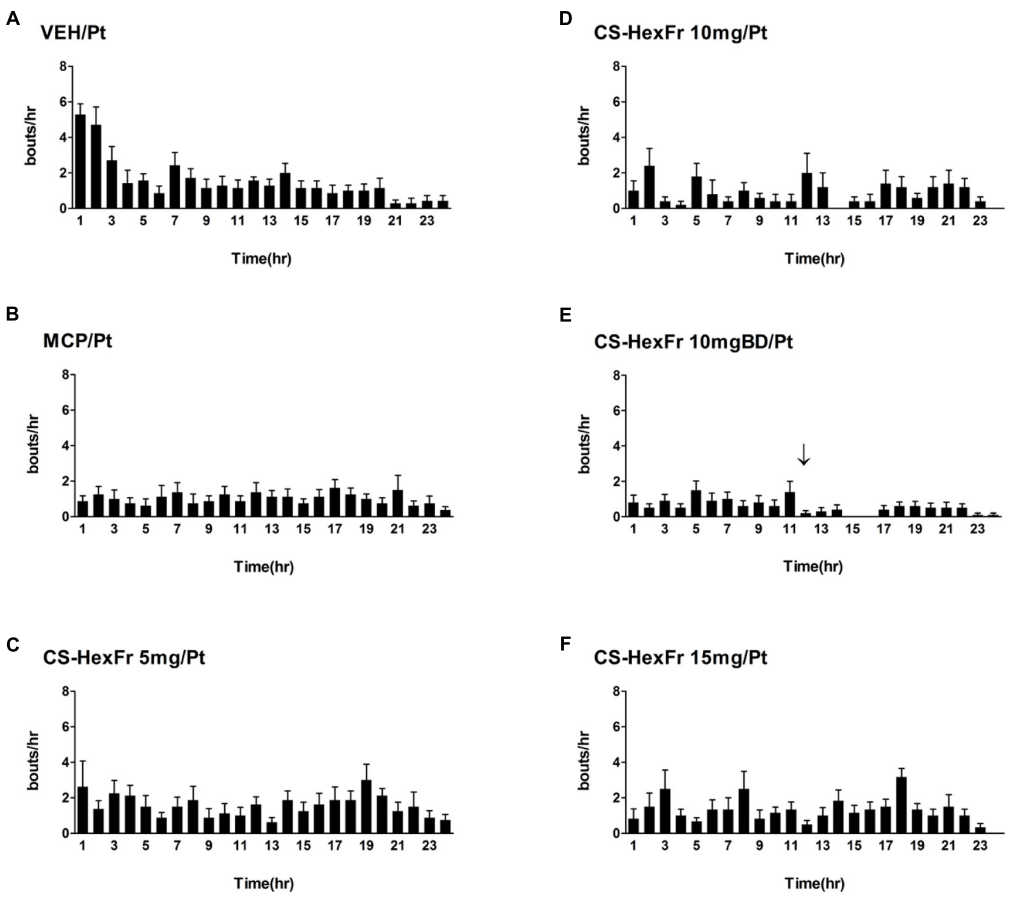


FIGURE 2 | Sketch of cisplatin-induced emesis in pigeons and the effect of treatments **(A–F)** on cisplatin induced emesis profile during a 24 h observation period. **(A)** Cisplatin control, **(B)** Metoclopramide treatment, **(C)** *Cannabis sativa* hexane fraction 5 mg treatment, **(D)** *Cannabis sativa* hexane fraction 10 mg treatment, **(E)** *Cannabis sativa* hexane fraction 10 mg BD treatment; the arrow indicates second dose administration, and **(F)** *Cannabis sativa* hexane fraction 15 mg treatment. Data represents the mean ± SEM of the total numbers of retches + vomits occurring during 1 h intervals ($n = 7$–8).

TABLE 1 | Effect of *Cannabis sativa* hexane fraction (CS-HexFr) or standard metoclopramide (MCP) on cisplatin-induced Retching plus Vomiting ($R + V$) and jerking during a 24 h observation period.

Drug treatment	Dose and route	Pigeons *n*/vomited	$R + V$ Episodes Mean ± SEM	Latency (min) Mean ± SEM	Jerks Mean ± SEM	Wt loss (%) Mean ± SEM
Vehicle + Cisplatin	2 ml/kg i.m. + 7 mg/kg i.v.	8/8	41 ± 2.4	67 ± 3.2	570 ± 63	15.1 ± 1.4
MCP + Cisplatin	30 mg/kg i.m. + 7 mg/kg i.v.	8/8	23 ± 1.1**	195 ± 41.2*	361 ± 25	11.9 ± 1.1
CS-HexFr + Cisplatin	5 mg/kg i.m. + 7 mg/kg i.v.	7/7	33 ± 5.9	185 ± 41	429 ± 69	9.1 ± 1.2
	10 mg/kg i.m. + 7 mg/kg i.v.	6/6	17 ± 3.4**	269 ± 114	301 ± 73	9.4 ± 1.7
	15 mg/kg i.m. + 7 mg/kg i.v.	8/8	27 ± 2.1	231 ± 39	435 ± 51	10.3 ± 1.1
	10 mg/kg i.m BD + 7 mg/kg i.v.	8/8	14.1 ± 2.9**	254 ± 70*	239 ± 59*	8.9 ± 1.0*

*$P < 0.05$, **$P < 0.01$ as compared to cisplatin control (ANOVA followed by Tukey's post hoc test).

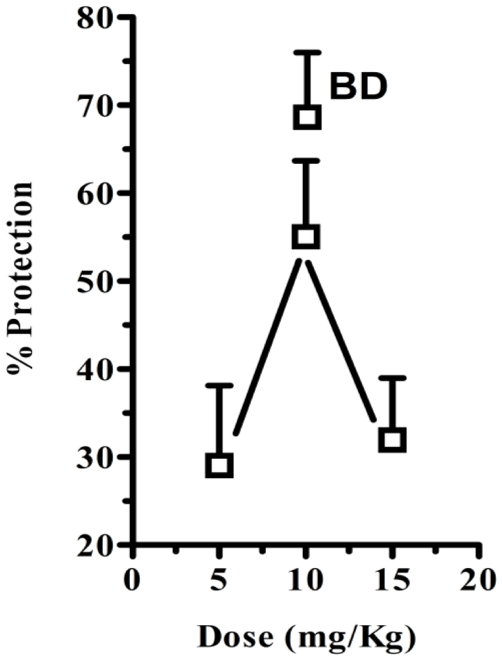


FIGURE 3 | Percent protection observed by either once daily dose of *Cannabis sativa* hexane fraction (OD; 5, 10, and 15 mg/kg) or twice daily (BD; 10 mg/kg) 30 min before cisplatin challenge. The values represent mean ± SEM of 5–8 determinations.

14.1 ± 2.9 (65.85% protection), respectively ($P < 0.01$; **Table 1**) during 24 h of observation period. The CS-HexFr was found to be effective as it suppressed $R + V$ up to 16 h of observation period while standard metoclopramide provided protection up to 8 h (**Figure 4**). CS-HexFr 10 mg BD and standard MCP increased the latency to first vomit significantly ($P < 0.01$).

None of the treatments induced vomiting when administered alone.

Effect of MCP or CS-HexFr on Cisplatin-Induced Jerks and Weight Loss

In the cisplatin control group, animals lost ∼15% of their starting body weight. The body weight loss in the standard MCP (30 mg/kg) treated group was 11.9 ± 1.1%, while CS-HexFr (10 mg/kg BD) reduced body weight loss up to 8.9% ($P < 0.05$, **Table 1**). All other treatments failed to reduce body weight loss significantly. The jerking behavior (reflects the vomiting intensity in pigeons; one vomiting episode may contain 2–80 jerks) observed in cisplatin control and standard MCP groups were 570 ± 63 and 361 ± 25, respectively, while no treatment decreased the jerking behavior up to the observation period (24 h) except CS-HexFr (10 mg/kg BD) where the jerking episodes were reduced significantly (570 ± 63 → 239 ± 59; $P < 0.05$, **Table 1**).

Effect of Standard MCP or CS-HexFr on Basal Level of Neurotransmitters and Their Metabolites in the Brain Areas and Small Intestine

The standard MCP treatment reduced the concentration of 5HIAA in the area postrema ($P < 0.05$) and brain stem ($P < 0.001$) as compared to basal level. In addition, the decrease in the concentration of HVA was also observed in the area postrema ($P < 0.05$, **Table 2**). As depicted in **Table 2**, treatment with CS-HexFr (10 mg/kg) had no significant effects on NA, DA and its metabolites DOPAC and HVA, 5HT and its metabolite 5HIAA in the brain areas (AP and BS) and intestine. Though, the concentration of DA at the level of AP and intestine was increased significantly ($P < 0.001$) as compared to basal level.

Effect of Standard MCP or CS-HexFr on the Level of Neurotransmitters and Their Metabolites in the Brain Areas and Small Intestine at 3rd Hour After Cisplatin Administration

Cisplatin treatment significantly increased the concentration of 5-hydroxytryptamine (5HT) in the brainstem and intestine ($P < 0.001$; **Table 3**) as compared to basal level, while a non-significant increase was observed in the area postrema. In addition, cisplatin also caused a significant increase in the concentration of 5HIAA in the area postrema ($P < 0.05$), brain stem ($P < 0.001$), and intestine ($P < 0.001$). The treatment with standard MCP at the dose of 30 mg/kg failed to change the concentration of NA, DOPAC, DA, and HVA in all the brain areas (AP and BS) and intestine, but reduced the concentration of 5HT in the area postrema, brain stem, and intestine significantly ($P < 0.05$–0.001) as compared to cisplatin control (**Table 3**). In addition to its inhibitory effects on 5HT, MCP also decreased

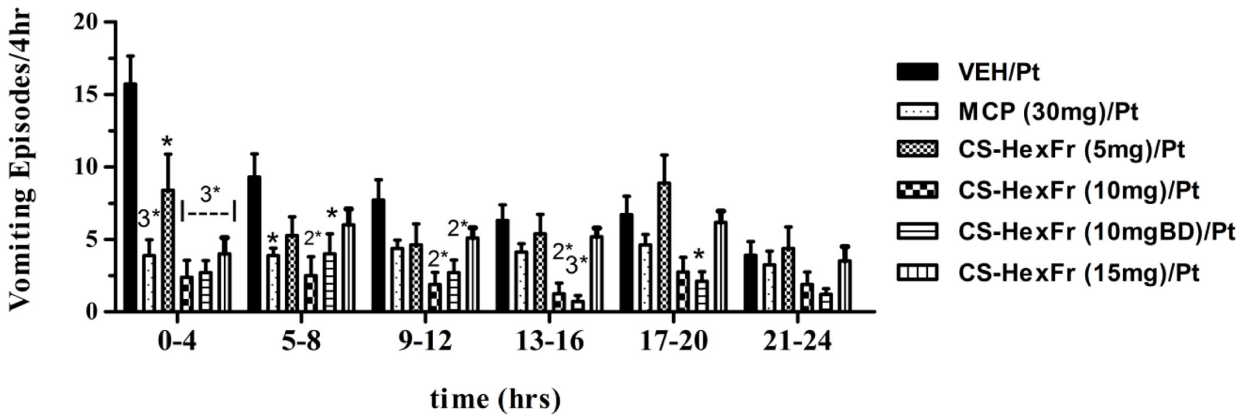


FIGURE 4 | The vomiting suppression time profile in pigeons of standard metoclopramide (MCP; 30 mg/kg) or *Cannabis sativa* hexane fraction (CS-HexFr; 5, 10, and 15 mg/kg) against cisplatin-induced vomiting during a 24 h observation period; each bar represents the mean ± SEM of vomiting episodes occurring during 4 h periods ($n = 5$–8). Values significantly different from cisplatin control are denoted as $^*P < 0.05$, $2^*P < 0.01$, $3^*P < 0.001$ (ANOVA followed by Tukey's *post hoc* test).

TABLE 2 | Effect of standard metoclopramide (MCP) or *Cannabis sativa* hexane fraction (CS-HexFr) on basal level of neurotransmitters (ng/mg tissue wet weight) and their metabolites in brain areas and the small intestine of pigeons.

Treatment	NA	DOPAC	DA	5HIAA	HVA	5HT
Area postrema						
Vehicle	0.590 ± 0.011	0.401 ± 0.101	0.530 ± 0.046	0.141 ± 0.021	0.793 ± 0.067	0.059 ± 0.010
MCP 30 mg	0.020 ± 0.003	0.013 ± 0.002	0.013 ± 0.010	0.004 ± 0.001*	0.119 ± 0.033*	0.011 ± 0.001
CS-HexFr 10 mg	0.579 ± 0.500	0.094 ± 0.026	1.888 ± 0.547***	0.260 ± 0.087	1.335 ± 0.323	0.126 ± 0.106
Brain stem						
Vehicle	0.089 ± 0.030	0.059 ± 0.013	0.164 ± 0.065	0.063 ± 0.020	0.040 ± 0.001	0.010 ± 0.010
MCP 30 mg	0.127 ± 0.073	0.034 ± 0.004	0.061 ± 0.020	0.005 ± 0.001***	0.071 ± 0.024	0.013 ± 0.001
CS-HexFr 10 mg	0.012 ± 0.003	0.098 ± 0.002	0.342 ± 0.039	0.038 ± 0.003	0.015 ± 0.000	0.031 ± 0.000
Intestine						
Vehicle	0.121 ± 0.039	0.053 ± 0.010	0.070 ± 0.050	0.048 ± 0.049	0.051 ± 0.024	0.071 ± 0.023
MCP 30 mg	0.153 ± 0.029	0.061 ± 0.021	0.060 ± 0.017	0.089 ± 0.012	0.124 ± 0.100	0.057 ± 0.011
CS-HexFr 10 mg	0.021 ± 0.001	0.063 ± 0.029	1.291 ± 0.273***	0.219 ± 0.045	0.102 ± 0.042	0.011 ± 0.002

*$P < 0.05$, ***$P < 0.001$ as compared to saline (ANOVA followed by Tukey's post hoc analysis; n = 6–8).

5HIAA concentration in both the brain areas (AP and BS) and intestine significantly ($P < 0.01$–0.001, **Table 3**). CS-HexFr (10 mg/kg) significantly reduced the 5HIAA and 5HT concentrations in the brain areas (AP and BS) and intestine ($P < 0.001$) while no effects were seen on the levels of NA and DOPAC. On the contrary, CS-HexFr (10 mg) treatment caused an increase in the concentration of DA in AP, BS, and intestine that was significant ($P < 0.001$) as compared to the cisplatin control (**Table 3**).

Effect of Standard MCP or CS-HexFr on the Level of Neurotransmitters and Their Metabolites in the Brain Areas and Small Intestine at 18th Hour After Cisplatin Administration

Cisplatin increased the level of DA significantly ($P < 0.001$) in the AP, while a non-significant trend toward increase was observed in the brain stem (**Table 4**). 5HT concentrations were also raised in the area postrema ($P < 0.01$), brain stem ($P < 0.001$) and intestine ($P < 0.001$), without effecting the levels of NA, DOPAC, 5HIAA, HVA (**Table 4**). Treatment with standard metoclopramide (MCP; 30 mg/kg) significantly decreased the upsurge of DA in the area postrema ($P < 0.001$; **Table 4**). Furthermore, a decrease in the concentration of 5HT was also observed in the area postrema ($P < 0.01$), brain stem and intestine ($P < 0.001$) and 5HIAA concentration ($P < 0.01$) in the area postrema as compared to cisplatin control (**Table 4**). *Cannabis sativa* hexane fraction (CS-HexFr) at the dose of 10 mg/kg decreased significantly ($P < 0.001$) the upsurge in the concentration of DA in the brain area of AP ($P < 0.001$) while decrease in 5HT was observed in the brain stem and intestine ($P < 0.001$; **Table 4**).

DISCUSSION

In the present study, we screened *n*-hexane fraction of *Cannabis sativa* (CS-HexFr) against cisplatin-induced retching and vomiting ($R + V$) in the pigeon vomiting model, where it was found to be effective to attenuate cisplatin-induced $R + V$.

TABLE 3 | Effect of standard metoclopramide (MCP) or *Cannabis sativa* hexane fraction (CS-HexFr) on neurotransmitters (ng/mg tissue wet weight) and their metabolites in brain areas and small intestine 3 h after cisplatin treatment in pigeons.

Treatment	NA	DOPAC	DA	5HIAA	HVA	5HT
Area postrema						
Vehicle	0.605 ± 0.298	0.217 ± 0.100	0.618 ± 0.218	0.087 ± 0.039	0.805 ± 0.166	0.113 ± 0.060
Cisplatin	1.799 ± 1.101	0.302 ± 0.091	0.091 ± 0.021	0.401 ± 0.109$^{\#}$	0.459 ± 0.139	0.265 ± 0.101
MCP 30 mg	0.110 ± 0.078	0.142 ± 0.050	0.310 ± 0.137	0.026 ± 0.006**	0.040 ± 0.021	0.030 ± 0.005*
CS-HexFr 10 mg	0.265 ± 0.034	0.638 ± 0.133	2.142 ± 0.387***	0.045 ± 0.012***	1.140 ± 0.162	0.030 ± 0.010**
Brain stem						
Vehicle	0.119 ± 0.043	0.031 ± 0.030	0.253 ± 0.152	0.013 ± 0.001	0.090 ± 0.016	0.018 ± 0.000
Cisplatin	0.081 ± 0.021	0.161 ± 0.127	0.029 ± 0.001	0.040 ± 0.003$^{\#\#\#}$	0.027 ± 0.002	0.147 ± 0.010$^{\#\#\#}$
MCP 30 mg	0.041 ± 0.021	0.039 ± 0.003	0.013 ± 0.002	0.021 ± 0.001***	0.023 ± 0.001	0.008 ± 0.000***
CS-HexFr 10 mg	0.026 ± 0.001	0.013 ± 0.001	0.436 ± 0.020***	0.009 ± 0.001***	0.013 ± 0.004	0.010 ± 0.003***
Intestine						
Vehicle	0.337 ± 0.045	0.087 ± 0.035	0.133 ± 0.031	0.032 ± 0.010	0.089 ± 0.046	0.048 ± 0.051
Cisplatin	0.301 ± 0.012	0.011 ± 0.010	0.031 ± 0.004	0.305 ± 0.016$^{\#\#\#}$	0.041 ± 0.010	0.545 ± 0.105$^{\#\#\#}$
MCP 30 mg	0.109 ± 0.040*	0.029 ± 0.001	0.246 ± 0.183	0.031 ± 0.006***	0.067 ± 0.030	0.041 ± 0.005***
CS-HexFr 10 mg	NA	0.067 ± 0.039	0.920 ± 0.130***	0.003 ± 0.001***	0.030 ± 0.012	0.001 ± 0.000***

*Neurotransmitter or metabolite levels significantly different from cisplatin control are denoted by *P < 0.05, **P < 0.01, ***P < 0.001, while values significantly different from basal levels are denoted by $^{\#}$P < 0.05, $^{\#\#\#}$P < 0.001 (ANOVA followed by Tukey's post hoc analysis; n = 6–8).*

TABLE 4 | Effect of standard metoclopramide (MCP) or *Cannabis sativa* hexane fraction (CS-HexFr) on neurotransmitters (ng/mg tissue wet weight) and their metabolites in brain areas and the small intestine at 18 h after cisplatin treatment in pigeons.

Treatment	NA	DOPAC	DA	5HIAA	HVA	5HT
Area postrema						
Vehicle	0.494 ± 0.063	0.337 ± 0.138	0.491 ± 0.169	0.219 ± 0.030	0.854 ± 0.121	0.010 ± 0.001
Cisplatin	0.279 ± 0.063	0.061 ± 0.011	5.066 ± 1.301$^{\#\#\#}$	0.201 ± 0.014	0.548 ± 0.121	0.160 ± 0.041$^{\#\#}$
MCP 30 mg	0.182 ± 0.092	0.062 ± 0.030	0.098 ± 0.029***	0.019 ± 0.002**	0.322 ± 0.178	0.008 ± 0.003**
CS-HexFr 10 mg	0.392 ± 0.052	0.254 ± 0.154	0.818 ± 0.232***	0.104 ± 0.016	0.294 ± 0.144	0.105 ± 0.011
Brain stem						
Vehicle	0.072 ± 0.005	0.074 ± 0.015	0.070 ± 0.030	0.118 ± 0.021	0.027 ± 0.014	0.001 ± 0.000
Cisplatin	0.079 ± 0.010	0.011 ± 0.001	0.163 ± 0.031	0.032 ± 0.030	0.010 ± 0.001	0.119 ± 0.011$^{\#\#\#}$
MCP 30 mg	0.008 ± 0.003	0.002 ± 0.001	0.019 ± 0.030	0.011 ± 0.002	0.080 ± 0.042	0.011 ± 0.001***
CS-HexFr 10 mg	0.138 ± 0.021	0.033 ± 0.014	0.116 ± 0.045	0.022 ± 0.005	0.210 ± 0.122	0.024 ± 0.006***
Intestine						
Vehicle	0.289 ± 0.181	0.119 ± 0.053	0.162 ± 0.071	0.001 ± 0.000	0.033 ± 0.020	0.053 ± 0.025
Cisplatin	0.203 ± 0.034	0.060 ± 0.001	0.159 ± 0.051	0.341 ± 0.061	0.071 ± 0.005	0.503 ± 0.078$^{\#\#\#}$
MCP 30 mg	0.167 ± 0.047	0.013 ± 0.001	0.023 ± 0.020	0.021 ± 0.010	0.421 ± 0.402	0.040 ± 0.005***
CS-HexFr 10 mg	0.392 ± 0.052	0.254 ± 0.154	0.818 ± 0.232	0.104 ± 0.016	0.294 ± 0.144	0.105 ± 0.011***

*Neurotransmitter and metabolites values significantly different from cisplatin control are denoted by **P < 0.01, ***P < 0.001, while values significantly different from basal level are indicated by $^{\#\#}$P < 0.05, $^{\#\#\#}$P < 0.001 (ANOVA followed by Tukey's post hoc analysis; n = 6–8).*

CS-HexFr at the dose of 10 mg/kg once and twice daily dosing provided up to 58.53% (17 ± 3.4 episodes) and 65.85% (14.1 ± 2.9 episodes) protection, respectively (**Table 1**). The *n*-hexane extract has been reported to contain cannabis major active constituent Delta-9-tetrahydrocannabinol (Δ^9- THC) which has been in use for the treatment of various diseases including management of CIV in clinics and the enhancement of appetite. Δ^9- THC is also found to have anti-inflammatory, spasmolytic, analgesic, and anti-glaucoma activity (Carlini, 2004). Furthermore, Sallan et al. (1975) have shown that the active component of *CS* (Δ^9- THC) has anti-emetic effects, by its ability to stimulate presynaptic cannabinoid CB_1 receptors (Darmani, 2001) and subsequent inhibition of monoamine neurotransmitters release (Darmani et al., 2003).

Metoclopramide (MCP), a clinically relevant anti-emetic with dopamine and 5-HT_3 receptor antagonist properties (Al-Zubaidy and Mohammad, 2005), was used as a positive control. The dose of MCP that we selected is higher than that required to antagonize cisplatin-induced emesis in other species (Zhang et al., 2006), and was based on a previous study in the pigeon showing activity against reserpine-induced emesis (Coronas et al., 1975). The metoclopramide was selected as standard drug because of the intrinsic emetic activity of $5HT_3$ receptor antagonists in pigeon (unpublished data).

Cisplatin which belongs to the highly emetogenic class of cancer chemotherapeutic agents is in use for the screening of anti-emetic potential of current anti-emetic agents. Cisplatin (4–10 mg/kg) has been used by several investigators to induce vomiting in pigeons (Feigenbaum et al., 1989b; Wolff and Leander, 1995). However, Tanihata et al. (2000) used a lower dose of 4 mg/kg and longer observation periods (∼72 h). In fact, our colony of pigeons had shown a reliable vomiting response at 7 mg/kg up to 24 h of observation period (Ullah et al., 2014, 2015) and we therefore, used the dose of 7 mg/kg to induce emesis.

The current evidences about the involvement of neurotransmitters implicate the overlap of serotonergic, dopaminergic, and neurokininergic systems in the whole time course of cisplatin-induced vomiting (Darmani et al., 2009). The neurotransmitters especially 5HT (serotonin) and dopamine are considerable mediators of vomiting induced by cancer chemotherapy treatments (Johnston et al., 2014) and the detection of neurotransmitter metabolites in biological samples suggest the involvement of serotonin and dopamine in the triggering of vomiting response (Veyrat-Follet et al., 1997; Gralla et al., 1999). Furthermore, substance P also plays an important role in the mediation of the vomiting response. Since increased turnover of 5-HT, dopamine and substance P occur during both phases of vomiting in the brainstem and intestine in vomit-competent animals as well as humans (Darmani et al., 2009), further investigations are needed to investigate the effects of substance P in both the phases of vomiting. At 3rd h, the reduction in the concentration of 5HT and the metabolite 5HIAA by CS-HexFr (10 mg) in the brain areas (AP and BS) and intestine (**Table 3**) correlate well with the suppression of the vomiting response, where serotonin has been reported to be the mediator of acute vomiting response of cisplatin-induced vomiting (Higgins et al., 2012; Ullah et al., 2014). Similarly, the reduction in the concentration of dopamine in the area postrema and 5HT concentration in the brain areas and intestine at 18th h of cisplatin administration (**Table 4**) further support the anti-emetic action of CS-HexFr later in the emetic episode. At 3rd h of cisplatin administration, dopamine concentration has been quantified as significantly high ($P < 0.001$) in the area postrema, brain stem and intestine, which is paradoxical with regard to the anti-emetic effect of CS-HexFr. The paradox can be hypothesized of (1) Switching of efficacy from agonist to antagonist of cannabis active constituent (2) Differential interaction with Gi or Gs signal transduction proteins (3) Pharmacokinetic factors, etc. (Darmani, 2010).

A number of studies suggest the involvement of CB_1 receptor activation for the anti-emetic action of *Cannabis sativa* (Δ^9-THC) (Darmani, 2001; Van Sickle et al., 2003) against various emetogenic agents. The CB_1 receptors are co-localized with $5HT_3$ receptors in the nucleus tractus solitarius (NTS) in the brain stem and Gastrointestinal tract (GIT) (Hermann et al., 2002), where the action of THC on these receptors inhibit the release of monoamines, especially 5HT, in the least shrew model (Darmani and Johnson, 2004) and Pigeon model (present study). Our study provides further evidence for the involvement of serotonin and dopamine (and their metabolites) in the control of cisplatin-induced emesis over a 24 h period in the pigeon. Our current data in **Figure 1** (VEH/Pt) does not support the time periods as acute- and delayed-emetic phases, which indicate that the acute emetic phase is probably between first and second hour post-cisplatin injection, and there is no identifiable delayed phase since the frequency of emesis/jerks gradually declines and there is no upsurge of emesis per hour later on through 24 h observation period. The two time points, i.e., 3rd and 18th h post-cisplatin administration have been selected to find a clue for any mechanistic differences in the mediation of cisplatin-induced vomiting throughout the observation period in pigeons.

In summary, this study provides evidence for the involvement of serotonin and dopamine differentially at the two different time points in the triggering of vomiting response by cisplatin in pigeons. Furthermore, the suppression of the behavioral signs of cisplatin-induced vomiting by CS-HexFr is supported by attenuation of the cisplatin-induced 5HT upsurge at acute time point (3rd h) and dopamine and 5HT upsurge at delayed time point (18th h).

AUTHOR CONTRIBUTIONS

IU conceived the project, performed experimental work, data collection, analysis, literature search, and manuscript preparation. FS supervised research work, helped in study design, and drafted the final version of the manuscript. MS, JA, and MA helped in project design, behavioral experiments, performed statistical analysis, and corrected the final version of the manuscript. All authors read and approved the final manuscript for publication.

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Endocannabinoid System and Migraine Pain: An Update

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The trigeminovascular system (TS) activation and the vasoactive release from trigeminal endings, in proximity of the meningeal vessels, are considered two of the main effector mechanisms of migraine attacks. Several other structures and mediators are involved, however, both upstream and alongside the TS. Among these, the endocannabinoid system (ES) has recently attracted considerable attention. Experimental and clinical data suggest indeed a link between dysregulation of this signaling complex and migraine headache. Clinical observations, in particular, show that the levels of anandamide (AEA)—one of the two primary endocannabinoid lipids—are reduced in cerebrospinal fluid and plasma of patients with chronic migraine (CM), and that this reduction is associated with pain facilitation in the spinal cord. AEA is produced on demand during inflammatory conditions and exerts most of its effects by acting on cannabinoid (CB) receptors. AEA is rapidly degraded by fatty acid amide hydrolase (FAAH) enzyme and its levels can be modulated in the peripheral and central nervous system (CNS) by FAAH inhibitors. Inhibition of AEA degradation via FAAH is a promising therapeutic target for migraine pain, since it is presumably associated to an increased availability of the endocannabinoid, specifically at the site where its formation is stimulated (e.g., trigeminal ganglion and/or meninges), thus prolonging its action.

Keywords: migraine, inflammation, trigeminal hyperalgesia, endocannabinoid system, FAAH inhibitors

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INTRODUCTION

Migraine is one of the most disabling painful conditions and a very common disorder (Global Burden of Disease, 2015). Although the pathophysiology of migraine is still largely elusive, the trigeminovascular system (TS) activation and the neurogenic inflammation of the dura mater are widely recognized as two key mechanisms underlying migraine attacks (Moskowitz, 1993). TS activation causes neuropeptide release from trigeminal endings in proximity of the meningeal vessels. Meningeal release of mediators produces peripheral sensitization, which is aggravated by central sensitization when the attacks recur more frequently. Calcitonin gene-related peptide (CGRP) and other inflammatory mediators, released from sensory nerve terminals (Moskowitz, 1993), irritate and further dilate blood vessels, thus resulting in the release of additional neuropeptides from the sensory neurons and an increase of pain impulses that are transmitted to the nucleus trigeminalis caudalis (NTC). The activated NTC relays in turn pain signals to higher brain centers, including thalamus and cortex. In this circuitry, another interesting player is nitric oxide (NO), which contributes to the perivascular sensory afferent nerve fibers

activation in the meninges and to neuropeptides release or NO formation by neuronal NO synthase (nNOS) (Messlinger et al., 2000; Alaşehirli et al., 2013; Ramachandran et al., 2014). Evidence suggests that the origin of migraine attacks is the interaction of internal or external triggers with dysfunctional central structures (brainstem, thalamus) involved in the transmission and regulation of pain sensation (Goadsby, 2002; Knight et al., 2005; Coppola et al., 2013).

Current standards of care for migraine have a moderate effectiveness at best and, in some cases, limited tolerability. Specifically, prophylactic treatments (beta blockers, antiepileptic drugs) may induce weight gain, depression, behavioral or cognitive disturbances. Triptans, 5-HT1-Receptor agonists, used for acute treatment, may cause a serious long-term side effects such us chest pain, neck and limbs with paresthesias and hot or cold sensations. Analgesics combinations and non-steroidal anti-inflammatory drugs, for acute migraine can lead to gastrointestinal and cardio-renal side effects (Antonaci et al., 2016). The endocannabinoid system (ES) has recently received attention in regard to pain control, after the availability of probes capable of modulating its activity via the interaction with endocannabinoid catabolic enzymes (Chiou et al., 2013). In this review, we summarize results collected in studies aimed at evaluating the role of the endocannabinoids (ECs) in migraine, with a specific focus on fatty acid amide hydrolase (FAAH) inhibitors.

ENDOCANNABINOID SIGNALING

EC signaling in the nervous system is different from those of the classic neurotransmission systems, where the depolarization of the presynaptic neuron causes neurotransmitters release which in turn activates their receptors on the postsynaptic neuron. The ECs act as retrograde or local neurotransmitters, and are produced and released from neurons upon demand. They bind to CB1-type cannabinoid (CB_1) receptors, which are localized on presynaptic terminals of excitatory and inhibitory neurons throughout the brain and spinal cord (Alger and Kim, 2011). CB_1 receptors are seven trans-membrane domain proteins that belong to the rhodopsin family of G protein-coupled receptors, specifically those of the Gi/o family (GPCRs). Recent crystallographic studies reported that extracellular surface of CB1 receptor is different from other lipid-activated GPCRs with a critical part of the ligand binding pocket (Hua et al., 2016; Shao et al., 2016). CB_1 receptors are found in neuroanatomical regions involved in pain processing, and inhibit the release of neurotransmitters such as γ-aminobutyric acid, glutamate, dopamine, noradrenaline and acetylcholine (Katona and Freund, 2008). Though abundant in neurons of the central nervous system (CNS), CB_1 receptors are also expressed in peripheral neurons and many non-neural cells (Kendall and Yudowski, 2017). A second cannabinoid receptor subtype, CB_2, is found primarily in immune cells (Gerdeman et al., 2002). Furthermore AEA and 2-arachidonoylglycerol (2-AG), the best characterized ECs, are produced in structures involved in nociception, such as the skin, dorsal root ganglia, spinal cord, periaqueductal gray matter (PAG), and rostral ventromedial medulla (RVM) (Katona and Freund, 2008). Through activation of CB_1 receptors, AEA and 2-AG can influence a variety of physiological processes, including energy balance, cognition and pain (Bellocchio et al., 2008; Kano et al., 2009).

In neurons, as in other cells, the ECs are not stored in vesicles but are enzymatically produced upon demand from membrane glycerophospholipid precursors. Enzymes involved in AEA and 2-AG formation are N-acylphosphatidylethanolamine-phospholipase D (NAPE-PLD) and diacylglycerol lipase (DGL), respectively (Bisogno et al., 2003; Okamoto et al., 2007). However, other pathways through which AEA can be produced have been described (Liu et al., 2006; Jin et al., 2007). Moreover, several enzymes involved in ECs biosynthesis, such as NAPE-PLD, give rise not only to AEA but also to structurally similar lipid messengers that do not bind and activate CB_1, i.e., oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) (Gaetani et al., 2010). AEA acts primarily on CB_1 receptors, though pharmacological actions on other receptors, such as transient receptor potential (TRP) V1, have been described (Puente et al., 2011), TRPV2, TRPA1, and TRPM8 (Di Marzo and De Petrocellis, 2010).

2-AG production also occurs via multiple biosynthetic pathways, in which diacylglycerol (DAG), produced by the action of either phospholipase C (PLC) or phosphatidic acid phosphohydrolase, acts as a common precursor. DAG is transformed into 2-AG by DGL; alternatively, phospholipase A_1 may convert phosphatidyl-inositol into lyso-phosphatidyl-inositol, which may be transformed to 2-AG by PLC.

The ECs are quickly deactivated by uptake into cells followed by intracellular hydrolysis (Urquhart et al., 2015). Transporter proteins remove AEA from the extracellular space; successively AEA is mostly degraded by FAAH, releasing arachidonic acid (AA) and ethanolamine. 2-AG is hydrolyzed mainly by the serine hydrolase, monoacylglycerol lipase (MGL), which produces AA and glycerol. However, it may be also degraded by α,β-hydrolase-6 or converted to bioactive oxygenated products by COX2. Thus, the enzymes responsible for the biosynthetic, as well as degradative pathways are essential in the regulation and modulation of EC levels in the CNS. Moreover, differential cellular distribution of the synthesizing and degrading enzymes may control of EC activity. Thus, selective pharmacological or genetic manipulations of FAAH and MGL activities can be used to evaluate the functions of each EC in animal model.

RELATIONSHIP BETWEEN ES DYSREGULATION AND MIGRAINE: HUMAN AND EXPERIMENTAL STUDIES

The ES may modulate the cerebrovascular tone, through interaction with serotonergic system, NO synthesis, and neuropeptides release (Pertwee, 2001), neurotransmitters that play a crucial role in migraine pathogenesis. CB_1 receptors have been localized in potential generators of migraine pain, like PAG, RVM, and NTC (Moldrich and Wenger, 2000). There are reports

that frequency of migraine headache may decrease in persons using medical cannabis (Rhyne et al., 2016). ECs may interact with and modulate several pathways related to migraine, such as opioids, or involved in the mechanism of action of anti-migraine drugs such as triptans (Akerman et al., 2013; Baron, 2015). AEA and other CB agonists have also been demonstrated to inhibit effects on serotonin type 3 receptors, which provide yet another effect when considering that nausea and vomiting are frequent and bothersome accompaniments of migraine (Fan, 1995; Park et al., 2008). CB agonists inhibit the serotonin-induced current in a concentration dependent manner in the rat nodose ganglion neurons by 5-HT3 receptor ion-channel (Fan, 1995). Moreover, they may also act on brain areas involved in emesis, such as the dorsal motor nucleus of the vagus (Van Sickle et al., 2001), where there is a high density of 5-HT3 receptors (Miquel et al., 2002). 5-HT3 inhibition can modulate neurotransmitters, including dopamine, GABA, substance P, and acetylcholine. The anti-migraine effects of the ES are not fully known, although some hypotheses were proposed. **Table 1** shows the potential modulatory effects of ECs on migraine pain.

Clinical observations show that women migraine without aura or episodic tension-type headache have increased FAAH and endocannabinoid membrane transporter (EMT) activities in platelets, which is consistent with reduced AEA levels (Cupini et al., 2006). In addition, women with episodic migraine have increased CB_1 receptor binding during the interictal period, as assessed by positron emission tomography; this increase is especially evident in brain regions that exert top-down influences to modulate pain (Van der Schueren et al., 2012). Variants in the CB_1 receptor gene increase the risk of migraine attack with nausea in life stress exposed subjects (Juhasz et al., 2017). Recently Gouveia-Figueira et al. (2017) failed to detect significant changes in the plasma levels of AEA and other fatty acid ethanolamides between patients with episodic migraine and controls. These contrasting findings may be related to higher inter-subject variability of EC levels in the evaluated cohorts or to a different migraine load on the populations investigated.

More consistent are the findings regarding the involvement of the ES in chronic migraine (CM). Subjects with CM with and without medication overuse headache (MOH) showed reduced activities of FAAH and EMT in platelets when compared to either controls or episodic migraine (Cupini et al., 2008). In another study, 2-AG and AEA platelet levels were significantly lower in MOH and CM patients compared to controls, without significant differences between the two patient groups (Rossi et al., 2008). These findings suggest an adaptive behavior induced by chronic headache *per se*, while medication overuse is apparently not related with EC activity. Interestingly, serotonin levels were reduced in the MOH and CM patients, with lower values detected in females as compared to males (Rossi et al., 2008) and that serotonin levels were also associated with 2-AG tone, with a higher correlation coefficient for MOH patients. This latter finding suggests a possible role for 2-AG, together with serotonin, in the "addiction" aspect of MOH. In this frame, it is worth mentioning that successful detoxification of MOH subjects is accompanied by a reduction in FAAH activity in platelets. This biochemical change is associated with the normalization of neurophysiological parameters that indicated a status of sensitization of the pain pathways (Perrotta et al., 2012). A reduction in AEA and an increase in PEA levels was also found in the cerebrospinal fluid of both CM and MOH patients (Sarchielli et al., 2007), pointing to a central alteration of ES in these subjects.

Inflammation and nerve injury cause changes in local AEA levels (Jhaveri et al., 2007). As mentioned before, AEA is produced on demand during inflammatory conditions and it is rapidly degraded by FAAH activity. Thus, AEA tone can be modulated by FAAH activity in both periphery and CNS. Increased activation of the TS may theoretically lead to reduced levels of AEA, which may, in turn, lead to an increased CGRP and NO release. AEA indeed inhibits the neurogenic dural vasodilatation, as well as CGRP-induced and NO-induced dural vessel dilation (Akerman et al., 2004). The CB_1 receptor antagonist, AM251, reversed this inhibitory activity, suggesting that CB_1 receptors may be implicated in the relationship between headache and dural blood vessel dilation and migraine mediators. Cortical spreading depression (CSD) is a self-propagating wave of neuronal hyperexcitability that has a role in migraine (Goadsby, 2007). WIN55,212-2, a CB1 receptor agonist, inhibited the amplitude, duration and velocity of CSD propagation, while JWH 133, a CB2 receptor agonist, was devoid of any effect (Kazemi et al., 2012).

The trigeminal firing in the trigeminocervical complex induced by AEA inhibition is reversed after CB_1 receptor antagonism, thus suggesting that the central effects of AEA are principally CB_1-mediated (Akerman et al., 2007). CB_1 receptor activation in the ventrolateral PAG, obtained with the administration of WIN55,212-2, attenuates the activity evoked by dural stimulation in A-fiber neurons and the basal spontaneous

TABLE 1 | Potential effects of endocannabinoids on migraine pain.

Target	Effects	References
Trigeminovascular activation	Substance P ↓ CGRP/nitric oxide ↓ Cyclooxygenase ↓ PGE-2 synthesis ↓ glutamate release ↓	Pertwee, 2001; Akerman et al., 2004; Sarchielli et al., 2007; La Rana et al., 2008; Chiou et al., 2013
Serotonergic system	Serotonin release ↓ platelets aggregation ↓ 5-HT2A ↑	Volfe et al., 1985; Ohuoha et al., 1994; Boger et al., 1998; Rossi et al., 2008; Parker et al., 2011; Mendiguren et al., 2018
Brainstem	NF-κB activation ↓ kynurenine pathway modulation	Kelly and Chapman, 2001; Nagy-Grócz et al., 2016
Hypothalamus	Glutamate release ↓	Di et al., 2005
Periaqueductal gray	Proenkephalin expression ↑	Manzanares et al., 1998

activity in the trigeminocervical complex of rodent. These findings suggest that, in the brainstem, ECs may provide to descending modulation upon basal trigeminovascular neuronal tone and A-fiber dural-nociceptive responses, (Akerman et al., 2013). Changes in FAAH and MGL activities were found in the brainstem and hypothalamus of rats treated with nitroglycerin (NTG) (Greco et al., 2010b), a recognized animal model of migraine (Buzzi and Tassorelli, 2010). NTG in rat causes an increased sensitivity to nociceptive tests and c-fos protein expression in brain areas nuclei involved in migraine pain transmission, such as NTC (Greco et al., 2011a). The use of this model by us and other groups has allowed the in-depth exploration of the mechanisms underlying the modulation of the ECs and the nociceptive activation of the TS during a migraine attack. In particular, we reported an increased FAAH activity in the hypothalamus and in the medulla area, where NTC neurons are located, and an up-regulation of CB_1 receptor binding sites in the same areas (Greco et al., 2010b), suggesting a key role of AEA in the cephalic pain.

Our findings also show that AEA pretreatment significantly reduces NTG-induced behavioral nocifensive and NTG-induced neuronal activation in the NTC (Greco et al., 2011a); moreover, AEA may modulate central sensitization through TRPV1, COX2 expression and NF-κB inhibition in NTC (Nagy-Grócz et al., 2016). The CB2 receptors activation in pain modulation has been considered in the past, showing analgesic activity in several models of pain (Nackley et al., 2003, 2004; Quartilho et al., 2003). In our migraine model, we have also shown that CB_2 receptor activation significantly decreases nocifensive behavior of rats made hyperalgesic by NTG (Greco et al., 2014). Likewise, MGL inhibition, and the subsequent increase in central and/or peripheral levels of 2-AG, reduces NTG-induced hyperalgesia at the nociceptive tests, and attenuates c-Fos protein expression in brain areas implicated in the transmission or integration of cephalic pain (Greco et al., 2017).

RECENT ADVANCES ON FAAH INHIBITION IN MIGRAINE PAIN

Though the analgesic effects of cannabinoids are fairly well established, their use in therapy remains limited by their psychoactive properties (Borgelt et al., 2013). Recent safety concerns about FAAH inhibitors turned out to be ungrounded, and due to off-target effects. Clearly, the successful development of compounds that modulate ECs tone for the pain relief in humans will hinge on the ability to separate psychotropic effects from therapeutic ones, and to control for potential off-target interactions. Positive allosteric modulation of CB_1 receptor signaling may represent a safe analgesic alternative strategy that lacks tolerance, dependence and abuse liability (Khurana et al., 2017; Slivicki et al., 2017). Several studies show that also increasing ECs levels through the inhibition of catabolic enzymes, FAAH in particular, would decrease cannabimimetic side effects (Piomelli et al., 2006; Booker et al., 2012).

Besides AEA, FAAH degrades other fatty acid amides, which have several biological functions and mechanisms of action (Ahn et al., 2008). FAAH is contained in intracellular membranes of postsynaptic somata and dendrites of the mammalian brain (Gulyas et al., 2004). In many cerebral structures FAAH and CB_1 receptors cellular co-localization in cell bodies or dendrites in proximity of CB_1-expressing fibers (Egertová et al., 1998). Manipulations of full-length and transmembrane-truncated FAAH variants have offered a characterization of mechanisms of action (McKinney and Cravatt, 2005). In particular, these studies showed that, unlike most serine hydrolases, which use a histidine residue as a catalytic base, FAAH recruits a lysine to hydrolyze both amides and esters at equivalent rates (Patricelli and Cravatt, 1999). Numerous FAAH inhibitors have been developed and tested in animal models of disease (Jayamanne et al., 2006; Kinsey et al., 2009). In particular, the FAAH inhibition induces anti-inflammatory effects *in vivo* (Jayamanne et al., 2006; Booker et al., 2012; Wilkerson et al., 2017). In addition, mutant mice for FAAH enzyme in non-neuronal cells, but with FAAH activity conserved in peripheral and central neurons, have a phenotype in which basal nociceptive transmission is connected to the reduced responsiveness to pro-inflammatory mediators (Cravatt et al., 2004). Researchers suggest that AEA regulates nociceptive transmission primarily at the peripheral level (Calignano et al., 1998; Clapper et al., 2010; Piomelli and Sasso, 2014).

Numerous studies have shown that FAAH inhibition causes analgesia and reduces inflammation in animal models of acute inflammatory pain (Kinsey et al., 2010; Lodola et al., 2015; Nasirinezhad et al., 2015), but there is little information on their effects in migraine. Recently, it was reported that AEA modulates the analgesic activity in the orofacial area and that endomorphin-2-induced antinociception is mediated by μ and CB_1 receptors (Zubrzycki et al., 2017). Nozaki et al. (2015) demonstrated that NTG-induced mechanical allodynia and c-Fos protein in the NTC is abolished in FAAH-deficient mice or after URB597 treatment, a global FAAH inhibitor, via maintenance of central and peripheral AEA levels. When considering that NTG is thought to activate meningeal trigeminovascular terminals via the local NO formation (Reuter et al., 2001; Greco et al., 2011b), it is probable that URB597 interferes with this mechanism of peripheral sensitization. Accordingly, we have shown that a peripherally restricted FAAH inhibitor, the compound URB937, inhibits NTG-induced nocifensive behaviors (plantar and orofacial formalin test, tail flick test), neuronal activation in the NTC and locus coeruleus (Greco et al., 2015). In agreement with these data, URB937 decreases the c-Fos expression induced by plantar formalin injection in spinal cord regions involved in nociceptive processing by the CB_1 receptors (Clapper et al., 2010).

Thus, since URB937 acts only peripherally, it seems reasonable to hypothesize that its mechanism of action relies on the maintenance of higher levels of AEA released by nervous terminal located in the injured peripheral tissues (hindpaw, upper lip, tail) (Agarwal et al., 2007) or in the dura, with consequent CB_1 receptor activation in trigeminovascular endings. An additional mechanism, is probably represented by the blockade of NTG-induced inflammatory pathway mediated by NO in dura mater and/or trigeminal ganglia. In agreement

with this hypothesis, *in vitro* studies have shown that increased AEA tone, through the inhibition of its degradation or uptake, decreases the cytokines and NO levels (Correa et al., 2009, 2010).

OUTLOOK

Pain is a heterogeneous condition and it should be treated as such. With its lack of sensitivity to standard analgesic medications (Ong and De Felice, 2017), migraine pain is a case in point and—perhaps better than most other forms of pain—underscores the need for tailored therapies. The human data and preclinical studies reviewed here confirm the importance of FAAH-regulated AEA signaling in the processing of nociceptive signals outside the CNS (Greco et al., 2010a; Piomelli and Sasso, 2014) and specifically point to peripheral FAAH inhibition as a possible therapeutic opportunity for migraine pain. Future experiments should be aimed at unlocking the precise cellular mechanisms and neural circuits through which peripheral FAAH blockade exerts its analgesic effects in migraine pain, further exploring the ground for potential clinical trials.

AUTHOR CONTRIBUTIONS

RG: designed this review; CD and AZ: contributed to cited data from our group; DP and CT: revised the final version of the manuscript.

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MINI REVIEW
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Emerging Role of (Endo)Cannabinoids in Migraine

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In this mini-review, we summarize recent discoveries and present new hypotheses on the role of cannabinoids in controlling trigeminal nociceptive system underlying migraine pain. Individual sections of this review cover key aspects of this topic, such as: (i) the current knowledge on the endocannabinoid system (ECS) with emphasis on expression of its components in migraine related structures; (ii) distinguishing peripheral from central site of action of cannabinoids, (iii) proposed mechanisms of migraine pain and control of nociceptive traffic by cannabinoids at the level of meninges and in brainstem, (iv) therapeutic targeting in migraine of monoacylglycerol lipase and fatty acid amide hydrolase, enzymes which control the level of endocannabinoids; (v) dual (possibly opposing) actions of cannabinoids via anti-nociceptive CB1 and CB2 and pro-nociceptive TRPV1 receptors. We explore the cannabinoid-mediated mechanisms in the frame of the Clinical Endocannabinoid Deficiency (CECD) hypothesis, which implies reduced tone of endocannabinoids in migraine patients. We further discuss the control of cortical excitability by cannabinoids via inhibition of cortical spreading depression (CSD) underlying the migraine aura. Finally, we present our view on perspectives of Cannabis-derived (extracted or synthetized marijuana components) or novel endocannabinoid therapeutics in migraine treatment.

Keywords: migraine, cannabinoids, CGRP, nociception, marijuana, cannabinoid receptor, TRPV1

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INTRODUCTION

Migraine is a debilitating disorder most commonly characterized by a unilateral hemicranial pulsating headache often accompanied by a great variety of other symptoms such as sensory disturbances and nausea (Pavlovic et al., 2014; Russo, 2016). The full list of migraine criteria is provided in the latest version of Headache Classification (ICHD-3 beta, 2013). Due to its high prevalence and disruptive nature, the mechanisms contributing to migraine headache have been intensely studied over many decades but remain debatable. The current consensus states that migraine pain is caused by lowering of the threshold of nociceptive signal processing in response to release of pro-inflammatory agents. Migraine attack's initiation has been linked to both environmental and hormonal triggers (Pavlovic et al., 2014), which lead to pathophysiological changes due to a sterile neurogenic inflammation in meninges and activation of trigeminal sensory nerves (Pietrobon and Moskowitz, 2013; Gouveia-Figueira et al., 2017).

The multifaceted nature of migraine makes it difficult to define the exact criteria for clinical assessment, and may underlie the vast variability in the ways in which migraine patients respond to existing modes of treatment. Additionally, many of the anti-migraine therapies carry

adverse effects, a challenge which has caused discontinuation of research and development of potential anti-migraine drugs (Russo, 2015). For these reasons, introduction of new, more inclusive and effective modes of therapy is urgently needed.

Different parts of *Cannabis sativa* plant have been utilized for centuries in treatment of multitude of health conditions, and consumption of this plant is often associated with psychotropic effects such as mood fluctuations, intoxication, euphoria, increased heart rate, physical dependence upon long-term use, and cognitive impairment (Niyuhire et al., 2007). Regarding migraine pathology, the vital characteristics justifying the proposed use of medical cannabis include anticonvulsive (Rosenberg et al., 2015), analgesic, antiemetic (Parker et al., 2011), and anti-inflammatory effects (Nagarkatti et al., 2009). Mainly due to their potent analgesic action, marijuana-derived exogenous cannabinoids are currently being used for symptomatic and prophylactic treatment in many pain conditions (Oláh et al., 2017), including migraine-associated pain (Chakrabarti et al., 2015). The use of exogenous cannabinoids has been greatly debated as a mode of therapy during past years, but the recent changes in legislation have facilitated their use in several countries. Following the push by the public for increasing cannabinoid availability, the demand for research on cannabinoid substances has also escalated.

This review aims to take a look at the recent publications on the effectiveness and safety of cannabinoid-based migraine treatment, as well as studies of the mechanisms underlying therapeutic effects of these compounds. Based on our experience in experimental studies of migraine, we discuss our own and other available data on the potential applications of cannabinoid therapy in migraine treatment.

ENDOCANNABINOID SYSTEM: EXOGENOUS AND ENDOGENOUS AGONISTS

Endocannabinoid system (ECS) is a comprehensive signaling system present in virtually every cell type and playing a critical role in maintaining body homoeostasis (Aizpurua-Olaizola et al., 2017). ECS' numerous components include the enzymes responsible for synthesis of endocannabinoids (eCBs), specific receptors of eCBs, and the post-activity neutralizing pathways (Marco et al., 2012). Here we provide only a short overview of this complex system related to discussion of migraine pathology.

To date, several major and many less explored components of the ECS have been identified (Chakrabarti et al., 2015). The most prevalent eCBs are 2-arachidonoylglycerol (2-AG) and arachidonoylethanolamine (anandamide, AEA) (**Figure 1**). Overall, 2-AG is considered the primary signaling molecule and is abundantly expressed throughout the brain (Sugiura et al., 2002). The action of eCBs is mimicked by the main pharmacological components of marijuana, namely phytocannabinoids (pCBs), including the psychotropic Δ9-tetrahydrocannabinol (THC) and the non-psychotropic cannabidiol (CBD) (Oláh et al., 2017; **Figure 1**).

The ECS signals are relayed primarily by two receptors: type 1 cannabinoid receptor (CB1), which is one of the most abundant G-protein coupled receptor in the brain (Smith et al., 2017), and type 2 cannabinoid receptor (CB2), which is functionally related to CB1 but is expressed primarily in peripheral tissues (Chakrabarti et al., 2015). Both CB1 and CB2 are natively activated by eCBs 2-AG and AEA, but they also respond to binding of pCBs. Thus, THC is thought to act primarily via its potent activation of CB1 and CB2. The mechanism of action is less clear for CBD, which has been reported to affect more than 65 discrete molecular targets and to have varied effects outside of ECS (Bih et al., 2015).

One important issue remaining unsolved is how exactly eCBs are released from cells. The traditional dogma states that bioactive eCBs, unlike other neurotransmitters such as acetylcholine and dopamine, are produced "on-demand" (Marsicano et al., 2003). An alternative view suggests that eCBs may be pre-synthesized and stored, much like neurotransmitters (Maccarrone et al., 2010; Fonseca et al., 2013; Chakrabarti et al., 2015).

Endocannabinoid system is active in stress-responsive parts of central and peripheral nervous system, functioning to reduce pain and to alleviate neurodegenerative and inflammatory damage (Preedy, 2017; Smith et al., 2017). Short-term effects induced by eCBs have been shown to involve plastic changes in many brain areas affecting pain sensation (Oláh et al., 2017). All these mechanisms are linked, directly or indirectly, to the migraine pathology.

MAPPING ECS EFFECTS IN MIGRAINE MODELS – CENTRAL VS. PERIPHERAL

The importance of the trigeminovascular system (TGVS) in migraine pathophysiology is widely recognized by experts in the field. During a migraine attack, prolonged activation of the TGVS – comprising meningeal trigeminal nerves and vessels along with dural mast cells (MC) (**Figure 1**) — ultimately causes sensitization of higher order neurons in the central nervous system (CNS), leading to the persistent nociceptive signaling (Burstein et al., 2015). Furthermore, the resulting sensitization has been found to stimulate TGVS activity, creating a positive feedback loop (Eroli et al., 2017). The main migraine mediator associated with the TGVS system is the neuropeptide calcitonin gene-related peptide (CGRP), which promotes vasodilation and contributes to the sterile meningeal inflammation associated with sensitization of nociceptive pathway (Giniatullin et al., 2008; Villalón and Olesen, 2009; Pietrobon and Moskowitz, 2013; Dux et al., 2016; **Figure 1**). All three key meningeal structures (nerves, vessels and MC) can act as targets for the action of pCBs or eCBs. Several papers from P. Goadsby lab have shown that CGRP-induced dilation of dural blood vessels and neuronal pro-nociceptive activity could be reduced by AEA (Akerman et al., 2003, 2007). MC, populating the TGVS in large quantities and responding to CGRP with degranulation (**Figure 1**), likely play a triggering role in migraine (Levy, 2010; Kilinc et al., 2017). In particular serotonin, a major component of mast cell granules, is able to produce a robust activation of trigeminal

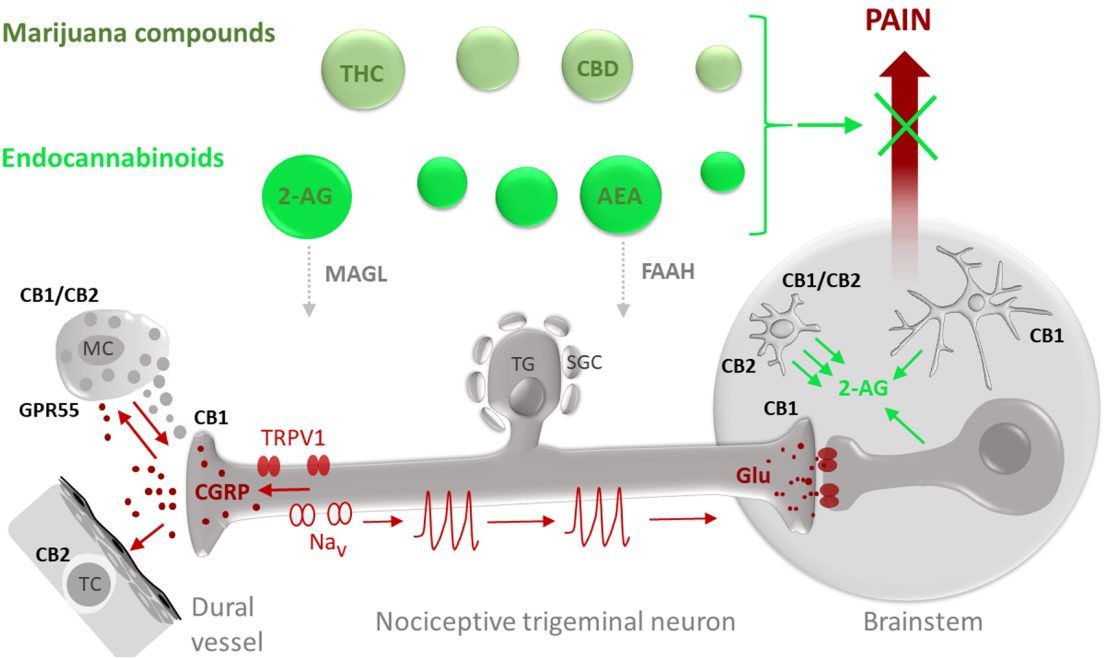


FIGURE 1 | Potential targets for the anti-nociceptive action of phyto-cannabinoids (marijuana compounds) and eCBs in migraine pain. Migraine-associated pain (pain propagating pathways are marked in brown) is generated in the TGVS comprising meningeal mast cells (MC), dural vessels and nociceptive trigeminal nerve fibers. Activation of pro-nociceptive TRPV1 receptors in sensory neurons which cell bodies are located in the trigeminal ganglion (TG) surrounded by satellite glial cells (SGC), results in release of the migraine mediator CGRP which can degranulate MC, provide a strong vasodilatory effect and target T-cells (TC). Degranulation of MC is associated with release of multiple pro-inflammatory compounds (5-HT, histamine, cytokines) supporting local neuroinflammation and sensitization of nociceptive fibers. Sensitized nerve fibers, via activation of certain subtypes of sodium channels (Nav), generate nociceptive firing (nociceptive spikes) propagated to the brainstem and, later, to the higher pain centers where this nociceptive traffic is perceived as migraine pain. eCBs and exocannabinoids (marked in green) including anandamide (AEA) and 2-arachidonoylglycerol (2-AG) as well as their exogenous counterparts THC and CBD, respectively, are promising agents to provide the anti-nociception in migraine. The anti-nociceptive effect of eCBs depends on their local concentrations, determined by the balance between the synthesis and degradation as well as availability and subtypes of their target receptors. The degradation of 2-AG is controlled by MAGL whereas hydrolysis of AEA is determined by the activity of FAAH. As some eCBs, such as AEA, can also activate TRPV1 receptors, migraine-associated pain is affected by a delicate balance between anti-nociceptive effects of CBs on specific cannabinoid receptors and pro-nociceptive effects on TRPV1 receptors. At peripheral site in meninges, CB1 receptors expressed in peripheral trigeminal nerve endings can contribute to anti-nociception by reducing probability of spike generation and reducing release of CGRP. Cannabinoid receptor type 1 (CB1) in the CNS is mainly expressed in neurons with predominant presynaptic location providing the inhibitory action of glutamate release in the brainstem. CB1 subtype is also expressed in astrocytes (Metna-Laurent and Marsicano, 2015). Cannabinoid receptor type 2 (CB2) and GPR55 are primarily expressed in cells of immune origin such as peripheral MC or T-cells as well as in microglial cells in the brain. Recent data also suggest heteromerization of CB1 and CB2 receptors in activated microglia. Notably, microglia are much more efficient than astrocytes and neurons (Walter et al., 2003) in producing the major eCB 2-AG which, in a paracrine way, can control neurons and, in autocrine manner, activate microglia.

afferents in meninges (Kilinc et al., 2017; **Figure 1**). Notably, eCB operating via CB1 receptors can stabilize MC (Sugawara et al., 2012) and this effect also contributes to the anti-migraine action of these compounds. However, other data suggest a role for CB2 and the orphan receptor GPR55 in the stabilizing action of cannabinoids on mast cell HMC-1 line (Cantarella et al., 2011). It is yet to be studied using migraine models, but similar mast cell stabilizing effect in meninges could potentially contribute to the anti-migraine action of cannabinoids (**Figure 1**).

Cannabinoid effects on the CNS are mediated primarily by inhibitory CB1 receptors, located throughout CNS as well as in afferent neurons (Marco et al., 2012). Both within CNS and peripherally, eCBs act as retrograde messengers or synaptic modulators for their respective target cells (Gabral et al., 2015). Thus, one of the main functions of the eCB 2-AG, degraded by the enzyme monoacylglycerol lipase (MAGL, Aaltonen et al., 2016), is to serve as a mediator of retrograde signaling to downregulate neurotransmitter release (Smith et al., 2017). Unlike the selective presynaptic inhibitory effect of adenosine on excitatory glutamatergic terminals (Safiulina et al., 2005), activation of CB1 receptor by eCB inhibits the release from presynaptic terminals of both inhibitory and excitatory neurotransmitters (Gabrielli et al., 2015; Iseger and Bossong, 2015).

CB2 receptor, being, like CB1 receptor, highly sensitive to 2-AG, possesses an individual set of expression patterns and characteristic functions. Thus, CB2 expression is higher in peripheral organs than in the CNS and is mostly restricted to the immune system cells including B and T lymphocytes (**Figure 1**). Endocannabinoid system contributes to both innate and adaptive immune responses, functioning as a preventative force against the onset of pro-inflammatory responses (Nagarkatti et al., 2009; Oláh et al., 2017). CB2 receptors are primarily responsible for exerting immunosuppressive effects in the periphery. During

an inflammatory reaction, which is expected in most severe or chronic forms of migraine, more of CB2 receptors are made available for activation (Gabral et al., 2015). In our recent study, familial migraine was found to be associated with enhanced concentrations of key inflammatory cytokines detected in blood (Khaiboullina et al., 2017). Thus, cannabinoids may act by correcting the dysregulation of cytokine production (Nagarkatti et al., 2009). Taken together, these studies suggest that the less explored CB2 receptors possessing the anti-inflammatory potential (Gabral et al., 2015) represent a promising target to counteract migraine (Scherma et al., 2016).

Besides their independent functions, CB1 and CB2 receptors have been shown to work together by forming hetero-receptor complexes (Callen et al., 2012). This type of receptor-receptor interaction has been shown recently for brain-residing immune cells such as microglia. Thus, it has been recently shown that, alongside CB2 receptors, the CB1-CB2 heteroreceptor complexes are expressed in microglia (Smith et al., 2017; Navarro et al., 2018; **Figure 1**). Microglia could play a part in the pathogenesis of migraine with aura, since the cortical spreading depression (CSD) associated with this type of migraine effectively activates microglia (Shibata and Suzuki, 2017). CSD also releases ATP (Karatas et al., 2013), which is a major driver of microglia, promoting release of the eCB 2-AG (Walter et al., 2003). Notably, the ability of microglia to secrete 2-AG is about 20-times higher than that of astrocytes and neurons (Walter et al., 2003). In view of the recent data, this link appears to be even more intriguing as microglia are essential for initiation of CSD (Pusic et al., 2014). Interestingly, this positive feedback loop could be disrupted by agonists of CB1 (but not of CB2 receptors), which block CSD (Kazemi et al., 2012). Consistent with growing interest to the medications targeting receptor heteromers, a study using the bivalent CB1 antagonist specifically affecting dimerized CB1 receptors, showed pain-alleviating effects (Zhang et al., 2010). Overall, di- and oligomerization of GPCRs within CNS represent an attractive therapeutic target in pain conditions (Borroto-Escuela et al., 2013; Fuxe and Borroto-Escuela, 2015).

In peripheral migraine mechanisms, activation of TRPV1 receptor, a non-selective cation channel expressed in trigeminal nociceptors, leads to massive CGRP release (Kageneck et al., 2014; **Figure 1**). Our and other studies indicate an important contribution of TRPV1 receptors to migraine pathology (Zakharov et al., 2015; Dux et al., 2016). Stimulation of dural sensory nerves by capsaicin was found to cause vasodilation modulated by CGRP via TRPV1 receptor (Dux et al., 2016). As the TRPV1 channels can also bind eCB AEA (Chakrabarti et al., 2015), this may result in unwanted pro-nociceptive action of cannabinoids, causing neuroinflammation in meninges. This complexity may explain why increased doses of cannabinoids diminished their analgesic effect (Kandasamy et al., 2018). It further creates an incentive for development of new synthetic CBs with minimal activity on TRPV1 receptors, or specific MAGL inhibitors, which, apart from triggering the accumulation of anti-nociceptive 2-AG, can decrease the level of the pro-nociceptive arachidonic acid (AA) and reduce pain (Aaltonen et al., 2016). MAGL inhibitors may also reduce the pro-nociceptive downstream products of AA such as endovanilloids, agonists of TRPV1 receptors (Hwang et al., 2000). Interestingly, the inhibition of fatty acid amide hydrolase (FAAH) degrading AEA is also anti-nociceptive in migraine models (Greco et al., 2015).

CLINICAL ENDOCANNABINOID DEFICIENCY (CECD) HYPOTHESIS

Endocannabinoid system's role in homeostatic upkeep highlights the importance of this system in maintaining overall health. Disruptions in supply or functionality of eCB ligands have been connected to numerous mental state disturbances and, particularly, to migraine. Migraine, along with comorbid conditions such as fibromyalgia and irritable bowel syndrome, share symptomatic commonalities of hyperalgesia as well as treatment resistance, likely stemming from common pathophysiological phenomenon: CECD. The CECD hypothesis suggests a correlation between deficient levels of eCB and pain (Russo, 2016).

Since the initial proposal of the CEDC in 2001, the importance of maintaining regular eCB levels was shown in a study comparing CB1- and CB2-KO mice that experienced inflammation, to mice lacking FAAH (and thus having elevated AEA) with reduced inflammation responses (Oláh et al., 2017). The lowered inhibitory activity of eCS in migraine, possibly due to reduced CB1 and CB2 receptor expression, serves as an assertion for the compensatory therapy with exogenous cannabinoids. According to the CECD hypothesis, treatment of migraine using exogenous cannabinoids could be achieved with low doses due to predisposition for elevated neuronal excitability. The CECD-causing deficiencies can appear for congenital reasons, or can be acquired.

PRO AND CONTRA OF CANNABINOIDS IN MIGRAINE TREATMENT

There is a long history of using cannabinoids for effective treatment of pain conditions. Due to their long-standing status of out-lawed substances (Baron, 2015), it is worth taking a look at the arguments still standing in the way of legalization. Overall, targeting ECS with peripherally acting drugs is a promising strategy for development of safe migraine treatments. However, there are still many insufficiently explored issues that may be detrimental for this seemingly harmless treatment.

Regularly experiencing chronic migraine pain can have adverse impacts on social relationships and job status which can lead into psychological distress (Ramsden et al., 2015). As it stands, the first 'pro' is that the treatment with pCB can acutely alleviate the resulting stress, in addition to tackling the initial cause by pain reduction.

In a study of the cannabis use for self-medication in Germany, Austria and Switzerland, 10.2% of patients reported using it

for migraine headache symptoms (Kandasamy et al., 2018). Another group found that the self-treatment outcome was highly variable, with low doses tending to alleviate migraine while higher doses even triggering headaches (Lu and Anderson, 2017). These findings call for creating a highly specific prescription for individual patients, which would be required for safe and successful treatment plan.

One of the main problems arising from the long-term usage of cannabis is the physical reliance on the pCBs, mainly THC. Moreover, there is evidence that patients can develop tolerance for pCBs (Kandasamy et al., 2018). Carelessly establishing a reliance on any form of medication may carry more ill effects on the patient's mentality, and may even lead to weakening or loss of pain relief.

A crucial point when considering cannabinoid treatment is that smoking marijuana is the most common method of pCB self-administration. When self-administering pCBs via smoking, the relief seekers often use marijuana mixed with tobacco leaves. In view of the recently established crosstalk between nicotinic cholinergic and ECS (Scherma et al., 2016), the nicotinic cholinergic system has been proposed as a molecular target for treating cannabis dependence (Scherma et al., 2016). Particularly interesting is the ability of the endogenous nicotinic antagonist kynurenic acid to counteract the addictive effects of CBs (Justinova et al., 2013). Notably, new derivatives of kynurenic acid were suggested recently as promising medicines for migraine (Greco et al., 2017) opening a new perspective for combined CB+antinicotinic therapy of this disorder. Interestingly, the main migraine mediator CGRP can reduce the activity of nicotinic receptors (Giniatullin et al., 1999), suggesting that the migraines associated with enhanced level of endogenous CGRP are 'pre-conditioned' to respond better to CB treatments.

Cannabidiol (CBD), the second most prevalent pCB, should also be explored in relation to migraine treatment. Unlike THC with its characteristic CB1 receptor affinity, CBD does not have intoxicating and psychoactive effects linked with CB1 receptor activation. Yet, CBD possesses anxiolytic (anxiety-reducing) and antipsychotic properties that have been suggested to be inflicted via interactions with TRPV1 and non-endocannabinoid GPR55 receptor (Bih et al., 2015). Recently, the US Food and Drug Administration (FDA) accepted an application for Epidiolex® (active agent CBD) in treatment of seizures prominent in Lennox-Gastaut syndrome (LGS) and Dravet syndrome (GW Pharmaceuticals, 2017). This stands as an important milestone paving the way for possible repurposing of this CBD-based drug for treating migraine, as well as other related neurological conditions.

CONCLUSION

In summary, cannabinoids – due to their anticonvulsive, analgesic, antiemetic, and anti-inflammatory effects – present a promising class of compounds for both acute and prophylactic treatment of migraine pain. In view of the rapidly unfolding changes in the legal status of cannabis, research on (endo)cannabinoids has become pertinent once again. Formal approval of a cannabinoid-based drug for other pathologies opens a possibility for repurposing these agents also to treat migraine. The abundance of CB1 receptors in the brain makes them an attractive target for treatment of migraine via blocking not only peripheral but also the central nociceptive traffic and reducing the pathologically enhanced cortical excitability predisposing to CSD. CB2 receptors in immune cells can be targeted to reduce the inflammatory component associated with severe forms of migraine. Exogenous compounds lacking the unwanted peripheral pro-nociceptive component or eCBs generated via inhibited degradation pathways and combined with other supportive agents are most desirable for this aim. Moreover, primary stratification of patients to identify and predict the effectiveness of cannabinoid treatment can greatly improve the efficiency of this approach.

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REVIEW
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Cannabidiol as a Promising Strategy to Treat and Prevent Movement Disorders?

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Movement disorders such as Parkinson's disease and dyskinesia are highly debilitating conditions linked to oxidative stress and neurodegeneration. When available, the pharmacological therapies for these disorders are still mainly symptomatic, do not benefit all patients and induce severe side effects. Cannabidiol is a non-psychotomimetic compound from *Cannabis sativa* that presents antipsychotic, anxiolytic, anti-inflammatory, and neuroprotective effects. Although the studies that investigate the effects of this compound on movement disorders are surprisingly few, cannabidiol emerges as a promising compound to treat and/or prevent them. Here, we review these clinical and pre-clinical studies and draw attention to the potential of cannabidiol in this field.

Keywords: cannabidiol, movement disorders, Parkinson's disease, Huntington's disease, dystonic disorders, cannabinoids

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CANNABIDIOL (CBD)

Cannabidiol (CBD) is one of the over 100 phytocannabinoids identified in *Cannabis sativa* (ElSohly and Gul, 2014), and constitutes up to 40% of the plant's extract, being the second most abundant component (Grlic, 1976). CBD was first isolated from marijuana in 1940 by Adams et al. (1940) and its structure was elucidated in 1963 by Mechoulam and Shvo (1963). Ten years later, Perez-Reyes et al. (1973) reported that, unlike the main constituent of cannabis Δ^9-tetrahydrocannabinol (Δ^9-THC), CBD does not induce psychological effects, leading to the suggestion that CBD was an inactive drug. Nonetheless, subsequent studies demonstrated that CBD modulates the effects of Δ^9-THC and displays multiple actions in the central nervous system, including antiepileptic, anxiolytic and antipsychotic effects (Zuardi, 2008).

Interestingly, CBD does not induce the cannabinoid tetrad, namely hypomotility, catalepsy, hypothermia, and antinociception. In fact, CBD mitigates the cataleptic effect of Δ^9-THC (El-Alfy et al., 2010). Clinical and pre-clinical studies have pointed to beneficial effects of CBD on the treatment of movement disorders. The first studies investigated CBD's actions on dystonia, with encouraging results. More recently, the studies have been focusing on Parkinson's (PD) and Huntington's (HD) diseases. The mechanisms whereby CBD exerts its effects are still not completely understood, mainly because several targets have been identified. Of note, CBD displays anti-inflammatory and antioxidant actions (Campos et al., 2016), and both inflammation and oxidative stress are linked to the pathogenesis of various movement disorders, such as PD (Farooqui and Farooqui, 2011; Niranjan, 2014), HD (Sánchez-López et al., 2012), and tardive dyskinesia (Zhang et al., 2007).

It is noteworthy that, when available, the pharmacological treatments for these movement disorders are mainly symptomatic and induce significant side effects (Connolly and Lang, 2014; Lerner et al., 2015; Dickey and La Spada, 2017). Nonetheless, despite its great clinical relevance, the studies evaluating CBD's role on the pharmacotherapy of movement disorders are surprisingly few. Here, we will review the clinical and pre-clinical evidence and draw attention to the potential of CBD in this field.

CBD'S MECHANISMS OF ACTION

CBD has several molecular targets, and new ones are currently being uncovered. CBD antagonizes the action of CB_1 and CB_2 receptors agonists, and is suggested to act as an inverse agonist of these receptors (Pertwee, 2008). Moreover, recent evidence point to CBD as a non-competitive negative allosteric modulator of CB_1 and CB_2 (Laprairie et al., 2015; Martínez-Pinilla et al., 2017). CBD is also an agonist of the vanilloid receptor TRPV1 (Bisogno et al., 2001), and the previous administration of a TRPV1 antagonist blocks some of CBD effects (Long et al., 2006; Hassan et al., 2014). In parallel, CBD inhibits the enzymatic hydrolysis and the uptake of the main endocannabinoid anandamide (Bisogno et al., 2001), an agonist of CB_1, CB_2 and TRPV1 receptors (Pertwee and Ross, 2002; Ross, 2003). The increase in anandamide levels induced by CBD seems to mediate some of its effects (Leweke et al., 2012). Moreover, in some behavioral paradigms the administration of an inhibitor of anandamide metabolism promotes effects similar to CBD (Pedrazzi et al., 2015; Stern et al., 2017).

CBD has also been shown to facilitate the neurotransmission mediated by the serotonin receptor 5-HT_{1A}. It was initially suggested that CBD would act as an agonist of 5-HT_{1A} (Russo et al., 2005), but the latest reports propose that this interaction might be allosteric or through an indirect mechanism (Rock et al., 2012). Although this interaction is not fully elucidated, multiple CBD's effects were reported to depend on 5-HT_{1A} activation (Espejo-Porras et al., 2013; Gomes et al., 2013; Pazos et al., 2013; Hind et al., 2016; Sartim et al., 2016; Lee et al., 2017).

The peroxisome proliferator-activated receptor γ (PPARγ) is a nuclear receptor involved in glucose metabolism and lipid storage, and PPARγ ligands have been reported to display anti-inflammatory actions (O'Sullivan et al., 2009). Data show that CBD can activate this receptor (O'Sullivan et al., 2009), and some of CBD effects are blocked by PPARγ antagonists (Esposito et al., 2011; Dos-Santos-Pereira et al., 2016; Hind et al., 2016). CBD also up-regulates PPARγ in a mice model of multiple sclerosis, an effect suggested to mediate the CBD's anti-inflammatory actions (Giacoppo et al., 2017b). In a rat model of Alzheimer's disease, CBD, through interaction with PPARγ, stimulates hippocampal neurogenesis, inhibits reactive gliosis, induces a decline in pro-inflammatory molecules, and consequently inhibits neurodegeneration (Esposito et al., 2011). Moreover, in an *in vitro* model of the blood-brain barrier, CBD reduces the ischemia-induced increased permeability and VCAM-1 levels—both effects are attenuated by PPARγ antagonism (Hind et al., 2016).

CBD also antagonizes the G-protein-coupled receptor GPR55 (Ryberg et al., 2007). GPR55 has been suggested as a novel cannabinoid receptor (Ryberg et al., 2007), but this classification is controversial (Ross, 2009). Currently, the phospholipid lysophosphatidylinositol (LPI) is considered the GPR55 endogenous ligand (Morales and Reggio, 2017). Although only few studies link the CBD effect to its action on GPR55 (Kaplan et al., 2017), it is noteworthy that GPR55 has been associated with PD in an animal model (Celorrio et al., 2017) and with axon growth *in vitro* (Cherif et al., 2015).

More recently, CBD was reported to act as inverse agonist of the G-protein-coupled orphan receptors GPR3, GPR6, and GPR12 (Brown et al., 2017; Laun and Song, 2017). GPR6 has been implicated in both HD and PD. Concerning animal models of PD, GPR6 deficiency was related to both diminished dyskinesia after 6-OHDA lesion (Oeckl et al., 2014), and increased sensitivity to MPTP neurotoxicity (Oeckl and Ferger, 2016). Moreover, Hodges et al. (2006) described decreased expression of GPR6 in brain of HD patients, compared to control. GPR3 is suggested as a biomarker for the prognosis of multiple sclerosis (Hecker et al., 2011). In addition, GPR3, GPR6, and GPR12 have been implicated in cell survival and neurite outgrow (Morales et al., 2018).

CBD has also been reported to act on mitochondria. Chronic and acute CBD administration increases the activity of mitochondrial complexes (I, II, II-III, and IV), and of creatine kinase in the brain of rats (Valvassori et al., 2013). In a rodent model of iron overload—that induces pathological changes that resemble neurodegenerative disorders—CBD reverses the iron-induced epigenetic modification of mitochondrial DNA and the reduction of succinate dehydrogenase's activity (da Silva et al., 2018). Of note, multiple studies associate mitochondrial dysfunctions with the pathophysiology of PD (Ammal Kaidery and Thomas, 2018).

In parallel, several studies show anti-inflammatory and antioxidant actions of CBD (Campos et al., 2016). CBD treatment decreases the levels of the pro-inflammatory cytokines IL-1β, TNF-α, IFN-β, IFN-γ, IL-17, and IL-6 (Watzl et al., 1991; Weiss et al., 2006; Esposito et al., 2007, 2011; Kozela et al., 2010; Chen et al., 2016; Rajan et al., 2016; Giacoppo et al., 2017b), and increases the levels of the anti-inflammatory cytokines IL-4 and IL-10 (Weiss et al., 2006; Rajan et al., 2016). In addition, it inhibits the expression of iNOS (Esposito et al., 2007; Pan et al., 2009; Chen et al., 2016; Rajan et al., 2016) and COX-2 (Chen et al., 2016) induced by distinct mechanisms. CBD also displays antioxidant properties, being able to donate electrons under a variable voltage potential and to prevent the hydroperoxide-induced oxidative damage (Hampson et al., 1998). In rodent models of PD and HD, CBD up-regulates the mRNA levels of the antioxidant enzyme superoxide dismutase (Garcia-Arencibia et al., 2007; Sagredo et al., 2007). In accordance, CBD decreases oxidative parameters in *in vitro* models of neurotoxicity (Hampson et al., 1998; Iuvone et al., 2004; Mecha et al., 2012). Of note, the anti-inflammatory and antioxidant effects of

CBD on lipopolysaccharide-stimulated murine macrophages are suppressed by a TRPV1 antagonist (Rajan et al., 2016). It has also been shown that CBD can affect the expression of several genes involved in zinc homeostasis, which is suggested to be linked to its anti-inflammatory and antioxidant actions (Juknat et al., 2012).

CBD's mechanisms of action are summarized in **Figure 1**.

PARKINSON'S DISEASE (PD)

PD is among the most common neurodegenerative disorders, with a prevalence that increases with age, affecting 1% of the population over 60 years old (Tysnes and Storstein, 2017). The disease is characterized by motor impairment (hypokinesia, tremors, muscle rigidity) and non-motor symptoms (e.g., sleep disturbances, cognitive deficits, anxiety, depression, psychotic symptoms) (Klockgether, 2004).

The pathophysiology of PD is mainly associated with the loss of midbrain dopaminergic neurons in the substantia nigra *pars compacta* (SNpc), with consequent reduced levels of dopamine in the striatum (Dauer and Przedborski, 2003). When the motor symptoms appear, about 60% of dopaminergic neurons is already lost (Dauer and Przedborski, 2003), hindering a possible early diagnosis. The most effective and used treatment for PD is L-DOPA, a precursor of dopamine that promotes an increase in the level of dopamine in the striatum, improving the motor symptoms (Connolly and Lang, 2014). However, after a long-term treatment the effect of L-DOPA can be unstable, presenting fluctuations in symptoms improvement (on / off effect) (Jankovic, 2005; Connolly and Lang, 2014). In addition, involuntary movements (namely L-DOPA-induced dyskinesia) appear in approximately 50% of the patients (Jankovic, 2005).

The first study with CBD on PD patients aimed to verify CBD's effects on the psychotic symptoms. Treatment with CBD for 4 weeks decreased the psychotic symptoms, evaluated by the Brief Psychiatric Rating Scale and the Parkinson Psychosis Questionnaire, without worsening the motor function or inducing adverse effects (Zuardi et al., 2009). Later, in a case series with four PD patients, it was verified that CBD is able to reduce the frequency of the events related to REM sleep behavior disorder (Chagas et al., 2014a). In addition, although not ameliorating PD patients' motor function or their general symptoms score, treatment with CBD for 6 weeks improves PD's patients quality of life (Chagas et al., 2014b). The authors suggest that this effect might be related to CBD's anxiolytic, antidepressant and antipsychotic properties (Chagas et al., 2014b).

Although the studies with patients with PD report beneficial effects of CBD only on the non-motor symptoms, CBD has been shown to prevent and/or reverse increased catalepsy behavior in rodents. When administered before the cataleptic agents haloperidol (antipsychotic drug), L-nitro-N-arginine (non-selective inhibitor of nitric oxide synthase) or WIN 55-212,2 (agonist of cannabinoid receptors), CBD hinders the cataleptic behavior in a dose-dependent manner (Gomes et al., 2013). A possible role of the activation of serotonin receptors 5-HT_{1A} in this action has been proposed, because this effect of CBD is blocked by the pre-treatment with the 5-HT_{1A} antagonist

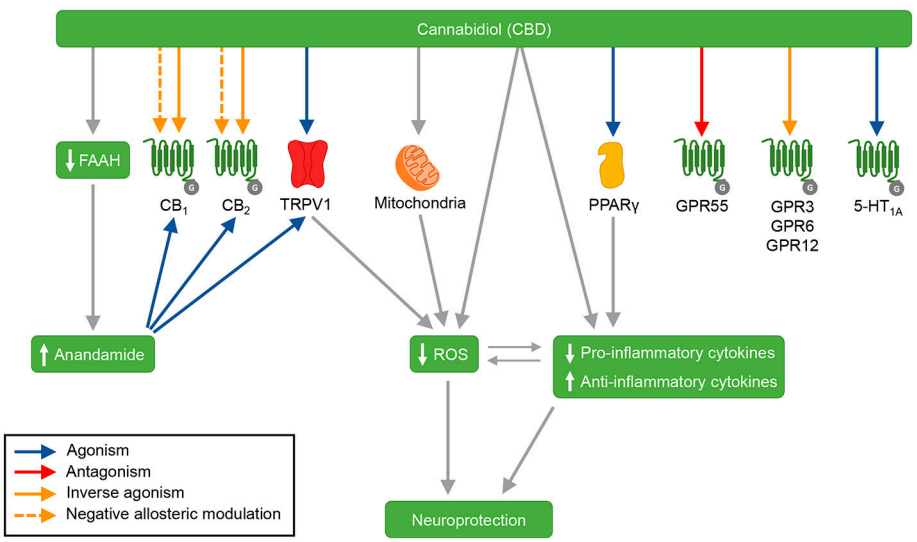


FIGURE 1 | CBD's mechanisms of action. CBD acts as agonist of the receptors TRPV1, PPARγ, and 5-HT_{1A}, and as antagonist of the receptor GPR55. CBD is an inverse agonist of the receptors GPR3, GPR6, and GPR12. Moreover, CBD antagonizes the action of CB_1 and CB_2 receptors agonists, and is suggested to act as an inverse agonist and a negative allosteric modulator of these receptors. CBD also inhibits FAAH, which results in increased anandamide levels. Anandamide activates CB_1, CB_2, and TRPV1 receptors. By acting on mitochondria, CBD increases the activity of mitochondrial complexes. In addition, CBD displays antioxidant and anti-inflammatory effects—that are partially mediated by CBD's actions on TRPV1, mitochondria and PPARγ. 5-HT_{1A}, serotonin receptor 1A; CB_1, cannabinoid receptor type 1; CB_2, cannabinoid receptor type 2; FAAH, fatty acid amide hydrolase; GPR3, G-protein-coupled receptor 3; GPR6, G-protein-coupled receptor 6; GPR12, G-protein-coupled receptor 12; GPR55, G-protein-coupled receptor 55; PPARγ, peroxisome proliferator-activated receptor gamma; ROS, reactive oxygen species; TRPV1, transient receptor potential vanilloid type 1.

WAY100635 (Gomes et al., 2013). In accordance, Sonego et al. (2016) showed that CBD diminishes the haloperidol-induced catalepsy and c-Fos protein expression in the dorsal striatum, also by a mechanism dependent on 5-HT_{1A} activation. Moreover, CBD prevents the increased catalepsy behavior induced by repeated administration of reserpine (Peres et al., 2016).

In addition, pre-clinical studies in animal models of PD have shown neuroprotective effects of CBD. The unilateral injection of the toxin 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle promotes neurodegeneration of nigrostriatal dopaminergic neurons, being used to model PD (Bové et al., 2005). When inside the cell, the neurotoxin 6-OHDA oxidizes in hydrogen peroxide and paraquinone, causing death mainly of catecolaminergic neurons (Breese and Traylor, 1971; Bové et al., 2005). This neurodegeneration leads to depletion of dopamine and decrease in tyrosine hydroxylase activity in caudate-putamen (Bové et al., 2005; Lastres-Becker et al., 2005). Treatment with CBD during the 2 weeks following 6-OHDA administration prevents these effects (Lastres-Becker et al., 2005). In another study, it was observed that CBD's protective effects after 6-OHDA injury are accompanied by elevation of mRNA levels of the antioxidant enzyme Cu,Zn-superoxide dismutase in substantia nigra (Garcia-Arencibia et al., 2007). The protective effects of CBD in this model do not seem to depend on the activation of CB_1 receptors (Garcia-Arencibia et al., 2007). In addition to preventing the loss of dopaminergic neurons—assessed by tyrosine hydroxylase immunostaining –, the administration of CBD after 6-OHDA injury attenuates the activation of microglia in substantia nigra (Garcia et al., 2011).

In an *in vitro* study, CBD increased the viability of cells treated with the neurotoxin N-methyl-4-phenylpyrimidine (MPP+), and prevented the MPP+-induced increase in caspase-3 activation and decrease in levels of nerve growth factor (NGF) (Santos et al., 2015). CBD treatment was also able to induce cell differentiation even in the presence of MPP+, an effect that depends on trkA receptors (Santos et al., 2015). MPP+ is a product of oxidation of MPTP that inhibits complex I of the respiratory chain in dopaminergic neurons, causing a rapid neuronal death (Schapira et al., 1990; Meredith et al., 2008).

Data from clinical and pre-clinical studies are summarized in **Tables 1**, **2**, respectively.

HUNTINGTON'S DISEASE (HD)

HD is a fatal progressive neurodegenerative disease characterized by motor dysfunctions, cognitive loss and psychiatric manifestations (McColgan and Tabrizi, 2018). HD is caused by the inclusion of trinucleotides (CAG) in the exons of the huntingtin gene, on chromosome 4 (MacDonald et al., 1993; McColgan and Tabrizi, 2018), and its prevalence is 1–10,000 (McColgan and Tabrizi, 2018). Neurodegeneration in HD affects mainly the striatal region (caudate and putamen) and this neuronal loss is responsible for the motor symptoms (McColgan and Tabrizi, 2018). Cortical degeneration is seen in later stages, and huntingtin inclusions are seen in few cells, but in all patients with HD (Crook and Housman, 2011). The diagnosis of HD is based on motor signs accompanied by genetic evidence, which is positive genetic test for the expansion of the huntingtin gene or family history (Mason and Barker, 2016; McColgan and Tabrizi, 2018).

The pharmacotherapy of HD is still directed toward the symptomatic relief of the disease, i.e., the motor disorders believed to be due to dopaminergic hyperactivity. This treatment is often conducted with typical and atypical antipsychotics, but in some cases the use of dopaminergic agonists is needed (Mason and Barker, 2016; McColgan and Tabrizi, 2018). Indeed, the role of dopamine in HD is not fully elucidated yet. Regarding the cognitive deficits, none of the investigated drugs was able to promote improvements (Mason and Barker, 2016; McColgan and Tabrizi, 2018).

Recently, there has been a growing number of studies aiming to verify the therapeutic potential of cannabinoid compounds in the treatment of HD, mainly because some cannabinoids present hypokinetic characteristics (Lastres-Becker et al., 2002). In a controlled clinical trial, patients with HD were treated with CBD for 6 weeks. There was no significant reduction in the chorea indicators, but no toxicity was observed (Consroe et al., 1991).

The protective effects of CBD and other cannabinoids were also evaluated in a cell culture model of HD, with cells expressing mutated huntingtin. In this model, the induction of huntingtin promotes rapid and extensive cell death (Aiken et al., 2004). CBD and the other three cannabinoid compounds tested—Δ^8-THC, Δ^9-THC, and cannabinol—show 51–84% protection against the huntingtin-induced cell death (Aiken et al., 2004). These effects seem to be independent of CB_1 activation, since absence of CB_1 receptors has been reported in PC12, the cell line used (Molderings et al., 2002). The authors suggest that the cannabinoids exert this protective effect by antioxidant mechanisms (Aiken et al., 2004).

Regarding studies with animal models, treatment with 3-nitropropionic acid (3-NP), an inhibitor of complex II of the respiratory chain, induces striatal damage—mainly by calpain activation and oxidative injury –, being suggested as relevant to study HD (Brouillet et al., 2005). Sub-chronic administration of 3-NP in rats reduces GABA contents and the levels of mRNA for several markers of striatal GABAergic neurons projections (Sagredo et al., 2007). In addition, 3-NP diminishes the levels of mRNA for the antioxidant enzymes superoxide dismutase-1 (SOD-1) and -2 (SOD-2) (Sagredo et al., 2007). The administration of CBD reverses or attenuates these 3-NP-induced alterations (Sagredo et al., 2007). CBD's neuroprotective effects are not blocked by the administration of antagonists of the CB_1, TRPV1 or A_{2A} receptors (Sagredo et al., 2007).

More recently, clinical and pre-clinical HD studies started to investigate the effects of Sativex® (CBD in combination with Δ^9-THC in an approximately 1:1 ratio). In accordance with what previously seen with CBD alone, Sativex administration attenuates all the 3-NP induced neurochemical, histological and molecular alterations (Sagredo et al., 2011). These effects do not seem to be linked to activation of CB_1 or CB_2 receptors (Sagredo et al., 2011). Authors also observed a protective effect of Sativex in reducing the increased expression of iNOS gene induced by malonate (Sagredo et al., 2011). Malonate administration leads to

TABLE 1 | Clinical studies investigating the effects of CBD on movement disorders.

Disease	Main Findings	Duration of Treatment	Dose of CBD and route of administration	Patients characteristics	References
PD	Open-label pilot study. Treatment with CBD for 4 weeks diminished the psychotic symptoms. CBD did not worsen the motor function or induce adverse effects.	4 weeks	150 mg/day of CBD, increasing by 150 mg every week, depending on patients' clinical response. Oral route.	6 PD patients (4 men and 2 women) with psychosis—not controlled with reduction of antiparkinsonian medications—for at least 3 months before the beginning of the study. Patients were in stable doses of anti-PD medication for at least 7 days.	Zuardi et al., 2009
PD	Case series. CBD reduced the frequency of the events related to REM sleep behavior disorder.	6 weeks	75 mg/day (3 patients) or 300 mg/day (1 patient) of CBD. Oral route.	4 PD male patients with REM sleep behavior disorder, with at least two episodes of complex sleep-related behaviors per week.	Chagas et al., 2014a
PD	Exploratory double-blind trial. Treatment with CBD did not improve the motor function or the general symptoms score, but the higher dose (300 mg/kg) improved quality of life.	6 weeks	75 or 300 mg/day of CBD. Oral route.	21 PD patients (15 men and 6 women) in stable doses of anti-PD medication for at least 30 days before the beginning of the study.	Chagas et al., 2014b
HD	Controlled clinical trial (double-blind randomized crossover). Treatment with CBD did not improve the symptoms, but it was not toxic.	6 weeks	10 mg/kg/day of CBD. Oral route.	15 patients (8 men and 7 women) with mild or moderate progression of HD, not taking antipsychotic drugs for at least 2 weeks before the beginning of the study.	Consroe et al., 1991
HD	Double-blind, randomized, cross-over, placebo-controlled, pilot trial. Sativex did not induce severe adverse effects or clinical worsening. However, Sativex did not improve patients' symptoms or promoted molecular changes on biomarkers.	12 weeks	Increasing doses of Sativex (CBD:THC in approximately 1:1 ratio) up to 12 sprays/day. Intranasal route.	25 HD (14 men and 11 women) patients with stable baseline medication for at least 6 weeks before the beginning of the study.	López-Sendón Moreno et al., 2016
HD	Case report of HD patients treated with cannabinoid. Cannabinoids improved UHDRS motor score and dystonia subscore.	6 or 9 months	Sativex: 12 or 7 sprays/day. Intranasal route.	2 male HD patients with complains of severe dystonia. Duration of the disease: 14 and 16 years.	Saft et al., 2018
Dystonic movement disorders	Open label study. Treatment with CBD resulted on 20–50% improvement of the dystonic symptoms. Two patients with simultaneous PD's signs showed worsening of their hypokinesia and/or resting tremor when receiving the higher doses of CBD (over 300 mg/day).	6 weeks	Increasing doses of CBD from 100 to 600 mg/day. Oral route.	5 patients (4 men and 1 woman) with dystonic movements, 2 with simultaneous parkinsonian symptoms.	Consroe et al., 1986
Dystonic movement disorders	Case report. CBD improved the dystonic symptoms without inducing adverse effects.	One administration	CBD 200 mg. Oral route.	2 patients: one woman with idiopathic spasmodic torticollis and one man with generalized torsion dystonia.	Sandyk et al., 1986

CBD, cannabidiol; HD, Huntington's disease; PD, Parkinson's disease; REM, rapid-eye movement; THC, Δ^9-tetrahydrocannabinol.

striatal damage by apoptosis and inflammatory events related to glial activation, being used as an acute model for HD (Sagredo et al., 2011; Valdeolivas et al., 2012).

In a subsequent study, it was observed that the administration of a Sativex-like combination attenuates all the malonate-induced alterations, namely: increased edema, decreased number of surviving cells, enhanced number of degenerating cells, strong glial activation, and increased expression of inflammatory markers (iNOS and IGF-1) (Valdeolivas et al., 2012). Although the beneficial effects of Sativex on cell survival are blocked by both CB_1 or CB_2 antagonists, CB_2 receptors seem to have a greater role in the protective effect observed (Valdeolivas et al., 2012).

The beneficial effects of Sativex have also been described in the R6/2 mice, a transgenic model of HD. Treatment with a Sativex-like combination, although not reversing animal's deterioration in rotarod performance, attenuates the elevated clasping behavior, that reflects dystonia (Valdeolivas et al., 2017).

TABLE 2 | Pre-clinical studies investigating the effects of CBD on movement disorders.

Model	Main findings	References
Hamster model of idiopathic paroxysmal dystonia	The higher dose of CBD shows a trend to delay the progression of dystonia.	Richter and Loscher, 2002
PC12 cells expressing mutated huntingtin	CBD and the other three cannabinoid compounds tested—Δ^8-THC, Δ^9-THC, and cannabinol—show 51–84% protection against the huntingtin-induced cell death. These protective effects seem to be independent of CB_1 receptors.	Aiken et al., 2004
Rats lesioned by the toxin 6-OHDA	Treatment with CBD for 2 weeks subsequent to lesion by the toxin 6-OHDA prevents the 6-OHDA-induced depletion of dopamine and decrease in tyrosine hydroxylase activity in caudate-putamen.	Lastres-Becker et al., 2005
Rats lesioned by the toxin 6-OHDA	Treatment with CBD for 2 weeks subsequent to lesion by 6-OHDA prevents the 6-OHDA-induced depletion of dopamine and decrease in tyrosine hydroxylase activity in caudate-putamen. CBD promoted upregulation of mRNA levels for the antioxidant enzyme Cu,Zn-superoxide dismutase. These protective effects do not seem to depend on activation of CB_1 receptors.	Garcia-Arencibia et al., 2007
Rats treated with 3-nitropropionic acid (3-NP)	Sub-chronic administration of 3-NP reduces GABA contents, levels of mRNA for several markers of striatal GABAergic neurons projections, and the levels of mRNA for the antioxidant enzymes superoxide dismutase-1 (SOD-1) and—2 (SOD-2). CBD reverses or attenuates the 3-NP-induced alterations. CBD's neuroprotective effects are not blocked by antagonists of the CB_1, $TRPV_1$ or A_{2A} receptors.	Sagredo et al., 2007
Rats lesioned by the toxin 6-OHDA	Treatment with CBD for 2 weeks subsequent to lesion by 6-OHDA prevents the 6-OHDA-induced decrease in tyrosine hydroxylase immunostaining, as well as enhanced microglial activation in the substantia nigra.	Garcia et al., 2011
Rats treated with 3-nitropropionic acid (3-NP) or malonate	Sub-chronic administration of 3-NP reduces GABA contents, diminishes the number of Nissl-stained neurons, down-regulates the expression of CB1 receptor and IGF-1, up-regulates the expression of calpain, and reduces the expression of superoxide dismutase-1 (SOD-1). Sativex (CBD and Δ^9-THC in an approximately 1:1 ratio) attenuates all the 3-NP-induced alterations. This effect is not blocked by antagonists of CB_1 or CB_2 receptors. In addition, rats treated with malonate display increased expression of the iNOS gene, reversed by the administration of Sativex.	Sagredo et al., 2011
Rats treated with malonate	Malonate increases edema, decreases the number of surviving cells, enhances the number of degenerating cells, induces strong glial activation, and increases the expression of the inflammatory markers iNOS and IGF-1. Sativex-like combination attenuates all malonate-induced alterations. Sativex effect seems to depend on both CB_1 and CB_2 receptors.	Valdeolivas et al., 2012
Mice injected with cataleptic agents	Pre-treatment with CBD dose-dependently attenuates the increase in catalepsy behavior induced by haloperidol, L-nitro-N-arginine or WIN 55,212-2. CBD's anticataleptic effect is prevented by the administration of WAY100635 (antagonist of 5-HT_{1A} receptors).	Gomes et al., 2013
PC12 cells treated with the toxin MPP+	CBD increases cell viability and prevents the MPP+-induced increase in caspase-3 activation and decrease in levels of NGF. CBD treatment also induces cell differentiation even in the presence of MPP+. CBD's effects on neuritogenesis seem to depend on trkA receptors.	Santos et al., 2015
Mice treated with L-DOPA	CBD, when administered with capsazepine, an antagonist of $TRPV_1$ receptors, decreases L-DOPA-induced dyskinesia. These effects are blocked by antagonists of CB_1 and $PPAR\gamma$ receptors. Treatment with capsazepine and CBD also decreases the expression of inflammatory markers (COX-2 and NFkB).	Dos-Santos-Pereira et al., 2016
Rats injected with the cataleptic and dyskinesia-inducing agent reserpine	Repeated administration of reserpine induces catalepsy, hypolocomotion, oral dyskinesia and impairment in the discriminative avoidance memory task. Concomitant treatment with CBD prevents the increase in catalepsy behavior, the oral dyskinesia and the memory deficit.	Peres et al., 2016
Mice injected with the cataleptic agent haloperidol	CBD prevents haloperidol-induced catalepsy and increase in c-Fos protein expression in the dorsolateral striatum. CBD also reverses the increase in catalepsy behavior induced by haloperidol. These CBD effects are prevented by the administration of WAY100635 (antagonist of 5-HT_{1A} receptors). CBD's anticataleptic effect is also observed when CBD is injected into the dorsal striatum.	Sonego et al., 2016
R6/2 mice (transgenic mouse models of HD)	Treatment with Sativex-like combination (from 4th to 12th weeks after birth) attenuated the R6/2 mice increased clasping behavior (that reflects dystonia) and reduced metabolic activity in basal ganglia. Sativex also reversed some of animals' alterations in markers of brain integrity, but not the deterioration in rotarod performance.	Valdeolivas et al., 2017

Δ^8-THC, Δ^8-tetrahydrocannabinol; Δ^9-THC, Δ^9-tetrahydrocannabinol; 3-NP, 3-nitropropionic acid; 6-OHDA, 6-hydroxydopamine; CBD, cannabidiol; HD, Huntington's disease; IGF-1, insulin growth factor 1; iNOS, inducible nitric oxide synthase; MPP+, 1-methyl-4-phenylpyridinium; NGF, nerve growth factor; PD, Parkinson's disease; SOD, superoxide dismutase.

Moreover, treatment mitigates R6/2 mice reduced metabolic activity in basal ganglia and some of the alterations in markers of brain integrity (Valdeolivas et al., 2017).

In spite of the pre-clinical encouraging results with Sativex, a pilot trial with 25 HD patients treated with Sativex for 12 weeks failed to detect improvement in symptoms or molecular changes on biomarkers (López-Sendón Moreno et al., 2016). Nonetheless, Sativex did not induce severe adverse effects or clinical worsening (López-Sendón Moreno et al., 2016). The authors suggest that future studies, with higher doses and/or longer treatment periods, are in need. More recently, one study described the results of administering cannabinoid drugs to 7 patients (2 of them were treated with Sativex; the others received dronabinol or nabilone, agonists of the cannabinoid receptors): patients displayed improvement on UHDRS motor score and dystonia subscore (Saft et al., 2018).

Tables 1, **2** summarize data from clinical and pre-clinical studies, respectively.

OTHER MOVEMENT DISORDERS

Dystonias are the result of abnormal muscles tone, causing involuntary muscle contraction, and resulting in repetitive movements or abnormal posture (Breakefield et al., 2008). Dystonias can be primary, for instance paroxysmal dyskinesia, or secondary to other conditions or drug use, such as tardive dyskinesia after prolonged treatment with antipsychotic drugs (Breakefield et al., 2008).

Consroe et al. (1986) were the first to evaluate the effects of CBD alone in movement disorders. In this open label study, the five patients with dystonic movement disorders displayed 20–50% improvement of dystonic symptoms when treated with CBD for 6 weeks. Of note, two patients with simultaneous PD's signs showed worsening of their hypokinesia and/or resting tremor when receiving the higher doses of CBD. However, it should be noted that in two more recent studies with PD patients no worsening of motor function was seen (Zuardi et al., 2009; Chagas et al., 2014b). In accordance, Sandyk et al. (1986) reported improvement of dystonic symptoms in two patients—one with idiopathic spasmodic torticollis and one with generalized torsion dystonia—after acute treatment with CBD.

The effects of CBD on dystonic movements were also evaluated in pre-clinical studies. In a hamster model of idiopathic paroxysmal dystonia, the higher dose of CBD showed a trend to delay the progression of dystonia (Richter and Loscher, 2002). In addition, CBD prevents the increase in vacuous chewing movements, i.e., dyskinesia, promoted by repeated administration of reserpine (Peres et al., 2016). CBD's beneficial effects are also seen in L-DOPA-induced dyskinesia in rodents, but only when CBD is administered with capsazepine, an antagonist of TRPV1 receptors (Dos-Santos-Pereira et al., 2016). These effects seem to depend on CB_1 and PPARγ receptors (Dos-Santos-Pereira et al., 2016). In addition, treatment with capsazepine and CBD decreases the expression of inflammatory markers, reinforcing the suggestion that the anti-inflammatory actions of CBD may be beneficial to the treatment of dyskinesia (Dos-Santos-Pereira et al., 2016).

Moreover, Sativex has been used in the treatment of spasticity in multiple sclerosis. Spasticity is a symptom that affects up to 80% of patients with multiple sclerosis and is associated with poorer quality of life (Flachenecker et al., 2014). A significant portion of patients does not respond to the conventional anti-spasmodic therapies, and some strategies are invasive, posing risks of complications (Flachenecker et al., 2014; Crabtree-Hartman, 2018). Recent data point to Sativex as a valid and well-tolerated therapeutic option. Sativex is able to treat the spasms, improving the quality of life, and displays a low incidence of adverse effects (Giacoppo et al., 2017a).

Data from clinical and pre-clinical studies are summarized in **Tables 1**, **2**, respectively.

SAFETY AND SIDE EFFECTS

One important concern is whether CBD is a safe therapeutic strategy. Several preclinical and clinical reports show that CBD does not alter metabolic and physiological parameters, such as glycemia, prolactin levels, blood pressure, and heart rate. In addition, CBD does not modify hematocrit, leukocyte and erythrocyte counts, and blood levels of bilirubin and creatinine in humans. CBD also does not affect urine osmolarity, pH, albumin levels, and leukocyte and erythrocyte counts. Moreover, *in vitro* studies demonstrate that CBD does not alter embryonic development nor the vitality of non-tumor cell lines. The most reported side effects of CBD are tiredness, diarrhea, and changes on appetite. CBD does not seem to induce tolerance. For a broad review of CBD's side effects, see Bergamaschi et al. (2011) and Iffland and Grotenhermen (2017).

In the context of movement disorders with concomitant cognitive symptoms, as the ones discussed here, it is crucial to evaluate the potential motor and cognitive side effects of CBD. CBD does not induce catalepsy behavior in rodents—being even able to attenuate the effects of several cataleptic agents, as discussed above (El-Alfy et al., 2010; Gomes et al., 2013; Peres et al., 2016; Sonego et al., 2016). In accordance, CBD does not induce extrapyramidal effects in humans (Leweke et al., 2012).

With respect to cognitive effects, studies report that CBD does not impair cognition, being even able to improve it in some conditions. Pre-clinical data show that CBD restores the deficit in the novel object recognition task in mice treated with MK-801 (a protocol used to model schizophrenia) (Gomes et al., 2015), in rats submitted to neonatal iron overload (Fagherazzi et al., 2012), in a transgenic mice model for Alzheimer's disease (Cheng et al., 2014), and in a mice model for cerebral malaria (Campos et al., 2015). CBD also reverses impaired social recognition in a murine model for Alzheimer's disease (Cheng et al., 2014) and restores the deficits in the Morris water maze—a task that evaluates spatial learning—in rodent models for Alzheimer's disease (Martín-Moreno et al., 2011), brain ischemia (Schiavon et al., 2014) and cerebral malaria (Campos et al., 2015). In addition,

studies demonstrate that CBD *per se* does not modify animals' performance in cognitive tasks (Osborne et al., 2017; Myers et al., 2018) and does not induce withdrawal after prolonged treatment (Myers et al., 2018). In accordance, in one recent clinical trial using CBD as an adjunctive therapy for schizophrenia, CBD group displayed greater cognitive improvement (assessed by BACS—Brief Assessment of Cognition in Schizophrenia), although it fell short of significance (McGuire et al., 2018). CBD also improves facial emotion recognition in cannabis users (Hindocha et al., 2015).

It is noteworthy that in some cases, particularly concerning multiple sclerosis and HD clinical studies, CBD *per se* does not seem to be beneficial. However, when CBD is administered with Δ^9-THC in a 1:1 ratio, therapeutic effects are observed. Therefore, it is also important to evaluate the interactions between CBD and Δ^9-THC as well as the adverse effects of this mixture. Multiple reports point to deleterious effects of Δ^9-THC on human cognition, mainly on memory and emotional processing (Colizzi and Bhattacharyya, 2017). On the other hand, studies reveal that CBD can counteract Δ^9-THC detrimental cognitive effects in rodents and monkeys (Wright et al., 2013; Jacobs et al., 2016; Murphy et al., 2017). Nonetheless, this protective effect depends on the doses, on the interval between CBD and Δ^9-THC administration, as well as on the behavioral paradigm used. In fact, some pre-clinical studies do not observe the protective effect of CBD against the Δ^9-THC cognitive effects (Wright et al., 2013; Jacobs et al., 2016) or even show that CBD may potentiate them (Hayakawa et al., 2008). Limited clinical evidence indicate that CBD does not worse Δ^9-THC cognitive effects and, depending on the dose, may protect against them (Colizzi and Bhattacharyya, 2017; Englund et al., 2017; Osborne et al., 2017). Multiple clinical studies with Sativex have not observed motor or cognitive adverse effects (Aragona et al., 2009; Rekand, 2014; López-Sendón Moreno et al., 2016; Russo et al., 2016). Nevertheless, one recent open-label study compared multiple sclerosis patients who continued the treatment with Sativex to those who quitted and reported worse balance and decrease in cognitive performance in the continuers (Castelli et al., 2018). In line with these findings, in an observational study with a large population of Italian patients with multiple sclerosis, cognitive/psychiatric disturbances were seen in 3.9% of the cases (Patti et al., 2016).

CONCLUSIONS

The data reviewed here point to a protective role of CBD in the treatment and/or prevention of some movement disorders. Although the studies are scarce, CBD seems to be effective on treating dystonic movements, both primary and secondary. It is noteworthy that in some cases, particularly concerning multiple sclerosis and HD, the clinical beneficial effects are observed only when CBD is combined with Δ^9-THC in a 1:1 ratio (Sativex). In fact, these therapeutic effects are probably due to Δ^9-THC, since they are also seen with other cannabinoid agonists (Curtis et al., 2009; Nielsen et al., 2018; Saft et al., 2018). Nonetheless, CBD is shown to diminish the Δ^9-THC unwanted effects, such as sedation, memory impairments, and psychosis (Russo and Guy, 2006). Data regarding HD are scarce, but the results of using Sativex in multiple sclerosis are encouraging. Reviews of the clinical use of this compound in the last decade point to effectiveness in the treatment of spasticity as well as improvement in quality of life, with low incidence of adverse effects (Giacoppo et al., 2017a).

In respect to PD, although the pre-clinical studies are promising, the few studies with patients failed to detect improvement of the motor symptoms after treatment with CBD. There is a significant difference between the clinical and pre-clinical PD studies. In animals, the beneficial effects are seen when CBD is administered prior to or immediately after the manipulation that induces the PD-like symptoms. Of note, when treatment with CBD commences 1 week after the lesion with 6-OHDA, the protective effects are not seen (Garcia-Arencibia et al., 2007). These data suggest that CBD's might have a preventive role rather than a therapeutic one in PD. In clinical practice, PD is diagnosed subsequently to the emergence of motor symptoms—that appear up to 10 years after the beginning of neurodegeneration and the onset of non-motor symptoms (Schrag et al., 2015). When the diagnosis occur, approximately 60% of the dopaminergic neurons has already been lost (Dauer and Przedborski, 2003). The fact that in clinical trials CBD is administered only after this substantial progression of the disease might explain the conflicting results. Unfortunately, the early diagnosis of PD remains a challenge, posing difficulty to the implementation of preventive strategies. The development of diagnosis criteria able to detect PD in early stages would probably expand the CBD's applications in this disease.

The molecular mechanisms associated with CBD's improvement of motor disorders are likely multifaceted. Data show that it might depend on CBD's actions on 5-HT_{1A}, CB_1, CB_2, and/or PPARγ receptors. Moreover, all movement disorders are in some extent linked to oxidative stress and inflammation, and CBD has been reported to display an antioxidant and anti-inflammatory profile, *in vitro* and in animal models for movement abnormalities.

The studies investigating the role of CBD on the treatment of movement disorders are few. Furthermore, differences in the dose and duration of treatment as well as in the stage of the disease (for instance, PD patients are treated only in an advanced stage of the disease) among these studies (shown in detail in **Table 1**) limit the generalization of the positive effect of CBD and might explain the conflicting results. Notwithstanding, the beneficial neuroprotective profile of CBD added to the preliminary results described here are encouraging. Undoubtedly, future investigations are needed to endorse these initial data and to elucidate the mechanisms involved in the preventive and/or therapeutic potential of CBD on movement disorders.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest Statement: JH, and JC are co-inventors (Mechoulam R, JC, Guimaraes FS, AZ, JH, Breuer A) of the patent "Fluorinated CBD compounds, compositions and uses thereof. Pub. No.: WO/2014/108899. International Application No.: PCT/IL2014/050023" Def. US no. Reg. 62193296; 29/07/2015; INPI on 19/08/2015 (BR1120150164927). The University of São Paulo has licensed the patent to Phytecs Pharm (USP Resolution No. 15.1.130002.1.1). The University of São Paulo has an agreement with Prati-Donaduzzi (Toledo, Brazil) to "develop a pharmaceutical product containing synthetic cannabidiol and prove its safety and therapeutic efficacy in the treatment of epilepsy, schizophrenia, Parkinson's disease, and anxiety disorders." JH and JC have received travel support from and are medical advisors of BSPG-Pharm.

The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Cannabigerol Action at Cannabinoid CB_1 and CB_2 Receptors and at CB_1–CB_2 Heteroreceptor Complexes

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Cannabigerol (CBG) is one of the major phytocannabinoids present in *Cannabis sativa* L. that is attracting pharmacological interest because it is non-psychotropic and is abundant in some industrial hemp varieties. The aim of this work was to investigate in parallel the binding properties of CBG to cannabinoid CB_1 (CB_1R) and CB_2 (CB_2R) receptors and the effects of the compound on agonist activation of those receptors and of CB_1–CB_2 heteroreceptor complexes. Using [^{3}H]-CP-55940, CBG competed with low micromolar K_i values the binding to CB_1R and CB_2R. Homogeneous binding in living cells, which is only technically possible for the CB_2R, provided a 152 nM K_i value. Also interesting, CBG competed the binding of [^{3}H]-WIN-55,212-2 to CB_2R but not to CB_1R (K_i: 2.7 versus >30 μM). The phytocannabinoid modulated signaling mediated by receptors and receptor heteromers even at low concentrations of 0.1–1 μM. cAMP, pERK, β-arrestin recruitment and label-free assays in HEK-293T cells expressing the receptors and treated with endocannabinoids or selective agonists proved that CBG is a partial agonist of CB_2R. The action on cells expressing heteromers was similar to that obtained in cells expressing the CB_2R. The effect of CBG on CB_1R was measurable but the underlying molecular mechanisms remain uncertain. The results indicate that CBG is indeed effective as regulator of endocannabinoid signaling.

Keywords: cannabinoid receptor, cannabigerol, G-protein-coupled receptor, phytocannabinoid, TR-FRET, partial agonist

Abbreviations: Δ^8-THC, Δ^8-tetrahydrocannabinol; Δ^9-THC, Δ^9-tetrahydrocannabinol; Δ^9-THCA, Δ^9- tetrahy drocannabinolic acid; Δ^9-THCV, Δ^9-tetrahydrocannabivarin; 2-AG, 2-arachidonoyl glicerol; AEA, anandamide; CB_1R, cannabinoid receptor 1; CB_2R, cannabinoid receptor 2; CBC, cannabichromene; CBD, cannabidiol; CBDA, cannabidiolic acid; CBDV, cannabidivarin; CBG, cannabigerol; CBGA, cannabigerolic acid; CBN, cannabinol; CNS, central nervous system; DMR, dynamic mass redistribution; HEK, human embryonic kidney; HTRF, homogeneous time-resolved fluorescence; SNAP, protein used as a tag; it contains circa 180 amino acids and may be covalently labeled with different probes; Tb, terbium; TLB, Tag-lite labeling medium.

INTRODUCTION

Cannabinoid compounds bind and activate cannabinoid CB_1 (CB_1R) and CB_2 (CB_2R) receptors, which belong to the superfamily of G-protein-coupled receptors. There are many ways to classify them, but the most used distinguishes between endogenous molecules (endocannabinoids), phytocannabinoids and synthetic cannabinoids. Endocannabinoids and one of the most studied phytocannabinoids, Δ^9-tetrahydrocannabinol (Δ^9-THC), are agonists with more or less CB_1R/CB_2R selectivity. Furthermore, synthetic cannabinoids mainly act (as agonists or antagonists) by binding to the orthosteric site of receptors (Mechoulam, 2016). Indeed, there is a limited number of molecules, either synthetic or phytocannabinoids, that behave as allosteric modulators of cannabinoid receptor function.

Anandamide and 2-arachidonoyl glycerol (2-AG) are the two main endocannabinoids, being synthesized from membrane lipids and having an alkyl-amide chemical structure. They are retrograde effectors being produced in the post-synaptic neuron to act in the pre-synaptic neuron where they regulate the release of neurotransmitters (Diana and Marty, 2004).

Phytocannabinoids are phenolic terpenes biosynthesized in nature nearly exclusively in the *Cannabis sativa* L. plant. In the *Cannabis* plant, all cannabinoids are biosynthesized in the acid form, mainly Δ^9-THCA, CBDA, etc. CBGA is the first molecule formed in the biosynthetic pathway and the substrate of Δ^9-tetrahydrocannabinol-synthase and CBD-synthase (Fellermeier and Zenk, 1998). The pharmacologic effects of *Cannabis* components, traditionally consumed through inhalation, are attributed to the decarboxylated neutral products of above mentioned acids: Δ^9-THC, CBD, and CBG.

Synthetic cannabinoids are very different in chemical structure. For instance, they may be indoles like WIN-55,212-2, AM-1241 or JWH-018, or phenolic, phenols lacking the pyrene ring, like CP-55,940 or HU-308. All these compounds have been used in cannabinoid research and have helped to unveil pharmacological aspects of the endocannabinoid system. It should be noted that some of these compounds have recently arrived at the streets sold as *legal highs*, thus raising Public Health concerns (Adams et al., 2017; Weinstein et al., 2017).

The endocannabinoid system is constituted by the endogenous cannabinoids, the enzymes that produce and degrade them, and by the receptors that mediate their actions. Whereas endocannabinoids consist of molecules with aliphatic structure, AEA and 2-AG, the structure of natural cannabinoids, derived from *C. sativa* L., is fairly different [see (Lu and Mackie, 2016) and references therein]. Although it is well established that one of the main active components of the plant and one of the few that are psychoactive, namely Δ^9-THC, acts via cannabinoid receptors, there is controversy on whether these receptors mediate the action of phytocannabinoids such as CBN, CBD or CBG. As happened the last years for CBD, a new research and revision of the cannabinoid receptor pharmacology must be done with the rest of phytocannabinoids as CBG. A further phenomenon that may be considered to understand the action of molecules from *C. sativa* L. and its extracts is the fact that cannabinoid receptors may form heteromers, namely CB_1–CB_2 heteroreceptors, which display particular functional properties (Callén et al., 2012). It should be noted that in CNS those heteromers are mainly expressed in pallidal neurons (Lanciego et al., 2011; Sierra et al., 2015) and in activated microglia (Navarro et al., 2018a).

Cannabigerol was isolated, characterized and synthetized by the same researchers than reported the structure of the main psychotropic agent of *Cannabis*, Δ^9-THC (Gaoni and Mechoulan, 1964). Few years later *in vivo* assays showed that CBG was non-psychoactive (Grunfeld and Edery, 1969; Mechoulam et al., 1970). The lower concentration and the lack of psychoactivity was probably the cause that CBG was shadowed by Δ^9-THC. In fact, CBG has attracted less attention than Δ^9-THC and even than CBD, but nowadays is gaining interest among the scientific community. Some commercial hemp varieties have CBG and CBGA as main cannabinoids and, therefore, CBG is another of the phytocannabinoids to be considered by the unregulated market of hemp oils and derivatives. As recently pointed out, the increased therapeutic potential of *C. sativa* L. components requires a more in deep understanding of the pharmacology of phytocannabinoids other than Δ^9-THC, namely CBD, CBG, CBN, Δ^9-THCV, Δ^8-THC, CBC and CBDV (Turner et al., 2017).

Preliminary results using membranes from mice brain or from CHO cells expressing the human CB_2R led to postulate that CBG could be a partial agonist at both CB_1R and CB_2R with K_i values in the 300–500 nM range (Gauson et al., 2007; Pertwee, 2008). The first published data on the binding of CBG to human CB_1R and CB_2R were provided by (Rosenthaler et al., 2014) working with [^{3}H]CP-55,940 as radioligand and with preparations from Sf9 cells co-expressing one receptor and the Gαi3β1γ2 protein. The K_i values obtained in competition assays are 897 and 153 nM for CB_1R and CB_2R, respectively. CBG may modulate the activity of transient receptor potential channels of ankyrin type-1; however, the EC_{50} values lie in the micromolar range (De Petrocellis et al., 2008). It has been reported that CBG binds to CB_1R (K_i = 381 nM) from mouse brain membranes and CB_2R (K_i = 2.6 μM) from CHO cells expressing the human receptor; CBG at high concentrations (10 μM) antagonized [^{35}S]GTPγS binding in mouse brain membranes treated with AEA or CP-55940 (Cascio et al., 2010). Authors also reported CBG as α_2-adrenoceptor agonist at nanomolar levels (EC_{50} = 0.2 nM), and being also able to antagonize [^{35}S]GTPγS binding upon stimulation of the $5HT_{1A}$ receptor by 1 μM 8-OH-DPAT (Cascio et al., 2010). Other findings indicate that CBG can act as (i) agonist/desensitizer of TRPA1 (EC_{50} = 700 nM), (ii) agonist of TRPV1 (EC_{50} = 1.3 μM) (iii) agonist of TRPV2 (EC_{50} = 1.7 μM), (iv) antagonist of TRPM8 channels (IC_{50} = 160 nM) and v) inhibitor of AEA cell uptake (K_i = 11.3 μM) (De Petrocellis et al., 2011). More recently, the PPARγ has been reported as target of the phytocannabinoid CBG (K_i = 11.7 μM) that at high concentrations, in the 10–25 μM range, may enhance the PPARγ transcriptional activity (Granja

et al., 2012; Nadal et al., 2017). A recent review substantiates the complexity of the field and highlights that other players, GPR55 for instance, are also targeted by cannabinoids (Solymosi and Kofalvi, 2017).

The aim of this work was to characterize CBG pharmacology on the cannabinoid receptors using binding and measurement of different signal transduction mechanisms in living HEK-293T cells expressing human CB_1R, CB_2R, or CB_1–CB_2 heteroreceptor complexes. The results indicate that, in our experimental conditions, CBG mainly acts on CB_2R and behaves as a partial agonist.

MATERIALS AND METHODS

Reagents

ACEA, JWH133, and AEA were purchased from Tocris Bioscience (Bristol, United Kingdom), CBD and CBG analytical standard solutions were purchased from THCpharm (Frankfurt, DE). Concentrated (10 mM) stock solutions prepared in ethanol (CBG, ACEA, and AEA) or DMSO (JWH133 and CM-157) were stored at −20°C. In each experimental session, aliquots of concentrated solutions of compounds were thawed and conveniently diluted in the appropriate experimental solution. For non-radioactive binding assays, TLB was obtained from Cisbio Bioassays (LABMED; Codolet, France). The Tb derivative of O6-benzylguanine was synthesized by Cisbio Bioassays and is commercialized as SNAP-Lumi4-Tb (SSNPTBC; Cisbio Assays). The plasmid encoding for the SNAP-tagged human CB_2R used for transient transfection was obtained from Cisbio Bioassays (PSNAP-CB2). CB_2R agonist 3-[[4-[2-tert-butyl-1-(tetrahydropyran-4-ylmethyl)benzimidazol-5-yl]sulfonyl-2-pyridyl]oxy]propan-1-amine (CM-157) conjugated to a fluorescent probe was developed in collaboration with Cisbio Bioassays (Martínez-Pinilla et al., 2016).

Cannabinoid Isolation, Purification and Analysis

Cannabidiol was purified from dried leaves and inflorescences of the *Cannabis* variety SARA (CPVO file number: 20150098), CBG from the variety AIDA (CPVO file number: 20160167) following a previously described method (Nadal, 2016) that provides compounds with >95% purity. An Agilent liquid chromatography set-up (Model 1260, Pittsburgh, PA, United States) consisting of a binary pump, a vacuum degasser, a column oven, an autosampler and a diode array detector (DAD) equipped with a 150 mm length × 2.1 mm internal diameter, 2.7 μm pore size Poroshell 120 EC-C18 column was used for the quality control of the purified cannabinoids. The analysis was performed using water and acetonitrile both containing ammonium formate 50 mM as mobile phases. Flow-rate was 0.2 mL/min and the injection volume was 3 μL. Chromatographic peaks were recorded at 210 nm. All determinations were carried out at 35°C. All samples were analyzed in duplicate. The results of each cannabinoid purity, 96.04% for CBD and 99.9% for CBG, were calculated as weight (%) versus a commercial standard from THCpharm (CBD batch n° L01258-M-1.0; CBG batch n° L01260-M-1.0).

Radioligand Binding Assays

Cell Culture and Membrane Preparation

For radioligand binding experiments CHO cells, stably transfected with cDNA for human CB_1 or CB_2 cannabinoid receptors, were grown adherently and maintained in Ham's F12 containing 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μg/mL) and geneticin (G418, 0.4 mg/mL) at 37°C in a humid atmosphere of 5% CO_2. Membranes were prepared from cells washed with PBS and scraped off plates in ice-cold hypotonic buffer (5 mM Tris HCl, 2 mM EDTA, pH 7.4). The cell suspension was homogenized with a Polytron and then centrifuged for 30 min at 40,000 × *g*.

Saturation Binding Experiments

[^{3}H]-CP-55940 saturation binding experiments (specific activity 169 Ci/mmol, Perkin Elmer) were performed incubating different concentrations of the radioligand (0.03 – 10 nM) in binding buffer (50 mM Tris-HCl, pH 7.4, 2.5 mM EDTA, 5 mM $MgCl_2$ for CB_1R or 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$ for CB_2R) using CHO membranes expressing the human versions of CB_1R or CB_2R (10 μg protein/sample) at 30°C. Non-specific binding was determined in the presence of 1 μM WIN-55,212-2. At the end of the incubation period (90 min for CB_1R or 60 min for CB_2R) bound and free radioactivity were separated in a cell harvester (Brandel Instruments) by filtering the assay mixture through Whatman GF/B glass fiber filters. The filter-bound radioactivity was counted in a 2810 TR liquid scintillation counter (Perkin Elmer).

[^{3}H]-WIN-55,212-2 saturation binding experiments (specific activity 48 Ci/mmol, Perkin Elmer) were performed incubating different concentrations of the radioligand (0.5–100 nM for CB_1R or 0.2–40 nM for CB_2R) in binding buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$) with CB_1R- or CB_2R-containing CHO cell membranes (10 μg protein/sample) at 30°C. Non-specific binding was determined in the presence of 1 μM WIN-55,212-2. At the end of the incubation period (60 min) bound and free radioactivity were separated in a cell harvester (Brandel Instruments) by filtering the assay mixture through Whatman GF/B glass fiber filters. The filter-bound radioactivity was counted in a 2810 TR liquid scintillation counter (Perkin Elmer).

Competition Binding Experiments

[^{3}H]-CP-55940 competition binding experiments were performed incubating 0.3 nM of radioligand and different concentrations of the tested compounds with membranes obtained from CHO cells expressing human CB_1 or CB_2 receptors (10 μg protein/sample) for 90 min (CB_1R) or 60 min (CB_2R) at 30°C. Non-specific binding was determined in the presence of 1 μM WIN-55,212-2. Bound and free radioactivity were separated by filtering the assay mixture as above indicated. The filter bound radioactivity was counted using a Packard Tri Carb 2810 TR scintillation counter (Perkin Elmer).

Competition binding experiments were also performed incubating 3 nM [^{3}H]-WIN-55,212-2 and different concentrations of the tested compounds with membranes obtained from CHO cells transfected with human CB_1 or CB_2 receptors (10 μg protein/sample) for 60 min at 30°C. Non-specific binding was determined in the presence of 1 μM WIN-55,212-2. Bound and free radioactivity were separated by filtering the assay mixture as above indicated. The filter bound radioactivity was counted using a Packard Tri Carb 2810 TR scintillation counter (Perkin Elmer).

Homogeneous Binding Assays in Living Cells

Expression Vector

cDNAs for the human version of cannabinoid CB_2R without their stop codon were obtained by PCR and subcloned to SNAP-containing vector (PSNAP; Cisbio Bioassays) using sense and antisense primers harboring unique restriction sites for HindIII and BamHI generating the SNAP tagged CB_2R (CB_2R-SNAP).

Cell Culture and Transfection

For HTRF assays, HEK-293T cells were used. HEK 293T (HEK-293T) cells were grown in DMEM supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/mL penicillin/streptomycin, and 5% (v/v) FBS [all supplements were from Invitrogen, (Paisley, Scotland, United Kingdom)]. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and were passaged, with enzyme-free cell dissociation buffer (13151-014, Gibco®, Thermo Fisher, Waltham, MA, United States), when they were 80–90% confluent, i.e., approximately twice a week. Cells were transiently transfected with the PEI (Polyethylenimine, Sigma, St. Louis, MO, United States) method as previously described (Medrano et al., 2017; Navarro et al., 2018b). Experiments were carried out in cells expressing SNAP-tagged CB_2R in the presence or in the absence of CB_1R.

Labeling of Cells Expressing SNAP-Tagged CB_2R

Cell culture medium was removed from the 25-cm^2 flask and 100 nM SNAP-Lumi4-Tb, previously diluted in 3 mL of TLB 1X, was added to the flask and incubated for 1 h at 37°C under 5% CO_2 atmosphere in a cell incubator. Cells were then washed four times with 2 mL of TLB 1X to remove the excess of SNAP-Lumi4-Tb, detached with enzyme-free cell dissociation buffer, centrifuged 5 min at 1,500 rpm and collected in 1 mL of TLB 1X. Tag-lite-based binding assays were performed 24 h after transfection. Densities in the 2,500–3,000 cells/well range were used to carry out binding assays in white opaque 384-well plates.

Non-radioactive Competition Binding Assays

For competition binding assays, the fluorophore-conjugated CB_2R ligand (labeled CM-157), unconjugated CM-157 and CBG were diluted in TLB 1X. HEK-293T cells transiently expressing Tb-labeled SNAP-CB_2R with or without CB_1R were incubated with 20 nM fluorophore-conjugated CB_2R ligand, in the presence of increasing concentrations (0–10 μM range) of CBG or CM-157. Plates contained 10 μL of labeled cells, and 5 μL of TLB 1X or 5 μL of CBG or 5 μL CM-157 were added prior to the addition of 5 μL of the fluorescent ligand. Plates were then incubated for at least 2 h at room temperature before signal detection. Detailed description of the HTRF assay is found in Martínez-Pinilla et al. (2016).

Signal was detected using an EnVision microplate reader (PerkinElmer, Waltham, MA, United States) equipped with a FRET optic module allowing donor excitation at 337 nm and signal collection at both 665 and 620 nm. A frequency of 10 flashes/well was selected for the xenon flash lamp excitation. The signal was collected at both 665 and 620 nm using the following time-resolved settings: delay, 150 μs; integration time, 500 μs. HTRF® ratios were obtained by dividing the acceptor (665 nm) by the donor (620 nm) signals and multiplying by 10,000. The 10,000-multiplying factor is used solely for the purpose of easier data handling.

Functional Assays

Cell Culture and Transient Transfection

HEK-293T cells were grown in DMEM medium (Gibco, Paisley, Scotland, United Kingdom) supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, MEM Non-Essential Amino Acids Solution (1/100) and 5% (v/v) heat inactivated Foetal Bovine Serum (FBS) (Invitrogen, Paisley, Scotland, United Kingdom). Cells were maintained in a humid atmosphere of 5% CO_2 at 37°C. Cells were transiently transfected with the PEI (Polyethylenimine, Sigma, St. Louis, MO, United States) method as previously described (Medrano et al., 2017; Navarro et al., 2018b) and used for functional assays 48 h later (unless otherwise stated).

cAMP Determination

Signaling experiments have been performed as previously described (Navarro et al., 2010, 2016, 2018b; Hinz et al., 2018). Two hours before initiating the experiment, HEK-293T cell-culture medium was replaced by serum-starved DMEM medium. Then, cells were detached, resuspended in growing medium containing 50 μM zardaverine and placed in 384-well microplates (2,500 cells/well). Cells were pretreated (15 min) with CBG -or vehicle- and stimulated with agonists (15 min) before adding 0.5 μM forskolin or vehicle. Readings were performed after 15 min incubation at 25°C. HTRF energy transfer measures were performed using the Lance Ultra cAMP kit (PerkinElmer, Waltham, MA, United States). Fluorescence at 665 nm was analyzed in a PHERAstar Flagship microplate reader equipped with an HTRF optical module (BMG Lab Technologies, Offenburg, Germany).

ERK Phosphorylation Assays

To determine ERK1/2 phosphorylation, 50,000 HEK-293T cells/well were plated in transparent Deltalab 96-well microplates and kept at the incubator for 24 h. 2 to 4 h before the experiment, the medium was substituted by serum-starved DMEM medium. Then, cells were pre-treated at 25°C for 10 min with vehicle or CBG in serum-starved DMEM medium and stimulated for an additional 7 min with the specific agonists. Cells were then washed twice with cold PBS before addition of lysis

buffer (20 min treatment). 10 μL of each supernatant were placed in white ProxiPlate 384-well microplates and ERK 1/2 phosphorylation was determined using AlphaScreen®SureFire® kit (Perkin Elmer) following the instructions of the supplier and using an EnSpire® Multimode Plate Reader (PerkinElmer, Waltham, MA, United States).

Dynamic Mass Redistribution Assays (DMR)

Cell mass redistribution induced upon receptor activation was detected by illuminating the underside of a biosensor with polychromatic light and measuring the changes in the wavelength of the reflected monochromatic light. The magnitude of this wavelength shift (in picometers) is directly proportional to the amount of DMR. HEK-293T cells were seeded in 384-well sensor microplates to obtain 70–80% confluent monolayers constituted by approximately 10,000 cells per well. Previous to the assay, cells were washed twice with assay buffer (HBSS with 20 mM HEPES, pH 7.15) and incubated for 2 h with assay-buffer containing 0.1% DMSO (24°C, 30 μL/well). Hereafter, the sensor plate was scanned and a baseline optical signature was recorded for 10 min before adding 10 μL of CBG for 30 min followed by the addition of 10 μL of specific agonists; all test compounds were dissolved in assay buffer. The cell signaling signature was determined using an EnSpire® Multimode Plate Reader (PerkinElmer, Waltham, MA, United States) by a label-free technology. Then, DMR responses were monitored for at least 5,000 s. Results were analyzed using EnSpire Workstation Software v 4.10.

β-Arrestin 2 Recruitment

Arrestin recruitment was determined as previously described (Medrano et al., 2017; Navarro et al., 2018b). Briefly, BRET experiments were performed in HEK-293T cells 48 h after transfection with the cDNA corresponding to the CB_2R-YFP or CB_1R-YFP and 1 μg cDNA corresponding to β-arrestin 2-Rluc. Cells (20 μg protein) were distributed in 96-well microplates (Corning 3600, white plates with white bottom) and were incubated with CBG for 15 min and stimulated with the agonist for 10 min prior the addition of 5 μM coelenterazine H (Molecular Probes, Eugene, OR, United States). After 1 min of adding coelenterazine H, BRET between β-arrestin 2-Rluc and receptor-YFP was determined and quantified. The readings were collected using a Mithras LB 940 (Berthold Technologies, Bad Wildbad, Germany) that allows the integration of the signals detected in the short-wavelength filter at 485 nm and the long-wavelength filter at 530 nm. To quantify protein-RLuc expression luminescence readings were also performed 10 min of adding 5 μM coelenterazine H.

Data Handling and Statistical Analysis

Affinity values (Ki) were calculated from the IC_{50} obtained in competition radioligand binding assays according to the Cheng and Prusoff equation: $K_i = IC_{50}/(1 + [C]/K_D)$, where $[C]$ is the free concentration of the radioligand and K_D its dissociation constant (Cheng, 2001).

Data from homogeneous binding assays were analyzed using Prism 6 (GraphPad Software, Inc., San Diego, CA, United States). K_i values were determined according to the Cheng and Prusoff equation with K_D = 21 nM for CM-157 (Cheng, 2001). Signal-to-background (S/B ratio) calculations were performed by dividing the mean of the maximum value (μ_{max}) by that of the minimum value (μ_{min}) obtained from the sigmoid fits.

The data are shown as the mean ± SEM. Statistical analysis was performed with SPSS 18.0 software. The test of Kolmogorov–Smirnov with the correction of Lilliefors was used to evaluate normal distribution and the test of Levene to evaluate the homogeneity of variance. Significance was analyzed by one-way ANOVA, followed by Bonferroni's multiple comparison *post hoc* test. Significant differences were considered when $p < 0.05$.

RESULTS

Saturation and Competition Radioligand-Based Assays in Membranes Expressing CB_1R or CB_2R

The effect of CBG on radioligand binding to CB_1R or CB_2R was first tested using the classical radioligand-binding assay in membranes isolated from CHO cells expressing human CB_1R or CB_2R and incubated with radioligands: [^{3}H]-CP-55940 or [^{3}H]-WIN-55,212-2. Data obtained from binding isotherms using increasing [^{3}H]-CP-55940 or [^{3}H]-WIN-55,212-2 concentrations lead to a monophasic saturation curve. Saturation curves, receptor density (B_{max} values) and affinity (K_D values) are shown in **Figures 1A–D**. The affinity of the two radioligands was in the nanomolar range for both CB_1R and CB_2R. K_D for [^{3}H]-CP-55940 to CB_1R and CB_2R was similar with values around 0.3 nM. K_D values for WIN-55,212-2 were 9.4 and 3.2 nM for CB_1R and CB_2R, respectively (**Figures 1C,D**). Overall the results agree with previously reported data (McPartland et al., 2007; Merighi et al., 2010).

Competition binding assays of WIN-55,212-2 showed similar K_i values using the two radioligands to CB_1R and CB_2R and agreed with the K_D values for [^{3}H]-WIN-55,212-2 binding (**Table 1** and **Figures 1E,F**). **Table 1** reports the affinity values of CBG. K_i values of CBG obtained using [^{3}H]-CP-55940 as radioligand were in the low micromolar range in both CB_1R and CB_2R. The affinity value of CBG obtained using [^{3}H]-WIN-55,212-2 for CB_2R was 2.7 μM, about twofold higher than that obtained using [^{3}H]-CP-55940. Using [^{3}H]-WIN-55,212-2 in competition binding experiments on CB_1R, CBG was not able to displace the radioligand (**Figures 2A,B**). In summary, CBG displayed K_i values in the low micromolar range when competing for the binding to the CB_2R. Surprisingly, significant competition in the binding to the CB_1R was only observed when using [^{3}H]-CP-55940 as radioligand.

CBG Binds to the Orthosteric Site of Cannabinoid CB_2R at Nanomolar Concentrations

Competition experiments were performed using 20 nM of a fluorophore-conjugated selective CB_2R agonist (CM-157) and

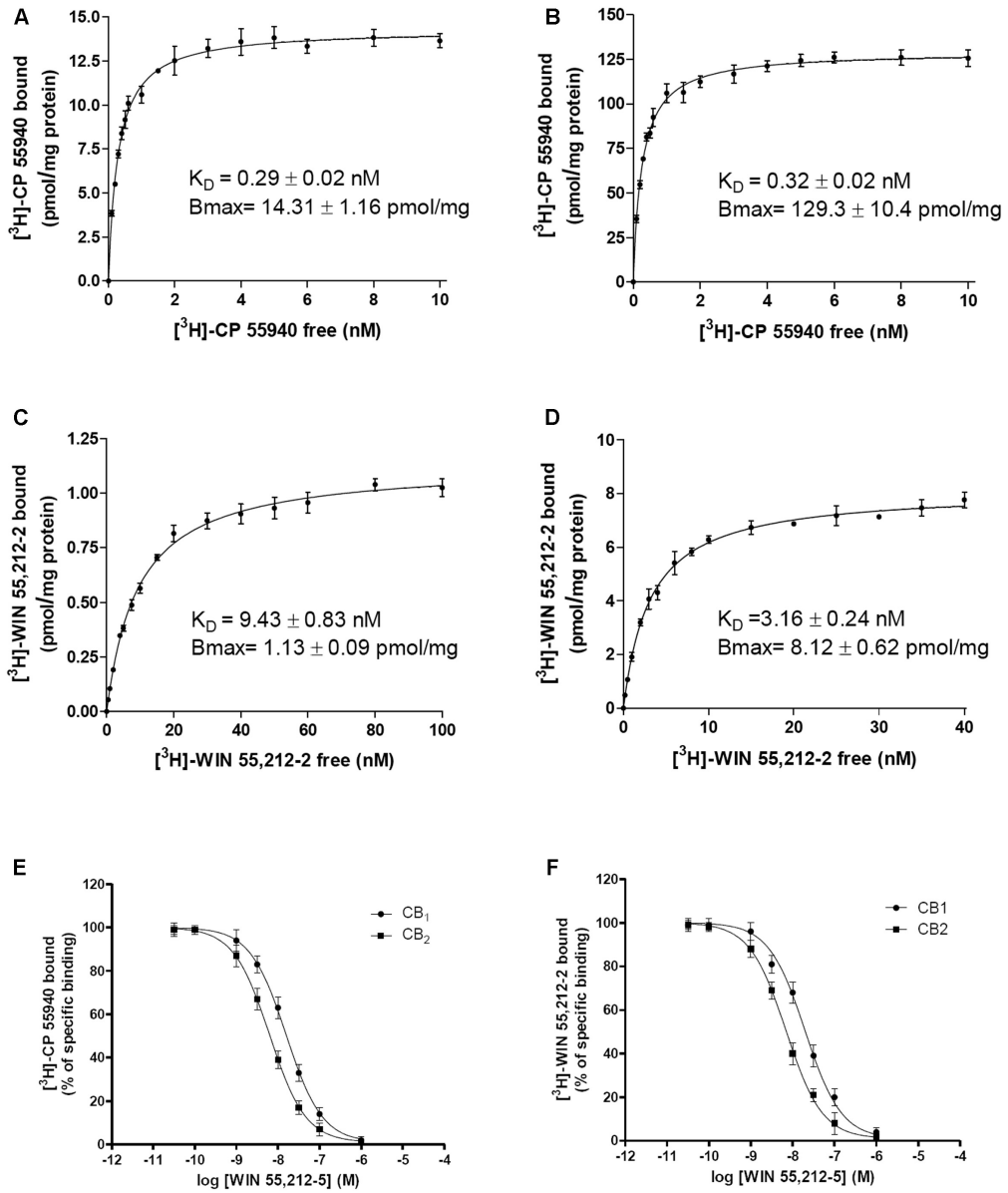


FIGURE 1 | Radioligand binding assays to CB_1R and CB_2R. **(A–D)** Saturation curves of either [^{3}H]-CP-55940 or [^{3}H]-WIN-55,212-2 binding on membranes from CHO cells stably expressing human CB_1R **(A,C)** or CB_2R **(B,D)**. **(E,F)** Competition curves for WIN-55,212-2 in radioligand-based assays using either [^{3}H]-CP-55940 **(E)** or [^{3}H]-WIN-55,212-2 **(F)** binding on membranes from CHO cells stably expressing human CB_1R or CB_2R. Data are expressed as the mean ± SEM of five independent experiments performed in duplicate. K_D (obtained from saturation isotherms) are shown in **Table 1**.

a homogeneous non-radioactive method performed in living cells expressing SNAP-CB_2R (details in Martínez-Pinilla et al., 2016; **Figure 2C**). Unfortunately, the equivalent fluorophore-conjugated selective CB_1R ligand is not available to perform HTRF assays in SNAP-CB_1R-expressing living cells. Competition assays were performed in HEK-293T cells expressing Lumi4-Tb-labeled CB_2R fused to the SNAP protein and incubated with a fixed amount of the fluorophore-conjugated agonist and different CBG concentrations. As observed in **Figure 2**, both the unlabelled selective agonist (CM-157) and CBG decreased the binding to SNAP-CB_2R in monophasic fashion and with K_i values in the nanomolar range (16 nM for CM-157 and of 152 nM for CBG; **Figures 2D,E**). The K_i obtained for CM-157 matches with previously reported dissociation constant K_D values (Martínez-Pinilla et al., 2016). These results indicate that CBG can significantly bind to the orthosteric site of cannabinoid CB_2R at nanomolar concentrations.

Similar experiments were carried out in HEK-293T cells expressing SNAP-CB_2R fusion protein and a similar amount of CB_1R, i.e., in cells that express CB_2R in a CB_1–CB_2 receptor heteromer context. In the presence of cannabinoid CB_1R the K_i for CM-157 was 19 nM (**Figure 2F**) and K_i for CBG was reduced

TABLE 1 | Affinity values of CB compounds obtained from radioligand binding assays.

	[^{3}H]-CP-55940 competition binding experiments		[^{3}H]-WIN-55,212-2 competition binding experiments	
	CB_1 – K_D (nM)	**CB_2 – K_D (nM)**	**CB_1 – K_D (nM)**	**CB_2 – K_D (nM)**
	0.29 ± 0.02	0.32 ± 0.02	9.43 ± 0.83	3.16 ± 0.24
	CB_1 – K_i (nM)	**CB_2 – K_i (nM)**	**CB_1 – K_i (nM)**	**CB_2 – K_i (nM)**
WIN-55,212-2	8.08 ± 0.65	3.22 ± 0.31	9.86 ± 0.84	3.48 ± 0.27
CBG	1,045 ± 74	1,225 ± 85	>30,000	2,656 ± 130
CBD	1,690 ± 110	1,714 ± 70	>30,000	4,019 ± 342

K_D values were obtained from saturation isotherms and K_i from data in competition assays using the indicated radiolabelled compounds ([^{3}H]-CP-55940 or [^{3}H]-WIN-55,212-2).

(56 nM, **Figure 2G**). These results indicate that in cells expressing both cannabinoid receptors, CB_1 and CB_2, CBG shows higher affinity for cannabinoid CB_2R.

CBG Effects on Cannabinoid Receptor-Agonist-Induced Effects

Previous reports Gauson et al. (2007), Cascio et al. (2010) suggest that CBG may be a partial agonist of cannabinoid receptors. To investigate this possibility, HEK-293T cells expressing CB_1R or CB_2R were treated with increasing concentrations of CBG (1 nM to 10 μM) and cAMP, MAPK, β-arrestin recruitment and dynamic mass cell redistribution (DMR) assays were developed. Interestingly, it was observed that in cells expressing CB_1R (**Figure 3**, blue curves), CBG induced a small decrease in forskolin induced cAMP levels and a small increase in β-arrestin recruitment (**Figures 3A,C**), while having no significant action on MAPK phosphorylation assay (**Figure 3B**). Consequently, CBG in label-free assays induced a slight effect in the DMR signal (**Figure 3D**) that is consistent with a G protein-dependent action on cAMP levels; label-free signal is based on optical detection of DMR following receptor activation and mainly reflects G-protein-coupling (Kebig et al., 2009; Schröder et al., 2009; Hamamoto et al., 2015). On the other hand, in HEK-293T cells expressing CB_2R (**Figure 3**, red curves), the action on forskolin-induced cAMP levels and on the DMR signal was small and similar to that exerted in CB_1R-expressing cells (**Figure 3A**). On the contrary, the activation of the MAP kinase pathway was notable (**Figure 3B**). Also noteworthy was the CBG-induced β-arrestin recruitment (**Figure 3C**). Taken together these data suggest that CBG is a poor agonist of CB_1R, whereas it acts as a partial agonist in some of the signaling pathways analyzed in cells expressing CB_2R.

To further examine the CBG effect over CB_1R, HEK-293T cells expressing CB_1R were treated with the endocannabinoid agonist, AEA, or with ACEA in the presence or in the absence of 100 nM or 1 μM CBG. In forskolin-induced cAMP assays we found that 100 nM or 1 μM CBG pretreatment induced a significant decrease in both, AEA and ACEA induced effects (**Figure 4A**). In contrast, CBG (100 nM or 1 μM) was unable to modify the agonist-induced MAPK phosphorylation and β-arrestin recruitment (**Figures 4B,C**). In label-free DMR assays the results were similar to those obtained in cAMP determination assays, i.e., CBG reduced the effect of the agonists (**Figure 4D**).

Cannabigerol (100 nM or 1 μM) was also tested in HEK-293T cells expressing CB_2R and using AEA and a receptor selective agonist, JWH133. Pretreatment with CBG reduced the effects of AEA and JWH133 in experiments of forskolin-induced cAMP levels, ERK1/2 phosphorylation and in label-free DMR read-outs (**Figure 4**). In contrast, CBG did not affect the recruitment of β-arrestin induced by agonists (**Figure 4G**). This last result may be due to the low sensitivity of the assay as β-arrestin recruitment BRET signal was virtually negligible. Energy transfer techniques completely depend on the correct orientation of the fusion proteins and the reduced signal may be due to poor recruitment of β-arrestin and/or to a high distance between BRET donor/acceptor in the putative β-arrestin-Rluc/CB_2R-YFP complex. Thus, CBG in cells activated by endocannabinoids or by selective agonists behaves as a partial agonist of the CB_2R.

CBG Effect in HEK-293T Cells Expressing CB_1R and CB_2R

Experiments were finally performed in cells co-expressing the two cannabinoid receptors, which are able to form heteromeric complexes. A CB_1–CB_2 receptor heteromer print consists of a negative cross-talk observed in Akt phosphorylation and neurite outgrowth; i.e., activation of one receptor reduces the signaling originated upon partner receptor activation (Callén et al., 2012). To characterize the CBG effect, experiments were performed in HEK-293T cells expressing the two cannabinoid receptors. Dose-effect curves were provided for cAMP level and ERK1/2 phosphorylation determination, and for label-free DMR signal and β-arresting recruitment. Interestingly, the effect on cAMP level determination and DMR assays was additive (**Figure 5**), i.e., the presence of CBG blunted the negative cross-talk in these signaling pathways. However, the negative cross-talk was still evident in both ERK1/2 phosphorylation and β-arrestin recruitment experiments (**Figures 5B,C**).

Finally, the effect of 100 nM CBG (100 nM) on AEA, ACEA and/or JWH133 actions was investigated in cells co-expressing CB_1R and CB_2R. CBG pretreatment led to significant effects, always reducing the effect of the agonists, in cAMP-related assays (**Figure 5E**). However, the effect in the other assay types was

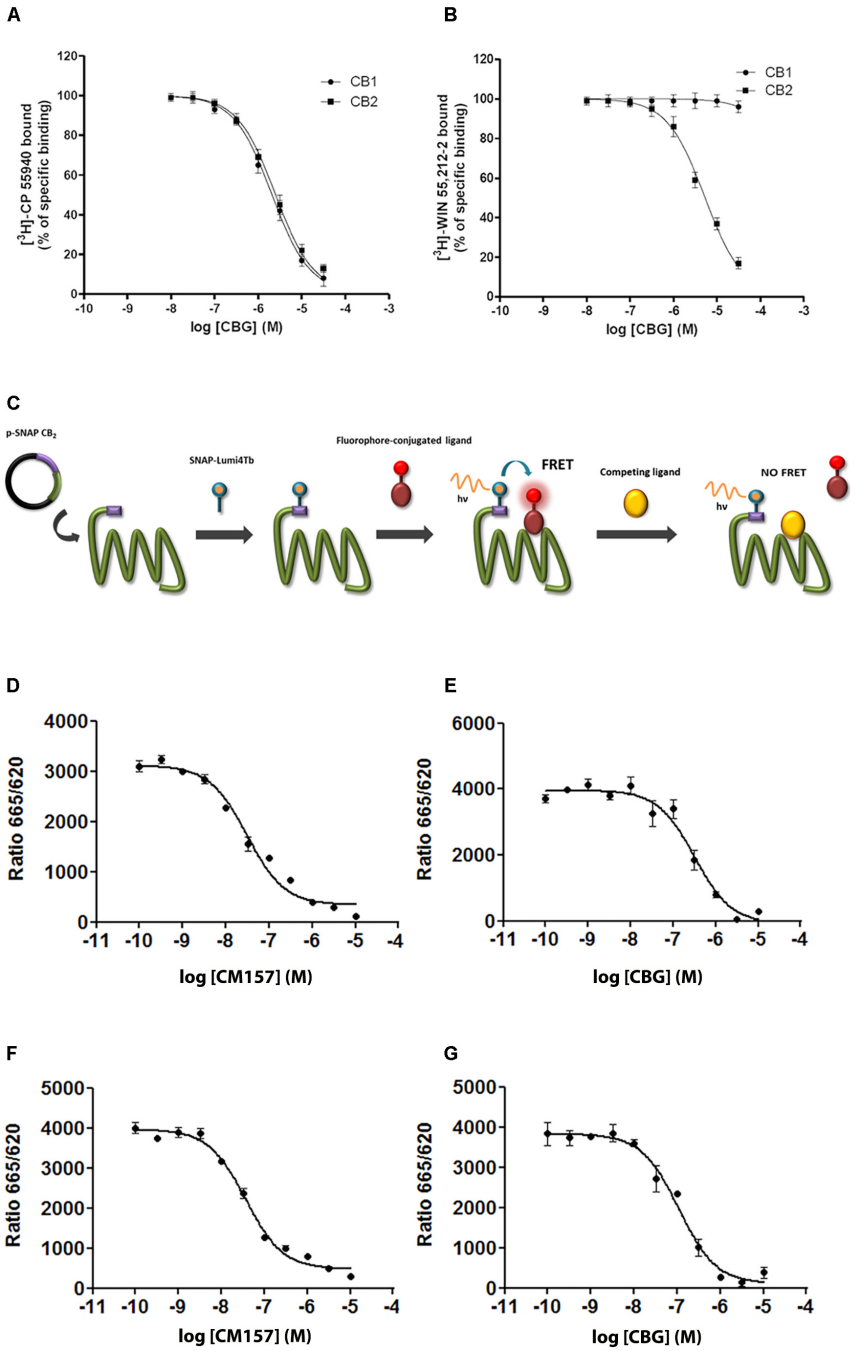


FIGURE 2 | Competition by CBG of agonist binding to CB_1R and/or CB_2R. **(A,B)** Competition curves for CBG in radioligand-based assays using either [^{3}H]-CP-55940 **(A)** or [^{3}H]-WIN-55,212-2 **(B)** binding on membranes from CHO cells stably expressing human CB_1R or CB_2R. **(C)** Scheme of the HTRF-based competitive binding assay. The GPCR of interest with the SNAP-tagged enzyme fused to its N-terminal domain is expressed at the cell surface. SNAP is a commercially available tag consisting of circa 180 amino acids, that can be labeled with fluorophores or other probes in a covalent fashion. The GPCR–SNAP-tagged cells are subsequently labeled with a Tb-containing probe (SNAP-Lumi4-Tb) through a covalent bond between the Tb and the reactive side of the SNAP enzyme. The Tb acts as FRET donor of an acceptor covalently linked to a selective CB2 receptor ligand. Thus, upon binding of a fluorophore-conjugated ligand (FRET acceptor) on the donor-labeled SNAP-tagged/GPCR fusion protein, an HTRF signal from the sensitized acceptor can be detected since the energy transfer can occur only when the donor and the acceptor are in close proximity. In competition binding assays using CM-157, the unlabelled specific ligand competes for receptor binding site with the fluorophore-conjugated ligand, leading to a decrease in the HTRF signal detected. **(D–G)** HEK-293T were transiently transfected with 1 μg cDNA for SNAP-CB_2R in the absence **(D,E)** or presence of 0.5 μg cDNA for CB_1R **(F,G)**. Competition curves of specific binding of 20 nM fluorophore-conjugated CM-157 using CM-157 (0–10 μM) **(D,F)** or of CBG (0–10 μM) **(E,G)** as competitors are shown. Data represent the mean ± SEM of five experiments in triplicates.

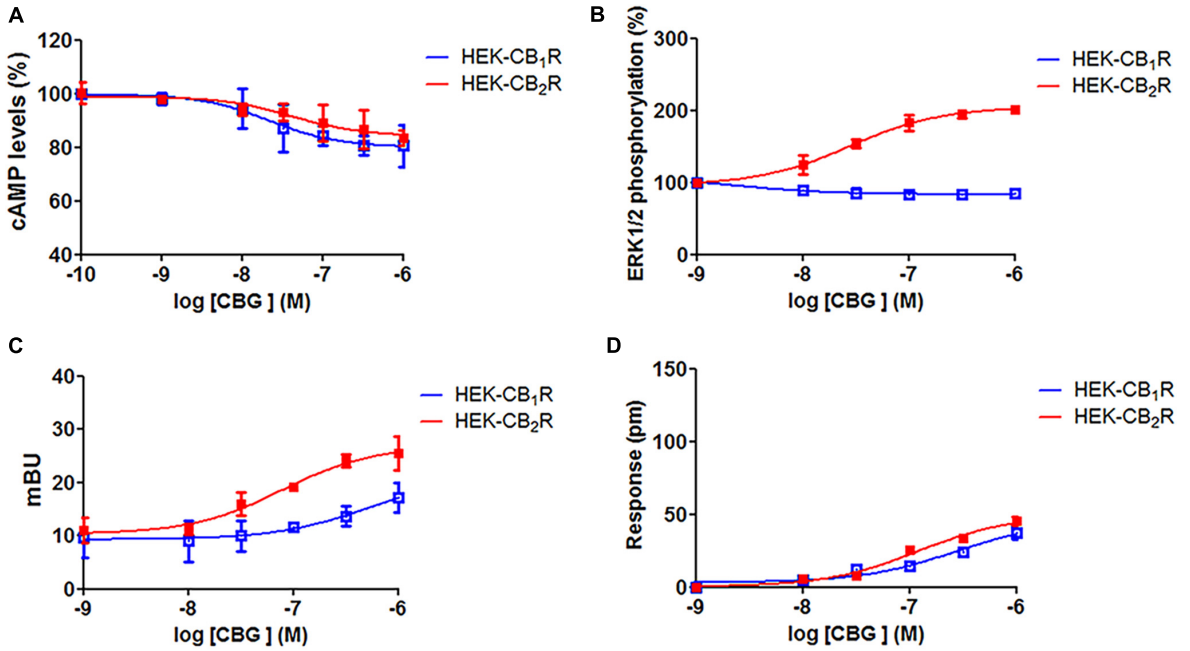


FIGURE 3 | Cannabigerol action in cells expressing CB_1R or CB_2R. HEK-293T cells were transfected with 0.75 μg cDNA for CB_1R (red line) or 1 μg cDNA for CB_2R (blue line). Dose–effect curves for cAMP production are expressed as % of levels obtained by 0.5 μM forskolin treatment **(A)**. Dose-effect curves for ERK1/2 phosphorylation are expressed as % respect to basal levels **(B)**. Dose-effect curves for β-arrestin recruitment **(C)** and label-free **(D)** assays are expressed, respectively, in mBRET units and pm. In β-arrestin-2 recruitment assays cells were transfected with 1 μg cDNA for β-arrestin-Rluc and either 0.75 μg cDNA for CB_1R-YFP or 1 μg cDNA for CB_2R-YFP. Data are the mean ± SEM of a representative experiment in triplicates (n = 6).

negligible except for the negative modulation of the ACEA effect on ERK1/2 phosphorylation and DMR, and of the AEA effect on DMR read-outs (**Figures 5F–H**). Therefore, CBG either blunted the cAMP-dependent signaling or did not significantly alter the negative cross-talk when other CB_1/CB_2-mediated signaling read-outs were determined (see **Figures 5B,C**). It should be noted that cross-talk at the intracellular signaling level, cannot be ruled out to partly explain some of the findings (Bayewitch et al., 1995; Wartmann et al., 1995; Mcguinness et al., 2009; Peters and Scott, 2009; Van Der Lee et al., 2009).

DISCUSSION

The aim of this paper was to comparatively address CBG pharmacology and effects on CB_1 and CB_2 receptors, and on CB_1–CB_2 heteroreceptor complexes. The binding experiments using radiolabelled- and non-radiolabelled-based approaches have provided relevant results. The results on CB_2R are clear an indicate that CBG acts as a competitive partial agonist ligand. There is, however, an interesting observation as the K_i values for competing both [^{3}H]-CP-55940 and [^{3}H]-WIN-55,212-2 are in the low micromolar range (**Table 1**), whereas displaying a value of 152 nM in HTRF-based assays. As pointed out in previous reports, the conditions of the approach using a fluorescent-conjugated CM-157 allows identification of different states of the receptor. Irrespective of the molecular mechanism, the marked differences in affinity constants suggest different ways to accommodate the ligand within the orthosteric center. To our knowledge this is the first report performed in parallel binding assays using three different ligands that reportedly bind to the orthosteric center of the CB_2R ([^{3}H]-CP-55940, [^{3}H]-WIN-55,212-2 and fluorescence-conjugated-CM-157). In summary, the most reasonable assumption is that CBG binds to the orthosteric center of CB_2R but with marked differences in affinity depending on the assay. It should be noted that differences in affinity may result from the fact that HTRF binding is performed in living cells whereas radioligand binding assays are performed in isolated membranes. The already existing data concerning CBG affinity for CB_1 and CB_2 receptors, all performed using [^{3}H]-CP-55940 also indicate that the affinity may vary depending on the context of the receptor, by *inter alia* the constraints of the membrane, heteromerization or interaction with G-proteins. Comparing our results with similar data using [^{3}H]-CP-55940, the affinity is higher for receptors expressed in HEK-293 cells or in brain membranes (Gauson et al., 2007; Pertwee, 2008; Pollastro et al., 2011) that in receptors expressed in CHO cells (**Table 1**). In competition assays of radioligand binding to CB_1R or to CB_2R, affinity for CBG is similar to that previously published (Gauson et al., 2007; Pertwee, 2008), except in the case of Sf9 cells (K_i: 897 and 153 nM for, respectively, CB_1R and CB_2R). This piece of data would indicate conformational changes induced by third molecules that affect the binding of the radioligand and/or of CBG. In fact, Sf9 are insect cells that do not express the cognate G_i protein and, therefore, Gαi3β1γ2 was heterologously expressed to perform the

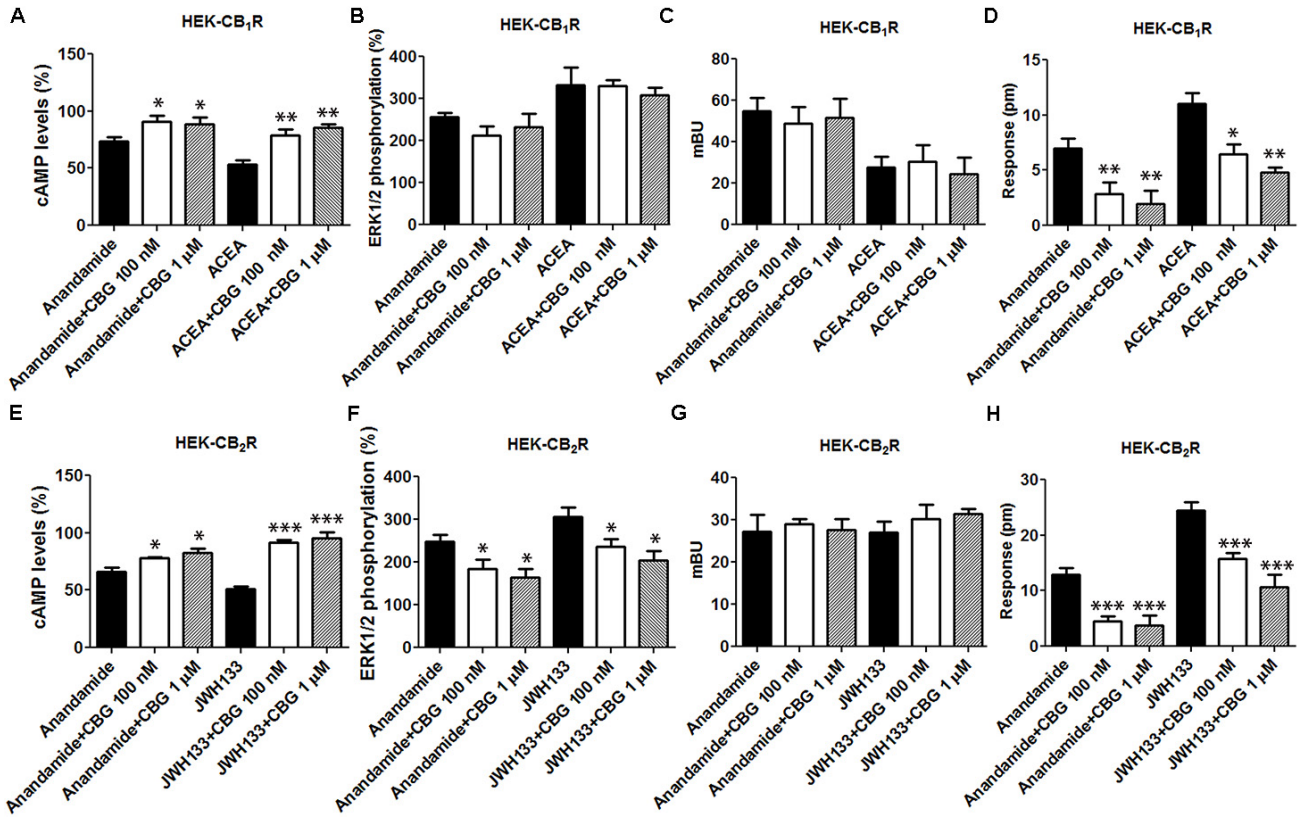


FIGURE 4 | Effect of CBG on the action of CB_1R and CB_2R agonists. **(A–D)** HEK-293T cells were transfected with 0.75 µg cDNA for CB_1R and treated with 100 nM AEA or a selective CB_1R ligand (100 nM ACEA) in the absence (black bars) or presence of 100 nM (white bars) or 1 µM (gray bars) CBG. **(E–H)** HEK-293T cells were transfected with 1 µg cDNA for CB_2R and treated with 100 nM AEA or a selective CB_2R ligand (100 nM JWH133) in the absence (black bars) or presence of 100 nM (white bars) or 1 µM (gray bars) CBG. cAMP production **(A,E)** is expressed as % of levels obtained by 0.5 µM forskolin. ERK1/2 phosphorylation data are expressed as % respect to basal levels **(B,F)**. In β-arrestin-2 recruitment assays cells were transfected with 1 µg cDNA for β-arrestin-Rluc and either 0.75 µg cDNA for CB_1R-YFP or 1 µg cDNA for CB_2R-YFP. Data for β-arrestin recruitment **(C,G)** and label-free **(D,H)** assays are expressed, respectively, in mBRET units and pm. Data represent the mean ± SEM of six different experiments performed with six replicates. One-way ANOVA and Bonferroni's multiple comparison *post hoc* test were used for statistical analysis (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; versus treatment with AEA, ACEA, or JWH133 alone).

binding assays that led to different affinities for CBG (897 and 153 nM for, respectively, CB_1R and CB_2R) (Rosenthaler et al., 2014).

The results from binding to the CB_1R are not very robust and more difficult to interpret. Unfortunately, there are no ligands available to perform HTRF binding to SNAP-CB_1R-expressing living cells, whereas the data from competition assays using [^{3}H]-CP-55940 or [^{3}H]-WIN-55,212-2 were contradictory. On the one hand, the K_i for binding to the CB_1R using [^{3}H]-CP-55940 was in the low micromolar range, as it occurred with data from radioligand binding to the CB_2R. However, CBG was unable to compete [^{3}H]-WIN-55,212-2 binding to the CB_1R. Taking into account that recognition sites for CP-55940 and WIN-55,212-2 are not identical in the CB_1R, one possibility is that CBG binds to the orthosteric center but displaying different equilibrium binding parameters depending on the radioligand. It was early observed that Lys^{192} in the CB_1R third transmembrane domain (TM3) was crucial for binding of CP-55940 and AEA but not for WIN-55,212-2 (Bonner et al., 1996; Chin et al., 1998). Later, *in silico* models pointed to an hydrophobic pocket for CP-55940 binding that involved residues in different transmembrane domains (not only in TM3) and in the second extracellular loop (Shim et al., 2003). Those models showed that WIN-55,212-2 not only binds to the hydrophobic pocket described for CP-55940 but to another hydrophobic region involving residues in TM2 and TM3 (Shim and Howlett, 2006). The structure of CBG is more similar to CP-55940 than to WIN-55,212,2, bearing an OH in the A ring that may interact with the TM3 Lys^{192} residue. In brief, CBG binds to the orthosteric center of CB_1R as indicated by the fact that CBG affects CP-55940 binding without affecting the binding of [^{3}H]-WIN-55,212-2. In other words, CBG was able to distinguish between two subregions of the CB_1R orthosteric center. We therefore suggest that pharmacological studies concerning the CB_1R should be run in parallel using radiolabelled CP-55940 and WIN-55,212-2. Interestingly CP-55940 and WIN-55,212-2 are able to fix the CB_1R in two different conformations (Georgieva et al., 2008) and, therefore, CBG would affect more the conformation and signaling arising from occupation of the CP-55940 binding site. Other possibilities cannot be ruled out and, in this respect,

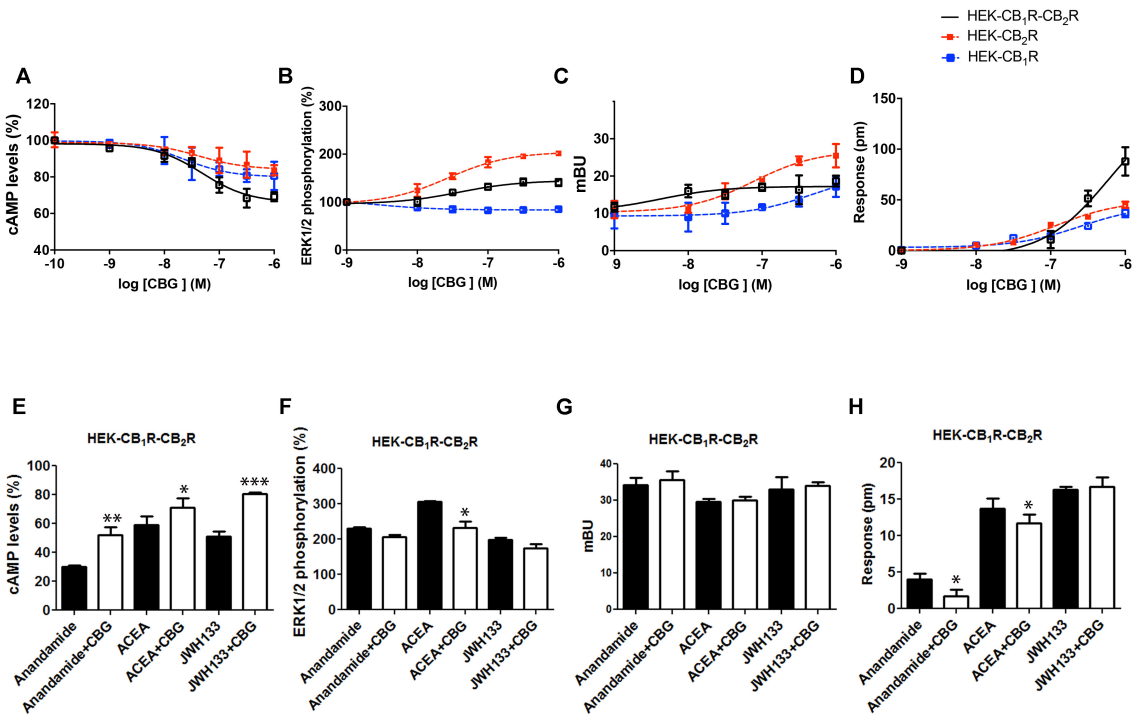


FIGURE 5 | Effect of CBG in cells expressing CB_1 and CB_2 receptors. **(A–D)** Effect of CBG in HEK-293T cells transfected with 0.75 μg cDNA for CB_1R and 1 μg cDNA for CB_2R **(A,B,D)** or 1 μg cDNA for β-arrestin-Rluc, 0.75 μg cDNA for CB_1R and 1 μg cDNA for CB_2R-YFP **(C)**. Dose–effect curves for cAMP production are expressed as % of levels obtained by 0.5 μM forskolin treatment **(A)**. Dose-effect curves for ERK1/2 phosphorylation are expressed as % respect of basal levels **(B)**. Dose-effect curves for β-arrestin recruitment **(C)** and label-free **(D)** assays are expressed, respectively, in mBRET units and pm. Dotted lines (red and blue) are the same than those shown in **Figure 3** and serve as a reference for differential effects in cells coexpressing both receptors. Data are the mean ± SEM of a representative experiment in triplicates ($n = 6$). **(E–H)** HEK-293T cells transfected with 0.75 μg cDNA for CB_1R and 1 μg cDNA for CB_2R **(E,F,H)** or 1 μg cDNA for β-arrestin-Rluc, 0.75 μg cDNA for CB_1R and 1 μg cDNA for CB_2R-YFP **(G)** were treated with 100 nM AEA or a selective CB_2R ligand (100 nM JWH133) in the absence (black bars) or presence (white bars) of 100 nM CBG. cAMP production **(E)** is expressed as % of levels obtained by 0.5 μM forskolin. ERK1/2 phosphorylation data are expressed as % respect of basal levels **(F)**. Data for β-arrestin recruitment **(G)** and label-free **(H)** assays are expressed, respectively, in mBRET units and pm. Data represent the mean ± SEM of six different experiments performed with three replicates. One-way ANOVA and Bonferroni's multiple comparison *post hoc* test were used for statistical analysis (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; versus treatment with AEA, ACEA, or JWH133 alone).

we assayed CBD in competition assays and obtained similar results than those obtained using CBG (**Table 1**). Accordingly, CBG could act on CB_1R (but not on CB_2R) as non-competitive (allosteric) modulator, as described for CBD (Laprairie et al., 2015).

When one compound binds to the orthosteric center and affects several signaling pathways with different potency as in the case of CBG in cells expressing CB_1R, the phenomenon is known as functional selectivity or biased agonism. In cells expressing CB_1R, CBG effect is skewed toward the G_i-mediated signaling pathway. This is in agreement with our finding of significant effect in label-free assays; often DMR signals correlate with effect on cAMP levels in the case of receptors coupled to G_i or G_s proteins (Grundmann and Kostenis, 2015a,b; Hamamoto et al., 2015). It is, however, intriguing that CBG was unable to displace the binding of [^{3}H]-WIN-55,212-2 to the CB_1R. Therefore, an action of CBG on a particular state of the receptor, which, in the case of CB_2R may be disclosed by HTRF binding in living cells (Martínez-Pinilla et al., 2016), cannot be ruled out. Taking together all results, an allosteric action of CBG on the CB_1R would not explain why it is able to engage G_i –mediating signaling. Another possibility, which was suggested for AM630, a previously considered CB_2R antagonist (Bolognini et al., 2012), is that CBG is a protean agonist displaying biased agonism.

Data from CB_2R-mediated functional assays were easier to interpret. First of all, the efficacy was lower compared to selective synthetic agonists and endocannabinoids. Also, CBG led to biased agonism as the effect on cAMP levels was small while being quite marked in ERK phosphorylation and β-arrestin recruitment. Therefore, CBG acted as a partial agonist and, as such, it was able to reduce the effects of other cannabinoid agonists. At 1 μM the effect of CBG on receptor activation by other agonists was similar to that exerted by 100 nM (**Figure 4**) thus suggesting that the *effective* affinity in living cells is that obtained in HTRF non-radioactive-based assays.

Due to the complex pharmacology of cannabinoids this research was undertaken to investigate whether CBG could be exerting a differential action on the CB_1–CB_2 receptor heteromers. Previous data have shown that the interplay between the two receptors in an heteromeric context is also complex. Whereas Callén et al. (2012) showed a negative cross-talk in

a heterologous expression system, the allosteric interaction in the CB_1–CB_2 heteroreceptor complex is synergistic in primary cultures of activated microglia activated with LPS and interferon gamma and in primary cultures of microglia from a transgenic model of Alzheimer's disease (Navarro et al., 2018a). Dose-effect experiments here undertaken in the HEK-293T-based heterologous expression system showed that CBG treatment in the absence of any other agonist, led to additive/synergistic effects on cAMP and label-free read-outs. In contrast, in ERK phosphorylation and β-arrestin recruitment, we found the negative cross-talk already described for this heteromer when full agonists are used to activate the receptors (Callén et al., 2012). These results suggest that partial agonism on the CB_2R is regulated by the presence of CB_1R; however, more complex alternative scenarios cannot be ruled out as CBG may act on the orthosteric site of the CB_2R protomer and as protean agonist of the CB_1R protomer. In cells expressing the two receptors, the overall effect of 100 nM CBG on agonist-induced activation is more consistent with acting on CB_2R than on CB_1R. In fact, the results in co-expressing cells, which likely express heteromers, are similar to those encountered in CB_2R-expressing cells. In summary, CBG significantly modulates CB_2R- or CB_1R/CB_2R-mediated endocannabinoid action, while the effects are weak in CB_1R-expressing cells. Our findings demonstrating the action of CBG on the cannabinoid receptors are in complete agreement and may explain the *in vitro* results, reporting the protection of macrophages against oxidative stress (Giacoppo et al., 2017), and the beneficial *in vivo* effects in a model of inflammatory bowel disease (Borrelli et al., 2013). In the first of these two studies CBG-mediated protection is blocked by AM630, a selective CB_2R ligand, whereas the CB_1R antagonist, SR141716A, had no effect on CBG action (Giacoppo et al., 2017). The second study reported that CBG may both reduce the histological and molecular changes of experimental colitis and nitrite release from macrophages after LPS stimulation; again these effects were seemingly mediated by CB_2R (Borrelli et al., 2013). These results can be explained by our findings; CBG acting as a partial agonist and exerting actions via CB_2R in macrophages (Giacoppo et al., 2017) or "antagonizing" the effects of endogenous or synthetic cannabinoids, as in LPS-stimulated macrophages (Borrelli et al., 2013). In conclusion, the results presented in this study reveal that the non-psychotropic phytocannabinoid, CBG, may exert beneficial actions with therapeutic potential via cannabinoid receptors.

AUTHOR CONTRIBUTIONS

XN and RF had the original idea, designed and coordinated actions in the different participating institutions, and wrote the initial manuscript. GN performed non-radiolabelled-based homogeneous binding assays, participated in the signaling experiments, and significantly contributed to manuscript preparation. IR-R participated in the signaling experiments and in writing methods. RR-S actively participated in data analysis and parameter calculation. EC supervised data analysis, provided pharmacological expertise, and insight into data interpretation. FV performed the radioligand binding experiments. KV and PB performed the radioligand binding data analysis and interpretation. SC selected the *Cannabis* varieties and supervised the production of the vegetal raw material used for the isolation and purification of cannabinoids. VSM performed the isolation and purification of cannabinoids. CS-CC and CF-V performed the analytical quality control to the purified cannabinoids. All co-authors critically revised, contributed to the editing, and approved the manuscript.

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Chemical Proteomic Analysis of Serine Hydrolase Activity in Niemann-Pick Type C Mouse Brain

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The endocannabinoid system (ECS) is considered to be an endogenous protective system in various neurodegenerative diseases. Niemann-Pick type C (NPC) is a neurodegenerative disease in which the role of the ECS has not been studied yet. Most of the endocannabinoid enzymes are serine hydrolases, which can be studied using activity-based protein profiling (ABPP). Here, we report the serine hydrolase activity in brain proteomes of a NPC mouse model as measured by ABPP. Two ABPP methods are used: a gel-based method and a chemical proteomics method. The activities of the following endocannabinoid enzymes were quantified: diacylglycerol lipase (DAGL) α, α/β-hydrolase domain-containing protein 4, α/β-hydrolase domain-containing protein 6, α/β-hydrolase domain-containing protein 12, fatty acid amide hydrolase, and monoacylglycerol lipase. Using the gel-based method, two bands were observed for DAGL α. Only the upper band corresponding to this enzyme was significantly decreased in the NPC mouse model. Chemical proteomics showed that three lysosomal serine hydrolase activities (retinoid-inducible serine carboxypeptidase, cathepsin A, and palmitoyl-protein thioesterase 1) were increased in Niemann-Pick C1 protein knockout mouse brain compared to wild-type brain, whereas no difference in endocannabinoid hydrolase activity was observed. We conclude that these targets might be interesting therapeutic targets for future validation studies.

Keywords: chemical proteomics, activity-based protein profiling, endocannabinoid system, hydrolases, Niemann-Pick type C

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INTRODUCTION

The endocannabinoid system (ECS) consists of the cannabinoid type 1 and 2 receptors (CB_1R and CB_2R) and their endogenous ligands: the endocannabinoids. The lipids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are the best characterized endocannabinoids (Di Marzo et al., 2004). The enzymes responsible for endocannabinoid biosynthesis and degradation are

Abbreviations: ABHD, α/β-hydrolase domain-containing protein; ABPP, activity-based protein profiling; AEA, anandamide; 2-AG, 2-arachidonoylglycerol; CB_xR, cannabinoid type x receptor; CTSA, cathepsin A; DAGL, diacylglycerol lipase; ECS, endocannabinoid system; FAAH, fatty acid amide hydrolase; FP, fluorophosphonate; GBA2, non-lysosomal glucosylceramidase; MAGL, monoacylglycerol lipase; NAPE, *N*-acylphospatidylethanolamine; NPC, Niemann-Pick type C; PLA2G4E, phospholipase A2 group 4E; PLD, phospholipase D; PPT1, palmitoyl-protein thioesterase 1; SCPEP1, retinoid-inducible serine carboxypeptidase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; THL, tetrahydrolipstatin.

also part of the ECS, with most endocannabinoid degrading enzymes belonging to the serine hydrolase family (**Figure 1**; Blankman and Cravatt, 2013). The combined activities of biosynthetic enzymes and degradative enzymes tightly regulate endocannabinoid concentrations in the brain. For example, 2-AG is mainly produced from 1-acyl-2-AG by the diacylglycerol lipases (DAGLs), DAGLα and DAGLβ, while it is degraded by monoacylglycerol lipase (MAGL) and to a minor extent by α,β-hydrolase domain-containing protein 6 (ABHD6) and α,β-hydrolase domain-containing protein 12 (ABHD12). Multiple pathways are known for the biosynthesis of AEA, but they all use *N*-acylphospatidylethanolamine (NAPE) as a central precursor, including the serine hydrolase α,β-hydrolase domain-containing protein 4 (ABHD4) and the β-metallolactamase *N*-acylphospatidylethanolamine-phospholipase D (NAPE-PLD). NAPE is synthesized by the calcium-dependent *N*-acyltransferase phospholipase A2 group 4E (PLA2G4E) (Ogura et al., 2016). Fatty acid amide hydrolase (FAAH) terminates AEA signaling by hydrolysis of its amide bond.

Endocannabinoid hydrolases that are part of the serine hydrolase superfamily can be studied using activity-based protein profiling (ABPP), a method which uses chemical probes for measuring enzyme activity in complex biological samples. Previously, we have used ABPP to compare serine hydrolase activity in wild-type and CB_1R knockout mice (Baggelaar et al., 2017). Comparative ABPP has also been successfully applied in the identification of new drug targets (Nomura et al., 2010a,b). With ABPP, enzyme activities that are deregulated in a certain pathophysiological state can be identified and the relative amount of active enzyme copies as compared to wild-type situations quantified. The classical fluorophosphonate (FP)-based probes (FP-TAMRA and FP-biotin; Liu et al., 1999) act as broad-spectrum serine hydrolase probes that label the endocannabinoid enzymes FAAH, MAGL, PLA2G4E (Ogura et al., 2016), ABHD6, and ABHD4 (Liu et al., 1999; Simon and Cravatt, 2010), whereas the tailor-made tetrahydrolipstatin (THL)-based probes (MB064 and MB108) label both DAGL isoforms (alpha and beta) and ABHD4, ABHD6, and ABHD12 (Baggelaar et al., 2013, 2015, 2017; Ogasawara et al., 2016). Of note, the fluorescent probe DH379 (Ogasawara et al., 2016) selectively targets DAGL and ABHD6 (Supplementary Figure S1).

Evaluation of endocannabinoid hydrolase activity in native tissue may provide insight in the role of the ECS in physiological and disease processes. Interestingly, endocannabinoid levels are elevated during neurodegeneration and neuroinflammation (van der Stelt and Di Marzo, 2005; Nomura et al., 2011; Chiurchiù et al., 2017). For example, endocannabinoid signaling is perturbed in various animal models of neurodegenerative diseases, including stroke (Hillard, 2008), traumatic brain injury (Schurman and Lichtman, 2017), Alzheimer's disease (Bisogno and Di Marzo, 2008), Huntington's disease (Maccarrone et al., 2007), Parkinson's disease (Di Marzo et al., 2000; Maccarrone et al., 2003), and multiple sclerosis (Baker et al., 2000). It is hypothesized that regulation of ECS activity may provide therapeutic benefit for these types of neurological diseases (Scotter et al., 2010).

Niemann-Pick type C (NPC) is a neurodegenerative lysosomal storage disorder, which is associated with mutations in either of the genes encoding Niemann-Pick C1 protein (NPC1) or NPC2 (Vanier and Millat, 2003). Both genes encode lysosomal proteins that are sequentially involved in cholesterol transport out of the lysosomes *via* a so far unknown mechanism. Defects in the function of the soluble NPC2 or the lysosomal membrane protein NPC1 leads to primary accumulation of cholesterol and secondary storage of sphingomyelin, sphingosine, and glycosphingolipids in lysosomes of multiple cell types, thereby leading to visceral complications such as enlarged liver and spleen combined with progressive neurological disease (Vanier and Millat, 2003).

A NPC mouse model is available. These NPC mice have previously been studied using ABPP with a retaining β-glucosidase probe (Marques et al., 2015). This study showed increased activity of the non-lysosomal glucosylceramidase (GBA2) in NPC1 knockout mice (and consistent increased abundance of the protein by Western blot). Importantly, pharmacological inhibition of GBA2 ameliorated the neuropathology of these mice (Marques et al., 2015). Miglustat is approved as a drug, and initially thought to work through substrate reduction by inhibiting glucosylceramide synthase (Nietupski et al., 2012). However, as we have shown before, the molecular mechanism does not involve glucosylceramide synthase, and we hypothesized that the therapeutic effect seems at least partly due to off-target inhibition of Miglustat on GBA2 (Marques et al., 2015). It has been suggested that accumulation of sterols in lysosomes impaired in NPC1 (or NPC2) causes a more general lysosome dysfunction involving multiple hydrolases, such as lysosomal glucocerebrosidase (GBA; Ferraz et al., 2016). Additionally, mutations in NPC1 or NPC2 genes result in severe progressive neurodegeneration. These observations led us to hypothesize that the hydrolases of the ECS might play a role in this disease. There is no treatment available for NPC patients. Additionally, there is no information available about the status of the ECS in Niemann-Pick. Therefore, we set out to measure endocannabinoid hydrolase activity in the NPC mouse model using ABPP.

MATERIALS AND METHODS

Animals

Npc1$^{-/-}$ mice, along with wild-type littermates (*Npc1*$^{+/+}$), were generated as published previously (Marques et al., 2015). Briefly, the heterozygous BALB/c Nctr-*Npc1*^{m1N}/J mice (stock number 003092) were obtained from The Jackson Laboratory (Bar Harbor, ME, United States). The mice used in the current study were crossed for six generations in c57/bl6 background. Mouse pups were genotyped according to published protocols (Loftus et al., 1997). The mice were housed at the Institute Animal Core Facility in a temperature- and humidity-controlled room with a 12-h light/dark cycle and given free access to food and water *ad libitum*. All animal protocols were approved by the Institutional Animal Welfare Committee of the Academic Medical Centre Amsterdam in Netherlands. At the age of

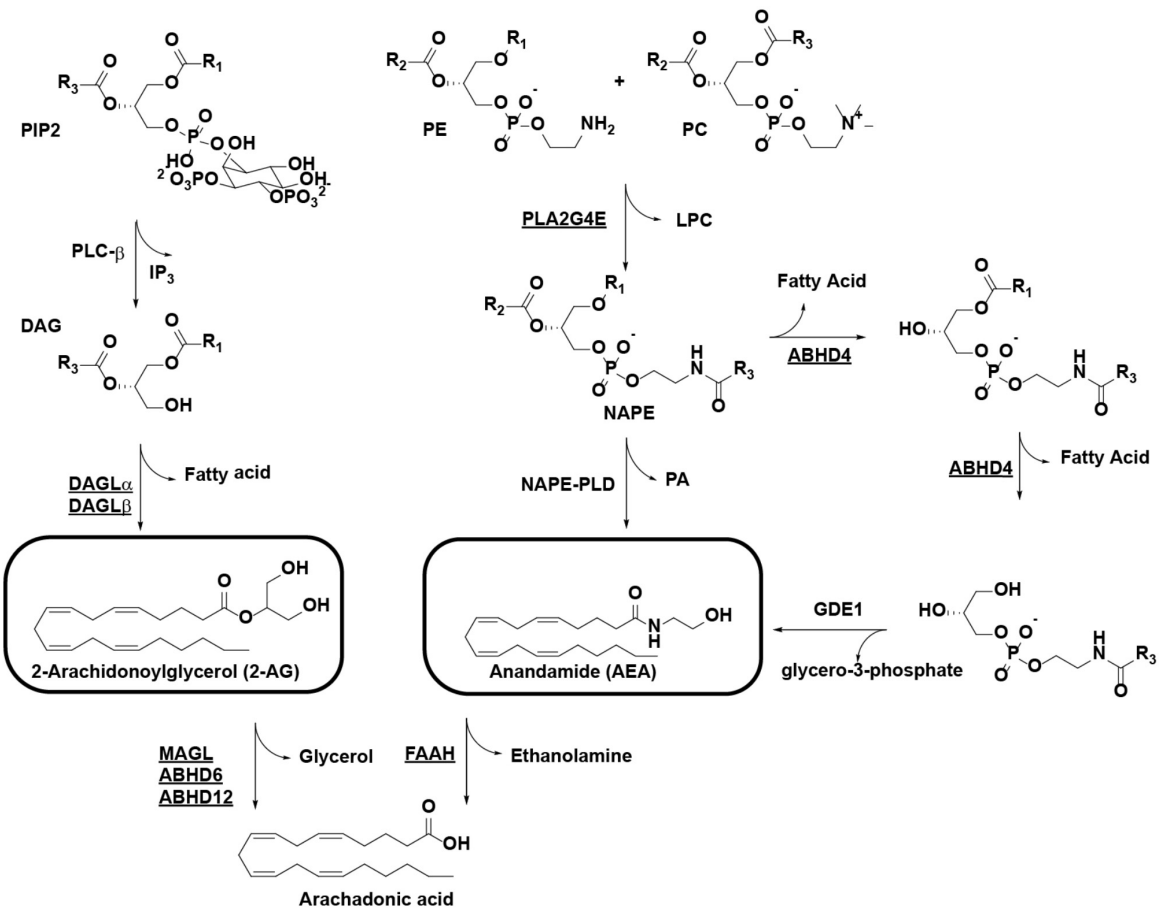


FIGURE 1 | Pathways for the biosynthesis and degradation of the endocannabinoids 2-AG and AEA. Enzymes with known activity-based probes are underlined. R1/R2, alkyl chains, R3, arachidonoyl. ABHD, α/β-hydrolase domain-containing protein; AEA, anandamide; 2-AG: 2-arachidonoylglycerol; DAG, diacylglycerol; DAGL, diacylglycerol lipase; FAAH, fatty acid amide hydrolase; GDE1, glycerophosphodiesterase 1; LPC, lysophosphatidylcholine; MAGL, monoacylglycerol lipase; NAPE, *N*-acylphosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PIP2, phosphatidylinositol 4,5-bisphosphate; PLA2G4E, phospholipase A2 group 4E; PLC, phospholipase C; PLD, phospholipase D.

69 days, animals were first anesthetized with a dose of Hypnorm (0.315 mg/mL fenyl citrate and 10 mg/mL fluanisone) and Dormicum (5 mg/mL midazolam) according to their weight. The given dose was 80 μL/10 g body weight. Blood was collected by a heart puncture followed by cervical dislocation. Brains were dissected, rinsed with phosphate-buffered saline (PBS), snap frozen in liquid N_2, and stored at −80°C for biochemistry. Brain tissue was obtained from six female mice, three wildtype and three knockout.

Preparation of Mouse Tissue Proteome

The mouse brains were cut in half with a scalpel through the midsection (sagittal plane). The mouse brain halves were slowly thawed on ice. The thawed mouse brain halves were dounce homogenized in 1.5-mL cold (4°C) lysis buffer (20 mM HEPES pH 7.2, 2 mM DTT, 1 mM $MgCl_2$, 25 U/mL benzonase) and incubated for 15 min on ice. The suspension was centrifuged (2500 *g*, 3 min, 4°C) to remove debris. The supernatant was collected and transferred to an ultracentrifuge tube. The debris was resuspended in 0.25-mL lysis buffer and resubjected to centrifugation. The combined supernatants were collected and subjected to ultracentrifugation (100,000 *g*, 45 min, 4°C, Beckman Coulter, Type Ti70 rotor). This yielded the membrane fraction as a pellet and the cytosolic fraction in the supernatant. The supernatant was collected and the membrane fraction was suspended in 1.5-mL storage buffer (20 mM HEPES pH 7.2, 2 mM DTT). The total protein concentration was determined with Quick Start Bradford assay (Bio-Rad). Membranes and supernatant fractions were both diluted to either 1.0 or 2.0 mg/mL (for proteomics and gel-based ABPP, respectively) and were used directly or flash frozen in liquid nitrogen and stored in aliquots at −80°C until use.

Activity-Based Protein Profiling

Gel-Based

Mouse brain cytosol or membrane fraction (2.0 mg/mL) was incubated with activity-based probe MB064 (250 nM), TAMRA-FP (500 nM), or DH379 (1 μM) (20 min, rt, 2.5% DMSO). For

the competition experiments with inhibitor DH376, this step was preceded by incubation with 100 nM inhibitor (30 min, rt, 2.5% DMSO). For the competition experiments with probe MB108, this step was preceded by incubation with 10 μM probe (30 min, 37°C, 2.5% DMSO). Laemmli buffer was added to quench the protein activity and the mixture was allowed to stand at rt for at least 5 min before the samples were loaded and resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel (10% acrylamide), together with PageRuler Plus Prestained Protein Ladder (Thermo Scientific). The gels were scanned using a ChemiDoc (Bio-Rad, Cy3 channel: expose 180 s for MB064, 60 s for DH379/TAMRA-FP, Cy5 channel 10 s for marker). After fluorescent scanning, the gels were stained with a Coomassie staining solution [0.25% (w/v) Coomassie Brilliant Blue in 50% MeOH, 10% AcOH, 40% MilliQ (v/v/v)]. The gels were scanned after destaining with MilliQ. Both the fluorescence and Coomassie images were analyzed using Image Lab 5.2. The Coomassie gel is used to determine protein loading (automatic lane/band detection, background subtraction not enabled, select whole lane as band). The fluorescence bands are quantified using automatic lane/band detection with background subtraction enabled. Using the Coomassie-corrected gel fluorescence values, the average is calculated for WT and KO and a two-sided Student's *t*-test is performed. The activities of proteins were relatively quantified by setting the average WT at 100%.

Western Blot

After the SDS-PAGE gel was resolved and imaged, proteins were transferred to 0.2-μm polyvinylidene difluoride membranes with a Trans-Blot Turbo^{TM} Transfer system (Bio-Rad). Membranes were washed with TBS (50 mM Tris, 150 mM NaCl) and blocked with 5% milk (w/v, Elk magere melkpoeder, FrieslandCampina) in TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween 20) for 1 h at rt. Membranes were then incubated with the primary antibody anti-DAGLα (1:1000, Cell Signaling Technology, #13626) in 5% BSA (w/v) in TBST (o/n, 4°C), washed with TBST, incubated with matching secondary antibody HRP-coupled-goat-anti-rabbit (1:5000, Santa Cruz, sc2030) in 5% milk in TBST (1 h, rt) and washed with TBST and TBS. Imaging solution (10 mL Luminol, 100 μL ECL enhancer, 3 μL H_2O_2) was added to develop membranes and chemiluminescence was detected on the ChemiDoc (Bio-Rad) using standard chemiluminescence settings. The signal was normalized to Coomassie staining and quantified with Image Lab 5.2.

Proteomics

Mouse brain membrane or soluble proteome (245 μL, 1.0 mg/mL) was incubated with 5 μL 0.5 mM MB108 (10 μM) or FP-Biotin (10 μM) for 1 h at rt. The labeling reaction was quenched and excess probe was removed by chloroform/methanol precipitation: 250 μL MilliQ, 666 μL MeOH, 166 μL $CHCl_3$, and 150 μL MilliQ added subsequently to each sample with a brief vortex after each addition. After centrifugation (4000 rpm, 10 min), the top and bottom layer surrounding the floating protein pellet was removed. 600 μL MeOH was added and the pellet was resuspended by sonication with a probe sonicator (10 s, 30% amplitude). After centrifugation (14,000 rpm, 5 min), the methanol was removed and the protein pellet was redissolved in 250 μL 6 M urea/25 mM ammonium bicarbonate and allowed to incubate for 15 min. 2.5 μL 1 M DTT was added and the mixture was heated to 65°C for 15 min. The sample was allowed to cool to rt (∼5 min) before 20 μL 0.5 M iodoacetamide was added and the sample was alkylated for 30 min in the dark. 70 μL 10% (wt/vol) SDS was added and the proteome was heated for 5 min at 65°C. The sample was diluted with 2 mL PBS. 50 μL of 50% slurry of Avidin-Agarose from egg white (Sigma-Aldrich) was washed with PBS and added to the proteome sample in 1 mL PBS. The beads were incubated with the proteome for 3 h, while rotating. The beads were isolated by centrifugation (2500 *g*, 2 min) and washed with 0.5% (wt/vol) SDS in PBS (1×) and PBS (3×). The proteins were digested overnight with 500 ng sequencing grade trypsin (Promega) in 250 μL on-bead digestion buffer (100 mM Tris pH 8, 100 mM NaCl, 1 mM $CaCl_2$, 2% ACN) at 37°C with vigorous shaking. The pH was adjusted with formic acid to pH 3 and the beads were removed. The peptides were isotopically labeled by on-stage tip dimethyl labeling. Wild-type and knockout samples were differently labeled with isotopic dimethyl labeling and combined after labeling to allow comparison (wild-type light and knockout heavy).

On-Stage Tip Dimethyl Labeling

The stage tips were made by inserting C18 material in a 200-μL pipet tip. The step-wise procedure given in the **Table 1** was followed for stage tip desalting and dimethyl labeling. The solutions were eluted by centrifugal force and the constitutions of the reagents are given below. For label-free quantification samples, steps 6 and 7 are omitted.

Stage tip solution A is 0.5% (vol/vol) FA in H_2O. Stage tip solution B is 0.5% (vol/vol) FA in 80% (vol/vol) ACN in H_2O. Dimethyl labeling reagents: phosphate buffer (50 mM; pH 7.5) with $NaBH_3CN$ 0.03 M containing either 2% (vol/vol) CH_2O (Light) or CD_2O (Medium). After the final elution step, the desired heavy and light samples were combined and concentrated on a Speedvac to remove the ACN. The residue was reconstituted in 95/3/0.1 H_2O/ACN/FA (vol/vol) before LC/MS analysis. Tryptic peptides were measured either on an Orbitrap or Synapt mass spectrometer.

Orbitrap

Tryptic peptides were analyzed on a Surveyor nanoLC system (Thermo) hyphenated to a LTQ-Orbitrap mass spectrometer (Thermo) as previously described. Briefly, emitter, trap and analytical column (C18, 120Å) were purchased from Nanoseparations (Nieuwkoop, Netherlands) and mobile phases (A: 0.1% formic acid/H_2O, B: 0.1% formic acid/ACN) were made with ULC/MS grade solvents (Biosolve). General mass spectrometric conditions were: electrospray voltage of 1.8–2.5 kV, no sheath and auxiliary gas flow, capillary voltage 40 V, tube lens voltage 155 V, and ion transfer tube temperature 150°C. Polydimethylcyclosiloxane (m/z = 445.12002) and dioctyl phthalate ions (m/z = 391.28429) from the environment were used as lock mass. Some 10 μL of the samples was pressure

TABLE 1 | Step-wise on-stage tip dimethyl labeling procedure.

Step	Aim	Solution	Centrifugation
1	Conditioning	Methanol (50 μL)	2 min 600 g
2	Conditioning	Stage tip solution B (50 μL)	2 min 600 g
3	Conditioning	Stage tip solution A (50 μL)	2 min 600 g
4	Loading	Load samples on stage tips	2.5 min 800 g
5	Washing	Stage tip solution A (100 μL)	2.5 min 800 g
6	Dimethyl labeling (5×)	20/20/40/40/30 μL L or M reagent	5 min 400 g
7	Washing	Stage tip solution A (100 μL)	2.5 min 800 g
8	Elution	Stage tip solution B (100 μL)	2.5 min 800 g

loaded on the trap column for 5 min with a 10-μL/min flow and separated with a gradient of 35 min 5–30% B, 15 min 30–60% B, and 5 min A at a flow of 300 μL/min split to 250 nL/min by the LTQ divert valve. Full MS scans (300–2000 m/z) acquired at high mass resolution (60,000 at 400 m/z, maximum injection time 1000 ms, AGC 106) in the Orbitrap was followed by three MS/MS fragmentations in the LTQ linear ion trap (AGC 5 × 103, max inj time 120 ms) from the three most abundant ions. MS/MS settings were: collision gas pressure 1.3 mT, normalized collision energy 35%, ion selection threshold of 750 counts, activation $q = 0.25$, and activation time 30 ms. Ions of $z < 2$ or unassigned were not analyzed and fragmented precursor ions were measured twice within 10 s and were dynamically excluded for 60 s. Data analysis was performed using Maxquant with acetylation (protein N term) and oxidation (M) as variable modifications. The false discovery rate was set at 1%, and the peptides were screened against reviewed mouse proteome (Uniprot). Serine hydrolases that were identified in at least two repetitive experiments and for which at least one unique peptide and two peptides in total were identified were considered as valid quantifiable hits. For proteins identified by both probes, the normalized ratios from Maxquant were combined for further analysis. The binary logarithm of each ratio was compared to 0 with a Student's t-test. The resulting p values were subjected to a Benjamini–Hochberg correction, setting the false discovery rate at 10% ($q = 0.1$). Briefly, the p values of all quantifiable hits were ordered from lowest to highest, and the Benjamini–Hochberg statistic was calculated as $q \times$ (position in the list) divided by the number of tests. Subsequently, the proteins for which the p value is smaller than the BH statistic are controlled for a FDR of $q \times 10\%$.

Synapt

The peptides were measured as described previously for the NanoACQUITY UPLC System coupled to SYNAPT G2-Si high definition mass spectrometer. A trap-elute protocol, where 1 μL of the digest is loaded on a trap column (C18 100Å, 5 μM, 180 μM × 20 mm, Waters) followed by elution and separation on the analytical column (HSS-T3 C18 1.8 μM, 75 μM × 250 mm, Waters). The sample is brought onto this column at a flow rate of 10 μL/min with 99.5% solvent A for 2 min before switching to the analytical column. Peptide separation is achieved using a multistep concave gradient based on the gradients used in Distler et al. (2016). The column is re-equilibrated to initial conditions after washing with 90% solvent B. The rear seals of the pump are flushed every 30 min with 10% (vol/vol) ACN. [Glu^1]-fibrinopeptide B (GluFib) is used as a lock mass compound. The auxiliary pump of the LC system is used to deliver this peptide to the reference sprayer (0.2 μL/min). A $UDMS^e$ method is set up as described in Distler et al. (2016). Briefly, the mass range is set from 50 to 2,000 Da with a scan time of 0.6 s in positive, resolution mode. The collision energy is set to 4 V in the trap cell for low energy MS mode. For the elevated energy scan, the transfer cell collision energy is ramped using drift time specific collision energies (Distler et al., 2014). The lock mass is sampled every 30 s. Raw data are processed in PLGS (v3.0.3) and ISOQuant v1.5.

RESULTS

In 95% of the patients afflicted by NPC, mutations in *NPC1* are observed. Therefore, the role of the ECS in this disease was investigated by comparison of endocannabinoid hydrolase activity between $Npc1^{+/+}$ and $Npc1^{-/-}$ mouse brains. First, labeling profiles of MB064, DH379, and FP-TAMRA in wild-type and knockout mouse brains was evaluated (**Figure 2A**). Membrane and soluble fractions of both wild-type and knockout tissue were labeled with each probe separately, resolved on SDS-PAGE and visualized using in gel fluorescence scanning. Coomassie staining was used as a protein loading control. The fluorescence intensity of the bands corresponding to DAGLα (∼120 kDa), ABHD12 (∼50 kDa), ABHD6 (∼35 kDa), and FAAH (∼60 kDa) were quantified to determine the relative enzyme activity between knockout and wildtype (**Figure 2B**). Using the FP-TAMRA probe, the two bands corresponding to MAGL (∼35 kDa) were observed, but due to band overlap with ABHD6 these cannot be accurately quantified. Labeling of DAGLα significantly decreased in the knockout mice as compared to wildtype, while ABHD6 and ABHD12 activity was the same. FAAH labeling was slightly decreased in the knockout, but this decrease was not statistically significant. In the cytosolic fraction, an increase in intensity of a 75-kDa band as labeled by MB064 and FP-TAMRA was observed in the knockout brains. As reported previously (Gao et al., 2010; Baggelaar et al., 2017), DAGLα is identified as two separate bands. Remarkably, only the fluorescent band corresponding to the higher molecular weight was significantly decreased in the $Npc1^{-/-}$ mice as quantified

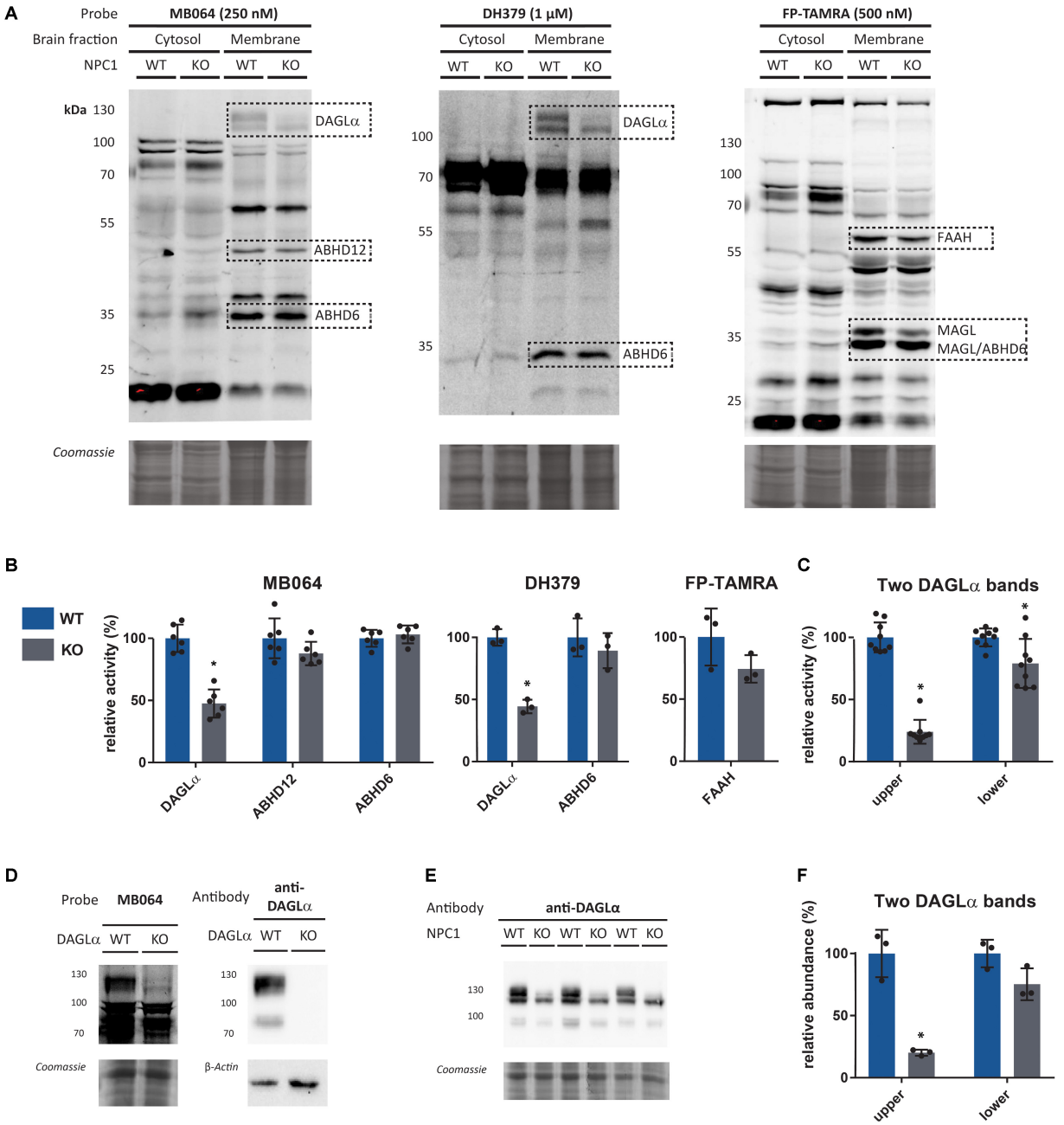


FIGURE 2 | Gel-based *Npc1*$^{+/+}$ (WT) vs. *Npc1*$^{-/-}$ (KO) comparison. **(A)** Enzyme activity in the brain fractions as measured by the probes MB064, DH379, and FP-TAMRA. **(B)** Quantification of relative enzyme activity in WT and KO for probe targets (average WT is set to 100%). $n = 3$ (mice), $n = 2$ (MB064), or $n = 1$ (DH379 and FP-TAMRA). **(C)** Quantification of probe labeling of the two observed bands for DAGLα as measured by MB064 and DH379 [gels: **(A)**]. Three mice per condition ($N = 3$) and three probe labeling experiments per lysate ($n = 3$). **(D)** DAGLα activity and abundance in DAGLα WT and KO mouse brain membrane proteome as measured by the probe MB064 and an anti-DAGLα antibody. **(E)** DAGLα abundance in NPC1 WT and KO mouse brain membrane proteome. **(F)** Quantification of antibody labeling of the two observed bands for DAGLα as measured by anti-DAGLα [blot: **(E)**]. Mean ± standard deviation is shown. *$p < 0.05$ (Student's *t*-test).

by two separate probes MB064 and DH379 (**Figure 2C**). Both bands can be labeled with a DAGLα antibody and are absent in DAGLα KO mice (**Figure 2D**). To see if this observation was due to a decrease of protein abundance, a Western blot against DAGLα was performed (**Figure 2E**). The signal of antibody labeling was quantified (**Figure 2F**). The same pattern was observed for relative abundance as for relative activity: only the upper band corresponding to DAGLα is significantly decreased (**Figures 2C,F**).

Although several endocannabinoid enzymes can be quantified with gel-based ABPP, multiple bands remain unidentified (**Figure 2A**). Additional enzymes can be identified using a

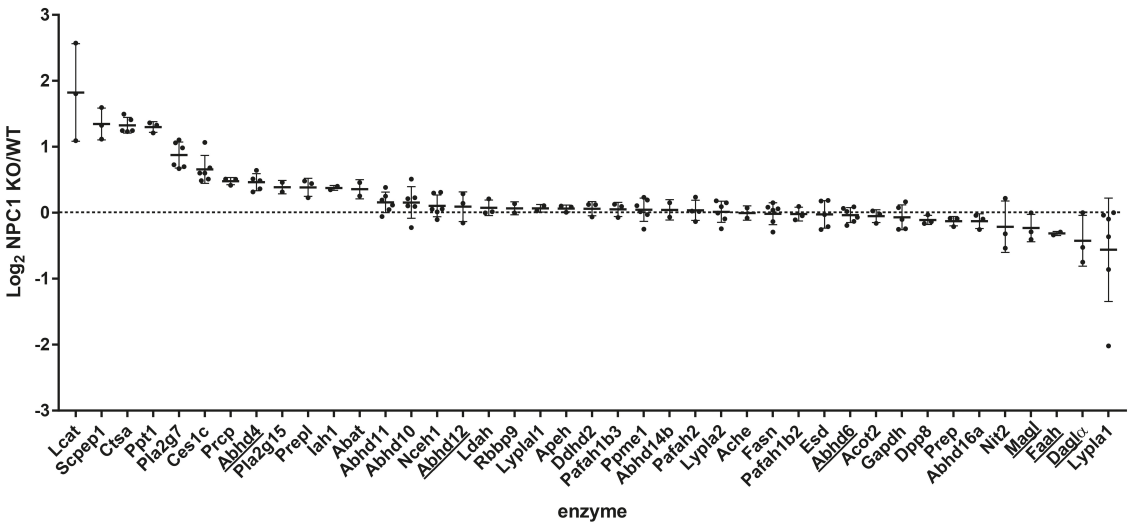


FIGURE 3 | Chemical proteomics comparison of *Npc1*$^{+/+}$ (WT) vs. *Npc1*$^{-/-}$ (KO). Log_2 ratio of enzyme activity in *Npc1*$^{-/-}$ brain proteome compared to *Npc1*$^{+/+}$. Activity was measured using FP-biotin or MB108 (both 10 μM). The data from both probes were combined for both the cytosolic and membrane fraction. The mean and standard deviation are shown. Endocannabinoid related enzymes are underlined. Statistical analysis by means of Student's *t*-test (each ratio was compared to a log_2 ratio of 0) and the resulting *p* values were subjected to Benjamini–Hochberg correction, setting the false discovery rate at 10% (* indicates significant difference).

sensitive mass spectrometry-based method for ABPP (chemical proteomics). Therefore, to study a broader range of serine hydrolases and to confirm our observations from the gel-based assay, we also employed a chemical proteomics method using FP biotin and MB108 to assess the role of the ECS in NPC1. The enzymatic activity of 41 hydrolases in NPC1 knockout mouse brain were identified and compared with wild-type mouse brains (**Figure 3**). In line with the gel-based ABPP, we did not find any significant difference in the endocannabinoid hydrolase activity (ABHD12, ABHD6, FAAH, and MAGL) in NPC1 wild-type vs. knockout mice brain proteomes. The activity of endocannabinoid hydrolase ABHD4 is also unaltered in knockout compared to wildtype. Remarkably, and in contrast to the gel-based ABPP results, DAGLα activity in knockout mice brain proteomes was not decreased compared to wild-type mice. Of note, in a control experiment, biotinylated probe MB108 competes with both DAGLα bands labeled by the fluorescent probe MB064. Additionally, DAGL inhibitor DH376 did reduce DAGLα labeling in the chemical proteomics assay (Supplementary Figure S2). Finally, three hydrolases were significantly increased in knockout mouse brains compared to wild-type brains: retinoid-inducible serine carboxypeptidase (SCPEP1), cathepsin A (CTSA), and palmitoyl-protein thioesterase 1 (PPT1) (**Figure 3**).

DISCUSSION

We used ABPP to quantify the activity of the endocannabinoid hydrolases FAAH, ABHD6, ABHD12, ABHD4, and MAGL in NPC1 knockout mice and found that their activity is not affected in wild-type vs. NPC1 knockout mice. DAGLα activity seems to be decreased in the knockout mice based on the gel-based ABPP results. However, we found a discrepancy in the gel-based ABPP assay and the chemical proteomics assay in DAGLα activity between the NPC1 knockout mouse brains compared to wild-type mice. This discrepancy can possibly be caused by technical factors inherent to the two applied methodologies. In the gel-based ABPP assay, only the labeling of the upper DAGLα-band was abolished in the NPC1 knockout brain proteome. It could be that the peptides from the protein corresponding to the upper band from the wild-type mouse proteome are not detected in the mass spectrometer due to post-translational modifications, such as phosphorylation, acetylation, methylation, palmitoylation, or glycosylation. Peptides with such modifications are not found in our assay. If this would be true, a decrease in DAGLα activity in the NPC1 knockout mice would not be detected. Further experiments, such as direct quantification of 2-AG levels, are required to confirm a decrease in DAGLα activity in Niemann-Pick mice. In addition, it would be interesting to find the origin and role of the altered modification of DAGLα in NPC. If the gel-based ABPP results are confirmed, it would be worthwhile to measure 2-AG levels and test the hypothesis that decreased DAGL activity might represent a neuroprotective response by lowering the formation of pro-inflammatory prostaglandins (Ogasawara et al., 2016). Alternatively, it is known that 2-AG reduces cytotoxic edema in traumatic brain injury (Panikashvili et al., 2001), therefore boosting 2-AG brain levels by inhibition of MAGL may constitute a neuroprotective response.

Finally, we found that the activities of three non-endocannabinoid hydrolases, namely, CTSA, PPT1, and SCPEP1, were significantly elevated in NPC1 knockout mouse brains. These enzymes are lysosomal proteins, which is in line with the important role of NPC1 in lysosomes. SCPEP1 shows homology with CTSA (Kollmann et al., 2009) and a study with double

knockout of SCPEP1 and CTSA suggests they share the same peptide substrate (Pan et al., 2014). Single knockout of SCPEP1 in mice resulted in viable mice without lysosomal impairment (Kollmann et al., 2009). Mutations in the CTSA gene cause galactosialidosis in humans (Galjart et al., 1990) and secondary deficiencies of beta-galactosidase and neuraminidase. This same phenotype is mirrored in a CTSA knockout mice mouse model (Korah et al., 2003). The protective role of the enzyme for galactosidase and neuraminidase is a structural function of the enzyme: mice with catalytic serine-to-alanine mutation have normal levels of β-galactosidase and neuraminidase (Seyrantepe et al., 2008). CTSA inhibitors have been tested in humans (Tillner et al., 2016), and CTSA is a therapeutic target for the treatment of cardiovascular diseases (Schreuder et al., 2014; Petrera et al., 2016). Thus, it would be interesting to test CTSA inhibitors in Niemann-Pick models. PPT1 is also a lysosomal enzyme (Lu et al., 2002) and the dysregulation of this enzyme causes a lysosomal storage and neurodegenerative disorder; infantile neuronal ceroid lipofuscinosis (Tillner et al., 2016). This enzyme removes palmitoyl modifications from proteins that are being degraded in the lysosome. In PPT1 knockout mice, cholesterol catabolism is altered (Ahtiainen et al., 2007). PPT1 has been proposed to hydrolyze 2-AG and might therefore be involved in the ECS (Wang et al., 2013). *In vivo* active inhibitors for PPT1 (Cognetta et al., 2015) have been identified and could therefore also be tested in Niemann-Pick models. To conclude, we have found no altered activity of endocannabinoid hydrolases in a NPC mouse model using our chemical proteomics assay. Three hydrolases were identified with upregulated activity in NPC1 knockout mice, which might be interesting therapeutic target for future studies.

DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics datasets generated for this study have been deposited to the ProteomeXchange Consortium *via* the PRIDE (Vizcaíno et al., 2016) partner repository with the dataset identifier PXD008979.

AUTHOR CONTRIBUTIONS

EvR and AvE carried out the experiments and analyzed gel-based results. EvR analyzed the mass spectrometry data and supervised by MB. HD synthesized DH379 and supervised sample preparation. BF assisted with LC/MS measurements. AM and RO performed mice experiments and harvested tissue. EvR and MvdS wrote the manuscript, supported by RB and HO. MvdS and JA conceived the presented study. All authors provided critical feedback.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2018.00440/full#supplementary-material

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Long-Term Stress and Concomitant Marijuana Smoke Exposure Affect Physiology, Behavior and Adult Hippocampal Neurogenesis

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Marijuana is a widely used recreational drug with increasing legalization worldwide for medical purposes. Most experimental studies use either synthetic or plant-derived cannabinoids to investigate the effect of cannabinoids on anxiety and cognitive functions. The aim of this study was to mimic real life situations where young people smoke cannabis regularly to relax from everyday stress. Therefore, we exposed young adult male NMRI mice to daily stress and concomitant marijuana smoke for 2 months and investigated the consequences on physiology, behavior and adult hippocampal neurogenesis. Animals were restrained for 6-h/day for 5-days a week. During the stress, mice were exposed to cannabis smoke for 2 × 30 min/day. We burned 2 "joints" (2 × 0.8 g marijuana) per occasion in a whole body smoking chamber. Cannabinoid content of the smoke and urine samples was measured by HPLC and SFC-MS/MS. Body weight gain was recorded daily and we did unrestrained, whole body plethysmography to investigate pulmonary functions. The cognitive performance of the animals was evaluated by the novel object recognition and Y maze tests. Anxiety-related spontaneous locomotor activity and self-grooming were assessed in the open field test (OFT). Adult neurogenesis was quantified *post mortem* in the hippocampal dentate gyrus. The proliferative activity of the precursor cells was detected by the use of the exogenous marker 5-bromo-2′-deoxyuridine. Treatment effects on maturing neurons were studied by the examination of doublecortin-positive neurons. Both stress and cannabis exposure significantly reduced body weight gain. Cannabis smoke had no effect on pulmonary functions, but stress delayed the maturation of several lung functions. Neither stress, nor cannabis smoke affected the cognitive functioning of the animals. Results of the OFT revealed that cannabis had a mild anxiolytic effect and markedly increased self-grooming behavior. Stress blocked cell proliferation in the dentate gyrus, but cannabis had no effect on this parameter. Marijuana smoke however had a pronounced impact on doublecortin-positive neurons influencing their number,

morphology and migration. In summary, we report here that long-term stress in combination with cannabis smoke exposure can alter several health-related measures, but the present experimental design could not reveal any interaction between these two treatment factors except for body weight gain.

Keywords: body weight, BrdU, *Cannabis sativa*, chronic stress, cognitive function, doublecortin, hippocampus, self-grooming

INTRODUCTION

Marijuana is the most widely used illicit drug, as about 2.5% of the world population consume cannabis regularly (UNODC, 2017). Cannabis is increasingly legalized worldwide for medical and recreational purposes (Carliner et al., 2017; EMCDDA, 2017; Webster, 2018), which results in an increasing consumption while the long-term consequences on health are not well understood (e.g., Volkow et al., 2014, 2016; Levine et al., 2017). Thus, it is important to investigate the outcomes of prolonged use.

Advocates argue that marijuana is a safe and natural alternative for the treatment of a variety of medical and mental health conditions, but ambiguous data are reported in the literature on the health risks imposed by chronic cannabis use. Cannabidiol (CBD), a major constituent of *Cannabis sativa* and several components of the endocannabinoid system are increasingly viewed as potential 'druggable' targets for the treatment for anxiety-related disorders (Blessing et al., 2015; Lee et al., 2017; Patel et al., 2017). Indeed, anxiety is among the top five medical symptoms for which North Americans report using medical marijuana while its anxiolytic effectiveness is still not well documented (Turna et al., 2017). The correlation between cannabis use and cognitive enhancement or impairment is also ambiguous. While numerous clinical and preclinical data suggest a strong correlation between marijuana exposure and impaired cognition, it does not conclusively demonstrate that cannabis consumption alone is sufficient to cause these deficits in humans (Broyd et al., 2016; Curran et al., 2016; Volkow et al., 2016; Levine et al., 2017). At the same time, there are reports on positive effect of cannabis use on various cognitive and executive functions (Osborne et al., 2017; Gruber et al., 2018; Tervo-Clemmens et al., 2018).

The consequences of prolonged marijuana inhalation on respiratory health and lung cancer is also debated (Gates et al., 2014; Martinasek et al., 2016; Chatkin et al., 2017; Stone et al., 2018). Similarly, there is conflicting data on the effect of cannabis use on body weight. Cannabis is known to stimulate appetite and potentially promote weight gain in patients suffering from human immunodeficiency virus or cancer, whereas, findings of the large epidemiological studies in the general population, consistently indicate that users of marijuana tend to have lower body mass indices (Sansone and Sansone, 2014).

Adult neurogenesis in the hippocampal dentate gyrus is a unique form of neuroplasticity that has received substantial attention during the recent years. Adult-born neurons in the dentate play an essential role in normal cognitive functioning and in specific forms of learning (Denny et al., 2014; Anacker and Hen, 2017). Stress is a potent inhibitor of the proliferative activity of the precursor cells and also blocks the survival of the newly generated neurons (Gould et al., 1997; Czéh et al., 2001, 2002; Cameron and Schoenfeld, 2018). In consequence, stress-induced disruption of adult neurogenesis may play a role in the development of various psychiatric disorders, including depression, anxiety, and schizophrenia (Santarelli et al., 2003; Snyder et al., 2011; Surget et al., 2011; Kim et al., 2012; Schoenfeld and Cameron, 2015).

Cannabinoid receptors are highly expressed in the hippocampus, and recent studies suggest that facilitation of the cannabinoid signaling in the hippocampus may prevent stress-induced behavioral changes (Campos et al., 2013; Scarante et al., 2017; Fogaça et al., 2018). Numerous studies investigated the effect of various cannabinoids on adult hippocampal neurogenesis (Prenderville et al., 2015), but these studies used either plant-derived extracts or synthetic cannabinoids. The results of these studies are also inconsistent. Some studies report on positive, stimulatory effect (Jiang et al., 2005; Palazuelos et al., 2006; Marchalant et al., 2009; Wolf et al., 2010; Rivera et al., 2011; Suliman et al., 2018), while others document negative, inhibitory effect on adult neurogenesis (Realini et al., 2011; Abboussi et al., 2014; Lee et al., 2014; Steel et al., 2014), or no effect at all (Kochman et al., 2006; Maćkowiak et al., 2007; Steel et al., 2014).

Most of the preclinical studies use either synthetic cannabinoid agents or cannabis extracts to investigate the physiological and behavioral consequences. Typically, animals are injected with various doses of the synthetic compound in an acute or chronic (10–14 days) treatment protocol. The aim of the present study was to mimic a real life situation where cannabis is smoked by adolescents on a daily basis to ease everyday stress. We used young adult mice and subjected them to daily stress over a 2-month period and during the stress exposure the animals were also exposed to cannabis smoke. To investigate the health risks of such a treatment protocol, we examined body weight gain, pulmonary functions, emotional responses and cognitive performance, as well as cellular changes in adult hippocampal neurogenesis. The *Cannabis sativa* used in this experiment was obtained from the local Hungarian Police Superintendancy. To determine the amount of tetrahydrocannabinol (Δ9-THC), cannabinol (CBN), and cannabidiol (CBD) content of our sample, we did an HPLC analysis of the marijuana smoke and metabolites of these compounds were measured in urine samples collected from the animals.

MATERIALS AND METHODS

Animals

Young male NMRI mice, weighing 20 ± 3 g (4 weeks of age; n = 36) at the beginning of the experiment, were used. In mice, the sexual maturity, i.e., the first vaginal estrus starts during puberty at postnatal day 25–50 depending on the mouse strain (Drickamer, 1981). This indicates, that the mice enrolled in this experiment were at their "late puberty" when the stress and cannabis treatment protocol started. Animals were purchased from "Toxi-Coop" Toxicological Research Center Ltd. (Budapest, Hungary), and kept (group-housed) in a temperature and humidity-controlled animal facility and maintained on a standard 12 h light/dark cycle (lights on at 8 AM). Food and water were available *ad libitum* in the home cages.

Ethical Considerations

The experimental procedures were carried out according to the 1998/XXVIII Act of the Hungarian Parliament on Animal Protection and Consideration Decree of Scientific Procedures of Animal Experiments (243/1988). The studies were approved by the Ethical Committee on Animal Research of the University of Pécs according to the Ethical Codex of Animal Experiments, and license was given (License No. BA02/2000-35/2016).

Experimental Design

The experimental design including the animal groups, behavioral tests and the timeline of the procedures are depicted on **Figure 1**. First, the animals were allowed to habituate to the new housing conditions for 10 days. Afterward the animals were divided into four experimental groups according to the stress and drug treatment protocols: Control, Control + Cannabis, Stress, and Stress + Cannabis (n = 9/group). The long-term stress experiment lasted for 8 weeks. Animals were exposed to restraint stress every day, 5-days a week, but on the weekends they had rest. All the behavioral and the respiratory function tests were performed on weekends, when the animals were not subjected to stress or cannabis treatment. The behavioral testing started at 8:00 a.m. and measurement of one animal usually took 5–10 min. We tested the mice in an alternating order using a predefined sequence to minimize the potential effect of the circadian rhythm on the performance of mice from the different experimental groups.

Restraint Stress Procedures

Animals were immobilized daily for 6 h, between 08:00 a.m. and 14:00 p.m. During restraint, the animals were placed in well ventilated polypropylene tubes (internal diameter, 3 cm; length, 11.5 cm) according to our pervious protocol (Scheich et al., 2017). During immobilization stress, mice did not have access to food or water. Control mice were not subjected to any kind of stress except daily handling. We used this restraint stress protocol because it has been demonstrated that this protocol is stressful for rodents and results in pronounced structural changes in the hippocampus, including dendritic atrophy and reduced adult neurogenesis in the dentate gyrus (McLaughlin et al., 2007; Veena et al., 2009; Yun et al., 2010).

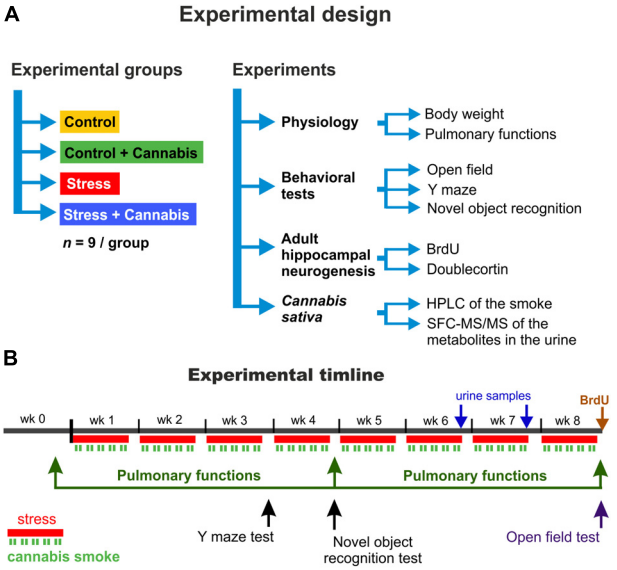


FIGURE 1 | The experimental design and procedures. **(A)** Mice were divided into four experimental groups according to the stress and drug treatment protocol: Control, Control + Cannabis, Stress, and Stress + Cannabis (n = 9/experimental group). Various experimental tests and measurements were done to examine treatment effects on physiology, behavior and adult hippocampal neurogenesis. To determine the amount of Δ9-THC, CBD and CBN in our *Cannabis sativa* sample, we did a HPLC analysis of the marijuana smoke and SFC-MS/MS analysis was used to measure metabolites of these compounds in urine samples collected from the animals. **(B)** Timeline of the experimental procedures. First, the animals were allowed to habituate for 1 week. The long-term stress experiment lasted for 8 weeks. Animals were exposed to restraint stress every day for 6 h, 5-days a week (red lines). On weekends they had rest. During the restraint stress, mice were exposed to the cannabis smoke for 2 × 30 min/day (green lines). The cannabis smoke exposure was done at the end, i.e., during the last 2 h of the 6 h long restraint stress procedure and the smoke was applied simultaneously to all animals. We burned 2 × 2 "joints" (4 joints/day; 20 joints/week) in a manual smoking system and the animals had a whole body smoke exposure. All the behavioral tests were done on the weekends when the animals were not exposed to stress and/or marijuana smoke.

Chronic Whole Body Cannabis Smoke Exposure

Dried marijuana was provided by the Hungarian Police Superintendancy after the necessary permission was obtained from the Drug Licensing Department operating at the Office of Health Authorization and Administrative Procedures (Budapest, Hungary; License No. 15259/2016/KAB).

The marijuana was chopped, and cigarettes containing 0.77 ± 0.03 g marijuana were prepared. Mice were exposed to whole body marijuana smoke in a two-port TE-2 smoking chamber (TE-2 Teague Enterprises) from the age of 5 week for 8 weeks, twice a day for 30–30 min. Two joints were burned per occasion (4 joints/day; 20 joints/week; 154.97 ± 5.18 mg total particulate material/m^3) for 10 min with a puff duration

of 2 s and a puff frequency of 1/min/joint. This was followed by a 30 min long exposure period during which the smoke was driven into the chamber. After the smoke exposure there was a 20 min long ventilation period, during which the smoke was drawn out of the chamber by a vent hood, and then, the smoking session was repeated. Stressed mice were exposed to the cannabis smoke at the end, i.e., during the last 2 h of the 6 h long restraint stress procedure. Home cages containing the control animals, as well as mice subjected to the restraint stress were all placed in the smoking chamber simultaneously, therefore all mice were exposed to the marijuana smoke at the same time-point of their diurnal rhythm.

HPLC Equipment and Chromatographic Conditions

Two cigarettes containing 0.77 ± 0.03 g marijuana were prepared and burned down in a smoking system. The formed marijuana smoke was pumped through a methanol containing gas-washing bottle by a vacuum pump. Methanol was removed by using a rotary evaporator. The sample was dissolved in 5 cm^3 of methanol and filtered through a membrane filter (0.2 μm). The HPLC system used consisted of a Dionex P680 gradient pump (Dionex Corp., Sunnyvale, CA, United States), a helium degassing system, a Rheodyne 8125 injector valve with a 20 μL loop (Rheodyne Europe GmbH, Bensheim, Germany), and a Dionex 340D UV–vis diode array detector (Dionex Corp., Sunnyvale, CA, United States). The eluate was monitored at ambient temperature at different wavelengths, 209, 210, and 220 nm, respectively, where the investigated cannabinoids have their absorbance maxima. Chromatographic separations were achieved using Kinetex, C18 reversed phase column (2.6 μm, 2.1 mm × 150 mm i.d.). A Chromeleon data management software (Version 6.60 SP3 Build 1485, Dionex Corp., Sunnyvale, CA, United States) was used to control the equipment and for data evaluation. Peaks were identified by retention times and by UV spectrum of the respective compounds in comparison with those of the references. Quantification was carried out using the peak areas method. All measurements were made in triplicate.

The gradient consisted of two eluents: (A) water/methanol (90:10; v/v); (B) water/methanol (10:90; v/v) containing 50 mM of ammonium formate (adjusted to pH 5.20). The gradient profile was 0.0–20.0 min from 80% to 100% B, 20.0–25.0 min 100% B. After maintaining this condition for 5 min, the coloumn was set to initial condition in 1 min and re-equilibrated under this condition for 4 min. The total runtime was 30 min. Flow rate was set to 0.2 mL/min, the injection volume was 20 μL. All experiments were carried out at 25°C.

Measurement of Cannabinoid Content of the Urine by SFC-MS/MS

Since cannabinoids have 97% plasma protein binding, penetrate to all tissues, and have high accumulation and urinary excretion (Nahas et al., 1981; Nahas and Latour, 1992), we determined the concentration of Δ9-THC, 11-nor-9-carboxy-THC, CBD and CBN in the urine. Urine samples (200–350 μl per mouse) were collected from all mice exposed to marijuana smoke by gently applying lower abdominal pressure on the 6th and 7th week of the experimental protocol (**Figure 1**). Samples were stored at 4°C and analyzed within 5 days.

After salting-out assisted liquid-liquid microextraction measurements were performed by an ACQUITY UPC^2 supercritical fluid chromatography system (Waters) coupled with a Xevo TQ-S Triple Quadrupole Mass Spectrometer (Waters). Data were recorded by MassLynx software (V4.1 SCN950) and evaluated by TargetLynx XS software. Separation of compounds was performed on a 3.0 mm × 100 mm, 1.7 μm particle size, ACQUITY UPC^2 Torus DIOL analytical column (Waters). The mobile phase consisted of the mixture of carbon dioxide (A) and 5 mM ammonium hydroxide in methanol (B) with flow rate of 1.2 mL/min. The following gradient profile was used: 99.9% A at 0.0 min and 82.0% A at 4.5 min. Pre-equilibration period lasting 2 min was applied before each injection. Constant 200 bar back pressure was used to maintain the supercritical state. The temperature was set at 45°C and the volume of injection was 1 μL. To sustain a suitable electrospray, additional solution consisted of 5 mM ammonium hydroxide in methanol was applied with flow rate of 0.1 mL/min. This makeup solvent was delivered by a Waters 515 HPLC Pump.

The MS measurement was performed in positive ion mode. The ESI source was operated with a spray voltage of 3.00 kV, cone voltage was 30 V. The source was set at 150°C. Both desolvation and cone gasses were nitrogen delivered at 300 and 150 L/min, respectively. Desolvation gas was temperatured at 300°C. Collision gas was argon with flow rate of 0.13 mL/min. MS/MS experiments were performed in MRM (multiple reaction monitoring) mode, monitoring five fragments with optimal collision energies.

Pulmonary Function Tests

Pulmonary functions were measured by unrestrained whole body plethysmography individually in conscious, spontaneously breathing, freely moving animals repeatedly in a self-controlled manner. We did a baseline measurement on Week 0 (before the stress and cannabis exposure started) and subsequent measurements on Week 4 and Week 8 of the experimental procedures (**Figure 1**). Mice were placed in the chamber of a whole body plethysmograph (PLY 3211, Buxco Europe Ltd., Winchester, United Kingdom). The flow transducers (TRD5700, Buxco Europe Ltd., Winchester, United Kingdom) were connected to the preamplifier module, which digitized the signals via an analog-to-digital converter (MAX2270 Buxco Europe Ltd.). Ventilation parameters frequency (f), tidal volume (TV), time of inspiration (Ti), time of expiration (Te), minute ventilation (MV), peak inspiratory flow (PIF), and relaxation time (RT) were measured every 10 s for 2 min and averaged by the BioSystem XA Software for Windows (Buxco Research Systems). We also determined the enhanced pause (Penh) value, which is a calculated parameter [(expiratory time/relaxation time)-1]/(max. expiratory flow/max. inspiratory flow). This parameter characterize bronchoconstriction, which was induced by nebulizing 50 μl of 22 mM carbachol (a muscarinic acetylcholine receptor agonist). Acquisitions were taken in a

2-min-long baseline period aerosolized with saline and then 15 min after carbachol inhalation.

Y Maze Test

The Y maze test was performed at the end of the 3rd experimental week (**Figure 1**) in an open Y-shaped maze placed on the ground with three 35 cm long gray plastic arms with 5 cm inner width (labeled A, B, and C) at 120° angle from each other lit from above. Mice were placed in the center of the maze and were allowed to freely explore the three arms for 5 min, their behavior was recorded with a video camera. Since mice generally prefer visiting a new arm of the maze and show a tendency to enter the less recently explored arm, the number of entries and triads (three consecutive visits of alternating arms) to calculate the spontaneous alternation were assessed afterward. Entries were counted when all four paws entered the arm.

Novel Object Recognition Test

The novel object recognition test (NOR) was performed on the 4th week of the experimental procedures (**Figure 1**) in an open arena (45 × 45 cm with 30-cm-high surrounding walls) lit from above. The test lasted for 3 days: on the first day animals were habituated to the new environment for 10 min. On the second day two identical objects smaller than the mice were placed in the arena and mice were allowed to explore the objects for 5 min. On the last day, exactly 24 h later one of the identical objects was replaced with another object with different shape and color, and the behavior of the mice was recorded with a video camera for 5 min. The parameters of discrimination index [(time spent with novel object – time spent with familiar object)/time spent with both objects] and recognition index (time spent with novel object/time spent with both objects × 100) were assessed afterward.

Open Field Test

The open field test (OFT) was performed at the end of the experimental procedures (**Figure 1**) in an open arena (45 × 45 cm with 30-cm-high surrounding walls) lit from above with a floor divided into 25 equal squares (9 × 9 cm). The test started by placing each mice individually into the center of the arena and then their behavior was recorded with a video camera for 5 min. The following parameters were analyzed afterward: time spent in the central fields (reciprocally proportional to the level of anxiety), locomotor activity (the time spent with moving), velocity (the ratio of the number of crossed squares/time spent with moving), time spent with grooming and grooming latency (time spent until the first grooming).

BrdU Injection, Fixation and Post Mortem Processing of the Brain Tissue

The thymidine analog 5-bromo-2′-deoxyuridine (BrdU) was purchased from Sigma (Sigma–Aldrich, St. Louis, MO, United States). BrdU was dissolved in sterile 0.9% saline (containing 0.007 N NaOH) at a concentration of 15 mg/ml. To study cell proliferation, a single BrdU injection (200mg/kg, ip) was administered on the last experimental day. Animals were then perfused 24 h after the BrdU injection.

At the end of the experiment, mice were anesthetized deeply with a mixture of ketamine (Calypsol inj. 50 mg/ml, Richter Gedeon) and xylazine (Sedaxylan® inj. 20 mg/ml, Eurovet Animal Health BV) 100/10 mg/kg administered intraperitoneally and then perfused transcardially with ice cold physiological saline, followed by freshly prepared ice cold 4% paraformaldehyde in 0.1 M phosphate buffer.

Brains were removed from the perfused animals, postfixed in perfusate overnight and sectioned on a Leica VT1200 S fully automated vibrating blade microtome. Serial coronal sections were cut throughout the entire hippocampal formation along the septo-temporal axis. The 50-μm sections were collected in series and stored in 0.1 M phosphate buffer (pH = 7.4) with 0.05% sodium azide at 4°C until staining.

BrdU Immunocytochemistry

Every third section was slide-mounted on Superfrosts slides (Menzel-Glaser, Braunschweig, Germany), and coded to ensure objectivity before processing for immunocytochemistry. Immunohistochemistry was performed according to our standard protocol (Czéh et al., 2001, 2002, 2007). The sections were first washed in 0.1M Tris-buffer and then the DNA was denaturized in 0.1M citric acid at pH 6.0 and 95°C for 20 min. After treating the sections with 1% H_2O_2 in Tris for 20 min, the samples were washed in 0.1M Tris, and then, the cellular membranes were permeabilized with 0.1% trypsin in 0.1M Tris for 10 min. After thorough washing in Tris, the sections underwent acidification in 2N HCl in Tris for 30 min. After repeated rinsing in 0.1M Tris and PBS, non-specific binding was prevented by incubating the sections for 1h in 5% normal goat serum (NGS; Vector Laboratories, Burlingame, CA, United States) in PBS containing 0.5% Triton X-100 at 4°C. Subsequently, the sections were incubated for one night at 4°C with 1:5000 mouse anti-BrdU (DAKO, Clone Bu20a, Catalog # M074401) in the incubation solution (1% NGS, 0.5% Triton X-100 and 0,5% TWEEN-20 in PBS). After washing, the sections were incubated with biotinylated goat anti-mouse IgG (1:200, Vector Laboratories, Catalog # BA-9200) for 1 h at 4°C, thoroughly washed, incubated in avidin-biotin-horseradish peroxidase (1:500; Vectastaine Elite ABC Kit, Vector Laboratories) for 2 h at 4°C and then washed again. BrdU-labeled cells were visualized in 0.025% 3,30-diaminobenzidine (Sigma–Aldrich) and 0.01% H_2O_2 in PBS for 10 min. Sections were washed in PBS and counterstained with cresyl violet. After overnight drying at room temperature, the sections were dehydrated in graded alcohol, cleared in xylene and coverslipped with Eukitt.

Doublecortin (DCX) Immunocytochemistry

Immunohistochemistry was performed according to our standard protocol (Csabai et al., 2016). Free-floating sections were washed in 0.1M PBS and then treated with 3% H_2O_2 for 30 min. After thorough washing in PBS, non-specific binding

was prevented by incubating the sections for 1 h in 3% normal goat serum (NGS; Vector Laboratories) in PBS containing 0.5% Triton X-100. Subsequently, the sections were repeatedly rinsed in PBS for 1 h and incubated for one night at 4°C with a rabbit anti-DCX antibody (1:3000 Cell Signaling Technology Catalog # 4604). After thorough washing, the sections were incubated with anti-rabbit biotinylated secondary antibody (1:200; Vector Laboratories) for 2 h, washed and incubated in avidin-biotin-horseradish peroxidase (1:100; Vectastaine Elite ABC Kit, Vector) for 2 h. Labeled cells were visualized in 0.025% 3,30-diaminobenzidine (Sigma–Aldrich) and 0.01% H_2O_2 in PBS for 10 min. The sections were mounted, dried overnight and then dehydrated in graded alcohol, cleared and coverslipped with Eukitt. Slides were coded before quantification to ensure objectivity. Images were acquired on a Nikon Eclipse Ti-U workstation.

Quantification of Adult Born Cells in the Dentate Gyrus

Cell were counted manually. A single experimenter (KR) who was blind to the group identification of each animal performed the data collection. The code was not broken until the cell counting analyses were completed. Cell counting was done using the Neurolucida (Version 7) reconstruction system (Microbrightfield, Colchester, VT, United States) attached to a Nikon Eclipse bright field microscope.

The quantitative analysis was carried out using a modified unbiased stereology protocol that has been reported to successfully quantify adult-born neurons in the dentate gyrus (Eisch et al., 2000; Czéh et al., 2001, 2002; Csabai et al., 2016). The BrdU+ and DCX+ cells were counted in a systematic manner in complete series of 50 μm thick sections starting at a random position along the entire septo-temporal axis of the hippocampal formation (from −0.94 to −3.88 relative to Bregma, according to the atlas of Franklin and Paxinos (2008). Every third section throughout the dentate gyrus was examined, yielding a mean of 25 sections per animal. All BrdU+ and DCX-labeled cells in the granule cell layer together with the subgranular zone, defined as a two-cell-body-wide zone along the border of the granule cell layer, were counted regardless of size or shape. Cells were examined under ×200 magnification, omitting cells in the outermost focal plane. The total number of BrdU+ or DCX+ cells was estimated by multiplying the number of cells counted in every third section by three. Neuron numbers are reported here as total neuron number/hemisphere.

DCX+ neurons were quantified similarly to the BrdU+ cells as described earlier (Csabai et al., 2016). We not only counted DCX+ cells, but also analyzed their morphology and cell migration. To examine cell migration, the neurons were selectively counted in the hilus, in the subgranular zone (sgz) and in the granule cell layer (gcl). The ratio of cells located in these three different cellular layers was expressed in percentage. Furthermore, we also counted the number of ectopic DCX+ neurons, i.e., cells which migrated out of the gcl into the molecular layer of the dentate gyrus. The morphological features of the DCX+ cells were quantified in two different ways. First, we did a semi-quantitative analysis in the following manner: we analyzed every 3rd serial section from each animal yielding 25 ± 3 sections/animal. After thorough visual inspection, every section was marked in which we observed abnormal looking DCX+ neurons (i.e., cells with irregular, disarrayed dendritic arbor). Data are reported as the number of sections including cells with abnormal morphology/animal. Afterward, we quantified the DCX+ neurons which had the most pronounced atypical appearance, i.e., bipolar DCX+ cells and DCX+ neurons with basal dendrites.

Statistical Analysis

Results are expressed as the mean ± SEM. The data on body weights and pulmonary function tests were analyzed with three-way ANOVA (time × stress × cannabis treatment) followed by Tukey's multiple comparisons *post hoc* test. The rest of the data was analyzed with two-way ANOVA (stress × cannabis treatment) followed by Tukey's multiple comparisons *post hoc* test.

RESULTS

Analysis of the Marijuana Smoke and Urine Samples

We performed a HPLC analysis to determine the Δ9-THC, CBD and CBN content of the marijuana smoke used in this study. Furthermore, we collected urine samples from the mice that were exposed to marijuana smoke on the 6th and 7th week of the experimental protocol (**Figure 1**). Concentration of Δ9-THC, 11-nor-9-carboxy-THC, CBD and CBN was determined by SFC/MS-MS in the urine. Data are reported in **Table 1**. The measurements revealed that the marijuana smoke had about 10× higher concentration of Δ9-THC than CBD. This ratio was even higher (30×) in the urine samples. The amount of 11-nor-9-carboxy-THC (a major metabolite of Δ9-THC) was 1.8 ± 0.2 in the urine (**Table 1**).

Stress and Cannabis Exposure Reduced the Body Weight Gain of the Animals

Body weights were determined every day and then, the daily values were grouped and reported here as mean body weight/week (**Figure 2**). Analysis with a three-way ANOVA (time × stress × cannabis treatment) revealed that all three factors had a significant main effect: time: $F(8,8) = 62.48$, $P < 0.001$; stress: $F(1,8) = 13.81$, $P < 0.002$; and cannabis treatment: $F(1,8) = 66.71$, $P < 0.001$. Furthermore, there were significant interactions between the following two factors: time × cannabis treatment: $F(8,8) = 2.11$, $P < 0.001$ and stress × cannabis treatment: $F(1,8) = 30.3$, $P < 0.01$. This indicates that cannabis treatment increasingly inhibited body weight gain as the animals grew and that cannabis smoke consumption could alleviate the stress-induced inhibitory effect on body weight gain. For the results of the *post hoc* comparisons on group differences please see **Figure 2**.

TABLE 1 | Cannabinoid concentrations in the marijuana smoke and urine samples.

	Δ9-THC	CBD	CBN	11-nor-9-carboxy-THC
Marijuana smoke* (mg/mL)	0.427 ± 0.002	0.0358 ± 0.0011	0.258 ± 0.008	–
Urine samples (ng/mL)	65.6 ± 22.8	2.4 ± 0.6	41.9 ± 17.6	1.8 ± 0.2

**Chemical content of the smoke produced by burning 2 "joints" (2 × 0.77 g) of marijuana. Data are reported as mean ± SEM.*

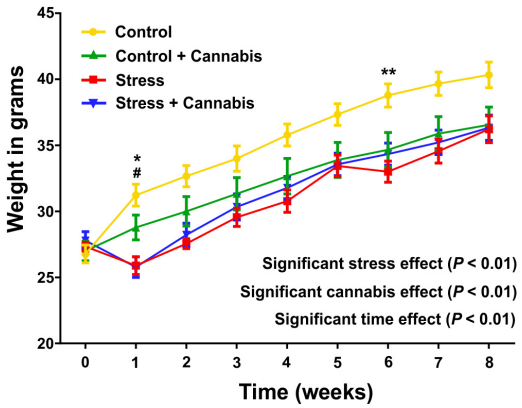


FIGURE 2 | Repeated stress and cannabis exposure reduced body weight gain of the animals. Body weights were measured every day and then, the daily values were grouped and reported here as weekly means. Statistics: three-way ANOVA (time × stress × cannabis treatment) followed by Tukey's multiple comparisons *post hoc* test. All three factors had highly significant main effect ($P < 0.01$) and there were significant interactions between time and cannabis treatment as well as between the stress and cannabis treatment. This indicates that cannabis treatment increasingly inhibited body weight gain as the animals grew and that cannabis smoke consumption could alleviate the stress-induced inhibitory effect on body weight gain. Tukey's multiple comparisons revealed further group differences at specific time points: Control *versus* Stress group: $^{*}P < 0.05$, $^{**}P < 0.01$; Control *versus* Stress + Cannabis group: $\#P < 0.05$.

Stress Hindered the Maturation of Several Pulmonary Functions, but Marijuana Smoke Did Not Affect Lung Functions

We did repetitive lung plethysmography measurements in order to examine the effect of long-term stress and cannabis treatment on pulmonary functions. We hypothesized that long-term cannabis smoke exposure might affect lung functions. A baseline measurement was done on Week 0 (before the stress and cannabis exposure started) and subsequent measurements were done on Week 4 and Week 8 of the experimental procedures (**Figure 1**). Results of the plethysmography measurements are presented on **Figure 3**. Data were analyzed with a three-way ANOVA (time × stress × cannabis treatment) followed by Tukey's multiple comparisons *post hoc* test. The main effects of the three-way ANOVA are presented in **Table 2**. Time (age) had the most pronounced effect on the pulmonary functional parameters. As the animals grew bigger all values of the pulmonary functions increased, except inhalation frequency, which decreased during maturation. Chronic stress altered the development of several lung parameters, i.e., the frequency, minute ventilation, peak inspiratory flow, time of expiration, relaxation time and tidal volume. For these parameters the three-way ANOVA showed a significant main effect of stress as well a significant time × stress interaction (**Table 2**). Chronic marijuana smoke exposure had no effect on any of the pulmonary functions except the carbachol-induced enhanced pause in breathing test (**Table 2**). The carbachol-induced enhanced pause is a functional parameter for the analysis of the bronchial hyper-reactivity of the lungs, correlating to the extent of airway inflammation. In this test bronchoconstriction is provoked by 50 μl nebulized carbachol (22 mM).

Neither Stress, nor Marijuana Exposure Influenced Cognitive Behavior

The cognitive performance of the animals was evaluated using two different learning tests. We did a Y maze test at the end of Week 3 and a novel object recognition test at the end of Week 4 (**Figure 1**). Results of the cognitive tests are presented on **Figure 4**. At these time points, neither stress, nor marijuana exposure had any effect on the cognitive performance of the animals. In the Y maze, both the stressed and/or cannabis treated mice tended to make more errors, i.e., they entered to arms which have been recently visited, but statistically there were no difference between the groups (**Figure 4A**). In the novel object recognition test, the recognition index (time spent with novel object/time spent with both objects × 100) was similar for all experimental groups (**Figure 4B**). The cannabis treated animals had the tendency to have a lower discrimination index [(time spent with novel object – time spent with familiar object)/time spent with both objects], however, statistically this was not significant either (**Figure 4A**).

Anxiety-Related Spontaneous Locomotor Activity and Self-Grooming in the Open Field Test

We performed an OFT at the end of the experiment to evaluate the anxiety-related spontaneous locomotor activity and self-grooming behavior of the animals (**Figure 1**). Stressed mice spent less time in the center of the arena (**Figure 5A**). Two-way ANOVA (stress × cannabis treatment) revealed a significant main effect of stress [$F(1,32) = 6.33$, $P < 0.05$], but Tukey's *post hoc* test found no further group differences. Measurement of the velocity of the animals, i.e., the number of squares crossed during the time spent with movement, also revealed that stressed mice showed significantly reduced exploratory activity

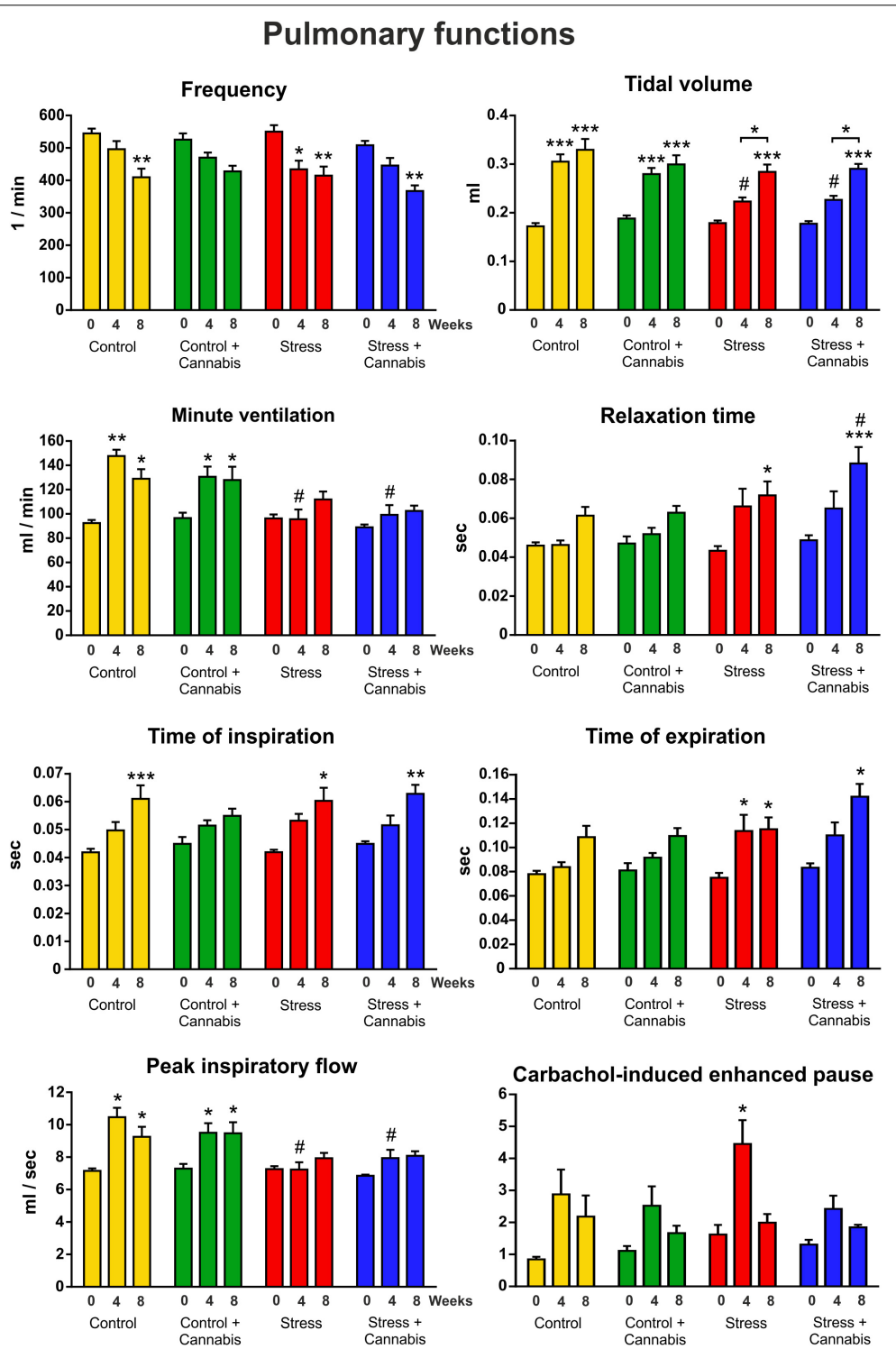


FIGURE 3 | The effect of repeated stress and cannabis exposure on pulmonary functions. We did repetitive whole body plethysmography measurements on freely moving animals. Baseline values were recorded on Week 0, before the stress and cannabis exposure started and then subsequent measurements were done on Week 4 and Week 8 of the experimental procedures. Time (age) had the most pronounced effect on lung functions. As the animals grew bigger almost all values increased. Chronic stress significantly blocked the maturation of several lung parameters (minute ventilation, peak inspiratory flow and tidal volume) whereas, the relaxation time and the time of expiration were increased. Results of the three-way ANOVA (time × stress × cannabis treatment) showed a significant main effect of stress and significant time × stress interaction for these parameters. Marijuana smoke exposure affected only the carbachol-induced enhanced pause in breathing test. Tukey's multiple comparisons revealed further group differences: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared to the value of Week 0 of the same treatment group. $\#P < 0.05$ *versus* the Control group at the same experimental week.

TABLE 2 | Summary of the three-way ANOVA analysis (time × stress × cannabis treatment) of the pulmonary functional tests.

	Time (age)		Stress		Cannabis treatment		Interaction (time × stress)	
f	$F = 35.95$	$P < 0.001$	$F = 4.38$	$P < 0.05$	n.s.	n.s.	n.s.	n.s.
MV	$F = 18.94$	$P < 0.001$	$F = 33.1$	$P < 0.001$	n.s.	n.s.	$F = 9.35$	$P < 0.001$
Penh	$F = 17.38$	$P < 0.001$	n.s.	n.s.	$F = 3.99$	$P < 0.05$	n.s.	n.s.
PIF	$F = 17.55$	$P < 0.001$	$F = 26.24$	$P < 0.001$	n.s.	n.s.	$F = 6.45$	$P < 0.01$
RT	$F = 20.50$	$P < 0.001$	$F = 12.71$	$P < 0.001$	n.s.	n.s.	$F = 3.46$	$P < 0.05$
Te	$F = 25.69$	$P < 0.001$	$F = 10.28$	$P < 0.01$	n.s.	n.s.	n.s.	n.s.
Ti	$F = 31.44$	$P < 0.001$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
TV	$F = 100$	$P < 0.001$	$F = 20.33$	$P < 0.001$	n.s.	n.s.	$F = 7.16$	$P < 0.01$

*Only the main effects of the three-way ANOVA are presented here. The results of the pulmonary functional tests are shown on **Figure 3**. f, frequency; MV, minute ventilation; n.s., non-significant; Penh, enhanced pause (induced by 22 mM carbachol); PIF, peak inspiratory flow; RT, relaxation time; Te, time of expiration; Ti, time of inspiration; TV, tidal volume.*

(**Figure 5B**). Two-way ANOVA (stress × cannabis treatment) yielded a significant main effect of stress [$F(1,32) = 5.07$, $P < 0.05$]. Cannabis treatment had no effect on these parameters, although animals exposed to marijuana smoke tended to spend more time in the center of the arena. However, cannabis treatment had a pronounced effect on self-grooming behavior (**Figures 5C,D**). Both the stressed and cannabis treated mice spent significantly more time with grooming. Two-way ANOVA (stress × cannabis treatment) revealed a significant main effect of stress [$F(1,32) = 4.17$, $P < 0.05$], and a significant main effect of cannabis treatment [$F(1,32) = 6.11$, $P < 0.05$], but no interaction between the two factors. Furthermore, Tukey's multiple comparisons *post hoc* test revealed a significant difference between the Control and Stress + Cannabis treated groups ($q = 4.51$, $P < 0.05$) (**Figure 5C**). In addition to that, stressed and/or cannabis treated mice started the self-grooming much earlier than control mice (**Figure 5D**). We measured grooming latency and two-way ANOVA (stress × cannabis treatment) revealed a significant main effect of stress [$F(1,32) = 4.71$, $P < 0.05$], and a significant main effect of cannabis treatment [$F(1,32) = 6.89$, $P < 0.05$], but no interaction between the two factors. Tukey's *post hoc* test revealed a significant difference between the Control and Control + Cannabis groups ($q = 4.02$, $P < 0.05$), as well as between the Control and Stress + Cannabis treated groups ($q = 4.60$, $P < 0.05$) (**Figure 5D**).

The Stress-Induced Inhibition of Cell Proliferation in the Dentate Gyrus Was Not Influenced by Cannabis Smoke Exposure

Cell proliferation in the dentate gyrus was visualized with use of the exogenous proliferation marker BrdU (**Figures 6A–D**). Stress significantly reduced the number of BrdU-positive cells in the dentate gyrus (**Figure 6E**). Two-way ANOVA (stress × cannabis treatment) revealed a significant main effect of stress [$F(1,32) = 4.43$, $P < 0.05$]. The number of BrdU-positive cells were also fewer in the dentate gyrus of mice of the Control + Cannabis group, but cannabis treatment had no statistically significant effect on cell proliferation.

Cannabis Smoke Exposure Had a Pronounced Effect on the Number, Morphology and Migration of the Doublecortin-Positive Immature Neurons

The population of immature neurons was visualized with DCX-immunohistochemistry (Brown et al., 2003; Rao and Shetty, 2004, **Figure 7**). Stress had no effect, but cannabis treatment significantly reduced the number of DCX-positive neurons in the dentate gyrus (**Figure 8A**). Two-way ANOVA (stress × cannabis treatment) revealed a highly significant main effect of cannabis treatment [$F(1,32) = 18.86$, $P = 0.0001$]. Tukey's *post hoc* test revealed further differences between the Control and Control + Cannabis ($q = 4.50$, $P < 0.05$) and between the Stress and Stress + Cannabis treated groups ($q = 4.19$, $P < 0.05$) (**Figure 8A**).

During the quantitative analysis of the DCX+ neurons we noticed that in the dentate gyrus of cannabis treated mice, DCX+ cells often had an unusual or abnormal appearance (**Figure 9**). For example, cannabis treatment significantly altered the dendritic morphology of the DCX+ cells. Many DCX+ cells lost their dendritic DCX-expression (**Figure 9B**) and occasionally we observed bipolar DCX+ neurons (**Figure 9E**), or cells with basal dendrites (**Figures 9B,G**). In order to quantify these morphological changes of the DCX+ cells, we quantified the number of sections/animal where we could find DCX+ neurons with abnormal appearance (**Figure 8B**). Two-way ANOVA (stress × cannabis treatment) revealed that cannabis treatment had a significant main effect [$F(1,32) = 8.35$, $P = 0.01$] indicating that the incidence of DCX+ cells with abnormal appearance was significantly higher in the cannabis treated animals (**Figure 8B**).

We also quantified cell migration of the DCX+ cells. In control mice, the majority, i.e., >90% of the DCX+ immature neurons, were located in the germinative subgranular zone (sgz). A small percentage, i.e., <5% of the cells, migrated out of the sgz either to the granule cell layer (gcl) (**Figure 8C**), or some cells were also found in the hilus (**Figure 8D**). This ratio was significantly altered by the marijuana exposure. In the cannabis treated animals, >15% of the DCX+ cells were found outside of the sgz (**Figure 8E**). Two-way ANOVA (stress × cannabis treatment) yielded a highly significant main effect of cannabis treatment [$F(1,32) = 15.87$, $P < 0.001$], a significant main effect

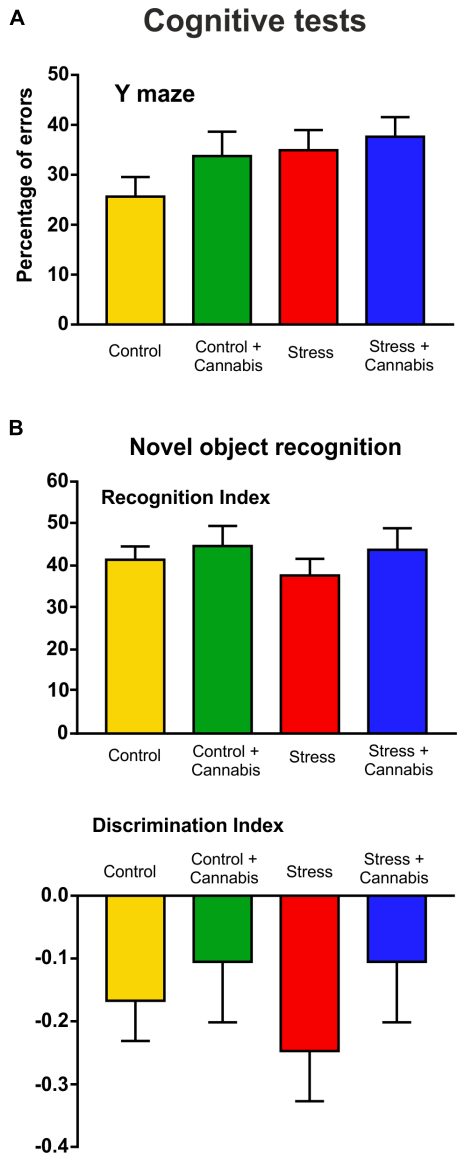


FIGURE 4 | Results of the cognitive tests: Neither stress, nor marijuana exposure affected the cognitive abilities of the animals. Cognitive performances were measured in the Y maze **(A)** and with the novel object recognition test **(B)**. **(A)** Stressed and/or cannabis treated mice tended to make more errors in the Y maze test, i.e., they entered to arms which have been recently visited, but statistically there was no difference between the groups. **(B)** The recognition indexes (time spent with novel object/time spent with both objects × 100) were also similar for all experimental groups. Marijuana treated animals had the tendency to have a lower discrimination index [(time spent with novel object – time spent with familiar object)/time spent with both objects], but statistically this was not significant. Data were analyzed with a two-way ANOVA (stress × cannabis treatment).

of stress [$F(1,32) = 5.46$, $P < 0.05$], and a significant interaction between the two factors [$F(1,32) = 5.96$, $P < 0.05$]. Furthermore, Tukey's *post hoc* test revealed significant differences between the Control and Control + Cannabis ($q = 6.42$, $P < 0.001$), Stress *versus* Control + Cannabis treated groups ($q = 6.32$, $P < 0.001$), and between the Stress + Cannabis and Control + Cannabis treated groups ($q = 4.78$, $P < 0.01$) (**Figure 8E**).

Quantitative data on the incidence of DCX+ cells with abnormal appearance is shown on **Figure 10**. Cannabis treatment significantly increased the percentage of bipolar DCX+ cells. Two-way ANOVA (stress × cannabis treatment) yielded a highly significant main effect of cannabis treatment [$F(1,32) = 41.36$, $P < 0.001$], and Tukey's *post hoc* test revealed significant differences between the Control and Control + Cannabis ($q = 5.75$, $P < 0.01$), Control *versus* Stress + Cannabis groups ($q = 6.90$, $P < 0.001$), and between the Stress and Stress + Cannabis groups ($q = 4.26$, $P < 0.05$) (**Figure 10A**). Cannabis treatment also increased the frequency of ectopic DCX+ cells, i.e., the incidence of cells that were found in the molecular layer of the dentate gyrus (**Figures 9G**, **10B**). The number of DCX+ neurons with basal dendrites was also much higher in the cannabis treated animals (**Figure 10C**). Two-way ANOVA (stress × cannabis treatment) revealed a highly significant main effect of cannabis treatment [$F(1,32) = 22.45$, $P < 0.001$], a significant main effect of stress [$F(1,32) = 19.75$, $P < 0.001$], and also a significant interaction between the two factors [$F(1,32) = 21.10$, $P < 0.001$]. Furthermore, Tukey's *post hoc* test revealed further significant group differences between the Control and Control + Cannabis treated groups ($q = 8.87$, $P < 0.001$), between the Stress and Control + Cannabis groups ($q = 8.47$, $P < 0.001$), and between the Control + Cannabis and Stress + Cannabis treated groups ($q = 8.47$, $P < 0.001$).

DISCUSSION

The principal aim of the present study was to model regular marijuana consumption of humans in an experimental setting. Since most of the users are young people who claim that they consume cannabis to relax from everyday stress therefore, we exposed young experimental mice to daily stress and concomitant cannabis smoke. While most experimental studies inject synthetic cannabinoids, we decided to use marijuana smoke exposure, because smoking is the most typical route of application in humans. Furthermore, we did a long-term, repeated exposure for 8 weeks, using daily restraint stress for 6 h/day and exposing animals to the smoke of 4 "joints"/day during the end of the restraint stress. Our results demonstrate that both stress and cannabis exposure significantly reduced body weight gain of the animals. However, cannabis could alleviate the stress-induced reduction of body-weight gain. We expected to see marijuana-induced changes in pulmonary functions, but instead it was the chronic stress which inhibited the maturation of most of the lung functions. In the behavioral tests measuring the cognitive performance of the animals, neither stress, nor cannabis treatment had any effect after 3 or 4 weeks of exposure. In the OFT, where we evaluated the anxiety related behavior of the animals after 8 weeks of treatment, stress had an anxiogenic effect, while cannabis had only a mild tendency to reverse the stress-induced anxiety. Marijuana however had a strong effect on self-grooming. Cannabis treated mice started to groom themselves

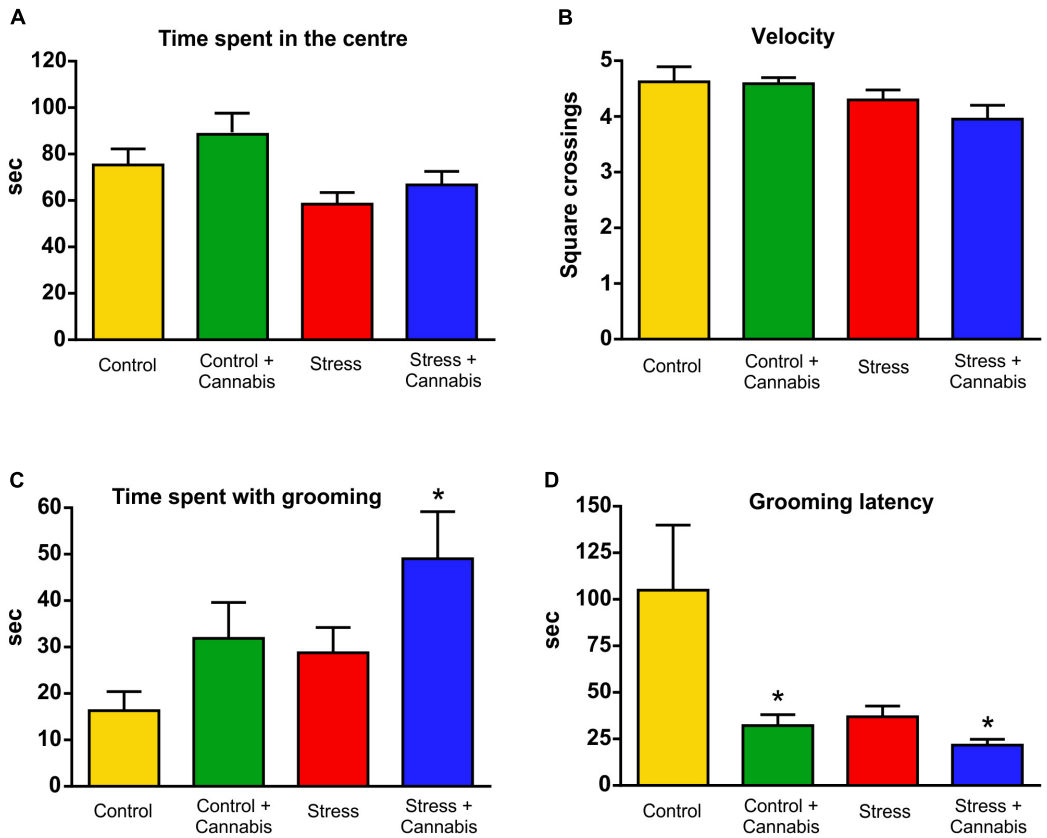


FIGURE 5 | Results of the open field test. We quantified anxiety-related spontaneous locomotor activity and self-grooming in the open field test. **(A)** Stressed mice spent significantly less time in the center of the arena. Two-way ANOVA (stress × cannabis treatment) revealed a significant main effect of stress ($P < 0.05$), but Tukey's *post hoc* test found no further group differences. Cannabis treated mice tended to spend more time in the center of the arena, but statistically this was not significant. **(B)** The velocity of the animals was evaluated by counting the number of squares that were crossed during the time spent with movement. Stressed mice had reduced exploratory activity. Two-way ANOVA (stress × cannabis treatment) yielded a significant main effect of stress ($P < 0.05$). Cannabis had no effect. **(C)** Cannabis had a pronounced effect on self-grooming. Both the stressed and cannabis treated mice spent significantly more time with self-grooming. Two-way ANOVA (stress × cannabis treatment) revealed a significant main effect of stress ($P < 0.05$), and of cannabis treatment ($P < 0.05$). Tukey's *post hoc* test revealed a significant difference between the Control and Stress + Cannabis groups ($^*P < 0.05$). **(D)** Stressed and/or cannabis treated mice started to groom themselves significantly sooner than control mice. Two-way ANOVA (stress × cannabis treatment) revealed a significant main effect of stress ($P < 0.05$), and of cannabis treatment ($P < 0.05$). Tukey's *post hoc* test revealed further significant differences between the Control and Control + Cannabis groups and between the Control and Stress + Cannabis treated groups ($^*P < 0.05$).

much sooner than controls and also spent significantly more time with grooming. Finally, we examined adult hippocampal neurogenesis and we found that chronic stress – as expected – blocked progenitor cell proliferation in the dentate gyrus, but marijuana smoking had no influence on that. In contrast to that, cannabis smoke exposure had a pronounced effect on the doublecortin-positive immature neurons. Cannabis significantly reduced the number of DCX+ neurons in the dentate gyrus, stimulated their migration and the dendritic morphology of the cells was also profoundly altered by the marijuana treatment. In sum, with this experimental design we found that long-term exposure to cannabis smoke had either no effect or negative impact on various health-related measures.

Cannabis contains over 500 different compounds (ElSohly and Gul, 2014), but the potency of marijuana is usually judged based on the Δ9-THC content of the preparation. We did a HPLC analysis to assess the major components of the cannabis smoke which was used in this experiment. Results of this analysis revealed that the Δ9-THC content was 10-fold higher than its CBD content (**Table 1**). This difference was even more pronounced in the urine samples, where the Δ9-THC content was 30-fold higher than the CBD content. Several reports indicate that the potency of marijuana sold on the streets has increased dramatically over the past few decades (Mehmedic et al., 2010; ElSohly et al., 2016) and it has been suggested that this increase in potency has been the reason for the rising emergency department visits involving marijuana use in the US (Data Spotlight, 2012).

Our principal aim was to mimic the regular cannabis consumption of humans. However, it is very difficult to define the average frequency of cannabis use in the general population.

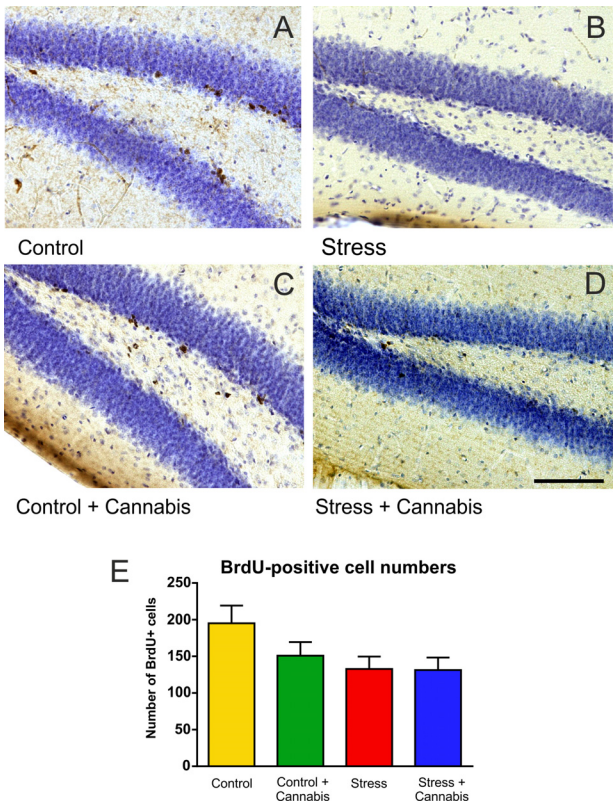


FIGURE 6 | Stress inhibited progenitor cell proliferation in the dentate gyrus. Representative images demonstrating BrdU-immunohistochemistry labeling in the dentate gyrus of the animals from the different treatment groups **(A–D)**. **(E)** The total number of BrdU-positive cells in the dentate gyrus of one hemisphere. Stress significantly reduced the number of BrdU-positive cells in the dentate gyrus. Two-way ANOVA (stress × cannabis treatment) revealed a significant main effect of stress ($P < 0.05$), but cannabis treatment had no statistically significant effect on cell proliferation. Scale bar: 100 μm for all images.

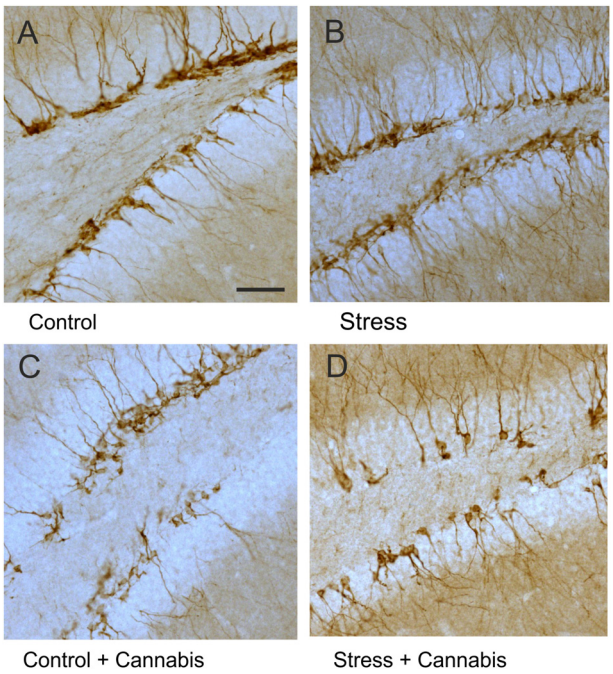


FIGURE 7 | DCX-positive immature neurons in the dentate gyrus. Representative images demonstrating DCX-immunohistochemistry staining in the dentate gyrus of animals from the different treatment groups **(A–D)**. Note that DCX-positive cells were often disarrayed in the dentate gyrus of cannabis treated mice **(C,D)**. Scale bar: 50 μm for all images.

A recent US survey documented that 3–4% of all primary care patients in Washington State report on a daily use, and 10–14% of them consume cannabis at least once a week or month (Lapham et al., 2017). Young adults (aged 18–29 years) use cannabis much more frequently and 6–12% of them report to consume it on a daily basis and 25–30% of them use it at least once a week or month (Lapham et al., 2017). US citizens, who consume cannabis regularly, report to use an average of 9.4 ± 9.7 joints weekly (1–60 joints/week) (Goodwin et al., 2008). From this perspective the regimen of cannabis exposure that we used, i.e., 4 joints/day (20 joints/week) was rather strong. Furthermore, we determined the THC/CBD/CBN and carboxy-THC concentrations in the urine of the mice. Our present cannabis treatment protocol resulted in a low (2 ng/mL) urine concentration of carboxy-THC. According to the guidelines of the Mayo Clinic, the presence of carboxy-THC in human urine samples at concentrations > 15.0 ng/mL is a strong indicator that the patient has used marijuana. The presence of carboxy-THC in urine > 100.0 ng/mL indicates relatively recent use, probably within the past 7 days. Levels of >500.0 ng/mL suggest chronic and recent use[1]. In this comparison, our cannabis exposure regimen was modest. However, it should be emphasized that the drug metabolism rate of mice is much higher compared to humans. We are not aware of any study that evaluated the amount of marijuana smoke exposure that is necessary to reach serum or urine concentrations of THC/CBD/CBN and carboxy-THC comparable to the human samples. It has been documented that rats have to be exposed to the smoke of 60 cigarettes/day to reach serum levels of nicotine and cotinine that is comparable to human cigarette smokers (Small et al., 2010; Bruijnzeel et al., 2011). However, to apply such a high dose would be technically impossible for us and also we would not get an ethical permission to expose animals to e.g., 60 joints/day over a 2-month period.

The clinical findings describing the effects of marijuana on the body and mind are often conflicting. For example, cannabis is known to stimulate appetite and potentially promote weight gain in patients with cancer of HIV (Abrams and Guzman, 2015; Kramer, 2015) whereas, the large epidemiological studies involving the general population consistently report that users of marijuana tend to have lower body mass indices (Sansone and Sansone, 2014). Therefore, it is important that the well-controlled experiments should aim to mimic real life situations as much as possible. In our experiment, both cannabis smoke and stress

[1]https://www.mayomedicallaboratories.com/test-catalog/Clinical+and+Interpretive/8898

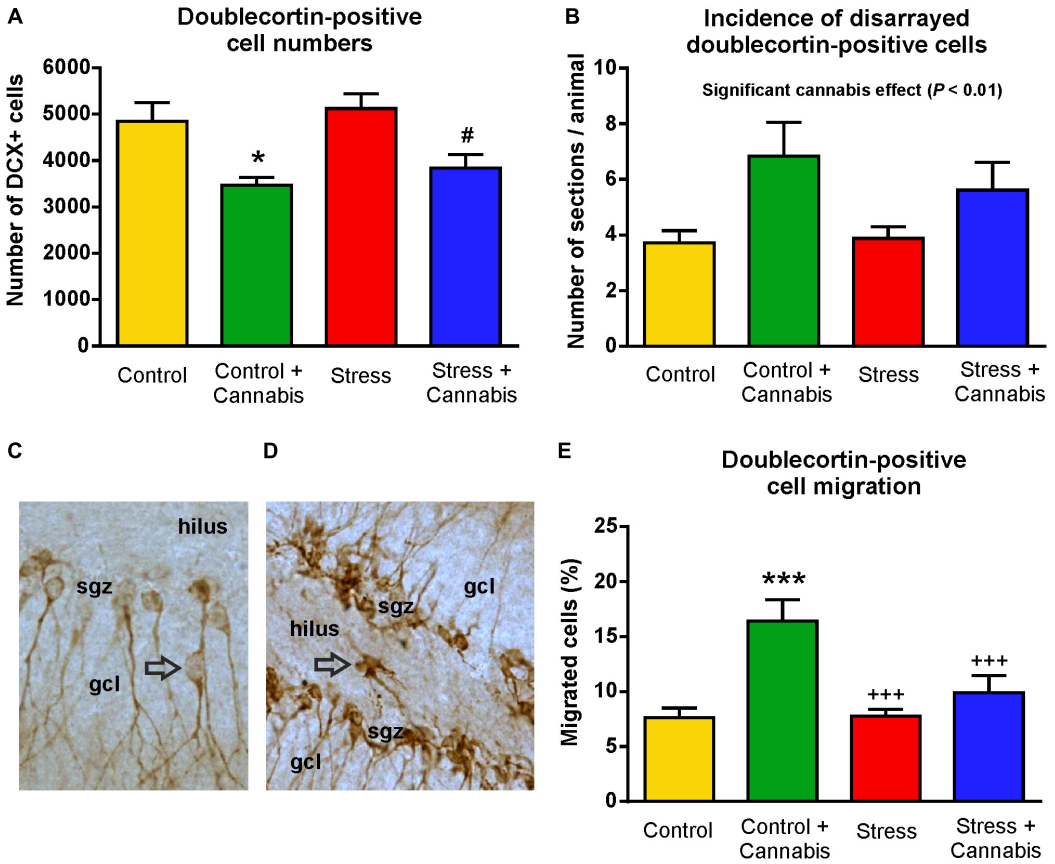


FIGURE 8 | Marijuana smoke exposure had a pronounced effect on the number, migration and morphology of doublecortin-positive immature neurons. **(A)** Cannabis treatment significantly reduced the number of DCX-positive neurons in the dentate gyrus. Two-way ANOVA (stress × cannabis treatment) revealed a highly significant main effect of cannabis treatment ($P < 0.001$). Tukey's *post hoc* test revealed further differences between the Control and Control + Cannabis groups (*$P < 0.05$) and between the Stress and Stress + Cannabis treated groups (#$P < 0.05$). **(B)** The number of sections/animal in which we observed disarranged DCX-positive cells. Two-way ANOVA (stress × cannabis treatment) revealed a significant main effect of cannabis treatment ($P < 0.01$). **(C)** In control mice, the majority of the DCX+ immature neurons were located in the subgranular zone (sgz) and a small percentage, i.e., <5% of the cells, migrated either to the granule cell layer (gcl) or a few cells were found in the hilus **(D)**. This ratio was significantly altered by the cannabis exposure. **(E)** In the marijuana treated animals >15% of the DCX+ cells were found in the granule cell layer, hilus or occasionally in the molecular layer. Two-way ANOVA (stress × cannabis treatment) revealed a significant main effect of stress ($P < 0.05$) and cannabis treatment ($P < 0.001$), and a significant interaction between the factors ($P < 0.05$). Tukey's *post hoc* test found significant differences between the groups: ***$P < 0.001$ *versus* Control; +++$P < 0.001$ *versus* the Control + Cannabis treated group.

reduced the body weight gain of the animals, and in this case we found a significant interaction between these two factors. This means that cannabis treatment could alleviate the stress-induced inhibitory effect on body weight gain.

Marijuana smoke contains many of the same toxins and carcinogens as tobacco smoke and thus, irritates the respiratory system similarly to tobacco. Clinical studies indicate that regular cannabis smoking alone is associated with airway inflammation (Jett et al., 2018). In our recent experimental study, daily marijuana inhalation for 4 months resulted in inflammation, tissue destruction, and emphysema (Helyes et al., 2017). In the present study, cannabis smoke did not affect any of the pulmonary functions. Notably, a large clinical study in the US could not find any adverse spirometric changes with cumulative lifetime marijuana use of up to 20 joint-years either (Kempker et al., 2015). In our present study, it was the repeated stress exposure that inhibited the development of several pulmonary functions. To our best of knowledge, ours is the first study to reveal such negative effect of chronic stress on pulmonary functions. We could not find any comparable experimental data in the literature. However, human studies document that experiencing psychological stress in children is significantly associated with asthma morbidity (Chen et al., 2006). Preclinical studies reported that chronic stress in mice can result in pneumonia (Kiank et al., 2008) and can worsen allergic airway inflammation (Okuyama et al., 2007).

Several research groups investigated the behavioral effects of marijuana smoke or specific cannabinoid molecules in the OFT (e.g., Bruijnzeel et al., 2016; Murphy et al., 2017; Tomas-Roig et al., 2018). These studies report on mixed results. Repeated daily injection of Δ9-THC into adolescent or adult CD1 mice had no behavioral consequences in the OFT (Murphy et al., 2017). Injection of the CB1/CB2 receptor agonist WIN 55,212-2 to C57Bl6/J mice resulted in reduced locomotor activity, but

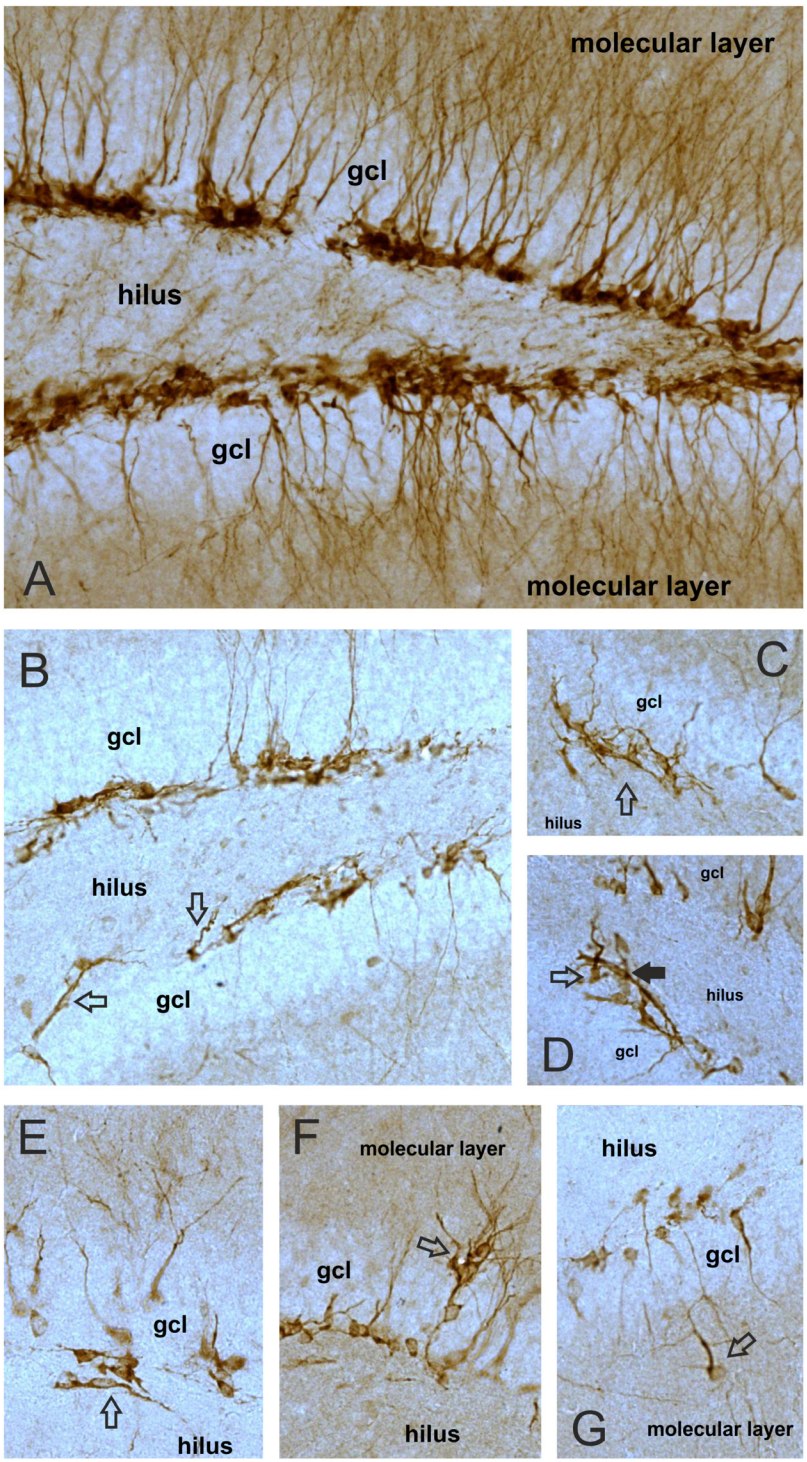


FIGURE 9 | Long-term cannabis exposure alters the morphology of DCX-positive neurons. Representative images demonstrating DCX+ immature neurons with normal appearance in a control animal **(A)** and cells with abnormal morphology in cannabis treated mice **(B–G)**. **(A)** In control mice almost all the DCX+ neurons were located in the subgranular zone, i.e., in a cell layer between the hilus and granule cell layer (gcl). Normal DCX+ cells had DCX-expressing dendrites projecting through the gcl and arborized in the molecular layer. The principal dendrites were typically running parallel to each other in the gcl giving an orderly, well-aligned appearance to the neurons. **(B)** DCX+ neurons in the cannabis treated animals often lost their dendritic DCX-expression. Arrows point to neurons with abnormal dendritic projections into the hilus. **(C)** DCX+ neurons with dendrites projecting into all directions, giving a disorganized, chaotic appearance to the neurons. **(D)** Open arrow points to a neuron with basal dendrites, i.e., with dendritic projections into the hilus. Black arrow points to a cell with bipolar appearance. **(E)** A bipolar DCX+ neuron in the hilus with dendritic projections running parallel to the gcl. **(F)** Neurons migrating out of the gcl into the molecular layer. **(G)** An ectopic DCX+ neuron with basal dendrite that migrated completely out of the gcl into the molecular layer. All images were taken with the same magnification (20× objective).

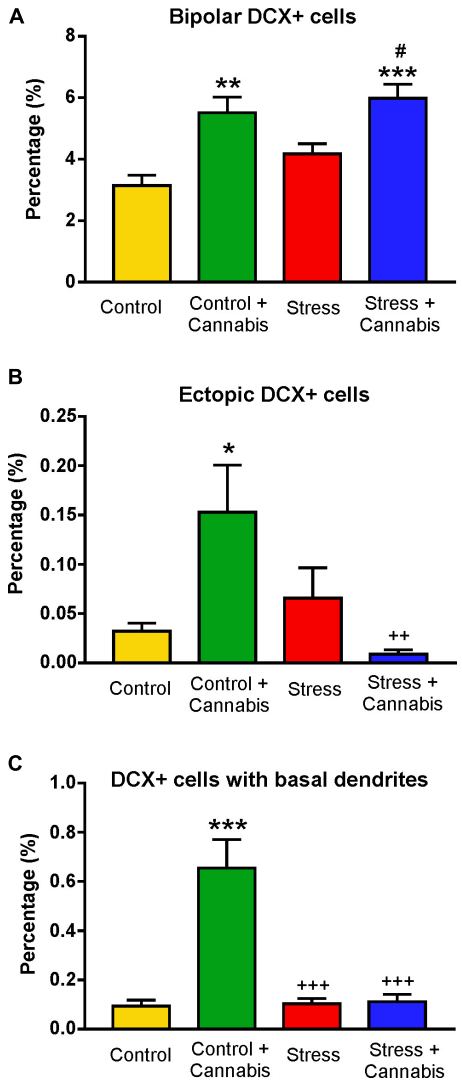


FIGURE 10 | The incidence of doublecortin-positive neurons with unusual or abnormal morphology. **(A)** Cannabis treatment significantly increased the percentage of bipolar DCX+ cells. Two-way ANOVA (stress × cannabis treatment) revealed a highly significant main effect of cannabis treatment ($P < 0.001$). Tukey's *post hoc* test revealed further significant group differences: $^{**}P < 0.01$ *versus* Control; $^{***}P < 0.001$ *versus* Control; $^{\#}P < 0.05$ *versus* Stress. **(B)** Cannabis treatment significantly increased the percentage of ectopic DCX+ cells, i.e., DCX+ cells which were found in the molecular layer of the dentate gyrus (see **Figure 9G**). Statistics: Two-way ANOVA followed by Tukey's *post hoc* test. $^{*}P < 0.05$ *versus* Control; $^{++}P < 0.01$ *versus* the Control + Cannabis treated group. **(C)** Cannabis treatment significantly increased the incidence of DCX+ cells with basal dendrites. Statistics: Two-way ANOVA followed by Tukey's *post hoc* test. $^{***}P < 0.001$ *versus* Control; $^{+++}P < 0.001$ *versus* the Control + Cannabis treated group.

had no effect on distance traveled in the center of the open field (Tomas-Roig et al., 2018). In rats, acute cannabis smoke exposure induced a brief increase in locomotor activity which was then followed by a prolonged decrease in locomotor activity and rearing (Bruijnzeel et al., 2016). In our study, cannabis smoke exposure had no effect on locomotor activity or on anxiety-related behavior. Marijuana smoke also did not alter the anxiogenic effect of repeated stress. However, cannabis smoke had a pronounced effect on self-grooming. Both stressed and cannabis treated mice spent significantly more time with self-grooming and they also started to groom themselves significantly sooner than control mice. Self-grooming is a complex innate behavior of rodents which is regulated by several neural circuits. Aberrant self-grooming has been described in animal models of several neuropsychiatric disorders including substance abuse (Kalueff et al., 2016). The same review also suggested that self-grooming has a great value for translational neuroscience, since each disease model may result in a distinct grooming phenotype (Kalueff et al., 2016). Our present study supports the notion that the microstructure of self-grooming should be investigated in more detail, because abnormal grooming behavior could signal the stress or anxiety level of the animals in the specific models (Kalueff et al., 2007).

The effect of cannabis use on the cognitive performance of the individual is probably the most ambiguous issue. Recent data suggest that cannabis use can improve various cognitive and executive functions (Osborne et al., 2017; Tervo-Clemmens et al., 2018; Gruber et al., 2018), while others claim that it impairs cognition (Volkow et al., 2014, 2016; Broyd et al., 2016; Curran et al., 2016). In this experiment, we could not detect any (positive or negative) effect of marijuana smoke exposure on the cognitive performance of the animals in the novel object recognition and Y maze tests. It has been argued that a potential explanation for the conflicting data in the literature is the different ratio of Δ9-THC and CBD content in the marijuana samples used in the experiment (Fadda et al., 2004). Data suggests that high Δ9-THC or low CBD cannabis results in greater cognitive impairment (Colizzi and Bhattacharyya, 2017; Murphy et al., 2017). In our case, the cognitive tests were done at the end of the 3rd and 4th week (**Figure 1**) and it might be that the cannabis exposure was too short at these time points to result in a detectable difference. Probably, it might have been better to do the cognitive testing at the end of the experiment which would have also allowed us to directly compare the cognitive performance of the animals with the findings on adult neurogenesis. The reason why we did not do the cognitive testing at the end of the experiment was that it is known that learning can affect the process of adult neurogenesis (Gould et al., 1999) and we wanted to avoid too many testing at the end of the experiment that could interfere with each other and with neuroplasticity.

Neurogenesis in the adult hippocampal dentate gyrus is a special form of neuroplasticity that has been implicated in various physiological and pathophysiological conditions including substance abuse (Eisch et al., 2000; Mandyam et al., 2008; Noonan et al., 2010; Bayer et al., 2015). It is well documented that the endocannabinoid system and the CB1 cannabinoid receptor mediate neural progenitor cell proliferation and neurogenesis (e.g., Aguado et al., 2005, 2007). Numerous studies investigated the effect of cannabinoids on adult hippocampal neurogenesis (reviewed recently by Prenderville et al., 2015), but to our best of knowledge, none of these studies used cannabis smoke exposure, instead they

all injected synthetic cannabinoids or plant-derived extracts to the experimental animals. There have been numerous reports on a positive, stimulatory effect of cannabinoid treatment on adult neurogenesis (Jiang et al., 2005; Palazuelos et al., 2006; Marchalant et al., 2009; Wolf et al., 2010; Rivera et al., 2011; Suliman et al., 2018). Furthermore, several reports suggested that facilitation of the cannabinoid signaling in the hippocampus may prevent stress-induced behavioral changes (Campos et al., 2013; Scarante et al., 2017; Fogaça et al., 2018). In our present study, chronic stress – as expected – reduced cell proliferation in the dentate gyrus, but cannabis smoke had no influence on this effect of stress. However, marijuana smoke had a distinct effect on the DCX+ cells, by reducing their number and affecting their morphology and migration. In the literature, one can find controversial data on the influence of cannabinoids on DCX+ neurons. In non-stressed Swiss mice, repeated administration of CBD at a lower dose (3 mg/kg) increased the number of DCX+ cells, but at a higher dose (30 mg/kg) it had a negative effect (Schiavon et al., 2016). Another study demonstrated that when CBD was fed to female C57Bl/6 mice it stimulated adult neurogenesis, whereas Δ9-THC had no such effect (Wolf et al., 2010). Yet, more recently, it was shown that injection of Δ9-THC to rats can stimulate adult neurogenesis (Suliman et al., 2018). Another study reported that daily injection of the cannabinoid agonist WIN 55,212-2 for 3 weeks to adolescent rats reduced the number of newly generated neurons in hippocampus (Abboussi et al., 2014).

To our best of knowledge, so far there was only one research group which carried out experiments where stress was combined with cannabinoid treatment and the consequences on behavior and hippocampal neuroplasticity were investigated. Guimarães and co-workers used chronic unpredictable stress (CUS) in combination with repeated injection of pure CBD and reported on a pronounced anxiolytic effect of CBD treatment which could also normalize the stress-induced inhibition of hippocampal progenitor cell proliferation and neurogenesis, i.e., it reversed the stress-induced reduction of BrdU- and DCX-positive cell numbers (Campos et al., 2013). In their more recent study, they replicated and extended these findings by demonstrating that CBD treatment can prevent the stress-induced inhibitory effects on adult neurogenesis via the activation of CB1/CB2 receptors (Fogaça et al., 2018). Our present experiment could not replicate these findings. There are several possible explanations for this: (1) The Guimarães group used a different stress protocol which lasted only for 14 days (Campos et al., 2013; Fogaça et al., 2018). It might be, that our stress protocol was too aggressive and/or lasted too long, as it was 4× longer than the protocol of the Guimarães group. (2) The Guimarães group treated their animals with pure CBD, whereas in our case the marijuana smoke included hundreds of molecules and had high Δ9-THC content (10:1 ratio of Δ9-THC : CBD content). Thus, it might be that in our case the high Δ9-THC content concealed the beneficial effects of the CBD.

In the present experiment, marijuana reduced the number DCX+ cells, stimulated their migration and significantly altered their dendritic architecture. Future investigations are needed to determine whether these cellular changes should be considered as positive or negative effects. Previous studies reported that CBD application can increase DCX+ cell numbers in various experimental conditions (Schiavon et al., 2016; Mori et al., 2017). Another study comparing the effects of Δ9-THC with CBD found that Δ9-THC can reduce DCX+ cell numbers (Wolf et al., 2010, but see also Suliman et al., 2018). High Δ9-THC content of our sample might explain our finding on reduced DCX+ cell numbers. Other research group also documented that CBD treatment can stimulate DCX+ cell migration from the subgranular zone to the granular zone of dentate gyrus (Fogaça et al., 2018). This finding is in harmony with the knowledge that endocannabinoids play an important role during brain development regulating cell proliferation, migration, differentiation and survival (Harkany et al., 2007). A recent developmental study provided further evidence that prenatal exposure to the CB1/CB2 receptor agonist WIN 55,212-2 can alter the migration of early-born neurons in the cerebral cortex (Saez et al., 2014). To our best of knowledge our present data is the first to report on altered dendritic architecture of DCX+ cells in response to cannabinoid treatment. Normal DCX+ cells have DCX-expressing dendrites which project through the granule cell layer to the molecular layer. In contrast to that, we observed numerous DCX+ cells which lost their dendritic DCX-expression in the marijuana treated animals. Furthermore, in the cannabis treated animals, we observed an increased occurrence of bipolar DCX+ cells and cells with basal dendrites. It is known from the literature that basal dendrites on dentate granule cells can be induced by epileptic seizures (Ribak et al., 2012; Hester and Danzer, 2014) thus, the presence of such cells suggests abnormal neuronal circuitry, or cellular niche. We also found higher frequency of ectopic DCX+ cells in the cannabis treated animals. One possible explanation for the fact that previous studies did not report on such abnormal looking cells after cannabinoid treatment is that our analysis was much more careful and thorough compared to the previous studies. Most studies examine only 5–6 sections/animal, whereas we examined every 3rd sections from the serially cut hippocampus, yielding in average 25 analyzed sections/animal.

However, the present study has some limitations. As we already discussed, it is very difficult to find a marijuana dosage that is truly comparable to the human situation. Human consumption habits are highly variable and mice have a much higher metabolic rate compared to humans which makes it very difficult to reach comparable serum or urine concentrations of cannabinoid metabolites. Not to mention the fact that street marijuana samples have highly variable potency and efficacy. We cannot rule out the possibility that using a higher dose of cannabis, or a different sample having a different potency/efficacy could result in a different outcome in a similar experimental setting. We do not question that treatment with CBD has a positive effect in stressed animals as it has been well documented by others (e.g., Campos et al., 2013; Fogaça et al., 2018). Furthermore, employing other type of stress protocols may also yield different results. It is possible that in our study the stress procedures were too severe for the animals and that was the

reason why cannabis treatment could not alleviate the stress-induced effects.

To our best of knowledge, this is the first experimental study to combine chronic stress exposure with concomitant cannabis smoke inhalation. We report here that long-term exposure to these factors can influence several health-related measures, but our present experimental design could not reveal any significant interaction between these two factors (except for body weight gain). We report here for the first time, that chronic cannabis smoke can significantly alter the morphology of DCX+ neurons in the dentate gyrus and this finding deserve further investigations. In the present experiment, chronic cannabis smoke had either no effect or negative impact on the physical and mental condition of the animals. We should however, emphasize that modeling cannabis smoke consumption in experimental animals is a difficult issue, because (1) human smoking habits can be extremely variable, (2) rodents have a much higher metabolic rate, therefore, it is very difficult to apply cannabis smoke in dosage that results in a serum or urine concentrations of cannabinoid metabolites that are comparable to humans. Finally, the present study cannot rule out the possibility that application of different doses of cannabis smoke or pure CBD treatment may yield a different outcome in a similar experimental setting.

AUTHOR CONTRIBUTIONS

BC, ZH, and KC had the experimental concepts and designed the experiments. KR did the behavioral tests, DCX-immunohistochemistry and all cell counting, and prepared the figures for the paper. KC carried out the treatment procedures, behavioral tests, and data analysis. ZV analyzed the behavioral data of the NOR and OFTs. DC did the BrdU-immunohistochemistry. ÁB did the HPLC analysis on the cannabis smoke. MM and ZK measured metabolites in the urine samples. All authors contributed to the writing of the paper and/or revising it critically for important intellectual content. All authors approved the final version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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REVIEW
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Cannabidiol as a Therapeutic Alternative for Post-traumatic Stress Disorder: From Bench Research to Confirmation in Human Trials

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Post-traumatic stress disorder (PTSD) is characterized by poor adaptation to a traumatic experience. This disorder affects approximately 10% of people at some point in life. Current pharmacological therapies for PTSD have been shown to be inefficient and produce considerable side effects. Since the discovery of the involvement of the endocannabinoid (eCB) system in emotional memory processing, pharmacological manipulation of eCB signaling has become a therapeutic possibility for the treatment of PTSD. Cannabidiol (CBD), a phytocannabinoid constituent of *Cannabis sativa* without the psychoactive effects of Δ^9-tetrahydrocannabinol, has gained particular attention. Preclinical studies in different rodent behavioral models have shown that CBD can both facilitate the extinction of aversive memories and block their reconsolidation, possibly through potentialization of the eCB system. These results, combined with the currently available pharmacological treatments for PTSD being limited, necessitated testing CBD use with the same therapeutic purpose in humans as well. Indeed, as observed in rodents, recent studies have confirmed the ability of CBD to alter important aspects of aversive memories in humans and promote significant improvements in the symptomatology of PTSD. The goal of this review was to highlight the potential of CBD as a treatment for disorders related to inappropriate retention of aversive memories, by assessing evidence from preclinical to human experimental studies.

Keywords: post-traumatic stress disorder, endocannabinoid system, cannabidiol, aversive memories, fear conditioning paradigm

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INTRODUCTION

Post-traumatic stress disorder (PTSD) is a chronic psychiatric condition that may develop after experiencing a potentially traumatic event. The disorder manifests itself at different levels, through symptoms such as sleep disturbances; changes in cognition (e.g., repeated recall of the event), mood (e.g., depression, anxiety), and emotion (e.g., psychological instability); and reduced social

Abbreviations: 2-AG, 2-arachidonylglycerol; AEA, anandamide; BLA, basolateral amygdala; CBD, cannabidiol; CNS, central nervous system; CS, conditioned stimulus; DSM, Diagnostic and Statistical Manual of Mental Disorders; eCB, endocannabinoid; FAAH, fatty acid amide hydrolase; i.c.v., intracerebroventricular; i.p., intraperitoneally; IL, infralimbic; LTD, long-term depression LTD; LTP, long-term potentiation; mPFC, medial prefrontal cortex; PL PFC, prelimbic prefrontal cortex; PTSD, post-traumatic stress disorder; SSRIs, selective serotonin reuptake inhibitors; THC, tetrahydrocannabinol; US, unconditioned stimulus.

skills. Through the fourth edition of the DSM-IV, post-traumatic stress was classified as an anxiety disorder; however, the latest edition, DSM-V, includes PTSD in a new category called "trauma- and stress-related disorders.". In this brand-new category, we consider disorders with poor adaptation to a traumatic experience. Maladaptive responses to trauma may trigger, among others, PTSD (Passie et al., 2012; Berardi et al., 2016).

At some point in their lives, approximately 10% of people will be affected by PTSD, resulting in an enormous economic and social impact. This impact is aggravated by the scarcity of psychological and, above all, pharmacological approaches to PTSD treatment (Hidalgo and Davidson, 2000; Yule, 2001; Jurkus et al., 2016). At present, approved treatments for PTSD involve anxiolytics and antidepressants, which are inefficient and have considerable side effects (Berger et al., 2009; Shin et al., 2014; Bernardy and Friedman, 2015).

The eCB system can provide more efficient and better tolerated alternatives to the standard treatments for PTSD. The eCB system plays an important role in the regulation of emotional behavior and is essential for synaptic processes that determine learning and emotional responses, especially those related to potentially traumatic experiences (Castillo et al., 2012; Riebe et al., 2012). Among the possible alternative approaches, the use of components from *Cannabis sativa* such as CBD is particularly promising. Recent reviews have reported promising results of CBD treatment of several neuropsychiatric disorders, including PTSD (Mechoulam, 2005; Izzo et al., 2009; Passie et al., 2012). What began as a possibility discovered in a study of an animal model of aversive conditioning (Bitencourt et al., 2008) gained strength through results obtained in humans (Das et al., 2013) (see **Figure 1** for a brief history of CBD in PTSD). Because the compound has been proved to be well tolerated by humans, both in overall safety and possible side effects (Bergamaschi et al., 2011), CBD is now considered a new therapeutic possibility for treating PTSD.

This paper reviews the therapeutic potential of CBD in the treatment of PTSD. It starts from the first evidence obtained in animal studies ("bench research") and proceeds to knowledge gathered in human trials ("confirmation in human trials").

CANNABINOIDS AND TRAUMA-RELATED DISORDER

Cannabis sativa contains over 100 compounds called phytocannabinoids. Two of them demonstrate considerable therapeutic potential: Δ9-tetrahydrocannabinol (Δ^9-THC), considered the main component responsible for the psychoactive effects of the plant, and CBD, the main non-psychotomimetic constituent of *Cannabis* (Adams et al., 1940; Mechoulam and Shvo, 1963; Gaoni and Mechoulam, 1964). CBD constitutes about 40% of the active substances of the plant (Crippa et al., 2009). However, its pharmacological effects are different from, and often even opposite to, those of Δ9-THC, and are not related to the development of tolerance and withdrawal syndrome (Mechoulam et al., 2007; Bergamaschi et al., 2011).

In this context, it is also important to highlight the eCB system, discovered in the 20th century and responsible for a revolution in the understanding of numerous neuropsychological functions related to the modulation of emotional responses (Lutz, 2009). The eCB system comprises two different cannabinoid receptors, their endogenous ligands, and enzymes involved in the synthesis and degradation thereof (Di Marzo, 2009). eCB signaling is distributed throughout the CNS and peripheral tissues, regulating presynaptic release of both excitatory and inhibitory neurotransmitters. Cannabinoid type 1 (CB1) receptors are expressed by peripheral and central neurons, particularly in the central regions known to play important roles in anxiety and aversive learning, such as the amygdala, hippocampus, and cerebral cortex (Childers and Breivogel, 1998). In contrast, CB2 receptors are expressed mostly in immune cells, while also being present in the brain (Van Sickle et al., 2005).

The two major endogenous ligands for CB1 and CB2 receptors are AEA and 2-AG. These eCBs are synthesized on demand, mainly postsynaptically, and act as retrograde messengers regulating the presynaptic release of various neurotransmitters, as mentioned above. AEA acts as a partial agonist of both CB1 and CB2 receptors, with a higher affinity for the former. In the CNS, 2-AG is the most abundant eCB, and non-selectively activates CB1 and CB2 receptors. AEA, 2-AG, and Δ^9-THC, have been shown to exert their effects mainly through activation of CB1 receptors (Di Marzo, 2009; Castillo et al., 2012). In the case of eCBs, the effects are rapidly terminated through carrier-mediated uptake followed by intracellular enzymatic degradation. AEA and 2-AG are metabolized by monoacylglycerol lipase and FAAH, respectively. The eCBs regulate neuronal activity and plasticity by depolarization-induced suppression of inhibition or excitation (Wilson and Nicoll, 2002). Both phenomena are forms of short-term synaptic plasticity that contribute to the regulation of a number of physiological functions, including memory and emotion. Additionally, eCBs appear to modulate the memory process by changing synaptic plasticity and mediating more persistent forms of synaptic plasticity (e.g., LTP and depression) in several brain areas (Maldonado et al., 2006; Di Marzo, 2009; Sidhpura and Parsons, 2011).

These findings have established the importance of the eCB system in a number of neurophysiological functions and led to an emerging interest in the eCB-mediated modulation of emotionality. The first study to address the role of the eCB system in fear memory, specifically in its extinction, was published at the beginning of the last decade by Marsicano et al. (2002). In this study, the authors showed that genetic deletion of the CB1 receptor or its pharmacological blockade strongly impaired extinction of auditory-conditioned fear and that eCBs were released in the BLA during extinction. This discovery revealed that the eCB system has a central function in the extinction of aversive memories and may therefore be a promising target for the treatment of disorders related to inappropriate retention of such memories (for details see Marsicano et al., 2002). Precisely what processes underlie this function of the eCB system is presently unclear, raising the question whether CBD exerts its effects through a different pathway(s). Previous reports on eCB system involvement in CBD-induced effects have been equivocal.

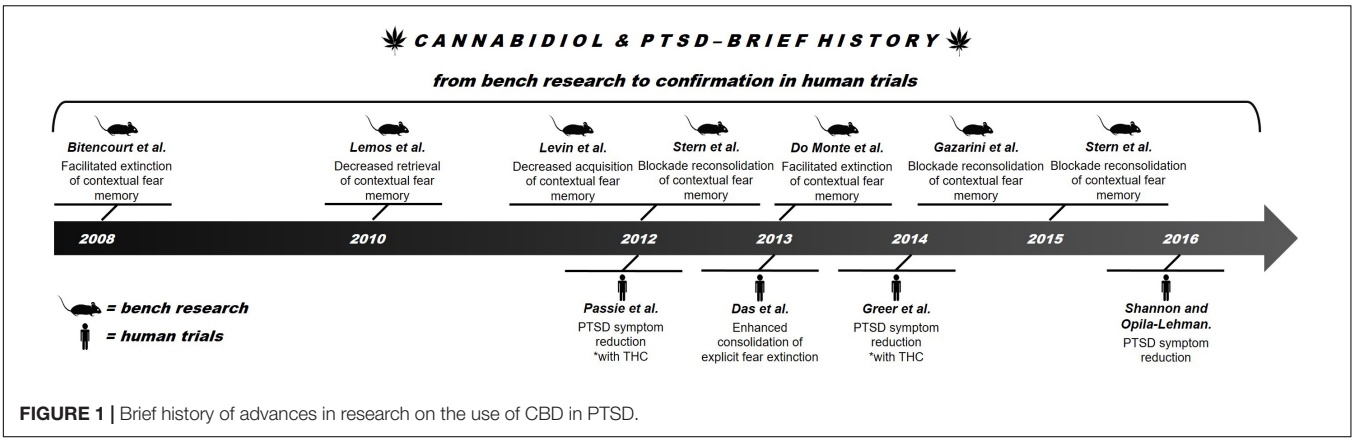


FIGURE 1 | Brief history of advances in research on the use of CBD in PTSD.

Because the endogenous ligands (e.g., AEA) and Δ^9-THC act directly on the CB1 receptor, it is possible that some of the effects of CBD are also mediated by this receptor, albeit indirectly. Indeed, CBD may exert its therapeutic effect on PTSD through inhibition of the uptake or enzymatic degradation of eCBs (Bisogno et al., 2001), as suggested by some recent studies (Bitencourt et al., 2008; Do Monte et al., 2013; Elmes et al., 2015; Stern et al., 2015). However, if this is the case, what is the advantage of using CBD over agents that act directly on the CB1 receptor? The answer is simple: fewer complications (specifically, anxiogenic side effects). Agents that target the eCB system directly, such as THC, CB1 agonists, and FAAH inhibitors, have a biphasic effect, in which low doses are anxiolytic, but higher doses can be anxiogenic, in both preclinical models and humans. In contrast, CBD, when administered in acute systemic doses in models of general anxiety, does not cause anxiety even at high doses. However, few studies have examined chronic dosing effects of CBD in models of generalized anxiety, and such studies are needed for the safe long-term use of CBD (Blessing et al., 2015).

Trauma-related disorders may involve dysregulation of the learning process of aversive memories. This process is fundamental for the individual to survive, because through it we avoid potentially dangerous situations without having to respond in a way that damages mental health (Quirk and Mueller, 2008). Consistent with this concept, neural circuits that support fear conditioning are related to circuits that are affected in clinical conditions such as PTSD (Davis and Whalen, 2001). That the available drugs (such as antidepressants and anxiolytics) do not specifically target the memory process may be one of the reasons that pharmacological treatment of PTSD is so difficult (Singewald et al., 2015). Currently approved treatments for PTSD include SSRIs and serotonin/noradrenaline reuptake inhibitors, both with low efficacy (Bernardy and Friedman, 2015). The response rate for SSRIs rarely exceeds 60%, of which less than 30% represents complete remission (Berger et al., 2009). Moreover, the available treatments have considerable side effects, which may limit tolerance or even decrease adherence to the treatment (Shin et al., 2014). In this regard, interventions that act on the eCB system have shown promise since they can affect both the emotional (e.g., relieve PTSD symptoms) and cognitive (e.g., increase the efficiency of psychological approaches) aspects of the disorder (Izzo et al., 2009; Steckler and Risbrough, 2012; Trezza and Campolongo, 2013).

Two important observations led to the consideration of cannabinoids for the treatment of PTSD: (i) patients with PTSD appear to be more likely to smoke *Cannabis*; and (ii) patients with PTSD have increased levels of cannabinoid receptors and reduced peripheral levels of AEA, suggesting that the CB1 receptor upregulation may be a result of low receptor occupancy caused in turn by the deficiency of AEA (Hauer et al., 2013; Hill et al., 2013a; Neumeister, 2013; Neumeister et al., 2013; Loflin et al., 2017). Consistent with these observations, studies have made use of treatment with cannabinoids in animal models of traumatic event exposure to reduce the appearance of PTSD-like behavioral responses. These studies have demonstrated great potential of cannabinoids in the mitigation of maladaptive responses to trauma (for a more detailed review, see Zer-aviv et al., 2016).

When administered after a traumatic situation, cannabinoids may interfere with the acquisition and consolidation of memories of the event, thus mitigating the risk of subsequent symptoms. However, intervention at this stage may be inadvisable because not all people exposed to a traumatic situation will later manifest PTSD. Alternatively, cannabinoids may reduce traumatic memory by affecting its retrieval or reconsolidation, or by stimulating the process of aversive memory extinction. The latter mechanism may hold the most therapeutic promise, especially when taking into account exposure-based psychotherapies, which extinction mechanisms are thought to be engaged (reviewed in Berardi et al., 2016).

Additionally, studies have shown CBD, in its isolated form, to be a constituent of *Cannabis* with enormous therapeutic potential not only for trauma-related disorders, but also for various other psychiatric and neurological disorders (Campos et al., 2012b). Among the advantages of CBD are its high efficacy, lack of psychotomimetic properties or anxiogenic effects caused by eCB transmission activation, inability to induce tolerance and dependence, and safety at high doses both in humans and in animals (Bergamaschi et al., 2011). However, an alternative view must also be considered, according to which the therapeutic effects of *Cannabis* result from the interaction of all the compounds present in the plant (particularly THC and CBD) rather than the isolated action of a single compound. Such

interaction, called in the pharmacology the "entourage effect" (Ben-Shabat et al., 1998), still needs to be better studied (for further discussion of the use of the term "entourage effect" on plants effect, see Rosenberg et al., 2015). In this review, we will focus on CBD effects in isolation.

CBD AND PTSD: "FROM BENCH RESEARCH. . ."

The fear-conditioning paradigm has been widely used in animals to better understand the processes of acquisition, consolidation, retrieval, reconsolidation, and extinction of aversive memories (LeDoux, 2000; Maren and Quirk, 2004). Parallels can be drawn between the expression of fear and anxiety in humans (e.g., those suffering from PTSD) to the expression of conditioned fear in animals (Brewin, 2001). Many studies use variations of this model (e.g., contextual fear conditioning) to better understand the effects of CBD on behavioral responses related to the recall of traumatic events. Briefly, this model involves the pairing of a neutral stimulus (called CS) with an aversive US, usually a mild foot shock. After successive rounds of pairing (or, in some cases, a single pairing), the animal learns that the CS precedes the US, leading to a series of physiological (e.g., cardiovascular responses) and behavioral (e.g., freezing) responses (for a more complete description of the fear conditioning paradigm, see Myers and Davis, 2007; Maren, 2008; de Bitencourt et al., 2013).

Intervention in the processes of acquisition and consolidation of aversive memories is not promising, since this approach can only be effective when closely following the traumatic event, that is, when it is not yet possible to know if the event will result in a disorder. However, intervention in the processes of retrieval, reconsolidation, and, especially, extinction may be a more promising alternative. Briefly, when reactivated by re-exposure (retrieval), an aversive memory enters a transitional state, where the original memory trace can be reconsolidated or extinguished. This process may be influenced pharmacologically (e.g., by administration of CBD) in order to block reconsolidation or facilitate extinction. The process involves repeated exposure to the CS without the US, which will lead to the formation of a new, US-free memory trace that will override the old (CS + US) trace and, consequently, cause a decrease in the behavioral and physiological responses related to fear (reviewed in Berardi et al., 2016).

Animal studies have shown that CBD can affect every stage of the process of aversive conditioning. In addition, exposure to traumatic stress is essential for the development of PTSD, and CBD is effective in reducing both the cardiovascular responses and anxiogenic effects caused by stress (Resstel et al., 2006, 2009; Gomes et al., 2011; Campos et al., 2012a, 2013). For example, CBD lowered responses related to trauma when administered before the acquisition (Levin et al., 2012) or retrieval (Lemos et al., 2010) of aversive memories. CBD also proved to be effective in reducing responses to aversive memories by blocking the process of reconsolidation (Stern et al., 2012). However, the most promising alternative, suggested by exposure-based psychotherapies, may be through enhancement of the extinction process by CBD (Bitencourt et al., 2008; Do Monte et al., 2013). Before discussing the facilitating effects of CBD on the extinction of aversive memories, it is necessary to highlight the role of the eCB system in this process.

The process of extinguishing an aversive memory requires the participation of CB1 receptors, which was discovered in a classic study by Marsicano et al. (2002). The authors showed that blocking the action of CB1 receptors, either by pharmacological antagonism or genetic deletion, in previously conditioned mice resulted in strongly impaired short- and long-term extinction in fear-conditioning tests (the function of CB1 receptors in the process of extinction of aversive memories is detailed in Wotjak, 2005; Lutz, 2007). This finding raised a new question: Would potentialization of the eCB system facilitate the extinction process? The answer was not surprising, and several studies were published showing that eCB system potentialization could in fact facilitate fear extinction in different behavioral tasks (Chhatwal et al., 2005; Bitencourt et al., 2008, 2014; de Oliveira Alvares et al., 2008; Pamplona et al., 2008; Abush and Akirav, 2009; Lin et al., 2009). From the answer to the previous question, another arose in Takahashi's lab – one that would raise CBD as a therapeutic possibility for the treatment of trauma-related disorders: Given that, according to Bisogno et al. (2001), CBD acts through potentialization of the eCB system, could CBD alone also facilitate the extinction of aversive memories? The answer once again was affirmative, and since then studies have shown that CBD can facilitate the extinction of aversive memories not only in animals (Bitencourt et al., 2008; Do Monte et al., 2013), but also in humans (Das et al., 2013). However, it is important to note that some studies suggest that the reduced expression of fear caused by CBD may result mostly from blocked reconsolidation of an aversive memory than its increased extinction (Stern et al., 2012, 2015; Gazarini et al., 2015). Regardless of which stage of aversive memory processing CBD affects, it appears that, at least in animals, this compound interferes with memory processing in a way that potentially mitigates damaging responses.

In addition to the possibility of CBD affecting different processes involved in aversive memory, animal studies also show favorable effects of this compound in the control of other frequent manifestations of PTSD symptomatology, such as sleep disorders. Studies in rats indicate that CBD may contribute to an increase in sleep duration and depth, and a decrease in anxiety responses induced by sleep disturbance (Monti, 1977; Hsiao et al., 2012; Chagas et al., 2013). In the case of anxiety, another frequent manifestation of PTSD symptomatology, therapeutic potential of CBD has also been reported. However, a thorough review of CBD and anxiety lies beyond the scope of this paper. The interested reader may want to see recent reviews by Blessing et al. (2015) and Lee et al. (2017). Even when all evidence from animal studies suggests an enormous therapeutic potential of CBD CBD for treating PTSD symptoms (for a summary, see **Table 1**), it will still be limited if the results are not replicated in humans. Research has also been moving in this direction, as we will see in the next section.

CBD AND PTSD: ". . .TO CONFIRMATION IN HUMAN TRIALS"

Confirming animal study results in humans is essential for the validation of any strategy that demands a pharmacological therapy. Some studies have shown that, in the case of CBD as a therapeutic alternative for PTSD, this translation is possible (for a summary, see **Table 2**).

In a study published by Das et al. (2013), CBD increased the consolidation of aversive memory extinction in healthy humans. Using inhaled CBD (at a dose of 32 mg), a study in a model of aversive conditioning showed that the compound caused a reduction in the skin conductance response as well as in the expectation levels for the CS during new exposure. Consistent with results of animal studies, these findings show that CBD may be a pharmacological complement to be used in exposure-based therapy. An important consideration in relation to this study is that CBD facilitated the extinction of aversive conditioning only when administered immediately after, and not before, the process. Therefore, understanding at which moment exposure-based therapy with CBD should start is one of several issues that still need to be resolved (Das et al., 2013).

A case report published in 2016 by Shannon and Opila-Lehman described a 10-year-old child who developed PTSD after being sexually abused before the age of five. The child showed significant relief of the symptomatology using CBD oil. Before the CBD therapy, the child underwent standard pharmacological treatment for the condition, which produced short-lasting partial relief, as well as significant side effects. However, CBD oil (given at a dose of 12–25 mg once a day) appeared to relieve key symptoms, such as anxiety and sleep disturbance, while inducing minimal side effects. Although CBD is considered safe (Bergamaschi et al., 2011), the long-term effects were not evaluated in this study and need to be better elucidated (Shannon and Opila-Lehman, 2016).

In two other studies conducted in patients diagnosed with PTSD (Passie et al., 2012; Greer et al., 2014), chronic use of *Cannabis* significantly decreased the symptoms. However, it is not possible to analyze the proportion of CBD and THC in the plant used by the patients in these studies. Patients with PTSD may use *Cannabis* as a form of self-medication (Hill et al., 2018) in an attempt to reduce their symptoms through the anxiolytic and sedative effects (Bremner et al., 1996; Bonn-Miller et al., 2007), and also to induce sleep (Russo et al.,

TABLE 1 | CBD and PTSD: "from bench research. . .".

References in chronological order	Animal/test(s) used	Effective dose/route of administration	Effect	Possible mechanisms of action
Bitencourt et al., 2008.	Rats/Contextual fear conditioning	CBD 2 μg/μl i.c.v.	Facilitated extinction of contextual fear memory	Via CB_1 receptors
Lemos et al., 2010.	Rats/Contextual fear conditioning	CBD 10 mg/kg i.p. and CBD 30 nmol into the PL PFC.	Decreased retrieval of contextual fear memory	Not shown, but highlights the involvement of the PL PFC
Levin et al., 2012.	Rats/Contextual fear conditioning	CBD 1 mg/kg i.p.	Decreased acquisition of contextual fear memory	Not shown
Stern et al., 2012.	Rats/Contextual fear conditioning	CBD 10 mg/kg i.p.	Blockade reconsolidation of contextual fear memory	Via CB_1 receptors
Do Monte et al., 2013.	Rats/Contextual fear conditioning	CBD 0.4 μg/side intra-IL cortex	Facilitated extinction of contextual fear memory	Via CB_1 receptors and highlights the involvement of the IL cortex
Stern et al., 2015.	Rats/Contextual fear conditioning	CBD 1 mg/kg i.p. + THC 0.1 mg/kg i.p.	Blockade reconsolidation of contextual fear memory	Not shown
Gazarini et al., 2015.	Rats/Contextual fear conditioning	CBD 10 mg/kg i.p.	Blockade reconsolidation of contextual fear memory	Not shown

CBD, cannabidiol; i.c.v., intracerebroventricular; i.p., intraperitoneally; PL PFC, prelimbic prefrontal cortex; IL, infralimbic.

TABLE 2 | CBD and PTSD: "...to confirmation in human trials."

References in chronological order	Subjects/test(s) used	Effective dose/route of administration	Effect	Possible mechanisms of action
Passie et al., 2012.	19 year-old male with PTSD (case report)	CBD + THC (cannabis resin from Turkey – 1/1 proportion)/smoked	Patient experienced reduced stress, less involvement with flashbacks and a significant decrease of anxiety.	Not shown
Das et al., 2013.	Healthy humans/Pavlovian fear-conditioning paradigm	CBD 32 mg inhaled	Enhanced consolidation of explicit fear extinction	Not shown
Greer et al., 2014.	80 patients with PTSD	CBD + THC (cannabis – unknown proportion)/smoked	Cannabis (CBD + THC) is associated with PTSD symptom reduction.	Not shown
Shannon and Opila-Lehman, 2016.	10 year-old girl with PTSD (case report)	CBD oil at least 25 mg daily for 5 months/oral capsules	Maintained decrease in anxiety and a steady improvement in the quality and quantity of the patient's sleep.	Not shown

CBD, cannabidiol; THC, tetrahydrocannabinol.

2007). Recent studies also point to a link between *Cannabis* use, possibly as a form of self-medication, and the occurrence of trauma-related events both in adolescents (Bujarski et al., 2012) and adults (Cougle et al., 2011). The more severe the traumatic experience, the greater the plant consumption (Kevorkian et al., 2015). These findings may reinforce the theory that the entourage effect may be more important to the therapeutic effects of the plant than any single compound used in isolation. To confirm this theory, more studies are required (for a review of *Cannabis* use in people with traumatic experiences, see Zer-aviv et al., 2016).

MULTIPLE MECHANISMS OF CBD ACTION: HOW DOES IT WORK, ANYWAY?

The mechanisms of CBD action in behavioral responses related to trauma are still unclear. Understanding the mechanisms underlying CBD action, for example on the expression of aversive memories, is important because a better understanding of this phenomenon may lead to the possibility of more effective interventions in traumatic memories in PTSD. Several mechanisms of action have been proposed to explain the pharmacology of CBD and, as we shall see, they are far from universally accepted.

Within the eCB system, CBD weakly binds to CB1 and CB2 receptors (Pertwee, 2008), and some evidence suggests that it may inhibit both the uptake and hydrolysis (by FAAH) of AEA, an eCB ligand. Thus, CBD may activate CB1 receptors indirectly, by potentiating the eCB system (Bisogno et al., 2001; De Petrocellis et al., 2011; Leweke et al., 2012; Elmes et al., 2015). Based on this assumption and taking into account different studies showing that the activation of CB1 receptors decreases the expression of behaviors related to aversive memories in rats (Chhatwal et al., 2005; Pamplona et al., 2006, 2008; Bitencourt et al., 2008), the action of CBD on such memories may be attributable to indirect potentiation of the eCB system.

A recent review by Hill et al. (2018) proposed that a state of eCB deficiency might represent a stress endophenotype predisposing the individual to the development of trauma-related psychopathology. This work lends further credence to the possibility of CBD enhancing eCB signaling as a possible explanation for the therapeutic effects of CBD and, consequently, its potential to treat PTSD. Furthermore, animal studies have confirmed the importance of the CB1 receptor in mediating the effects of CBD on behavioral responses related to potentially traumatic memories (Bitencourt et al., 2008; Stern et al., 2012, 2015; Do Monte et al., 2013; Gazarini et al., 2015).

However, other research has shown that the answer will not be that simple. In a systematic search of the extant literature for original articles on the molecular pharmacology of CBD, we found a study by Ibeas Bih et al. (2015), which suggested that CBD was unlikely to exert its effects in neurological diseases through modulation of the eCB system. The authors show that CBD can act through 65 discrete, specific molecular targets, including 10 receptors, 32 enzymes, 10 ion channels, and 13 transporters. With regard to the possible modulation of the eCB system, a study published by Massi et al. (2008) showed that CBD stimulated (rather than inhibited, as previously proposed) FAAH, which is involved in the catabolism of AEA; reports of CBD effects on this target are conflicting in the literature. In addition, inhibition of FAAH by CBD *in vitro* is only manifested at high concentrations, which may be difficult to achieve *in vivo*, given the relatively poor bioavailability of CBD (Ibeas Bih et al., 2015). Nevertheless, because FAAH activity appears to be increased by chronic restraint stress in animal models as well as by anxiety-like behaviors (Hill et al., 2013b), FAAH inhibition by CBD appears to us as a possible alternative to explain the CBD effects in aversive memories. In any case, a great deal of caution is needed when interpreting *in vitro* assays and, especially, when extrapolating *in vitro* results to the *in vivo* effects of CBD. Taking into account a possible inhibitory effect of high doses of CBD on the FAAH transporter, it appears likely that incomplete inhibition of FAAH by CBD underlies at least some of its effects *in vivo* (Bisogno et al., 2001; De Petrocellis et al., 2011). This mechanism may be the most promising possibility to explain at a molecular level the inhibitory effects of CBD on behavioral responses related to the recall of traumatic events and it is worth further investigation.

While reports of CBD effects on the eCB system have been contradictory, another molecular target that appears consistent with some of the stress-attenuating effects of CBD may involve serotoninergic transmission via 5-HT receptors (Campos and Guimarães, 2008; Campos et al., 2012a; Gomes et al., 2012). Seven different types of serotoninergic receptors have been identified (5-HT_{1-7}), and the 5-HT_1 class is further subdivided into five other subclasses (5-$HT_{1A,B,D,E,\text{and}F}$). Of the latter, 5-$HT_{1A}$ is the main receptor related to CBD effects, with CBD facilitating $5HT_{1A}$-mediated neurotransmission by acting as an agonist (Russo et al., 2005), promoting anxiolytic effects (Campos and Guimarães, 2008; Gomes et al., 2011; Campos et al., 2012b), mitigating stress responses (Resstel et al., 2009), and, most importantly, reducing the expression of contextual fear conditioning (Gomes et al., 2012). However, controversy regarding the effects of CBD on serotonergic transmission remains. A study by Rock et al. (2012) showed that CBD was not a $5HT_{1A}$ receptor agonist as originally proposed. In this study, the authors suggested that the $5HT_{1A}$-mediated effects of CBD might involve allosteric interactions with the receptor binding site or interference with intracellular pathways (Rock et al., 2012). The possible interaction of CBD with the serotonergic receptor, also observed in the eCB system, has not been confirmed *in vivo* (Ibeas Bih et al., 2015).

Another molecular target, still less explored, that may mediate, at least in part, the effects of CBD on the expression of aversive memories, is the adenosinergic system. Domingos et al. (2018) showed that specific pharmacological or genetic blockade of the P2X7R adenosinergic receptor promoted anxiogenic-like effects, along with deficits in extinction learning. It has now been established that the blocking of the eCB system leads to an increase in the expression of fear responses, whereas eCB system stimulation causes a decrease in such responses. Drawing parallels between eCB and adenosinergic signaling, adenosinergic receptor stimulation (direct or indirect) may represent an alternative treatment for trauma-related psychiatric disorders.

Moreover, indirect stimulation of the adenosinergic system may explain the effects of CBD on aversive memories. Carrier et al. (2006) showed, also *in vitro*, that CBD decreased the uptake of adenosine and, therefore, might increase endogenous adenosine signaling. Given the precariousness of the extrapolation of *in vitro* results to *in vivo* effects, the potential role of the adenosinergic system in the CBD-induced inhibition of aversive memory expression requires further investigation.

We are still far from reaching a consensus regarding the possibility of other molecular targets mediating the effects of CBD on aversive memories. Precisely for this reason, great care must be taken when interpreting the existing literature as well as proposing new experiments. For a detailed review of the pharmacological mechanisms underlying CBD action, see McPartland et al. (2015), Campos et al. (2016), Lee et al. (2017), and especially Ibeas Bih et al. (2015), who reviewed dozens of potential molecular targets of CBD, questioning its action on the eCB system.

In addition to the behavioral changes induced by treatment with CBD, some of which possibly mediated by the CB1 receptor, studies have also shown that chronic CBD treatment may facilitate neurogenesis in the hippocampus, a structure well known for its important role in processing memories (Wolf et al., 2010; Campos et al., 2013), and that is found reduced in patients with PTSD (Shin et al., 2006). Among the brain areas implicated in the effects of CBD, it is also important to highlight the amygdala, which is hyperactive in patients with PTSD and may be related to the severity of the symptoms (Shin et al., 2006; Patel et al., 2012; Etkin and Wager, 2007). CBD attenuated the level of blood oxygenation in the amygdalae of healthy subjects exposed to different levels of anxiety (Fusar-Poli et al., 2010), and decreased c-fos protein expression in the mouse amygdala (Todd and Arnold, 2016). Reduction in the hyperactivity of the amygdala may also explain, in part, the therapeutic effects of CBD against the symptoms caused by PTSD (Passie et al., 2012). The activity of the mPFC, a brain structure that plays an important role in the effects of CBD on the regulation of aversive responses (Lemos et al., 2010; Do Monte et al., 2013), is also reduced in patients with PTSD (Lanius et al., 2003).

Finally, CBD-induced reduction of trauma-related responses raises a wide spectrum of possibilities involving multiple pharmacological and neural circuit mechanisms. Understanding how these mechanisms work is just one more of the various challenges in the study of cannabinoids as potential treatment for neuropsychiatric disorders.

CONCLUSION AND FUTURE PERSPECTIVES

Human and animal studies suggest that CBD may offer therapeutic benefits for disorders related to inappropriate responses to traumatic memories. The effects of CBD on the different stages of aversive memory processing make this compound a candidate pharmacological adjunct to psychological therapies for PTSD. CBD also shows an action profile with fewer side effects than the pharmacological therapy currently used to treat this type of disorder. In addition, even at high doses, CBD does not show the anxiogenic profile of compounds that directly activate eCB transmission.

However, even in the face of evidence pointing to the modulation of the eCB system, more studies are needed to develop a better understanding of the neurobiological mechanisms involved in CBD responses. Additional controlled studies showing the efficacy of CBD for PTSD in humans are also needed. Although much remains to be discovered about the effects of CBD on PTSD symptoms many steps have already been taken in this direction, which may yield a formulation of CBD for the treatment of patients with trauma and stress-related disorders.

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Inhibition of Diacylglycerol Lipase Impairs Fear Extinction in Mice

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Elucidating the underlying molecular mechanisms regulating fear and extinction learning may offer insights that can lead to novel treatments for debilitating anxiety and trauma-related disorders including posttraumatic stress disorder. The endocannabinoid (eCB) system is a retrograde inhibitory signaling pathway involved in regulating central responses to stress. The eCB 2-arachidonoylglycerol (2-AG) has recently been proposed to serve as a homeostatic signal mitigating adverse effects of stress exposure, however, less well understood is 2-AG's role in fear learning and fear extinction. In this study, we have sought to explore 2-AG's role in fear conditioning and fear extinction by disrupting 2-AG synthesis utilizing the DAGL inhibitor (DO34) and DAGLα knock-out mice (DAGLα$^{-/-}$). We found that DAGLα$^{-/-}$ mice, and male and female C57B6/J mice treated with DO34, exhibited impairment in extinction learning in an auditory cue fear-conditioning paradigm. DO34 did not increase unconditioned freezing. Interestingly, inhibition of fatty-acid amide hydrolase was not able to restore normal fear extinction in DO34-treated mice suggesting increased Anandamide cannot compensate for deficient 2-AG signaling in the regulation of fear extinction. Moreover, augmentation of CB1R signaling with tetrahydrocannabinol also failed to restore normal fear extinction in DO34-treated mice. Overall, these data support the hypothesis that DAGLα plays an important role in fear extinction learning. Although genetic and pharmacological disruption of DAGL activity causes widespread lipidomic remodeling, these data combined with previous studies putatively suggest that deficient 2-AG signaling could be a susceptibility endophenotype for the development of trauma-related psychiatric disorders.

Keywords: FAAH, endocannabinoid, fear, stress, extinction, cannabinoid, 2-arachidonoylglycerol

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INTRODUCTION

Over the past 25 years, studies have shown that the endocannabinoid (eCB) system is a key regulator of an organism's response to stress and plays an important role in facilitating recovery after exposure to stress (Lutz et al., 2015; Patel et al., 2017; Hill et al., 2018). Dysregulation of fear learning, fear extinction learning, and the abnormal retention of a heightened fear response have been implicated in many anxiety-related mental illnesses including posttraumatic stress disorder (PTSD) (Parsons and Ressler, 2013; Maren and Holmes, 2016; Deslauriers et al., 2017). Understanding the molecular mechanisms involved in fear learning, fear extinction, and the development of generalized anxiety with persistent hyper responsiveness to stressful

situations, could lead to important insights into the pathophysiological mechanisms underlying fear adaptations and potentially novel treatment approaches for stress-related mental disorders. In this study, we investigate the role of eCBs in fear learning and fear extinction via pharmacological and genetic modulation of 2-arachidonoylglycerol (2-AG) synthesis in male and female mice.

The eCB system is a retrograde inhibitory signaling pathway composed of the presynaptic cannabinoid CB1 receptor (CB1R) and its endogenous ligands arachidonoylethanolamine (AEA) and 2-AG (Herkenham et al., 1990; Matsuda et al., 1990; Devane et al., 1992; Piomelli, 2003; Ohno-Shosaku and Kano, 2014). 2-AG is the most abundant eCB in the brain and is acutely increased by stress exposure (Kondo et al., 1998; Patel et al., 2005, 2009; Dubreucq et al., 2012; Bedse et al., 2017). It is hypothesized that this stress-induced increase in 2-AG signaling serves to counteract some of the adverse behavioral consequences of stress exposure (Hohmann et al., 2005; Evanson et al., 2010; Hill et al., 2011; Wang et al., 2012; Bluett et al., 2014, 2017; Bedse et al., 2017). Conversely, AEA is decreased in response to stress, and plays a role in activating the stress response within the HPA-axis (Dubreucq et al., 2012; McLaughlin et al., 2012; Wang et al., 2012; Gray et al., 2015, 2016). Studies have shown that augmenting 2-AG reduces stress-induced anxiety-like and depressive-like behaviors and can promote resilience to the adverse effects of acute and repeated stress (Kinsey et al., 2011; Sciolino et al., 2011; Sumislawski et al., 2011; Roberts et al., 2014; Zhang et al., 2015; Bedse et al., 2017; Bluett et al., 2017; Heinz et al., 2017). 2-AG augmentation can also increase active fear responses to threats (Heinz et al., 2017). Surprisingly, 2-AG augmentation promotes the expression of conditioned freezing and impairs conditioned fear extinction learning (Llorente-Berzal et al., 2015; Hartley et al., 2016). This surprising contradiction highlights the complexity of eCB signaling in the brain and motivated us to further investigate the role of 2-AG signaling in the regulation of fear learning and extinction.

The DAGLα is a key enzyme responsible for 2-AG synthesis in the postsynaptic neuron in response to increased synaptic activity (Bisogno et al., 2003; Tanimura et al., 2010; Shonesy et al., 2014). Repeated homotypic stress results in increased 2-AG production via DAG hydrolysis by DAGLα (Patel et al., 2009). DAGLα$^{-/-}$ mouse models show reduced levels of 2-AG [and in some cases AEA (Tanimura et al., 2010; Jenniches et al., 2016)], increases in anxiety associated behaviors and increased susceptibility to adverse behavioral consequences of stress exposures (Shonesy et al., 2014; Jenniches et al., 2016; Bluett et al., 2017). These studies are consistent with work demonstrating reducing 2-AG levels via MAGL overexpression also increases anxiety-like behaviors (Guggenhuber et al., 2015). In addition, acute treatment with the DAGL inhibitor DO34 increases innate anxiety levels and promotes susceptibility to the adverse behavioral consequences of stress in mice (Bedse et al., 2017; Bluett et al., 2017). Interestingly, DAGLα$^{-/-}$ also exhibit impairment in extinction of conditioned fear responses (Jenniches et al., 2016). Here, we aim to replicate and extend these findings to gain insight into how DAGL regulates fear extinction learning using a combination of pharmacological and genetic approaches in male and female mice. Overall, our convergent pharmacological and genetic data demonstrate an important role for DAGL in the regulation of fear extinction and further suggest deficient 2-AG-mediated eCB signaling may be an important susceptibility endophenotype subserving risk for the development of trauma-related psychiatric disorders.

MATERIALS AND METHODS

Animals

The 9- to 12-week-old male and female C57BL/6J were used as subjects (Jackson Laboratory, Bar Harbor, ME, United States). Male and female DAGLα$^{-/-}$ mice on a C57BL/6J background were bred in house as described previously and used in one experiment (Shonesy et al., 2014; Bluett et al., 2017). All mice were habituated for one week at Vanderbilt Murine Neurobehavior Core prior to behavior testing. Three to five mice were housed per cage in a temperature and humidity controlled housing facility under a 12-h light/dark cycle, with access to food and water *ad libitum*. Behavior experiments were performed during dark cycle under red light. All studies were approved by the Vanderbilt University Animal Care and Use Committees, and were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Drug Treatment

Mice were given an intraperitoneal (IP) injection of DO34 (50 mg/kg) synthesized as previously described (Ogasawara et al., 2016) dissolved in an 18:1:1 solution of saline, ethanol, and kolliphor EL (Sigma–Aldrich, St. Louis, MO, United States), or vehicle alone (18:1:1 solution of saline, ethanol and kolliphor EL) 2 h prior to behavioral testing at a volume of 10 ml/kg. In one experiment, mice were treated with a combination of DO34 (50 mg/kg) and PF-3845 (FAAH Inhibitor) at 1 mg/kg (A gift from Pfizer Central Research), via IP injection 2 h prior to behavioral testing, or just DO34 (50 mg/kg) alone. In two experiments, mice were treated with a combination of IP injection of DO34 (50 mg/kg) 2 h prior to extinction training and Tetrahydrocannabinol (THC; CB1R partial agonist) at 0.3 mg/kg (in one experiment) or 0.6 mg/kg (Cayman Chemical Company-18:1:1 solution of saline, ethanol and kolliphor EL), via IP injection 30 min prior to behavioral testing, or just DO34 (50 mg/kg) alone. Doses utilized in this study were similar to those previously described in (Bedse et al., 2017).

Fear-Condition and Extinction Paradigm

Freezing behavior was measured using video analysis software (Video Freeze-Med Associates) during all of the fear conditioning and extinction trials exactly as described previously (Hartley et al., 2016). The mice were placed in an auxiliary room directly adjacent to the testing room and allowed to acclimate for 30 min prior to each trial under red light, with consistent temperature and humidity conditions between the auxiliary and trial room. At the end of each protocol, the mice were placed in their respective home cages in the auxiliary room and later returned to the housing facility.

On testing day 1, the mice were placed in a square Plexiglas chamber (dimensions: 30.5 × 24.1 × 21.0 cm) housed in a sound proofing box developed by Med. Associates. Context A consists of a bare metal grid floor, no insert along the walls, and no added scent. Baseline measurements were taken for 90 s. After the baseline, six 30-s tones – conditioned stimulus (CS), were played through a chamber wall mounted speaker, each tone was followed by followed by a 2-s 70 mA shock (unconditioned stimulus-US). There was a 30-s delay between each tone. All mice who failed to freeze at least 50% by final CS on conditioning day were removed from analysis except in **Figure 2** where effects of DO34 on conditioning *per se* were examined.

On testing days 2 and 3, mice were placed in the conditioning chamber in Context B for extinction training. Context B has a smooth white plastic floor insert to cover the metal grid, as well as a curved plastic insert along the side and back walls made of the same white plastic material as the floor. The wall insert has perforations that align with the wall mounted speaker in order to ensure the sound quality of the (CS). Additionally, a paper towel soaked in vanilla extract was placed under the floor insert and grid as a novel olfactory queue, specific to Context B. A baseline measure of freezing behavior was recorded for 30 s followed by a series of 20 30-s tones with a 30-s delay between each tone. No shock was administered during the extinction training.

On day 4, the mice were put back in Context B for CS recall. No drug was administered prior to CS recall. Baseline measure was taken for 2.5 min followed by five 30-s tones with a 30-s delay between each tone. A final measure of extinction recall was taken after the final tone for 2.5 min.

Statistical Analysis

The freezing data for each group was analyzed using a repeated measures two-way ANOVA factoring trial block (time) and drug treatment/or genotype. Total freezing time was entered as reported by the video analysis software. All statistical analyses were conducted using Prism GraphPad 7. $P < 0.05$ was considered significant throughout. *F*- and *P*-values for significant effects of drug treatment or genotype can be found within figure panels. Effect size was calculated using formula for η^2 to reflect the proportion of total variability in the freezing behavior that is accounted for by variation in genotypes or drug treatment during each trial (Tabachnick and Fidell, 2007).

RESULTS

DAGLα$^{-/-}$ Mice Have Impaired Fear Extinction

Given that it has been previously shown that global DAGLα$^{-/-}$ mice exhibit impaired fear extinction (Jenniches et al., 2016), we first aimed to replicate these findings in our line of global DAGLα$^{-/-}$ mice in a fear conditioning and extinction protocol we and others have previously utilized extensively (Hartley et al., 2016) (**Figure 1A**). There was no significant difference in freezing to tone during fear-conditioning on day 1 between DAGLα$^{-/-}$ and WT littermate controls (**Figures 1B,C**). However, freezing behavior in response to tone presentation during extinction was increased in both male and female DAGLα$^{-/-}$ mice compared to WT littermate controls [**Figures 1B,C**, female day 2 and 3: $P = 0.0154/0.0124$, $F(1,7) = 6.422/11.18$, $\eta^2 = 0.23/0.32$, males day 2 and 3: ($P = 0.0390/0.0261$, $F(1,7) = 10.13/7.901$, $\eta^2 = 0.26/0.30$)]. Freezing levels were also significantly higher during extinction recall in DAGLα$^{-/-}$ male and female mice compared to WT mice during both baseline and tone presentation [females day 4:$P = 0.0389$, $F(1,7) = 6.431$, $\eta^2 = 0.28$, males day 4: $P = 0.0045$, $F(1,7) = 16.87$, $\eta^2 = 0.59$].

Pharmacological DAGL Inhibition Does Not Affect Acquisition of Conditioned-Fear

In order to confirm the effects observed in DAGLα$^{-/-}$ mice were mediated via impaired enzymatic activity during adulthood and to gain insight into the temporal regulation of fear learning and extinction by DAGL, we utilized a pharmacological inhibitor of DAGL, DO34 (Ogasawara et al., 2016). We first tested whether acquisition of conditioned fear was regulated by DAGL activity via administration of DO34 or vehicle 2 h prior to fear conditioning on day one in Context A (**Figure 2A**). We observed a very small but significant decrease in freezing behavior in DO34-treated male mice compared to vehicle-treated controls during conditioning [**Figure 2B**, day 1: $P = 0.050$, $F(1,38) = 8.875$, $\eta^2 = 0.025$] suggesting a slight delay in the acquisition of conditioned freezing behavior. In contrast, there was no significant difference in subsequent freezing behavior during tone presentation during extinction training on days 2–3 nor during extinction recall on day 4 in the drug-free states.

Pharmacological DAGL Inhibition Impairs Fear Extinction

Data derived from global DAGLα$^{-/-}$ mice suggest a potential role for 2-AG signaling in the regulation of fear extinction; however, limitations of global knock-out models make conclusive interpretations in this regard difficult (Jenniches et al., 2016). To circumvent these limitations, we next determined whether acute depletion of 2-AG using the pharmacological DAGL inhibitor DO34 would also inhibit fear extinction learning in male and female mice (**Figure 3A**). We utilized a dose of 50 mg/kg which we have shown causes a near-complete elimination of measurable 2-AG throughout the brain (Bedse et al., 2017; Bluett et al., 2017). Prior to conditioning, mice were assigned to vehicle or DO34 treatment groups. There were no differences in freezing response to tone-sock pairings between groups (**Figure 3B**), confirming similar conditioning efficiency in both treatment groups. On day two, DO34 or vehicle was injected 2 h prior to extinction training. DO34-treated male and female mice showed a significant increase in percent freezing time during tone presentation on both extinction day 2 and extinction day 3 [**Figures 3B,C**, day 2 and 3: female $P = 0.0023/0.0006$, $F(1,38) = 10.69/14.13$, $\eta^2 = 0.097/0.142$, male $P = 0.0015/0.0023$, $F(1,33) = 11.96/10.93$, $\eta^2 = 0.11/0.11$]. During the extinction recall test on day 4, performed under drug-free conditions, previously DO34-treated mice showed a sensitized freezing response during baseline and subsequent

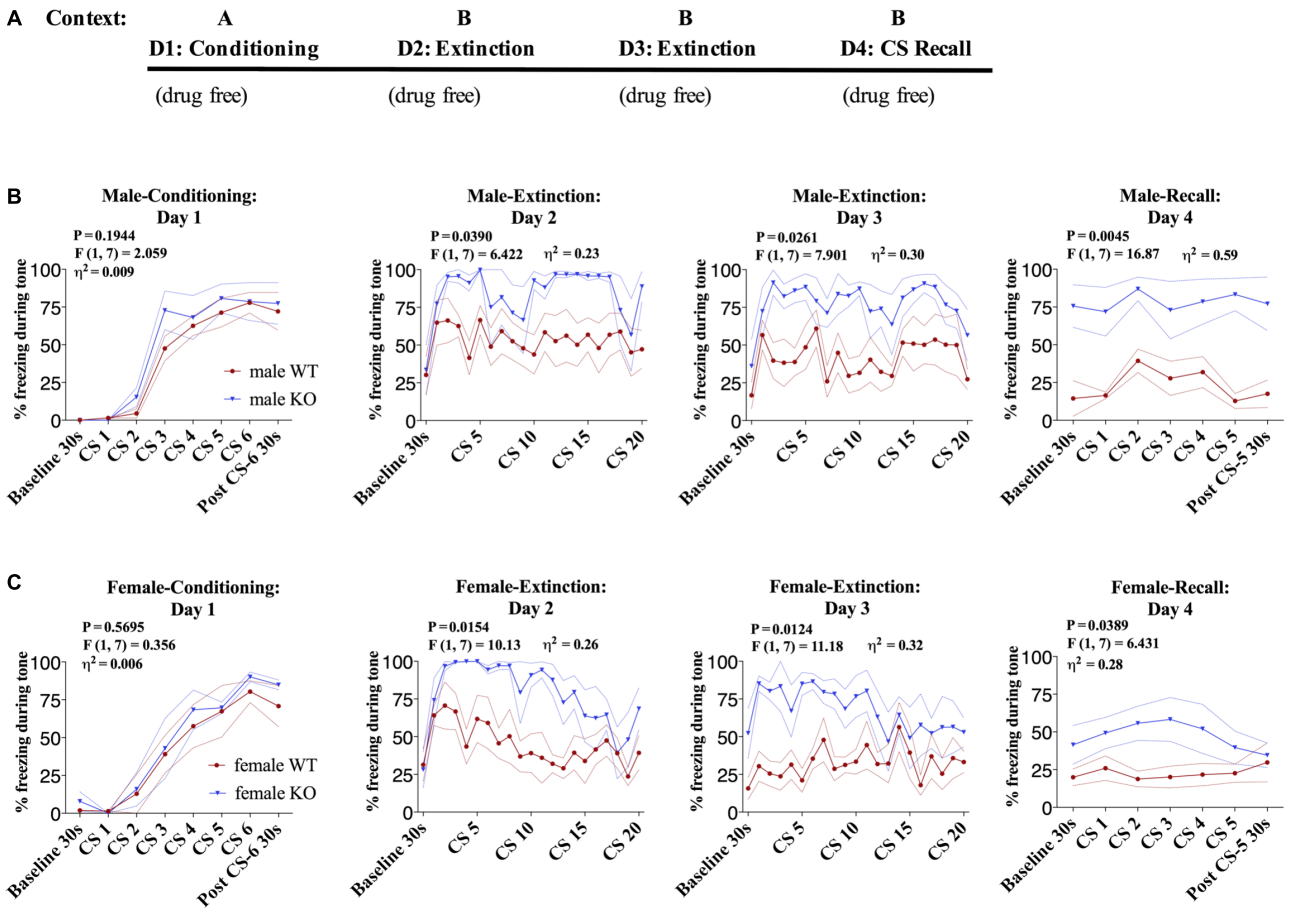


FIGURE 1 | Male and female DAGLα$^{-/-}$ mice have impaired fear extinction. **(A)** Schematic diagram of the experimental paradigm. **(B)** (Far left panel) % freezing by male DAGLα$^{-/-}$ and WT mice during acquisition of cue-conditioned fear. (Middle panels) % freezing during auditory cue during extinction training days 2 and 3. (Far right panel) % freezing during extinction recall (n = 5 WT, n = 4 KO mice). **(C)** (Far left panel) % freezing by female DAGLα$^{-/-}$ and WT mice during acquisition of cue-conditioned fear. (Middle panels) % freezing during auditory cue during extinction training days 2 and 3. (Far right panel) % freezing during extinction recall (n = 5 WT, n = 4 KO mice). F- and P-values and η^2 for genotype effect shown in each panel. All values are presented as mean ± SEM.

tone presentation relative to vehicle-treated mice [**Figures 3B,C**, day 4: female P = 0.0005, $F(1,36)$ = 14.5, η^2 = 0.195, male P = 0.0015, $F(1,33)$ = 11.96, η^2 = 0.11]. Similar effects were observed in female mice (**Figure 3C**). The effect of DO34 treatment during extinction training was similar to that observed in mice that did not undergo extinction training compared to those that did [**Supplementary Figure S1**, day 4: P = 0.0075, $F(1,28)$ = 8.136, η^2 = 0.166], indicating DAGL inhibition during extinction training reduces the effectiveness of fear extinction training, mirroring effects obtained in mice which had not undergone extinction training at all.

Pharmacological DAGL Inhibition Does Not Affect Unconditioned Freezing

Given that DAGLα inhibition can increase unconditioned anxiety (Shonesy et al., 2014; Jenniches et al., 2016), we wanted to rule out the possibility that DO34 increased freezing behavior independent of a fear-conditioning. To explicitly test this, mice were tested in a sham conditioning paradigm where, on day one in Context A, mice were exposed to tone without successive shocks (i.e., CS only). On days 2–3, mice were injected with DO34 2 h prior to sham fear extinction training (**Figure 4A**). Freezing behavior was not different between DO34-treated and vehicle-treated mice on days 2–3 of sham extinction, or on day 4 of sham extinction recall (**Figure 4B**). These data indicate that DO34 does not increase freezing behavior independent of conditioning.

AEA Augmentation Does Not Reverse Impaired Extinction After DAGL Inhibition

In order to determine whether augmentation of AEA signaling could compensate for deficient 2-AG synthesis and promote successful fear extinction, we blocked AEA degradation using the FAAH inhibitor PF-3845 concomitantly with DO34 treatment during extinction training (**Figure 5A**). We found no significant difference between the groups' freezing behaviors when mice were given DO34 + FAAH Inhibitor or DO34 alone, prior to fear extinction training on days 2 or 3 (**Figure 5B**).

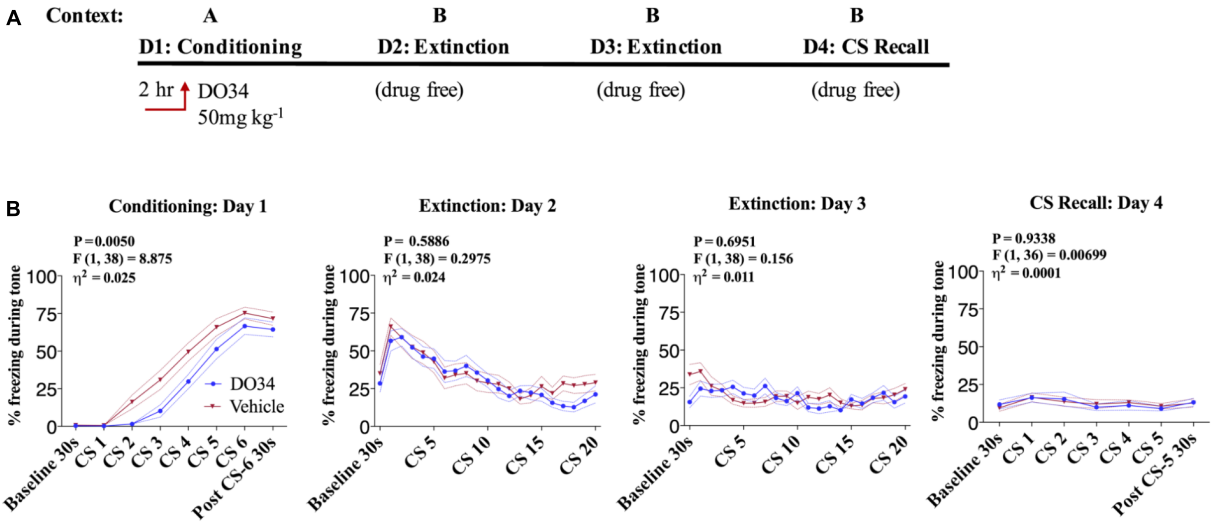


FIGURE 2 | Pharmacological DAGL inhibition does not affect acquisition of conditioned-fear. **(A)** Schematic diagram of the experimental paradigm. **(B)** (Far left panel) % freezing by C57BL/6J male mice during acquisition of cue-conditioned fear when DO34 (50 mg kg^{-1}) was injected IP 2 h prior to trial. (Middle panels) % freezing during auditory cue during extinction training days 2 and 3. (Far right panel) % freezing during extinction recall (n = 20 male mice per condition). F- and P-values and η^2 for main effect of drug treatment show in each panel. All values are presented as mean ± SEM.

CB1R Partial Agonist THC Does Not Reverse Impaired Extinction After DAGL Inhibition

In order to determine whether activation of CB1R receptors could compensate for deficient 2-AG synthesis and promote successful fear extinction, we injected THC (0.3–0.6 mg/kg) 30 min before extinction training on days 2 and 3. We found no significant difference between the groups' freezing behaviors when mice were given DO34 + THC or DO34 alone, prior to fear extinction training on days 2 or 3 (**Supplementary Figure S2**).

DISCUSSION

The eCB signaling is a well-established regulator of fear extinction, however, the role of 2-AG in the regulation of these processes has only recently been studied due to previously limited pharmacological and genetic tools to interrogate 2-AG signaling *in vivo* (Llorente-Berzal et al., 2015; Hartley et al., 2016; Jenniches et al., 2016). Generation of pharmacological tools for 2-AG augmentation and depletion (Ogasawara et al., 2016), and development of global and conditional DAGLα$^{-/-}$ mice (Shonesy et al., 2014; Bluett et al., 2017), has significantly enhanced our ability to examine the role of 2-AG within multiple stress-related biological processes. In this context, we aimed to utilize convergent pharmacological and genetic modulation of DAGL to elucidate the role of 2-AG signaling in fear-learning and extinction. The main findings of the present study are that (1) global life-long DAGLα deletion results in impaired extinction of conditioned fear, (2) acute pharmacological DAGL inhibition impairs extinction of conditioned fear behavior, and (3) neither FAAH inhibition nor THC were able to correct these deficits in fear extinction caused by acute DAGL inhibition. These data provide further support for the notion that 2-AG deficiency states could predispose to the development of stress-related psychopathology and that pharmacological approaches aimed at counteracting this deficiency could represent novel approaches to the treatment of an array of anxiety and trauma-related disorders (Bedse et al., 2017; Bluett et al., 2017; Hill et al., 2018).

With regard to the effects of 2-AG modulation on acquisition of conditioned fear behaviors, our pharmacological data suggest 2-AG signaling may be important for aversive learning as DO34 administered prior to conditioning slowed the acquisition of conditioned freezing behavior. This finding is consistent with data showing accelerated acquisition of conditioned freezing in a trace fear conditioning paradigm after 2-AG augmentation with the monoacylglycerol lipase (MAGL) inhibitor JZL184 (Xu et al., 2014). These data suggest 2-AG signaling may be important for optimal cognitive function consistent with the proposed nootropic effects of CB1 receptor stimulation in aging (Bilkei-Gorzo et al., 2017). However, cognitive impairment of CB1 stimulation have also been demonstrated (Kruk-Slomka et al., 2017), suggesting a potentially complex and/or divergent roles of eCB signaling relative to exogenous cannabinoid agonist administration.

Our findings that both genetic and pharmacological inhibitions of DAGL impair fear extinction are consistent with a recent study showing impaired long-term fear extinction in DAGLα$^{-/-}$ mice (Jenniches et al., 2016). Our studies extend these findings by demonstrating impaired fear extinction after acute pharmacological DAGL inhibition, suggesting findings in DAGLα$^{-/-}$ mice are not a consequence of developmental abnormalities or compensatory adaptations resulting from life-long DAGLα deletion. We further show that the effects

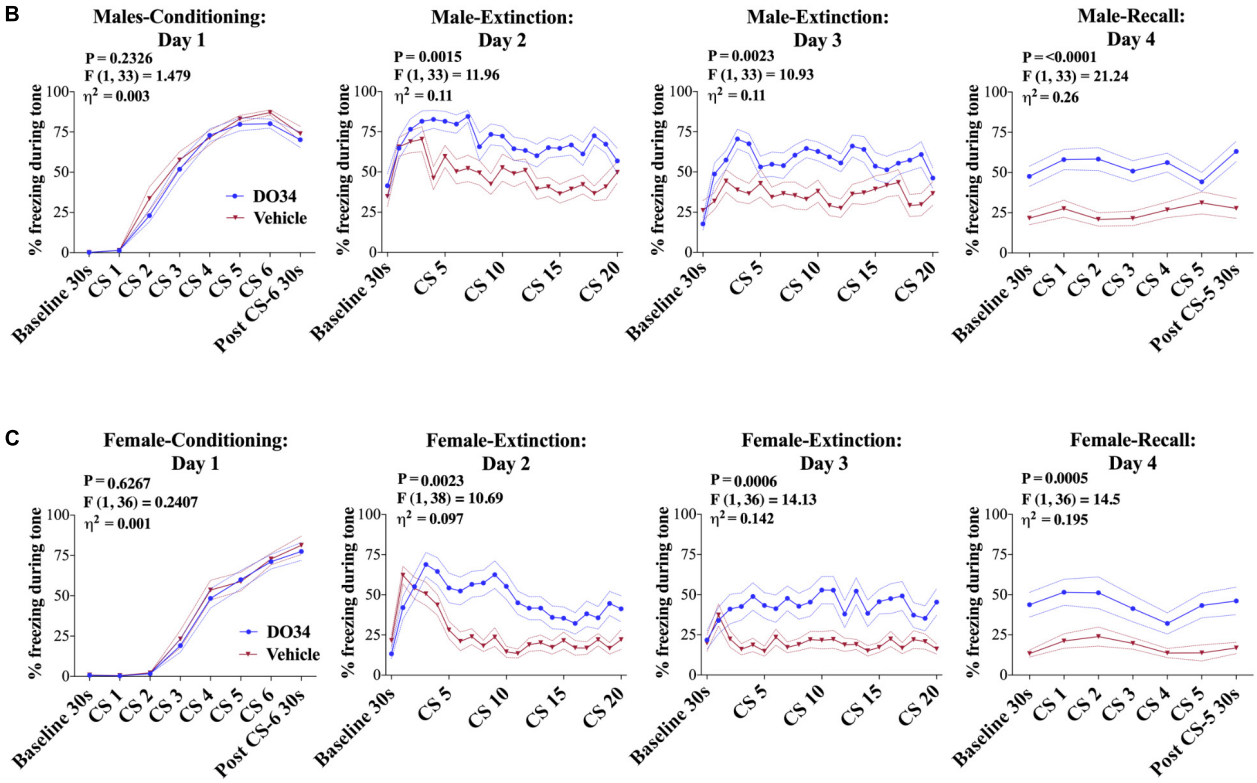


FIGURE 3 | Pharmacological DAGL inhibition impairs fear extinction. **(A)** Schematic diagram of the experimental paradigm. **(B)** (Far left panel) % freezing by C57BL/6J male mice during acquisition of cue-conditioned fear. (Middle panels) % freezing during auditory cue by mice during extinction training days 2 and 3 when D034 (50 mg kg^{-1}) was injected IP 2 h prior to trial. (Far right panel) % freezing during extinction recall (n = 17 DO34-treated male mice, n = 18 vehicle-treated male mice). **(C)** (Far left panel) % freezing by C57BL/6J female mice during acquisition of cue-conditioned fear when DO34 (50 mg kg^{-1}) was injected IP 2 h prior to trial. (Middle panels) % freezing during auditory cue during extinction training days 2 and 3. (Far right panel) % freezing during extinction recall (n = 18 DO34-treated female mice n = 20 vehicle-treated female mice). F- and P-values and η^2 for main effects of drug treatment shown in each panel. All values are presented as mean ± SEM.

of DAGL inhibition are not confounded by increases in unconditioned freezing. Overall, these data are consistent with long-standing findings that blockade or genetic deletion of CB1 receptors robustly impairs fear extinction (see (Lutz, 2007; Ruehle et al., 2012; Rabinak and Phan, 2014; Hill et al., 2018) for review), supporting the notion that 2-AG depletion secondary to DAGL inhibition may be the cause of the behavioral effects observed here. However, DAGL inhibition causes widespread changes in lipidomic networks and thus lack of ability to conclusively ascribe 2-AG deficiency as causally related to the observed phenotypes remains a limitation of the work. Lastly, extinction impairing effects of DO34 were only observed when relatively high shock intensities were used for the US (0.7 mA), but not lower (0.4 mA) intensities (data not shown). This caused significant fear generalization as evidence by unconditioned freezing during the baseline period in all mice on days 2, 3, and 4. Whether the effects of DAGL inhibition are explicitly dependent on US intensity remains to be determined.

That acute pharmacological DAGL inhibition is associated with impaired extinction of conditioned fear is also globally consistent with the increased unconditioned anxiety and increased stress-susceptibility observed after DAGL inhibition and in DAGL$\alpha^{-/-}$ mice (Shonesy et al., 2014; Jenniches et al., 2016; Bedse et al., 2017; Bluett et al., 2017). These data are also consistent with the anxiolytic effects of 2-AG augmentation in a variety of unconditioned anxiety models and the ability or 2-AG augmentation to reduce, and promote resilience to, the adverse effects of stress exposure (Busquets-Garcia et al., 2011; Kinsey et al., 2011; Sciolino et al., 2011; Sumislawski et al., 2011; Zhang et al., 2015; Morena et al., 2016;

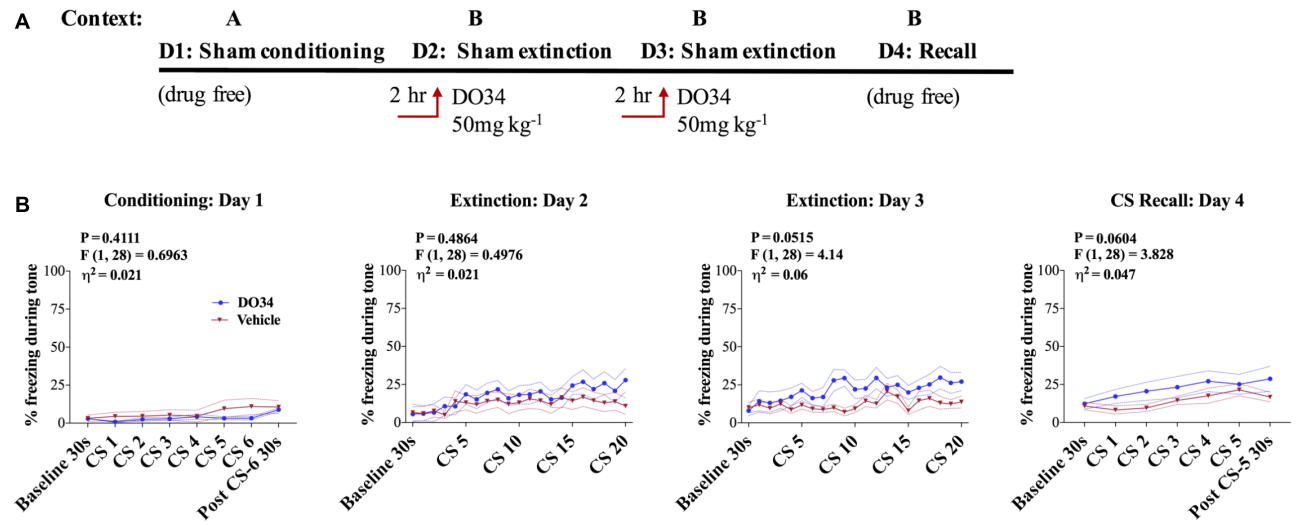


FIGURE 4 | Pharmacological DAGL inhibition does not affect unconditioned freezing. **(A)** Schematic diagram of the experimental paradigm. **(B)** (Far left panel) % freezing by C57BL/6J male mice during sham-conditioning assay-no shock was administered after tone. (Middle panels) % freezing during auditory cue during extinction training days 2 and 3. (Far right panel) % freezing during extinction recall (n = 15 male mice per condition). F- and P-values and η^2 for main effect of drug treatment shown in each panel. All values are presented as mean ± SEM.

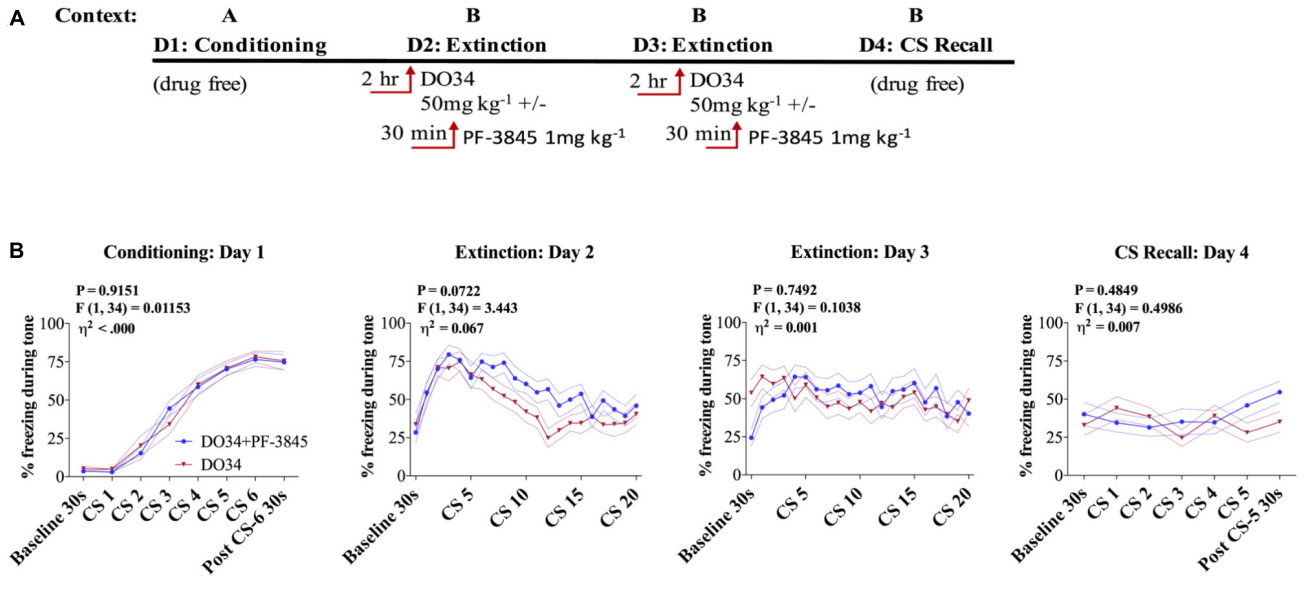


FIGURE 5 | AEA augmentation does not reverse impaired extinction after DAGL inhibition. **(A)** Schematic diagram of the experimental paradigm. **(B)** (Far left panel) % freezing by C57BL/6J male mice during acquisition of cue-conditioned fear. (Middle panels) % freezing during auditory cue by mice during extinction training days 2 and 3 when DO34 (50 mg kg^{-1}) was injected alone or in addition to PF-3845 (FAHH inhibitor 1 mg kg^{-1}) IP 2 h prior to trial. (Far right panel) % freezing during extinction recall (n = 18 male mice per condition). F- and P-values and η^2 for main effect of drug shown in each panel. All values are presented as mean ± SEM.

Bluett et al., 2017). Taken together, these recent and compelling findings suggest 2-AG is an important signaling molecule involved in reducing unconditioned anxiety and the adverse effects of stress, and facilitating appropriate extinction of aversive memories. They also suggest 2-AG deficiency could represent a susceptibility endophenotype predisposing to anxiety and trauma-related psychiatric disorders (see Hill et al., 2018). A corollary to these conclusions is that 2-AG augmentation may represent a novel approach for the treatment of anxiety and stress-related neuropsychiatric disorders, however, there are some contradictory findings in this regard. Specifically, as noted above (**Figure 2**), 2-AG augmentation increased the acquisition of conditioned fear responses, increased the expression of conditioned fear (Llorente-Berzal et al., 2015), and impairs fear extinction (Hartley et al., 2016). Thus, there appears to be an emerging distinction between the effects of

2-AG augmentation on conditioned versus unconditioned fear behaviors, with only the latter being consistently reduced in response to pharmacological 2-AG augmentation.

A critical question that arises from these findings is how both augmenting and depletion of 2-AG results in similar fear extinction deficits. The fear promoting effects of 2-AG augmentation are absent in mice with GABA neuron-specific CB1 deletion, supporting the importance of 2-AG acting on GABAergic neurons to impair extinction and promote conditioned fear (Llorente-Berzal et al., 2015). The retrograde inhibition of GABA release may regulate important circuits within the basolateral amygdala thereby enhancing neuronal output to the central amygdala controlling freezing behavior. It is also important to point out that CB1 deletion from forebrain glutamatergic neurons itself impairs fear extinction (Llorente-Berzal et al., 2015). Based on these data, we propose the extinction-impairing and anxiogenic effects of 2-AG deficiency are due to reduced activity at CB1 on limbic glutamatergic terminals, which may be tonically suppressed by 2-AG signaling. In contrast, the fear promoting and extinction impairing effects of MAGL inhibition are mediated via 2-AG accumulation, synaptic spillover, and subsequent activation of CB1 on GABAergic neurons controlling freezing behavior. We hypothesize that these synapses may physiologically be under less tonic inhibition by 2-AG than glutamatergic CB1, thus 2-AG depletion does not significantly change CB1 activity on GABAergic cells and does not produce anxiolytic effects *per se*. A critical assumption in this model is the differential tonic inhibition of glutamate and GABA release mediated via 2-AG-CB1 signaling, an effect which remains to be tested experimentally. Additionally, that 2-AG augmentation reduces unconditioned anxiety suggests 2-AG may be acting on distinct neural circuits to affect excitation/inhibition balance to ultimately differentially affect conditioned versus unconditioned behaviors.

With regard to the therapeutic potential of eCB signaling, pharmacological AEA augmentation via inhibition of FAAH has also been demonstrated to have anxiolytic and anti-stress effects (Piomelli et al., 2006; Gunduz-Cinar et al., 2013a; Hill et al., 2018), and to facilitate extinction of conditioned fear in some models (Gunduz-Cinar et al., 2013b; Llorente-Berzal et al., 2015). Furthermore, we have recently demonstrated that FAAH inhibition can prevent the unconditioned anxiety associated with acute 2-AG depletion (Bedse et al., 2017). However, FAAH inhibition at the same dose used in Bedse et al. (2017) was unable to enhance fear extinction in mice treated with DO34, suggesting AEA cannot compensate for all aspects of DAGL inhibition. Furthermore, the addition of the CB1 partial agonist, THC was unable to overcome 2-AG-deficiency-induced impairments in fear extinction at dose that has mitigated anxiety like behaviors in non-fear conditioning paradigms (Bedse et al., 2017). In Bedse et al. (2017), when animals were treated with 0.3 mg/kg dose of THC + 50 mg/kg of DO34, they showed decreases in anxiety like behaviors versus animals treated with DO34 alone. In this study, we tested both 0.3 and 0.6 mg/kg doses and found no significance between doses in total freezing behavior or the ability to reverse fear extinction impairments caused by DAGL inhibition. Whether direct full CB1 agonists or CB1 positive allosteric modulators, or alternative doses of THC, could overcome DAGL inhibition-induced impairments in fear extinction is a critical open question in the field of cannabinoid therapeutics development. Lastly, our data might suggest the possibility that the impairment if fear extinction after DAGL inhibition is not mediated by 2-AG deficiency, but rather other mechanisms secondary to the widespread lipidomic changes induced by DAGL inhibition.

CONCLUSION

By utilizing genetic and pharmacological approaches, we have demonstrated that DAGL activity plays an important role in fear extinction learning in both male and female mice. We have also shown that augmentation of AEA levels during fear extinction cannot reverse fear extinction deficits caused by disrupting the molecular mechanisms regulating 2-AG synthesis. Our data combined with data in previous studies highlights the intriguing paradoxical findings that both depletion and augmentation of 2-AG levels impairs fear extinction. There are some limitations to the present work that should be considered in the context of the above interpretations. For example, DAGL inhibition decreases levels of several monoacylglycerols in addition to 2-AG and reduces arachidonic acid levels (Shonesy et al., 2014; Ogasawara et al., 2016), both of which could affect physiology and behavior independent of 2-AG-mediated eCB signaling. In addition, both genetic and pharmacological approaches utilized here are systemic, therefore, the specific brain regions and circuits responsible for the behavioral effects of DAGL inhibition are not currently known. Despite these limitations, the present data provide a solid framework from which to test critical hypotheses regarding the potential therapeutic benefits of eCB modulating compounds on stress-related behavioral outcomes.

AUTHOR CONTRIBUTIONS

VC conducted the behavioral experiments with assistance from AG, DP, and PJ. VC and SP contributed to the research design, data interpretation, and analysis in the laboratory of SP. JU contributed to the generation of reagents in the laboratory of LM. VC and SP wrote the manuscript with input from all the authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2018.00479/full#supplementary-material

FIGURE S1 | DO34 treatment prior to extinction training mimics the effect of no fear extinction training. **(A)** Schematic diagram of the experimental paradigm. **(B)** (Far left panel) % freezing by C57BL/6J male mice during conditioning assay (Middle panels) % freezing during auditory cue during extinction training days 2 and 3. "No extinction" group remained in home cage during extinction trials day 2–3. (Far right panel) % freezing during extinction recall (n = 15 male mice per condition). F- and P-values and η^2 for main effect of condition (extinction versus no extinction) shown in relevant panels. All values are presented as mean ± SEM.

FIGURE S2 | THC does not reverse impaired extinction after DAGL inhibition. **(A)** Schematic diagram of the experimental paradigm. **(B)** (Far left panel) % freezing by C57BL/6J male mice during acquisition of cue-conditioned fear. (Middle panels) % freezing during auditory cue by mice during extinction training days 2 and 3 when DO34 (50 mg kg^{-1}) was injected IP 2 h prior to trial, alone or with an additional injection of THC 30 min prior to extinction training (THC 0.6 mg kg^{-1}) (Far right panel) % freezing during auditory cue during CS recall (n = 14 DO34 + THC-treated male mice, n = 13 DO34 + vehicle = treated male mice, n = 14 vehicle + vehicle-treated male mice). F- and P-values and η^2 obtained by repeated measures two-way ANOVA representing effect of DAGL + THC treatment, DO34 + vehicle treatment, and vehicle only treated animals, prior to fear extinction training. A separate ANOVA was conducted to compare DO34 + THC and DO34 + vehicle-treated animals, and is shown. Colored dots corresponding to treatment legend delineates which treatments are represented in each analysis. All values are given as mean ± SEM. **(C)** In a separate experiment, there was no significant difference in total freezing behavior between two doses of THC (THC 0.3 and 0.6 mg kg^{-1}) on extinction days 2 and 3. Significance values obtained by Student's t-test of total freezing behavior during the entire trial.

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Cannabidiol (CBD) Is a Novel Inhibitor for Exosome and Microvesicle (EMV) Release in Cancer

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Exosomes and microvesicles (EMV) are lipid bilayer-enclosed structures, released by cells and involved in intercellular communication through transfer of proteins and genetic material. EMV release is also associated with various pathologies, including cancer, where increased EMV release is amongst other associated with chemo-resistance and active transfer of pro-oncogenic factors. Recent studies show that EMV-inhibiting agents can sensitize cancer cells to chemotherapeutic agents and reduce cancer growth *in vivo*. Cannabidiol (CBD), a phytocannabinoid derived from *Cannabis sativa*, has anti-inflammatory and anti-oxidant properties, and displays anti-proliferative activity. Here we report a novel role for CBD as a potent inhibitor of EMV release from three cancer cell lines: prostate cancer (PC3), hepatocellular carcinoma (HEPG2) and breast adenocarcinoma (MDA-MB-231). CBD significantly reduced exosome release in all three cancer cell lines, and also significantly, albeit more variably, inhibited microvesicle release. The EMV modulating effects of CBD were found to be dose dependent (1 and 5 μM) and cancer cell type specific. Moreover, we provide evidence that this may be associated with changes in mitochondrial function, including modulation of STAT3 and prohibitin expression, and that CBD can be used to sensitize cancer cells to chemotherapy. We suggest that the known anti-cancer effects of CBD may partly be due to the regulatory effects on EMV biogenesis, and thus CBD poses as a novel and safe modulator of EMV-mediated pathological events.

Keywords: exosomes, microvesicles (MVs), cannabidiol (CBD), peptidylarginine deiminase (PAD), cancer, inflammation, mitochondria, combinatory treatment

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INTRODUCTION

Extracellular vesicles released from cells are classified into exosomes, microvesicles and apoptotic bodies (György et al., 2011). Exosomes and microvesicles (EMVs) are lipid-bilayer structures that carry molecules characteristic of their parental cells to recipient cells, mediating intercellular communication and affecting various physiological and pathological processes including cell

migration, differentiation and angiogenesis (Ansa-Addo et al., 2010; Muralidharan-Chari et al., 2010; Turola et al., 2012; Colombo et al., 2014; Batrakova and Kim, 2015; Kholia et al., 2016).

Microvesicles (MVs) are phospholipid-rich cell-membrane derived vesicles (100–1000 nm) released as part of normal physiology as well as during apoptosis or upon stimulation (Piccin et al., 2007; Inal et al., 2013). The release of MVs can be mediated via calcium ion influx through stimulation of cation channels such as the ATP-gated P2X7, through pores created by sublytic complement, or via calcium released by the endoplasmic reticulum (Turola et al., 2012; Raposo and Stoorvogel, 2013; Stratton et al., 2015a,b). This increase in cytosolic calcium results in cytoskeletal reorganization and membrane asymmetry, followed by subsequent MV blebbing (Inal et al., 2012, 2013; Kholia et al., 2015; Kosgodage et al., 2017; Tricarico et al., 2017). MV formation can also be caused by mitochondrial stress, which leads to increased membrane permeability and leakage of reactive oxygen species (ROS), cytochrome C and apoptosis inducing factor into the cytoplasm. This results in the formation of the apoptosome – which, during pseudoapoptosis, can be formed into MVs for the export of hazardous agents (Inal et al., 2013).

Exosomes (30–100 nm) are generated intracellularly as they are formed after the invagination of the endosome membrane, resulting in intraluminal vesicle formation and the appearance of multivesicular endosomes, which then release exosomes from the plasma membrane via exocytosis (Kowal et al., 2014; van Niel et al., 2018). Crucial cellular components for exosomal biogenesis are ESCRT (endosomal sorting complexes required for transport), sphingolipid ceramide, syntetin and syndecan, and tetraspanins (Théry et al., 2002; Baietti et al., 2012; Colombo et al., 2013; Costa, 2017; Hessvik and Llorente, 2018). The secretion of exosomes is also affected via purinergic receptors such as P2X7 (Qu et al., 2007), by microenvironmental pH (Federici et al., 2014) and calcium (Savina et al., 2003; Kramer-Albers et al., 2007).

Exosome and microvesicles are emerging as novel therapeutic targets in treatment of disease as they have been shown to contribute to inflammatory processes (Foers et al., 2017) and the progression of numerous pathologies including autoimmune diseases (Antwi-Baffour et al., 2010; Withrow et al., 2016; Perez-Hernandez et al., 2017), cancers (Luga et al., 2012; Jorfi et al., 2015; Kholia et al., 2015; Stratton et al., 2015a; Sung et al., 2015; Tkach and Théry, 2016; Moore et al., 2017; Sung and Weaver, 2017) and neurodegenerative diseases (Colombo et al., 2012; Gupta and Pulliam, 2014; Porro et al., 2015; Basso and Bonetto, 2016). In cancer patients, elevated EMV levels have for example been demonstrated in the blood (Ginestra et al., 1998; Kim et al., 2003; Zwicker et al., 2009) and EMVs can also aid tumor spread and survival as they transport various micro RNAs, pathological growth factor receptors and soluble proteins (Muralidharan-Chari et al., 2010; Inal et al., 2012; Hoshino et al., 2015). Circulating EMVs in various body fluids such as cerebrospinal fluid, urine and blood, may in addition serve as reliable biomarkers of pathophysiological processes (Piccin et al., 2007; Inal et al., 2012, 2013; Porro et al., 2015). Besides contributing to disease pathology, EMVs are being considered as therapeutic vehicles themselves (György et al., 2015; Moore et al., 2017).

It has been shown that EMV shedding from cancer cells aids increased active drug efflux and thus contributes to their resistance to chemotherapeutic agents (Bebawy et al., 2009; Tang et al., 2012; Jorfi and Inal, 2013; Pascucci et al., 2014; Jorfi et al., 2015; Saari et al., 2015; Soekmadji and Nelson, 2015; Aubertin et al., 2016; Koch et al., 2016; Muralidharan-Chari et al., 2016). Recent studies on pharmacological inhibition of EMV release have shown that such interventions could be a new strategy to render cancer cells more susceptible to anticancer drug treatment (Tang et al., 2012; Federici et al., 2014; Jorfi et al., 2015; Koch et al., 2016; Muralidharan-Chari et al., 2016; Kosgodage et al., 2017). Such approaches have recently been shown to be effective *in vivo*, demonstrating that the application of EMV inhibitors can effectively sensitize tumors to chemotherapy (Jorfi et al., 2015; Koch et al., 2016; Muralidharan-Chari et al., 2016), reduce drug efflux (Federici et al., 2014; Koch et al., 2016; Muralidharan-Chari et al., 2016) and reduce the dose of anti-cancer drug required to limit tumor growth *in vivo* (Jorfi et al., 2015). Pharmacological non-toxic agents that can selectively manipulate extracellular vesicle release may thus be relevant not only to cancer but also to other pathologies involving EMV release (Lange et al., 2017).

Cannabidiol (CBD) (Mechoulam et al., 2002), a phytocannabinoid derived from *Cannabis sativa*, is anxiolytic (Blessing et al., 2015) and has analgesic, anti-inflammatory, antineoplastic and chemo-preventive activities (Martin-Moreno et al., 2011; Pisanti et al., 2017). CBD has been shown to have a plethora of molecular targets, including the classical endocannabinoid system, while effects that do not involve the classical cannabinoid system are also gaining increased attention (Ibeas Bih et al., 2015; Pisanti et al., 2017). CBD is generally safe at therapeutic doses, shows biphasic effects on the immune system, and has demonstrated anti-cancer activity *in vivo* (Bergamaschi et al., 2011; Massi et al., 2013; Haustein et al., 2014; Velasco et al., 2016). Critically, CBD has been shown to be effective in various EMV-linked pathologies (Velasco et al., 2016), and seems to modulate mitochondrial function, including ATP, ROS and proton leak, as well as uptake and release of calcium (Ryan et al., 2009; Mato et al., 2010; Rimmerman et al., 2013; Cui et al., 2017). These observations may be relevant as mitochondria are key in modulating calcium signaling (Szabadkai and Duchen, 2008; Rizzuto et al., 2012) and importantly, altered calcium signaling and mitochondrial function are hallmarks of many cancers (Boland et al., 2013; Stefano and Kream, 2015; Monteith et al., 2017). This study therefore aimed to investigate putative modulatory effects of CBD on EMV release and to further establish whether CBD had combinatory effects with the recently described EMV-inhibitor Cl-amidine (Luo et al., 2006; Kholia et al., 2015; Kosgodage et al., 2017). For proof of principle we used three cancer cell lines, prostate cancer (PC3), hepatocellular carcinoma (HEPG2) and breast adenocarcinoma (MDA-MB-231). Here we show effects of CBD on EMV release, on mitochondrial function, as well as on STAT3 expression, which amongst other is associated with mitochondrial respiration and Ca^{2+} regulation in the mitochondrion (Wegrzyn et al., 2009;

Yang et al., 2015; Yang and Rincon, 2016), alongside modulatory effects on prohibitin, a pleiotropic protein involved in cellular proliferation and mitochondrial housekeeping (Peng et al., 2015; Ande et al., 2017). Our findings suggest a new link between the emerging understanding of anti-cancer effects of CBD and its modulatory effects on EMV biogenesis in cancer cells, described here for the first time.

MATERIALS AND METHODS

Cell Cultures

Human prostate adenocarcinoma (PC3 and ECACC), human hepatocellular carcinoma (HEPG2 and ECACC) and human breast adenocarcinoma (MDA-MB-231; a kind gift from Dr T. Kalber, UCL) cell lines were maintained at 37°C/5% CO_2, in growth medium containing 10% EMV-free Foetal Bovine Serum (FBS) and RPMI (Sigma, United Kingdom). The cells were split every 3–5 days, depending on confluence, washed twice with EMV-free Dulbecco's Phosphate Buffered Saline (DPBS), prepared as described before (Kosgodage et al., 2017) and detached by incubation for 10–15 min at 37^{o}C with 0.25% (v/v) trypsin/EDTA, followed by two washes by centrifugation using EMV-free DPBS at 200 *g*/5 min. Before the start of every experiment, cell numbers and viability were determined by Guava ViaCount assay (Guava Millipore) and exponentially growing cells with viabilities of ≥95% were used.

Cell Viability Assays

The Guava EasyCyte 8HT flow cytometer (Millipore) and ViaCount assay (Guava Millipore) were used to count and determine viability of cells before the start of every experiment and to assess cell viability after treatment with EMV inhibitors, as previously described (Jorfi et al., 2015; Kosgodage et al., 2017). Cell viability after cisplatin treatment (see 2.9) was assessed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, performed according to the manufacturer's instructions (Sigma, United Kingdom).

Effects on EMV Biogenesis Using CBD and Cl-Amidine

For assessment of effects of CBD and Cl-amidine on EMV generation, PC3, HEPG2 and MDA-MB-231 cells were seeded at a density of 3.8×10^5 cells/well, in triplicate, in 12-well microtiter plates, using pre-warmed serum- and EMV-free RPMI 1640 (Sigma-Aldrich, United Kingdom). To ensure that the medium was EMV free, it was centrifuged at 70,000 *g*/24 h and filtered through a 0.22 μm pore size membrane before use. For testing of putative inhibitory or modulatory effects on EMV release, the cells were then incubated with CBD (1 or 5 μM), Cl-amidine (50 μM) or with a combination of CBD (5 μM) and Cl-amidine (50 μM), for 60 min at 37°C/5% CO_2, while control cells were treated with either DMSO (0.001%) or PBS for CBD and Cl-amidine, respectively. The following concentrations of CBD (GW Pharmaceuticals, United Kingdom) were used: 1 or 5 μM in 0.001% DMSO, based on clinically relevant doses for CBD (Bergamaschi et al., 2011); while Cl-amidine (a kind gift from Prof P.R. Thompson, UMASS) was used at 50 μM concentration (in PBS) as previously determined as an optimal dose for maximum EMV inhibition in several cancer cell lines (Kholia et al., 2015; Kosgodage et al., 2017). For testing of a combinatory effect on EMV release, CBD was applied at 5 μM together with Cl-amidine at 50 μM concentrations. After the 1 h incubation period, the supernatants from each well were collected from the cell preparations, transferred to sterile 1.5 ml Eppendorf tubes (kept on ice) and centrifuged at 200 *g* for 5 min at 4°C to remove the cell debris. The resulting supernatants were kept on ice and subsequently treated for isolation of EMVs, as described below, to include both exosomes and MVs based on previously established protocols (Lötvall et al., 2014; Kholia et al., 2015; Kosgodage et al., 2017; Witwer et al., 2017).

Isolation of EMVs

Exosome and microvesicles were isolated from the CBD, Cl-amidine, and CBD plus Cl-amidine treated cell culture supernatants, as well as from the control treated cells (DMSO or PBS), by differential centrifugation as follows: First, whole cells were removed by spinning at 200 *g*/5 min at 4°C. The supernatants were then collected and further centrifuged at 4,000 *g* for 60 min at 4°C, to remove cell debris. The resulting supernatants were thereafter collected and centrifuged again at 25,000 *g* for 1 h at 4°C. The resulting EMV pellets were collected and the supernatants were discarded. Next, the isolated EMV pellets were resuspended in sterile-filtered (0.22 μm) EMV-free Dulbecco's PBS (DPBS) and thereafter centrifuged again at 25,000 *g* for 1 h at 4°C to remove proteins that may have bound to the EMV surface. The DPBS supernatant was thereafter discarded and the resulting isolated EMV pellets were resuspended in 200 μl of sterile EMV-free DPBS for further nanoparticle tracking analysis (NTA), using the Nanosight (LM10; Nanosight, Amesbury, United Kingdom). Each experiment was repeated three times and performed in triplicate.

Nanoparticle Tracking Analysis (NTA, NanoSight LM10)

To determine size distribution of isolated EMVs, nanoparticle tracking analysis (NTA), based on the Brownian motion of vesicles in suspension (Soo et al., 2012), was used. A Nanosight LM10, equipped with a sCMOS camera and a 405 nm diode laser, was used to enumerate the EMVs. The NTA software 3.0 was used for data acquisition and processing according to the manufacturer's instructions (Malvern). The ambient temperature was set at 23°C, while background extraction and automatic settings were applied for the minimum expected particle size, minimum track length and blur. For calibration, silica beads (100 nm diameter; Microspheres-Nanospheres, Cold Spring, NY) were used. The samples were diluted 1:50 in sterile-filtered, EMV-free DPBS. To maintain the number of particles in the field of view approximately in-between 20 and 40, the minimum concentration of samples was set at 5×10^7 particles/ml. For capturing, the screen and camera gain were set at 8 and 13, respectively; while for processing, the settings were at nine

and three for screen gain and detection threshold, respectively, as according to the manufacturer's instructions (Malvern). Five × 30 s videos were recorded for each sample, measurements with at least 1,000 completed tracks were used for analysis and the resulting replicate histograms were averaged. Each experiment was repeated three times and performed in triplicate.

For verification of the presence of exosomes within the 30–100 nm sized vesicle peak, according to NTA analysis, and MVs within the 101–900 nm sized vesicle peak, according to NTA analysis (**Supplementary Figures 1A,B**), MVs were pelleted first, from the EMV supernatants, by centrifugation at 11,000 *g* for 30 min at 4°C, and thereafter the presence of MVs was assessed by flow cytometry for Annexin V-FITC binding as a measure of phosphatidylserine exposition characteristic for MVs (**Supplementary Figure 1C**). The remaining supernatant was further centrifuged for the isolation of the smaller sized exosomes (<100 nm) at 100,000 *g* for 1 h at 4°C, using the Beckman-Coulter Type 60 Ti rotor. Exosomes were then characterized by Western blotting for the exosome marker CD63 (**Supplementary Figure 1D**). Exosomes and MVs were also verified by transmission electron microscopy (**Supplementary Figures 1A,B**) according to previously described methods and recommendations (Ansa-Addo et al., 2010; Lötvall et al., 2014; Stratton et al., 2015a; Kosgodage et al., 2017; Witwer et al., 2017).

Western Blotting Analysis for Changes in Exosome-Associated CD63 Expression

HEPG2, PC3, and MDA-MB231 cells were grown as a monolayer in T75 flasks (Nunc, United States) until approximately 80% confluent. The media was removed, the cells washed in DPBS and fresh medium added, containing 5 μM CBD or 0.001% DMSO as control treatment. After 1 h incubation with CBD or DMSO, respectively, the media containing EMVs was removed and first centrifuged at 4000 *g* for 30 min at 4°C for removal of cell debris. The resulting supernatant was thereafter ultra-centrifuged for 1 h at 100,000 *g* at 4°C, collecting the resulting EMV pellet, which was washed in 500 μl DPBS and subsequently ultra-centrifuged again at 100,000 *g* for 1 h at 4°C. The isolated EMV pellets were thereafter subjected to protein extraction, using 50 μl RIPA buffer (Sigma, United Kingdom; supplemented with protease inhibitor cocktail P8340, Sigma United Kingdom), per pellet, by pipetting up and down 20 times and thereafter incubating the pellets in RIPA+ buffer on a shaking platform for 1 h on ice. Thereafter, extracted proteins were isolated by centrifugation at 16,000 *g* for 20 min at 4°C, collecting the protein containing supernatant. The corresponding HEPG2, PC3, and MDA-MB231 cells were also collected from each flask for internal comparison of cell amount (as estimated by β-actin) versus vesicles released between CBD treatment and DMSO controls. Cell pellets were treated with equal amounts of RIPA+ buffer using 50 μl buffer per pelleted cells from each flask. Cell protein isolates were then prepared in the same way as EMV protein isolates. The resulting EMV and cell protein preparations were then reconstituted 1:1 in 2× Laemmli sample buffer (BioRad, United Kingdom) containing 5% β-mercaptoethanol (BioRad) and boiled at 100°C for 5 min before protein separation on 4–20% Mini-Protean TGX gels (BioRad). For each EMV sample 20 μl were loaded, while for each cell lysate preparation, 10 μl were loaded per lane. For immunoblotting, proteins were transferred to 0.45 μm nitrocellulose membranes (BioRad) using semi-dry Western blotting at 15 V constant for 1 h, even transfer was assessed using Ponceau S staining (Sigma) and the membranes were blocked in 5% bovine serum albumin (Sigma) in tris-buffered-saline (TBS) containing 0.01% Tween-20 (Sigma) for 1 h at room temperature. Incubation with anti-human CD63 (ab68418, Abcam, United Kingdom, 1/1000 in TBS-T) was carried out overnight at 4°C, thereafter the blots were washed three times for 10 min in TBS-T and incubated thereafter in secondary antibody (HRP-conjugated anti rabbit IgG1, 1/4000, BioRad) for 1 h at room temperature. The blots were washed five times for 10 min in TBS-T, followed by one wash in TBS before visualization with ECL (Amersham, United Kingdom). Membranes were imaged using the UVP transilluminator (UVP BioDoc-ITTM System, United Kingdom). For quantitative comparison of CD63 positive vesicles released from each cell line in the presence of CBD versus DMSO control, the amount of β-actin (ab20272, Abcam, 1/5000 in TBS-T) expression in the corresponding cell preparations was compared by densitometry, using ImageJ. The absence of actin in exosome samples was also tested to verify a lack of contamination by cellular debris in the exosome isolates.

Western Blotting Analysis for Cellular Changes in Mitochondrial Associated Prohibitin and STAT-3 Expression in Response to CBD Treatment

Protein isolates from HEPG2, PC3 and MDA-MB231 cells were prepared, separated by SDS–PAGE and immunoblotted as described above (2.6). To assess changes in two mitochondrial associated proteins, prohibitin and STAT3, following CBD treatment, the membranes were incubated with anti-prohibitin antibody (ab75771, Abcam; 1/2000 in TBS-T) and anti-STAT3 (phospho Y705) antibody (ab76315, Abcam; 1/2000 in TBS-T). The secondary antibody was HRP-conjugated anti rabbit IgG1 (BioRad; 1/4000). For internal loading control, β-actin (ab20272, Abcam, 1/5000 in TBS-T) was used, and detection of prohibitin and STAT3 expression was normalized against β-actin expression by densitometry analysis using ImageJ.

Cellular Respiration and Mitochondrial Function Analysis

Cellular respiration was measured in MDA-MB-231 and PC3 cancer cells using the Seahorse Bioanalyser according to the manufacturer's instructions (Seahorse Biosciences, United States). The sensor cartridge was hydrated with Seahorse sensor media (Seahorse Biosciences) 18 h prior to the assay. In brief, mitochondrial respiration, as determined by oxygen consumption rate (OCR) was measured by seeding cells 2.5×10^4 cells/well (for MDA-MB-231) or 4×10^4 cells/well (for PC3) in specific 24 well Seahorse Bioanalyser plates (Seahorse Biosciences), 24 h prior to the cell respiration assay. Cells were treated with CBD (1 or 5 μM) for 1 h, followed by washing in Seahorse Assay medium (Seahorse

Biosciences) supplemented with glucose and 1% sodium pyruvate, pH 7.4 at 37°C. Thereafter, oligomycin, carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP, 0.2 μM) and antimycin/rotenone (0.25 μM) were added to the sensor plate prior to the commencement of calibration and the assay. Calculations were normalized to protein level, as calculated by Bradford assay directly after the experimental procedure. Each experiment was repeated 3–5 times, with technical replicates of four per plate.

Effect of CBD on Cisplatin-Mediated Apoptosis of HEPG2 and MDA-MB231 Cancer Cells

HEPG2 and MDA-MB231 cells were grown as a monolayer in T75 flasks (Nunc, United States) until 80% confluent. The media was removed, the cells washed in DPBS and fresh medium added, containing 1 or 5 μM CBD, for 24 h. Medium containing 0.001% DMSO was used as control treatment. After 1 h incubation with the compounds, the media was removed, cells gently washed with DPBS and incubated with 100 μM cisplatin (Sigma, United Kingdom), dissolved in culture media, for further 24 h. Cell viability assessment was carried out by MTT assay. The optical density was measured as a percentage of untreated cells and repeated 3–5 times per cell type for experimental replicates, with five technical replicates per plate.

Statistical Analysis

Graphs were prepared and statistical analysis performed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA, United States). A one-way ANOVA was performed with Tukey's *post hoc* analysis. Differences were considered significant for $p \leq 0.05$ ($^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$).

RESULTS

Effects of CBD on Cancer Cell Viability

Cancer cell viability was not significantly affected by the levels of CBD used in these experiments after 1 h incubation (**Figure 1**). In PC3 cells, 1 μM CBD resulted in a 5.6% decrease in cell viability ($p = 0.1583$), and 5 μM CBD in a 2.2% decrease in cell viability ($p = 0.7247$) compared to DMSO treated control cells. The same was observed for HEPG2 cells, with both 1 and 5 μM CBD causing 1.18% decreased cell viability ($p = 0.1890$ and $p = 0.2746$, respectively) compared to DMSO treated control cells. CBD did also not affect MDA-MB-231 cell viability significantly compared to DMSO treated control cells, with a 3.5% decrease observed in 1 μM CBD ($p = 0.7090$) and 5.4% decrease in 5 μM CBD treated cells ($p = 0.3081$). In comparison, cell viability was affected to some extent by Cl-amidine (50 μM), which so far has proven our most effective EMV inhibitor with the lowest toxicity levels compared to other inhibitors previously tested (Kosgodage et al., 2017). Cell viability for PC3 cells was reduced by 20% ($p = 0.0005$), by 11% for HEPG2 ($p = 0.0033$) and by 5.3% for MDA-MB-231 ($p = 0.0353$) in the presence of 50 μM Cl-amidine compared to PBS treated control cells (**Figure 1**). In addition, longer-term (24 h) treatment effects of CBD on cancer cell viability was further assessed for HEPG-2 and MDA-MB-231 cells, showing dose-depended reduction in cell viability compared to control DMSO treated cells as follows: In HEPG2 cells, 1 μM CBD resulted in a 38.8% decrease in cell viability ($p < 0.001$), and 5 μM CBD in a 47.2% decrease in cell viability ($p < 0.001$) compared to DMSO treated control cells. In MDA-MB-231 cells, 1 μM CBD resulted in a 12.9% decrease in cell viability ($p < 0.05$), and 5 μM CBD in a 35.8% decrease in cell viability ($p < 0.01$) compared to DMSO treated control cells (**Supplementary Figure 2**).

EMV Release Profiles Vary Between PC3, HEPG2, and MDA-MB-231 Untreated Cancer Cells

A range in the total amount of EMVs (<900 nm) released from the three cancer cell lines used in this study varied considerably under normal control conditions (untreated cells in the absence of CBD, Cl-amidine or DMSO; **Supplementary Figure 3**). Differences were observed in the proportions of exosomes (<100 nm) and microvesicles (MVs; 100–900 nm) released from control treated cells (absence of EMV inhibitors CBD and/or Cl-amidine). While PC3 cells released the highest amount of EMVs and similar proportions of exosomes and MVs, both HEPG2 and MDA-MB-231 released a higher proportion of MVs versus exosomes (**Supplementary Figures 3A,B**). In addition, a range in the proportional release of the two MV subsets at 100–200 nm and 201–500 nm were observed between the three cell lines, particularly regarding the 201–500 nm subset which was proportionally highest in HEPG2 compared to PC3 and MDA-MB-231 cells (**Supplementary Figure 3B**).

CBD Effectively Inhibits Exosome and Microvesicle Release From HEPG2 Cells

Pre-treatment of HEPG2 with both 1 and 5 μM CBD, for 60 min before EMV isolation, resulted in a significant reduction of total EMV release compared to the DMSO treated control cells (86.7%; $p = 0.0001$ and 97.9%; $p = 0.0002$, respectively) and was more potent than for Cl-amidine (61.9%; $p = 0.0002$) compared to control. When using CBD (5 μM) in combination with Cl-amidine, a significantly higher inhibition was observed compared to Cl-amidine alone ($p = 0.0058$). Compared to control treated cells the combinatory treatment resulted in a 91.9% reduction of EMVs ($p = 0.0002$; **Figure 2A**).

Further analysis of the NTA data, based on size exclusion, was performed to elucidate the inhibitory effects of CBD on the release of exosome-sized vesicles (<100 nm) or MV-sized vesicles (≥100 nm) (**Figures 2B,C**). The total EMV vesicles collected at 25,000 *g* had been confirmed to be comprised of MVs and exosomes, as confirmed by separate isolation of MVs (centrifugation at 11,000 *g*) and of exosomes (100,000 *g*) as identified by the expression of CD63 (strong in exosomes, negligible in MVs), by phosphatidylserine exposition (higher in MVs compared to exosomes), and by electron microscopy (MVs ≥ 100 nm; exosomes <100 nm) according to previously

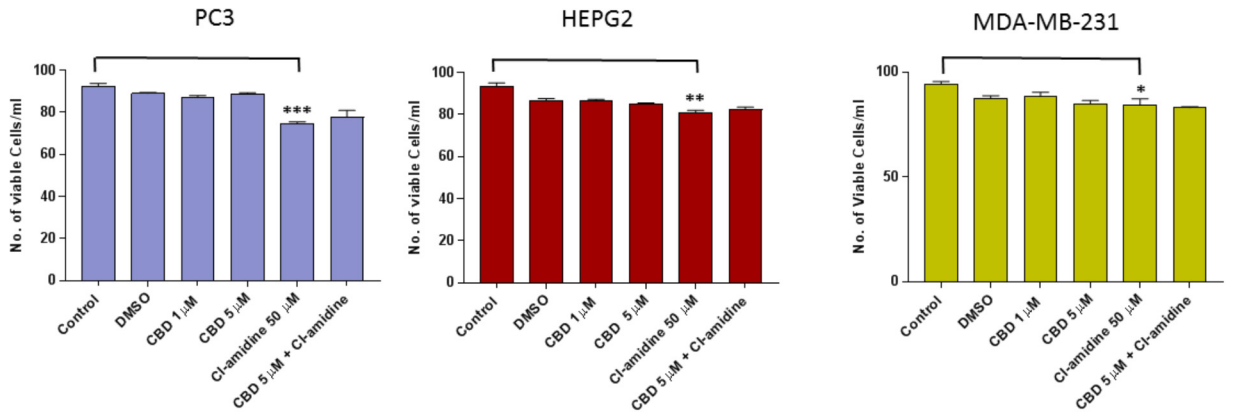


FIGURE 1 | CBD does not affect cell viability of PC3, HEPG2, and MDA-MB-231 cells after 1 h treatment. The Guava EasyCyte 8HT flow cytometer (Millipore) and ViaCount assay were used to count and determine viability of CBD treated cells compared to EMV inhibitor Cl-amidine and DMSO treated control cells after 1 h incubation (*$p \leq 0.05$;**$p \leq 0.01$;***$p \leq 0.001$).

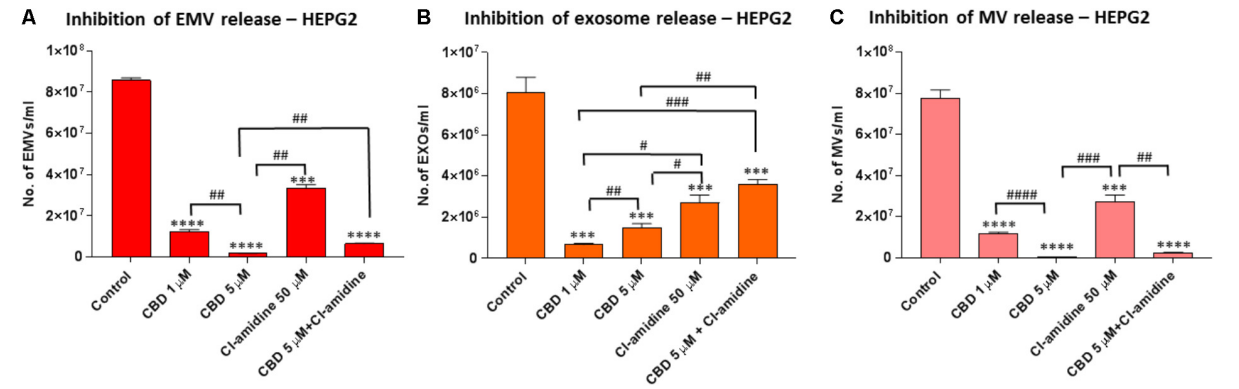


FIGURE 2 | CBD significantly inhibits total EMV, exosome and MV release from HEPG2 cells. Inhibitory effects of CBD alone and in combination with Cl-amidine on extracellular vesicle release from HEPG2 cancer cells are presented as histograms which are based on size exclusion analysis by Nanosight Tracking Analysis (NTA). EMVs represent all vesicles 0–900 nm **(A)**; exosomes are vesicles <100 nm **(B)**; and microvesicles (MV) are 100–900 nm **(C)**. The experiments were repeated three times and the data presented are mean ± SEM of the results (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$ versus Control; Differences between CBD and Cl-amidine treatment group is further indicated as #$p \leq 0.05$; ##$p \leq 0.01$; ###$p \leq 0.001$; ####$p \leq 0.0001$).

established protocols [11,14,15, 81,82; see **Supplementary Figure 1**].

Analysis of inhibitory effects on exosome sized vesicles (<100 nm) showed that both concentrations of CBD (1 and 5 μM) were more effective (91.6%; p = 0.0005 and 84.0%; p = 0.0009; respectively) than Cl-amidine (68.4%; p = 0.0026). The lower dose of CBD (1 μM) was the most potent inhibitor of exosome release in this cancer cell type. Combinatory treatment with 5 μM CBD and Cl-amidine resulted in less exosome inhibition (57.9% compared to control; p = 0.0039) than any of the single inhibitor treatments, albeit not statistically significantly different from Cl-amidine treatment alone (p = 0.1134), while significantly less compared to CBD alone (p = 0.0039 for 1 μM CBD; p = 0.0025 for 5 μM CBD; **Figure 2B**).

The inhibitory effect of CBD on MV-sized vesicle release (≥100 nm) was significant in HEPG2 cells for both 1 μM (86.1%; p = 0.0001) and 5 μM (99.6%; p = 0.0001) concentrations of CBD compared to control cells, with 5 μM CBD being significantly more effective (p = 0.0001). MV inhibitory effects of Cl-amidine in comparison were 61.1% compared to control (p = 0.0007), while combinatory treatment of CBD (5 μM) and Cl-amidine showed a similar effect on total MV release (96.2%; p = 0.0001) as CBD alone (**Figure 2C**).

The histograms from the NTA analysis showed a notable reduction in the approximately 300 nm (201–400 nm range) peak in CBD pre-treated HEPG2 cells (**Supplementary Figure 4**), a feature also observed in the combinatory treatment with CBD and Cl-amidine, while this 300 nm (201–400 nm range) peak was present in Cl-amidine treated cells (**Supplementary Figure 4**). Thus the effect of CBD and Cl-amidine on MV release in the 100–200 and 201–500 nm ranges was further assessed in all three cell lines used in this study (**Figure 5**).

While CBD had a strong inhibitory effect on both MV subsets, Cl-amidine had a much stronger inhibitory effect on the smaller (100–200 nm) than larger (201–500 nm) MV subset compared to control treated HEPG2 cells (**Figures 5A,B**). In the smaller MV

subset, 5 μM CBD showed a stronger inhibitory effect (99.4%; $p = 0.0003$) than 1 μM CBD (82.0%; $p = 0.0006$) compared to control. Cl-amidine reduced this smaller subset by 98.1% ($p = 0.0004$) compared to control, and combinatory treatment of 5 μM CBD and Cl-amidine had a 98.2% inhibitory effect ($p = 0.0003$) compared to control treated cells (**Figure 5A**).

For the shedding of the larger 201–500 nm sized vesicles, CBD was a more effective inhibitor in HEPG2 cells than Cl-amidine, with 1 μM CBD showing 97.1% ($p = 0.0001$) and 5 μM CBD 100% ($p = 0.0002$) inhibitory effect, respectively, compared to controls. In this larger MV subset, Cl-amidine showed only 30% inhibition ($p = 0.0356$) compared to control. The combinatory application of 5 μM CBD and Cl-amidine had a similar inhibitory effect (99.7%; $p = 0.0001$) as 5 μM CBD alone (**Figure 5B**).

CBD Effectively Inhibits Exosome and Microvesicle Release From PC3 Cells

Pre-treatment of PC3 with both 1 and 5 μM CBD, for 60 min before EMV isolation, resulted in a significant reduction of total EMV release compared to the DMSO treated control cells (44.5 and 98.1% reduction of EMV release for 1 and 5 μM CBD, respectively; $p = 0.0149$; $p = 0.0008$, respectively) (**Figure 3A**). The inhibitory effect by 5 μM CBD on total EMV release was greater than observed with our previously most efficient EMV inhibitor Cl-amidine, which was used for comparison ($p = 0.0001$), while Cl-amidine had a significantly stronger EMV inhibitory effect than 1 μM CBD ($p = 0.0001$). When using CBD (5 μM) in combination with Cl-amidine no additive change in total EMV inhibition was found compared to single inhibitors (**Figure 3A**).

Analysis of inhibitory effects on exosome sized vesicles (<100 nm), showed that both CBD and Cl-amidine significantly reduced the number of vesicles released compared to control, untreated PC3 cells (98.0 versus 66.1%; $p = 0.0001$ and $p = 0.0001$, respectively compared to control). A significantly stronger inhibitory effect was observed for 5 μM CBD than with Cl-amidine ($p = 0.0001$), while 1 μM CBD was less effective, inhibiting exosome release by 51.3% compared to control ($p = 0.0002$). Combinatory treatment with 5 μM CBD and Cl-amidine gave similar results as single CBD (5 μM) inhibitor treatment (96.6%; $p = 0.0001$ compared to control) (**Figure 3B**).

The inhibitory effect of CBD on MV-sized vesicle release (≥100 nm) was significant for both 1 μM (38.5%; $p = 0.0009$) and 5 μM (98.1%; $p = 0.0001$) concentrations of CBD, compared to non-treated control cells, although 5 μM CBD was significantly more effective than 1 μM CBD ($p = 0.0002$). The effect of 5 μM CBD alone was similar in reducing in MV release as seen for Cl-amidine compared to control cells (95.6%; $p = 0.0001$) while combinatory treatment of CBD (5 μM) and Cl-amidine did not show a further significant additive effect on MV release (93.6%; $p = 0.0001$ compared to control) (**Figure 3C**).

Next, the effect of CBD and Cl-amidine on MV release in the 100–200 and 201–500 nm ranges was further assessed for PC3 cells (**Figures 5C,D**). MV count in the size range of 100–200 nm was significantly reduced at similar levels by 5 μM CBD, Cl-amidine, and CBD (5 μM) in combination with Cl-amidine, compared to DMSO treated controls (98.8, 95.9, and 94.4%, respectively; $p = 0.0001$ for all groups compared to control). CBD at 1 μM also showed significant inhibition compared to control (71.8%; $p = 0.0007$), but significantly less inhibition on this MV subset than 5 μM CBD alone, Cl-amidine alone or CBD (5 μM) and Cl-amidine in combination ($p = 0.0001$, $p = 0.0001$ and $p = 0.0002$, respectively; **Figure 5C**).

For the shedding of the larger MV subset of 201–500 nm sized vesicles, CBD was more effective at the lower dose of 1 μM than at 5 μM ($p = 0.0001$). Cellular release of this MV subset was reduced by 92% in 1 μM CBD treated cells ($p = 0.0001$), and by 81.2% in 5 μM CBD treated cells ($p = 0.0001$) compared to controls, while Cl-amidine alone reduced this subset of MVs by 64.0% ($p = 0.0002$). When used in combination, 5 μM CBD with Cl-amidine did not show significant inhibition of this MV subset compared to control (4% inhibition; $p = 0.4250$) (**Figure 5D**).

CBD Effectively Inhibits Exosome and Microvesicle Release From MDA-MB-231 Cells

Pre-treatment of MDA-MB-231 with both 1 and 5 μM CBD for 60 min before EMV isolation resulted in a significant reduction of total EMV release compared to the control treated cells (53.4%; $p = 0.0001$ and 42.9%; $p = 0.0001$, respectively) but was a less potent total EMV inhibitor than Cl-amidine (75.9%; $p = 0.0001$ compared to control). When using CBD (5 μM) in combination with Cl-amidine, a significantly ($p = 0.0052$) higher inhibition was observed compared to 5 μM CBD alone, while there was no significant difference compared to 1 μM CBD treatment ($p = 0.2474$). Compared to control treated cells the combinatory treatment resulted in a 55.1% reduction of total EMVs ($p = 0.0006$) (**Figure 4A**)

Analysis of inhibitory effects on exosome sized vesicles (<100 nm) showed that both concentrations of CBD (1 and 5 μM) were similarly potent at being more effective inhibitors (97.5%; $p = 0.0001$ and 99%; $p = 0.0001$; respectively) than Cl-amidine (46.7%; $p = 0.0001$) compared to control treated MDA-MB-231 cells. Combinatory treatment with 5 μM CBD and Cl-amidine resulted in similar effects on exosome inhibition as CBD alone (99.5%; $p = 0.0001$) (**Figure 4B**).

The inhibitory effect of CBD on MV-sized vesicle release (≥100 nm) was significant for both concentrations, albeit less effective than Cl-amidine. CBD showed 34.4% ($p = 0.0001$) inhibition at 1 μM, and 56.5% ($p = 0.0001$) inhibition at 5 μM, compared to control, with 5 μM CBD being a significantly more effective ($p = 0.0007$) total MV inhibitor. In comparison, MV inhibitory effects of Cl-amidine were higher, at 77.8% compared to control ($p = 0.0001$), while combinatory treatment of CBD (5 μM) and Cl-amidine showed a similar effect on total MV release (52.7%; $p = 0.0001$) as 5 μM CBD alone (**Figure 4C**)

The effect of CBD and Cl-amidine on MV release in the 100–200 and 201–500 nm ranges was further assessed in MDA-MB-231 cells (**Figures 5E,F**). While both concentrations of CBD showed a significant decrease on the smaller (100–200 nm) MV subset, the 5 μM CBD concentration showed a stronger inhibitory effect (77.0%; $p = 0.0007$) than 1 μM CBD (41.7%;

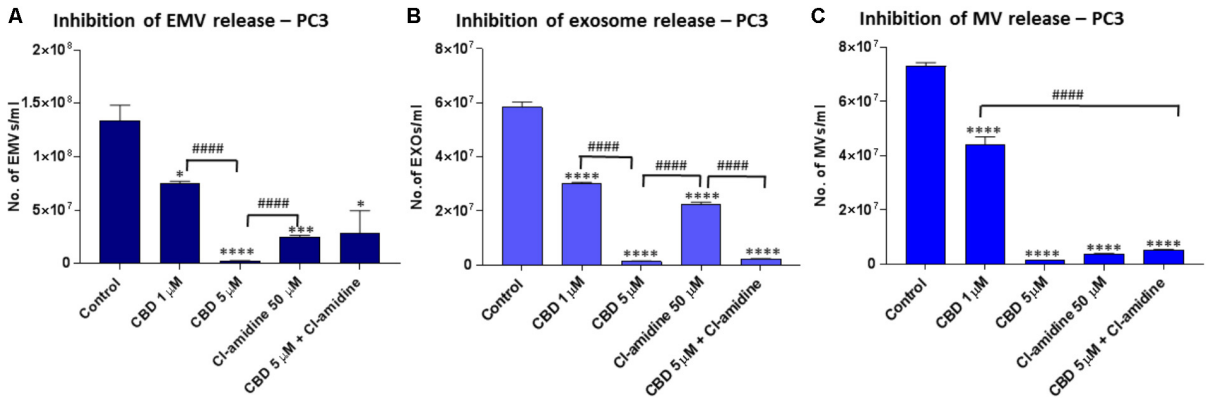


FIGURE 3 | CBD significantly inhibits total EMV, exosome and MV release from PC3 cells. Inhibitory effects of CBD alone and in combination with Cl-amidine on extracellular vesicle release from PC3 cancer cells are presented as histograms which are based on size exclusion analysis by Nanosight Tracking Analysis (NTA). EMVs represent all vesicles 0–900 nm **(A)**; exosomes are vesicles <100 nm **(B)**; and microvesicles (MV) are 100–900 nm **(C)**. The experiments were repeated three times and the data presented are mean ± SEM of the results (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$ versus Control; Differences between CBD and Cl-amidine treatment group is further indicated as #$p \leq 0.05$; ##$p \leq 0.01$; ###$p \leq 0.001$; ####$p \leq 0.0001$).

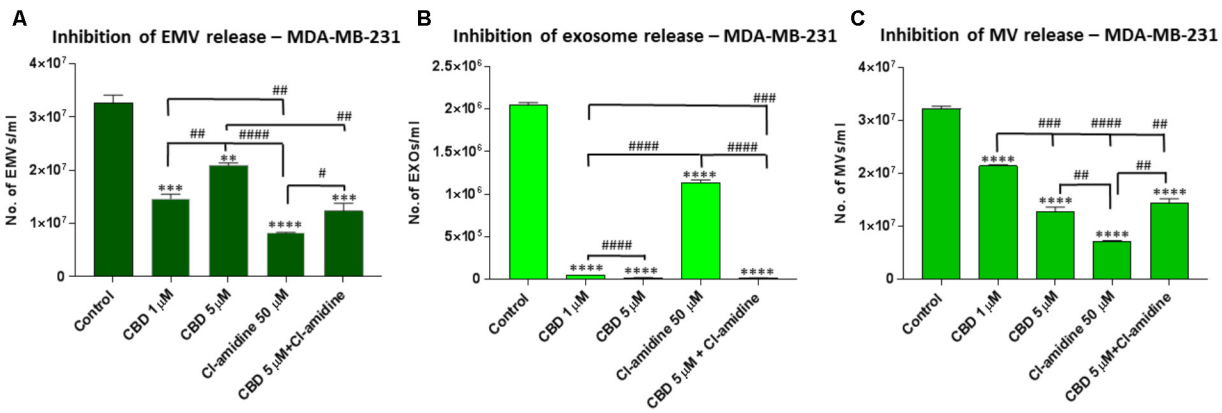


FIGURE 4 | CBD significantly inhibits total EMV, exosome and MV release from MDA-MB-231 cells. Inhibitory effects of CBD alone and in combination with Cl-amidine on extracellular vesicle release from MDA-MB-231 cancer cells are presented as histograms which are based on size exclusion analysis by Nanosight Tracking Analysis (NTA). EMVs represent all vesicles 0–900 nm **(A)**; exosomes are vesicles <100 nm **(B)**; and microvesicles (MV) are 100–900 nm **(C)**. The experiments were repeated three times and the data presented are mean ± SEM of the results (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$ versus Control; Differences between CBD and Cl-amidine treatment group is further indicated as #$p \leq 0.05$; ##$p \leq 0.01$; ###$p \leq 0.001$; ####$p \leq 0.0001$).

$p = 0.0174$), compared to control. Cl-amidine had the strongest inhibitory effect on this MV subset (84.8%; $p = 0.0004$), while the combination of CBD and Cl-amidine showed no significant change ($p = 0.4238$) compared to 5 μM CBD alone, reducing this MV subset by 61.0% compared to control ($p = 0.0089$) (**Figure 5E**).

For the shedding of the larger 201–500 nm sized vesicles, less inhibitory effects were observed for CBD. Neither CBD alone nor in combination with Cl-amidine, showed any inhibitory effects, while Cl-amidine alone reduced the release of this MV population by 25.7% ($p = 0.0004$). Contrary to what was observed in the other two cancer cell lines, CBD increased the release of this MV subpopulation by 10.5% ($p = 0.0143$) at 1 μM concentration and by 84.2% ($p = 0.0001$) at 5 μM concentration compared to control – an effect that was somewhat counteracted in the combinatory treatment with Cl-amidine, where this CBD-mediated increase was reduced by 42.3% ($p = 0.0001$), bringing it down to similar levels as the control treated cells (**Figure 5F**).

CBD Modulates CD63 Expression in HEPG2, PC3, and MDA-MB-231 Cells

Findings from the NTA analysis, showing significant reduction in EMV release, particularly exosome release, was further assessed by Western blotting of CD63 expression in all three cancer cell lines following CBD treatment (5 μM). The expression of CD63 was reduced in all three cell lines following 1 h CBD treatment (**Figure 6**), thus confirming the NTA results, showing significant reduction in exosome biogenesis in response to CBD treatment in HEPG2 (**Figure 6A**), PC3 (**Figure 6B**) and MDA-MB-231 (**Figure 6C**) cancer cells. The absence of actin in exosome samples

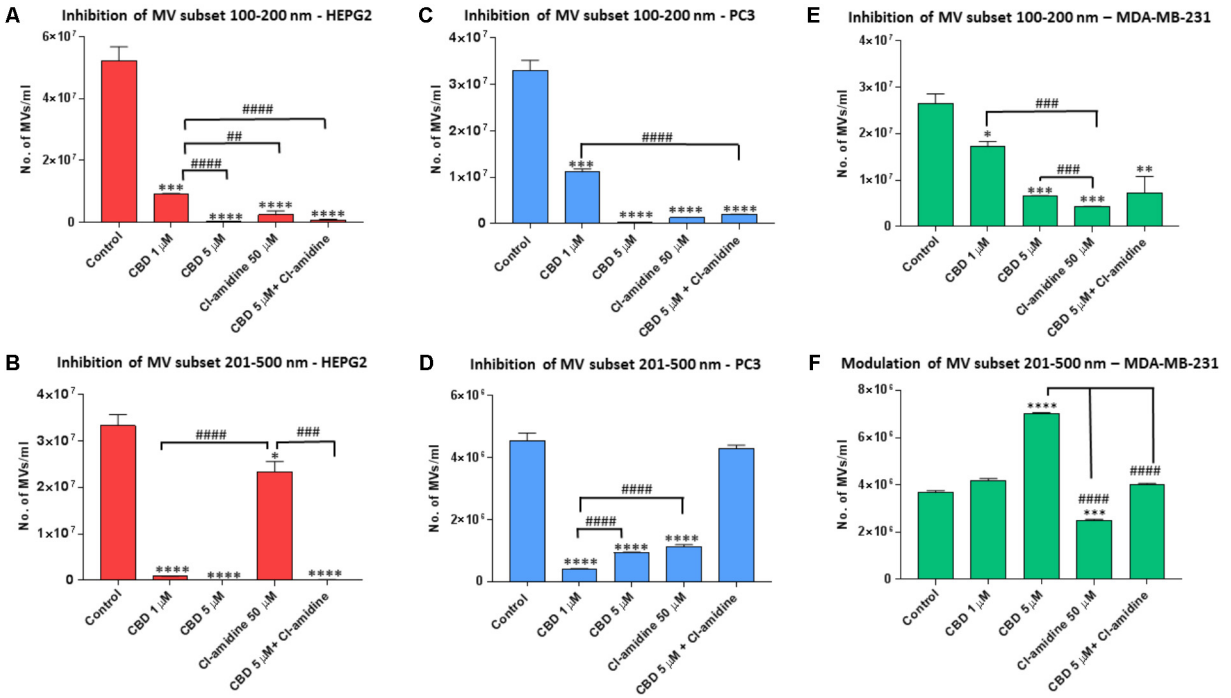


FIGURE 5 | CBD modulates different MV subpopulations released from PC3, HEPG2 and MDA-MB-231 cells. Inhibitory effects of CBD alone, Cl-amidine or CBD in combination with Cl-amidine on 100–200 nm and 201–500 nm sized microvesicles, based on size exclusion analysis by Nanosight Tracking Analysis (NTA). Inhibition of 100–200 nm MV release in **(A)** PC3; **(C)** HEPG2; and **(E)** MDA-MB-231 cancer cells. Inhibition of 201–500 nm MV release in **(B)** PC3; **(D)** HEPG2; and **(F)** MDA-MB-231 cancer cells. The experiments were repeated three times and the data presented are mean ± SEM of the results (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$ versus Control; Differences between CBD and Cl-amidine treatment group is further indicated as #$p \leq 0.05$; ##$p \leq 0.01$; ###$p \leq 0.001$; ####$p \leq 0.0001$).

was also confirmed to exclude contamination by cellular debris in the exosome isolates (not shown).

Mitochondrial Function Alteration Analysis in MDA-MB-231 and PC3 Cells Following CBD Treatment

Mitochondrial analysis, using the Seahorse Bionalayser, measured mitochondrial respiration along with several key mitochondrial factors associated with mitochondrial function through oxygen consumption (**Figure 7**). In MDA-MB-231 cells, basal mitochondrial OCR (oxygen consumption rate) was significantly increased, compared to non-treated controls (50.4 ± 13.5 pMoles/min), following 1 h CBD treatment at 1 μM (104.1 ± 23.7 pMoles/min; $p \leq 0.05$) and 5 μM CBD (129.6 ± 36.4 pMoles/min; $p \leq 0.05$) (**Figure 7A**). In PC3 cells, basal mitochondrial OCR showed a decreasing trend with increased dose of CBD, following 1 h CBD treatment at 1 μM (124.2 ± 9.6 pMoles/min; $p \leq 0.05$) and 5 μM CBD (116.3 ± 13.1 pMoles/min), while not statistically significant compared to non-treated control (146.8 ± 12.9 pMoles/min) (**Figure 7D**).

Dose dependent changes in relative ATP production levels were observed in both cancer cell lines in response to CBD treatment compared to untreated cells. Compared to untreated MDA-MB-231 cells (37.5 ± 8.9 pMoles/min), in 1 μM CBD treated MDA-MB-231 cells ATP production levels were 60.5 ± 12.8 pMoles/min ($p \leq 0.05$) and in 5 μM CBD treated cells ATP production levels were 69.2 ± 16.8 pMoles/min ($p \leq 0.05$) (**Figure 7B**). PC3 cells showed a decreasing trend in ATP with increased dose of CBD, albeit not statistically significant compared to control treated PC3 cells (106.0 ± 9.4 pMoles/min). In 1 μM CBD treated PC3 cells ATP production levels were 87.8 ± 9.1 pMoles/min and in 5 μM CBD treated cells ATP production levels were 81.2 ± 11.9 pMoles/min (**Figure 7E**).

A significant dose dependent increase in proton leak was observed for both concentrations of CBD in MDA-MB-231 cells as follows: 1 μM CBD: 21.6 ± 3.2 pMoles/min ($p \leq 0.01$), and 5 μM CBD: 32.2 ± 9.5 pMoles/min ($p \leq 0.01$), compared to untreated cells (5.8 ± 3.3 pMoles/min) (**Figure 7C**). In PC3 cells proton leak was somewhat, but not significantly, reduced in the presence of 1 μM (36.4 ± 3.6 pMoles/min) and 5 μM CBD (35.1 ± 2.8 pMoles/min) compared to untreated cells (40.9 ± 3.7 pMoles/min) (**Figure 7F**).

CBD Modulates Expression of Mitochondrial Associated Proteins Prohibitin and STAT3

Protein isolates from HEPG2, PC3 and MDA-MB231 cells were further assessed for changes in two mitochondrial associated proteins; prohibitin and STAT3, following CBD (5 μM)

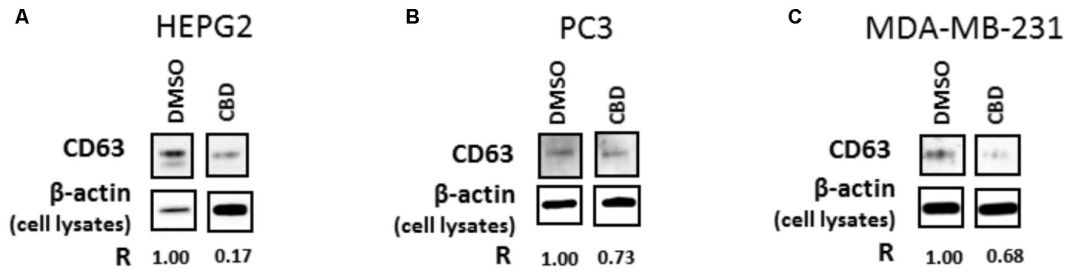


FIGURE 6 | CD63 exosomal marker is reduced following 1 h CBD treatment in HEPG2, PC3, and MDA-MB-231 cancer cells. The results from the NTA analysis were confirmed by Western blotting for the exosomal CD63 marker, which was reduced in all three cell lines following 1 h treatment with 5 μM CBD: **(A)** CD63 expression is reduced in CBD treated versus DMSO control treated HEPG2 cancer cells; **(B)** CD63 expression is reduced in CBD treated versus DMSO control treated PC3 cells; **(C)** CD63 expression is reduced in CBD treated versus DMSO control treated MDA-MB-231 cells. All EMV preparations were performed in equal buffer volume (50 μl) and all cell lysates were prepared in equal buffer volume (50 μl) between all samples, for accurate presentation of amounts of vesicles isolated and amounts of cells grown and collected per flask. For EMV isolates, 20 μl of sample was loaded per lane, while for β-actin detection in the corresponding cell lysates, 10 μl of sample was loaded per lane. The relative detection of CD63 in EMVs released from the corresponding cell preparation is indicated by "R," in relation to β-actin detection in the corresponding cell isolate, for comparison between CBD treatments versus DMSO control.

treatment and compared to DMSO treated controls. In all three cancer cell lines, levels of prohibitin were reduced, although more marked changes were noted in the PC3 (**Figure 8A**) and HEPG2 (**Figure 8B**) cells compared to the MDA-MB-231 cells (**Figure 8C**). In all three cancer cell lines, STAT3 (phospho Y705) was also reduced after 1 h CBD (5 μM) treatment; again this reduction was higher in PC3 and HEPG2 cells (**Figures 8D,E**), compared to the MDA-MB-231 cells (**Figure 8F**).

CBD Sensitizes HEPG2 and MDA-MB-231 Cancer Cells to on Cisplatin-Mediated Apoptosis

In both HEPG2 and MDA-MB-231 cancer cells, CBD increased cisplatin-mediated apoptosis (**Figure 9**). In HEPG2 cells, compared to untreated control cells, cisplatin treatment alone resulted in 57.3% cell viability ($p < 0.01$). However, this effect was significantly enhanced ($p < 0.001$) if cells were first treated with 1 and 5 μM CBD (54.5 and 39.1%, respectively), prior to cisplatin (**Figure 9A**). In MDA-MB-231 cells, compared to untreated control cells, cisplatin treatment alone resulted in 47.3% cell viability ($p < 0.001$), while 21.3 and 8.3% cell viability was observed for cells treated with 1 or 5 μM CBD prior to cisplatin treatment ($p < 0.01$). CBD treatment alone led to significant changes in cell viability, but to a much lesser extent than those observed when cells were first treated with CBD followed by cisplatin (**Figures 9A,B**).

DISCUSSION

This study reveals a novel finding for CBD; it can selectively inhibit the release of subsets of EMVs, from cancer cell lines. The different cancer cell lines tested here (prostate cancer PC3, hepatocellular carcinoma HEPG2 and breast adenocarcinoma MDA-MB-231) varied in the proportional amounts of total EMVs, MVs and exosomes released under standard conditions (**Supplementary Figure 3**). Nonetheless, across this range of EMV release profiles, we found that CBD consistently inhibited exosome release significantly and also had significant, albeit more variable, modulating effects on MV release. This novel function of CBD on EMV release, revealed here for the first time, may be of high relevance for optimized therapeutic application in various EMV-mediated pathologies.

There is a considerable interest in using EMV inhibitors to sensitize cancers to chemotherapy. Previous work, using the calpain inhibitor calpeptin for MV inhibition, in combination with chemotherapy drugs fluorouracil and docetaxel, reduced the effective chemotherapeutic dose needed by 100-fold to produce comparable reduction in tumor volume *in vivo*. The same study also showed that methotrexate is released from cancer cells in MVs (Jorfi et al., 2015). Similar findings of drug efflux and sensitisation to gemcitabine in response to MV inhibition were established in pancreatic cancer *in vitro* and *in vivo* upon MV inhibition via ERK-mediated pathways (Muralidharan-Chari et al., 2016); and to doxorubicin and pixantrone treatment upon exosome inhibition via inhibition of ATP-transporter A3 in B-cell lymphoma models (Koch et al., 2016). Also, chemotaxis of cancer cells has been shown to be promoted by exosome secretion, but to be diminished by knockdown of the exosome regulator Rab27a (Sung and Weaver, 2017). Inhibition of exosome secretion has been shown to cause defective tumor cell migration (Sung et al., 2015), while exosomes isolated from gastric tumor cells were shown to induce tumor cell migration and promotion in receiving cells (Wu et al., 2016). Previously, our work identified a novel pathway of MV release involving peptidylarginine deiminases (PADs) and the effective inhibition of PAD-mediated EMV release using Cl-amidine (Kholia et al., 2015; Kosgodage et al., 2017), suggesting implications in a number of pathologies (Lange et al., 2017). In addition, we have also recently shown that several new candidate EMV inhibitors, including bisindolylmaleimide-I, imipramine and Cl-amidine, are more potent EMV inhibitors than calpeptin (Kosgodage et al., 2017) and those they sensitize cancer cells to chemotherapeutic agents. This further call for the identification of novel EMV inhibitors, which are safe for application *in vivo*, such as CBD now

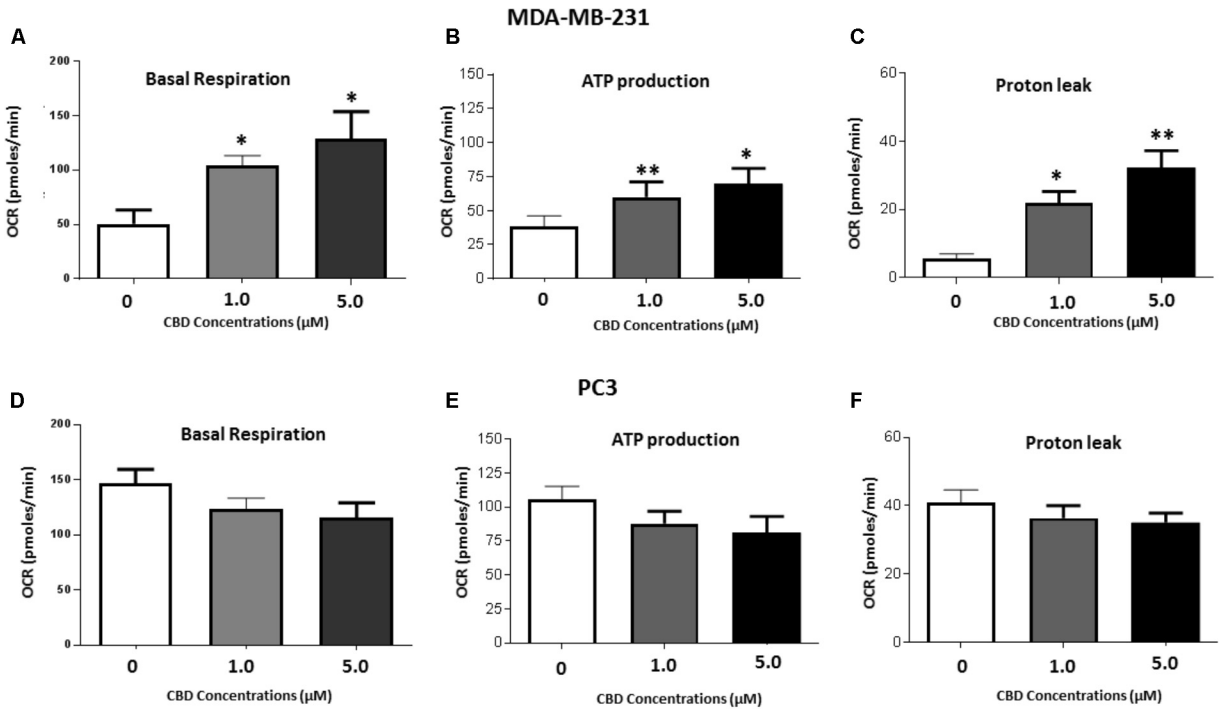


FIGURE 7 | Mitochondrial function alteration following 1 h CBD treatment in MDA-MB-231 and PC3 cancer cells. MDA-MB-231 and PC3 cells were treated with 1 or 5 μM CBD for 1 h prior to mitochondrial functional analysis using the Seahorse Bioanalyser for the following parameters: **(A,D)** Basal mitochondrial respiration; **(B,E)** Quantification of ATP production; **(C,F)** Proton leak. Data shown is repeated three times (for MDA-MB-231 cells) or five times (for PC3 cells) with four technical replicates per plate. Data is represented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus untreated control cells.

identified here. Indeed, as we have shown here, by significantly increasing cisplatin mediated apoptosis, CBD showed a similar ability to other EMV inhibitors of sensitizing cancer cells to chemotherapy.

CBD-mediated inhibition of EMV release, observed in the present study, was more effective for some EMV subsets and cancer cells than Cl-amidine, our most potent EMV inhibitor to date (Kosgodage et al., 2017). One intriguing finding in our study is the selectivity of CBD on different EMV subsets in the three different cancer cell lines, which also varied with concentration (1 and 5 μM). In PC3 cells, 5 μM of CBD was the most effective inhibitor of total EMVs, exosomes, total MVs and the smaller MV subset (100–200 nm), while 1 μM CBD was most effective at inhibiting the larger MV subset (201–500 nm). In the HEPG2 hepatocellular carcinoma cells 5 μM CBD had the main impact on total EMV and MV release, while 1 μM CBD most significantly affected exosome release. Overall, the potency of CBD to inhibit all subsets of EMVs tested here was most marked in the HEPG2 cells. In MDA-MB-231 cells the inhibitory effect of CBD was particularly marked for exosome release, while total MV release was less inhibited by CBD compared to Cl-amidine. Recent studies in this invasive breast cancer cell line have suggested an active role for exosomes in increased cell movement and metastasis (Harris et al., 2015). The increase in MVs released in the size range of 201–500 nm in response to CBD treatment was specific for the MDA-MB-231 cells. This may indicate a higher sensitivity of this particular cancer cell line to CBD and may also be a sign of pseudoapoptotic responses, where increased membrane permeability and leakage of reactive oxygen species (ROS) and other apoptotic factors is still low enough for the cell to turn the apoptosome into MVs for export of hazardous agents (Mackenzie et al., 2005; Inal et al., 2013). Indeed, a dose-dependent increase in ROS levels in response to 1 h CBD treatment (**Supplementary Figure 5**) alongside a dose-dependent increase in proton leak, mitochondrial respiration and ATP levels (**Figures 7A–C**) were observed in this cancer cell line in particular. In the PC3 cells on the other hand, the reduced EMV release observed in all EMV subsets, tallied in with a trend of reduced ATP production and reduced proton leak as well as lowered mitochondrial respiration, indicating an absence of pseudoapoptotic responses, as clearly reflected also in the significant reduction of the 201–500 nm MV subset in the PC cancer cells.

In the current study we have found that while reducing EMVs, CBD also modulates mitochondrial function and the expression of mitochondrial associated proteins prohibitin and STAT3. Although studies on direct links between EMV release and mitochondrial changes are relatively limited, requiring further investigation, EMV generation has previously been linked to this organelle (Mackenzie et al., 2005; Qu et al., 2007; Lopez et al., 2008; Morel et al., 2010; Dubyak, 2012; Soto-Heredero et al., 2017). Both changes in mitochondrial calcium buffering and dynamics, including ROS, ATP and proton leak, have previously been shown to be linked to MV formation (Mackenzie

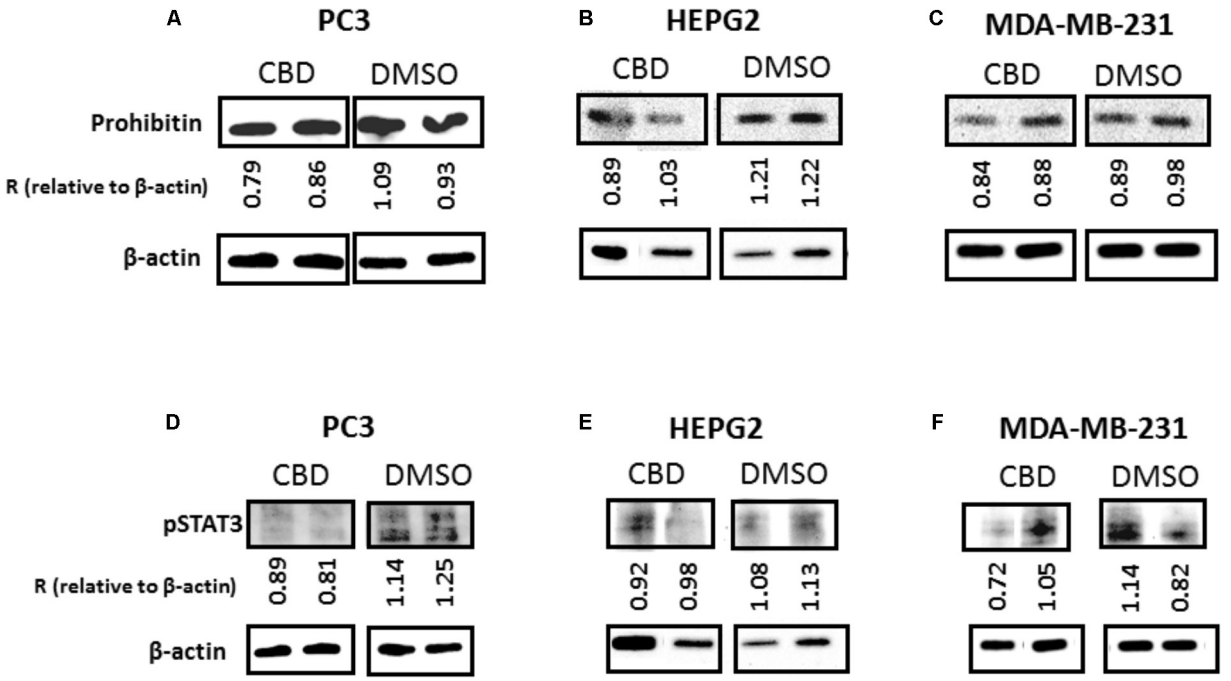


FIGURE 8 | CBD modulates expression of prohibitin and STAT3 in cancer cells. PC3, HEPG2, and MDA-MB-231 cells were tested for changes in mitochondrial associated prohibitin and STAT3 expression following 1 h treatment with CBD (5 μM). Levels of prohibitin were reduced in all three cancer cell lines while this reduction was more marked in PC3 **(A)** and HEPG2 **(B)** cells compared to MDA-MB-231 **(C)**. STAT3 (phospho Y705) was also reduced after 1 h CBD (5 μM) in both PC3 **(D)** and HEPG2 cells **(E)**, while MDA-MB-231 cells showed a similar, albeit less marked trend **(F)**. Beta-actin is shown as an internal loading control and "R" indicates the change of prohibitin and pSTAT3 expression relative to β-actin levels, respectively, for comparison between CBD treatment and DMSO control.

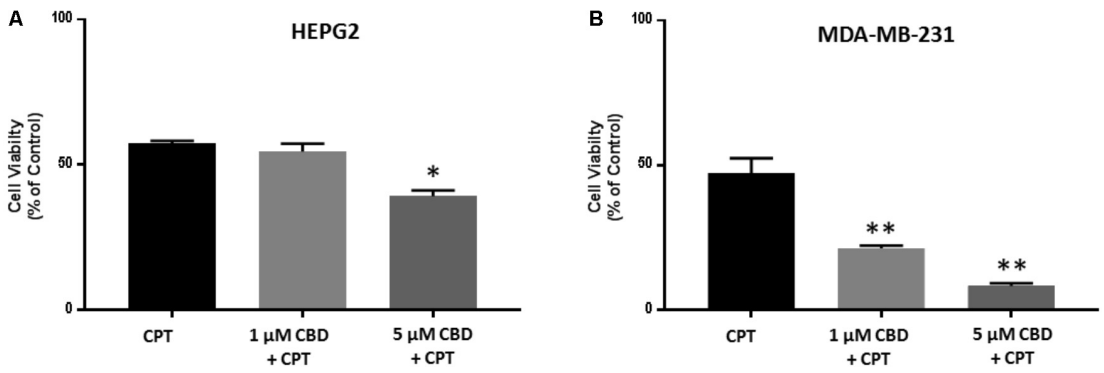


FIGURE 9 | CBD sensitizes HEPG2 and MD-MB-231 cancer cells to cisplatin-mediated apoptosis. HEPG2 **(A)** and MDA-MB-231 **(B)** cells were treated with 1 or 5 μM CBD for 24 h prior to further 24 h incubation with cisplatin (CSP, 100 μM). Cell viability was assessed by MTT assay. Data shown is repeated three times with three technical replicates per plate. Data is represented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ versus untreated control cells.

et al., 2005) and to affect ATP-mediated release of MVs and exosomes (Qu et al., 2007; Dubyak, 2012). Mitochondrial stress can also lead to MV formation via pro-apoptotic Bax and Bak, which insert into the mitochondrial outer membrane resulting in its depolarisation and increased membrane permeability. This leads to ROS, cytochrome C (Cyt C) and apoptosis inducing factor (AIF) leakage into the cytoplasm and eventual apoptosis. Where apoptosis is triggered by the extrinsic pathway, such as stimulation of FasL, activation of caspase 8 leads to cleavage of Bid, tBid then translocating to the mitochondrial membrane, mediating Cyt C release; this causes cytoskeletal degradation and formation of the apoptosome (Dale and Friese, 2006; Lopez et al., 2008; Inal et al., 2012). In scenarios of minimal damage the cell can use the apoptosome to form a MV and export the hazardous agents via pseudoapoptosis (Inal et al., 2012). Furthermore, pseudoapoptosis has been shown to involve rapid reversible mitochondrial depolarization, mitochondrial swelling and changes in mitochondrial and cytosolic calcium (Mackenzie et al., 2005). In cancer cells, a previous study has for example shown a ten-fold increase in the release of a 333–385 nm MV

subset in pseudoapoptotic response to sublytic C5b-9 stimulation (Stratton et al., 2015a). Mitochondrial permeability has been shown to be important also for MV shedding from platelets, where the natural phenol and Bax activator gossypol promoted mitochondrial depolarization, PS exposure and MV release (Dale and Friese, 2006). As it is now thought that many oncogenes and tumor suppressors control calcium flow into the mitochondrion, one key emerging target in cancer treatment is mitochondrial control of calcium signaling (Danese et al., 2017). Previously, effects of CBD on modulating mitochondrial calcium buffering and mitochondrial function have been described (Ryan et al., 2009; Mato et al., 2010; De Petrocellis et al., 2011; Shrivastava et al., 2011; Rimmerman et al., 2013; Fisar et al., 2014; Cui et al., 2017), including on mitochondrial swelling, ROS production and mitochondrial potential (Mato et al., 2010). STAT3 is indeed implicated in mitochondrial calcium control (Yang et al., 2015; Garama et al., 2016; Yang and Rincon, 2016) and the reduction in STAT3 in cancer cells observed here following CBD treatment may thus have modulatory effects on EMV release. A reduction of STAT3 by CBD has previously been shown in glioblastoma cells where it was for example related to the inhibition of self-renewal (Singer et al., 2015). Prohibitin is ubiquitously expressed in many cell types and involved amongst other in energy metabolism, proliferation and apoptosis (Peng et al., 2015; Ande et al., 2017). It acts as a scaffold protein in the inner mitochondrial membrane and is thus important for the regulation of mitochondrial architecture (Merkwirth et al., 2012). Prohibitin is critical for mitochondrial house-keeping including mitochondrial dynamics, morphology and biogenesis as well as stabilizing the mitochondrial genome (Peng et al., 2015). Here we show, for the first time, that prohibitin is reduced in cancer cells following CBD treatment. The slight variability in reduction of prohibitin in response to CBD between the cancer cell lines tested here tallies in with the observed differences in effectivity of CBD to inhibit EMVs from these different cancer cells. A similar correlation was found between expression changes in STAT3 and inhibition of EMV release following CBD treatment, as both PC3 and HEPG2 cells showed more reduction in STAT3 levels following CBD treatment, alongside a more pronounced inhibitory effect on total EMV release, compared to MDA-MB-231 cells; which, while showing overall significant reduction in EMVs and reduced levels of STAT3 and prohibitin, these effects were somewhat less marked than in the other two cancer types. The EMV modulatory effects of CBD could thus be partly mediated by the above observed mitochondrial changes. In addition, CBD has also been shown for example to stimulate mitochondrial uptake of calcium, followed by a decrease and a matching sudden increase in intracellular calcium (Ryan et al., 2009), indicating thus also putative dynamic effects on EMV release. Notably, in PC3 cells, a CBD-dose-dependent trend was observed for reduced ATP production, which correlated with the overall reduction observed in total EMVs, exosomes and MVs in response to CBD treatment, compared to DMSO treated control cells. Furthermore, prohibitin has previously been shown to protect cancer cells from ER stress and chemotherapy-induced cell death (Cheng et al., 2014; Tortelli et al., 2017). Prohibitin accumulation in mitochondria and *de novo* accumulation has been shown to cause chemoresistance, while knock-down of prohibitin sensitized cancer cells to chemotherapeutic treatment (Tortelli et al., 2017). Inhibition of prohibitin has also been shown to repress cancer cell malignancy progression in hypoxia (Cheng et al., 2014). The observed reduction in prohibitin observed here, following CBD treatment, may thus be an important factor in contributing to the sensitisation of cancer cells to chemotherapeutic agents, as previously shown for CBD in glioblastoma (Torres et al., 2011), in addition to affecting EMV release due to changes in mitochondrial function caused partly by prohibitin and STAT3 downregulation following CBD exposure.

Using a combined application of CBD (5 μM) with Cl-amidine resulted in different effects on the various EMV subsets and varied between the three cancer cell lines. In general, Cl-amidine did not have additive effects on the inhibitory effect of EMV release compared to CBD alone, while the combinatory treatment was more effective on some subsets than Cl-amidine alone, as was observed on exosome release in PC3 cells and on MV release in HEPG2 cells. However, the difference between cancer cell types to combinatory treatment did not significantly affect the larger MV subset in PC3 cells, while both CBD and Cl-amidine alone did. Similarly, combinatory treatment did not show more effect than CBD or Cl-amidine alone on exosome release from HEPG2 cells. Interestingly, in MDA-MB-231 cells, Cl-amidine counteracted the increased CBD-mediated release observed for the larger MV subset (201–500 nm), when used in combination, bringing the amount of vesicles release down to similar levels as for the control treated cells. Overall our results suggest that the two EMV inhibitors act on different pathways involved in MV and exosome release. While previously, Cl-amidine has been shown to act on MV biogenesis via increased cytoskeletal actin deimination and nuclear PAD translocation, indicative for changes in histone deimination (Kholia et al., 2015), CBD may act in part through modulation of mitochondrial metabolism as described here. Accordingly, and depending on which EMV subset is being targeted, our results indicate that tailored approaches for selective EMV inhibition could be developed for various EMV mediated pathologies. The expanded repertoire of EMV inhibiting agents, including CBD now revealed here, along with its sensitizing effects on cancer cells to cisplatin-mediated apoptosis, indicates a therapeutic potential for sensitisation of cancer cells to chemotherapy, as has been demonstrated for other promising EMV inhibitors (Tang et al., 2012; Federici et al., 2014; Jorfi et al., 2015; Koch et al., 2016; Muralidharan-Chari et al., 2016; Kosgodage et al., 2017). Importantly, such EMV-modulating agents could be used to allow for lower dose of chemotherapeutic drug for effective inhibition of tumor growth *in vivo* (Jorfi et al., 2015; Muralidharan-Chari et al., 2016). The ability of CBD to inhibit EMV release may indeed be a hitherto overlooked contributing factor in the beneficial effects of CBD observed in cancer therapy, where the exact mechanisms still remain to be unraveled (Torres et al., 2011; Ramer et al., 2012, 2014; Massi et al., 2013; Vara et al., 2013; Haustein et al., 2014; Velasco et al., 2016; Pisanti et al., 2017), as for example in glioma models, where CBD has been shown to enhance effects of temozolomide (Torres et al., 2011). Modulating EMV release may thus be an important therapeutic approach, also to prevent

metastasis, where tumor derived exosomes have been shown to be involved in preparation of the pre-metastatic niche (Hoshino et al., 2015).

CONCLUSION

A new mode of action for CBD in cancer, via modulation of EMV release, is revealed here for the first time. The findings presented in this study serve as a first proof of principle for CBD-mediated inhibition and modulation of EMV biogenesis, and shows cancer-type and dose specific effects. As CBD modulation of mitochondrial functions is well established, the effects observed here on changes in EMV release, mitochondrial function and mitochondrial associated proteins, alongside sensitisation of cancer cells to cisplatin mediated apoptosis, provide a platform for further research on detailed mechanistic pathways of CBD's mode of action on EMV biogenesis and cellular communication. Furthermore, this work opens up wide ranging research into novel therapeutic avenues in EMV-mediated pathologies.

AUTHOR CONTRIBUTIONS

UK, RM, AH, and SL carried out the experiments. AN, GG, ET, JI, JB, and SL contributed to experimental design and data analysis. SL and AN wrote the manuscript. All authors contributed equally to the critical reviewing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2018.00889/full#supplementary-material

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BRIEF RESEARCH REPORT
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Self-Reported Effectiveness and Safety of Trokie® Lozenges: A Standardized Formulation for the Buccal Delivery of Cannabis Extracts

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Therapeutic use of cannabinoids, the main active ingredients of *Cannabis sativa* L., is often hindered by their limited bioavailability and undesirable psychoactivity. We conducted an observational study in December 2016 and another one in February 2018 to investigate respectively: (i) the effectiveness of Trokie® lozenges, a standardized formulation containing cannabis extracts, to deliver cannabinoids via buccal absorption and (ii) its long-term safety. Participants were members of the Palliative Care Corporation health clinic, registered California cannabis patients, and had a diagnosis of chronic non-cancer pain. For the effectiveness study, 49 participants were asked to self-report pain perception before and after 1–12 weeks of taking Trokie® lozenges, using an 11-point pain intensity numeric rating scale (PI-NRS). A mean reduction in PI-NRS score of 4.9 ± 2.0 points was observed. Onset of analgesia typically varied between 5 and 40 min, which seems consistent with, at least partial, buccal absorption. In the safety study, 35 participants were asked to complete a questionnaire about adverse events (AEs) associated with Trokie® lozenges. AEs were reported by 16 subjects (46%), the most common being dizziness/unsteadiness (N = 7), bad taste (N = 5), and throat irritation/dry mouth (N = 4). None of the self-reported AEs resulted in a serious medical situation and most of them had limited impact on daily functions. Despite the AEs, 90% of participants reported being "satisfied" or "very satisfied" with the product. These observations suggest that buccal administration of standardized extracts via Trokie® lozenges may represent an efficacious and safe approach to cannabis administration.

Keywords: cannabis, effectiveness, safety, adverse events, trokie® lozenges, standardized, buccal administration, polyethylene glycol

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INTRODUCTION

In recent years, many countries have passed legislation permitting the use of cannabis for medical reasons. This has given patients access to both herbal material and cannabis-based products, bypassing the strict regulatory procedures that usually apply to pharmaceutical development (Fitzcharles and Eisenberg, 2018). Further, legalization of cannabis for recreational purposes

Abbreviations: AEs, Adverse events; CBD, Cannabidiol; IRB, institutional review board; PCC, Palliative Care Corporation; PEG, polyethylene glycol; PI-NRS, Pain intensity numeric rating scale; THC, Δ^9-tetrahydrocannabinol.

in several states of the Union as well as in internationally-relevant countries, such as Canada, conferrers cannabis an intriguing dual status, both as a pharmaceutical substance and a mere commodity, thus creating a complex regulatory scenario. Manufacturers of cannabis products, irrespective of their purpose, are met alike with the limitations that the lipophilic nature of cannabinoids, the main active substances in *Cannabis sativa* L., poses to their absorption and distribution in the human body (Huestis, 2005). Moreover, the psychoactive effects induced by Δ^9-tetrahydrocannabinol (THC), the primary active ingredient present in cannabis, need to be: (i) minimized for patients to maximize the therapeutic index of cannabis medications, and (ii) carefully controlled for adult consumers in order to reduce both the risk of acute intoxication and the impact on public health and safety. Economic reasons also play a role since increasing the bioavailability of cannabinoids could allow for a reduction in the amount of active ingredient per dose, thus lowering the cost of the final product. Current efforts to achieve efficient, reliable dosing aim at exploring different routes of administration (e.g., pulmonary, oral, mucosal, transdermal) and delivery vectors (e.g., inhaling powders, nanovehicles) to optimize the pharmacokinetic profile of cannabinoids (van Drooge et al., 2005; Conte et al., 2017). Given the current lack of regulation in the medical and adult markets, cannabis-based products are readily available for the general population to acquire and consume without medical supervision. For some researchers, this represents a waste of valuable clinical information that could be extremely useful if adequately collected.

Sativex® (USAN: nabiximols), an ethanol-based oromucosal preparation with 2.7 mg of THC and 2.5 mg cannabidiol (CBD) per spray, was the first cannabis product to attain regulatory approval as a pharmaceutical in 29 countries, having met the required standards of safety, efficacy and consistency (MacCallum and Russo, 2018). Nevertheless, it presents some adverse events (AEs) associated either with the pharmacology of cannabinoids (e.g., dizziness, drowsiness, dry mouth) or the detrimental effects of alcohol upon the oral mucosa (Scully, 2007). An alternative to ethanol could be muco-inert polymeric coatings such as polyethylene glycol (PEG), a hydrophilic, non-ionic, biocompatible polymer considered the gold standard in engineering mucus-penetrating surfaces. PEG grafting reduces adhesion to mucin fibers, allowing nanoparticles to quickly diffuse through the interstitial fluids enabling sustained mucosal drug delivery (Huckaby and Lai, 2018). Here, we have assessed the ability of a standardized cannabis formulation containing PEG (Trokie® lozenges) to deliver cannabinoids via the buccal/oral mucosa as well as associated AEs.

MATERIALS AND METHODS

Research Population

We conducted two observational studies with members of the Palliative Care Corporation (PCC), a health clinic in Huntington Beach, CA, which distributes Trokie® lozenges. Admission criteria for the health clinic include: (i) willingness to incorporate cannabis into current medication regime; (ii) not having known allergies to cannabinoid drugs (dronabinol, nabilone, nabiximols); (iii) not having a cardiac arrhythmia; and (iv) not being pregnant. At the time of enrollment, members were asked to rate their pain on a pain intensity numeric rating scale (PI-NRS). This study was carried out in accordance with the principles of the Declaration of Helsinki and followed the relevant institutional and national guidelines. The study met the criteria set forth in 45 CFR §46.101(b) (4) and was therefore exempted from institutional review board (IRB) approval by Quorum Review IRB (Seattle, WA, United States).

Cannabis Self-Administration Protocol

New members joining PCC, especially if cannabis-naive (which represent approximately 60%), were instructed on how to properly self-administer Trokie® lozenges, embracing the "start low, go slow" motto proposed by clinicians for the use of herbal cannabis (MacCallum and Russo, 2018), starting with 1/4th of a 50 mg CBD lozenge (12.5 mg) two or three times a day for 3 days. After this initial period, members are coached by PCC clinical personnel on how to slowly adjust their intake of THC and CBD to achieve optimal symptom relief, with follow-up calls on days 4, 7, 14, and each time they place a new order, under the guiding principle of "primum non nocere" (Fitzcharles and Eisenberg, 2018).

Preparation of Trokie® Lozenges

The following 4-dose lozenges were available to members at PCC: 50 mg (only CBD), 40 mg (1:1 ratio CBD:THC), 64 mg (1:15 ratio CBD:THC), 120 mg (1:15 ratio CBD:THC), and 120 mg (only THC). Trokie® lozenges were prepared as described in U.S. Patent No. 62/018,484 (Crowley, 2017). After preparation, batch samples were sent to a third-party laboratory for analysis (CannaSafe Analytics, Murrieta, CA Unites States). Batches were quarantined until laboratory results allowed release for packaging and labeling.

Study 1: Effectiveness of the Delivery System

On December 2016, members of the PCC with a diagnosis of chronic non-cancer pain who had been enrolled for at least 12 weeks were invited to participate in a phone interview conducted by KC. The time frame (1–12 weeks) was selected to maximize the number of members actively enrolled in the clinic at the time of the study. Participants were asked: (i) to score their current pain on a PI-NRS from 0 to 10 (Farrar et al., 2001), (ii) answer a global self-assessment of feeling better, same or worse, and (iii) to estimate the onset of analgesia after taking Trokie® lozenges. Participants using opiate medication were questioned about any modifications on their intake from the time of enrollment.

Study 2: Assessment of Adverse Events

To assess the safety of Trokie® lozenges, we conducted a survey on February 2018 among PCC members with chronic non-cancer pain that had been enrolled for a sustained period of time, between 4 and 60 weeks. This time frame was selected to capture

AEs associated to mid- to long-term use of Trokie® lozenges. Participants were asked to complete a questionnaire to report adverse drug reactions (de Vries et al., 2013), slightly modified to focus on AEs caused by Trokie® lozenges.

Analyses

Pain intensity was calculated as the difference between PI-NRS values reported on December 2016 and those reported at enrollment, and it is expressed as mean ± standard deviation of the mean. Significance of differences was determined using a paired *t*-test (PI-NRS before vs. PI-NRS after). Differences were considered significant if $P < 0.05$. Statistical analyses were conducted using GraphPad Prism Version 7.0 (San Diego, CA, United States). Patient-reported AEs were analyzed descriptively using IBM SPSS Statistics version 23 (Armonk, New York, NY, United States).

RESULTS

Study 1: Effectiveness of the Delivery System

A total of 49 participants (15 males/34 females) with an average age of 59.9 years completed the study (descriptive statistics in **Supplementary Table 1**). An average reduction in PI-NRS score of 4.9 ± 2.0 points (from 7.4 ± 1.3 to 2.4 ± 1.8) was observed (**Figure 1A**). Also, among 31 participants using opiates, 26 (84%) voluntarily reduced or discontinued their use of opiate

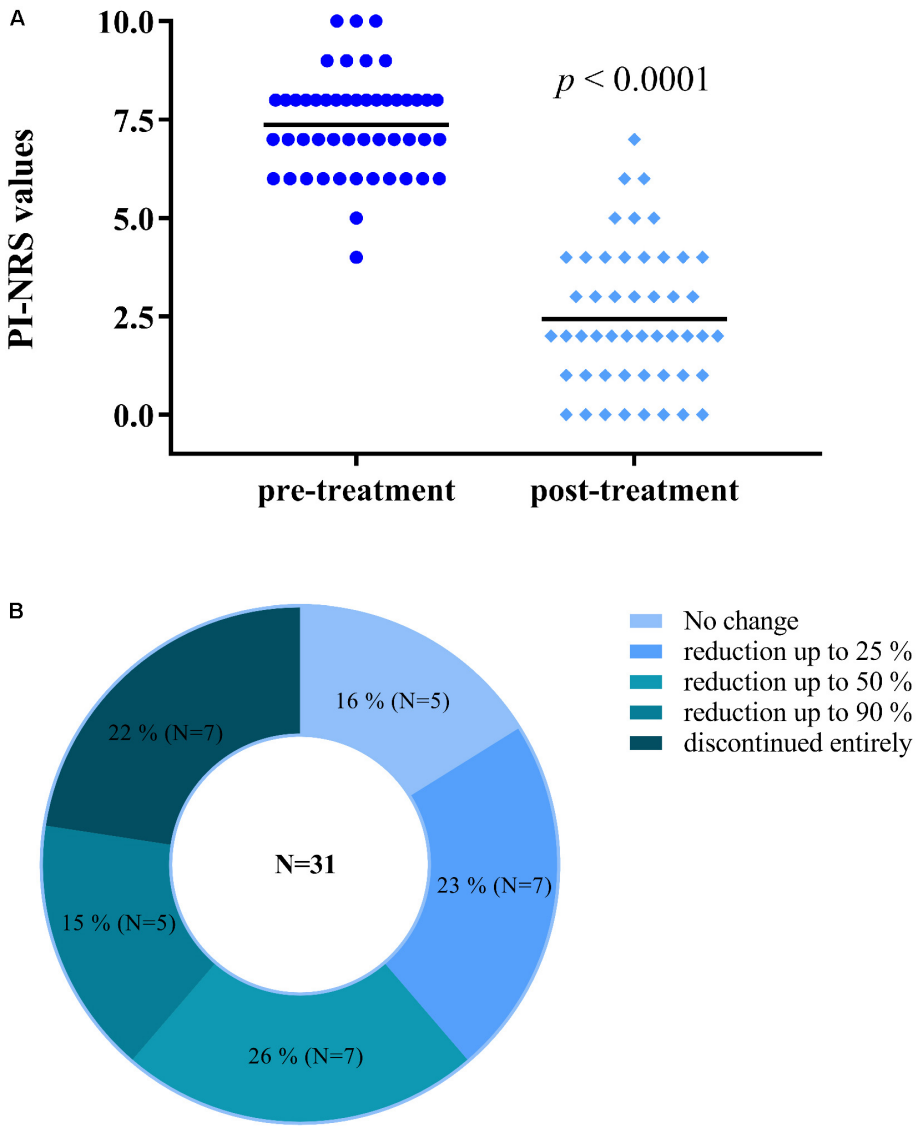


FIGURE 1 | Participants using Trokie® lozenges reported a mean reduction in pain intensity of 4.9 ± 2.0 points **(A)**, from a median value of 7.4 (pre-treatment, dark blue circles) to 2.4 (post-treatment, light blue diamonds) on an 11-point scale (0 = no pain, 10 = worst pain imaginable). Among 31 participants using opiates, 26 (84%) reduced in some degree or completely discontinued their use of opiate medication **(B)**.

medication (**Figure 1B**). These reductions were completed by participants with no reported symptoms of opiate withdrawal. Lastly, all participants reported feeling an improvement in their condition (100%) and an onset of analgesia between 5 and 40 min.

Study 2: Assessment of Adverse Events

The adverse drug reaction questionnaire was completed by a total of 35 participants (7 males/28 females) with an average age of 65.3 years (descriptive statistics in **Supplementary Table 1**). Twenty AEs were reported by 16 participants (46%), with most reporting one single AE (N = 12; 75%) and four reporting two AEs. Descriptions given by participants corresponded to 7 AEs (with some of them relating to more than one AE) which were: dizziness/unsteadiness (N = 7), bad taste (5), throat irritation/dry mouth (N = 4), drowsiness/fatigue (N = 3), impaired consciousness/high feeling (N = 2), nausea (N = 1), and palpitations (N = 1). There were no reports of damage to the oral mucosa, gums, or teeth. None of the self-reported AEs resulted in a serious medical situation and most of them had only limited impact on daily functioning. Of notice, AEs associated with CBD-only Trokie® lozenges were not related to any psychoactivity and were restricted to bad taste (4) and throat irritation/dry mouth (2). The remainder of AEs were associated with THC-containing lozenges, alone or in combination with opiates or other THC-containing products. Despite these AEs, 90% of participants reported being "satisfied" or "very satisfied" with the product (**Table 1**).

TABLE 1 | Nature of the reported adverse events (AEs).

	N (%)
First time experiencing the AE	
Today	0 (0)
Yesterday	0 (0)
2–7 days ago	0 (0)
Between 1 week and 1 month ago	0 (0)
Between 1 and 6 months ago	1 (5)
Between 6 and 12 months ago	3 (15)
More than 12 months ago	16 (80)
How much bothersome	
Not at all	0 (0)
Only a bit	7 (35)
Somewhat	6 (30)
Quite a lot	6 (30)
Very much	1 (5)
Influence daily functioning	
None	10 (50)
Only a bit	4 (20)
Somewhat	2 (10)
Quite a lot	3 (15)
Very much	1 (5)
Drug causing the AE	
One drug, namely:	18 (90)
Trokie® lozenges CBD	6
Trokie® lozenges CBD and THC	5
Trokie® lozenges THC	7
More than one drug	2 (10)
Trokie® lozenges THC + Opiates	1
Trokie® lozenges THC + THC capsules	1
Participants' certainty about the relationship between the reported AE and Trokie®	
Very sure	18 (90)
Quite sure	2 (10)
Not very sure	0 (0)
Very unsure	0 (0)
Satisfaction with Trokie® lozenges	
Very satisfied	9 (45)
Satisfied	9 (45)
Neither satisfied or dissatisfied	0 (0)
Dissatisfied	1 (5)
Very dissatisfied	1 (5)

DISCUSSION

Trokie® lozenge is a cannabis-based product currently available in six states of the Union (Arizona, California, Florida, Iowa, Nevada, and Minnesota) and Puerto Rico. Two observational studies were conducted with California-certified cannabis patients to assess the effectiveness and safety of Trokie® lozenges. First, we focused on the effectiveness of Trokie®lozenges to deliver cannabinoids, namely THC and CBD, through the buccal mucosa: *does it work in practice?* (Haynes, 1999). Our findings indicate that the use of Trokie® lozenges is associated with a self-reported pain reduction in chronic, non-cancer pain patients, a condition for which the efficacy of cannabis has been previously described (Lynch and Ware, 2015). Of note, reported time to onset was between 5 and 40 min which, considering lozenges take 20–25 min to dissolve, seems consistent with, at least partial, buccal absorption (Karschner et al., 2011). However, it is reasonable to assume that it can also be swallowed with saliva. Correct placement in the mouth appeared to be critical for minimizing saliva production. Next, we aimed at assessing the safety of Trokie® lozenges and the kind of AEs that could be associated with its long-term use: *is it safe to use?* Seven different AEs were reported by participants, one of them related to the organoleptic qualities of the product (bad taste) and the rest being common to other cannabis products containing THC, such as dizziness or dry mouth, none of which resulted in a serious medical situation and only had limited impact on daily functioning.

Interestingly, our results are in strong agreement with a recent study performed in Israel on a large cohort of elderly (over 900 participants, 74.5 ± 7.5 years) reporting a reduction of pain levels from a median of 8 to a median of 4 on a scale of 0–10 after 6 months of cannabis treatment. Further, most common AEs were dizziness (10%), dry mouth (7%), and 18% of participants stopped using opiate analgesics or reduced their dose (Abuhasira et al., 2018). In our case, the proportion of participants reducing or discontinuing opiate analgesics was significantly larger (84%), similar to what has been previously found in a study based on patient self-reports (Reiman et al., 2017). This may be explained

by the extensive use of opiate medication in the United States and the fact that many PCC members are seeking cannabis treatment because they "want to get off opiates and don't want to get high" (personal communications with PCC staff).

Legalization of medical and recreational cannabis has bypassed the usual drug regulatory procedures in jurisdictions worldwide. Pending sound evidence for its effects in many conditions, physicians face the challenge of continuing to provide competent, compassionate care with an emphasis in harm reduction. Nevertheless, this regulatory scenario creates unprecedented opportunities to study the clinical impact of cannabinoids in human health and behavior. An illustrative example of how commodities can revolutionize the way we perform biomedical research are consumer physical activity monitors, which have raised enormous interest to physiology and psychopathology research because of their ability to measure activity continuously under real-life conditions and because they are already widely used by consumers (Wright et al., 2017). We believe the present results provide valuable information in terms of route validation, dosage selection and expected AEs, while cognizant of the severe limitations of our design due to the nature of the research, such as biased participant selection, lack of blinding or absence of placebo control. Therefore, these results should not be interpreted to establish any causality between the use of Trokie® lozenges and the improvement in participants well-being. However, the findings support the need for conducting a phase 1 clinical trial to formally characterize the pharmacokinetic profile of Trokie® lozenges in humans.

AUTHOR CONTRIBUTIONS

KC and GM-S designed the studies. KC coordinated the field research and conducted interviews. GM-S and SdV analyzed the data and generated results of the effectiveness and safety studies, respectively. GM-S wrote the manuscript with the aid of KC and SdV.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2018.00564/full#supplementary-material

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Conflict of Interest Statement: KC is an inventor on U.S. Patent No. 62/018,484, describing buccal and sublingual cannabinoid formulations and method of making the same, which are the basis for the preparation of Trokie® lozenges.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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The Endocannabinoid/Cannabinoid Receptor 2 System Protects Against Cisplatin-Induced Hearing Loss

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Previous studies have demonstrated the presence of cannabinoid 2 receptor (CB2R) in the rat cochlea which was induced by cisplatin. In an organ of Corti-derived cell culture model, it was also shown that an agonist of the CB2R protected these cells against cisplatin-induced apoptosis. In the current study, we determined the distribution of CB2R in the mouse and rat cochleae and examined whether these receptors provide protection against cisplatin-induced hearing loss. In a knock-in mouse model expressing the CB2R tagged with green fluorescent protein, we show distribution of CB2R in the organ of Corti, stria vascularis, spiral ligament and spiral ganglion cells. A similar distribution of CB2R was observed in the rat cochlea using a polyclonal antibody against CB2R. *Trans*-tympanic administration of (2-methyl-1-propyl-1H-indol-3-yl)-1-naphthalenylmethanone (JWH015), a selective agonist of the CB2R, protected against cisplatin-induced hearing loss which was reversed by blockade of this receptor with 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone (AM630), an antagonist of CB2R. JWH015 also reduced the loss of outer hair cells (OHCs) in the organ of Corti, loss of inner hair cell (IHC) ribbon synapses and loss of Na^+/K^+-ATPase immunoreactivity in the stria vascularis. Administration of AM630 alone produced significant hearing loss (measured by auditory brainstem responses) which was not associated with loss of OHCs, but led to reductions in the levels of IHC ribbon synapses and strial Na^+/K^+-ATPase immunoreactivity. Furthermore, knock-down of CB2R by *trans*-tympanic administration of siRNA sensitized the cochlea to cisplatin-induced hearing loss at the low and middle frequencies. Hearing loss induced by cisplatin and AM630 in the rat was associated with increased expression of genes for oxidative stress and inflammatory proteins in the rat cochlea. *In vitro* studies indicate that JWH015 did not alter cisplatin-induced killing of cancer cells suggesting this agent could be safely used during cisplatin chemotherapy. These data unmask a protective role of the cochlear endocannabinoid/CB2R system which appears tonically active under normal conditions to preserve normal hearing. However, an exogenous agonist is needed to boost the activity of endocannabinoid/CB2R system for protection against a more traumatic cochlear insult, as observed with cisplatin administration.

Keywords: endocannabinoids, CB2 receptor, cisplatin, hearing loss, ribbon synapse

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INTRODUCTION

Cisplatin is one of the main chemotherapeutic agents used to treat different solid tumors including head and neck cancer, testicular and ovarian cancer. However, cisplatin chemotherapy results in dose-limiting toxicity to the cochlea, kidney, and peripheral neurons. Hearing loss derives from the generation of significant oxidative stress in the cochlea which results in part from the activation and induction of a cochlear-specific NADPH oxidase isoform, NOX3 (Banfi et al., 2004). The oxidative stress coupled to the subsequent inflammatory processes and DNA damage occurring in the cochlea promotes apoptosis and necrosis of the sensory outer hair cells (OHCs) at the basal and middle regions of cochlea (Estrem et al., 1981; Laurell and Bagger-Sjoback, 1991; van den Berg et al., 2006; Rybak et al., 2007), damage to the stria vascularis (SV) (Meech et al., 1998) and loss of spiral ganglion neurons (SG) (Zheng et al., 1995; van Ruijven et al., 2005). Currently, there are no drugs which are currently approved by the US FDA for treating cisplatin-mediated hearing loss (Rybak et al., 2009). We believe that a better understanding of the mechanisms involved in ototoxicity and endogenous protective signaling pathways could stimulate the development of effective and novel otoprotective drugs.

Cannabinoid (CB) receptors include cannabinoid receptor 1 (CB1R), cannabinoid receptor 2 (CB2R), non-CB1/CB2Rs, such as transient potential vanilloid 1 receptor (TRPV1), and G protein-coupled receptor 55 (GPR55) (Console-Bram et al., 2012). High levels of CB1R are expressed in the central nervous system (CNS), while CB2R are predominantly expressed in immune cells of the body including macrophages, leucocytes, dendritic cells and natural killer cells (Console-Bram et al., 2012). CB2R regulate the release of pro-inflammatory cytokines such as interleukins (IL-1β, Il-6), tumor necrosis factor-α (TNF-α), cyclooxygenase-2 (COX-2) and chemokines (CXCL8, CCL2) from leucocytes and the differentiation of B and T cells (Klein, 2005). CBs also suppress the activity of helper T cells (Th1), increase the activity of Th2 cells (Klein, 2005) and reduce leucocyte recruitment to the site of inflammation (Klein, 2005; Pacher et al., 2006). Activation of CB2R regulates the cell growth and proliferation of immune cells and suppresses macrophage growth and phagocytosis (Ashton and Glass, 2007). Activation of endogenous cannabinoid receptors protects against inflammatory diseases such as colonic inflammation in colitis (Massa et al., 2004), rheumatoid arthritis (Zurier et al., 2003) and autoimmune diabetes (Weiss et al., 2008). Endogenous ligands for CB receptors, termed endocannabinoids, comprise anandamide and 2-arachidonoyl glycerol (2-AG).

Previous studies have documented expression of CB2R in immortalized House Ear Institute - Organ of Corti cell line (HEI-OC1) which protected against cisplatin-induced cell death (Jeong et al., 2007). A recent report showed that CB2Rs are expressed in the rat cochlea and are induced by cisplatin treatment (Martin-Saldana et al., 2016). However, no functional role of these receptors was demonstrated. In other disease models, upregulation of CB2R has been shown to mediate an "autoprotective" role (Pertwee, 2009). Increased expression of CB2R was observed in neuropathic pain models (Zhang et al., 2003) and in rat sensory neurons during peripheral nerve injury (Wotherspoon et al., 2005).

In the current study, we provide evidence that CB2R plays a central role in regulating normal hearing, as inhibition or knockdown of this receptor results in hearing loss. Furthermore, *trans*-tympanic application of an agonist of CB2R protected against cisplatin-induced hearing loss. These findings support the conclusion that drugs targeting the CB2R could boost the endocannabinoid system and play an important role in protecting against hearing loss.

MATERIALS AND METHODS

Drugs and Reagents

CB2 agonist, (2-methyl-1-propyl-1H-indol-3-yl)-1-naphthalenylmethanone (JWH015), and antagonist, 6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone (AM630), were purchased from Tocris (#1341 and #1120, respectively). The different antibodies used for this project were purchased from different vendors and are as follows: CB2R (#Ab45942) and CB2R blocking peptide (#Ab45941) from Abcam, antibody for Tuj1 was obtained from Covance (#MMS-435P), while anti-CtBP2 was obtained from BD Biosciences (#612044) and Anti-GluR2 was obtained from Millipore (#MAB397). Antibody against Na^+/K^+-ATPase-α1 was obtained from Santa Cruz Biotechnology (#sc21712) while Alexa Fluor^{TM} 488 Phalloidin antibody was obtained from Thermo Fisher Scientific (#A12379). Two different myosin VIIa antibodies were used for different purposes: Mouse IgG1 anti-myosin-VIIa antibody was obtained from Developmental Studies Hybridoma Bank (#138-1), while rabbit anti-myosin-VIIa was purchased from Proteus Biosciences (#25-6790). Alexa Fluor 647 goat anti-rabbit antibody (#A21244), Alexa Fluor 488 goat anti-rabbit antibody (#A11008), Alexa Fluor 647 goat anti-mouse IgG1 antibody (#A21240), Alexa Fluor 568 goat anti-mouse IgG2a antibody (#A21134) and Alexa 568 goat anti-mouse IgG1 antibody (#A21124) were obtained from Life Technologies. Donkey anti-rabbit IRDye 680RD (#926-68073) and goat anti-mouse IRDye 800RD (#926-32214) were from LI-COR biosciences. Detailed description of the primary antibodies used is provided in the **Supplementary Table S1**. For culturing cells, RPMI1640 media was ordered from Gibco Life Technologies (#11875085).

Animal Procedures

All the animals were housed in the Division of Laboratory Animal Medicine (DLAM) facility of SIU School of Medicine and they were provided with easy access to commercial food and water. The rooms had controlled temperature and normal light/dark cycles (12 h light/12 h dark). All experiments performed on animals were approved and monitored by the Southern Illinois University School of Medicine Laboratory Animal Care and Use Committee.

Mice expressing the CB2R tagged with green fluorescent protein (GFP) were kindly provided by Dr. Cecilia J. Hillard (Department of Pharmacology and Toxicology, Medical College

of Wisconsin). The mice (5–6 weeks old) were anesthetized using isoflurane and sacrificed. Different organs including cochlea, spleen and cerebellum were isolated and fixed in 4% paraformaldehyde for 4–6 h in 4°C, followed by washing in 10 mM PBS and stored at 4°C until use.

Adult male Wistar rats (weight 200–250 g, 2–3 months old) were purchased from Envigo (Indianapolis, IN, United States). Rats were anesthetized with a mixture of ketamine (90 mg/kg) and xylazine (17 mg/kg) and the depth of anesthesia was determined by the absence of reflex to toe pinch which was performed routinely during the duration of the procedure. If the depth of anesthesis was determined to be insufficient, the rats were administered additional dose of the mixture. Auditory brainstem responses (ABRs) were performed on anesthetized rats in a radio frequency concealed sound chamber just prior to *trans*-tympanic JWH015 or AM630 treatments (for experimental group) or PBS of pH 7.2 (for control groups). Cisplatin (11 mg/kg) was administered via the intraperitoneal route and post-treatment ABRs were measured 72 h later. At this point, the animals were anesthetized using the same procedure mentioned before and decapitated. One cochlea from each animal was fixed using cochlear perfusion of 4% paraformaldehyde for immunohistochemistry studies and the other cochlea was snap-frozen for RNA extraction.

Auditory Brainstem Responses

Auditory evoked potentials were recorded as described previously (Sheehan et al., 2018). Briefly the stainless steel electrodes were positioned as follows: the ground electrode in the rear flank, the positive electrode between the two ears directly atop the skull, and the negative electrodes below the pinna of each ear. Acoustic stimuli were delivered through inserted earphones as a 5 ms tone burst at 8, 16, and 32 kHz and stimulus intensities were determined as decibel sound pressure level (dB SPL) which began at 10 dB SPL and reached ultimately 90 dB SPL with a 10 dB step size. Threshold was defined as the lowest sound intensity capable of evoking a reproducible, visually detectable response with two distinct waveforms with minimum amplitudes (II and III) of 0.5 μV. Threshold shift represents the difference in threshold measured after treatment compared with threshold obtained prior to treatment. Wave I was identified from the neural responses and wave I amplitudes were documented from the ABRs obtained from the different treatment groups.

Trans-Tympanic Administration of Drugs and siRNA

Rats were anesthetized with ketamine/xylazine mixture. A single *trans*-tympanic injection of the drug or siRNA was made in the anterior–inferior region of rat tympanic membrane using a 28–30 gauge needle (Sheehan et al., 2018). The position of the needle was visualized using a Zeiss operating microscope and 50 μl of solution was injected into the middle ear (siRNA was resuspended in 50 μl of sterile water to get the desired concentration). A similar procedure was performed 30 min later on the other ear.

siRNA Sequences

The rat CB2R siRNA was purchased from Dharmacon as an ON-TARGETplus SMARTpool siRNA which includes a combination of four siRNA sequences against CB2R RNA. The target sequence of CB2R siRNA included in this combination are as follows: 5′-GCUUGGAGUUCAACCCUAU-3′; 5′-CCAAUCAGCCUCCCUAAUA-3′; 5′-CUUGUUAACU CCAUGAUCA-3′ and 5′-UGACCGCUGUUGACCGAUA-3′. ON-TARGETplus Non-targeting Control siRNAs (scramble), also purchased from Dharmacon, was used as negative control.

Cochlear Whole-Mount Preparation

Isolated mice and rat cochleae were perfused with 4% paraformaldehyde and kept overnight at 4°C in the same solution for fixation. Cochleae were then decalcified in 100 mM EDTA (pH 7.3) with stirring at room temperature for 3 weeks for rat cochlea and 4 days for mouse cochlea, respectively. The decalcified cochleae were then microdissected into basal, middle and apical turns for immunolabeling.

Immunohistochemistry

Decalcified cochleae were dissected and whole mount sections were blocked, permeabilized in a mixture of 1% Triton X-100, 1% BSA and 10% horse or goat serum depending on the host species used for primary antibodies at room temperature for 1 h. They were then incubated with primary antibodies diluted in a solution containing 0.1% Triton X-100, 1% BSA and 10% horse or goat serum. All primary antibodies were incubated at 4°C overnight on a rotator. The cochleae were then washed three times and then placed in secondary antibodies, washed, mounted on slides and used for imaging with Zeiss LSM800 scanning confocal microscope. Mid-modiolar sections (10 μm thickness) of fixed and decalcified cochlea were obtained by using a cryostat, which were then dehydrated by incubating in 100% ethanol at −20°C for 20 min, followed by rehydration. These sections were stained as above. Fluorescence intensity of the staining was quantified using ImageJ software.

Hair Cell and Ribbon Synapse Count

The whole mount preparations of the cochlea samples were labeled with antibodies against myosin VIIa, CtBP2, and GluR2 for labeling of hair cells, presynaptic RIBEYE and post-synaptic glutamate receptors, respectively. The average hair cell counts in each region were determined by manually counting cells in the basal turn (100 μm length) of the cochlea. Normal counting yield was 39–42 OHCs per 100 μm. The average number of OHCs per three such regions taken at random from the base and averaged to give the mean OHC count per animal per treatment group. Functional ribbon synapses were defined as the number of complexes showing both CtBP2 + GluR2 immunolabeling apposing each IHC. At least 30 IHCs were used per sample to provide an estimate of functional synapses per rat cochlea. At least 3–4 animal cochleae were counted per group and used for statistical comparisons.

RNA Isolation and Real-Time RT-PCR

Total RNA was isolated from the cochlea as described previously (Mukherjea et al., 2011). The purity and concentration of RNA was determined by measuring the optical densities at 260, 280, and 230 nm by a nanodrop spectrophotometer. The purified RNA sample (260/230 ratios ranged from 1.8 to 2.0) were used for PCR. PCR was performed using 500 ng of total RNA for gene specific cDNA synthesis by using iScript Select cDNA Synthesis Kit (Bio-Rad). The procedure used for real-time RT-PCR was described previously (Mukherjea et al., 2011). The cycle threshold (*C*t) was determined by the number of cycles at which the samples reached threshold fluorescent intensity. Negative controls were set up for both the target and housekeeping gene, GAPDH, for all reaction groups and no template cDNA was added to this mixture. The relative change in mRNA levels between the vehicle and treated group was quantified by the following formula: $2^{\Delta\Delta C_t}$ (Soong et al., 2001). The nucleotide sequences of rodent primer sets were based on homologous sequences of rat and mouse cDNA sequence. The primers were purchased from Sigma Genosys (St. Louis, MO, United States) and the primer sets used are listed below:

Rodent-GAPDH (sense): 5′-ATGGTGAAGGTCGGTGTGAAC-3′ (antisense): 5-TGTAGTTGAGGTCAATGAAGG-3′ Rodent-TRPV1 (sense): 5′- CAAGGCTGTCTTCATCATCC-3′ (antisense): 5-AGTCCAGTTTACCTCG TCCA-3′ Rodent-NOX3 (sense): 5′ GTGAACAAGGGAAGGCTCAT-3′ (antisense): 5′-GACCCACAGAAGAACACGC-3′ Rodent-TNF-α (sense): 5′-CAGACCCTCACACTCAGATCA-3′ (antisense): 5′-TGAAGAGAACCTGGGAGTAGA-3′ Rodent-COX-2 (sense): 5′-TGATCGAAGACTACGTGCAAC-3′ (antisense): 5′-GTAC TCCTGGTCTTCAATGTT-3′ and Rodent-iNOS (sense): 5′-CATTCTACTACTACCAGATC-3′ (antisense): 5′-ATGT GCTTGTCACCACCAG-3′ and Rodent KIM-1 (sense): 5′-TTCAAGTCTTCATTTCAGGCC-3′(antisense): 5′-CTGCTC CGATAGGTGACTTGG-3′.

Cell Cultures

Immortalized mouse organ of Corti cells UB/OC-1 cells were kindly provided by Dr. Matthew Holley (Institute of Molecular Physiology, Western Bank, Sheffield, United Kingdom). Cells were cultured in RPMI-1640 media supplemented with 10% Fetalclone II serum (Hyclone), 5% penicillin–streptomycin (Invitrogen) and normocin (InvivoGen). Cultures were grown at 33°C in a humidified incubator with 10% CO_2. Head and neck cancer cell line [University of Michigan squamous carcinoma cells (UMSCC)10B], ovarian cancer cell (HeyA8) and colon cancer cells (HCT116 WT) were cultured in RPMI-1640 media supplemented with 10% FBS (Atlanta Biologicals) and 5% penicillin-streptomycin at 37°C and 5% CO_2. Confluent monolayer cells were used for experiments and cells were cultured thrice a week for passaging.

Plasmid Transfection in UB/OC-1 Cells

Wild type CB2 receptor expressing pcDNA3.1 plasmids were purchased from cDNA resource center. *E. coli* bacteria were transformed with this plasmid and the transformed colonies were selected by ampicillin resistance. DNA was isolated from the transformed bacteria by maxi-prep (Qiagen) and transfected into UB/OC1 cells by using Lipofectamine 3000 reagent (Invitrogen) following the vendor's protocol.

Immunocytochemistry

To detect the expression of CB2R in cells, UB/OC-1 cells were plated in 24 well dishes on coverslips in complete media. The confluent monolayer of cells was washed three times with ice-cold 1X PBS and fixed with 4% paraformaldehyde for 15–20 min at room temperature. The staining procedure was the same as mentioned above for immunohistochemistry. The slides were imaged by Zeiss LSM800 scanning confocal microscope.

MTS Assay for Cell Viability

In vitro cell viability of cancer cells following different drug treatments was measured by using CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (Promega). HeyA8 (2,000 cells per well), UMSCC10B and HCT116 WT (2,500 cells per well) cells were plated in 96 well plate. The cells were treated with JWH015 (10 μM) for 30 min followed by cisplatin (10 μM) for 48 h. At the end point, 20 μl of Cell Titer Aqueous One solution reagent was added to each well containing 100 μl media. The cells were incubated for at least 45 min in 33°C and checked for any color development and the plates were read at a wavelength of 490 nm by Fluoroskan Ascent™ FL Microplate Fluorometer plate reader. For each cell line, experiments were repeated independently at least three times and averages from independent repeats were used for statistical analyses. The percentage of cell viability was normalized against vehicle treated cells.

Statistical Analyses

The statistical significance differences were evaluated by using either student's *t*-test for two groups or Analysis of Variance (ANOVA) with Tukey's *post hoc* test for multiple treatment groups using Graph Pad Prism software 6.0.

RESULTS

CB2 Receptors Are Expressed in the Mouse and Rat Cochlea

CB2R immunolabeling in the rat cochlea has been reported previously using commercially available CB2R antibody (Martin-Saldana et al., 2016). However, the specificity of this antibody is controversial (Baek et al., 2013). We therefore validated the distribution of CB2R in the cochlea using a GFP-tagged CB2R conditional knock-in mouse model using a commercially available antibody. In the knock-in mice model, GFP was inserted within exon 3 of the CB2R, and the expression of GFP was driven by the endogenous CB2R promoter (**Figure 1A**). In mid-modiolar sections of cochleae obtained from these mice, we show endogenous GFP fluorescence in the organ of Corti (OC), spiral ganglion (SG) neurons, stria vascularis (SV), and spiral ligament (SL) (**Figure 1B**). We demonstrate the expression of GFP-CB2R in the three rows of OHC and inner hair cells (IHC) in mid-modiolar sections using co-immunolabeling of hair cells with

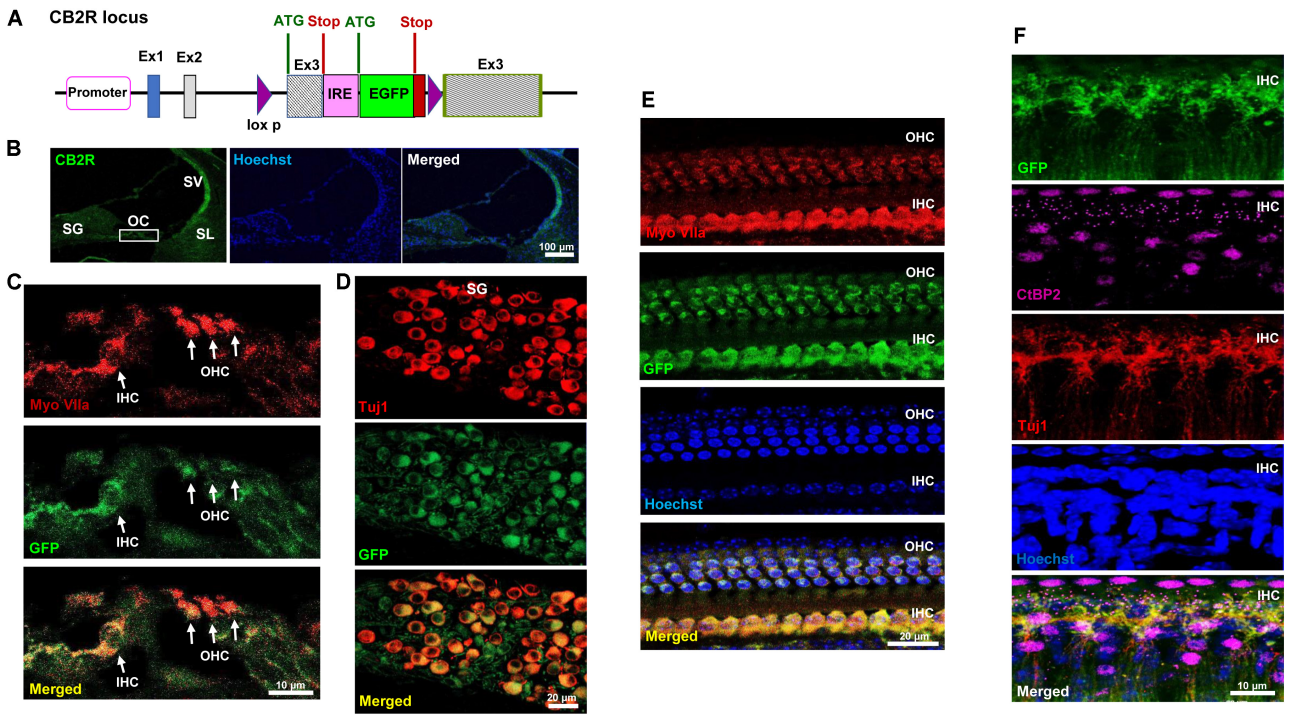


FIGURE 1 | Distribution of the GFP-tagged CB2R knock-in mice. **(A)** The genetic background of the knock-in mice is shown where the GFP reporter gene was inserted within exon 3 of CB2R and the expression of GFP was driven by internal ribosomal entry (IRE) promoter. Cochleae isolated from these GFP-CB2R co-expressing knock-in mice were fixed in 4% paraformaldehyde followed by decalcification and then sectioned. **(B)** The representative mid-modiolar section shows endogenous GFP fluorescence (green) in different anatomical regions of the cochlea including organ of Corti (OC), stria vascularis (SV), spiral ligament (SL), and spiral ganglion (SG) neuron. **(C)** Immunolabeling of GFP-tagged CB2R (green) along with hair cell marker, Myo VIIa (red), show colocalization of CB2R in both, the OHCs and IHCs. **(D)** Co-labeling of these cochleae with Tuj1 (red), a neuronal marker, show the distribution of GFP-CB2R (green) in spiral ganglion neurons. **(E)** Whole mount sections of cochleae from GFP-CB2R co-expressing knock-in mice show co-localization of GFP-CB2R (green) and myosin VIIa (red) in the OHCs and IHCs of OC. **(F)** GFP-CB2R whole mounts sections were also co-stained with neuronal marker, Tuj1 (red), and pre-synaptic ribbon marker, CtBP2 (magenta), showing pre-synaptic localization of CB2R. Cell nuclei (blue) are stained with Hoechst stain in **(B,E,F)**. Studies reported here were repeated in at least three different animals and similar results were obtained.

myosin VIIa, a hair cell marker (**Figure 1C**). To demonstrate the distribution of CB2R in cochlear neurons we used Tuj1, a mouse monoclonal antibody for class III β-tubulin which labels both Type I and Type II SG neurons in the rodent cochlea (Flores-Otero et al., 2007; Lallemend et al., 2007; Barclay et al., 2011). Immunolabeling with Tuj1 showed co-localization of GFP-CB2R with Tuj1 in the soma or cell bodies (**Figure 1D**) and neurites (**Figure 1F**) of SG neurons. Whole mount preparations show co-localization of GFP-CB2R and myosin VIIa in the organ of Corti (**Figure 1E**). Presynaptic localization of CB2R has previously been shown in the autaptic hippocampal neurons (Atwood et al., 2012). Therefore, we investigated the synaptic localization of CB2R in the cochlea. Labeling with CtBP2, a presynaptic marker for the IHCs, we show co-localization of endogenous GFP-tagged CB2R and CtBP2 (**Figure 1F**).

CB2R from adult male Wistar rat cochleae were characterized using a polyclonal antibody raised against amino acid residues 200–300 of this protein. This antibody was previously characterized using blocking peptide controls, inclusion of which resulted in no discernable signals in immunolabeling studies (**Supplementary Figure S1A**). Using this antibody, we observed high levels of immunolabeling in the rat spleen (which is rich in CB2R), while low levels were detected in the hippocampus (which normally show low levels of CB2R) (data not shown). In addition, higher levels of CB2R were detected in Chinese hamster ovary (CHO) cells which were transiently transfected with the CB2R expression plasmid, as compared to cells transfected with the empty plasmid vector alone (data not shown). Increased CB2R immunoreactivity was also demonstrated in organ of Corti-derived UB/OC-1 cells which were transiently transfected with the CB2R plasmid (**Supplementary Figure S1B**). Using this antibody for immunolabeling of rat cochlear sections, we showed CB2R immunoreactivity in both the OHCs and IHCs, inner (IPC) and outer pillar cells (OPC) (**Figures 2A,B**) and SG neurons (**Figure 2D**). Mid-modiolar sections were co-labeled with antibodies against Na^+/K^+-ATPase α1 subunit and CB2R to investigate the expression of this receptor in the SV. Intense CB2R immunolabelling was observed in the basal cells of SV, as compared to the intermediate and marginal cells (**Figure 2C**). CB2Rs were also distributed in Type II fibrocytes of the SL (**Figure 2C**). Co-labeling of CB2R and myosin VIIa was also detected in OHCs and IHCs of cochlear whole mount preparations prepared from the rat cochleae (**Figure 2E**).

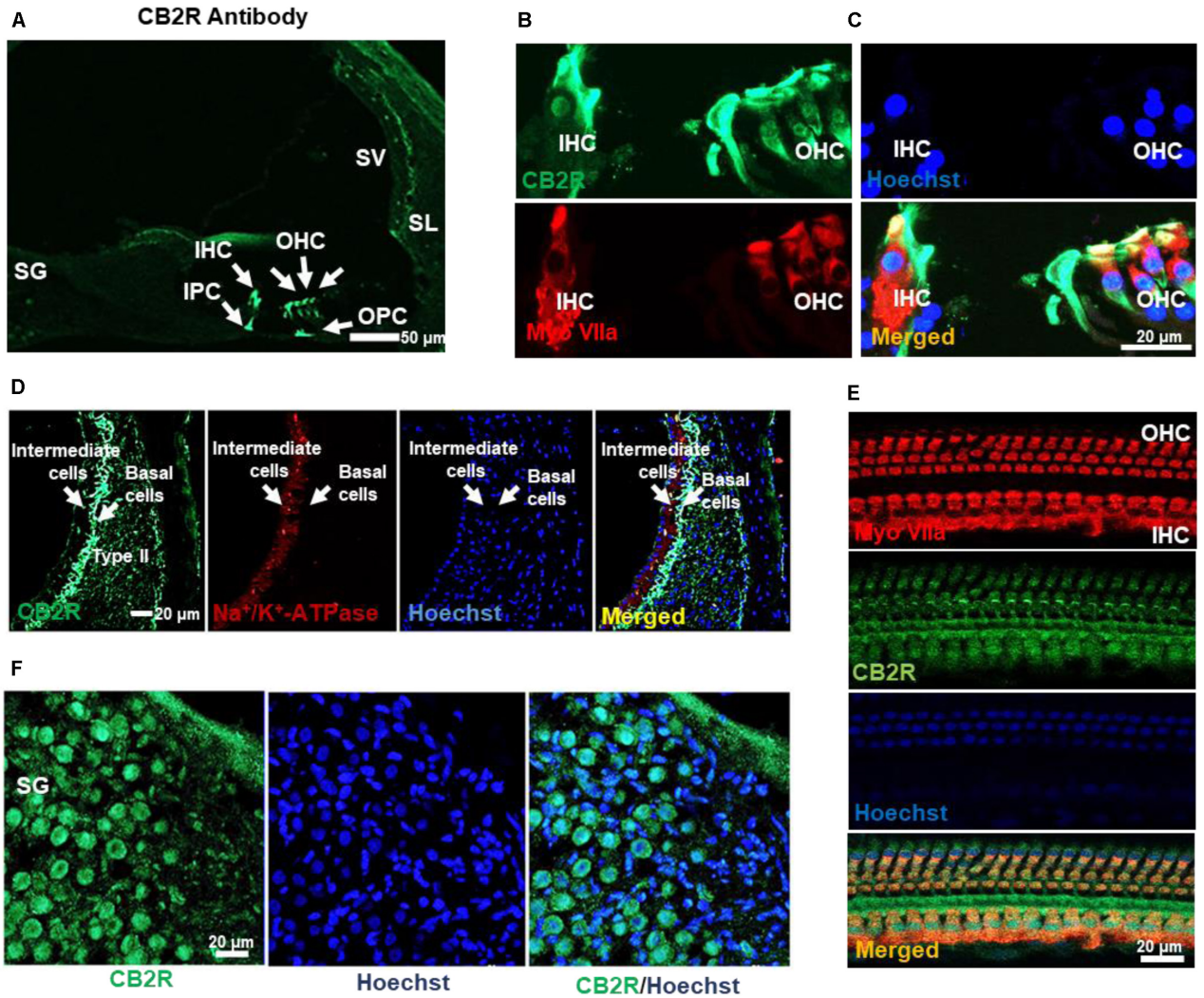


FIGURE 2 | Distribution of the CB2R in the rat cochlea. **(A)** Immunolabeling of mid-modiolar cochlear sections with CB2R antibody shows labeling of cells in the organ of Corti (green). Labeling was observed in the outer hair cells (OHC), inner hair cells (IHC), inner pillar cell (IPC), and outer pillar cell (OPC). **(B)** Co-labeling of the organ of Corti with antibodies for CB2R (green) and myosin VIIa (red) showed co-localization of these proteins in the OHC and IHC. **(C)** Staining of CB2R was also observed in the intermediate and basal cells of the SV and type II fibrocytes in SL. CB2R (green) showed some overlap with Na^+/K^+-ATPase α1 subunit (red) in the SV. **(D)** CB2R was localized to the plasma membranes of SG. Approximately 60% of cells in this section were stained with both CB2R (green) and Hoechst (blue). **(E)** Co-labeling of CB2R and myosin VIIa in OHCs and IHCs in whole mount preparations of the organ of Corti shows that distribution of CB2R (green) is more widespread than that of myosin VIIa (red). Hoechst was used as a nuclear stain (blue) in **(B–E)**. Studies reported here were repeated in at least three different animals and similar results were obtained.

Trans-Tympanic Administration of CB2R Agonist Attenuated Cisplatin-Mediated Hearing Loss and Loss of OHCs

Male Wistar rats were administered vehicle or JWH015 (2.5 nmoles in PBS per ear) by *trans*-tympanic injections, followed 30 min later by intraperitoneal cisplatin (11 mg/kg) administration. This dose of JWH015 produced optimal otoprotection than lower doses of JWH015 tested and is calculated to achieve a low micromolar range in the perilymph, assuming a 1–10% entry of the drug from the middle ear to the perilymph. Administration of vehicle produced no change in ABR thresholds when compared to pre-treatment ABRs (**Figure 3A**), attesting to the efficiency of the *trans*-tympanic drug delivery. Cisplatin increased ABR thresholds by 5.0 ± 1.5, 15.0 ± 2.5, and 27.0 ± 1.7 dB at 8, 16, and 32 kHz, respectively. Pretreatment with JWH015 diminished ABR threshold shifts in all the three frequency regions. The ABR threshold shifts were 0.6 ± 0.6, 1.9 ± 1.0, and 5.6 ± 1.5 dB at 8, 16, and 32 kHz, respectively, for the JWH015 + cisplatin treatment group. This protective action was completely blocked by *trans*-tympanic administration of AM630 (2.5 nmoles in PBS per ear), an antagonist of the CB2R (Morgan et al., 2009). ABR threshold shifts induced by cisplatin in rats pretreated with AM630 + JWH015 were 5.6 ± 1.2, 15.6 ± 2.4, and

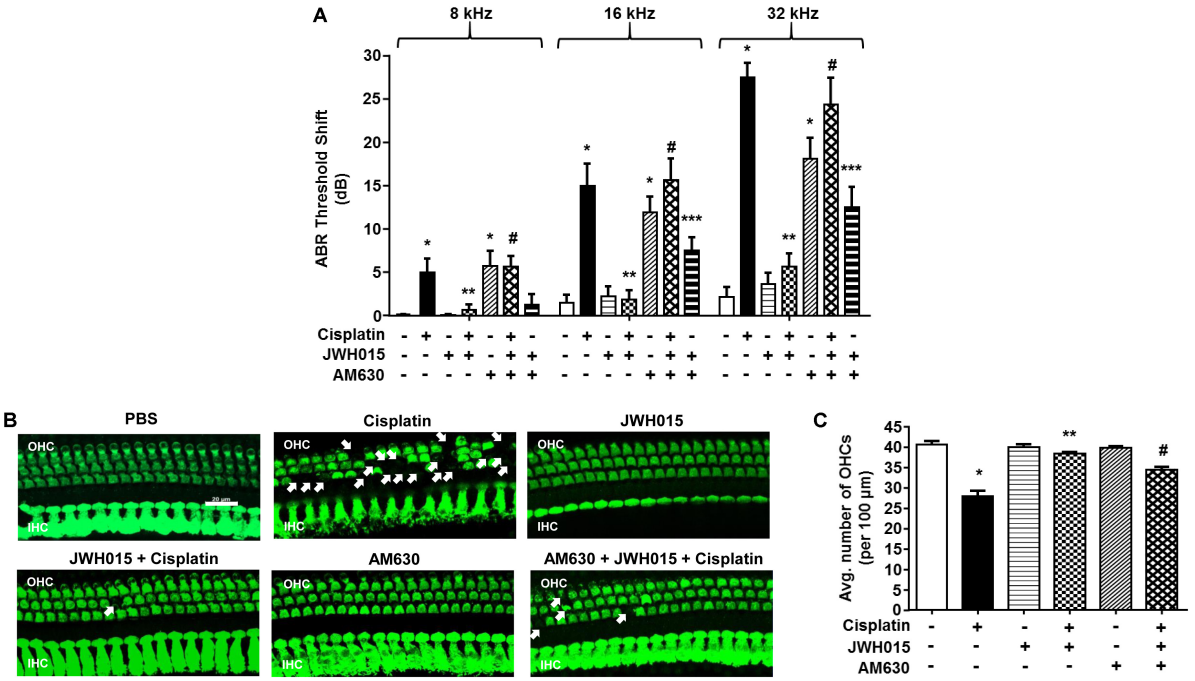


FIGURE 3 | *Trans*-tympanic administration of JWH015 prevents cisplatin-induced hearing loss. **(A)** Baseline ABR thresholds were recorded in Wistar rats, which were then pre-treated with either *trans*-tympanic AM630 (2.5 nmoles) for 30 min followed by *trans*-tympanic JWH015 (2.5 nmoles) or individually with *trans*-tympanic AM630 or JWH015 for 24 h. Cisplatin (11 mg/kg) was then administered (i.p.) after day 1 and post-ABR was performed after 72 h. Pre-treatment with JWH015 diminished cisplatin-induce ABR threshold shift in all the three frequency regions. The protective action of JWH015 was completely blocked by AM630. *Trans*-tympanic administration of AM630 alone significantly elevated by ABR thresholds. Data (threshold shift) is plotted as mean ± SEM (n = 12). **(B)** The animals were sacrificed at the end point and cochleae were collected, fixed by 4% PFA in PBS followed by decalcification for 3 weeks in 100 mM EDTA. The base of the cochlea were dissected out and stained with hair cell marker, myosin VIIa, to determine the hair cell loss. Representative images are shown. Scale bar is 20 µm. **(C)** The bar graph represents the average number of hair cells per 100 µm area shown in **(B)** ($n \geq 3$). Pre-treatment with JWH015 reduced cisplatin-induced outer hair cell loss. Administration of AM630 produced no hair cell loss. $^{*}p < 0.05$ vs. vehicle; $^{**}p < 0.05$ vs. cisplatin; $^{\#}p < 0.05$ vs. JWH015 + cisplatin; and $^{***}p < 0.05$ vs. JWH015 (one-way ANOVA).

24.3 ± 3.1 dB at 8, 16, and 32 kHz, respectively, which were similar to those obtained with cisplatin alone. An unexpected finding was that *trans*-tympanic administration of AM630 alone significantly elevated ABR thresholds by 5.8 ± 1.7, 11.9 ± 1.8, and 18.1 ± 2.4 dB at 8, 16, and 32 kHz, respectively, compared to vehicle-treated rats. Cisplatin-induced hearing loss was associated with a reduction in OHC number, assessed at the basal turn (**Figures 3B,C**). This loss of OHCs was reduced by *trans*-tympanic JWH015 administration. Administration of AM630 alone did not produce any loss of OHCs nor did it enhance the cell loss induced by cisplatin (**Supplementary Figure S2**). This finding suggests that endogenous activation of the cochlear CB2R (by endocannabinoids) helps in maintaining normal hearing and that blockade of this tonic stimulation results in hearing loss. Moreover, exogenous CB2 ligand can prevent cisplatin-induced hearing loss.

CB2R Agonist Protected Against Cisplatin-Induced Loss of IHC Ribbon Synapse

Significant loss of ribbon synapses has been reported following exposure to loud noise or treatment with aminoglycosides (Kujawa and Liberman, 2009; Chen et al., 2012; Zhang et al., 2013). Loss of ribbon synapses following noise exposure is mediated by glutamate (Puel et al., 1998). A functional ribbon synapse complex comprises a presynaptic ribbon containing RIBEYE and post-synaptic glutamate receptors, such as GluR2 (**Figure 4A**). RIBEYE can be labeled by antibodies against C-terminal binding protein 2 (CtBP2). In a recent report, we demonstrated that CtBP2 immunoreactivity was decreased following cisplatin treatment in rats and that this was abrogated by pretreatment with EGCG (Borse et al., 2017). Here we show that CB2R could also protect against cisplatin-induced loss of ribbon synapses. Functional ribbon synapses were identified as co-localization of both CtBP2 and GluR2 (**Figure 4B**). We show a significant loss of functional ribbon synapse in the basal turn from 20.9 ± 0.1 per IHC to 14.5 ± 0.2 per IHC, following cisplatin treatment (**Figures 4C,D**). Pre-treatment with JWH015 by *trans*-tympanic injection significantly reduced cisplatin-mediated loss of functional synapses. The mean number of functional synapses in JWH015 + cisplatin treated animal was 18.5 ± 0.8. This finding suggests that CB2R protects both the sensory hair cells and ribbon synapses, at least in the basal turn of the cochlea, which could account for its overall efficacy in reducing cisplatin ototoxicity. AM630 administered alone or

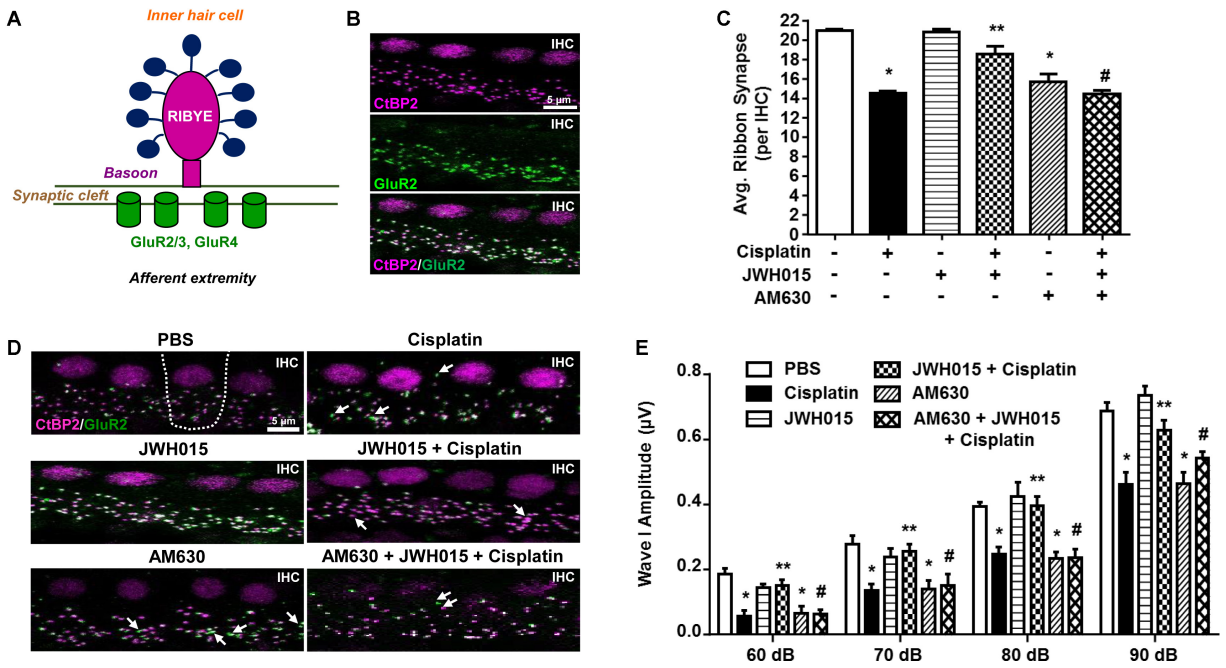


FIGURE 4 | Activation of CB2R protects against cisplatin induced loss of ribbon synapse. **(A)** The cartoon represents a typical ribbon synapse at the inner hair cell (IHC). It is primarily composed of two major components: presynaptic RIBEYE and glutamate receptors (GluR) at the post-synapse. **(B)** Representative images shows whole mount preparations co-stained with presynaptic CtBP2 (for RIBEYE, magenta) and postsynaptic GluR2 (green). Functional ribbon synapses are complexes showing both CtBP2 and GluR2 immunolabeling (white) apposing each IHC. **(D)** The whole mount preparation form the basal region of cochleae from treated animals were dissected and stained for CtBP2 and GluR2. Representative images are shown. White arrows indicate single staining of either CtBP2 or GluR2. Functional synapse complexes (CtBP2 + GluR2) were quantified by averaging the numbers from 30 IHCs per samples. The bar graph **(C)** represents the average number of synaptic complexes (mean ± SEM) per IHC ($n \geq 4$). Pre-treatment with JWH015 protected against cisplatin-induced loss of functional synapse. CB2R antagonist, AM630, administered alone or in combination with JWH015 + cisplatin produced significant reductions in functional ribbon synapses. **(E)** ABR wave I amplitude of different treatment groups at 60, 70, 80, and 90 dB SPL (32 kHz) was computed from sorted ABR wave forms. The bar graph represents mean wave I amplitude ± SEM ($n = 12$). Cisplatin decreases wave I amplitude indicating synaptopathy which was completely reversed by pre-treatment with JWH015. Blockade of CB2R activation by AM630 resulted in decrease in wave I amplitude. $^{*}p < 0.05$ vs. vehicle; $^{**}p < 0.05$ vs. cisplatin; and $^{\#}p < 0.05$ vs. JWH015 + cisplatin (one-way ANOVA).

in combination with JWH015 + cisplatin produced significant reductions in the number of ribbon synapses (15.6 ± 0.8 and 14.4 ± 0.4, respectively), compared to vehicle controls (**Figures 4C,D**). These data suggest that synaptic ribbons are a potential target of AM630 for mediating hearing loss, as this drug did not produce any loss in OHCs. Thus, it appears that one of the functions of endogenous cannabinoids in the cochlea is to maintain the integrity of ribbon synapses which could contribute significantly toward preserving normal hearing.

Synapse loss after acoustic trauma results in reductions in supra-threshold amplitudes of wave I of the ABR (Bharadwaj and Shinn-Cunningham, 2014). This phenomenon is considered to be a good indicator of cochlear synaptopathy (Kujawa and Liberman, 2009). Therefore, we determined whether cisplatin-mediated cochlear synaptopathy is associated with change of wave I supra-threshold amplitude. ABR wave I amplitude elicited by 60–90 dB SPL tones delivered at 32 kHz were significantly reduced by 33% (from 0.69 ± 0.03 µV to 0.46 ± 0.04 µV) following cisplatin treatment ($p < 0.0001$, $n = 12$) (**Figure 4E**). These decreases in wave I amplitudes were attenuated in rats pretreated with *trans*-tympanic JWH015 prior to administration of cisplatin (mean amplitude of wave I was 0.63 ± 0.03 µV), suggesting that activation of CB2R in the cochlea regulates cisplatin-induced cochlear loss of wave I amplitude. Rats treated with AM630 alone or in combination of JWH015 + cisplatin showed significantly diminished wave I amplitudes (mean amplitude 0.46 ± 0.03 µV), compared to the control animals, suggesting that CB2R activation provides tonic maintenance of normal cochlear physiology.

Anti-inflammatory Role of CB2R in the Cochlea

CB2Rs are primarily distributed in the immune cells where they play a key role in the regulating both humoral and innate immunity (Klein et al., 1998). These receptors suppress the growth of macrophages and phagocytes and modulate the release of inflammatory cytokines from leukocytes by regulating the release of pro- and anti-inflammatory cytokines from helper T cells, Th1 and Th2, respectively (Ashton and Glass, 2007). Inflammation plays a critical role in cisplatin ototoxicity which involves induction of pro-inflammatory cytokines by STAT1 (Kaur et al., 2011, 2016). Cisplatin increased the expression of STAT1-regulated genes, such as *TRPV1*, *NOX3*, *TNF*-α, *COX2*,

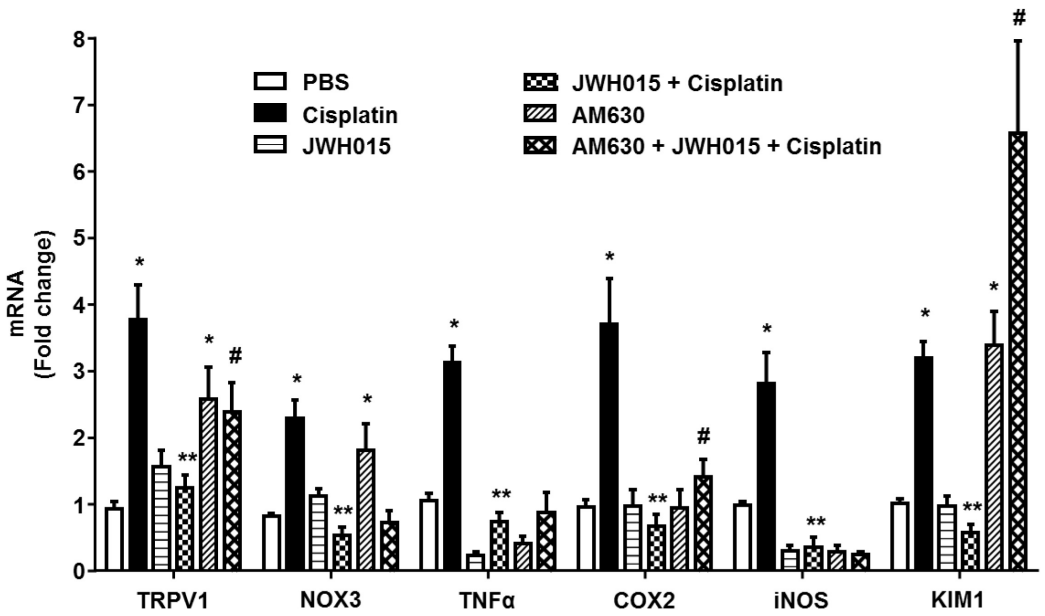


FIGURE 5 | Activation of CB2R by JWH015 mitigates cisplatin-induced inflammation in the rat cochlea. Snap frozen cochlea from treated animals were used for total RNA extraction which was then converted to cDNA and used for real-time RT-PCR for the expression of *TRPV1*, *NOX3*, *TNF-α*, *COX2*, *iNOS,* and *KIM1*. Housekeeping gene, *GAPDH,* was used for normalization. Cisplatin significantly increased the expression of all the genes tested. The expression of these genes were significantly reduced in rats pre-treated with JWH015 followed by cisplatin. Administration of CB2R antagonist, AM630, alone increased the expression of *TRPV1*, *NOX3,* and *KIM1*. Data is plotted as mean fold change ± SEM ($n \geq 5$). $^{*}p < 0.05$ vs. vehicle; $^{**}p < 0.05$ vs. cisplatin; and $^{\#}p < 0.05$ vs. JWH015 + cisplatin (one-way ANOVA).

iNOS, and *KIM1* by 3.8 ± 0.5-, 2.3 ± 0.3-, 3.1 ± 0.3-, 3.7 ± 0.7-, 2.8 ± 0.5-, and 3.2 ± 0.2-fold, respectively, compared to vehicle treated animals (**Figure 5**). These genes were significantly reduced in rats pre-treated with JWH015 followed by cisplatin. Interestingly, application of AM630 alone led to increased expression of *TRPV1*, *NOX3,* and *KIM1* by 2.6 ± 0.5, 1.8 ± 0.4 and 3.4 ± 0.1-fold, respectively, possibly indicating a functional role of endocannabinoids targeting CB2Rs in dictating the basal expression of these genes.

CB2R Maintains the Levels of Na^+/K^+-ATPase in the Stria Vascularis

Na^+/K^+-ATPase present in the SV and SL fibrocytes plays a critical role in maintaining endocochlear potential (EP) in the cochlea (van Benthem et al., 1994; Wangemann, 2002). Recently we have shown that cisplatin reduces the levels of this protein in SV and SL (Borse et al., 2017) which might contribute to disruption of EP and hearing loss. In this study, we determined whether activation of CB2R could also attenuate cisplatin-induced loss of Na^+/K^+-ATPase using an antibody which labels the α1 subunits of this enzyme. We show that cisplatin reduced the level of Na^+/K^+-ATPase in the SV which was abrogated by CB2R activation (**Figures 6A,B**). Cisplatin reduced Na^+/K^+-ATPase α1 immunoreactivity to 26.4 ± 5.2% of vehicle-treated controls, which was attenuated in rats pretreated with JWH015 (labeling was reduced to 96.9 ± 21.1% of vehicle-treated controls). No significant change was observed in Na^+/K^+-ATPase α1 immunoreactivity in SV from rats treated with JWH015 alone. Surprisingly, AM630 added alone reduced Na^+/K^+-ATPase α1 immunoreactivity to an extent which was comparable to cisplatin (21.4 ± 3.6%). In addition, administration of AM630 blocked the increase in Na^+/K^+-ATPase α1 immunoreactivity produced by JWH015 in presence of cisplatin (38.4 ± 10%). These data suggest that Na^+/K^+-ATPase could serve as another target of cisplatin for inducing hearing loss which is regulated by CB2R activation. Furthermore, the findings with AM630 lend support to the conclusion that the basal expression and/or distribution of this enzyme in the SV is strictly dependent on CB2R.

Knockdown of CB2R in the Cochlea Exacerbates Cisplatin-Induced Hearing Loss

Trans-tympanic administration of CB2R siRNA produced dose-dependent reduction in total cochlear CB2R mRNA within 48 h, as assessed by RT-PCR (**Figure 7A**). Approximately 80% reduction was observed at the highest dose of siRNA (0.9 μg) used. Lower levels of siRNAs were less effective. At 48 h post siRNA administration, animals were administered vehicle or cisplatin (11 mg/kg) by the intraperitoneal route. Post-treatment ABRs were assessed 3 days later at 8, 16, and 32 kHz. As observed previously, cisplatin produced significant ABR threshold shifts which increased with increasing sound frequencies. Rats which were treated with CB2R siRNA, followed by cisplatin, showed significantly increased threshold shifts

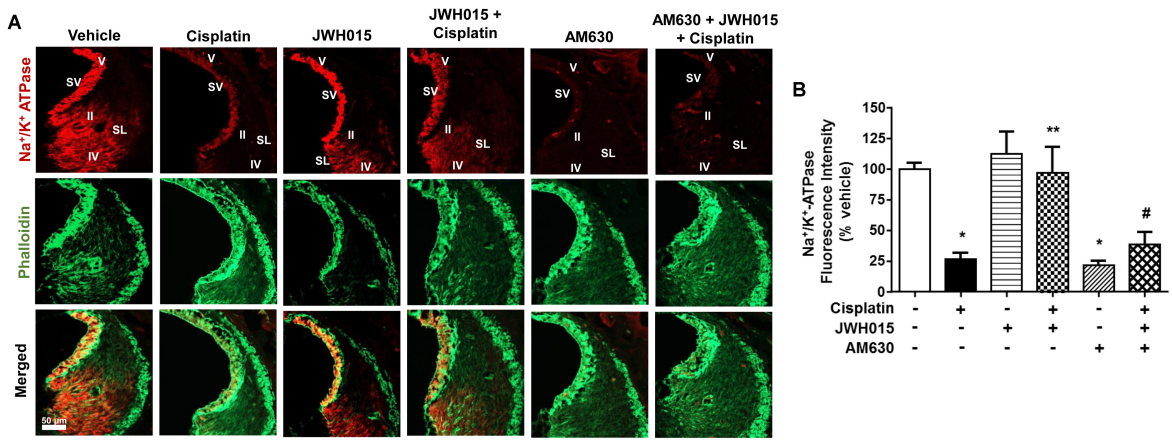


FIGURE 6 | Activation of CB2R by JWH015 mitigates cisplatin-induced loss of Na^+/K^+-ATPase-α1 levels in the rat cochlea. **(A)** Mid-modiolar sections of the rat cochlea were co-immunolabeled with Na^+/K^+-ATPase-α1 and phalloidin. The sections were imaged using Zeiss LSM800 scanning confocal microscope. Representative images from the base of the cochlea are shown ($n \geq 3$). **(B)** Bar graph represents % Na^+/K^+-ATPase fluorescence intensity ± SEM. Cisplatin decreases expression of Na^+/K^+-ATPase-α1 in stria vascularis (SV) and spiral ligament (SL) and this is attenuated by pre-treatment with JWH015. AM630 added alone also reduced Na^+/K^+-ATPase immunoreactivity which was similar to cisplatin. $^*p < 0.05$ vs. vehicle; $^{**}p < 0.05$ vs. cisplatin; and $^{\#}p < 0.05$ vs. JWH015 + cisplatin (one-way ANOVA).

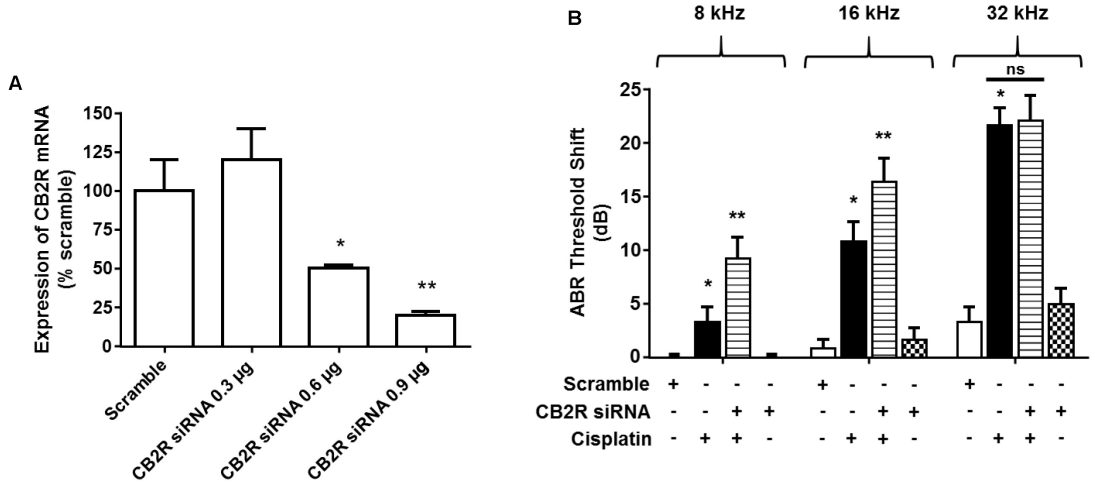


FIGURE 7 | Knock-down of CB2R expression by siRNA increases the sensitivity to cisplatin-induced hearing loss. **(A)** Rats were administered either scramble siRNA (0.9 µg) or increasing doses of CB2R siRNA (0.3, 0.6, and 0.9 µg) by *trans*-tympanic injections. The animals were sacrificed 48 h later and mRNA was assessed for CB2R expression by real-time RT-PCR. CB2R siRNA knocked down CB2R gene expression by ∼50 and ∼80% using 0.6 µg and 0.9 µg, respectively. Data are represented as % expression ± SEM ($n = 3$). $^*p < 0.05$ vs. scramble siRNA and $^{**}p < 0.05$ vs. CB2R siRNA 0.6 µg. **(B)** Baseline ABR was recorded in rats administered with either a scramble siRNA or CB2R siRNA by *trans*-tympanic injections for 48 h. Cisplatin (11 mg/kg, i.p.) was then administered and post-ABR was recorded 72 h later. There was no significant change in ABR threshold shift in the animals treated with scramble siRNA or CB2R siRNA alone. Pre-treatment with CB2 siRNA (0.9 µg) followed by cisplatin significantly potentiated cisplatin-induced hearing loss at 8 and 16 kHz. Data are represented as mean ± SEM. $^*p < 0.05$ vs. scramble siRNA and $^{**}p < 0.05$ vs. cisplatin ($n = 6$, one-way ANOVA).

at 8 and 16 kHz over those observed in the scrambled siRNA plus cisplatin group. No difference was observed at the 32 kHz frequency (**Figure 7B**). These results are consistent with the conclusion that the endocannabinoid/CB2R system in the cochlea can partially counter cisplatin-induced ototoxicity in the 8 and 16 kHz frequency range. The lack of effect at the high frequency (32 kHz) region of the cochlea could reflect greater toxicity in the base of the cochlea where cisplatin's concentrations are the greatest (Hellberg et al., 2013) and which overwhelms the protection afforded by CB2R.

JWH015 Does Not Interfere With Cisplatin Anti-cancer Efficacy in Cancer Cells

Previous studies have shown that endogenous and synthetic cannabinoids produce anti-cancer properties. For example,

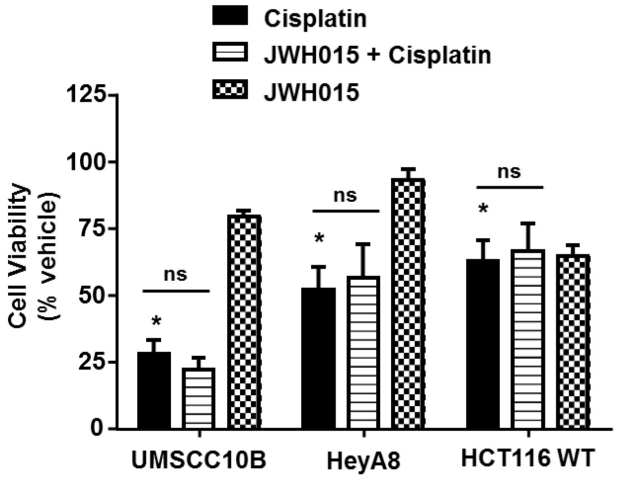


FIGURE 8 | Pre-treatment with JWH015 does not interfere with cisplatin anti-cancer efficacy *in vitro*. Different cancer cells (UMSCC10B, HeyA8, and HCT116 WT) were treated with JWH015 (10 μM) for 30 min followed by cisplatin (10 μM) for 48 h. The cell viability was measured by MTS assay. The percent cell viability is represented in the bar graph as mean ± SEM of at least four independent experiments. $*p < 0.05$ represent significant difference from vehicle-treated group at 100% (one-way ANOVA).

activation of CBRs induce apoptosis in cancer cells by the classical cell death pathways (Olea-Herrero et al., 2009), induce autophagy-mediated cell death (Salazar et al., 2009), arrest the cell cycle and cell division (Laezza et al., 2006) or inhibit neovascularization (Blazquez et al., 2003). We decided to study whether CB2R agonists could interfere with cisplatin chemotherapeutic effects in the event that these agents need to be administered systemically to treat hearing loss. Several cell lines derived from tumors known to be responsive to cisplatin were examined. These include head and neck cancer cells (UMSCC10B), ovarian cancer cells (HeyA8) and colon cancer cells (HCT116 WT). Administration of 10 μM cisplatin to cell cultures produced significant reductions in cell viability which averaged 28.0 ± 5.1%, 52.1 ± 8.6%, and 62.6 ± 7.8% of control cells, respectively, in UMSCC10B, HeyA8, and HCT116 WT cells (**Figure 8**). In cells pre-treated with 10 μM JWH015, the percent cell viability in the JWH-015 + cisplatin group were 22.0 ± 4.5, 56.4 ± 12.5, and 66.4 ± 10.6%, respectively, in UMSCC10B, HeyA8, and HCT116 WT cells. These values were not significantly different from those obtained with cisplatin added alone, suggesting a lack of interference of cisplatin's anti-cancer response in presence of this CB2R agonist.

DISCUSSION

The current study demonstrates that CB2Rs are distributed throughout the cochlea, including sensory hair cells, SG neurons, SV and SL. Activation of CB2R by JWH015 protected against cisplatin-induced hearing loss and this protective effect could be completely blocked by AM630, an antagonist of CB2R. Surprisingly, AM630 potentiated the ototoxicity produced by cisplatin, especially at the low frequency range, and produced significant hearing loss when administered alone. These data suggest that activation of CB2Rs (by endocannabinoids) plays a tonic protective role under normal condition and that interference with these receptors or the endogenous cannabinoid system could compromise hearing. In this regard, we observed that knockdown of CB2R in the cochlea by siRNA exacerbated cisplatin-induced hearing loss (at lower frequencies). In addition, this study identified different cochlear targets of the CB2R agonist, JWH015, which could account for its otoprotective actions. These include the OHCs, ribbons synapses and stria vascularis (Na^+/K^+-ATPase), regional targets which are shown to express the CB2R subtype.

Systemic administration of cisplatin to the male Wistar rat elevated ABR thresholds, which is especially evident in the basal region of the cochlea. This loss of hearing in the high frequency range was associated with a reduction in OHCs in the basal turn of the cochlea and was attenuated by *trans*-tympanic administration of JWH015, an agonist of the CB2R. JWH015 also protected against hearing loss in the lower frequency range, even though these regions did not experience much discernable damage or loss of OHCs. Thus, other mechanisms could account for the "additional" protection afforded by CB2R in the low frequency range.

Other targets of cisplatin's action and CB2R-mediated protection include functional ribbon synapses associated with the IHCs. These synapses communicate the original auditory signal generated by the IHCs to SG neurons. Alteration in the number and/or function of these synapses would alter the auditory transmission to SG neurons and central auditory pathways. The reductions in ribbon synapses, or synaptopathy, observed in cochleae obtained from cisplatin-treated rats would therefore contribute to the overall elevations in ABR thresholds which were observed. The basis for this synaptopathy induced by cisplatin which has previously been described (Borse et al., 2017) is not clear, but could result from glutamate excitotoxicity (Puel et al., 1998). Cochlear synaptopathy resulting from noise exposure has been previously described (Liberman, 2016). Previous studies have reported that synapse loss in noise and age-induced hearing loss (Sergeyenko et al., 2013; Espejo-Porras et al., 2015; Liberman and Kujawa, 2017) which was not associated with loss of OHCs or ABR threshold shifts. This type of cochlea synaptopathy is known as "hidden hearing loss" (Liberman and Kujawa, 2017) and is detected by a reduction in wave I supra-threshold amplitude (Sredni et al., 2016; Liberman and Kujawa, 2017). The present study revealed an important "tonic" role of the endocannabinoid/CB2R system to preserve synaptic integrity under normal condition which was unmasked by the application of CB2R antagonist. Since noise-induced temporary threshold shift and resulting synaptopathy is associated with reductions in wave I amplitudes (Liberman, 2016), it is possible that CB2R agonists could similarly protect against this deficit. Whether activation of the CB2R could initiate regeneration of synapses, following trauma, similar to neurotrophin 3 (Suzuki et al., 2016), is not yet known. Unlike noise, cisplatin-induced trauma results in permanent peripheral deficits which are not restored by subsequent administration of protective agents. We

observed a significant decrease in wave I amplitudes at 60, 70, 80, and 90 dB in the high frequency region (32 kHz) in both cisplatin and AM630 treatment groups which reflect synapse loss. The mechanism underlying the preservation of synaptic integrity by pretreatment with CB2R agonist is not clear. One possibility is that activation of presynaptic CB2R reduces the release of excessive glutamate and this limits activation of postsynaptic glutamate receptors and damage to the afferent fibers. In support of this hypothesis, we observed co-localization of CB2R and CtBP2 protein in the presynaptic nerve terminal in the mouse cochlea. Previous studies have demonstrated a role of presynaptic CB2R in autaptic hippocampal neurons which mediate presynaptic inhibition of neurotransmitter release (Atwood et al., 2012). In other disease models, upregulation of CB2R has been shown to mediate an "autoprotective" role (Pertwee, 2009). Increased expression of CB2R was observed in neuropathic pain models (Zhang et al., 2003) and in rat sensory neurons during peripheral nerve injury (Wotherspoon et al., 2005).

While CB1Rs are expressed in the cochlea (unpublished data), whether they are expressed in the IHC synapse and to what extent they modulate afferent neurotransmission is unknown. Nevertheless, in the CNS, CB1R plays a major role in regulating presynaptic release of excitatory and inhibitory neurotransmitters, such as GABA and glutamate (Pertwee, 2009).

A significant finding of this study is the observation that perturbation of the endocannabinoid system in the cochlea contributes to hearing loss. We showed that inhibition or knockdown of CB2R resulted in elevations in ABR thresholds. These findings suggest that the endocannabinoid system provides a tonic level of protection to the cochlea which is only revealed upon inhibition of this system with AM630. In addition to the CBRs, we could detect labeling of the endocannabinoid 2-AG synthetic enzyme, diacylglycerol lipase, to similar locations in the cochlea (unpublished data). As such, 2-AG released by cells in the cochlea would be able to activate CB2R on the same or neighboring cells. Such an endogenous system could contribute to cytoprotection under normal physiological activity but could be overwhelmed by ototoxic drugs, such as cisplatin. In such a situation, exogenously administered CB2R agonist could be required to boost the protection afforded by the endocannabinoid system. Another interesting observation is that knockdown of CB2R by siRNA partly mimicked the response to AM630, in that this perturbation sensitized these animals to cisplatin-induced hearing loss at the lower frequencies. These data would support the conclusion that endocannabinoids (probably by acting through CB2R) help to maintain the integrity of IHC ribbon synapse and protect hearing. Another explanation is that in the presence of CB2R blockade the full effects of endocannabinoids on other targets, such as CB1R and TRPV1 channels (Pertwee, 2009) are realized. Accordingly, activation of these targets by endocannabinoids could confer ototoxicity.

The hearing loss produced by AM630 was not associated with loss of OHCs and IHCs, but was more correlated with a loss of IHC ribbon synapses. This would suggest that protecting synaptic integrity and maintenance of EP are more important roles of endocannabinoids than protecting the sensory hair cells. Thus, the hearing loss produced by AM630 is partially different from that produced by cisplatin, as the latter also mediates apoptosis of hair cells. Changes in the EP have been associated with acquired hearing loss including chemotherapeutic drugs, age and noise-induced hearing loss (Schulte and Schmiedt, 1992; Tsukasaki et al., 2000; Hirose and Liberman, 2003). Na^+/K^+-ATPase present in the SV plays a critical role in generating high K^+ concentrations in the endolymph and in maintaining the EP (Hibino et al., 2010). Increased oxidative stress has been shown to decrease the activity of Na^+/K^+-ATPase by promoting its proteolytic degradation and endocytosis (Chen et al., 2012). Activation of CB2R protects against cisplatin-induced loss of OHCs in the basal turn of the cochlea. This protection is similar to that observed following activation of the adenosine A_1 receptor (Kaur et al., 2016), where it is linked to suppression of a NOX3 NADPH oxidase/STAT1 inflammatory pathway. It is believed that inhibition of STAT1 in these OHCs attenuated cisplatin-mediated p53 activation and apoptosis. Inhibition of STAT1 as an otoprotective strategy has been documented in several studies (Schmitt et al., 2009; Mukherjea et al., 2011; Kaur et al., 2016; Borse et al., 2017). We showed that JWH015 suppressed cisplatin-induced STAT1 activation *in vitro*, which could account for its anti-apoptotic role in UB/OC-1 cell cultures (unpublished data). A similar mechanism *in vivo* could account for the preservation of OHCs in the basal turn of the cochlea. In support of this, we show that JWH015 suppressed the induction of a number of STAT1-regulated inflammatory genes, such as *TNF*-α, *COX2,* and *iNOS* in whole cochlear extracts. The expression of *NOX3* has been positively linked to activation of STAT1, as would be expected from an increase in oxidative stress Kaur et al., 2016; Borse et al., 2017).

CB2R plays a major role in regulating the immune system. In addition to suppressing STAT1-dependent inflammatory cytokines and related genes, activation of CB2R suppress the recruitment of inflammatory cells to the site of inflammation. (Klein, 2005). Activation of CB2R also suppress the proliferation and phagocytotic function of macrophages (Ashton and Glass, 2007). CB2R agonist has also been shown to promote apoptosis of immune cells (Lombard et al., 2007). Therefore, yet unexplored actions of JWH015 in this study are its effects on the resident immune cells and the recruitment and apoptosis of circulating immune cells to the cochlea. Such actions would be expected to reduce overall cochlear inflammation and reduce hearing loss. We hope to explore these additional actions of CB2R in the cochlea in the future.

Stimulation of CB2R can exert different effects on cancer cells in a tissue and tumor specific manner (Velasco et al., 2016). Various clinical studies have evaluated the role of cannabinoids for palliative care in cancer patients to alleviate chemotherapy-induced nausea and vomiting (Rock and Parker, 2016) and cancer pain (Johnson et al., 2010). In this study, we show JWH015 does not interfere with cisplatin's anti-cancer efficacy in head and neck, ovarian and colon cancer cells. These findings need to be further studied in the xenograft mice model and other models before systemic use of JWH015 could be initiated.

In summary, our data support a role of the endocannabinoid/CB2R system in maintaining normal hearing

in the rat cochlea. Protection stems from endocannabinoid/CB2R interaction to maintain the integrity of OHCs, IHC ribbon synapses and Na^+/K^+-ATPases in the SV. The endocannabinoid/CB2R system is not fully able to protect against cisplatin ototoxicity and requires addition of exogenous CB2R agonist. This agonist protects against the loss of OHCs and ribbon synapses. *Trans*-tympanic administration of CB2R agonist provides effective protection against cisplatin-induced hearing loss and should produce limited side effects of this drug. Based on these results, we would recommend the use of localized administration of CB2R agonists for the treatment of cisplatin-induced hearing loss.

AUTHOR CONTRIBUTIONS

SG and VR developed the idea for the research mentioned in this article. SG, SS, and VR wrote the manuscript and edited the figures. KS, SG, SS, AD, and VB performed the experiments while SS, VB, and AD helped with data analysis. DM and LR critiqued and revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2018.00271/full#supplementary-material

FIGURE S1 | Characterization of the CB2R. **(A)** Specificity of CB2R immunolabeling was confirmed in mid-modiolar sections using a blocking peptide for the CB2R protein which revealed an absence of labeled proteins. **(B)** UB/OC1 cells were transiently transfected with a plasmid vector expressing CB2R. Cells were fixed in 4% paraformaldehyde, washed with PBS and then immunolabeled for CB2R (green). Labeling in the untransfected cells was confined to the plasma membrane and was substantially increased following transient transfection of CB2R.

FIGURE S2 | Administration of AM630 alone does not reduce OHC number and did not alter the effect of cisplatin. The animals were sacrificed at the end point and cochleae were collected, fixed by 4% PFA in PBS followed by decalcification in 100 mM EDTA for 3 weeks. The base of the cochlea were dissected out and stained with hair cell marker, myosin VIIa, to determine the hair cell loss. Representative images are shown. Scale bar is 20 μm.

TABLE S1 | Description of antibodies used.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Potential Clinical Benefits of CBD-Rich *Cannabis* Extracts Over Purified CBD in Treatment-Resistant Epilepsy: Observational Data Meta-analysis

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This meta-analysis paper describes the analysis of observational clinical studies on the treatment of refractory epilepsy with cannabidiol (CBD)-based products. Beyond attempting to establish the safety and efficacy of such products, we also investigated if there is enough evidence to assume any difference in efficacy between CBD-rich extracts compared to purified CBD products. The systematic search took place in February/2017 and updated in December/2017 using the keywords "epilepsy" or "Dravet" or "Lennox-Gastaut" or "CDKL5" combined with "Cannabis," "cannabinoid," "cannabidiol," or "CBD" resulting in 199 papers. The qualitative assessment resulted in 11 valid references, with an average impact factor of 8.1 (ranging from 1.4 to 47.8). The categorical data of a total of 670 patients were analyzed by Fischer test. The average daily dose ranged between 1 and 50 mg/kg, with treatment length from 3 to 12 months (mean 6.2 months). Two thirds of patients reported improvement in the frequency of seizures (399/622, 64%). There were more reports of improvement from patients treated with CBD-rich extracts (318/447, 71%) than patients treated with purified CBD (81/175, 46%), with statistical significance ($p < 0.0001$). Nevertheless, when the standard clinical threshold of a "50% reduction or more in the frequency of seizures" was applied, only 39% of the individuals were considered "responders," and there was no difference ($p = 0.52$) between treatments with CBD-rich extracts (122/330, 37%) and purified CBD (94/223, 42%). Patients treated with CBD-rich extracts reported lower average dose (6.0 mg/kg/day) than those using purified CBD (25.3 mg/kg/day). The reports of mild (158/216, 76% vs. 148/447, 33%, $p < 0.001$) and severe (41/155, 26% vs. 23/328, 7%, $p < 0.0001$) adverse effects were more frequent in products containing purified CBD than in CBD-rich extracts. CBD-rich extracts seem to present a better therapeutic profile than purified CBD, at least in this population of patients with refractory epilepsy. The roots of this difference is likely due to synergistic effects of CBD with other phytocompounds (aka Entourage effect), but this remains to be confirmed in controlled clinical studies.

Keywords: cannabinoids, cannabidiol (CBD), epilepsy, meta-analysis, refractory epilepsy, phytotherapy

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INTRODUCTION

Derivative products from the *Cannabis sativa* plant have historically been used for a number of neurological disorders, as broad as pain and appetite stimulation in oncological and HIV patients (1). The delicate balance between therapeutic and adverse effects in medicinal *Cannabis* yields controversial discussions in the literature (2–4), where the main psychoactive cannabinoid compound—delta-9-tetrahydrocannabinol (THC) is the major protagonist. More recently, another non-psychoactive cannabinoid–cannabidiol (CBD)—has received a lot of attention, since its promising pharmacological profile suggests a broader therapeutic index compared to THC. CBD has been described as a potential therapeutic compound to control seizures in humans in the 80's (5) and since then, several other studies have extended this data (6–15).

The natural source of CBD is a variety of *Cannabis* plants called "hemp" or "fiber-type *Cannabis,*" where one can find a high ratio between CBD and THC compounds, sometimes around 30:1 (CBD:THC), with negligible amounts of THC (16, 17). Fiber-type *Cannabis* are, by definition, *Cannabis* with <0.3% THC content, which is not considered controlled substance by the United Nations Office on Drugs and Crime (18). Hemp extracts became an internet buzz (19), with several anecdotal descriptions of therapeutic effects in children with treatment-resistant epilepsies, especially Dravet syndrome, starting to appear since 2013 (11, 20, 21). Preclinical evidence support anti-convulsant properties of CBD [reviewed in Hill et al. (22) and Devinsky et al. (23)]. Furthermore, a number of observational papers suggested good tolerability and therapeutic benefits in seizure control, with patients experiencing low frequency of side effects (6–15). Few randomized control trials in specific diseases have followed (24, 25) and the putative neuronal mechanism of action is still to be established, with the more likely candidates being inhibition of endocannabinoid uptake, allosteric modulation of CB1 receptors, activation of 5-HT1A serotoninergic receptors anti-inflammatory/anti-oxidant effects [reviewed in Bih et al. (26)].

The first CBD-based product was just recently registered for the treatment of treatment-resistant epilepsies (27). Meanwhile, the patients are using non-registered hemp extracts and derivative products that are considered "nutritional supplements" with high CBD content and often unknown THC concentration. These products are not considered controlled substances at the production countries and are being distributed in many countries via exceptional import mechanisms.

Despite several positive anecdotal pieces of evidence of patients and family members about the "CBD extracts," which are broadly publicized through several magazines and TV shows worldwide; until now, there is no consensus on the medical literature about the efficacy and safety of these products. Some observational studies are available on scientific literature, but there is a scarcity of clinical data acquired within the logic, rigor and organization necessary to the conduction of clinical studies destined to the registration of a pharmaceutical product.

The objective of the present study is to conduct a meta-analysis to investigate the available data about the clinical use of CBD-rich products for patients with treatment-resistant epilepsy. Whenever possible, we also tried to investigate if there was any difference of efficacy and side effects between "purified CBD" and CBD-rich extracts.

META-ANALYSIS SEARCH STRATEGY

Sources

A systematic search was performed on MEDLINE/PubMed (http://www.ncbi.nlm.nih.gov/pubmed) and Google Scholar (http://scholar.google.com) databases intending to identify original papers with clinical data (observational) on the use of *Cannabis* and its compounds on the treatment of refractory epilepsy. Main focus was on cannabidiol (CBD) and CBD-rich *Cannabis* extracts, whose use has been disseminated among infant and juvenile patients of treatment-resistant forms of epilepsy. The search was limited to papers published in English, with results obtained from human beings in parent surveys and proper medical records. The systematic search took place in February/2017 and updated in December/2017 using the keywords "child" and "epilepsy" or "Dravet" or "Lennox-Gastaut" or "CDKL5" combined with "Cannabis," "cannabinoid," "cannabidiol," or "CBD." We made every effort to include the available data, including searching through the paper references to identify additional sources, contacting the studies' authors and presenting a preliminary version of this study in two conferences to gather additional information that we could have missed in the first search. Papers containing only title and/or abstracts were not included, as well as unpublished results (at the time of the first search one in press article was included, but it is now officially published). Pre-print servers like PeerJ and BioRxiv were also used for the search, but did not reveal any forthcoming useful clinical study.

Study Selection

The titles, abstracts and full texts of all search results had their eligibility analyzed, considering inclusion and exclusion criteria. Inclusion criteria: studies containing observational clinical data in humans that could infer the efficacy and/or safety profile of the products containing cannabinoids for epilepsy. Exclusion criteria: Review and opinion papers, case studies, studies with no measurable data, and studies with no accessible numerical data. Papers describing studies in prospective and retrospective design were considered eligible, regardless of the kind and duration of treatment. Papers failing to present objective measurement of seizures and/or objective measurements of clinical improvement were disregarded. Papers presenting partial data (example: data for clinical improvement, but not for adverse effects) were included only in the appropriate sections of the meta-analysis study.

Treatment of Clinical Data and Statistical Analysis

Classical objective clinical outcomes in the research field of epilepsy were used to group the articles. Subjective clinical outcomes like "reported improvement" were considered, but the more objective "reduction of the number of seizures" was

preferred. Data regarding the reduction in the seizures frequency were grouped in two cumulative thresholds, (1) reduction in seizures frequency >50% (classically considered a "responder" to the treatment in epilepsy studies) and (2) reduction in seizures frequency >70% were considered for objective measurement of treatment efficacy in the pooled data (whenever available). The relative number of "responders" in each study was used as the main objective measurement to evaluate efficacy and for comparison between treatment types (purified CBD vs. CBD-rich *Cannabis* extracts). Data were pooled together in categorical variable format (proportion of patients) for combined analysis. Data from papers using continuous variable format (percentage, or individual frequency reduction) were inferred/estimated and transformed in categorical variable for further analysis. In two cases, the authors were contacted for additional information that could not be inferred from the paper. The transformed data were analyzed statistically by the Fischer test for categorical variables. The data of every paper was organized in tables and plotted in RawGraphs (http://app.rawgraphs.io/) for the scatterplot diagram. Direct comparisons were performed among different epileptic encephalopathies (Lennox-Gastaut patients vs. whole epileptic population; Dravet syndrome patients vs. whole epileptic population) and between "purified CBD" and "CBD-rich extracts" preparations, whenever possible (Fischer test, $p < 0.05$).

As clinical safety outcomes, all reported outcomes of adverse events were considered and grouped afterwards by similarity. The objective data considered were the "frequency of adverse events," categorized according to the symptoms and severity ("mild" or "severe"), according to the description in the original paper.

Clinical Studies Considered in the Meta-Analysis

The systematic search took place in February/2017 and updated on the 13th of December 2017 using the keywords "child" and "epilepsy" or "Dravet" or "Lennox-Gastaut" or "CDKL5" combined with "Cannabis," "cannabinoid," "cannabidiol," or "CBD" resulting in 199 papers. From these, 138 were duplicates and were removed. The remained 61 records were screened and 42 of these studies were excluded. Nineteen (19) papers were assessed for eligibility and 6 papers were excluded due to lack of observational clinical data (ex: preclinical studies). The qualitative assessment of 13 articles resulted in 11 valid references for analysis, with an average impact factor of 8.1 (ranging from 1.4 to 47.8) (**Figure 1**).

All the studies included are fairly recent, published between 2013 and 2017, showing how vivid this subject is in the scientific literature worldwide. Overall, the papers analyzed report observational clinical data from 670 patients, treated with CBD-rich *Cannabis* extracts or purified CBD, with average daily doses between 1 and 50 mg/kg, and duration of treatment from 3 to 12 months (average of 6.5 months), as shown in **Table 1** below.

From the selected studies, six (6) show a retrospective design (with a total of 447 patients) and 5 show a prospective design (with a total of 223 patients). The quality of evidence reported in the papers is a relevant variable: three (3) studies used research based on online questionnaires with family members and caretakers (179 patients) and 8 studies report evidence from proper medical history (491 patients). As for the type of treatment, five (5) studies report data from patients who used purified CBD (223 patients) and 6 studies report data from patients who used *Cannabis* extracts with high CBD content, whose composition is not standardized (466 patients). Noteworthy, these variables don't seem to constitute an obvious bias compromising interpretation of data, since the groups are relatively well balanced. The only remarkable difference is that all studies using purified CBD had a prospective design, while the studies using CBD-rich extracts had a retrospective design. All studies were conducted by medical centers experienced in conducting this type of study, at universities or internationally reputed research centers. Curiously, nine (9) out of the 11 studies were conducted or lead by universities or research centers in the United States (553 patients). One study was conducted in Israel (74 patients) and another in Mexico (43 patients). All studies used a heterogeneous population of epilepsy patients, and the segmentation in specific types of syndromes was eventually done afterwards (**Figure 1**).

The majority of the studied population consisted of children and adolescents, between 1 (one) and 18 (eighteen) years old with treatment-resistant epilepsy (refractory epilepsy), who tried between 4 (four) and 12 (twelve) other medications for 3 (three) years before trying CBD-based treatments. Needless to say, this population constitutes a very hard-to-treat population, diagnosed with treatment-resistant epileptic syndromes. Roughly, this affects one-third of the total population of epileptic patients.

RESULTS

The results of efficacy in the studied population suggest that treatment with CBD-based products significantly reduces seizure frequency, even for this otherwise treatment-resistant population. According to the analysis of "reported improvement," which means, any improvement reported in the selected papers, almost 2/3 of the patients had an observed reduction in seizure frequency (399/622, 64%), with individual studies rate ranging between 37 and 89% (**Table 2**; **Figure 2**). Notably, 6 out of 11 studies showed over 80% of the patients reporting improvement. There was a higher number of patients reporting improvement after using CBD-rich *Cannabis* extracts (318/447, 71%) than those treated with purified CBD (81/175, 46%), with valid statistical significance ($p < 0.0001$).

However, when the clinical threshold of "reduction of 50% or more in seizure frequency" was evaluated, only 39% of the individuals were considered responders (studies varying between 24 and 74%), and there was no difference ($p = 0.52$) between treatments with CBD-rich extracts (122/330, 37%) and purified CBD (94/223, 42%). The mean dose, regardless of treatment was 15.0 mg/kg/day of CBD equivalent. The average daily dose reported for purified CBD was 25.3 mg/kg/day, while the average daily dose of CBD equivalent reported for CBD-rich *Cannabis* extract was merely 6.0 mg/kg/day.

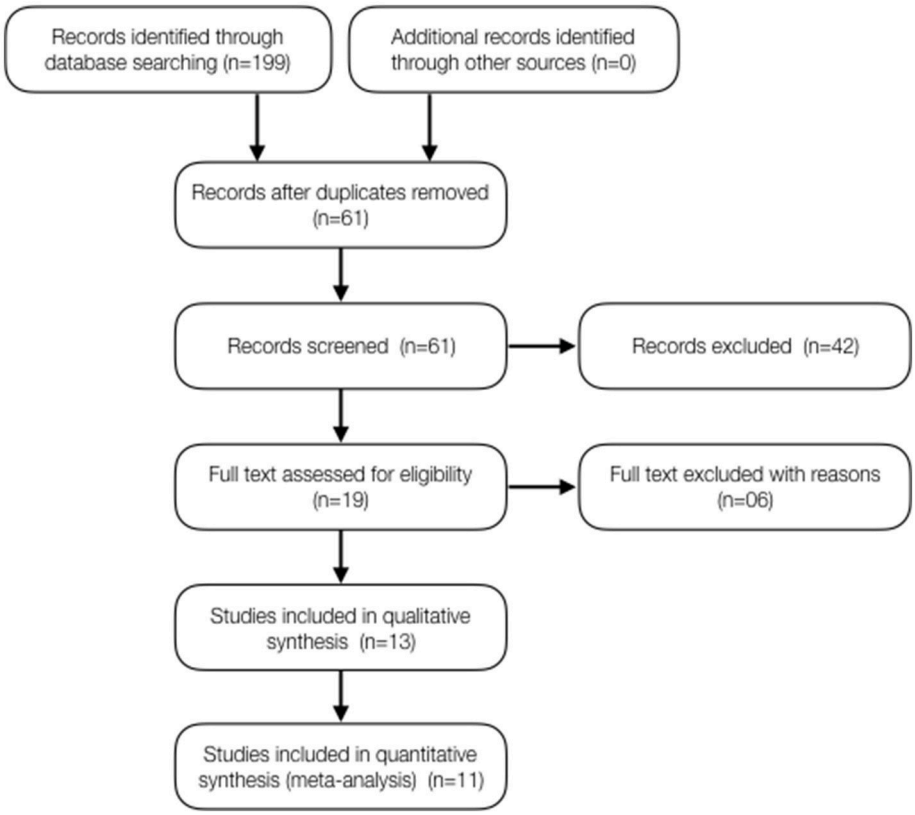


FIGURE 1 | FLOW diagram of the reference searches.

TABLE 1 | Information about the clinical studies included as valid reference in the current meta-analysis.

Treatment references	Medical center	Study design	Patients	Age (year)	Duration
CBD pure (6)	Langone Med. Center NY Univ. (USA)	Prospective medical record	137	10.5 year (1–30)	3 months
CBD pure (7)	Child Neurology, Child Hosp. Philadelphia (USA)	Prospective medical record	7	NR	12 months
CBD pure (8)	Pediatric Epilepsy, Mass. Gen. Hospital-Harvard (USA)	Prospective medical record	13	10.8 year (4–19)	2 months
CBD pure (9)	Pediatric Epilepsy, Mass. Gen. Hospital-Harvard (USA)	Prospective medical record	18	14 year (2–31)	∼9 months (6–12+)
CBD pure (10)	Langone Med. Center NY Univ. (USA)	Prospective medical record	48	11.7 year (1–30)	3 months
CBD-rich extract (11)	Neurology, Stanford (USA)	Parent survey (online)	19	6.2 year (2–16)	8 months
CBD-rich extract (12)	Pediatric Neurology, Univ. Calif. Los Angeles-UCLA (USA)	Parent survey (online)	117	6 year (0.4–NR)	6.8 months
CBD-rich extract (28)	Pediatrics and Neurology, Univ. Colorado (USA)	Retrospective medical record	75	7.3 year (0.5–18)	5.6 months
CBD-rich extract (13)	Pediatric Neurology, Sheba Medical Center (Israel)	Retrospective medical record	74	∼10 year (1–18)	6 months
CBD-rich extract (14)	Inst. Tec. Est. Sup. Monterrey (Mexico)	Parent survey (online)	53	∼9.4 year (0.8–18)	4.2 months (1–12)
CBD-rich extract (15)	Pediatrics and Neurology, Univ. Colorado (USA)	Retrospective medical record	119	7.5 year (0.1–18)	11.7 months

Average treatment duration.

TABLE 2 | Efficacy of treatments in the reduction of convulsive seizures (heterogeneous population).

References	Patients	Reported improvement	>50%	>70%	Mean daily dose (mg/kg/day)
Total reports	**670**	**399/622**	**216/553**	**83/311**	**(2–50 mg/kg)**
Mean	**100%**	**64%**	**39%**	**27%**	**15.0 mg/kg**
CBD pure (6)	137	37%	37%	22%	22.9 mg/kg
CBD pure (7)	7	86%	71%	57%	22 mg/kg
CBD pure (8)	13	85%	70%	46%	24.6 mg/kg
CBD pure (9)	18	72%	50%	22%	37.7 mg/kg
CBD pure (10)	48	NR	42%	NR	28.2 mg/kg
CBD-rich extract (11)	19	84%	74%	42%	7.0 mg/kg
CBD-rich extract (12)	117	85%	NR	NR	4.3 mg/kg
CBD-rich extract (28)	75	57%	33%	NR	NR
CBD-rich extract (13)	74	89%	34%	18%	<10 mg/kg
CBD-rich extract (14)	43	83%	67%	42%	3.2 mg/kg
CBD-rich extract (15)	119	49%	24%	NR	NR

Endpoints: any improvement reported, improvement > 50% ("clinical responder") and >70%, and average dose reported. NR, not reported; ?, inconclusive.

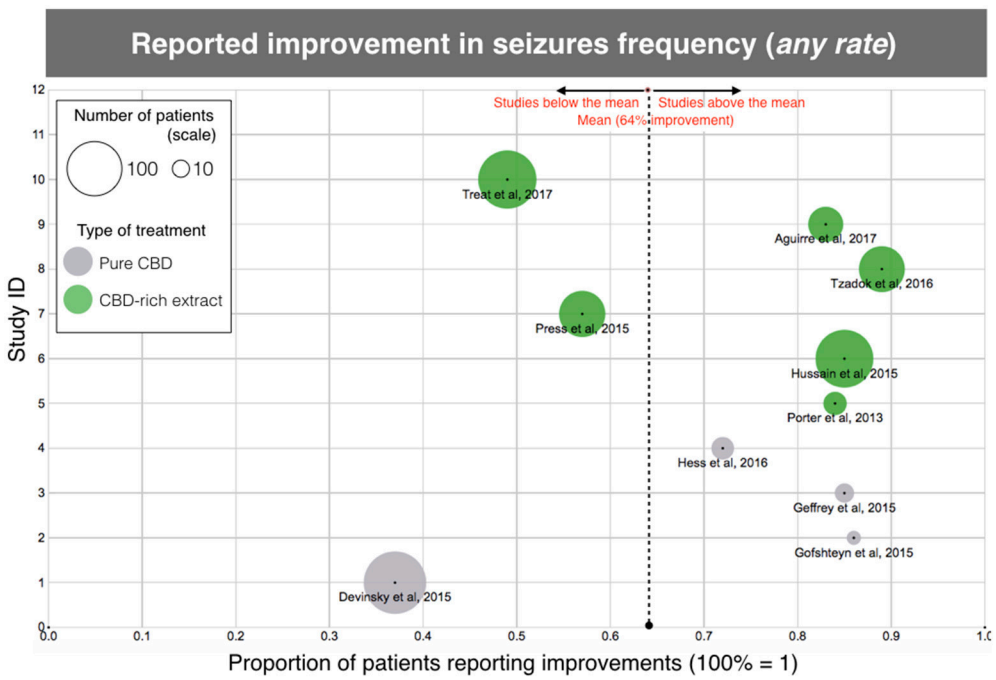


FIGURE 2 | Scatterplot diagram of treatment efficacy, according to the data of "clinical improvement" reported in the aforementioned clinical studies. The X-axis represents the rate of clinical improvement (from 0 to 1, 100% = 1). The Y-axis is arbitrary "Study ID." The size of each point represents the number of patients included in the study and gives an idea of the "weight" of each study. The dotted line is the average, regardless of treatment. Each type of treatment (Purified CBD vs. CBD-rich extracts) is coded with a different color, according to the legend. Same data as **Table 1**, except for one study (10) due to unreported data.

Moreover, there was no difference among subtypes of epileptic encephalopathies (Dravet and Lennox-Gastaut syndromes), although the data implies that patients from these two genetic-related epileptic syndrome are more sensitive to CBD treatment (**Supplementary Table 1**). At least 27% of all treated patients showed an "improvement >70%" in seizures frequency (83/311 patients) and the studies varied between 18 and 57% (**Table 2**). The "seizure free" endpoint was not used for the analysis because it is not a parameter used by a significant amount of the selected studies. The number of individuals that remained free of seizures was close to 10% in the papers reporting this endpoint, which is a relevant amount for a population that tried several prior anti-epileptic medications without success. The proportion of patients reporting any improvement and "classical" responders was also

TABLE 3 | Positive secondary effects* of treatment with CBD-rich *Cannabis* extracts and purified CBD described as secondary endpoints in the clinical studies.

Treatment references	Patients	Mood	Alertness	Behav.	Sleep	Language	Motor
Total reports	**508**	**151/226**	**275/508**	**91/224**	**150/447**	**51/149**	**28/268**
Mean	**100%**	**67%**	**54%**	**41%**	**34%**	**34%**	**10%**
CBD pure (9)	9/14	NR	86%	67%	NR	NR	NR
CBD pure (10)	47	57%	68%	42%	NR	67%	NR
CBD-rich extract (11)	19	79%	74%	32%	68%	NR	NR
CBD-rich extract (12)	117	63%	71%	NR	53%	NR	NR
CBD-rich extract (28)	75	NR	33%	33%	7%	11%	11%
CBD-rich extract (13)	74	NR	34%	34%	11%	15%	15%
CBD-rich extract (14)	43	83%	89%	NR	77%	NR	NR
CBD-rich extract (15)	119	NR	39%	NR	7%	NR	8%

**Only those reported by at least 5% of the study population are listed. NR, not reported.*

relatively high for a treatment-resistant population and is slightly above the number of responders commonly observed in studies of registered anti-epileptic drugs (29–31).

Beyond the direct therapeutic effect of CBD in reducing epileptic seizures, reports about improvement in "secondary" health aspects were very common. They shall not be negligible, since they provide a significant improvement in quality of life for the patients and their family members. Secondary endpoints were reported for 285 patients in the selected papers. Unfortunately, not all studies considered such endpoints during their development. The main secondary effects were improvements in awareness (147/285, 52%), quality of sleep (88/285, 31%), mood (87/285, 30%), behavior/aggression (56/285, 20%), language/cognition (19/285, 7%), and motor skills (19/285, 7%) (**Table 3**). There were also reports of other improvements, but for the sake of the current meta-analysis, only those affecting at least 5% of the studied population were considered. Arguably, these effects occurred as a consequence of the seizures reduction, but in many cases they occurred before or even in the absence of significant reductions of epileptic seizures (considering each case individually). There were no reports of secondary health aspects in studies of purified CBD (6–10). However, it's impossible to conclude that no improvements on secondary endpoints occurred with this type of treatment; rather, it is more likely that the study didn't focus on this clinical phenomenon. As demonstrated in one of the studies with 117 patients (12), in a direct comparison of the same population, the conventional antiepileptic drugs caused an improvement in these "secondary" parameters related to quality of life, but this effect occurred in a smaller scale than with CBD-based treatments. This is true, at least, for the effects in mood improvement, awareness, sleep quality, and self-control. This data suggests that secondary positive events described for CBD are attributed to this substance, and not only due to the reduction in the frequency of seizures (**Table 3**).

Although treatment with CBD products is regarded as at least equally safe in comparison to regular anti-epileptic drugs, CBD is not devoid of adverse effects (11). The studies mention the occurrence of adverse events on a relatively large portion of the population studied (217/422, 51%), even though the great majority of events are considered "mild." Severe events were reported by a smaller portion of patients (64/422, 15%) (11). Importantly, in this case, we are considering only patients of studies that mentioned the occurrence of adverse effects. To improve accuracy, if the study did not mention adverse events, we considered that it was not reasonable to assume that there were no adverse events and, therefore, the whole study was excluded of the analysis. Two studies containing only 20 patients were excluded according to this criterion (7, 8). Counter-intuitively, there is also an advantage of CBD-rich extracts in relation to purified CBD regarding the occurrence of adverse events. The reports of mild (158/216, 76% vs. 148/447, 33%, $p < 0.001$) and severe (41/155, 26% vs. 23/328, 7%, $p < 0.0001$) adverse effects were more frequent in products containing purified CBD that in CBD-rich extracts. The most common adverse events reported were appetite alteration, sleepiness, gastrointestinal disturbances/diarrhea, weight changes, fatigue, and nausea. Uncommon or rare adverse events include thrombocytopenia, respiratory infections and alteration of the liver enzymes (**Table 4**).

DISCUSSION

The present meta-analysis study confirms the anecdotal evidence that CBD treatment improves seizure control in patients with treatment-resistant epilepsy. Pooled together, the data from 11 studies provide strong evidence in support of the therapeutic value of high-CBD treatments, at least as far as this population of 670 patients is regarded. Important to say, not every study reported all the clinical parameters (e.g., % of responders, side effects, quality of life endpoints, etc.), therefore, the analysis might be skewed in some way that it's impossible to account for. The difference in the quality of the studies is also an important limitation that should be taken into consideration.

This said, it's clear that CBD works for this type of epilepsy, with over 60% of volunteers describing clinical improvement and nearly 40% being clinical responders at a hard threshold of

over 50% reduction in seizure frequency (**Table 2**, this study). With the observational non-blinded design, it's impossible to quantify how much of this response would be due to placebo effect. It's common to see placebo effects ranging from 15 to 25% in well-conducted epilepsy studies (24, 25), but a recent clinical study surprisingly showed a placebo effect as high as 40% (32). This might suggest a big impact of the belief in the current "fashionable" therapy using cannabinoids on reported therapeutic responses. More objective physiological measures would help to improve accuracy and are welcome in cannabinoid-related clinical studies.

One remarkable observation of this study is the difference in average dose reported by patients taking "plant-based" and "purified" CBD treatments. Curiously, even though treatment with CBD-rich extracts and purified CBD yielded similar, the patients treated with CBD-rich extracts reported a significantly lower average daily dose than patients using purified CBD. As described in the results section, the average dose described by patients taking CBD-rich *Cannabis* extracts was over 4 times lower than the dose reported by patients taking purified CBD (**Table 2**). This data suggests that CBD is 4 times "more potent" when administered in herbal form, probably because other minor compounds present in the extract may contribute to its therapeutic effect (33). The interpretation of higher potency of CBD in combination with other minor compounds is in line with previous reports of synergistic effects between cannabinoid and even non-cannabinoid compounds (34).

For instance, King et al. (35) described a clear synergistic effect of the combination between CBD and THC, where THC potentiates CBD effects in a mouse model of neuropathic pain in substantially smaller dose range than when CBD is given alone. This was already described in the classic study by Karniol and Carlini where CBD blocked certain effects of THC: catatonia in mice, corneal arreflexia in rabbits, increased defecation and decreased ambulation in rats in the open field after chronic administration, and aggressiveness in rats after REM-sleep deprivation. In contrast, CBD potentiated THC analgesia in mice and the impairment of rope climbing in rats (36). Similar examples of pharmacological interaction between these cannabinoids were summarized in Russo and Guy (37). Another interesting aspect of cannabinoid pharmacology is that CBD tends to block some of adverse events of THC, like anxiety and paranoia (38). Modern pharmacology suggests that these effects are due to allosteric modulation of CB1 cannabinoid receptors by CBD (39, 40). This means that CBD exerts a "fine-tuning" of the CB1 cannabinoid receptor affecting the interaction of other cannabinoids at the receptor level. Noteworthy, the original description of the physiology of allosteric modulation of these receptors was performed by the main author of the current paper (41). Whether or not this mechanism contributes to the anti-epileptic effects of cannabinoid remain to be established, but preliminary evidence of CBD/THC synergistic interaction in a mouse model of refractory epilepsy were recently reported in the congress of the International Cannabinoid Research Society [Anderson et al. (42) oral presentation in ICRS]. On top of this, minor plant cannabinoids like canabidivivarin (CBDV), tetrahydrocannabivivarin (THCV), and cannabinol (CBN) are also anti-convulsants (43–48).

TABLE 4 | Negative secondary effects of treatment with CBD-rich *Cannabis* extracts and purified CBD described as secondary endpoints in the clinical studies.

References	*n*	Mild AE	Serious AE	Total AE
Total reports	**663**	**308/663**	**64/483**	**326/663**
Mean	**100%**	**46%**	**13%**	**49%**
CBD pure (6)	137	79%	30%	128/137
CBD pure (9)	18	67%	0%	12/18
CBD pure (8)	13	77%	NR	10/13
CBD pure (10)	48	58%	NR	28/48
CBD-rich extract (11)	19	37%	0%	7/19
CBD-rich extract (12)	117	30%	0%	35/117
CBD-rich extract (28)	75	44%	13%	33/75
CBD-rich extract (13)	74	46%	18%	34/74
CBD-rich extract (14)	43	37%	0%	16/43
CBD-rich extract (15)	119	19%	NR	23/119

**Reporting adverse events in a study population does not necessarily mean that it is related to treatment. NR, not reported.*

When it comes to adverse events, the same pattern was true: patients treated with CBD-rich extracts tend to show less adverse events, regardless of its severity. This is counter-intuitive, and we believe that it might be secondary to the dose. Since patients taking CBD-rich extracts reported lower CBD dose, it's reasonable to expect lower side effects, including those related with the oil vehicle itself, like gastrointestinal discomfort or abdominal pain.

The most common adverse events reported were appetite alteration, sleepiness, gastrointestinal disturbances/diarrhea, weight changes, fatigue, and nausea. Uncommon or rare adverse events include thrombocytopenia, respiratory infections, and alteration of the liver enzymes. There was a worsening of the seizure burden in some cases, but this is uncommon and cannot be necessarily attributed to the treatment. Uncommon or rare events reported occurred in combination with other anti-epileptic medication, particularly valproic acid and clobazam, and may be related to drug interaction, and not due to direct CBD toxicity. Data from a recent study suggested that CBD tends to reduce the occurrence of adverse events, in general, when used as an add-on therapy to other anti-convulsants (12). In that cohort with 117 patients, CBD reduced the occurrence of fatigue, sleepiness, irritability, insomnia, appetite loss, aggressiveness, nausea, dizziness, anxiety, confusion, weight loss, vomiting, and obsessive behavior in 5–10 times. Among an extensive list, the only adverse events that actually increased with the addition of CBD were weight gain and increased appetite (about 2 times higher).

In conclusion, this meta-analysis suggests that treatments using CBD are effective and safe, at least in the population of patients with treatment-resistant epilepsy, considering risks, and benefits inherent to the treatment of this severe neurological

condition. A considerable share of patients obtains benefits from this treatment, and the adverse events, when they occur, are fairly mild. Apparently, CBD-rich *Cannabis* extracts are more potent and have a better safety profile (but not higher efficacy) than products with purified CBD. The lack of standardization among *Cannabis* extracts does not allow us to infer which characteristics of the product provide this therapeutic advantage. However, considering the scientific literature describing the "entourage effect" in plant compounds (34–37) and in the endocannabinoid system (41, 49), it's reasonable to suggest that the higher potency of the CBD-rich *Cannabis* extracts over purified CBD is related to other plant compounds acting synergistically to CBD, as discussed above. Controlled studies with standardized *Cannabis* extracts are necessary to confirm if these compounds contribute *per se* or synergistically for the anticonvulsive effect of *Cannabis* and its derivatives.

AUTHOR CONTRIBUTIONS

FP gathered data, analyzed results, and wrote the manuscript. LdS and AC helped with data organization and manuscript writing.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2018.00759/full#supplementary-material

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Anandamide Effects in a Streptozotocin-Induced Alzheimer's Disease-Like Sporadic Dementia in Rats

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Alzheimer's disease (AD) is characterized by multiple cognitive deficits including memory and sensorimotor gating impairments as a result of neuronal and synaptic loss. The endocannabinoid system plays an important role in these deficits but little is known about its influence on the molecular mechanism regarding phosphorylated tau (p-tau) protein accumulation – one of the hallmarks of AD –, and on the density of synaptic proteins. Thus, the aim of this study was to investigate the preventive effects of anandamide (*N*-arachidonoylethanolamine, AEA) on multiple cognitive deficits and on the levels of synaptic proteins (syntaxin 1, synaptophysin and synaptosomal-associated protein, SNAP-25), cannabinoid receptor type 1 (CB_1) and molecules related to p-tau degradation machinery (heat shock protein 70, HSP70), and Bcl2-associated athanogene (BAG2) in an AD-like sporadic dementia model in rats using intracerebroventricular (icv) injection of streptozotocin (STZ). Our hypothesis is that AEA could interact with HSP70, modulating the level of p-tau and synaptic proteins, preventing STZ-induced cognitive impairments. Thirty days after receiving bilateral icv injections of AEA or STZ or both, the cognitive performance of adult male Wistar rats was evaluated in the object recognition test, by the escape latency in the elevated plus maze (EPM), by the tone and context fear conditioning as well as in prepulse inhibition tests. Subsequently, the animals were euthanized and their brains were removed for histological analysis or for protein quantification by Western Blotting. The behavioral results showed that STZ impaired recognition, plus maze and tone fear memories but did not affect contextual fear memory and prepulse inhibition. Moreover, AEA prevented recognition and non-associative emotional memory impairments induced by STZ, but did not influence tone fear conditioning. STZ increased the brain ventricular area and this enlargement was prevented by AEA. Additionally, STZ reduced the levels of p-tau (Ser199/202) and increased p-tau (Ser396), although AEA did not affect these alterations. HSP70 was found diminished only by STZ, while BAG2 levels

were decreased by STZ and AEA. Synaptophysin, syntaxin and CB_1 receptor levels were reduced by STZ, but only syntaxin was recovered by AEA. Altogether, albeit AEA failed to modify some AD-like neurochemical alterations, it partially prevented STZ-induced cognitive impairments, changes in synaptic markers and ventricle enlargement. This study showed, for the first time, that the administration of an endocannabinoid can prevent AD-like effects induced by STZ, boosting further investigations about the modulation of endocannabinoid levels as a therapeutic approach for AD.

Keywords: endocannabinoid, CB_1, HSP70, BAG2, phosphorylated tau, fear conditioning, object recognition memory, prepulse inhibition

INTRODUCTION

Alzheimer's disease (AD) is characterized by multiple cognitive deficits, such as impairments of sensorimotor gating and emotional, spatial and working memory (España et al., 2010; Santos et al., 2012; Yassine et al., 2013; Cheng et al., 2014b). Besides the presence of amyloid plaques, accumulation of phosphorylated tau (p-tau) protein is one of the pathological hallmarks of AD being responsible for the destabilization of neuronal microtubules (Kadavath et al., 2015) and neuronal death (Pristerà et al., 2013). This accumulation might occur due to failures in the p-tau degradation machinery such as ubiquitin-independent proteasome pathway, which is mediated by heat shock protein 70 (HSP70) and Bcl2-associated athanogene 2 (BAG2). Although less usual, this later pathway is remarkably efficient and is disrupted during AD (Petrucelli et al., 2004; Carrettiero et al., 2009). BAG2 is also involved in several physiopathological mechanisms related to AD such as thermoregulation (de Paula et al., 2016), nicotinic receptors activation (de Oliveira et al., 2016), and the effects of $A\beta_{1-42}$ on cell viability (Santiago et al., 2015).

HSP70 does not only play a role on tau degradation but is also involved in other mechanisms of neuroprotection, and it has been shown to interact with the endocannabinoid system. HSP70 has a high affinity for anandamide (N-arachidonoylethanolamine – AEA), functioning as a cytosolic carrier of this endocannabinoid (Oddi et al., 2009). Under pathological conditions, the enhancement of AEA levels promotes an increase in HSP70 levels and reduces dendritic loss and amyloid deposition (Tchantchou et al., 2014).

AEA (and all the endocannabinoid system) is recognized to influence the progress of AD, by regulating neurogenesis, cognitive and neuroinflammatory processes during senescence (Koppel and Davies, 2008; Marchalant et al., 2012; Bedse et al., 2014). Decreased levels of AEA have been found in the brain of AD transgenic mice and patients with AD, and were correlated with the cognitive deficits of the subjects (Jung et al., 2012; Maroof et al., 2014). Interestingly, the increase in AEA levels *in vitro* was reported to reduce tau phosphorylation through the inhibition of the activity of protein kinases (Lin et al., 2016).

In the course of AD, changes occur in the enzymatic pathways of endocannabinoids synthesis (Mulder et al., 2011) and degradation (Pascual et al., 2014) as well as in the density of cannabinoid receptor type 1 receptors (CB1R) (Farkas et al., 2012). The decreased density of CB1R, which is mostly located in synapses (Bouskila et al., 2012), is accompanied by a lower density of different synaptic markers (Canas et al., 2014), also observed in AD patients (Shimohama et al., 1997), in accordance with the hypothesis that AD begins as a synaptic dysfunction (Selkoe, 2002).

Although sporadic AD (SAD) (multifactorial AD) is the most prevalent form of the pathology, corresponding to 95% of all AD cases (Lecanu and Papadopoulos, 2013), most of the above cited studies were performed using transgenic animal models, that are more correlated to familial AD observed in humans. Deficits of non-emotional memories were widely explored using SAD models, by classical memory tasks such as the new object recognition (NOR) and Morris' water maze (Santos et al., 2012; Yassine et al., 2013) as well as non-associative emotional memories, evaluated by the escape latency in the elevated plus maze (EPM) (Sachdeva et al., 2014). However, impairments of associative emotional memories – classical fear conditioning (España et al., 2010) and sensorimotor gating (PPI) (Cheng et al., 2014b; Koppel et al., 2014) were mostly investigated in transgenic AD animals.

Intracerebroventricular (icv) injection of streptozotocin (STZ) is a SAD model based on brain resistance to insulin (Mayer et al., 1989; Grünblatt et al., 2007; Espinosa et al., 2013; Salkovic-Petrisic et al., 2013), which mimics many of physiopathological aspects of SAD in human, like memory impairment, changes of glucose metabolism, oxidative stress and phosphorylation of tau protein (Santos et al., 2012; Peng et al., 2013; Yang et al., 2014).

The modulation of the endocannabinoid system in the development of SAD was not investigated yet, but using STZ intraperitoneally as a model for neuropathic diabetes, it was observed an increase in the hippocampal levels and activation of CB1R (Duarte et al., 2007). The endocannabinoid system seems to be involved in cerebral glucose metabolism as well, given that CB2R participate in neuronal glucose uptake, mediated by AEA (Köfalvi et al., 2016). In accordance with the tight connection between glucose metabolism and AD, which is argued to be a type 3 diabetes (De Felice, 2013; Ahmed et al., 2015), this STZ-induced AD-like sporadic dementia model seems suitable to study the endocannabinoid modulation of cognitive and molecular aspects of SAD.

Therefore, our main objective was to explore cognitive processes that are disrupted in SAD and also to investigate the effects of AEA on cognitive impairments induced by icv-STZ administration and alterations of different synaptic markers

(syntaxin, synaptophysin and SNAP-25) and p-tau degradation mechanisms in the hippocampus (p-tau, HSP70, and BAG2 levels). Given the physical interaction between AEA and HSP70, our hypothesis is that AEA could modulate HSP70 levels, exerting neuroprotective effects on p-tau degradation, neuroplasticity and cognitive changes induced by STZ.

MATERIALS AND METHODS

Animals and Drug Treatments

Male Wistar rats aged 3–4 months (350–400 g) were obtained from the Animal Experimentation Laboratory of National Institute of Pharmacology of the Federal University of São Paulo (LEA/INFARUNIFESP). Animals were housed in groups of four per cage and maintained under controlled temperature (23 ± 2°C), light/dark period of 12/12 h and with free access to food and water. All procedures were in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA) and were approved by the Committee on Ethics in Animal Use of Federal University of ABC (CEUA–UFABC), protocol 008/13.

In our STZ-induced AD-like sporadic dementia model, rats were first anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) and placed in a stereotaxic apparatus. After an incision in the skin, the skull was exposed and two holes were made bilaterally, following coordinates measured from bregma (–0.8 mm on the anteroposterior, ±1.4 mm on the medial-lateral and –3.6 mm on the dorso-ventral axis, in accordance with a rat brain atlas; Paxinos and Watson, 2005).

Both STZ (Sigma) and AEA (Sigma) were diluted in citrate buffer and injected by an automated microinjection system, consisting of a microtube of polyethylene (PE-10) attached to an injection needle and to a microsyringe (Hamilton) coupled to an infusion pump (Model Bi2000 – Insight Equipment LTDA).

Each animal received two icv bilateral injections of 2 μL (1 μL/min): the first one was citrate (vehicle) or AEA (100 ng) (Fraga et al., 2009), followed by another injection of vehicle or STZ (2 mg/kg) (Motzko-Soares et al., 2018) 5 min later. After each infusion, the injection needle remained for at least 2 min in place, in order to prevent reflux of the administered solution. After surgery, animals received intramuscular injections of an antibiotic (pentabiotic, 24,000 UI/kg) and an anti-inflammatory and analgesic (meloxicam, 1 mg/kg). Also, 2 mL of saline solution were administered subcutaneously for rehydration and the animals were maintained in thermal blankets with controlled temperature until the recovery. Animals were kept in individual home cages for 5 days after surgery and body weight, food, water intake and animals well-being were accompanied daily.

Before and after surgery, animals were weighted and their blood glucose was measured. Thirty days after surgery, one set of animals (n = 12–16 per group) was submitted to the NOR task (days 30–31), followed by contextual and tone fear conditioning (CFC and TFC, respectively) (days 34–36) and another set (n = 10–11 per group) was submitted to escape latency in the EPM (days 30-31), followed by pre-pulse inhibition (days 34–35). Cages and objects were always cleaned with 15% ethanol in between testing. After all behavioral tests, animals were euthanized and their brains were removed for histological or Western blotting analysis (**Figure 1**).

New Object Recognition (NOR)

Hippocampus-dependent non-emotional memory was examined using the NOR task, using an adapted protocol after standardization tests for short-term memory evaluation (Ennaceur and Delacour, 1988; Carey et al., 2009; Botton et al., 2010; Espinosa et al., 2013). In the first phase (habituation; day 30), animals were allowed to freely explore a circular arena (60 cm in diameter) for 10 min; during training phase II (day 31), objects A and B (a pair of identical LEGO® towers) were placed on opposite sides of the arena 1 cm from the walls and finally in phase III, performed 10 min later, animals returned from their home cage to the arena, where object B was replaced by a new object C (a plastic bottle) placed at the same position.

Experiments were recorded by a camera positioned on the ceiling for latter analysis of the videos. Animal's movement throughout the arena was analyzed by Ethovision software (Noldus Information Technology Inc., Leesburg, VA, United States) and in the training and test sessions, the time that animals spent exploring each object was manually recorded. The exploration of each object was defined as the period in which the animal remained in physical and visual contact with the object (or no more than 0.5 cm far from the object), with frontal and active exploration (sniffing or manipulation, for example). The recognition index was calculated by the formula: [Time exploring object C or B/ (Time exploring object A + Time exploring object C or B)] in the training and the test sessions to evaluate the short-term recognition memory performance. In healthy animals, a higher recognition index is expected in the test compared to the training session, indicating that the animals learned the task.

Contextual and Tone Fear Conditioning (CFC and TFC)

To evaluate associative emotional memory, animals were submitted to CFC and TFC training in conditioning box (Med-Associates, Inc., St. Albans, VT, United States), following a protocol adapted from previous studies (Blanchard and Blanchard, 1969; LeDoux, 1993; Wood and Anagnostaras, 2011; Bueno et al., 2017). The training of CFC and TFC and also the CFC test (days 34 and 35, respectively) were carried out in the context A, consisting of a conditioning box with striped walls, grid floor and bright light. TFC test (day 36) was performed in context B, a box with semicircular white walls, flat floor, light off and cleaned with acetic acid 30%. During training, after 120 s of habituation, a tone (2 kHz, 90 dB) was emitted for 30 s and in the last second, a foot-shock (1 s, 1 mA) was delivered (unconditioned stimulus-US). One minute later, the animal was placed back in its home cage.

For CFC test, animals were re-exposed 24 h after training to the context A for 240 s, without any other stimulus. During TFC test animals were placed 24 h after CFC test the context B and 2 min after habituation, the conditioned stimulus (tone)

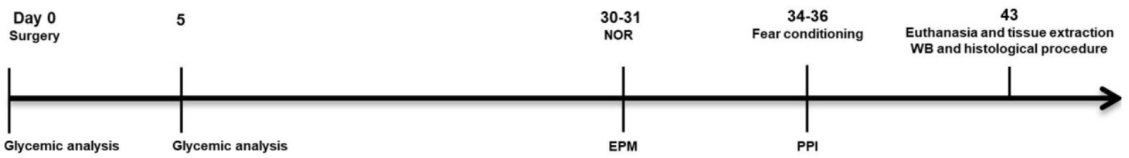


FIGURE 1 | Timeline of the experimental design. Thirty days after receiving AEA (100 ng) and/or STZ (2 mg/kg), rats were submitted to behavioral tasks to detect early deficits of emotional and non-emotional memories and sensorimotor gating. One week after the end of behavioral tasks, the animals were sacrificed for either neurochemical analysis of their hippocampi by Western Blotting (WB) or histological analyses of their brains.

was presented twice, for 30 s after 120 s and for 30 s after 180 s (adapted from Simões et al., 2016). During all sessions, the animals' behavior was video-taped for latter analysis using the Video Freeze software (Version 1.12.0.0, Med-Associates). The freezing time in test sessions was measured as a parameter of fear memory retention.

Escape Latency in the Elevated Plus Maze (EPM)

The EPM used in the present study was made of white wood and consisted of a central square (10 × 10 cm) from which four arms (10 × 50 cm each) radiated outward, two of them with walls around the edge 40 cm high (closed arms), and two of them without walls (open arms). The arms were arranged so that the two open arms were opposite to each other. Arms were elevated to a height of 55 cm off the floor. The EPM is classically used for evaluation of locomotion and anxiety, but a modified version of the EPM have been also used for testing memory (Sachdeva et al., 2014; Hill et al., 2015; Mutlu et al., 2015). The experimental procedure was carried out as described by Sachdeva et al. (2014), allowing also the study of a memory parameter, measuring the retention of latency to escape to a closed arm in the test, compared to the training session.

In the training session (day 30), each animal was placed in an open arm, facing outwards and was allowed to explore the EPM for 90 s. The time taken by the rat to enter in a closed arm for the first time was measured (initial escape latency – L1) and movement parameters were recorded by a camera and analyzed by Ethovision software (Noldus Information Technology Inc., Leesburg, VA, United States). During test (day 31), animals were allowed to explore the EPM, identically as in the training session and the final escape latency was recorded (L2). The retention of escape latency [(L2/L1) × 100] represents the learning of non-associative emotional memory, related to the natural tendency of rodents to prefer dark and closed places.

Prepulse Inhibition (PPI)

Two startle chambers were used to perform the PPI test (SR-LAB, San Diego Instruments, San Diego, CA, United States). Each box contained a loudspeaker located 24 cm above a Plexiglas cylinder fixed on a platform. The startle responses were detected within the cylinder and digitized by a piezoelectric accelerometer positioned below this platform. The startle amplitude was calculated by the mean of 100 samples during the first 100 ms after the onset of the stimulus (1/ms) and presented in arbitrary units (AU). Five days before to surgery, the animals were submitted to a PPI matching session and homogeneously distributed among the groups based on their natural percentage of PPI.

After surgery (day 34 or 35), animals were submitted to a new PPI session identical to the matching procedure (adapted from Rodrigues et al., 2017). The test session started with an acclimatization period of 5 min with a constant background white noise (60 dB). Then, the animals were exposed to various combinations of white noise pulses with random intertrial intervals of 10–20 s for 20 min. The stimuli could be one of four types: pulse alone (40 ms, 120 dB), prepulse alone (20 ms, 70, 75, or 80 dB), pulse preceded by prepulse (100 ms interval) or no stimulus, except the background noise. The different types of stimuli were presented pseudorandomly so that no combination was presented twice consecutively. The PPI percentages were calculated by the relation between the means of acoustic startle response (ASR) amplitudes to prepulse-pulse trials to the pulse alone ones. According to the formula: 100 × (ASR to pulse - ASR to prepulse-pulse)/ASR to pulse.

Protein Extraction and Western Blotting

One week after all behavioral tests, animals randomly assigned for Western Blotting analysis were euthanized by decapitation. Their brains were quickly dissected to isolate the hippocampus, which was frozen in liquid nitrogen and stored at –80°C. For protein extraction, hippocampal tissue was homogenized, and proteins were extracted with RIPA buffer (100 mM Tris, pH 7.4, 10 mM EDTA, 10% SDS, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 1 mM DTT, 1 mM PMSF, 1% protease inhibitor cocktail from Sigma-Aldrich, St. Louis, MO, United States).

The preparation of synaptosomes was carried out as previously described (Canas et al., 2009). Briefly, hippocampi were homogenized with ice-cold buffered sucrose solution (pH 7.4, 0.32 M) and then the homogenate was centrifuged at 3,000 × *g* for 10 min at 4°C. The supernatant was collected and centrifuged at 14,000 × *g* for 12 min at 4°C. Next, the supernatants were discarded and the pellets resuspended in 45% Percoll, before being centrifuged (16,000 × *g*, 2 min at 4°C). The top layer was collected and resuspended in Krebs-HEPES-Ringer solution and centrifuged again (16,000 × *g*, 2 min at 4°C). The pellet was finally resuspended in lysis buffer for subsequent analysis of synaptophysin, syntaxin-I, SNAP-25 and CB1R. The quantification of protein levels was performed using

BCA colorimetric assay, using bovine serum albumin (BSA) as standard, to allow equalizing the protein concentration of all samples by adding sample buffer. Samples were then denatured for 20 min at 70°C and stored at –20°C for further use.

Total cell lysates or synaptosomes (10 or 20 μg, depending on the protein) were separated by 12% SDS-PAGE and transferred to nitrocellulose membrane. Membranes were blocked with Tris-buffered saline (TBS, 5% non-fat milk) for 1 h at 20°C, followed by incubation for 24 h at 4°C with the primary antibodies: mouse anti-total tau (1:1,000, Genway), rabbit anti-p-tau Ser199/202 (1:1,000, Sigma), rabbit anti-p-tau Ser396 (1:1,000, Abcam), rabbit anti-BAG2 (1:1,000, Novus Biologicals), rabbit anti-Hsp70 (1:1,000, Sigma), goat anti-CB1R (generously supplied by Ken Mackie, Indiana University), mouse anti-synaptophysin (1:20,000, Sigma), mouse anti-syntaxin-I (1:20,000, Sigma), mouse anti-SNAP-25 (1:20,000, Sigma), mouse anti-α-tubulin (1:10,000, Sigma) and mouse anti-β-actin antibody (1:2,000, Novus Biologicals). After washing with TBS with Tween 20 (TBS-T), membranes were incubated with the appropriate secondary antibody (1:5,000, Thermo Fisher Scientific, Waltham, MA, United States), conjugated to peroxidase, for 2 h at 20°C. Membranes were then washed in TBS-T for 20 min and incubated with chemiluminescent reagent. Quantification of the optical density of the bands was performed using Image Lab 6.0 (Bio-Rad Laboratories, Inc. United States) and values were normalized by β-actin or α-tubulin density and expressed as percentage related to control samples (Veh/Veh).

Perfusion and Histological Procedure

Animals randomly assigned for histological analysis were overdosed with urethane and transcardially perfused initially with phosphate-buffered saline (PBS 0.01 M, pH 7.4) and then with 4% paraformaldehyde in 0.01 M PBS, pH 7.4, for fixation. After perfusion, brains were removed and stored in the same fixative solution for 24 h. Brains were then transferred to a 30% sucrose solution in 0.01 M PBS, frozen in dry ice and stored at −80°C until being sliced in a cryostat (–20°C) into 40 μm coronal sections, which were mounted on gelatinized blades and stained with cresyl violet. Slices were photographed using a light microscope (Carl Zeiss Microscopy, Thornwood, NY, United States) for the qualitative and quantitative analysis of the cross-sectional area of the lateral ventricles (LV), which was used as a general index of alteration of brain morphology, often associated with neuronal damage.

Statistical Analysis

Treatment was considered the categorical factor and one-way ANOVA was used for statistical analysis of movement, anxiety and memory parameters in EPM and fear conditioning training as well as for the measurements of ventricle area and the densities in the Western blotting. Body weight, blood glucose levels, memory parameters in the NOR, CFC, TFC, %PPI, and ASR were analyzed by repeated measures two-way ANOVA. Data were expressed as means ± SEM and Duncan's test was used as *post hoc* in all experiments. Differences were considered statistically significant when $p \leq 0.05$ and all analysis were performed using Statistica 7.0 Stat Soft, Inc. 2004.

RESULTS

Behavioral Analysis

Physiological Parameters

No differences in body weight and plasma levels of glucose were found between the different treatments, before and after surgery (data not shown). This suggests that the impact of icv-administered STZ seems restricted to the brain and does not cause evident peripheral alterations. These lack of alterations also indicate that the recovery from surgery was not affected by the treatments.

AEA Prevents STZ-Induced MEMORY Impairment in the NOR

None of the treatments interfered with the distance traveled and velocity during the habituation phase or with the total time spent exploring both objects in training phase of the NOR test, indicating that AEA and/or STZ did not modify spontaneous locomotion (**Figures 2A,B**) and exploration patterns (**Figure 2C**). Considering recognition memory performance, the interaction between treatment and sessions had a significant effect [$F_{(3,51)} = 3.5611$, $p = 0.020$] and *post hoc* comparisons showed that Veh/Veh, AEA/Veh and AEA/STZ presented a higher NOR index in the test compared to the training session ($p < 0.001$ in all cases), while only Veh/STZ did not show difference between the training and the test sessions ($p = 0.792$). These results indicate that Veh/STZ animals did not learn the task and AEA prevented this cognitive impairment in AEA/STZ animals (**Figure 2D**).

AEA Does Not Alter STZ-Induced Deficits in Fear Memory

The measures of average motion index during the fear conditioning training showed that none of the treatments affected spontaneous locomotion before the delivery of the footshock (**Figure 2E**). The CFC test revealed no differences related to treatment, time or the interaction between treatment and time (**Figure 2F**). In the TFC test, treatment [$F_{(3,\ 54)} = 3.2241$, $p = 0.029$] and time [$F_{(3,\ 162)} = 69.985$, $p < 0.001$] showed significant differences. However, the interaction between these factors was not significant. *Post hoc* comparisons showed that freezing time was increased in the minutes after the tone emission compared to the period before tone ($p < 0.001$), confirming an association between the conditioned and unconditioned stimuli (**Figure 2G**). Further *post hoc* comparisons of treatment effect revealed that Veh/STZ and AEA/STZ presented less freezing time than Veh/Veh ($p = 0.030$ and $p = 0.031$, respectively) indicating that STZ disrupted memory performance and AEA was devoid of effect (**Figure 2G**).

AEA Partially Prevents STZ-Induced Memory Impairment in the EPM

In the EPM test, the spontaneous ambulation measured during training session, was not altered by any treatment (**Figure 3A**) and the same occurred with anxiety-like parameters such as the time spent in the open arms (**Figure 3B**) and the number of total entries (**Figure 3C**). Treatments altered the retention of

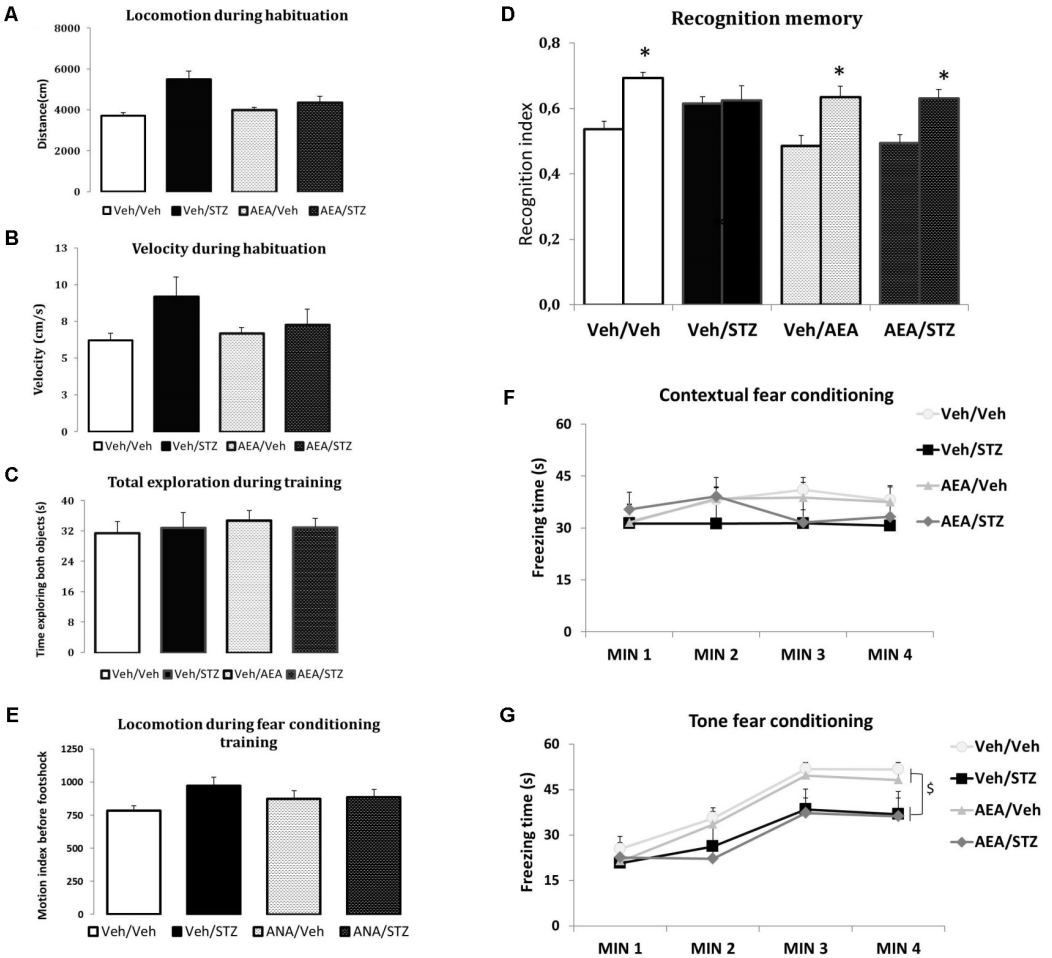


FIGURE 2 | Performance of Wistar rats icv-treated with AEA and/or STZ (Veh/Veh, Veh/STZ, AEA/Veh, and AEA/STZ) in ambulatory **(A,B),** exploratory **(C),** and memory **(D)** parameters in the NOR task carried out 30 days after the drug treatments, and in the CFC **(E)** and TFC **(F)** 34 days after treatment. **(A)** Traveled distance (in cm) and **(B)** velocity (in cm/s) during the habituation session, represented as means ± S.E.M. (n = 7–10 animals per group). No differences were detected between groups (one-way ANOVA). **(C)** Total time exploring objects **(A,B)** during the training and testing phases, represented as means ± S.E.M. (n = 13–14 animals per group). No differences were detected between groups (repeated measures ANOVA). **(D)** Recognition index in the training (first bar of each pair) and in the test (second bar of each pair) (represented as means ± S.E.M. (n = 13–14 animals per group). *$p < 0.05$, training is different from test (repeated measures ANOVA, followed by Duncan's post-test). **(E)** Shows the average motion index during 2 min of habituation in the CFC training, before the delivery of the footshock, presented as means ± S.E.M. (n = 12–17 animals per group). No differences were found between groups (repeated measures ANOVA). **(F)** Shows the freezing time during 4 min in the CFC test, 24 h after training, presented as means ± S.E.M. (n = 12–17 animals per group). No differences were found between groups (repeated measures ANOVA). **(G)** Freezing time during 4 min in the TFC test, presented as means ± S.E.M. (n = 12–17 animals per group). Freezing time of both groups treated with STZ was decreased. $^{\$}p < 0.05$, Veh/STZ and AEA/STZ are different from Veh/Veh and AEA/Veh (repeated measures ANOVA, followed by Duncan's post-test).

escape latency to a closed arm [$F_{(3,\ 39)} = 3.0160$, $p = 0.041$] and *post hoc* comparisons showed significant higher escape latency in Veh/STZ compared to Veh/Veh ($p = 0.021$), but not to AEA/STZ ($p = 0.166$). However, AEA/STZ was not different from Veh/Veh ($p = 0.279$), suggesting that AEA partially attenuated the impairment of non-associative emotional memory caused by STZ (**Figure 3D**).

STZ and/or AEA Did Not Alter Sensorimotor Gating Responses in the PPI

In the PPI test, no effect of treatments on ASR was detected. Data analysis revealed differences only regarding the type of the stimulus [$F_{(3,\ 90)} = 321.11$, $p < 0.001$] and *post hoc* comparisons showed lower ASR level of pulses preceded by prepulses of 70, 75, and 80 dB compared to pulse alone trials ($p = 0.006$, $p < 0.001$, and $p < 0.001$, respectively). The interaction between treatment and type of stimulus was also not significant (**Figure 3E**).

Differences in the percentage of PPI among treatments were also not significant. Yet, different intensities of prepulse were able to induce different %PPI [$F_{(2,\ 60)} = 24.308$, $p < 0.001$] and *post hoc* comparisons indicated that PPI elicited by 70 dB differs from 75 and 80 dB ($p < 0.001$ in both cases) and that the PPI values between 75 and 80 dB also differ ($p = 0.039$). The interaction between treatment and prepulse intensity was not significant (**Figure 3F**). Therefore neither STZ nor AEA modified sensorimotor gating responses.

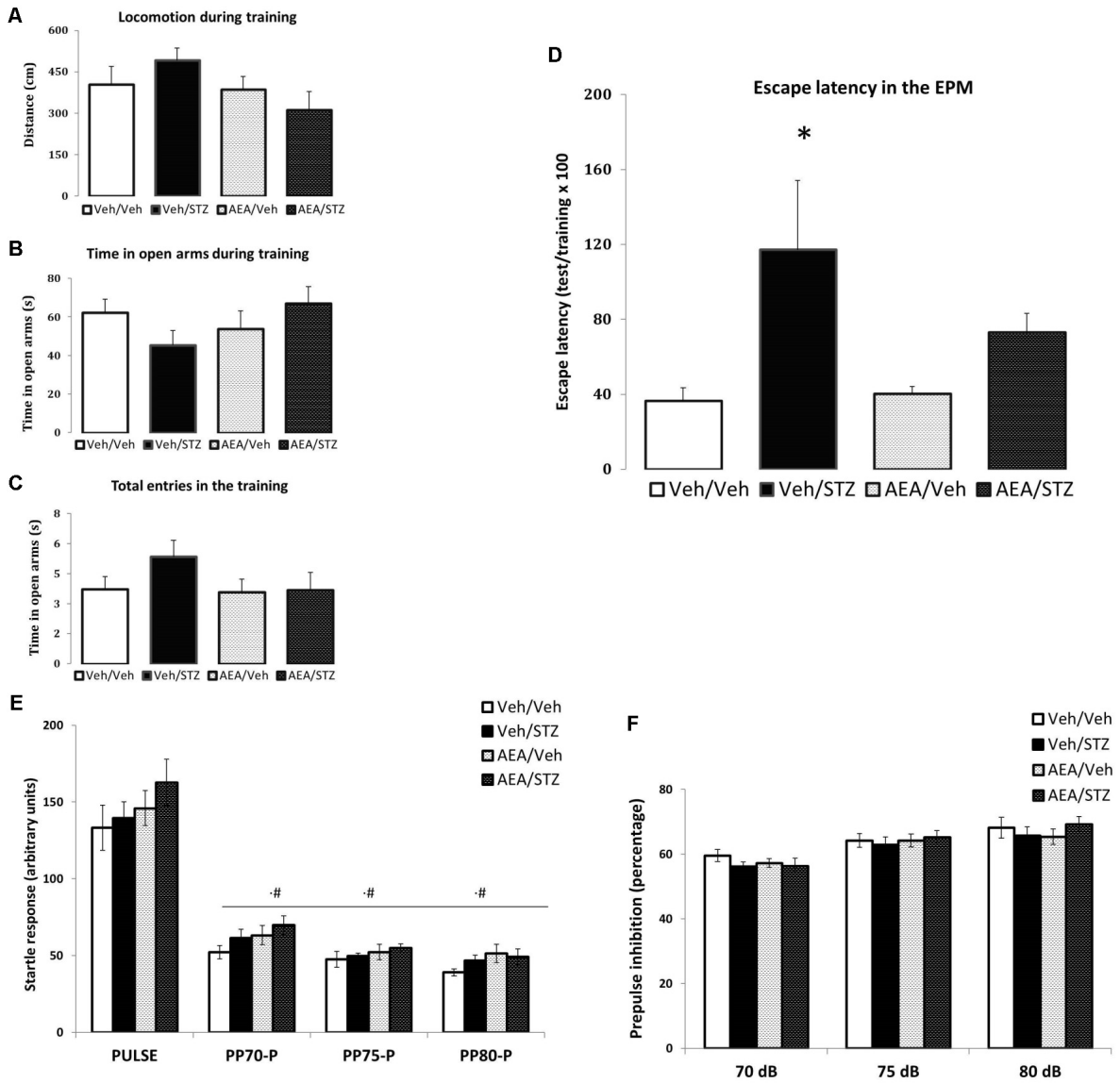


FIGURE 3 | Performance of Wistar rats icv-treated with AEA and/or STZ (Veh/Veh, Veh/STZ, AEA/Veh, and AEA/STZ) in anxiety **(A–C)** and memory **(D)** parameters in the escape latency test in the EPM 30 days after treatment and in startle **(E)** and prepulse inhibition responses **(F)**. **(A)** Distance (in cm), **(B)** time spent in open arms and **(C)** the number of total entries during the training session, represented as means ± S.E.M. (n = 56 animals per group). No differences were detected between groups (one-way ANOVA). **(D)** Percentage of the time needed to enter in one closed arm in the test session compared to training, represented as means ± S.E.M. (n = 11–12 animals per group. *$p < 0.05$, Veh/STZ is different from Veh/Veh (repeated measures ANOVA, followed by Duncan's post-test). **(E)** Startle response after a tone stimulus of 120 dB (pulse) preceded or not by a tone stimulus of 70, 75, or 80 dB (prepulse) presented in a pseudorandomic manner, represented as means ± S.E.M. (n = 7–10 animals per group). #$p \leq 0.05$, ASR response with pulse preceded prepulse of 70, 75, and 80 dB was lower than pulse alone (repeated measures ANOVA, followed by Duncan's post-test). **(F)** Percentage of inhibition of startle response caused by the prepulse + pulse (prepulse inhibition) compared with the response to the pulse alone represented as means ± S.E.M. (n = 7–10 animals per group). No differences were found between treatments.

Histological Analysis of the Area of Lateral Ventricle

The quantitative analysis of the area of the lateral ventricle (LV) (using a protocol adapted from Shoham et al., 2003) (**Figure 4**) showed an effect of the treatment [$F_{(2,9)}$ = 6.3582, p = 0.019] and *post hoc* comparisons detected an enlargement of the LV area in Veh/STZ when compared to Veh/Veh (p = 0.017) and AEA/STZ (p = 0.014). This suggests an ability of AEA to prevent STZ-induced ventricle enlargement, which is an indicative of neuronal loss, a common feature of AD-like sporadic dementia.

Neurochemical Analysis

Levels of Synaptic Proteins and CB1R

The levels of synaptophysin in synaptosomes were affected by treatment [$F_{(3,\ 12)}$ = 3.4400, p = 0.051] and *post hoc* comparisons

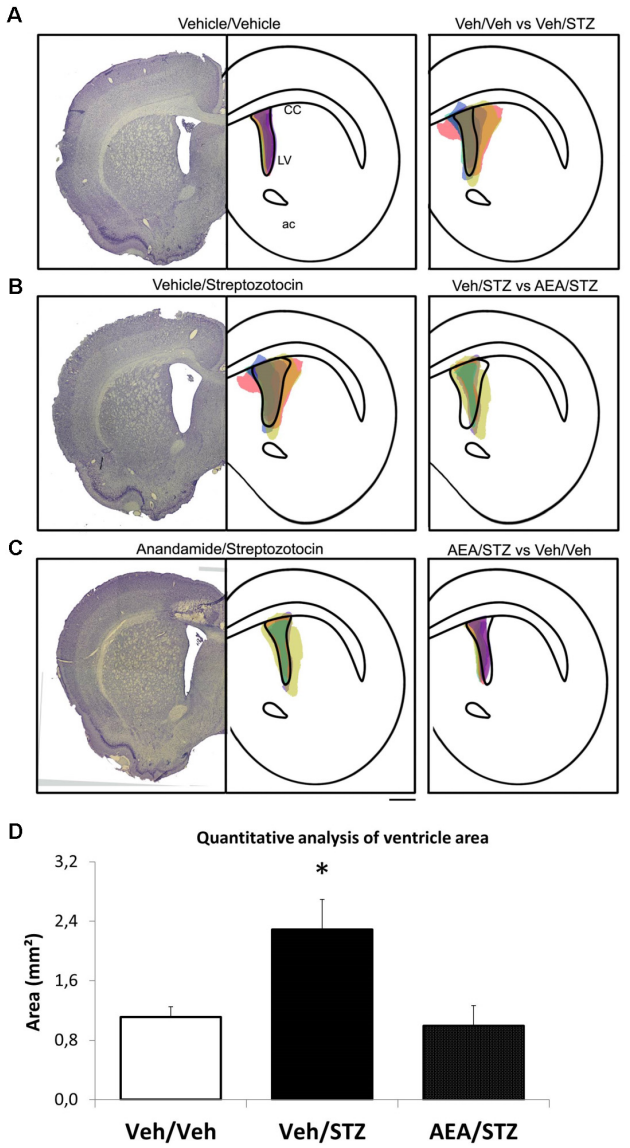


FIGURE 4 | Schematic representation and quantitative analysis of the cerebral ventricular area of Veh/Veh, Veh/STZ, and AEA/STZ animals. The left panel presents a representative histologic image of the lateral ventricle (LV) of the Veh/Veh groups in **(A)**, Veh/STZ in **(B)**, and AEA/STZ in **(C)**. The central panel represents schematically the shape of the histological figure from left, filled with colored spots corresponding to the ventricular area of each of the samples used in the respective groups (n = 3–5). The right panel represents the central figure with the shape of the ventricle of one group overlapped with the ventricular area of the samples from another group (**A**: Veh/Veh × Veh/STZ, **B**: Veh/STZ × AEA/STZ and **C**: AEA/STZ × Veh/Veh). **(D)** shows the quantitative analysis of ventricle area (mm^2), measured + 1.56 mm (anteroposterior) from bregma. *$p < 0.05$, area values are different from Veh/Veh and AEA/STZ (one-way ANOVA, followed by Duncan's post-test).

revealed that Veh/STZ and AEA/STZ display lower levels than Veh/Veh (p = 0.045 and p = 0.048, respectively) (**Figure 5A**). SNAP-25 levels remained unaltered by treatments (**Figure 5B**) while syntaxin levels were reduced [$F_{(3,\ 8)}$ = 7.8587, p = 0.009] in Veh/STZ compared to Veh/Veh (p = 0.003). AEA/STZ rescued this depletion of syntaxin levels, presenting higher optical density values than Veh/STZ (p = 0.007) (**Figure 5C**). Regarding to levels of CB1R, one-way ANOVA did not detect significant differences but given that we observed a tendency for a decrease of CB1R levels only in Veh/STZ compared to Veh/Veh, an analysis with independent samples t-test was performed and confirmed this tendency of difference between these two groups (p = 0.004) and also confirmed that AEA/STZ was not different from Veh/Veh (p = 0.538) and from Veh/STZ (p = 0.379), presenting an intermediary value (**Figure 5D**).

Levels of p-Tau, Total Tau, HSP70, and BAG2

One-way ANOVA revealed a significant effect of treatment on the p-tau Ser199/202/total tau ratio [$F_{(3,\ 8)}$ = 5.8893, p = 0.020] and *post hoc* comparisons showed that p-tau levels were decreased in Veh/STZ, AEA/Veh, and AEA/STZ, compared to Veh/Veh (p = 0.027, 0.006, and 0.017, respectively) (**Figure 5E**). In contrast, p-tau Ser396/total tau ratio remained unaltered (**Figure 5F**). HSP70 levels were also affected by treatment [$F_{(3,\ 8)}$ = 4.3391, p = 0.043] only in Veh/STZ compared to Veh/Veh (p = 0.009) (**Figure 5G**). BAG2 levels were decreased [$F_{(3,\ 8)}$ = 19.412, $p < 0.001$] in Veh/STZ, AEA/Veh and AEA/STZ compared to Veh/Veh (p = 0.025, $p < 0.001$, and $p < 0.001$, respectively). Moreover, AEA/Veh and AEA/STZ groups displayed even lower levels of BAG2 in relation to Veh/STZ (p = 0.004 and p = 0.015, respectively), showing that AEA *per se* decreased BAG2 levels (**Figure 5H**). Please see **Supplementary Figure 1** to check complete bands of all the proteins analyzed in this study.

DISCUSSION

The main finding of the present study is that AEA prevented memory impairments induced by STZ in two memory tasks. These tasks, especially NOR, are commonly used to validate the behavioral impairment in STZ-treated rats. Indeed, previous studies (Espinosa et al., 2013; Bassani et al., 2017), observed deficits of NOR and object location recognition 28 days after administration of icv-STZ (3 mg/kg) with different intervals between training and test sessions. Considering the evaluation of the escape latency as a parameter of emotional memory in the EPM, Sachdeva et al. (2014) and colleagues found STZ effects similar to our study: using also an interval of 24 h between acquisition and retention sessions, animals presented greater escape latency 20 days after icv-STZ (3 mg/kg) compared to control. Moreover, no alterations were found in our study regarding locomotion and anxiety-like parameters in the open field, EPM or fear conditioning training. A tendency for an increase in several anxiety-like behaviors was observed only in the Veh/STZ group, but it did not reach statistical significance.

Although several molecular alterations of AD arise only months after STZ administration (Knezovic et al., 2015), the deterioration of some types of memory seems to emerge even earlier in this model. Indeed different studies reported an impairment of spatial working memory in the Morris' water maze (MWM) 3 h and 15 days after icv administration of STZ (3 mg/kg) (Santos et al., 2012; Sachdeva et al., 2014).

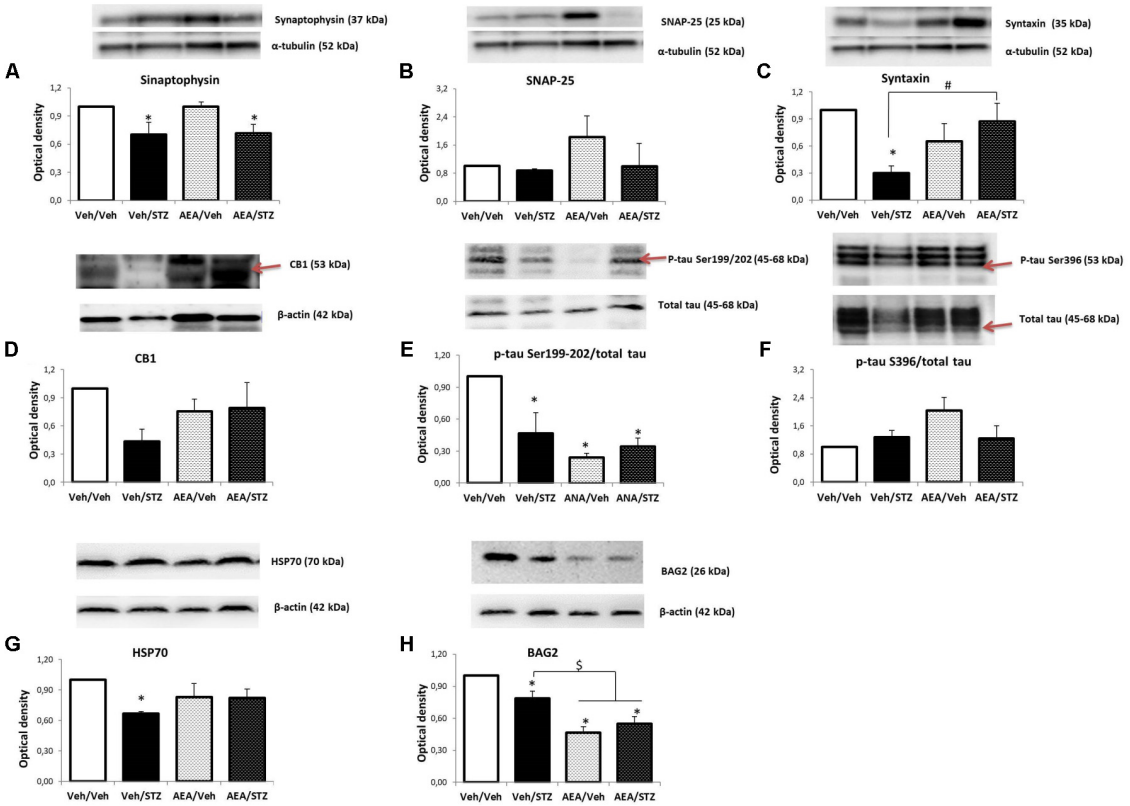


FIGURE 5 | Levels of synaptophysin **(A)**, SNAP-25 **(B)**, syntaxin **(C)**, CB1 receptor **(D)**, p-tau Ser199/202/total tau **(E)**, p-tau Ser396/total tau **(F)**, HSP70 **(G)**, and BAG2 **(H)** in the hippocampus of Wistar rats icv-treated with AEA and/or STZ (Veh/Veh, Veh/STZ, AEA/Veh, and AEA/STZ), quantified by Western blotting. Synaptophysin, SNAP-25 and syntaxin levels were normalized by α-tubulin levels. CB1 receptor, HSP70 and BAG2 levels were normalized by β-actin levels. P-tau Ser199/202 and Ser396 levels were normalized by total tau levels. Data are represented as means ± S.E.M of the percentage of groups treated with AEA and/or STZ compared to Veh/Veh, which was considered 1.0 (n = 3-5 animals per group), $^{*}p \leq 0.05$, optical density values are different from Veh/Veh, $^{\#}p \leq 0.05$, optical density values of AEA/STZ are different from Veh/STZ, $^{\$}p \leq 0.05$, optical density values of AEA/Veh and AEA/STZ are different than Veh/Veh and Veh/STZ (one-way ANOVA, followed by Duncan's post-test). Red narrows indicate the bands that were analyzed.

Most studies using icv-STZ generally use a dose of 3 mg/kg (Jayant et al., 2016) but we choose 2 mg/kg in order to trigger mild and non-generalized cognitive alterations. Given that we detected impairments of recognition, non-associative emotional and cued fear memory but not contextual fear memory and sensorimotor gating, our data seemingly accomplished this objective of building early and selective cognitive impairments in an AD-like model of sporadic dementia in rats. Clarifying selective changes in the first stages of AD might enable more specific diagnosis, allowing therapeutic interventions before the emergence of multiple irreversible damages.

Most studies using icv-STZ focused on the investigation of spatial memories, especially because of their dependence from hippocampus, which has been widely described to be compromised in SAD development (Braak and Braak, 1997; Shoham et al., 2003; Grünblatt et al., 2007). Likewise, inhibitory avoidance (IA) performance is also deteriorated at 3–9 months after icv-STZ (1–3 mg/kg) (Knezovic et al., 2015). Contextual fear memories, which are also emotional memories dependent on the hippocampus, have mostly been investigated in transgenic models of AD and the results are still not very clear. Deficits of retention of these memories seems to occur at later stages of AD, between 7 and 9 months in PS1-APP mutant mice (Roy et al., 2016) and only deficits of intra-session acquisition were found earlier, at 4 months of age in AβPPswe/PS1ΔE9 mice (Maroof et al., 2014). However, España et al. (2010) reported an increase in the freezing time of APPSwInd mice aged 12 months, diverging from the other studies. Our data show that the performance of contextual fear memory is not impaired in STZ-treated animals, at least during early evaluation (34–35 days post-treatment), corroborating most studies using transgenic animals.

We also observed an impairment of tone fear memory, an emotional memory which is unaffected by hippocampal lesion (Phillips and LeDoux, 1992) but it is dependent on the striatum (Schenberg et al., 2006; Ferreira et al., 2008): this suggests that after STZ, other brain areas involved in cognitive processes besides the hippocampus, also undergo an early disruption. Interestingly, STZ-icv injected animals showed, 30 days later, a marked increase in p-tau and Aβ peptide in basal ganglia (Santos et al., 2012) corroborating the idea that other forebrain structures and cognitive function related to them are disrupted in this model of SAD. Considering genetic models, impairment

of cued fear memories has been described previously in 5XFAD and TgCRND8 mice, which presented less freezing time than wild type during TFC test (Yang et al., 2017).

PPI, on the other hand, is an unlearned cognitive process that does not induce an associative memory between tone pulse and response, but instead a pre-attentional unconscious response (Lichtenberg et al., 2008) involving subcortical areas, such as brainstem nuclei that might also be damaged in AD (Arendt et al., 2015; Hormigo et al., 2017). Although it is known that PPI is compromised in AD (Salem et al., 2011), there is still no data about PPI performance after STZ-icv. Our study showed a lack of alterations in PPI and ASR in STZ-icv as was also observed in APPxPS1 mice aged 9 months (Cheng et al., 2014b). In contrast, another study rTg (tauP301L) 4,510 mice with a phenotype of psychotic AD, reported PPI deficits in animals aged 4–5 months (Koppel et al., 2014). Differences of PPI responses in different AD models could be explained by the anatomic patterns of the neuropathological progress of AD in different types of familial AD (represented by transgenic models) compared to SAD. This may be related to the expression of the different behavioral symptoms in different AD patients, some with depressive symptoms, other with psychotic behavior and others only with cognitive deficits.

The prevention by AEA of some of the cognitive processes disrupted by STZ is consonant to recent studies, which report a growing importance of the role of the endocannabinoid system in the course of AD (Bisogno and Di Marzo, 2008; Fowler et al., 2010; Bedse et al., 2014). This parallels the increasingly recognized therapeutic potential of cannabinoid compounds as potential therapies for many neurodegenerative diseases (Esposito et al., 2006; Carroll et al., 2012; Da Silva et al., 2014). Thus, animals that were administered with cannabidiol (CBD) for 3 weeks after β-amyloid icv injection are protected from the impaired performance in a spatial navigation task and displayed a reduction of the levels of neuroinflammatory mediators (Martín-Moreno et al., 2011). Long-term oral treatment with CBD for 8 months also prevented deficits of social recognition memory in transgenic AβPPSwe/PS1ΔE9 (AβPP × PS1) mice (Cheng et al., 2014c) and using the same model, but treating the animals daily with intraperitoneal injections of CBD for 3 weeks, Cheng et al. (2014a) reported that CBD reversed social and novel object recognition deficits without affecting anxiety-related behaviors. In addition, the presently reported ability of AEA to blunt the enlargement of the brain ventricles suggests an indirect preventive action against neuronal death, which is a well-reported neuroprotective feature of endocannabinoids (Rapino et al., 2017; Vrechi et al., 2018), including AEA (Xu et al., 2017). Further analysis of specific markers of neurodegeneration such as Fluorojade, caspase-3 and Bax, which are increased by icv-STZ (Espinosa et al., 2013; Song et al., 2014; Biswas et al., 2016), would be interesting to better understand the mechanisms behind the neuroprotective role of AEA against the ventricle enlargement.

Most of the studies mentioned above were performed using transgenic models and with intraperitoneal or oral administration of phytocannabinoids. Our study, besides using a model of SAD, tested an endocannabinoid, AEA, through a single icv injection. AEA is a less potent agonist of cannabinoid receptors and did not exert any effect on behavior *per se* as expected, given that all the behavioral procedures were carried out 1 month after the administration of the drugs. AEA was neuroprotective in the deficits of recognition and non-associative emotional memory, but not in associative emotional memory impairment. Although cannabinoid compounds are lipid molecules that can easily cross the brain, icv administration enables a greater control of the concentration of AEA in the brain which is required for an efficient preventive action of the compound. Further studies with other doses of AEA and with drugs that inhibit FAAH, enhancing AEA internal levels, are required for an even more precise evaluation of the relation between AEA levels and AD development. Our results further indicated a tendency for lower CB1R levels only in Veh/STZ. This might be a consequence of the tight interaction between endocannabinoids, CB1R and insulin receptors and their signaling that was previously reported in different systems and brain areas (Kim et al., 2011; Labouèbe et al., 2013; Osmanovic Barilar et al., 2015; Pinheiro et al., 2016). Yet, it is important to remind that AEA also activates CB2R, transient receptor potential vanilloid channels (Rodella et al., 2005) and has membrane and intracellular targets, since it is a hydrophobic molecule that can easily cross lipid membranes (Glaser et al., 2005). Thus, it cannot be ensured that AEA only activated CB1R and it is difficult to determine the pathways operated by AEA to afford its neuroprotective action.

We investigated the levels of CB1R, synaptophysin, SNAP-25 and syntaxin, to verify the existence of a putative synaptic dysfunction in early SAD so that later p-tau alterations that would mediate synaptic changes could be investigated. Synaptophysin and syntaxin were reduced by STZ while SNAP-25 remained unchanged. Notably, AEA only prevented the STZ-induced decrease of syntaxin levels. The brain of AD patients also displays a different alteration of different presynaptic markers, namely a 30% decrease of synaptophysin levels while syntaxin and SNAP-25 were reduced by 10%, probably because each of these presynaptic markers has a different subsynaptic localization and function. Although the present data are indicative of a synaptic dysfunction in this STZ model of SAD, further studies with different synaptic markers will be required to better characterize the pattern of synaptic alterations.

We further tested if the accumulation of hyperphosphorylated tau could be associated with synaptic modifications after icv-STZ. Our results agreed with previous studies that showed an early decrease of p-tau Ser199/202 (Osmanovic Barilar et al., 2015) and did not find differences regarding the levels of p-tau Ser396 between 0.5 and 1 month after STZ injection. Since Veh/STZ and AEA/STZ displayed the same pattern of alterations of p-tau levels it cannot be concluded that these changes are correlated with AEA preventive effect on cognitive deficits.

We also analyzed HSP70 and BAG2 levels and observed that HSP70 levels were only damped in Veh/STZ while all treatments diminished BAG2 levels, with a more pronounced reduction in AEA/Veh and AEA/STZ. Since recent evidence showed that endocannabinoids can be stored in defined reservoirs like the adiposomes and by intracellular trafficking performed by cytosolic carriers such HSP70 and albumin, it is possible that

AEA or its metabolites might have been stored and could still be present to modulate the levels of p-tau and BAG2 even long after their administration (Maccarrone et al., 2010). Curiously, another non-selective agonist of CB1R, curcumin (an antioxidant polyphenol) (Witkin et al., 2013) prevented cognitive deficits when administered for 30 consecutive days after icv-STZ injection (Bassani et al., 2017) and when tested *in vitro* curcumin up-regulated BAG2 levels and reduced p-tau levels (Patil et al., 2013). Although these studies reported that levels of BAG2 increased after CB1R activation, this study hints at the possibility that the activation of the endocannabinoid system might interfere with BAG2 levels in a manner dependent on many factors such as the type of cannabinoid administered, the duration of treatment and the physiopathological context. Since HSP70 and BAG2 can also afford neuroprotective actions through mechanisms independent of tau phosphorylation, other players in p-tau metabolism might be involved, such as CHIP, kinases and other forms of phosphorylated tau protein. Their future study might allow understanding the presently observed dissociation between tau phosphorylation and early memory deficits triggered by STZ.

Although the present study provides a first proof of concept for a potential of AEA to control memory dysfunction in SAD, it has several limitations. In fact, we optimized the icv-STZ model in adult rats whereas SAD is prevalent in aged individuals. Thus, further studies should aim at optimizing an icv-STZ model of SAD in aged rats. Also, we only tested male rats, whereas AD is actually prevalent in women, however, female rats are generally not used in studies with STZ because they seem to be resistant to the neurodegenerative effects of STZ, possibly due to the hormonal oscillations of the estral cycle (Bao et al., 2017). Finally, the consolidation of a role of AEA in SAD should require testing different doses of AEA administered both icv and systemically, in an acute manner as well as with different chronic schedules.

Altogether, our results showed, for the first time, that the administration of an endocannabinoid can prevent cognitive, synaptic and histopatological AD-like alterations induced by STZ, thus prompting endocannabinoids as a candidate therapeutic target in AD.

AUTHOR CONTRIBUTIONS

JdS-L performed the histological experiments and helped in the surgery procedures and data analysis. DM-S, DC, and TF conceived and planned the study. DM-S, DC, TF, RC, and MA provided the financial support. DM-S, AO, SR, JS-L and PC collected the data. DM-S, AO, SR, JdS-L, PC, RC, MA, DC, and TF analyzed and interpreted the data. DM-S, AO, SR, PC, RC, JdS-L, MA, DC, and TF wrote and reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2018.00653/full#supplementary-material

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Cannabis Therapeutics and the Future of Neurology

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Neurological therapeutics have been hampered by its inability to advance beyond symptomatic treatment of neurodegenerative disorders into the realm of actual palliation, arrest or reversal of the attendant pathological processes. While cannabis-based medicines have demonstrated safety, efficacy and consistency sufficient for regulatory approval in spasticity in multiple sclerosis (MS), and in Dravet and Lennox-Gastaut Syndromes (LGS), many therapeutic challenges remain. This review will examine the intriguing promise that recent discoveries regarding cannabis-based medicines offer to neurological therapeutics by incorporating the neutral phytocannabinoids tetrahydrocannabinol (THC), cannabidiol (CBD), their acidic precursors, tetrahydrocannabinolic acid (THCA) and cannabidiolic acid (CBDA), and cannabis terpenoids in the putative treatment of five syndromes, currently labeled recalcitrant to therapeutic success, and wherein improved pharmacological intervention is required: intractable epilepsy, brain tumors, Parkinson disease (PD), Alzheimer disease (AD) and traumatic brain injury (TBI)/chronic traumatic encephalopathy (CTE). Current basic science and clinical investigations support the safety and efficacy of such interventions in treatment of these currently intractable conditions, that in some cases share pathological processes, and the plausibility of interventions that harness endocannabinoid mechanisms, whether mediated via direct activity on CB_1 and CB_2 (tetrahydrocannabinol, THC, caryophyllene), peroxisome proliferator-activated receptor-gamma (PPARγ; THCA), 5-HT_{1A} (CBD, CBDA) or even nutritional approaches utilizing prebiotics and probiotics. The inherent polypharmaceutical properties of cannabis botanicals offer distinct advantages over the current single-target pharmaceutical model and portend to revolutionize neurological treatment into a new reality of effective interventional and even preventative treatment.

Keywords: cannabis, pain, brain tumor, epilepsy, Alzheimer disease, Parkinson disease, traumatic brain injury, microbiome

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INTRODUCTION

Cannabis burst across the Western medicine horizon after its introduction by William O'Shaughnessy in 1838 (O'Shaughnessy, 1838–1840; Russo, 2017b), who described remarkable successes in treating epilepsy, rheumatic pains, and even universally fatal tetanus with the "new" drug. Cannabis, or "Indian hemp," was rapidly adopted by European physicians noting benefits on migraine by Clendinning in England (Clendinning, 1843; Russo, 2001) and neuropathic pain, including trigeminal neuralgia by Donovan in Ireland (Donovan, 1845; Russo, 2017b). These developments did not escape notice of the giants of neurology on both sides of the Atlantic, who similarly adopted its use in these indications: Silas Weir Mitchell, Seguin, Gowers and Osler (Mitchell, 1874; Seguin, 1877; Gowers, 1888; Osler and McCrae, 1915). While

medicinal cannabis suffered a period of obscurity and quiescence, mainly attributable to quality control issues and political barriers, modern data on migraine (Russo, 2004, 2016b; Rhyne et al., 2016) and neuropathic pain, whether central or peripheral support its common application by affected patients (Rog et al., 2005; Nurmikko et al., 2007; Russo and Hohmann, 2013; Serpell et al., 2014), additionally supported by the National Academies of Science, Engineering and Medicine (National Academies of Sciences Engineering and Medicine (U.S.). Committee on the Health Effects of Marijuana: An Evidence Review and Research Agenda, 2017).

It has been noted for some time that muscle tone on the central level is mediated by the endocannabinoid system (Baker et al., 2003), but some additional years were necessary to bring this "aspirin of the 21st century" through Phase I–III Randomized Clinical Trials (RCTs; Novotna et al., 2011) and post-marketing assessment to demonstrate its safety, efficacy and consistency (Rekand, 2014; Fife et al., 2015; Maccarrone et al., 2017). That preparation, nabiximols (US Adopted Name; Sativex®) has currently attained regulatory approval in 30 countries for spasticity associated with multiple sclerosis (MS), and in Canada for central neuropathic pain in MS (Rog et al., 2005), and for opioid-resistant cancer pain (Johnson et al., 2010). Recent surveys find usage rates for cannabis of 20%–60% among MS patients (Rudroff and Honce, 2017). An earlier attempt to demonstrate neuroprotection in head trauma after intravenous administration of single doses of the non-intoxicating cannabinoid analog, dexanabinol, failed (Maas et al., 2006), but hope remains for other preparations in stroke and other brain insults (Latorre and Schmidt, 2015; Russo, 2015; Pacher et al., 2018). **Table 1** summarizes the current status of cannabis-based drugs in neurological conditions not discussed at length herein, including sleep disturbance (Russo et al., 2007; Babson et al., 2017), glaucoma (Merritt et al., 1980), lower urinary tract symptoms (LUTS; Brady et al., 2004; Kavia et al., 2010), social anxiety (Bergamaschi et al., 2011), Tourette syndrome (Müller-Vahl et al., 2002, 2003) and schizophrenia (Leweke et al., 2012; McGuire et al., 2018). This Perspective article will rather focus on several neurological syndromes that overlap in their pathophysiology or have yet to receive concerted attention in clinical trials of cannabis-based medicines.

This author has previously addressed the pathophysiology of migraine (Sarchielli et al., 2007), post-traumatic stress (Hill et al., 2013), Parkinson disease (PD; Pisani et al., 2005) and other conditions as putative clinical endocannabinoid deficiency disorders wherein disturbances in endocannabinoid tone have been demonstrated objectively (Russo, 2004, 2016b).

Various synthetic fatty acid amidohydrolase (FAAH) inhibitors have been investigated for neurological therapeutics (Nozaki et al., 2015), but none have advanced to Phase III clinical trials. This is a mechanism of action seemingly shared with cannabidiol (Bisogno et al., 2001).

CANNABIS AND EPILEPSY

After elucidation of phytocannabinoid structures in the 1960s, their pharmacology was slowly revealed (reviewed by Cascio and Pertwee, 2014; Pertwee and Cascio, 2014; Russo and Marcu, 2017; **Figure 1**). Various components were tested for anticonvulsant activities with findings of ED_{50} in mice of 80 mg/kg for tetrahydrocannabinol (THC), 120 mg/kg for cannabidiol (CBD) and 200 mg/kg for tetrahydrocannabinolic acid A (THCA-A), the carboxylic acid precursor to THC found in raw cannabis flowers (Karler and Turkanis, 1979). Although dose-response was tested, it is unclear that very low doses were assessed and given the biphasic tendencies of cannabinoids, it is possible that positive lower dose effects may have remained unnoticed. CBD was considered an excellent candidate for development based on its lack of untoward psychoactive sequelae. However, little work was done until a series of small human trials in Brazil in following decades (reviewed by Russo, 2017a).

Subsequent investigation demonstrated that seizure threshold is mediated by the endocannabinoid system (Wallace et al., 2003), and that THC produced a 100% reduction in seizures, whereas phenobarbital and diphenylhydantoin did not. Additionally, animal studies demonstrated both acute increases in endocannabinoid production and a long-term up-regulation of CB_1 production as apparent compensatory effects counteracting glutamate excitotoxicity, and that anticonvulsant effect was present at sub-sedating levels.

Sporadic case reports of successful utilization of THC in seizures associated with severe neurological conditions in children in Germany followed (Lorenz, 2004; Gottschling, 2011),

TABLE 1 | Neurological conditions for which cannabis-based treatments have been employed (revised, reformatted and supplemented from MacCallum and Russo, 2018).

Condition	Preparation	Level of evidence	Type of evidence
Multiple sclerosis (MS) spasticity	Nabiximols	Conclusive	Phase III RCTs, Regulatory approval
Epilepsy (Dravet and Lennox-Gastaut syndromes)	Cannabidiol (Epidiolex®)	Conclusive	Phase III RCTs, Regulatory approval
Chronic pain	THC, nabiximols	Substantial	Phase II RCTs
Schizophrenia, positive and negative symptoms	CBD	Substantial	Phase II RCTs
Sleep disturbance secondary to neurological symptoms	THC, nabilone, nabiximols	Moderate	Phase II–III RCTs
Glaucoma	THC, cannabis	Moderate	Phase II RCTs
Lower urinary tract symptoms (LUTS) in MS	Nabiximols	Moderate	Phase II RCTs
Tourette syndrome	THC, cannabis	Moderate	Phase II RCTs, observational studies
Dementia with agitation	THC, cannabis	Limited	Observational studies
Parkinson disease symptoms	THC, CBD, cannabis	Limited	Observational studies
Post-traumatic stress disorder	Cannabis	Limited	Observational studies
Social anxiety	CBD	Limited	Phase II RCT, observational studies

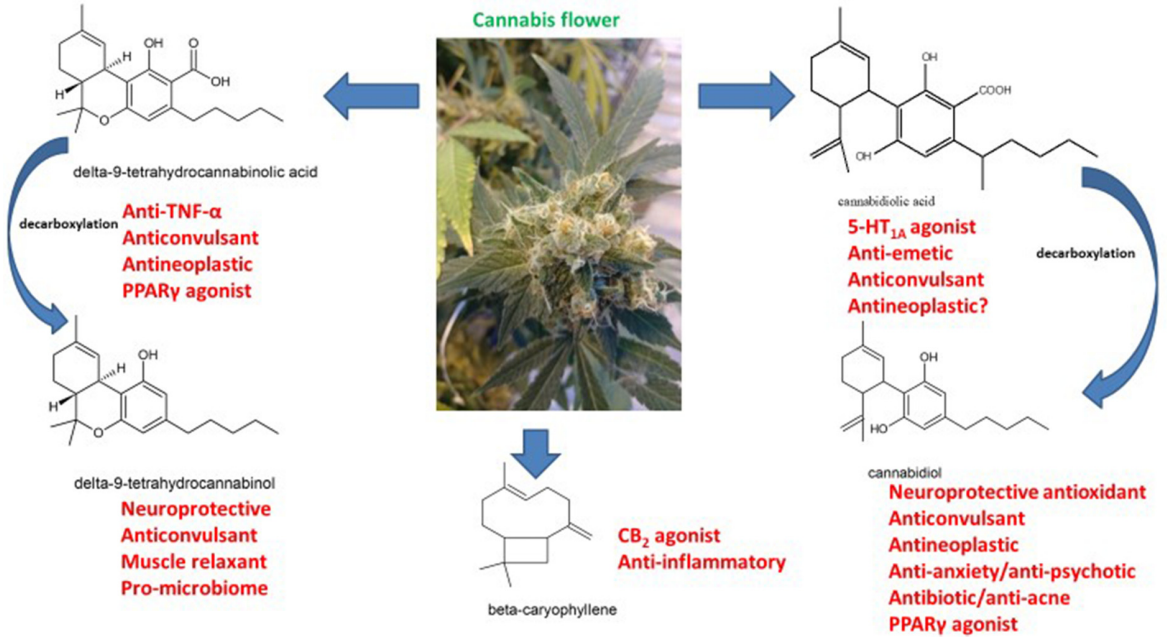


FIGURE 1 | The pharmacology of phytocannabinoids pertinent to treatment of neurodegenerative disorders (molecular structures drawn by ER with ACD/ChemSketch 2015.2.5).

but the prime focus returned to CBD due to strong anticonvulsant results in laboratory investigation (Jones et al., 2010), which led directly to a pharmaceutical development program. The lay public quickly became aware of these developments, with promotion of the concept by Project CBD[1] and publicity associated with the case of Charlotte Figi and significant improvement in seizures associated with Dravet syndrome, as portrayed on the *Weeds* documentary on Cable News Network (Maa and Figi, 2014). Positive survey results (Porter and Jacobson, 2013) were tempered, however, by studies suggesting strong ascertainment bias in parental reporting of seizure frequency: response rate for families moving to the state of Colorado for cannabidiol treatment was 47% vs. only 22% for those already living there, and were three-fold higher for those reporting >50% response (Press et al., 2015). More careful observational studies with a standardized cannabidiol oral extract with THC removed (Epidiolex®) provided more compelling results (Devinsky et al., 2016) with a 55% median reduction in seizures in Dravet and Lennox-Gastaut Syndrome (LGS) patients at high dose. Subsequent Phase III results in Dravet syndrome at CBD 20 mg/kg/d showed strong statistical significance in seizure frequency and Caregiver Global Impression of Change (Devinsky et al., 2017). More recent studies have bolstered evidence for safety and efficacy of the preparation in both conditions (Devinsky et al., 2018; Thiele et al., 2018). As a result, it received US Food and Drug Administration approval in June 2018.

Interestingly, extensive observations from other practitioners (Russo et al., 2015) seemed to indicate similar therapeutic successes with much lower doses of CBD when utilized in cannabis-based preparations with small concomitant amounts of THC, THCA and linalool, a terpenoid component of cannabis (Russo, 2017a; Sulak et al., 2017; Pamplona et al., 2018). Selective cannabis breeding via Mendelian techniques raises the possibility of producing chemovars with multiple anticonvulsant components that may produce synergistic benefits (Lewis et al., 2018). THCA is an intriguing issue, in that there is debate about whether it harbors CB_1 activity, or rather is due to spontaneous decarboxylation to THC (McPartland et al., 2017; **Figure 1**). Cannabidiolic acid (CBDA) was also recently reported to demonstrate anticonvulsant activity (Bonni Goldstein, personal communication), possibly attributable to its serotonergic activity (Bagdy et al., 2007), in that CBDA demonstrates 100-fold greater affinity for the 5-HT_{1A} receptor (Bolognini et al., 2013) as compared to CBD (Russo et al., 2005).

CANNABIS AND BRAIN TUMORS

Strong scientific evidence of cytotoxic benefit of phytocannabinoids has been available since 1975 (Munson et al., 1975) and highlighted three decades later (Ligresti et al., 2006), but the historical record suggests ancient use by Egyptian Copts (THC and/or THCA; Reymond, 1976; Russo, 2007) with similar claims by Renaissance herbalists in Europe (CBD and/or CBDA; Russo, 2007). Brain tumors are the subject of an excellent current review (Dumitru et al., 2018). To summarize available research, specific pro-apoptotic activity of THC in C6 glioma was reported (Sánchez et al., 1998), and shrinkage of *in situ* human glioma cell line tumors was observed with CBD (Massi et al., 2004). Intra-tumoral THC administration in glioblastoma

[1]https://www.projectcbd.org/

multiforme (GBM) produced slight life prolongation over expectations in nine human patients (Guzmán et al., 2006). Case reports from Canada documented total regression of residua in two pilocytic astrocytomata in children after smoked cannabis (Foroughi et al., 2011). Careful laboratory analysis has established synergistic benefits of combinations of THC, CBD and standard chemotherapy with temozolomide on glioma (Torres et al., 2011). Clinical application of the concept has been reported online in a Phase II randomized controlled trial (RCT) of 21 patients with recurrent GBM on temozolomide plus nabiximols up to 12 sprays per day (32.4 mg THC plus 30 mg CBD plus terpenoids) vs. placebo with an 83% 1-year survival vs. 53% in controls ($p = 0.042$) and survival exceeding 550 days vs. 369 for controls, and only two withdrawals in each group due to adverse events (AEs)[2].

Such encouraging results are supplemented by a recent report that THCA is a peroxisome proliferator-activated receptor-gamma (PPARγ) agonist (IC_{50} = 470 nM, K_i = 209 nM) > CBGA (517.7 nM) and ≫ than CBDA, CBD or THC (Nadal et al., 2017). THCA improved neuronal viability in an animal model of Huntington disease (HD), and decreased striatal neurodegeneration (blocked by PPARγ antagonist), and it was suggested as a therapeutic agent in HD. This finding, however, has much larger implications and could explain claims of therapeutic efficacy in epilepsy noted above (Sun et al., 2008), as well tumors, and perhaps even in major depression (Colle et al., 2017a,b). In contrast to other neutral cannabinoids and terpenoids, THCA is reported not to cross the blood-brain barrier (BBB), but if true, that hindrance may not be applicable in the context of chronic epilepsy (Oby and Janigro, 2006), or in brain tumors wherein that barrier is compromised.

As reviewed (Elrod and Sun, 2008), PPARs are ligand-binding transcription factors on nuclear membranes that affect adipogenesis, apoptosis and many other functions. PPARγ stimulation may kill cancer cells without toxicity to normal cells, such as astrocytes, and their effects are additive with other cytotoxic agents. Butyrate and capsaicin may be natural ligands. PPARγ has been identified in many cancers including those affecting the brain, where it regulates target gene transcription (Shen et al., 2016), and its activation inhibits tumor cell growth. These authors suggested that PPARγ agonist may prove useful in treating brain tumors, and may extend as well to "benign" lesions, such as meningioma, wherein pioglitazone demonstrated activity (Gehring et al., 2011; Shen et al., 2016).

Thus, a Type II cannabis preparation, with equal THC and CBD concentration, combining THC, CBD, THCA and even CBDA along with cytotoxic terpenoids such as limonene may prove extremely useful in cancer treatment (Lewis et al., 2018).

CANNABIS AND PARKINSON DISEASE (PD)

As early as 1888, Gowers noted benefits of "Indian hemp" on a parkinsonian syndrome (Gowers, 1888; Russo, 2007). Because of the density of cannabinoid receptors in basal ganglia, PD has been an area of active research, but with mixed results therapeutically. An oral THC:CBD extract showed no significant benefits on dyskinesia or other signs in 17 patients (Carroll et al., 2004), but CBD was helpful in five PD patients with psychosis (Zuardi et al., 2009) and 21 patients with more general symptoms (Chagas et al., 2014b) and more specifically on rapid eye movement sleep disorder in four patients (Chagas et al., 2014a). An observational study showed 22/28 patients tolerated smoked cannabis (presumably THC-predominant) and showed acute benefits on tremor, rigidity and bradykinesia (Lotan et al., 2014). Five of nine patients using cannabis reported great improvement, particularly on mood and sleep (Finseth et al., 2015).

A carefully crafted survey of 339 Czech patients using oral cannabis leaves reported significant alleviations of multiple symptoms (Venderová et al., 2004), particularly those using the treatment for three or more months, with improvement in general function ($p < 0.001$), resting tremor ($p < 0.01$), bradykinesia ($p < 0.01$), and rigidity ($p < 0.01$) with few side effects.

Whereas PD is commonly attributed to cell loss in the substantia nigra, with chronicity, widespread pathology is the norm. In common with Alzheimer disease (AD), tau proteins that regulate microtubule assembly, cytoskeletal integrity and axonal transport in neurons develop neurofibrillary tangles (Lei et al., 2010). Interestingly, nabiximols reduced such tangles in parkin-null human tau-expressing mice with improvement in dopamine metabolism, glial function and oxidative stress, as well as reducing anxiety and self-injury (Casarejos et al., 2013).

CANNABIS AND ALZHEIMER DISEASE (AD)

Recent reviews (Aso and Ferrer, 2014; Ahmed et al., 2015) have nicely summarized the pathophysiology of AD: a neurodegenerative disease with senile plaques formed of fibrillar β-amyloid (Aβ) from cleavage of the Aβ precursor protein (APP) by β- and γ-secretases and by presence of neurofibrillary tangles composed of hyper-phosphorylated and nitrated tau protein. The latter precedes Aβ deposition in sporadic cases. Once the process begins, deterioration is inexorable. Additional pathology includes functional mitochondrial defects, increased reactive oxygen species (ROS) and reactive nitrogen species (RNS), and failure of enzymes involved in energy production that, in turn, produces nerve cell exhaustion. Eventually, synapses and dendritic branching fail, with consequent progressive neuronal wastage. Dementia and cognitive decline develop, and no treatment arrests the process. Intervention must begin at an early preclinical stage to have any hope of success. Endocannabinoid function modulates the primary pathological processes of AD during the silent phase of neurodegeneration: protein misfolding, neuroinflammation, excitotoxicity, mitochondrial dysfunction and oxidative stress. CB_2 levels increase in AD especially in microglia around senile plaques, and its stimulation stimulates Aβ removal by macrophages.

[2]www.gwpharm.com

The epidemiology of AD is fascinating (Mayeux and Stern, 2012). North America and Western Europe have highest rates (6.4% and 5.4% at age 60), then Latin America (4.9%), and China (4%; ascertainment bias vs. mirroring economic development and Western diet?). Prevalence is lower for Africans in homelands, as opposed to higher rates in the Western European and American diaspora. Head trauma increases Aβ deposition and neuronal tau expression, and diabetes, obesity, trans-fats and head trauma all increase AD risk. Mediterranean diet (increased monounsaturated olive oil, and omega-3 from fish), education and physical activity reduce it.

No current pharmacotherapy is approved for agitation in AD. Commonly used anti-psychotics, antidepressants, anxiolytics and hypnotics are often associated with increased mortality in demented patients (Kales et al., 2007), with an FDA "Black Box Warning." Four acetylcholinesterase inhibitors are approved in the USA to improve memory: galantamine, donepezil, tacrine and rivastigmine. None show strong evidence of efficacy and are of limited benefit on a temporary basis. Various NMDA receptor antagonists in development have proven largely ineffective on disease progression or have proven toxic. In contrast, treatment with cannabinoids appears both more promising and benign. As demonstrated in 1998 (Hampson et al., 1998), and the subject of USA patent US09674028, CBD is a neuroprotective antioxidant, more potent than ascorbate or tocopherol, that works on the same NMDA target without attendant toxicity. Subsequently (Iuvone et al., 2004), CBD inhibited Aβ plaque formation, prevented ROS production and peroxidation of lipids in PC12 cells exposed to Aβ, limited neuronal apoptosis from caspase 3 reduction, and counteracted increases in intracellular Ca^{++} from Aβ. In an *in vivo* model (Esposito et al., 2006), CBD was anti-inflammatory via reduction in inducible nitric oxide synthase (iNOS) and IL-1β expression and release. It also inhibited tau protein hyper-phosphorylation in Aβ-stimulated PC12 neurons. Subsequently, it was shown that CBD's MOA seemed to be selectively mediated via PPARγ (Esposito et al., 2011): dose dependently antagonizing pro-inflammatory NO, tumor necrosis factor-alpha (TNF-α), and IL-1β. That effect was blocked by GW9662 (PPARγ antagonist), reducing reactive gliosis via selective PPARγ-related NFκB inhibition. Both AEA and CBD promoted neurogenesis after Aβ exposure.

In addition to its neuroprotective antioxidant effects (Iuvone et al., 2004), THC competitively inhibited acetylcholinesterase, increasing levels, and prevented Aβ aggregation via binding to the enzyme in a critical region affecting amyloid production (Eubanks et al., 2006).

On the clinical side, various trials of THC in AD have produced positive results. In 1997 (Volicer et al., 1997), in 15 institutionalized dementia patients refusing nutrition, an RCT 6-week crossover trial of THC (Marinol®) 2.5 mg twice daily led to increased body-mass index (BMI), with decreased Cohen-Mansfield Agitation Inventory (CMAI) scores, improved negative affect scores, and a notable carry-over effect when THC was administered first. In 2006 (Walther et al., 2006), an open-label 2-week study of five AD and one vascular dementia patient taking THC 2.5 mg at 19:00 h showed benefit noted on nocturnal motor activity, agitation, appetite, and irritability with no AEs. A 2015 study (van den Elsen et al., 2015) failed, however: an RCT in 50 demented patients with neuropsychiatric symptoms received 1.5 mg THC vs. placebo thrice daily for 3 weeks with no benefit noted to THC. A total lack of AEs indicated to the even the authors that the administered dosage was inadequate and that higher doses might be required.

Initial trials of herbal cannabis for AD have begun sporadically, with a more focused effort in a California nursing home (Hergenrather, 2017). Patients were treated with a variety of preparations: THC-predominant (2.5–30 mg/dose), CBD predominant, and THCA, mainly in tinctures and confections. Marked benefit was reported on neuroleptic drug sparing, decreased agitation, increased appetite, aggression, sleep quality, objective mood, nursing care demands, self-mutilation and pain control.

Based on its pharmacology (Russo and Marcu, 2017), cannabis components may provide myriad benefits on target symptoms in this complex disorder:

- Agitation: THC, CBD, linalool
- Anxiety: CBD, THC (low dose), linalool
- Psychosis: CBD
- Insomnia/Restlessness: THC, linalool
- Anorexia: THC
- Aggression: THC, CBD, linalool
- Depression: THC, limonene, CBD
- Pain: THC, CBD
- Memory: alpha-pinene (Russo, 2011; Russo and Marcu, 2017) + THC
- Neuroprotection: CBD, THC
- Reduced Aβ plaque formation: THC, CBD, THCA

Thus, an extract of a Type II chemovar of cannabis (THC/CBD) with a sufficient pinene fraction would seem to be an excellent candidate for clinical trials (Lewis et al., 2018).

CANNABIS AND TRAUMATIC BRAIN INJURY (TBI)/CHRONIC TRAUMATIC ENCEPHALOPATHY (CTE)

The neuroprotective antioxidant effects of the cannabinoids (Hampson et al., 1998) are particularly relevant in their ability to counteract "glutamate excitoxicity," which leads to neuronal demise after traumatic brain injury (TBI). Anecdotally, cannabis, particularly chemovars combining THC and CBD, have been extremely helpful in treatment of chronic traumatic encephalopathy (CTE) symptoms: headache, nausea, insomnia, dizziness, agitation, substance abuse, and psychotic symptoms. CTE, previously known as *dementia pugilistica*, or "punch-drunk syndrome" has garnered a great deal of attention due to its apparent frequency among long-term players of American football but including victims of repetitive head injury from causes as diverse as other contact sports, warfare and even "heading" in soccer. A recent study (Mez et al., 2017) showed 87% of autopsied American football players demonstrated CTE with tau aggregates in neurons and astrocytes, neurofibrillary tangles in superficial cortical layers

and hippocampus, α-synuclein and Aβ deposition. Microglia were present early in the course, whose premonitory symptoms include dementia, personality change, rage, and attention problems. Ninety-six percent demonstrated a degenerative course. Heretofore, this has been considered a post-mortem pathological diagnosis, but two current studies support the ability for pre-mortem identification. CCL11 protein is a chemokine associated with cognitive decline and enhances microglial production of ROS and excitotoxic cell death. CSF examination in CTE patients were elevated compared to controls and AD patients (p = 0.028), and correlated to years of football played (p = 0.04; Cherry et al., 2017), indicating CCL11 may be a premortem biomarker for the syndrome. Additionally, PET imaging binding levels in a CTE patient before death correlated with postmortem tau deposition (p = 0.02). The greatest tau concentration was observed in parasagittal and paraventricular cortical and brainstem areas (Omalu et al., 2018), allowing pre-mortem diagnosis and distinction from AD. Neuroprotective benefits of phytocannabinoids, particularly CBD, further outlined below, provide support for trials of these agents in post-traumatic syndrome and CTE prevention.

HUMAN NUTRITION, CANNABIS, THE ECS, "ACNE OF THE BRAIN" AND THE "GUT-SKIN-BRAIN AXIS"

Human gut harbors 100 trillion micro-organisms at a concentration of 10^{12} bacteria/ml, and exceeding the human genome 100-fold (Musso et al., 2010). This is termed the microbiome. Obese humans have lower Bacteroidetes and higher Firmicutes counts. Recent review (Clarke et al., 2012; Russo, 2016b) supports the efficacy of probiotics (supplemental beneficial gut lactic acid bacteria) in treating irritable bowel syndrome without AEs. Microbiota regulate 5-HT_{1A}, BDNF and NMDA expression (Sampson et al., 2016), and experimental transplantation of the microbiome of Parkinson patients to mice was demonstrated to increase their motor deficits, supporting the finding of a pro-inflammatory dysbiosis (microbiome imbalance) in that disorder (Keshavarzian et al., 2015).

Another recent review elucidates additional findings of pertinence to the current discussion (He and Shi, 2017). The combination of prebiotics (dietary fiber that serves as bacterial feedstock, reviewed by Russo, 2016a), and deficient in modern Western diets (Calame et al., 2008; Slavin, 2013) and probiotics may be termed, "synbiotics." Translocation of bacterial fragments produces "metabolic endotoxemia" from bacterial lipopolysaccharides (LPS). Probiotics may help control PPARγ, "the master regulator of adipogenesis" and TNF-α in inflammation. Additional research supports that prebiotic galacto-oligosaccharides (as from beans) decrease TNF-α, and interleukin production (He and Shi, 2017). GPR41 and GPR43 are orphan receptors for short-chain fatty acids (SCFA) that can increase release of 5-HT and other factors. Additionally, prebiotics change microbiota to reduce adipogenesis and stabilize the gut barrier. Furthermore, CB_2 levels correlate to *Lactobacillus* concentrations and negatively with potentially pathogenic *Clostridium* species.

Other experiments relate the microbiome to the ECS. A direct effect of *Lactobacillus acidophilus* NCFM strain via

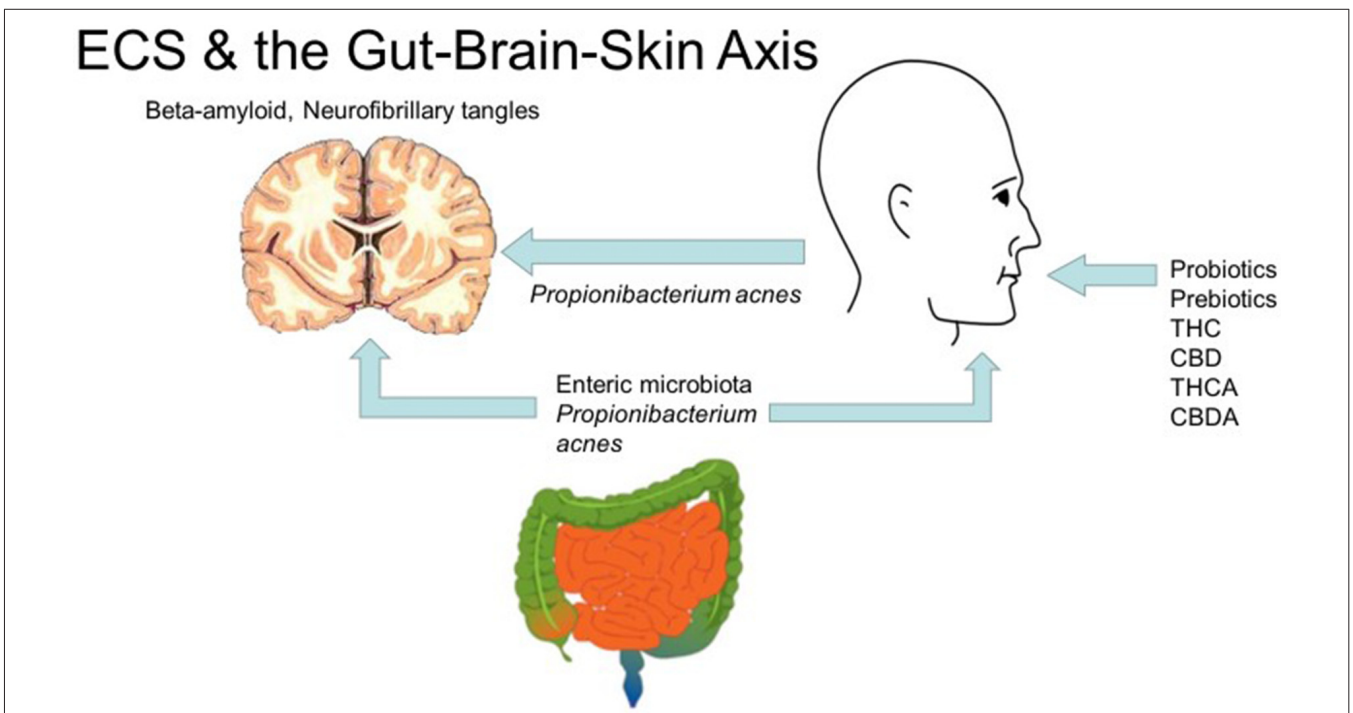


FIGURE 2 | Cannabis, the endocannabinoid system and the gut-brain-skin axis (diagrams of brain, gut by Mikael Hagstrom, face by Mouagip, all public domain).

oral administration to induce CNR2 (gene encoding the CB_2 receptor) mRNA expression above that of resting human HT-29 epithelial cells ($p < 0.01$) was demonstrated. An enhancement of morphine antinociceptive effect in rats ($p < 0.001$) was also demonstrated which was inhibited by administration of the CB_2 antagonist, AM-630 ($p < 0.001$; Rousseaux et al., 2007). Additionally, THC altered the microbiome balance in obese DIO mice affecting the Firmicutes: Bacteroidetes ratio ($p = 0.021$). Furthermore, THC prevented weight gain despite a high-fat diet (Cluny et al., 2015). This explains, perhaps, how the stereotype of the "skinny hippie" is more accurate than that of the lazy, obese "stoner."

Additional dietary factors include the function of bitter taste receptors (Tepper et al., 2014), present not only on the tongue, but in the gut, and hypothalamus (Herrera Moro Chao et al., 2016), wherein interaction with ECS appetite mechanisms seem to be operative.

Diet is also a key factor in *acne vulgaris*, whose pathophysiology and epidemiology are surprisingly relevant to this discussion. Acne was not observed in Inuit populations living a traditional lifestyle over 30 years, but became common with adoption of a Western diet and lifestyle (Cordain et al., 2002). Similarly, no acne was observed in Papua New Guinea or Paraguay among traditional indigenous peoples. Neither population demonstrated markers of insulin resistance, nor leptin elevations. The author then suggests that in many respects, the epidemiology of acne parallels that of AD. The relationship becomes more salient in light of recent findings (Emery et al., 2017) demonstrating that neuroinflammation is a stimulus to AD development and is triggered by infectious insults. Additionally, AD brains demonstrated 5–10× greater bacterial loads, especially with Actinobacteria, particularly *Propionibacterium acnes*, a gram-positive an aerobic resident of skin, mouth and gut and pathological agent of acne. *P. acnes* has been cultured from AD brains, can grow there, and stimulate alpha synuclein fibrillar formation in PD, amyloid fibrillization in AD, and biofilm formation, which is opposed by cannabinoids, and cannabis terpenoids limonene, alpha-pinene (Soni et al., 2015; Subramenium et al., 2015; Russo and Marcu, 2017).

An additional parallel pertains to the TRPV4 receptor (Zhang et al., 2013). TRPV4 is expressed in cerebral endothelial cells where it mediates Ca^{++} and influx acetylcholine-induced dilation. Cerebral hypoperfusion with impaired vessel dilation is a pathogenetic factor in AD. That function is impaired in a mouse model of AD and is sensitive to oxidative stress from Aβ, which is alleviated by antioxidants. The authors suggested TRPV4 as a target for AD treatment.

Cannabidiol, in addition to its anti-inflammatory and bacteriostatic effects, is a TRPV4 agonist that works as a sebostatic agent in acne (Oláh et al., 2014), while cannabis terpenoids limonene, linalool potently inhibited *P. acnes* and consequent TNF-α production (Kim et al., 2008). Alpha-pinene was also a potent inhibitor of the bacterium (Raman et al., 1995; reviewed by Russo, 2011).

The importance of these relationships becomes apparent as efforts are made to integrate disparate threads (Bowe and Logan, 2011). Mental health impairment scores in acne patients surprisingly exceed those with epilepsy and diabetes. Oral probiotics regulate inflammatory cytokines in skin. Intestinal microbiota, skin inflammation and psychiatric symptoms are thus intertwined in a "gut-brain-skin axis." The author posits that acne-induced processes could also affect PD, AD and CTE pathophysiology (**Figure 2**).

FUTURE TRENDS

It is the opinion of many that neurology is facing therapeutic brick walls. The current single target receptor model of pharmacotherapy has not proven universally salutary in the face of complex neurodegenerative diseases. Rather, reconsideration must be given to an older proven model of botanical synergy that may enable polytherapy in single preparations (Russo, 2011; Brodie et al., 2015; Russo and Marcu, 2017; Lewis et al., 2018). Such approaches, combined with nutritional and lifestyle management may make neurology a more preventative and therapeutic specialty, rather than merely diagnostic, and provide better treatment for epilepsy, tumors, AD, PD and TBI/CTE. Suggested strategies include:

- Aerobic activity (Raichlen et al., 2012; Schenkman et al., 2018)
- Education as a lifestyle
- Anti-inflammatory, prebiotic and probiotic diet emphasizing saturated and monounsaturated and omega-3 EFAs, bioflavonoids (berries), fermented foods, protein and minimizing carbohydrates (Fallon and Enig, 1999; Perlmutter and Loberg, 2015)
- Supplementation with cannabis extracts providing THC, CBD, THCA, CBDA, caryophyllene and other select terpenoids (**Figure 1**; Russo and Marcu, 2017; Lewis et al., 2018).

Legitimate concerns surround the psychoactive sequelae of THC, but as amply demonstrated by the nabiximols RCTs and supported by mitigating effects of cannabidiol and cannabis terpenoids (Russo, 2011; Russo and Marcu, 2017; Lewis et al., 2018; MacCallum and Russo, 2018), cannabis-based drugs portend to provide future safe and effective treatments for heretofore recalcitrant neurological conditions.

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The author confirms being the sole contributor to this work and has approved it for publication.

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Cannabis and the Anxiety of Fragmentation—A Systems Approach for Finding an Anxiolytic Cannabis Chemotype

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Cannabis sativa is a medicinal herb with a diverse range of chemotypes that can exert both anxiolytic and anxiogenic effects on humans. Medical cannabis patients receiving organically grown cannabis from a single source were surveyed about the effectiveness of cannabis for treating anxiety. Patients rated cannabis as highly effective overall for treating anxiety with an average score of 8.03 on a Likert scale of 0 to 10 (0 = not effective, 10 = extremely effective). Patients also identified which strains they found the most or least effective for relieving their symptoms of anxiety. To find correlations between anxiolytic activity and chemotype, the top four strains voted most and least effective were analyzed by HPLC-MS/MS to quantify cannabinoids and GC-MS to quantify terpenes. Tetrahydrocannabinol (THC) and trans-nerolidol have statistically significant correlations with increased anxiolytic activity. Guiaol, eucalyptol, γ-terpinene, α-phellandrene, 3-carene, and sabinene hydrate all have significant correlations with decreased anxiolytic activity. Further studies are needed to better elucidate the entourage effects that contribute to the anxiolytic properties of cannabis varieties.

Keywords: cannabinoids, terpenoids, THC, CBD, synergy, cannabis, anxiety, terpenes

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INTRODUCTION

Anxiety is an emotion characterized by an inner state of unease, most often in anticipation of future events. From a biological perspective, anxiety is a biochemical response to a perceived danger or threat in the future, as opposed to fear, which is a response to an immediate threat (Barlow, 2000). Anxiety is considered pathogenic when the emotional response is disproportionate, in duration, frequency, or intensity, to the cause, which hinders the patient to lead a normal life (American Psychiatric Association, 2013). Anxiety disorders may manifest in many different forms and durations, from continuous anxiety in daily life known as Generalized Anxiety Disorder, to sudden and debilitating episodes of extreme anxiety known as in Panic Attack Disorder (Rynn and Brawman-Mintzer, 2004). Anxiety can also be in response to the symptoms of other illnesses, such as Chronic Obstructive Pulmonary Disease (COPD), and asthma (Tselebis et al., 2016). It may also manifest from the possible negative outcomes of diseases such as the prospect of death for cancer patients (Mosher et al., 2016). An estimated 3.5 million (12.6%) of Canadians (Statistics Canada, 2015b) met the criteria for a mood disorder, 2.4 million of which have reported symptoms consistent with Generalized Anxiety Disorder (Statistics Canada, 2015a). Clinicians manage symptoms of anxiety by administering pharmacological drugs, allowing patients to return to a normal life.

Cannabis may hold many pharmacological benefits over conventional therapies for anxiety, which mostly include but are not limited to Benzodiazepines and Selective Serotonin Reuptake Inhibitors (SSRIs) (Patel et al., 2017; Turna et al., 2017). Anxiety is one of the most common symptoms for which medical marijuana users seek relief from in North America (Turna et al., 2017). Somewhat paradoxically, anxiety is also reported as a common adverse effect of cannabis use in medical and recreational contexts, with panic attacks or paranoia being reported after high doses of THC consumption (Hall and Solowij, 1998; Hoch et al., 2015). More than just molecular interactions must be considered as the "*set and setting*," which refers to the mindset of the user along with their physical and social environment, affects the individual's response to psychoactive drugs (Hartogsohn, 2017). The "set and setting" of cannabis use could also result in anxiety due to its illegality and social stigma. Nonetheless, cannabis has a complex pharmacology that could be harnessed for the treatment of anxiety.

Phytocannabinoids are a diverse group of over 150 terpenoid compounds and the most abundant secondary plant metabolites produced by the cannabis plant (Hanuš et al., 2016). The most abundant and widely studied phytocannabinoids are delta-9-Tetrahydrocannabinol (THC), which is responsible for the psychoactive effects of cannabis, and cannabidiol (CBD), a non-psychotropic but pharmacologically active substance, both of which act on the endocannabinoid system (Greydanus et al., 2013). Current molecular research indicates that the endocannabinoid system plays an important role in the pathophysiology of anxiety disorders and its modulation may be an effective treatment (Lisboa et al., 2017). Patients suffering from Post-Traumatic Stress Disorder (PTSD) exhibit higher expression of CB1 receptors but lower peripheral concentrations of anandamide or *N*-arachidonoylethanolamine (AEA), the endogenous ligand of CB1 (Lisboa et al., 2017). Likewise, peripheral concentrations of anandamide in PTSD patients have been shown to be inversely correlated with symptom severity (Hill et al., 2013). Moreover, baseline anxiety is also inversely correlated with peripheral AEA concentrations in healthy adult individuals (Dlugos et al., 2012). CB1 antagonists have also been shown to cause anxiety in humans, with the drug Rimonabant, removed from the market due to adverse effects, mainly anxiety and depression (Moreira and Crippa, 2009). Clinical trials have shown Nabilone, a synthetic THC analog and CB1 agonist, to be effective in the treatment of anxiety (Fabre and McLendon, 1981). Interestingly, synthetic THC (Dronabinol) which has been approved to treat pain, exhibits anxiety as a common side effect (Naef et al., 2003). Synthetic THC has been found to have a lower abuse potential compared to herbal cannabis because of its propensity to cause anxiety and dysphoria (Calhoun et al., 1998; McPartland and Pruitt, 1999). Altogether, this evidence suggests that the CB1 is involved in the pathophysiology of anxiety, and its activation appears to be inversely correlated to symptoms of anxiety. Moreover, pure THC appears to cause more anxiety than whole plant cannabis, and this may be due to the therapeutic and/or modulatory effects of other minor constituents such as terpenes.

To determine the effect of major cannabinoids on anxiety, studies conducted on mice and rats have shown THC and other CB1 agonists display a dose-dependent biphasic response curve whereby low doses result in anxiolytic effects and high doses in anxiogenic effects (Rubino et al., 2007). This biphasic response curve explains in part why cannabis is both sought for anxiety relief and also seems to cause anxiety as a side effect (Bhattacharyya et al., 2017). Furthermore, preclinical evidence suggests that CBD possesses both anxiolytic and antipsychotic properties (Guimarães et al., 1990; Bergamaschi et al., 2011). High doses of CBD (100 mg/kg) were ineffective in animal models of generalized anxiety, while low doses (10 mg/kg) were found to have anxiolytic-like effects (Silveira Filho and Tufik, 1981). Further studies have confirmed the anxiolytic-like effects of CBD at moderate doses (Onaivi et al., 1990; Long et al., 2010). In rat models of generalized anxiety, micro-injections of CBD in the dorsal periaqueductal gray is shown to produce anxiolytic effects via partial activation of the Serotonin 1A Receptor (5-HT_{1A}) receptors (Bitencourt et al., 2008; Soares et al., 2010). Experimentally induced anxiety in healthy human volunteers is reduced by administration of CBD (Crippa et al., 2004). Functional Magnetic Resonance Imaging (fMRI) studies show that CBD attenuates activity in the amygdala and the anterior cingulate (Fusar-Poli et al., 2009). The regions of the brain modulated in these studies reflect the anxiolytic action of conventional therapies such as Benzodiazepines (Crippa et al., 2004; Bitencourt et al., 2008).

It appears that the isolated cannabinoids, as well as herbal cannabis, can induce both anxiolytic and anxiogenic effects, and the mechanism of action responsible for such opposing effects may include both a dose dependent biphasic response and various "entourage effects" produced by different cannabis chemotypes. Recently, Gallily et al. demonstrated the greater efficacy of standardized cannabis plant extract over purified CBD isolate for pain and inflammation (Gallily et al., 2015). Purified CBD isolates were found to have a biphasic response curve whereas the whole cannabis extract showed a dose dependent response curve, with higher concentrations still exhibiting the therapeutic benefits of CBD (Gallily et al., 2015). Further research is necessary to elucidate the mechanisms of action of pure cannabiniod isolates vs. whole cannabis plant extract containing predominately THC and/or CBD along with many more phytocannabinoids and terpenoids. Altogether, this presents a complex situation in which cannabis can have both anxiolytic and anxiogenic properties which are not only dependent on pharmacological factors but also environmental ones. A large amount of study is required to elucidate the effects of different cannabis chemotypes on anxiety. Much of the research in humans has focused on using isolated forms of THC and CBD as medical treatments (Schrot and Hubbard, 2016); and not as they exist in the cannabis plant. These two major cannabinoids collect with other minor cannabinoids, terpenes, and other secondary plant metabolites inside glandular trichomes on the surface of cannabis leaves, creating a complex botanical mixture (Booth et al., 2017) that deserves to be researched separately from purified cannabinoids.

Terpenes are the pungent and volatile oils that give cannabis varieties (and other plants) their distinctive flavors and scents and are derived from isoprene units, which is also one of the precursors of phytocannabinoids (De Meijer et al., 2009). Terpenes comprise around 10% of trichome content by weight, making them a significant component of cannabis resin (Potter, 2009). Terpene concentrations >500 ppm are of pharmacological interest (Smith et al., 2005; Baser and Buchbauer, 2010; Pauli and Schilcher, 2010) as they can affect ion channels and various types of receptors to induce secondary messenger systems and signaling cascades (Baser and Buchbauer, 2010). Cannabis-derived terpenes may have anxiolytic properties, and these include D-limonene, myrcene, α-Pinene, linalool, β-Caryophyllene, humulene, trans-nerolidol, and many others (Russo, 2011). Linalool, a monoterpene common to both lavender and cannabis possesses potential anti-neoplastic, sedative, and anxiolytic properties via modulatory activity on glutamate and GABA neurotransmitter systems (Silva Brum et al., 2001; Pauli and Schilcher, 2010). β-Caryophyllene is one of the most abundant terpenes found in cannabis extracts and is postulated to be an anti-inflammatory analgesic (Basile et al., 1988). Recent evidence suggests that the cannabinoid receptor subtype 2 (CB2) is involved in regulation of mood and anxiety disorders (Hill and Gorzalka, 2009; Ashton, 2011) and β-Caryophyllene is a selective full agonist at CB2 receptor (Gertsch, 2008; Bahi et al., 2014), and when administered systemically in mice, it produces anxiolytic effects (Bahi et al., 2014). In addition, trans-nerolidol is a non-toxic sesquiterpene which demonstrated anxiolytic activity in mice (Goel et al., 2016). Overall, cannabis products retaining the full spectrum of cannabinoids and terpenes may provide a novel therapeutic approach for the treatment of anxiety. In addition to their own mechanisms of activity, terpenes are postulated to alter the effects of cannabinoids and contribute to the greater "entourage effect" (Russo, 2011). There is some research on the therapeutic effects of terpenes in humans, and there is evidence to show that the minor constituents of cannabis modulate the effects of the major cannabinoids. However, research demonstrating how different terpene profiles change the pharmacological activity of cannabis in humans is lacking.

Depending on the genetics and environmental growth conditions of a cannabis plant, different ratios of cannabinoids and terpenes are produced, and infinite variations are possible through selective breeding and blending of different chemotypes (Lewis et al., 2017). It is postulated that the unique mixture of active compounds within each variety or extract of cannabis may together form a collective activity which cannot be reduced to any singular component such as THC or CBD. This possible modulatory property of terpenes and other minor constituents in cannabis is termed the "entourage effect" (Russo, 2011). Whole plant cannabis extracts containing THC or CBD are found to exert different activities compared to pure cannabinoids in pre-clinical models (Carlini et al., 1974; Ryan et al., 2006). A recent meta-analysis of observational research on human patients using CBD for the treatment of epilepsy has shown that cannabis extracts enriched in CBD were more potent and caused less adverse effects than isolated CBD products (Pamplona et al., 2017). Altogether, current evidence indicates that whole plant extracts containing predominately THC and CBD exert an array of different effects than their respective isolated compounds. Additional research to elucidate the contribution that minor cannabinoids such as Cannabigerol (CBG), Cannabichromene (CBC), and terpenes make in the therapeutic effects of whole plant extracts is much needed. Therefore, in this study, the relationship between patient preference for certain cannabis strains for anxiety relief and their chemotype is investigated.

METHODS

Survey

A survey was conducted on *Typeform* (www.typeform.com), an online survey application, by *Whistler Medical Marijuana Corporation* (WMMC), a licensed producer of medical cannabis in Canada. The survey contained 90 questions which included, but were not limited to, participants' health conditions (e.g., diverse medical history and diagnosis), use patterns of medical cannabis, perceived effectiveness of cannabis at alleviating or managing their symptoms, and cannabis strain preference for treating different symptoms. A survey link was sent to patients via email after they received their medical cannabis from WMMC. Informed consent was obtained from survey participants. Access to the survey was restricted to one use only to avoid duplication of survey results. Participants rated strains as most effective or least effective for treatment of their anxiety, followed by rating the degree of effectiveness of cannabis overall for management of their anxiety on a Likert Scale of 10. Inclusion criteria included only registered WMMC patients who ordered either oil and dried cannabis products/strains from WMMC and are diagnosed with one or more health conditions by a certified healthcare professional.

Terpene and Cannabinoid Analysis

Samples from 21 different lots of dried cannabis (comprising three production lots of seven different strains) were analyzed for levels of cannabinoids and terpenes. The previous three production lots of each strain identified in the survey were analyzed. High Performance Liquid Chromatography coupled with Mass Spectrometry (MS) was used for detection and quantification of eight major cannabinoids in all samples of cannabis. Gas chromatography coupled with Mass Spectrometry (GC-MS) was used for detection and quantification of 29 terpenes in all samples. Analysis was done by independent testing laboratories (MB Labs and Anandia Labs). Anandia and MB Labs are private commercial laboratories and obtaining information regarding their specific procedures was not possible as it breached their intellectual property policies. Lots P051 and P069 were analyzed by MB labs, while all others were analyzed by Anandia Laboratories. Samples analyzed by MB Labs were not analyzed for trans-nerolidol, eucalyptol, γ-terpinene, and α-terpinene.

Data Analysis

Survey

Survey data from participants' responses were analyzed descriptively on Microsoft Excel 2016. Data analysis of self-reported health conditions, perceived cannabis effectiveness, and cannabis use were conducted.

Weighted Average Cannabinoid and Terpene Profiles

To find an "average" cannabinoid and terpene profile for strains which are considered most anxiolytic or least anxiolytic by patients of WMMC, a weighted average was calculated for each cannabinoid and terpene from the four most and four least anxiolytic strains. Potency data, including the values of THC, CBD, CBG, and CBC (given as total potential to include both acidic and neutral forms), along with 29 terpenes were collected from the last three production lots of each strain identified in the survey. For a given compound, each test result (three for each strain) was multiplied by the number of votes its strain received as a weighting factor, all values summed together, and then divided by the total of all the weights. Standard error was calculated as weighted standard deviation divided by the square root of the sample size. An example of the weighted average calculation is given below:

$$THC\ (WA) = \frac{[THC]\,s1,t1\ x\ Vs1 + [THC]\,s1,t2\ x\ Vs1 + [THC]\,s1,t3\ x\ Vs1 + \ldots + [THC]\,s4,t1\ x\ Vs4 + [THC]\,s4,t2\ x\ Vs4 + [THC]\,s4,t3\ x\ Vs4}{Total\ Number\ of\ Votes}$$

$$\frac{(16.6\%\ x\ 44) + (19.4\%\ x\ 44) + (21.0\%\ x\ 44) + \ldots + (19.8\%\ x\ 33) + (14.2\%\ x\ 33) + (20.1\%\ x\ 33)}{44 + 44 + 44 + \ldots + 33 + 33 + 33} = 18.0\%\ THC \quad (1)$$

s = strain, t = test, WA = weighted average, V= votes.

Cannabinoid and Terpene Correlation Coefficients

A difference of means test was used to find an association between chemotype and anxiolytic activity. Correlation coefficients were calculated by subtracting the least effective weighted average from the most effective and dividing by the sum as displayed below. Positive numbers indicate a correlation with increased anxiolytic activity and negative numbers with a decrease in anxiolytic activity. A weighted standard distribution was used to find standard error and *p*-values were calculated using a difference of means test between the two weighted averages. The null hypothesis is that the difference of means is zero, so a double-sided *t*-test was used to find significance.

$$THC\ (cc) = \frac{THC\ (wa,\ me) - THC\ \left(wa, le\right)}{THC\ (wa,\ me) + THC\ \left(wa, le\right)} \quad (2)$$

$$THC\,(\mathrm{cc}) = \frac{18.0 - 13.7}{18.0 + 13.7} = 0.137 \quad (3)$$

me = most effective, le = least effective, wa = weighted average, cc = correlation coefficient.

RESULTS

Sample Survey Population

A total of 442 participants, medical cannabis patients from WMMC, completed the online survey. Survey participants included 267 males, 173 females, and 2 unidentified sex. Median age range for both male and female participants were 40–59 years of age ($n = 113$; $n = 82$), followed by 20–39 years of age ($n = 109$; $n = 56$). Average rate of survey participants' perceived health was 6.28 on a Likert Scale of 9 (0 = Very Poor and 9 = Excellent).

Anxiety Specific Responses

Over half of the total respondents ($n = 266/442$, 60%) reported anxiety as a symptom for which they use medical cannabis. A smaller portion—15% of the participants—reported being diagnosed with a specific anxiety disorder. When asked about side effects, 14% of participants reported anxiety as an adverse reaction. Side effects as experienced by survey participants are listed in **Table 1**. Survey participants were asked about their perception of how cannabis helps alleviate their symptoms by rating its overall effectiveness on a Likert Scale from 0 (not effective) to 10 (extremely effective). **Figure 1** displays a histogram of survey participants' responses on a Likert Scale. The average score reported for treating symptoms of anxiety was 8.03, with a median response of 8 ($n = 260$), indicating patients found the use of cannabis quite effective overall for their anxiety.

To elucidate which cannabis varieties participants preferred for treating their wide range of health conditions, they were asked to identify the strain(s) or product(s) which had the most and least beneficial effect for the treatment of their conditions. Participants chose from a list of the 25 most common varieties provided by WMMC, and an "other" category for any strains not captured. Patients could select more than one strain to be either effective or least effective for management of their anxiety. A total of 219 and 189 patients responded for the most effective strain and the least effective, respectively. Excluding "others" as an option, the top four strains which were rated as the most effective were Bubba Kush ($n = 44$, 20.1%), Skywalker OG Kush ($n = 39$, 17.8%); Blueberry Lambsbread ($n = 36$, 16.4%); and Kosher Kush ($n = 33$ 15.1%). The top four least effective strains were Chocolope ($n = 22$, 11.6%) Blueberry Lambsbread ($n = 16$, 8.5%), CBD Shark ($n = 16$, 8.5%), and Tangerine Dream ($n = 15$, 7.9%). The most and least efective strains as rated can be found in **Tables 2**, **3** respectively.

Weighted Average Terpene and Cannabinoid Concentrations

To find a correlation between chemotype and effectiveness for treating anxiety, weighted averages were calculated for each cannabinoid and terpene in the groups of most effective and least effective strains. The number of votes each strain received in the most effective or least effective category was used as a weighting factor for each terpene and cannabinoid constituent in the strains. The weighted average terpene and cannabinoid concentrations for the groups of most and least effective strains can be seen in **Table 4** and **Figures 2A,B**, **3A,B**. In the most effective strains, trans-nerolidol was the most abundant terpene. In the least effective strains, myrcene was the most abundant terpene.

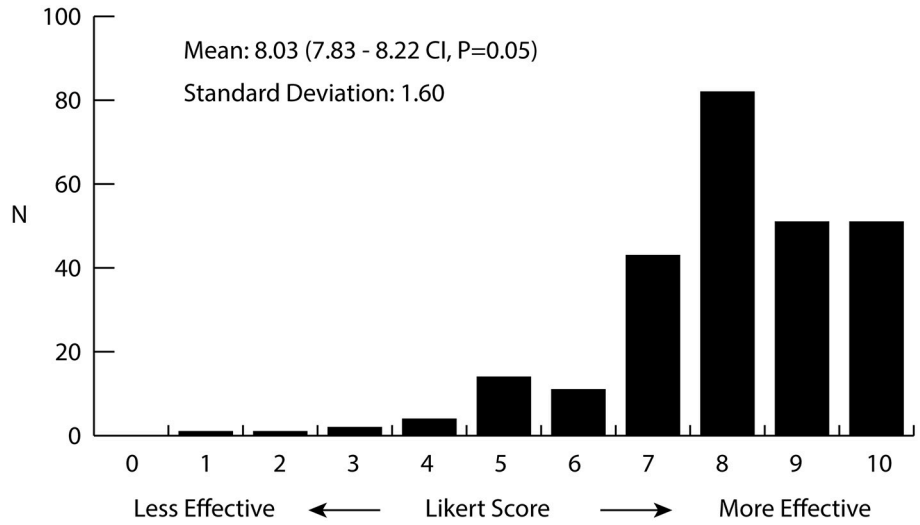


FIGURE 1 | Histogram of the perceived effectiveness of cannabis for the treatment of anxiety as rated by medical cannabis patients. Patients ($n = 260$) were asked to rate the effectiveness of cannabis for the treatment of anxiety on a Likert scale from 0 to 10, 0 being not effective at all and 10 being extremely effective. Average value was 8.03 (7.83–8.22 CI, $p = 0.05$) with a standard deviation of 1.60.

TABLE 1 | Side effects experienced by survey participants from consumption of WMMC strains and products ($n = 442$)*.

Side effect	No. of respondents	Percentage of respondents
Dry mouth	274	64.9
Short-term memory	144	34.1
Anxiety	60	14.2
Paranoia	56	13.3
Respiratory problems	25	5.9
Others	51	12.1

Survey participants experienced more than one possible side effect from consumption of WMMC strains and products.

TABLE 2 | Strain(s) or product(s) most effective for treating anxiety for survey participants.

Survey response	Votes (*n*)
Bubba Kush	44
Skywalker OG Kush	39
Blueberry Lambsbread	36
Kosher Kush	33

Participants (n = 219) were asked to identify which strain(s) or product(s) they found most effective for the treatment of anxiety.

TABLE 3 | Strain(s) or product(s) least effective for treating anxiety for survey participants.

Survey response	Votes (*n*)
Chocolope	22
Blueberry Lambsbread	16
CBD Shark	16
Tangerine Dream	15

Participants (n = 189) were asked to identify which strain(s) or product(s) they found least effective for the treatment of anxiety.

Cannabinoid and Terpene Correlation Coefficients

A difference of means test was used to find a correlation coefficient for each constituent using the weighted average concentrations of active compounds for the groups of most and least effective strains. The values for the least effective strains were subtracted from the most effective strains and divided by the sum of the two groups to find a final correlation coefficient for each terpene or cannabinoid constituent. Positive numbers indicate a correlation with more anxiolytic activity, negative numbers indicate a correlation with less anxiolytic activity. A weighted standard distribution was used to calculate the standard error for the correlation factor. A two-sided t-test was used to calculate the significance of each correlation coefficient. A complete list of all weighted averages, correlation coefficients, and p-values is shown in **Table 4**. Correlation coefficients for cannabinoids (total potential) and total terpenes is displayed in **Figure 4A**. THC was significantly correlated with increased anxiolytic activity, while CBD displayed a strong negative correlation, but this was only significant at $p = 0.09$. All the correlation factors for major terpenes (>0.05%) are displayed in **Figure 4B** and minor terpenes (<0.05%) in **Figure 4C**. Trans-nerolidol has a significant ($p < 0.05$) correlation with increased anxiolytic activity, while guaiol eucalyptol, γ-terpinene, α-phellandrene, 3-carene, and sabinene hydrate significant negative correlations. All raw data can be found in the **Supplementary Material**.

TABLE 4 | Weighted averages of all terpenes and cannabinoids for the top four most effective and least effective strains, their correlation coefficient, and *p*-values.

Constituent	Most effective weighted average (% w/w)	Least effective weighted average (% w/w)	Correlation coefficient	*p*-values
Δ^9-Tetrahydrocannabinol	18.01	13.67	0.137	0.013
Cannabidiol	0.075	2.62	−0.944	0.097
Cannabigerol	0.68	0.76	−0.059	0.690
Cannabichromene	0.13	0.21	−0.248	0.120
Total terpenes	1.850	1.621	0.066	0.402
α-Pinene	0.160	0.092	0.270	0.265
Camphene	0.011	0.009	0.110	0.365
Sabinene	0.042	0.014	0.498	0.141
β-Pinene	0.067	0.051	0.136	0.143
Myrcene	0.319	0.357	−0.057	0.703
α-Phellandrene	0.000	0.009	−1.000	0.046
3-Carene	0.000	0.006	−1.000	0.046
D-Limonene	0.211	0.150	0.168	0.237
Eucalyptol	0.000	0.005	−0.953	0.012
β-Ocimene	0.007	0.042	−0.712	0.091
Terpinolene	0.019	0.134	−0.755	0.072
a-Terpinene	0.000	0.006	−0.959	0.067
y-Terpinene	0.000	0.005	−0.954	0.022
Sabinene hydrate	0.000	0.005	−0.843	0.018
Fenchone	0.013	0.013	0.030	0.834
Linalool	0.109	0.108	0.004	0.976
Fenchol	0.032	0.024	0.133	0.271
Borneol	0.020	0.023	−0.074	0.391
α-Terpineol	0.061	0.059	0.018	0.892
Geraniol	0.000	0.000	N/A	N/A
y-Terpineol	0.013	0.000	1.000	0.374
Nerol	0.000	0.000	N/A	N/A
β-Caryophyllene	0.296	0.188	0.222	0.081
Caryophyllene oxide	0.010	0.006	0.260	0.311
Humulene	0.098	0.078	0.116	0.461
Valencene	0.003	0.000	1.000	0.332
trans-Nerolidol	0.444	0.146	0.503	0.018
Cedrol	0.000	0.000	N/A	N/A
Guaiol	0.000	0.069	−1.000	0.003
α-Bisabolol	0.077	0.039	0.335	0.108

The weighted average of the least effective was subtracted from the most effective and divided by their sum to calculate the correlation coefficient. Weighted standard distributions were used to calculate p-values with a two-sided t-test.

DISCUSSION

The Anxiety of Fragmentation

Over the past 70 years, scientists have attempted to study and treat anxiety, along with other mental illnesses, using a reductionist medical model (Andreasen, 1985). The focus of this model is on treating physical changes in brain structure and neurochemistry, which consequently assumes that there are appropriate drugs to effectively treat specific mental health issues (Bolton, 2008). This endeavor has failed to produce any clinically relevant biomarkers to diagnose any mental illness, nor has any drug been developed from genetic studies of mental illness (Dean, 2017). Reductionism is accepted as truth by many scientists despite the fact that there is little to no empirical data to support this conclusion, meanwhile, evidence for emergence and top-down modulation of living systems is abundant (Primas, 1991). The widespread reductionist philosophy has made our scientific, social, and even ecological landscapes increasingly fragmented over the past century (Watson, 1971; Haddad et al., 2015). In scientific research, investigators are increasingly specialized, becoming factionalized into smaller and smaller groups of experts to the detriment of a greater understanding between disciplines (Casadevall and Fang, 2014). The implications of fragmentation in science and society have been postulated to have

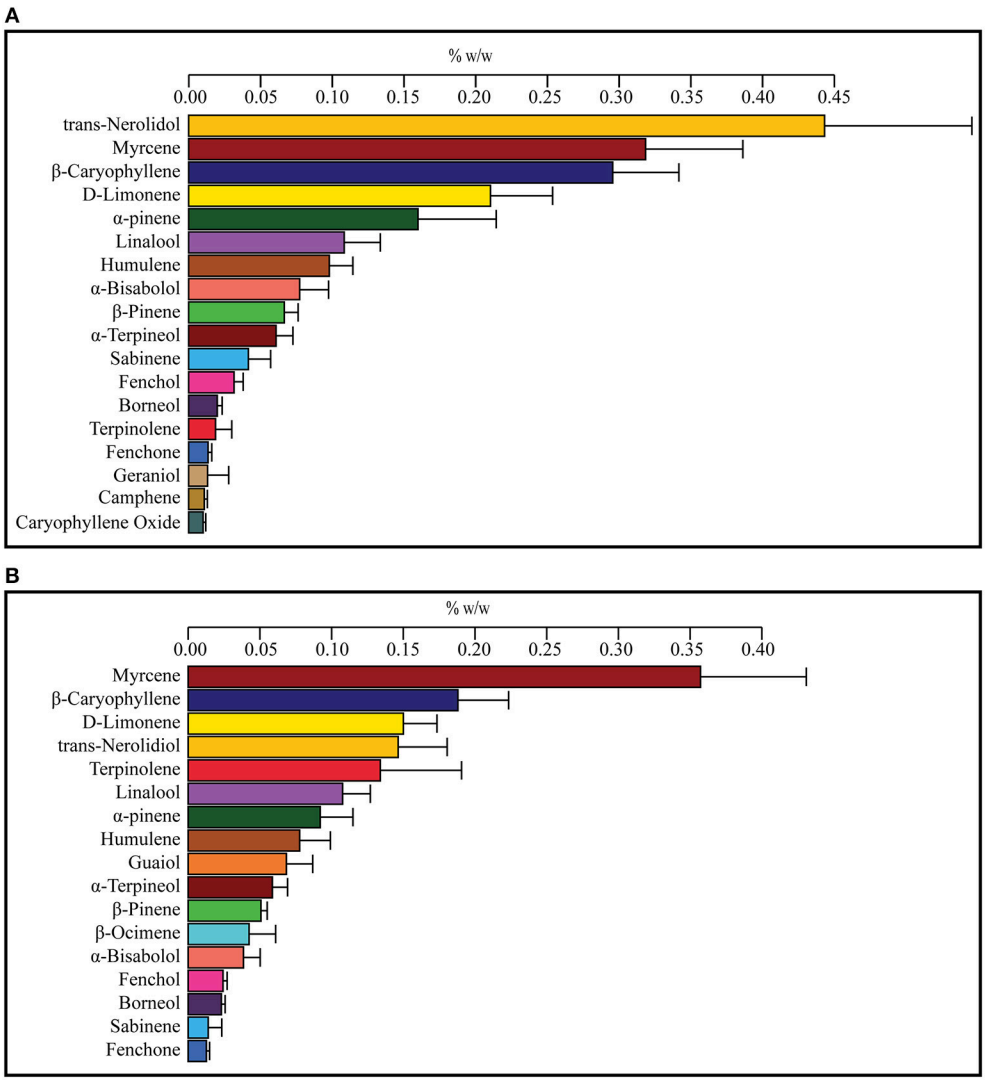


FIGURE 2 | Weighted average terpene concentrations for the top four most effective **(A)** and top four least effective **(B)** cannabis strains as rated by medical marijuana patients. Patients were asked to select the cannabis strain(s) that were the most effective or least effective for the treatment of their anxiety. Selected strains were analyzed for the concentration of cannabinoids and terpenes and weighted average values for the most and least effective groups calculated using the number of votes as a weighting factor. Values over 0.01% w/w are displayed.

wide ranging consequences as described by physicist David Bohm in his book *Wholeness and the Implicate Order* (Bohm, 2002). Bohm postulates that the fragmentation of science compounds our lack of sustainable solutions as findings of the scientific method are not being integrated to obtain a comprehensive holistic viewpoint.

In the case of anxiety, this fragmentation has segregated medical treatment and the drug discovery process from the study of many of the risk factors. In addition to genetic determinants of anxiety, there are also significant social, environmental, and experiential risk factors. Traumatic events and social determinants of health are risk factors for anxiety (National Research Council Institute of Medicine, 2009). Recent epigenetic research has demonstrated that early life traumatic events leave lasting changes to the epigenome, and there are even significant differences in DNA-methylation patterns between different socio-economic classes (Labonté et al., 2012; McGuinness et al., 2012).

Environmental factors are also important for mental illnesses. Access to green spaces is well established to have beneficial effects on mental health, and the magnitude of these effects is correlated to the biodiversity of those green spaces (Duarte-Tagles et al., 2015; Wheeler et al., 2015). The loss of local biodiversity in one's external environment leads to a loss of personal microbiome diversity with negative health consequences (Prescott et al., 2018). Currently, we are experiencing a loss of species 1,000 times greater than the background rate, and this is projected to increase if human activity continues in the current trend of pollution

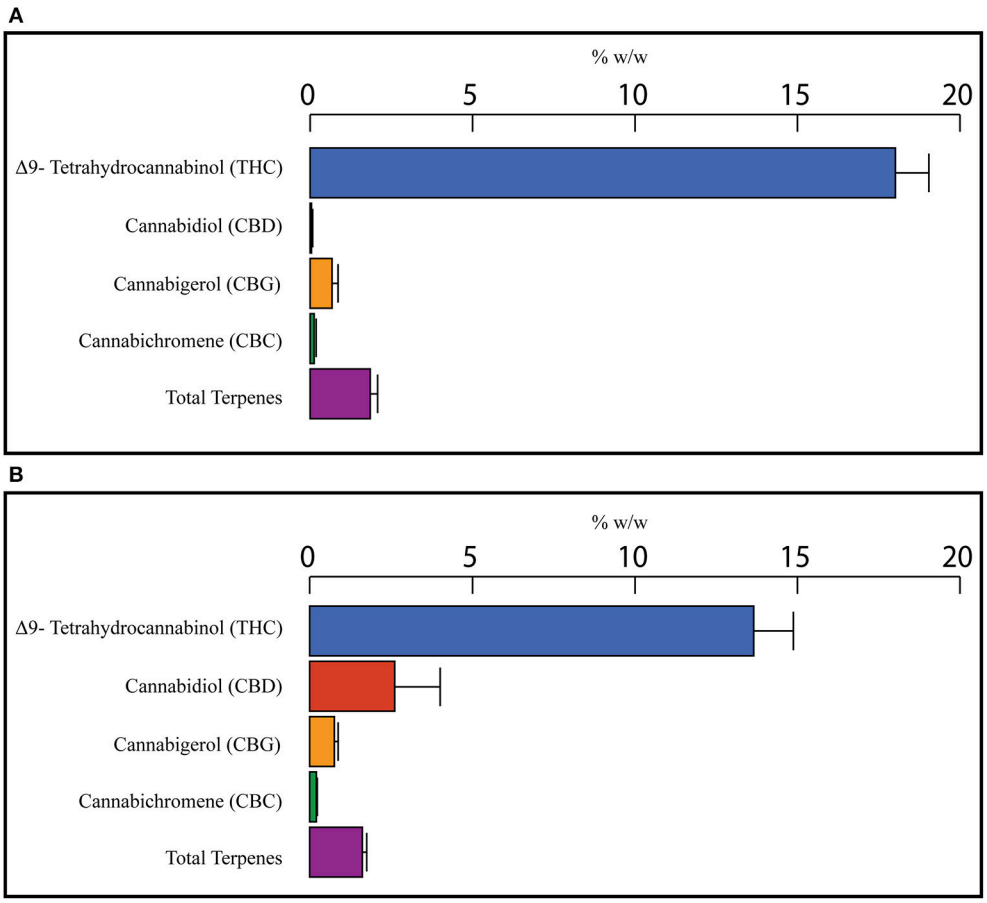


FIGURE 3 | Weighted average cannabinoid and total terpene concentrations for the top four most effective **(A)** and top four least effective **(B)** cannabis strains as rated by medical marijuana patients. Patients were asked to select the cannabis strain(s) that were the most effective or least effective for the treatment of their anxiety. Selected Strains were analyzed for the concentration of cannabinoids and terpenes and weighted average values for the most and least effective groups calculated using the number of votes as a weighting factor.

and habitat destruction (Díaz et al., 2006; Pimm et al., 2014), threatening the health of future generations (Díaz et al., 2006). Climate change is already associated with increased stress and anxiety, and this is only projected to increase with crop failures, economic perturbations, and habitat destruction (Fritze et al., 2008; Berry et al., 2010). The rise of chronic illness in humans and the destruction of the biosphere are intricately connected and cannot be viewed as separate. The refusal to integrate the evidence of these risk factors into treatment protocols or the drug discovery process is hurting our ability to make effective health interventions. The fragmentation of scientific research has separated those studying environmental and socioeconomic factors from those conducting clinical research or drug discovery. A holistic solution to anxiety and other mental illness represents an incredibly complex problem which requires social, economic, and ecological interventions on a large scale.

High Hopes for Holistic Healing

Holism, the opposing view to reductionism, is the idea that complex systems cannot be completely understood just in terms of their simplest components—as espoused by Aristotle *"The whole is greater than the sum of its parts."* Emergent phenomenon, such as living systems, display top-down modulation that cannot be explained just in terms of their simplest components (Kesić, 2016). Systems biology adopts a holistic stance in the current reductionist paradigm. Its proponents think it could lead to better medical outcomes, although they are usually still limited in scope to the human as a system, and not yet the entire biosphere (Small, 2017). In terms of cannabis, the holistic view is that each strain's medical activity is not reducible to one or a few components, but the result of an entourage effect from all its active components together. An even wider viewpoint can see the industrial and commercial uses of cannabis, along with its medical activity, as part of a path to better health for our entire biosphere and all its inhabitants.

Cannabis is becoming increasingly researched and recognized for the treatment of many health conditions including anxiety, stress, depression, and pain (Notcutt et al., 2004; Andreae et al., 2015). Furthermore, the industrial uses of cannabis can replace many polluting industries causing damage to our ecosystems, such as concrete and petroleum fuels (Arrigoni et al., 2017) (Prade et al., 2011) and can even be used to

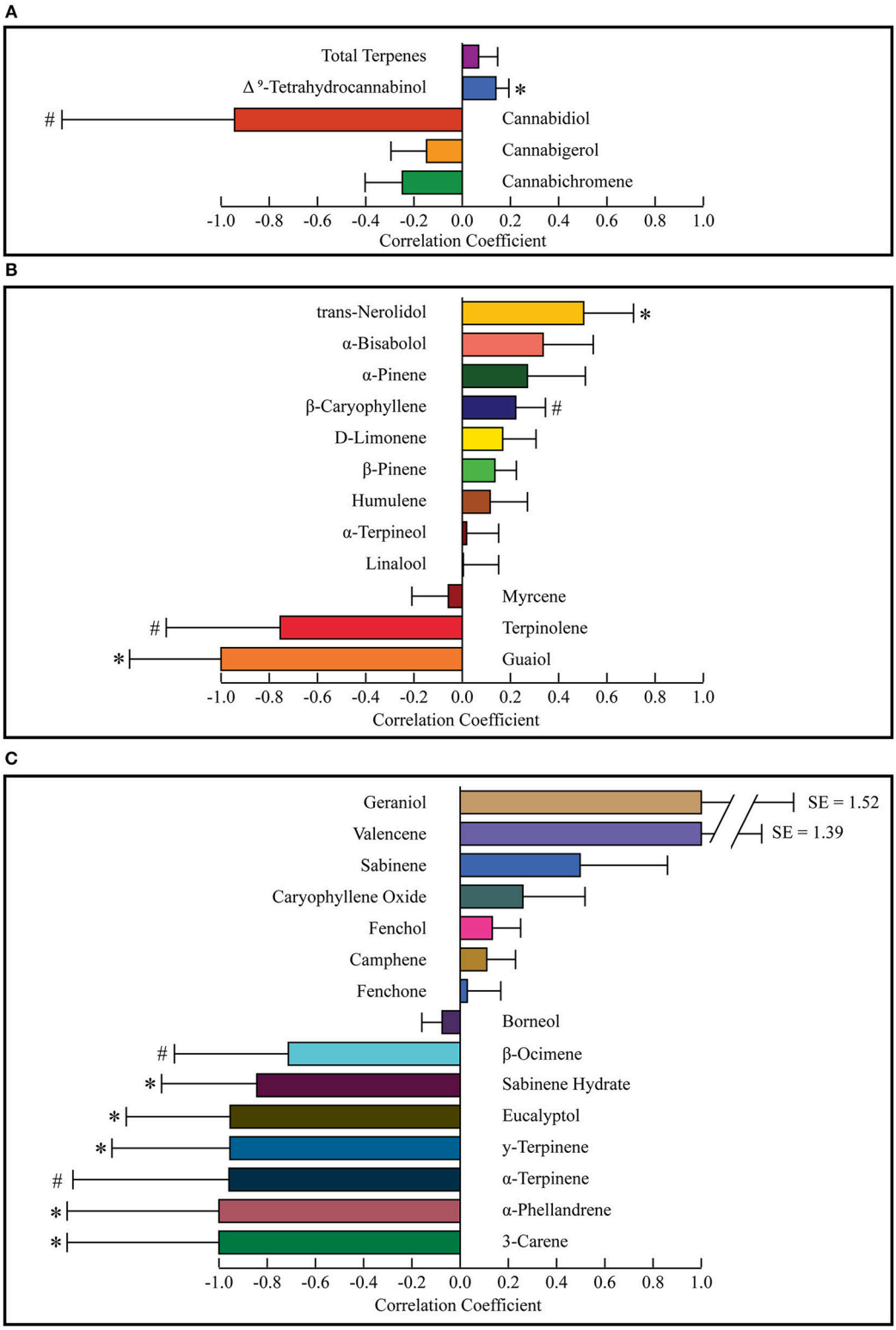


FIGURE 4 | Correlation coefficients for cannabinoids and total terpenes **(A)** major terpenes **(B)**, and minor terpenes **(C)**. Correlation coefficients were calculated for each constituent using a difference of means test. The weighted average of the least effective was subtracted from the most effective and divided by their sum to calculate the correlation coefficient. Weighted standard distributions were used to calculate *p*-values with a two-sided *t*-test. Values market with * indicate $p < 0.05$ and those marked with # indicate $0.05 < p < 0.10$. SE, Standard Error.

remediate contaminated soils (Poursafa et al., 2012). It has been a source of fiber, food, oil and medicine during the last 10 millennia (van Bakel et al., 2011), and during this time, humans expanded the natural range of cannabis from a small niche as a mountain herb in Central Asia to now having a growth range that spans more than half of the earth's land. This resulted in "landrace" varieties with diverse chemotypes adapted to local ecosystems (Hillig, 2005; Warf, 2014). This possible symbiotic relationship between humans and *Cannabis sativa* seems to have been beneficial to both parties, but has been violently interrupted with worldwide prohibition enacted in the last century (Miron, 1999). Altogether, *C. sativa*, through its many cultivars and uses, represents part of a holistic solution to many of our health problems, including anxiety. It can treat patients suffering today, and simultaneously help preserve the environment to prevent future illnesses. However, due to its diverse chemotypes, not all varieties of cannabis may be suitable to use as a pharmacological intervention for anxiety.

A Systems Approach for Finding an Anxiolytic Cannabis Chemotype

In this study, we describe the start of a holistic drug discovery process. This paper serves as a proof of concept for sustainable and equitable medicine production, while simultaneously investigating which cannabis chemotypes are optimal for treating anxiety. We looked for a correlation between different cannabis chemotypes and the subjective effectiveness of cannabis varieties to treat symptoms of anxiety. Most studies have focused on using an isolated cannabinoid or terpene, to investigate its activity in pre-clinical models. However, most medical cannabis patients use herbal cannabis products which are a complex mixture of active compounds instead of isolated compounds. Thus, we aim to study the effects of specific cannabis strains in patients who use herbal cannabis products as treatment for anxiety.

Currently, there is no standardization of cannabis cultivars due to its legal position in the past century. Cannabis varieties are referred to as "strains," but two producers can sell a cannabis "strain" with the same name, while the products have different chemotypes. To avoid these issues, we chose to work with a single medical cannabis producer, WMMC, based in Canada. Canada has a highly regulated medical cannabis industry, and WMMC is inspected by federal inspectors from Health Canada to ensure that cannabis medicines are produced under sanitary conditions and with proper systems of quality assurance. WMMC was the ideal medical cannabis producer because of its vertical integration, wide range of cannabis varieties, and full analytical testing for all produced lots. Canadian medical cannabis regulations dictate producers must sell cannabis directly to the patient registered with them, as opposed to from a dispensary which sells products from many producers. This vertical integration gives patients exposure to many products from a single producer, which allowed us to match a chemotype to each strain they used. WMMC is also a certified organic producer, using local agricultural inputs, demonstrating that sustainable and equitable production methods can be used to make safe, regulated, and effective medicines.

Using data from a survey conducted by WMMC on their patients along with the analytical test results of the cannabis they sold to patients, a model was constructed to determine which cannabis chemotypes are most useful for the treatment of anxiety. A focus on anxiety was chosen as it is one of the most common symptoms patients report using medicinal cannabis for, and quite prevalent in overall Canadian society. In our survey, 60% of the overall survey participants ($n = 266/442$) reported anxiety as a symptom and 15% of survey participants ($n = 67$) reported being diagnosed with an anxiety-related disorder. **Figure 1** shows that 227 of 260 (87%) respondents rated cannabis as 7 or higher in effectiveness for treating their anxiety, indicating it is quite effective for a large majority of respondents. Since the survey only relied on self-reporting and was only sent to current medical cannabis patients actively ordering products from WMMC, it is likely to exclude many people who may have tried medical cannabis for anxiety and stopped after finding it ineffective. Nonetheless, this data suggests a certain subset of patients suffering from anxiety are finding strong relief from using cannabis. Another point of consideration is that many of the survey participants have had months or years to find the cannabis strains which best treat their symptoms, so the effectiveness may be higher than for inexperienced patients who have only had access to one or few strains.

When asked which strains were most effective for treating anxiety, no single strain was overwhelmingly preferred by participants, with the top four strains only garnering 44, 39, 36, and 33 votes of 219 total respondents. It is important to note that survey participants could choose more than one strain. There was even less consensus on the least effective strains, with the top four least effective strains only having 22, 16, 16, and 15 votes out of a total of 189. The lower number of total responses for "least effective" strains, and the larger distribution over the answers seems to suggest that cannabis overall is effective for the treatment of anxiety, and the participants' dissatisfaction with particular strains may have more to do with unique personal reactions than a shared disease etiology. An important consideration is that what patients categorized as the "least effective strains" may still be effective anxiolytic agents, but just not as potent as the "most effective strains." Alternatively, these strains could also have a neutral or even anxiogenic effect, but this could not be ascertained from the current survey. When asked about side effects, 15% of respondents reported anxiety, indicating some patients experience angiogenic effects. Further research should try to classify strains as either anxiogenic, neutral, or anxiolytic to better understand the effects of different chemotypes. Furthermore, what makes some cannabis users experience anxiogenic instead of anxiolytic effects should be investigated to determine whether these experiences are the result of the environment ("set and setting"), the dosage, or the entourage effect.

All the strains chosen can be roughly grouped into three categories by their terpene profile. Three of the four most effective strains were "Kush" varieties, which all share a similar chemotype with high levels of trans-nerolidol, β-Caryophyllene, and D-limonene, and contain genetics from landrace strains found in the Kush mountain range in Central Asia (Hillig, 2005).

The large amount of terpinolene in the least effective weighted average is predominantly from one strain, Chocolope, which was the strain chosen most often as least effective. Myrcene dominant strains were found to be less effective and the terpinolene dominant strain the least effective. One myrcene dominant strain, Blueberry Lambsbread, was in the top four choices for the most and the least effective. This could be the result of different symptom etiology requiring different pharmacological interventions or may also arise from differences in personal biochemistry between patients with similar etiology. This overlap of strains in both categories and the lack of a clear consensus for anxiolytic strains suggests that *"a one size fits all"* approach to anxiety treatment with medical cannabis may not be the most effective therapeutic approach. The future of cannabis medicine could be one that is personalized, whereby a specific cannabis formulation is created based on each patient's unique disease etiology and biochemistry.

Depicted in **Figures 2A,B** are the weighted average terpene profiles for the top four most or least effective anxiolytic strains. Most of the same terpenes are present in both profiles (and most cannabis strains in general), with the one notable exception: guaiol only being present in the least effective strains and geraniol in the most effective, but both at low concentrations. The most abundant terpene in the least effective was myrcene, followed by β-caryophyllene and D-limonene. In the most effective strains, trans-nerolidol was the most abundant, followed by myrcene and β-Caryophyllene. Trans-nerolidol was the dominant terpene in three of the four most effective strains, while terpinolene was dominant in Chocolope. Chocolope had the most votes of any strain for least effective.

In **Figures 3A,B**, the weighted average cannabinoid profiles are shown. All the most effective strains and three of the least effective were high THC varieties. One type II strain was selected as a least effective strain, making that average of the least effective strains enriched in CBD and have a slightly lower THC concentration (**Figure 3B**). Similar research involving more type II/III strains could provide better data for cannabinoids.

Figure 4 shows correlation coefficients for all terpenes and cannabinoids. Standard error and *p*-values were calculated using the weighted standard deviations and a two-sided *t*-test. As seen in **Figure 4A**, THC is significantly correlated with increased anxiolytic activity, corroborating previous research. CBD displayed a correlation with decreased anxiolytic activity, but it was not significant. **Figure 4B** displays the correlation coefficients for major terpenes, which were all terpenes with over 0.05% w/w in either the most effective or the least effective weighted average. This level was chosen as previous research has indicated terpenes with over 0.05% w/w are of pharmacological interest (Baser and Buchbauer, 2010). Of the major terpenes only trans-nerolidol displayed a significant ($p = 0.018$) correlation with increased anxiolytic activity. In addition to trans-nerolidol, caryophyllene has a positive association with a *p*-value of 0.08, while α-pinene and D-limonene displayed non-significant correlations with increased anxiolytic activity, corroborating pre-clinical research on all these compounds (Guimarães et al., 1990; Lorenzetti et al., 1991; Komori et al., 1995; Long et al., 2010; Pauli and Schilcher, 2010; Goel et al., 2016; Bhattacharyya et al., 2017). Of importance is the fact that the zero line on the correlation coefficient is equal to the average anxiolytic activity of all the terpenes and does not imply no activity. Some terpenes with negative coefficients may still be anxiolytic, but certain terpenes with high negative coefficients, such as terpinolene or guaiol may be anxiogenic. Guaiol was the only major terpene with a "perfect" correlation. It was found in three of the least effective strains and in none of the most effective, giving a correlation coefficient of −1 and the highest significance of any compound ($p = 0.003$). Guaiol was only found in modest quantities (0.05–0.15%), and therefore may be just a chemotype marker, but could also be a compound with significant anti-analgesic activity. The analysis used assumes a normal distribution for constituents in each group, but many are found in bimodal distributions, like CBD and terpinolene, making their *p*-values less reliable. Since the only strain with a terpinolene dominant chemotype, chocolope, also received the most votes for least effective, we suspect terpinolene may have a stimulating activity which is anti-analgesic.

Figure 4C displays correlation coefficients for the minor terpenes (<0.05% content w/w). Eucalyptol, γ-terpinene ,α-phellandrene, 3-carene, and sabinene hydrate are significantly correlated with decreased anxiolytic activity. Since these minor terpenes are only present in minute quantities, they may be markers of certain chemotypes rather than important active ingredients. For example, the strain Chocolope, which is the only terpinolene dominant strain, is also the only strain to contain significant quantities of the minor terpenes with −1 correlations (3-carene, α-phellandrene, α-terpinene, and γ-terpinene). These minor terpenes could be markers of this terpinolene-dominant chemotype and offer little activity due to low concentrations. It is also possible that all these minor terpenes display a potent synergy and are important for the activity of this chemotype. Most terpenes are present in both groups, and the activity of each terpene is modulated by all the others, such that certain terpenes could be causing anxiolytic activity only when in the presence of certain others. More research is warranted to better understand which chemotypes will be the most appropriate or ideal for the treatment of anxiety. The survey nature of this study and its inherent error lead to many limitations and more controlled investigations are necessary to understand the anxiolytic activity of various cannabis chemotypes.

There were many limitations to this study. First, as this study is survey-based, recall bias may play a role in patients' self-reporting. Patients could be using strains of the same name from different licensed producers which have different chemical profiles than those offered by WMMC. Many patients will only have tried a few strains and have a smaller range of possible chemotypes to gauge effectiveness. We did not provide patients with any products for a specific duration. Our research was inherently exposed to selection bias as our participants were only WMMC patients; our results may be different if we had enrolled patients outside of WMMC. There is a possibility of response bias as participants may choose strains as effective or ineffective based on anecdotal reports or hearsay. Patients could have been using other medications at the same time as their medical cannabis which could confound their perceptions of cannabis effectiveness. Due to many confounding factors, we feel the overall power of this study to make definitive conclusions is low. Further pharmacological investigations with much

more controlled parameters are necessary to determine which cannabis chemotypes produce anxiolytic or anxiogenic effects. This preliminary data can be used to develop products with chemotypes directed toward anxiety and test their effectiveness in more controlled environments. Using repeated iterations of this process, cannabis chemotypes optimized for anxiolytic activity can be identified.

The results from this survey indicate that many patients find relief from the symptoms of anxiety by using medical cannabis. This study also demonstrates that effective medicines can be produced organically, locally and sustainably and still comply with strict quality specifications. Patients have a specific preference for certain strains of cannabis for treating anxiety, and strains which they find most effective have distinctly different chemotypes than those they find least effective. In contrast, some other patients experience anxiety as a side effect of using cannabis. Cannabis can be used as an effective anxiolytic agent, but further investigations are required to find which chemotypes or doses are anxiolytic, and which are anxiogenic. From a broader perspective, cannabis can be produced sustainably, and its many uses could help us to preserve the biosphere we all need to survive, relieving anxiety worldwide.

DATA AVAILABILITY

The raw data supporting the conclusions made in this manuscript is available on request.

ETHICS STATEMENT

This study was performed in accordance with the recommendations of the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans, Canadian Institutes of Health Research, Natural Sciences and Engineering Research Council of Canada, and Social Sciences and Humanities Research Council of Canada. This research used anonymized data from surveys on medical marijuana patients conducted for quality assurance purposes and was thus found to be exempt from IRB review and approved under the expedited review provisions of the Institutional Review Board Services standard operating procedures.

AUTHOR CONTRIBUTIONS

BK and DL created the online administered survey. FK, BK, and DL analyzed the results and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2018.00730/full#supplementary-material

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Behavioral and Pharmacokinetic Profile of Indole-Derived Synthetic Cannabinoids JWH-073 and JWH-210 as Compared to the Phytocannabinoid Δ^9-THC in Rats

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Synthetic cannabinoid compounds are marketed as "legal" marijuana substitutes, even though little is known about their behavioral effects in relation to their pharmacokinetic profiles. Therefore, in the present study we assessed the behavioral effects of systemic treatment with the two synthetic cannabinoids JWH-073 and JWH-210 and the phytocannabinoid Δ^9-THC on locomotor activity, anxiety-like phenotype (in the open field) and sensorimotor gating (measured as prepulse inhibition of the acoustic startle response, PPI), in relation to cannabinoid serum levels. Wistar rats were injected subcutaneously (sc.) with JWH-073 (0.1, 0.5, or 5 mg/kg), JWH-210 (0.1, 0.5, or 5 mg/kg), Δ^9-THC (1 or 3 mg/kg) or vehicle (oleum helanti) in a volume of 0.5 ml/kg and tested in the open field and PPI. Although JWH-073, JWH-210, Δ^9-THC (and its metabolites) were confirmed in serum, effects on sensorimotor gating were absent, and locomotor activity was only partially affected. Δ^9-THC (3 mg/kg) elicited an anxiolytic-like effect as suggested by the increased time spent in the center of the open field ($p < 0.05$). Our results further support the potential anxiolytic-like effect of pharmacological modulation of the endocannabinoid system.

Keywords: synthetic cannabinoids, Δ^9-THC, pharmacokinetics, behavior, JWH-073, JWH-210

INTRODUCTION

Synthetic cannabinoids (SCs) are substances referred to as cannabinoid CB1 and/or CB2 receptor ligands that were originally developed as research tools to assess the endocannabinoid system (ECS) pharmacology and to examine the cannabinoid CB1 and CB2 receptors (Wiley et al., 2012). Since the beginning of 2000s, they appeared on the drug market worldwide under exotic brand names such as "Spice," "Jamaican Spirits," or "K2" and have become popular for their psychoactive and euphoric cannabis-like effects and also for their ability to escape detection by standard cannabinoid screening tests (Fattore and Fratta, 2011).

Most synthetic cannabinoids are highly lipophilic compounds which easily cross the blood brain barrier, and they typically exhibit higher affinities (in some cases 100 times higher) for central and peripheral cannabinoid CB1 receptors than the psychoactive phytocannabinoid Δ9-tetrahydrocannabinol (Δ^9-THC; Ki = 41 ± 2 nM) (Huffman et al., 2005). Therefore, they could induce stronger cannabimimetic effects such as anti-nociception, catalepsy, hypothermia, cognitive impairment, altered sensory perception and psychotic reactions (Huffman et al., 2005; Kucerova et al., 2014; Fattore, 2016; Tait et al., 2016).

Unlike *cannabis*, which has a reputation as fairly benign substance, the SCs have been associated with systemic toxicities including myocardial infarction (Schwartz et al., 2015), ischemic strokes (Freeman et al., 2013; Takematsu et al., 2014), seizures (Schneir and Baumbacher, 2012; Schwartz et al., 2015), acute kidney injury (Buser et al., 2014) and sudden death; thus their abuse has become a substantial social and public health issue (Behonick et al., 2014; Castaneto et al., 2014, 2015; Tai and Fantegrossi, 2014).

One of the most frequently occurring SCs identified in specimens from users belongs to the group of indole-derivatives or aminoalkylindoles family (the "JWH" series) (Uchiyama et al., 2011; Carroll et al., 2012). Among these, JWH-073 (1-butyl-1H-indol-3-yl)-1-naphthalenyl-methanone) has four-fold higher binding affinity toward central CB1 receptors (Ki that ranging from 8.9 ± 1.8 to 12.9 ± 3.4 nM) than Δ^9-THC (Wiley et al., 1998; Aung et al., 2000; Brents et al., 2012) and is biotransformed *in vivo* into monohydroxylated metabolites that retain significant affinity and activity at cannabinoid CB1 receptors (Brents et al., 2012). *In vivo* animal studies report that JWH-073 reproduces the typical "tetrad" effects of Δ^9-THC such as hypothermia, analgesia, hypolocomotion, akinesia (Wiley et al., 1998; Brents et al., 2012; Marshell et al., 2014), as well as impaired sensorimotor responses, seizures and aggressiveness (Ossato et al., 2016). In human studies agitation, hallucinations, confusions and alterations in cognitive abilities have been reported (Papanti et al., 2013; Zawilska and Wojcieszak, 2014).

JWH-210 is a newer compound detected in the "marijuana alternatives", which has a high binding affinity toward central cannabinoid CB1 receptors (Ki = 0.46 ± 0.03 nM). In comparison to other cannabinoids, it has 20 times higher affinity to CB1 than JWH-073 and 100 times higher than Δ^9-THC; thus it reproduces a stronger "tetrad" effects in rodents as well as nausea, seizures and cardiovascular impairment in humans (Dogan et al., 2016; Hermanns-Clausen et al., 2016; Tait et al., 2016).

Given that these drugs have been found in severe poisonings in humans, including fatalities, we assessed the behavioral and pharmacokinetic profile of JWH-073 and JWH-210 as compared to Δ^9-THC in rats. More specifically, their potential anxiogenic- and/or anxiolytic-like effects were investigated in the open field test (OFT), an unconditioned test based on spontaneous behavior of animals which is usually used to assess anxiety, as well as exploration and locomotor activity (Micale et al., 2013b). Given that chronic *cannabis* use in healthy individuals or systemic treatment with CB1/CB2 agonists (i.e., Δ^9-THC or WIN55,212-2) in laboratory animals may affect sensorimotor gating (Kucerova et al., 2014), the prepulse inhibition (PPI) of the acoustic startle response (ASR) was also assessed (Micale et al., 2013a; Horsley et al., 2018). Alongside this, the pharmacokinetic profiles of JWH-073, JWH-210, Δ^9-THC (as well as Δ^9-THC metabolites 11-OH-THC and THC-COOH) in serum were also evaluated.

MATERIALS AND METHODS

Animals

All experiments were carried out on male Wistar rats (200–250 g) (VELAZ, Czech Republic). Animals were housed in pairs in a 12 h light/dark cycle regime at 22 ± 2°C and water and standard diet *ad libitum*. Before the behavioral testing, animals (n = 10 per group) were acclimatized for 7–10 days during which they were handled four times and weighed twice. Experiments and measurements were conducted during the light phase of the cycle (between 8:00 and 14:00 h). In order to minimize the total number of animals used across experiments, rats from behavioral experiments were subsequently used for pharmacokinetic analyses (n = 8 per one time point). All experiments respected the Guidelines of the European Union (86/609/EU) and the National Committee for the Care and Use of Laboratory Animals (Czech Republic), and were according to Guidelines of the European Union (86/609/EU). The protocol was approved by the National Committee for the Care and Use of Laboratory Animals (Czech Republic) under the number: MEYSCR-27527/2012-31.

Drugs and Chemicals

The SCs JWH-073 (1-butylindol-3-yl)-naphthalen-1-ylmethanone and JWH-210 (4-ethyl-1-naphtalenyl) (1-phenyl-1H-indol-3-yl)-methanone were purchased *via* the internet and subsequently purified by Alfarma s.r.o (Czech Republic). The resulting compounds were analyzed for purity, JWH-073 99.48% and JWH-210 97.84% (analyzed by infrared spectroscopy), and in the form of a free base were dissolved in pharmaceutical grade sunflower oil (oleum helanti) and administered subcutaneously (sc.) at the doses of 0.1, 0.5, or 5.0 mg/kg in a volume of 0.5 ml/kg. The phytocannabinoid Δ^9-THC 99.3% (THC-Pharm GmbH) was dissolved in oleum helanti and administered sc. at the dose of 1 or 3 mg/kg in a volume of 0.5 ml/kg. Control animals were treated with the corresponding amounts of sunflower oil as vehicle. The doses of the SCs were selected according to the reports from users on the internet and according to the potency of similar compounds that have been tested in preclinical experiments (Cha et al., 2015; Gatch and Forster, 2016; Ossato et al., 2016). The doses of Δ^9-THC were selected based on our previous results focusing on its behavioral and pharmacokinetic effects induced by different routes of administration (Micale et al., 2013a; Hlozek et al., 2017).

Pharmacokinetics

Determination of JWH-073 and JWH-210 Levels in Serum Samples

Different groups of rats (n = 8 per group) were treated sc. with JWH-073 (0.5 mg/kg), JWH-210 (0.5 mg/kg) and subsequently decapitated after 30 min, 1, 2, 4, 8, or 24 h. Serum samples were

collected and stored at −20°C. These samples were analyzed after extensive optimization and validation of the sample preparation procedure according to the 2001 FDA Guidance using LC-MS method. Serum sample preparation consists of a protein precipitation and was following: (1) 800 μL 0.1% solution of formic acid in acetonitrile (v/v) was cooled down for 30 min at −20°C; (2) 200 μL of serum was added to the cooled solution and immediately mixed in a Bullet Blender Storm homogenizer (Next Advance, United States) for 5 min (speed 4); (3) centrifugation for 10 min (14,000 RPM) at 5°C; (4) evaporation of 800 μL supernatant to dryness (Centrivap Concentrator); and (5) reconstitution with 0.1% formic acid in water/acetonitrile, 80/20 (v/v). Prior to analysis by LC-MS, all samples were vortexed and centrifuged. LC-MS analysis: the samples in this section were analyzed using UHPLC-MS/MS instrumentation (1,290 Infinity Agilent Technologies Agilent 6460 Triple Quadrupole LC/MS with Agilent Jet Stream electrospray ionization source). A column Agilent Zorbax Eclipse RRHD (50 × 2.1 mm, 1.8 μm) with a pre-column was used for a chromatographic separation with gradient elution in system of 0.1% (v/v) formic acid (mobile phase A) and acetonitrile (mobile phase B). Data were acquired in positive electrospray ionization (ESI) mode by a multiple reaction monitoring method (MRM). JWH-073 and JWH-210 were quantified using an external matrix-matched calibration (US FDA. Guidance for Industry: Bioanalytical Method Validation. US FDA, Center for Drug Evaluation and Research, MD, USA 2001). Limit of detection (LOD) and quantification (LOQ) were for both drugs 0.05 ng/ml and 1 ng/ml, respectively.

Determination of Δ^9-THC Levels in Serum Samples

Different groups of rats ($n = 6$ per group) were treated sc. with Δ^9-THC (3 mg/kg) and subsequently decapitated after 30 min, 1, 2, 4, 8, or 24 h. Serum were collected and stored at −20°C. Δ^9-THC were determined by an in-house validated and certified GC-MS method (certified by Police Presidium of the CR, ref. no.: PPR-31123-7/CJ-2015-990530/ evidence no.: 16/2015). A total of 10 μl of deuterated THC-d3/11-OH-THC-d3/THC-COOH-d3 (5 ng/μl) internal standard solution was added to each 1.0 ml sample of serum. Serum was diluted with a 4 ml sodium acetate buffer with a pH of 4.0 (0.01 mol/l). Serum phytocannabinoid Δ^9-THC was extracted with SPE columns (Bond-ELUT, 130 mg, Agilent Technologies), eluted with hexal/ethyl acetate (1:4 v/v) and dried under a nitrogen gas stream in a 400 μl glass insert placed in a 1.5 glass vial. The samples were derivatized with 100 μl of N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) for 20 min at 80°C. Quantification of extracted Δ^9-THC was performed by gas chromatography-mass spectrometry (GC-MS) (GC7860/5742CMSD, Agilent Technologies) using electron impact ionization in the selective ion mode (THC: m/z 386; THC-d3: m/z 389; 11-OH-THC: m/z 371; 11-OH-THC-d3: m/z 374; THC-COOH: m/z 371; THC-COOH-d3: m/z 374). Calibration curve ranges were prepared by spiking drug-free bovine serum at concentrations (1) 2–200 ng/ml THC, 11-OH-THC, and THC-COOH; (2) 100–1,000 ng/ml THC, 11-OH-THC and THC-COOH. Limit of detection (LOD) and quantification (LOQ) were 1 and 2 ng/ml, respectively. The spikes were vortexed and treated identically to the experimental samples (Hlozek et al., 2017).

Behavioral Experiments

All behavioral experiments were performed 1 h after sc. drugs administration.

Open Field

A square black plastic open field arena (68 × 68 × 30 cm) was placed in a soundproof and diffusely lit room. Each of animals was placed into the center of the arena, in a novel unfamiliar environment, and 1 h after drug administration the behavior was video-recorded for 30 min using the system EthoVision Color pro v. 3.0 (Noldus, NL). Locomotor activity was subsequently analyzed within 5 min blocks/time intervals (1–6). The calculation of the data was performed in the EthoVision software and corrected (smoothed) for movement deviations of <3 cm. Initially total distance traveled per time block was calculated and data were plotted in the graphs. To evaluate the spatial characteristics of the locomotor activity such as thigmotaxis and time spent in the center of arena, the arena was virtually divided into 5 × 5 identical square zones with 16 located peripherally and nine centrally. Frequency (f) of appearances of the animal in different zones of the arena was used to calculate thigmotaxis (i) ($i = \sum f_{peripheralzones} / \sum f_{allzones}$) which is a number (value varying from 0 to 1) indicating the probability of appearance in peripheral zones. Time spent in the center of the arena (T_{center}) was calculated $T_{center} = \sum time_{centralzones}$ (Balikova et al., 2014; Horsley et al., 2016; Palenicek et al., 2016; Tyls et al., 2016; Hlozek et al., 2017; Sichova et al., 2017; Stefkova et al., 2017).

Prepulse Inhibition (PPI) of Acoustic Startle Response (ASR)

The PPI of ASR took place in two ventilated startle chambers (SR-LAB, San Diego Instruments, California, USA) which were calibrated to ensure equivalent stabilimeter sensitivity between the chambers. The test consists of acclimatization and two sessions, as previuosly described (Direnberger et al., 2012; Palenicek et al., 2016; Tyls et al., 2016; Hlozek et al., 2017; Sichova et al., 2017; Stefkova et al., 2017). Briefly, acclimatization was performed 2 days before the test, when drug-free rats were habituated in 5 min session with five presentations of pulse alone stimuli (115 dB/20 ms) over background white noise (75 dB). On the day of test, the compounds or vehicle were administered sc. 1 h prior to PPI/ASR testing. After acclimatization (5 min with 75 dB background noise), the test started with a short session of six 40 ms 125 dB pulse trials to establish baseline ASR. It was followed by the second session consisting of trials presented in a pseudorandom order: (1) single pulse alone: 40 ms 125 dB; (2) trial of prepulse-pulse: 20 ms prepulse of 83 dB presented 30, 60, and 120 ms (average 70 ms) before 40 ms 125 dB pulse; (C) 60 ms no stimulus. Finally, six 40 s 125 dB pulse trials were delivered. Habituation was calculated by the percentage reduction in ASR from the initial six, to the final six pulse trials. PPI was calculated as: [100–(mean prepulse–pulse trials/mean pulse alone trials)*100]. ASR was derived from mean pulse alone trials. Animals with an AVG response lower than 10 were excluded from further analysis as non-responders.

Statistical Analysis

To evaluate the effect on the locomotion measured in 5 min intervals, two-way repeated measures ANOVA analysis (factor 1: drug; factor 2: time intervals) was used in software system IBM SPSS version 22. Significant main effects and interaction two-way repeated measures ANOVAs were followed with pairwise comparisons using independent *t*-test. For repeated measures ANOVAs, where Mauchly's test of sphericity was significant, Greenhouse-Geisser [Greenhouse-Geisser estimate of sphericity (ε) <0.75 or Huynh-Feldt (ε) >0.75] correction are reported. Degrees of freedom were rounded to whole number for presentational purposes. For independent *t*-test, where Levene's test for equality of variances was significant, statistics corrected for unequal variances are given $p < 0.05$ (two tailed) was considered the minimal criterion for statistical significance. For multiple comparisons, *t*-test was used with Bonferroni correction. The total length of the trajectory over 30-min, thigmotaxis and time in the center, ASR, habituation and PPI were analyzed by one-way ANOVA analysis by using software STATISTICA version 9.0. Where appropriate, ANOVA analyses were followed by the Tukey *post-hoc* test. Statistical significance was set at $p < 0.05$ for all analyses.

RESULTS

Pharmacokinetics

Δ^9-THC (3 mg/kg) and its metabolites 11-OH-THC and THC-COOH were detected within 24 h after sc. administration (**Figure 1A**). They reached the maximum serum concentration (Mean ± SEM; Δ^9-THC: 12.1 ± 3.06 ng/ml; 11-OH-THC: 2.08 ± 1.21 ng/ml; THC-COOH: 10.5 ± 7.27 ng/ml) 1 h after the treatment. Second peak of Δ^9-THC was observed after 8 h. JWH-073 (0.5 mg/kg) and JWH-210 (0.5 mg/kg) were detected within 24 h after sc. administration (**Figure 1B**). The maximum mean of JWH-073 serum concentration (1.84 ± 0.06 ng/ml) was attained 4 h after the treatment. JWH-210 reached the maximum serum concentration (4.20 ± 0.86 ng/ml) 1 h after administration and second peak of JWH-210 was detected after 4 h. 24 h after both cannabinoids JWH-073 (0.41 ± 0.19 ng/ml) and JWH-210 (0.38 ± 0.12 ng/ml) were slightly above the level of detection (LOD = 0.05 ng/ml; LOQ = 1 ng/ml).

Behavior

Open Field Test: Total Locomotor Activity

Mauchly's test of sphericity was significant and Greenhouse-Geisser correction is presented for repeated measures, Mauchly's $W_{(14)} = 0.44$, $p = 0.01$. Analyses of locomotion in 5 min time intervals following Δ^9-THC administration revealed a main effect of time interval [$F_{(4, 133)} = 160.02$, $p < 0.001$], but neither a main effect of drug nor drug × time interval interaction were found (**Figure 2A**). The locomotor activity of Δ^9-THC and vehicle treated rats gradually decreased over the course of the test session, indicative of normal habituation.

For JWH-073 and JWH-210 Mauchly's tests of sphericity were significant [JWH-073: $W_{(14)} = 0.35$, $p = 0.00$; JWH-210: $W_{(14)} = 0.50$, $p = 0.00$] and Greenhouse-Geisser correction are presented for repeated measures JWH-073 and Huynh-Feldt correction are presented for repeated measures JWH-210. The SCs JWH-073 and JWH-210 had significant effect on time intervals [JWH-073: $F_{(3, 156)} = 260.85$, $p < 0.001$; JWH-210: $F_{(4, 204)} = 261.91$, $p < 0.001$]. Furthermore, there was drug effect for JWH-073 [$F_{(3, 46)} = 3.76$, $p < 0.05$], but not for JWH-210. The interaction between drug and time interval was significant for JWH-073 [$F_{(10, 156)} = 1.88$, $p = 0.05$], but not for JWH-210 [$F_{(11, 174)} = 1.67$, $p = 0.08$]. The locomotor activity gradually decreased in all treated groups suggesting that habituation was not attenuated in any of the treatments used (**Figures 2B,C**). At the dose of 0.1 mg/kg, JWH-073 significantly increased locomotor activity at the 5-10 and 10-15 min time blocks, minimum [$t_{(28)} = 1.60$, $p < 0.05$], but not in the others time blocks (with 0, 15, 20, and 25 min onset). Similarly, at the dose of 0.5 mg/kg it increased locomotion at 0–5, 5–10, 10–15, and 20–25 min onset, minimum [$t_{(28)} = 2.44$, $p < 0.01$]. By contrast, JWH-073 (5 mg/kg) reduced the locomotor activity at 15–20 min time block as compared to vehicle-treated animals [$t_{(28)} = 1.74$, $p < 0.05$; **Figure 2B**]. As described in **Figure 2C**, JWH-210 (0.5 mg/kg) treated rats

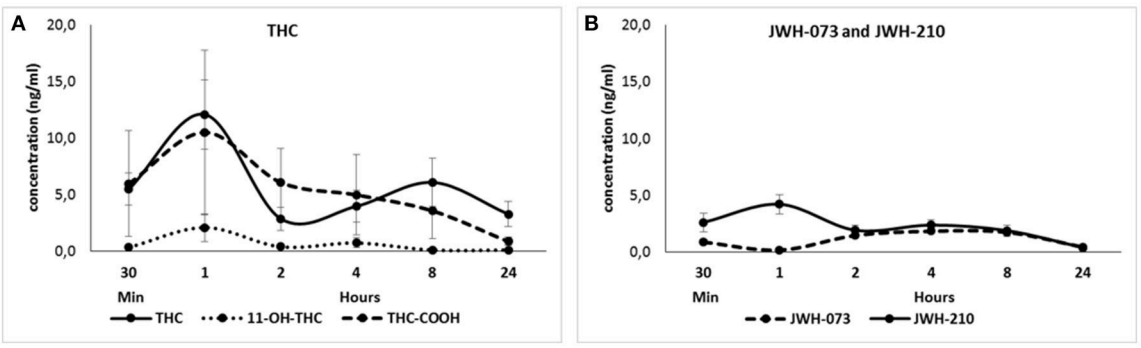


FIGURE 1 | (A) Pharmacokinetic profile of Δ^9-THC (3 mg/kg, sc.) and its metabolites 11-OH-THC and THC-COOH at different time point (30 min, 1, 2, 4, 8, or 24 h after treatment). Data are presented as mean ± SEM ($n = 6$ animals per group) of serum levels expressed in ng/ml. **(B)** Pharmacokinetic profile of JWH-073 (0.5 mg/kg, sc.) and JWH-210 (0.5 mg/kg, sc.) at different time point (30 min, 1, 2, 4, 8, or 24 h after treatment). Data are presented as mean ± SEM ($n = 8$ animals per group) of serum levels expressed in ng/ml.

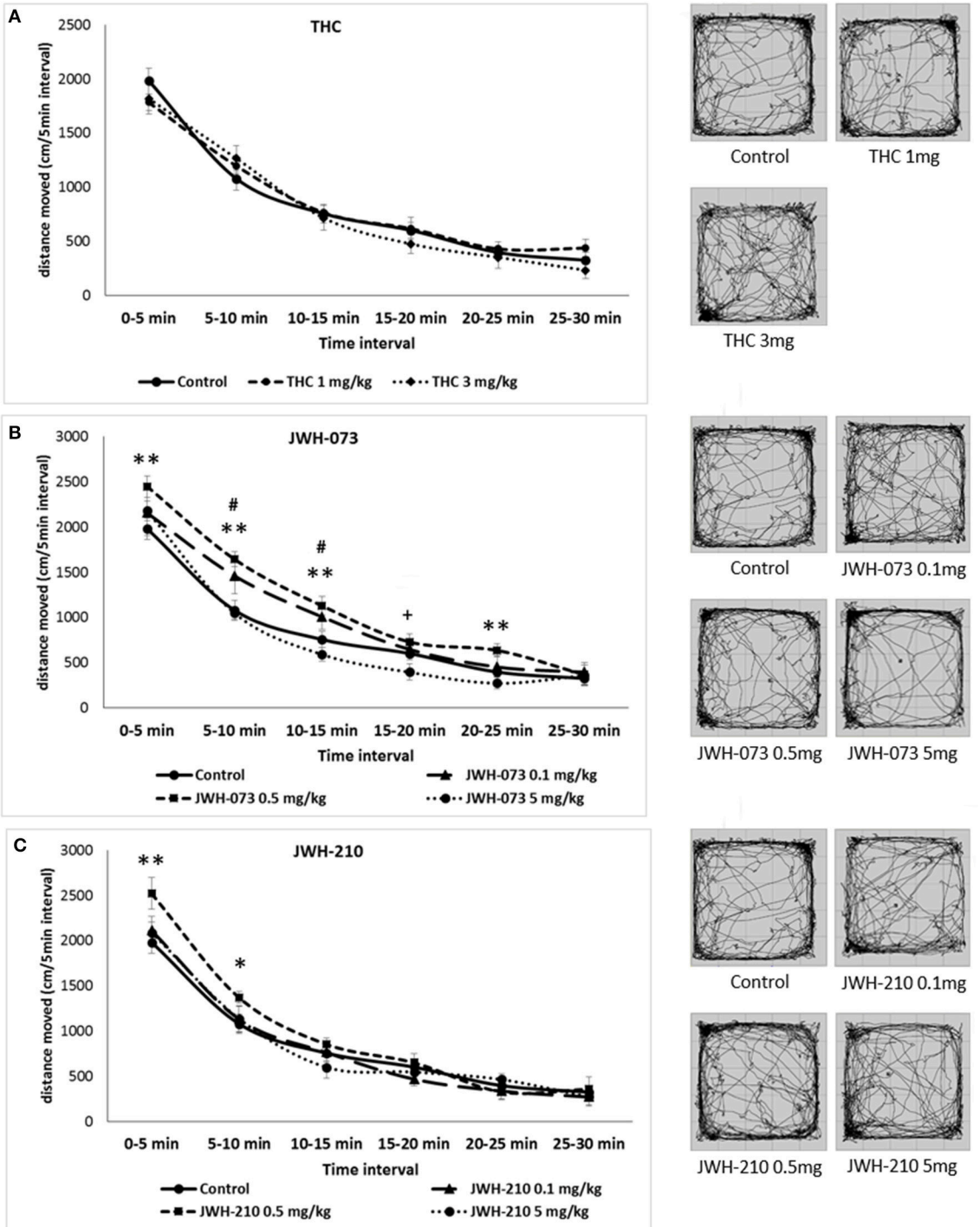


FIGURE 2 | Open field test (OFT): Trajectory length (divided into 5-min blocks) and trajectory pattern over the entire 30 min period test. **(A)** Δ^9-THC (1 or 3 mg/kg, sc.) error bars display ± 1 SEM. **(B)** JWH-073 (0.1, 0.5 or 5 mg/kg, sc.) error bars display ± 1 SEM. $^{\#}p < 0.05$ for JWH-073 0.1 mg/kg, sc., $^{**}p < 0.01$ for JWH-073 0.5 mg/kg, sc. and $^{+}p < 0.05$ for JWH-073 5 mg/kg, sc. vs. vehicle group. **(C)** JWH-210 (0.1, 0.5, and 5 mg/kg, sc.), error bars display ± 1 SEM. JWH-210 (0.5 mg/kg, sc.) $^{*}p < 0.05$; JWH-210 (0.5 mg/kg, sc.) $^{**}p < 0.01$ vs. vehicle group.

showed a significant increased locomotor activity at 0 to 5 and 5 to 10 min time blocks as compared to vehicle-treated group, minimum [$t_{(28)} = 1.79, p < 0.05$].

One-way ANOVA for total length of the trajectory over 30-min revealed a significant effect of JWH-073 treatment [$F_{(3, 46)} = 3.7562, p < 0.05$]. *Post-hoc* analysis showed that JWH-073 (0.5

mg/kg) significantly increased locomotor activity ($p < 0.05$) as compared to the control group (**Figure 3B**). Neither Δ^9-THC [$F_{(2, 37)} = 0.21685$, $p = 0.8$, **Figure 3A**] nor JWH-210 [$F_{(3, 46)} = 0.96950$, $p = 0.41$, **Figure 3C**] affected the total locomotor activity.

Open Field Test: Thigmotaxis and the Time Spent in the Center of Arena

Δ^9-THC significantly modified thigmotaxis [$F_{(2, 37)} = 6.4791$, $p < 0.05$] and the time in the central zones [$F_{(2, 37)} = 6.0172$, $p < 0.001$]. *Post-hoc* revealed that Δ^9-THC (3 mg/kg) increased time spent in central zone ($p < 0.01$, **Figure 4A**) and decreased thigmotaxis ($p < 0.01$, **Figure 4D**), as compared to control animals. By contrast, neither JWH-073 nor JWH-210 modified the thigmotaxis [JWH-073 $F_{(3, 46)} = 1.1572$, $p = 0.33$, **Figure 4E**; JWH-210 $F_{(3, 46)} = 1.8661$, $p = 0.14$, **Figure 4F**] or the time spent in the central zones [JWH-073 $F_{(3, 46)} = 1.1891$, $p = 0.32$, **Figure 4B**; JWH-210 $F_{(3, 46)} = 0.45117$, $p = 0.71$, **Figure 4C**].

Prepulse Inhibition (PPI) of Acoustic Startle Response (ASR)

ASR data were initially screened for non-responders (ASR < 10) leading to exclusion of following number of animals: JWH-073 ($n = 4$), JWH-210 ($n = 5$), Δ^9-THC ($n = 2$) and controls ($n = 3$). Subsequent analyses revealed that none of the tested compounds affected the ASR (**Figure 5A**). Similarly, habituation data showed no significant treatment effect.

Although Δ^9-THC treatment significantly affected PPI [$F_{(2, 32)} = 3.6635$, $p < 0.05$, **Figure 5B**], *post-hoc* analysis found a slight not significant decrease of PPI ($p = 0.06$) induced by Δ^9-THC at the dose of 1 mg/kg. Neither JWH-073 [$F_{(3, 39)} = 1.4218$, $p = 0.25$, **Figure 5C**] nor JWH-210 [$F_{(3, 38)} = 2.2994$, $p = 0.09$, **Figure 5D**] affected the PPI.

DISCUSSION

In this study, we evaluated the behavioral effects and the pharmacokinetic profile of acute treatment with JWH-073, JWH-210 and Δ^9-THC. The main findings of pharmacokinetic studies were as follows: JWH-073 had slow pharmacokinetics which peaked after 4 h, and was detectable at all measurement intervals with a temporary decrease 1 h after administration. JWH-210 had biphasic profile in serum, showing the highest peak and the second peak 1 and 4 h after administration, respectively. Furthermore, it was detectable at all intervals. The profile of Δ^9-THC in serum was very similar to that of JWH-210: biphasic, with the highest peak at 1 h post-administration but the second peak later, at 8 h after administration. Metabolites from Δ^9-THC (11-OH-THC and THC-COOH) had only one peak (1 h after the administration), and they were detectable as Δ^9-THC at all intervals.

The main behavioral effect was that JWH-073 at the dose of 0.5 mg/kg increased the total locomotor activity in the OFT. Furthermore, rats treated with Δ^9-THC (3mg/kg) spent more time in the center of OF arena, as index of anxiolytic-like effect. Sensorimotor gating, as measured by PPI, baseline startle (ASR) or habituation were not altered by the pharmacological treatment.

Pharmacokinetics

The phytocannabinoid Δ^9-THC (3 mg/kg) showed biphasic profile, in agreement with our previous results (Hlozek et al., 2017). A similar profile was also observed for JWH-210 and might be due to their partial release from subcutaneous tissue into the bloodstream, followed by their partial accumulation in adipose tissue; thus resulting in slow degradation over 24 h. By contrast, JWH-073 showed a major peak 4 h after the administration, suggesting its higher lipophilicity and slower release into the blood. Previous results have shown that Δ^9-THC elicited different behavioral effects as well as different serum levels based on route of administration (Leighty, 1973; Deiana et al., 2012; Hlozek et al., 2017). In animal studies focusing on the effects of different routes of administration (i.e., intraperitoneal, intravenous or pulmonary) on SCs pharmacokinetic profile more rapid peaks and higher concentrations were detected (Marshell et al., 2014; Kevin et al., 2017; Malyshevskaya et al., 2017). In

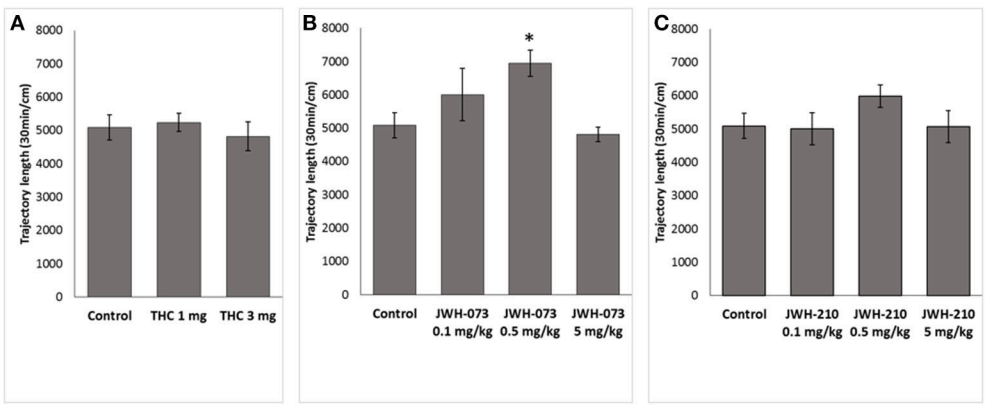


FIGURE 3 | Total locomotor activity. **(A)** Δ^9-THC (1 and 3 mg/kg, sc.), **(B)** JWH-073 (0.1, 0.5, and 5 mg/kg, sc.) and **(C)** JWH-210 (0.1, 0.5, and 5 mg/kg, sc.). Data are presented as mean ± SEM of distance traveled expressed in cm over the entire 30 min period test. *$p < 0.05$ vs. vehicle group.

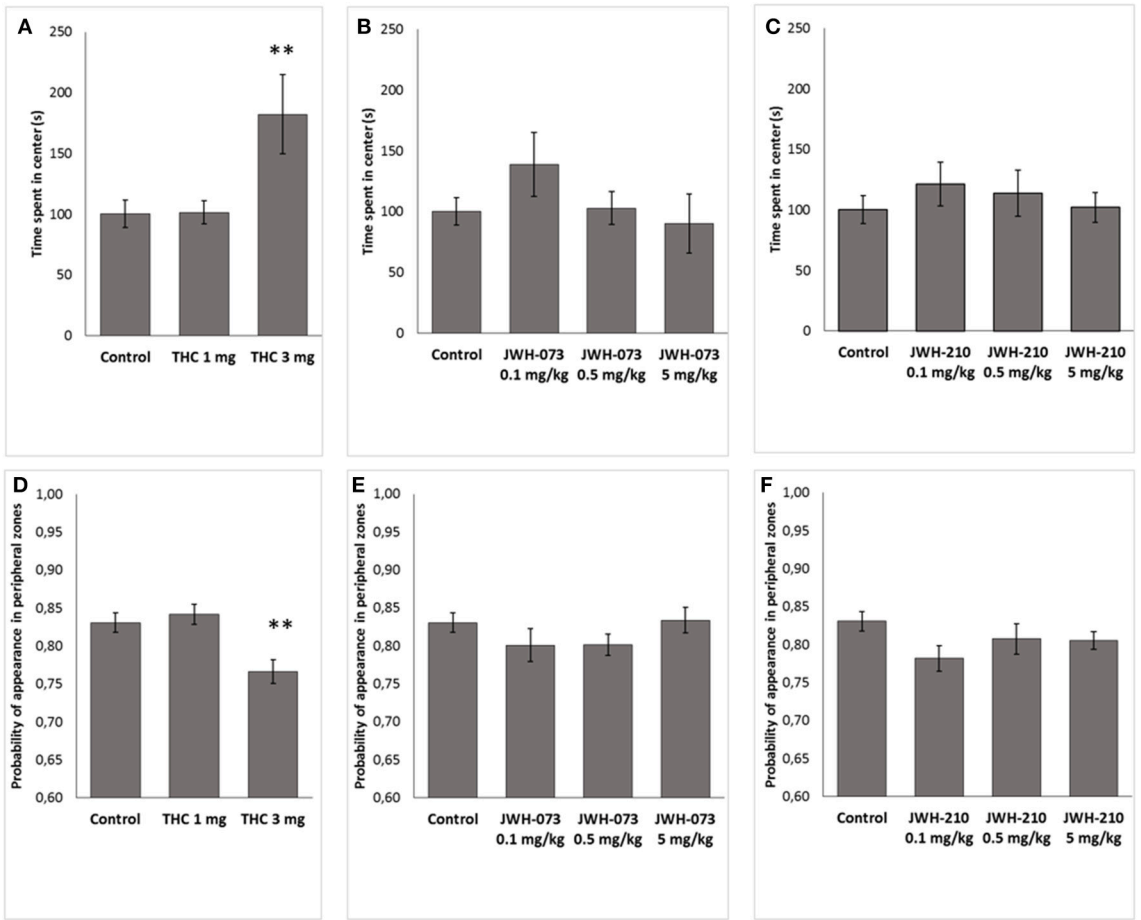


FIGURE 4 | Time (s) spent in the center of open field arena. **(A)** Δ^9-THC (1 and 3 mg/kg, sc.), **(B)** JWH-073 (0.1, 0.5, and 5 mg/kg, sc.) and **(C)** JWH-210 (0.1, 0.5, and 5 mg/kg, sc.). Probability of appearance in peripheral zones (thigmotaxis). **(D)** Δ^9-THC (1 and 3 mg/kg, sc.), **(E)** JWH-073 (0.1, 0.5 and 5 mg/kg, sc.) and **(F)** JWH-210 (0.1, 0.5, and 5 mg/kg, sc.). Data are presented as mean ± SEM. $^{**}p < 0.01$ vs. vehicle treated group.

our study the detection of cannabinoid serum levels 24 h after the treatment is in agreement with previous studies showing their detection even for longer period (Schaefer et al., 2014; Hasegawa et al., 2015).

Behavioral Effects: Open Field and PPI

We found that acute treatment with JWH-073 at dose of 0.5 mg/kg, but not of 0.1 or 5 mg/kg significantly increased total trajectory in the open field test. However, treatment with JWH-210 (0.1–5 mg/kg) did not result in locomotor activity change. Our results are not consistent with those previously described showing that treatment with JWH-210 (0.5–5 mg/kg; Gatch and Forster, 2016) or JWH-073 (3–30 mg/kg; Marshell et al., 2014) elicited a dose-dependent reduced locomotor activity. These discrepancies could be due to the species (rat *vs.* mice) difference in response to the treatment or to differences in experimental procedures (e.g., locomotor activity measured 1 h after sc. administration *vs.* measurement immediately after the intraperitoneal treatment). We also found that sc. treatment with Δ^9-THC (1 or 3 mg/kg) did not affect the total trajectory in the open field test, in line with our previous results (Hlozek et al., 2017).

However, spatial characterization of locomotor behavior showed that Δ^9-THC (3 mg/kg) increased the time spent in the center of the open field arena. Since this dose did not increase total locomotion, stimulatory effects do not likely account for this; increased exploration of the aversive central zone may therefore suggest an anxiolytic-like effect of this dose. Although there is contradictory literature about the behavioral effects of CB1 receptor activation in animal models of anxiety (as well as in humans), a general conclusion is that low and high doses of CB1 agonists induce anxiolytic and anxiogenic effects, respectively (Moreira and Wotjak, 2010). More specifically, in the elevated plus maze and in the light-dark box test, low doses of Δ^9-THC in rodents increased the time spent in open arms and the time in the light compartment, respectively (as index of anxiolytic-like effect), through a CB1 mediated mechanism (Berrendero and Maldonado, 2002; Patel and Hillard, 2006; Rubino et al., 2008). By contrast, higher doses of Δ^9-THC elicited anxiogenic-like responses in rodents (Patel and Hillard, 2006; Rubino et al., 2008; Hlozek et al.,

A

Measure		Vehicle	THC		JWH-073			JWH-210		
		----------	1 mg/kg	3 mg/kg	0.1 mg/kg	0.5 mg/kg	5 mg/kg	0.1 mg/kg	0.5 mg/kg	5 mg/kg
ASR (arbitary units)	Mean	195,01	120,28	222,49	105,56	179,94	119,31	124,09	202,13	340,69
	SEM	34,70	34,11	73,65	23,86	71,24	28,19	21,19	46,06	98,73
Percentage habituation	Mean	46,99	55,68	34,63	49,20	21,66	54,19	42,85	41,71	37,55
	SEM	8,88	7,40	11,50	7,36	12,85	8,30	5,94	9,53	16,29

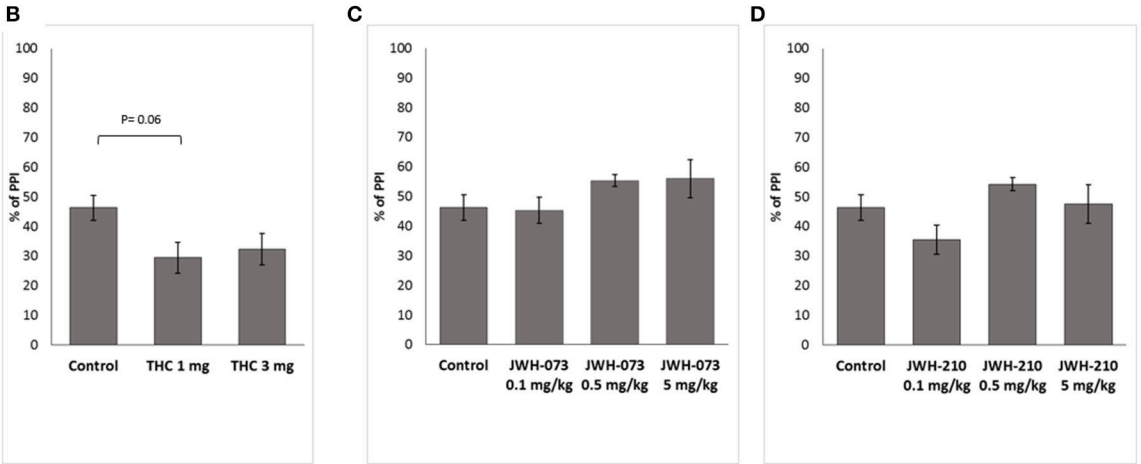


FIGURE 5 | (A) Effect of Δ^9-THC (1 or 3 mg/kg, sc.), JWH-073 (0.1, 0.5, and 5 mg/kg, sc.) and JWH-210 (0.1, 0.5 or 5 mg/kg, sc.) on acoustic startle response (ASR) and habituation. Effect of **(B)** Δ^9-THC (1 or 3 mg/kg, sc.), **(C)** JWH-073 (0.1, 0.5, or 5 mg/kg, sc.) and **(D)** JWH-210 (0.1, 0.5, or 5 mg/kg, sc.) on prepulse inhibition. Data are presented as mean ± SEM regarding percentage (%) of prepulse inhibition.

2017). The recent development of cell type specific genetic deletion of CB1 receptors has provided a new tool to better understand cannabinoid action, and assess the different role of the neuronal subpopulations of CB1-expressing neurons, such as GABAergic, glutamatergic and dopamine D1 terminals in the control of emotional behavior (Terzian et al., 2011, 2014; Micale et al., 2017). Given that CB1 receptors on GABAergic *vs.* glutamatergic terminals are required for the anxiogenic- *vs.* anxiolytic-like effects induced by high *vs.* low doses of the CB1 agonist CP55,940 (Rey et al., 2012), we cannot exclude that an anxiolytic-like effect of Δ^9-THC could be due to the specifically target the CB1 receptors on glutamatergic terminals. Further studies on animals with specific deletion of CB1 receptors in specific neuronal subpopulations are required to support this hypothesis.

In our study, we did not find significant alteration of ASR or PPI induced by acute treatment with Δ^9-THC, JWH-073 or JWH-210. It is noteworthy that previous studies describe controversial results in relation to the acute effects of cannabinoids on PPI. More specifically, in some studies Δ^9-THC and SCs (i.e., JWH-073, JWH-18, JWH-250, or WIN55212,2) dose-dependently decreased ASR (Levin et al., 2014; Ossato et al., 2016; Hlozek et al., 2017); as well as in other studies Δ^9-THC did not affect PPI (Malone and Taylor, 2006; Boucher et al., 2007; Long et al., 2010). Beyond the different route of administration or the different species (rats *vs.* mice) or stress sensitivities, we cannot also exclude that PPI alteration induced by cannabinoid exposure could be strain related, since spontaneously hypertensive rats (SHR), but not Wistar rats had a disturbed PPI induced by CB1/CB2 agonist (Levin et al., 2014).

CONCLUSIONS

Although JWH-073 and JWH-210 at the dose of 0.5 mg/kg had lowest and highest serum levels 1 h after the administration, respectively; our results suggest that their levels are not strictly related to their effects on locomotor activity in our experimental condition. Further evaluation of locomotor activity under different conditions (i.e., higher light intensity an index of aversive condition) is needed. By contrast, Δ^9-THC at the dose of 3 mg/kg induced anxiolytic-like effect, which seems to be related to its higher serum concentration. Overall, we cannot also exclude that the lack of more significant behavioral effects induced by SCs could be due to their lower serum concentration as compared to Δ^9-THC. Further behavioral tests are necessary to support the potential therapeutic of endocannabinoid system modulation in the treatment of anxiety disorders (Micale et al., 2013a).

AUTHOR CONTRIBUTIONS

All authors made a substantial contribution to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work. All authors were involved in drafting the work or revising it critically for important

intellectual contents. All authors gave final approval for the current version of the work to be published. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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The Endocannabinoid System and Oligodendrocytes in Health and Disease

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Cannabinoid-based interventions are being explored for central nervous system (CNS) pathologies such as neurodegeneration, demyelination, epilepsy, stroke, and trauma. As these disease states involve dysregulation of myelin integrity and/or remyelination, it is important to consider effects of the endocannabinoid system on oligodendrocytes and their precursors. In this review, we examine research reports on the effects of the endocannabinoid system (ECS) components on oligodendrocytes and their precursors, with a focus on therapeutic implications. Cannabinoid ligands and modulators of the endocannabinoid system promote cell signaling in oligodendrocyte precursor survival, proliferation, migration and differentiation, and mature oligodendrocyte survival and myelination. Agonist stimulation of oligodendrocyte precursor cells (OPCs) at both CB_1 and CB_2 receptors counter apoptotic processes via Akt/PI3K, and promote proliferation via Akt/mTOR and ERK pathways. CB_1 receptors in radial glia promote proliferation and conversion to progenitors fated to become oligodendroglia, whereas CB_2 receptors promote OPC migration in neonatal development. OPCs produce 2-arachidonoylglycerol (2-AG), stimulating cannabinoid receptor-mediated ERK pathways responsible for differentiation to arborized, myelin basic protein (MBP)-producing oligodendrocytes. In cell culture models of excitotoxicity, increased reactive oxygen species, and depolarization-dependent calcium influx, CB_1 agonists improved viability of oligodendrocytes. In transient and permanent middle cerebral artery occlusion models of anoxic stroke, WIN55212-2 increased OPC proliferation and maturation to oligodendroglia, thereby reducing cerebral tissue damage. In several models of rodent encephalomyelitis, chronic treatment with cannabinoid agonists ameliorated the damage by promoting OPC survival and oligodendrocyte function. Pharmacotherapeutic strategies based upon ECS and oligodendrocyte production and survival should be considered.

Keywords: 2-arachidonoylglycerol (2-AG), CP55940, HU210, multiple sclerosis (MS), neural stem cells (NSCs), oligodendrocyte precursor cells (OPCs), SR141716, WIN55212-2

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INTRODUCTION

Phytocannabinoid use in management of multiple sclerosis (MS) symptoms (Consroe et al., 1997) has led to clinical trial evidence for the efficacy of tetrahydrocannabinol (THC)/cannabidiol (CBD) oromucosal spray (Sativex) in controlling spasticity and pain (Wade et al., 2010; Giacoppo et al., 2017). MS, a demyelinating disease characterized by persistent neuroinflammation and progressive central nervous system (CNS) demyelination (Kutzelnigg and Lassmann, 2014), is only one of many demyelinating neurodegenerative diseases involving oligodendrocytes, the myelinating cells of the CNS. The endocannabinoid system (ECS) (Howlett et al., 2002; Pertwee et al., 2010) involvement in neuroprotection (Panikashvili et al., 2001; van der Stelt and Di Marzo, 2005; Martinez-Orgado et al., 2007; Sanchez and García-Merino, 2012) and the immune system in CNS diseases (Croxford and Yamamura, 2005; Rom and Persidsky, 2013; Chiurchiu et al., 2015; Olah et al., 2017) have been reviewed. Here, we address ECS effects on oligodendrocytes and their precursors, in order to evaluate the evolving research around cannabinoids in healthy development and in demyelinating neurodegenerative diseases.

CANNABINOIDS, OLIGODENDROCYTE PRECURSOR CELLS AND OLIGODENDROCYTES IN HEALTH

Oligodendrocytes, myelinating cells of the vertebrate CNS, enable neurons to signal more energy-efficiently and at higher speed due to saltatory conduction, and maintain axonal integrity through trophic and metabolic support (Michalski and Kothary, 2015; Simons and Nave, 2015). Generation of oligodendrocytes is an ongoing process, starting in embryonic development and continuing throughout life (Baumann and Pham-Dinh, 2001; Trotter et al., 2010; Dimou and Gallo, 2015; Michalski and Kothary, 2015). In brief, oligodendrocyte precursor cells (OPCs), also known as NG2-glia, O-2A progenitors, polydendrocytes, or synantocytes, arise from neural stem cells (NSCs), and preferentially populate distinct areas of the developing CNS in lineage- and time-specific waves (Richardson et al., 2006). Upon arrival, many undergo apoptosis, while many others either mature into myelinating oligodendrocytes or persist as progenitors and remain capable of self-renewal as well as production of mature oligodendrocytes well into adulthood (Dawson et al., 2003). These progenitors become distributed throughout gray and white matter and maintain their respective domains by continuously sampling their environment, able to expand to neighboring areas vacated by other OPCs (Kirby et al., 2006; Hughes et al., 2013).

Despite this interchangeability, it is becoming increasingly clear that oligodendrocyte precursors represent a heterogeneous group, distinct in their origin, signaling, and ability to revert differentiation to produce neurons and astrocytes (Trotter et al., 2010; Dimou and Gallo, 2015; Nishiyama et al., 2016; Vigano and Dimou, 2016). Regardless of the differences, all OPCs rely on the processes of proliferation, migration, and differentiation to become mature, functioning oligodendrocytes (Baumann and Pham-Dinh, 2001; Miron et al., 2011; Dubois et al., 2014; Sampaio-Baptista and Johansen-Berg, 2017). As these steps are differentially regulated (Marinelli et al., 2016), it is important to look at the effects of cannabinoid agonists on each (see **Figure 1**).

Survival

Reports of cannabinoid receptors in newborn rat white matter by immunostaining (Berrendero et al., 1999), led to subsequent studies exploring cells *in vitro*. OPCs were isolated from newborn Wistar rat forebrains and expanded by incubation in serum-free defined media with supplements including platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) (Molina-Holgado et al., 2002). OPCs could be differentiated into myelin basic protein (MBP)-producing mature oligodendrocytes by incubation in serum-free defined media in which PDGF and FGF were replaced by triiodothyronine (T3). As ascertained by RT-PCR, Western blot, and immunohistochemistry, both OPC and mature oligodendrocytes expressed both CB_1 and CB_2 cannabinoid receptors (Molina-Holgado et al., 2002). Because activation of cannabinoid receptors confers neuroprotection (van der Stelt and Di Marzo, 2005; Martinez-Orgado et al., 2007; Sanchez and García-Merino, 2012), the influence of cannabinoid agonists on viability of OPCs was investigated. Upon incubation in serum-free DMEM/F12 media for 12 h, nearly half of OPCs underwent apoptosis (Molina-Holgado et al., 2002). However, most were rescued by concurrent supplementation with CB_1 agonist arachidonyl-2′-chloroethylamide (ACEA, 25 nM) or CB_1/CB_2 agonists WIN55212-2 (25 nM) or HU210 (500 nM). Co-treatment with CB_1 antagonist SR141716 (1 μM) abolished the anti-apoptotic effect of ACEA, but not of WIN55212-2 or HU210. Both SR141716 plus CB_2 antagonist SR144528 (1 μM) were required to nullify the pro-survival effect of HU210. These results show that the activation of either CB_1 or CB_2 receptors

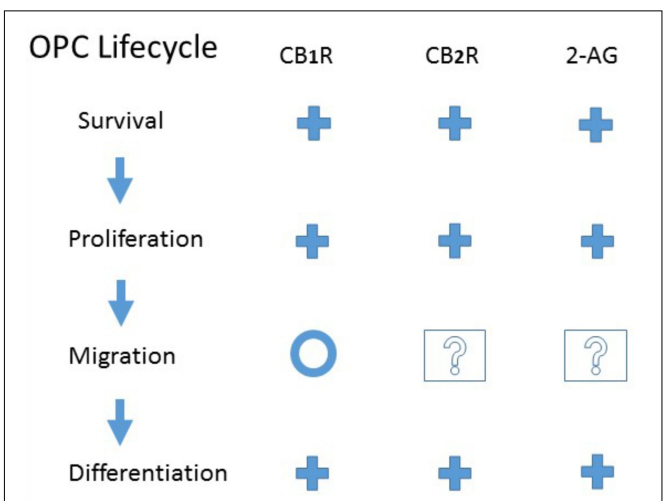


FIGURE 1 | Evidence for CB_1 or CB_2 receptor activation or endogenous 2-Arachidonoylglycerol modulation of Oligodendrocyte Precursor Cell Life Cycle. **+**, Promote; **O**, No effect; **?**, Unknown. See the text for details and original references.

could be sufficient in promoting OPC survival under conditions of trophic factor deprivation. The mechanism includes activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, a known modulator of OPC survival (Vemuri and McMorris, 1996; Ebner et al., 2000). Applying each of the three agonists correlated with increased Akt phosphorylation, while co-treatment with PI3K inhibitors LY294002 (10 μM) or wortmannin (100 nM) nullified the effects of WIN55212-2 and HU210 on both Akt phosphorylation and cell survival (Molina-Holgado et al., 2002). Thus, stimulation of Akt/PI3K pathways via CB_1 and CB_2 receptors present in OPCs can curtail apoptotic processes and promote survival.

Proliferation

Molina-Holgado and colleagues explored the ability of the endocannabinoid system to modulate OPC proliferation and self-renewal (Gomez et al., 2015). In cultured OPCs (Molina-Holgado et al., 2002), 24-h blockade of CB_1 receptors with AM281 (1 μM), CB_2 receptors with AM630 (1 μM), or synthesis of 2-arachidonoylglycerol (2-AG) by diacylglycerol lipase α and β (DAGLs) with RHC80267 (5 μM), led to reduction in PDGF/FGF-stimulated OPC proliferation. In the absence of PDGF and FGF, OPC proliferation increased in response to 24-h application of CB_1 agonist ACEA (0.5 μM), CB_2 agonist JWH133 (0.5 μM), 2-AG (1 μM), or JZL184 (1 μM) which blocks the 2-AG metabolizing enzyme monoacylglycerol lipase (MAGL). The effect depended on phosphorylation of Akt and mammalian target of rapamycin (mTOR), as their blockers LY294002 (5 μM) and rapamycin (3 nM), respectively (24 h), decreased the proliferative effects of ACEA, JWH133, and JZL184. Extracellular signal regulated-kinase (ERK) 1/2 phosphorylation was decreased in cells treated with the 2-AG synthesis inhibitor RHC80267, as well as with CB_1 and CB_2 antagonists AM281 and AM630, respectively. These results extend the impact of cannabinoid receptor activation from promoting OPC survival (Molina-Holgado et al., 2002) to increasing proliferation, while also implicating cannabinoid-mediated Akt/mTOR and ERK pathways, known to play a role throughout OPC development to myelination (Gonsalvez et al., 2016; Figlia et al., 2018).

Migration

NSCs of postnatal subventricular zone (SVZ) emigrate the neurogenic niche in the form of neuroblasts or OPCs (Marshall et al., 2003). In rat CNS, both myelination and SVZ gliogenesis peak at postnatal day (PD) 15 (Bjelke and Seiger, 1989; Hamano et al., 1996), making this an optimal time-point for exploring cannabinoid effects on OPC proliferation and migration from postnatal SVZ. Immunohistochemical analysis of PD7 and PD15 Wistar rat brain revealed that the CB_1 receptor was primarily expressed by radial glia (Aré-Martín et al., 2007), which can transform to NSCs (Merkle et al., 2004). However, the CB_2 receptor co-stained with poly-sialylated neural cell adhesion molecule (PSA-NCAM) (Aré-Martín et al., 2007), a marker of migration in OPCs and neuroblasts (Doetsch et al., 1999; Menn et al., 2006). The CB_1 agonist ACEA (escalating dose 1.25–2.5 mg/kg, SC daily PD1 to PD14) increased PD15 SVZ staining for 4A4, a marker of radial glia proliferation (Howard et al., 2006), and Olig2 (Aré-Martín et al., 2007), which is expressed in glial precursors fated to become oligodendrocytes (Mitew et al., 2014). Similar treatment with CB_2 agonist JWH056 (escalating dose 2.5–5.0 mg/kg, SC) increased SVZ staining for PSA-NCAM. Activation of both CB_1 and CB_2 receptors by WIN55212-2 (escalating dose 2.5–5.0 mg/kg, SC) increased MBP staining in the external capsule, which was inhibited below control values by concurrent administration of either SR141716 (CB_1) or SR144528 (CB_2) antagonists (escalating dose 2–4 mg/kg, SC) (Aré-Martín et al., 2007). These results support the involvement of CB_1 receptors in radial glia proliferation and conversion to oligodendrocytes, but CB_2 receptors in OPC migration, leading to functional oligodendrocytes in neonatal myelination.

Studies have examined cannabinoid agonist impact on rodent SVZ proliferation beyond the newborn stage. In juvenile (PD35-PD48) Lewis rats, WIN55212-2 (2 mg/kg, IP bid, 2-weeks) increased SVZ BrdU staining, without changing the ratio of progenitors committed to neuronal or OPC fates, or the number of cells undergoing caspase-3 mediated apoptosis (Bortolato et al., 2014). These results are consistent with findings that genetic ablation of CB_1 receptors decreased progenitor proliferation in adult mouse SVZ (Jin et al., 2004; Kim et al., 2006). In adult mice, cannabidiol (CBD; 3 mg/kg IP daily for 14 days) increased SVZ proliferative markers Ki67 and BrdU staining (Schiavon et al., 2016). However, CBD at 30 mg/kg decreased proliferation markers (Schiavon et al., 2016), highlighting the importance of dose.

Differentiation to Mature Oligodendrocytes

Molina-Holgado et al. (2002) investigated OPC differentiation using isolated OPCs differentiated with T3 (30 ng/mL, 48 h) in the absence of PDGF/FGF (Gomez et al., 2011). Activating either cannabinoid receptor (ACEA for CB_1 and JWH133 for CB_2, 0.5 μM) increased OPC branching and accumulation of MBP (Western blot). The CB_1/CB_2 agonist HU210 (0.5 μM) evoked the same responses, which could be abolished by either AM281 (CB_1) or AM630 (CB_2; 1 μM). The mechanism for HU210-mediated OPC arborization and production of MBP involved PI3K/Akt and mTOR pathways, as these effects were blocked with LY290042 (2.5 μM) and rapamycin (0.75 nM), respectively. Gomez et al. (2010) found the Western blot level of DAGLs to be higher in OPCs, whereas the level of MAGL was higher in mature oligodendrocytes, culminating in the finding that OPCs accumulated a greater content of 2-AG than mature oligodendrocytes. Levels of anandamide (AEA) were low and did not differ between the cell stages (Gomez et al., 2010). Differentiation, denoted by branching morphology and levels of MBP after 96 h, was increased by MAGL inhibitor JZL184 (1 μM), and decreased by DAGL inhibitor RHC80267 (5 μM), with exogenous 2-AG (2 μM) abolishing the effect of blocking its synthesis. Inhibition of the ERK pathway by the MEK blocker PD98059 (10 μM) abolished Western blot staining for MBP, implicating the ERK pathway in differentiation. In a CB_1

receptor mRNA-expressing human oligodendrocyte precursor line HOG16, WIN55212-2 (1 μM, 24 h) increased MBP mRNA expression, particularly in cells treated with T3-supplemented differentiating medium (Tomas-Roig et al., 2016). Collectively, these studies suggest that endogenous 2-AG in OPCs triggers the ERK pathway, leading to the maturation of arborized, MBP-producing oligodendrocytes.

THE ECS AND OPCS-OLIGODENDROCYTES IN MODELS OF DISEASE

The role of cannabinoids in OPCs and oligodendrocytes under stress has been evaluated in models of insults such as excitotoxicity (Shouman et al., 2006; Bernal-Chico et al., 2015), reactive oxygen species (ROS) toxicity (Ribeiro et al., 2013), KCl-induced depolarization (Mato et al., 2009), as well as in models of stroke (Fernández-López et al., 2010; Sun et al., 2013a,b), spinal cord injury (SCI) (Marsicano et al., 2003; Arevalo-Martin et al., 2010), and demyelination (Solbrig et al., 2010; Ribeiro et al., 2013; Bernal-Chico et al., 2015; Wen et al., 2015; Feliu et al., 2017).

Cannabinoids and OPC-Oligodendrocytes in Cytopathology Models

The ability of cannabinoid agonists to protect neurons from excitotoxicity is well known (Shen and Thayer, 1998; Hansen et al., 2002; Marsicano et al., 2003; van der Stelt and Di Marzo, 2005). Likewise, ECS involvement in OPC and oligodendrocyte excitotoxicity has recently been explored (Shouman et al., 2006; Bernal-Chico et al., 2015). Newborn (PD5) Sprague Dawley rats received AMPA/kainate agonist bromowillardine (15 μg, IC), immediately followed by AEA (10 mg/kg, IP) (Shouman et al., 2006). AEA significantly increased OPC density within the periventricular white matter lesion after 1 day, and increased MBP staining after 5 days. The ECS in excitotoxicity was also investigated in mature oligodendrocytes (Bernal-Chico et al., 2015). OPCs were isolated from the cerebral cortex of newborn wildtype or CB_1-KO Sprague-Dawley rats and differentiated with T3 minus growth factors. Oligodendrocytes were exposed to AMPA plus cyclothiazide (10 μM:100 μM) for 15 min, resulting in excessive cytosolic calcium, reactive oxygen species (ROS) production, and cell death (Bernal-Chico et al., 2015). A 30-min pretreatment with CB_1 agonist ACEA (25 nM), endocannabinoids AEA or 2-AG (1 μM), or MAGL inhibitor JZL184 (25 nM) reduced cell death (Bernal-Chico et al., 2015). No protection occurred in oligodendrocytes lacking CB_1 receptors, or pretreated with CB_2 agonist JWH133 (25 nM) or the inhibitor of AEA metabolizing enzyme fatty acid amide hydrolase (FAAH) URB597 (10 nM – 1 μM). These findings implicate endocannabinoids and CB_1 receptors in improved viability of oligodendrocytes in excitotoxic conditions.

Due to their high metabolic demand, oligodendrocytes are vulnerable to elevated levels of ROS (McTigue and Tripathi, 2008; Roth and Nunez, 2016), although less so than their precursors (Back et al., 1998). To investigate how cannabinoid agonists affect oligodendrocyte survival, precursors isolated from PD2-PD3 Sprague-Dawley rat brains were differentiated by T3 plus ciliary neurotrophic factor for 2 weeks, and exposed to a peroxynitrite generator SIN-1 (1 mM) (Zhang et al., 2006, 2007) for 2 h (Ribeiro et al., 2013). Concurrent treatment with CB_1/CB_2 agonists CP55940 (1–3 μM), WIN55212-2 (10 μM), or anandamide/THC hybrid CB52 (3–10 μM), reduced cell death. CB52's mechanism included activation of CB_2 receptors, as CB_2 antagonist AM630 (10 μM), but not CB_1 antagonist AM281 (10 μM) reduced its effectiveness, while CB_2 agonists AM1241 and JWH015 partially replicated it (Ribeiro et al., 2013).

The ability of CB_1 agonists to inhibit depolarization-dependent calcium channels, as observed in neurons (Howlett et al., 2002), was explored in oligodendrocytes (Mato et al., 2009). Precursors were isolated from optic nerve of PD12 Sprague-Dawley rats, differentiated by 2-day incubation in defined media, and exposed to KCl (50 mM, 1 min) to induce depolarization (Mato et al., 2009). Resulting calcium influx was decreased by CB_1 agonist ACEA (1 μM) or CB_1/CB_2 agonists THC (3 μM), CP55940 (3 μM), AEA (3 μM) or 2-AG (3 μM), but not CB_2 agonist JWH133 (3 μM). Oligodendrocytes from CB_1-KO mice were less responsive to AEA and ACEA, although not completely unresponsive, suggesting other targets exist such as transient receptor potential vanilloid receptor-1 (TRPV1) (Ross, 2003; Ruparel et al., 2011), expressed in oligodendrocytes (Gonzalez-Reyes et al., 2013). In contrast, CBD (100 nM, 20–30 min) reduced oligodendrocyte viability, in part through an increase in intracellular calcium (Mato et al., 2010). CBD (1 μM, 10 min) increased oligodendrocyte production of ROS, and induced apoptosis through mitochondria-mediated activation of caspase-8 and -9 and their downstream effector caspase-3, as well as poly-(ADP ribose) polymerase-1 (PARP-1), triggered by caspase-independent mitochondrial apoptosis-induced factor (AIF) (Hong et al., 2004).

ECS and Oligodendrocytes in Stroke and Traumatic Injury

Oligodendrocytes are vulnerable to ischemic conditions and their replacement via OPCs has been found to aid recovery (Dewar et al., 2003; Zhang et al., 2013, 2016). The impact of the ECS was studied in adult (Sun et al., 2013a,b) and neonatal (Fernández-López et al., 2010) rat models of middle cerebral artery occlusion (MCAO). In transient (2 h) MCAO (Sun et al., 2013a), WIN55212-2 (9 mg/kg, IP immediately after reperfusion and daily) increased staining for penumbral OPCs and mature oligodendrocytes at 4, 7, and 14 days post ischemia (DPI), and penumbral myelin density at 14 DPI. WIN55212-2 reduced penumbral expression of caspase-3 in OPCs at 7 DPI, which correlated with reduced ERK1/2 phosphorylation. These effects were ameliorated by CB_1 antagonist SR141716 (1 mg/kg, IV). In adult male Sprague-Dawley rats, WIN55212-2 (9 mg/kg, IV) was given within 2 h after permanent MCAO, with anoxia damage quantitated after 24 h (Sun et al., 2013b). WIN55212-2 treatment increased OPC proliferation within the penumbra and ipsilateral SVZ, and decreased penumbral OPC expression of tau-1, an oligodendrocyte marker of ischemic

stress (Dewar and Dawson, 1995; Irving et al., 1996). Both effects were partially due to CB_1 receptor activation (Sun et al., 2013b). In a permanent MCAO model in neonatal PD7 Wistar rats (Fernández-López et al., 2010), WIN55212-2 (1 mg/kg, IP daily for 7 days) increased ipsilateral SVZ OPC proliferation and number of penumbral OPCs at 7 DPI. WIN55212-2 increased the number of mature penumbral oligodendrocytes at 14 and 28 DPI, and accelerated complete MBP recovery. Consistent with earlier findings (Goncalves et al., 2008; Bortolato et al., 2014), WIN55212-2 increased SVZ OPC proliferation even in the absence of ischemia. Although preclinical results appear promising for cannabinoid pharmacotherapies for anoxic demyelination, SVZ neurogenic response to stroke varies greatly between rodents and humans (Kahle and Bix, 2013).

The endocannabinoid 2-AG has been shown to be neuroprotective after traumatic brain injury (Panikashvili et al., 2001), and 2-AG is elevated after spinal trauma (Marsicano et al., 2003; Garcia-Ovejero et al., 2009). Thus, the impact of 2-AG on oligodendrocyte survival was explored in a model of contusive SCI generated by a dropped weight in male adult Wistar rats (Arevalo-Martin et al., 2010). 2-AG (5 mg/kg, IP 30 min after injury) preserved myelin integrity and reduced oligodendrocyte death at the epicenter (1 day post-injury), with the same effects seen as far as 10 mm rostral of epicenter (1 and 7 days post-injury). Co-administration of both CB_1 and CB_2 antagonists (AM281 and AM630, respectively; 3 mg/kg, IP), but not by either alone, could reverse the effects of 2-AG. These results support the idea that improved oligodendrocyte survival and preserved white matter integrity underlie the cannabinoid-mediated improvement in SCI recovery (Arevalo-Martin et al., 2012).

ECS and Models of Demyelination

To investigate demyelination in a mouse model of experimental autoimmune encephalomyelitis (EAE), PD49 or PD56 C57BL/6 mice received a single injection of myelin oligodendrocyte glycoprotein peptide (MOG 35-55), followed by injections of pertussis toxin on the day of MOG inoculation and again 2 days afterward (Bernal-Chico et al., 2015). MAGL inhibitor JZL184 (8 mg/kg, IP daily for 3 weeks, starting on day 14 postinoculation) ameliorated the reduction in spinal cord white matter staining (Bernal-Chico et al., 2015). Similar results in the EAE model were achieved by THC (Moreno-Martet et al., 2015), CBD (Giacoppo et al., 2015), cannabigerol quinone (Carrillo-Salinas et al., 2014), and CB_2 agonist HU308 (Shao et al., 2014). The effects of CB52 on demyelination EAE (Ribeiro et al., 2013) showed that when initiated before symptom development (3 days) or after clinical disease onset (12 or 20 days), CB52 (2 mg/kg, IP daily) ameliorated the loss of staining for spinal cord myelin and mature oligodendrocytes at day-30. In contrast to CB52's action on cultured oligodendrocytes *in vitro* (Ribeiro et al., 2013), both of its effects *in vivo* were blocked by CB_1 antagonist AM281 (2 mg/kg), but not by CB_2 antagonist AM630 (2 mg/kg).

Microglia are an integral part of demyelinating diseases' neuroimmune complex (Gonzalez et al., 2014). In microglia, CB_1 receptors are expressed at low levels constitutively; however, CB_2 receptors become upregulated when microglia become activated (Cabral et al., 2008). Endocannabinoids 2-AG and AEA have been shown to drive microglia toward alternative, anti-inflammatory activation state, M2, and away from classic, pro-inflammatory polarization, M1, which in turn causes microglia to upregulate its own 2-AG synthesizing enzymes (Mecha et al., 2015). Because microglial 2-AG has been shown to promote OPC differentiation (Miron et al., 2013), blocking its degradation could be of use in counteracting demyelination. This has been explored in a mouse model of EAE (Wen et al., 2015), by inhibiting the 2-AG hydrolyzing microglial enzyme ABHD6 (Li et al., 2007; Marrs et al., 2010; Murataeva et al., 2014) with WWL70 (10 mg/kg, IP daily starting at the onset of clinical symptoms on day-11 postinoculation). WWL70 increased cerebral 2-AG at day-21, and ameliorated the loss of staining of spinal cord myelin and mature oligodendrocytes in wildtype mice on day-28 (Wen et al., 2015). These results were not seen in CB_2-KO mice, nor when WWL70 was co-administered with CB_2 antagonist AM630 (3 mg/kg), suggesting that microglial 2-AG accumulation is dependent upon CB_2 receptor signaling. Co-administration with CB_1 antagonist AM281 failed to interfere with WWL70's effects.

OPC gliogenesis in Borna Disease Virus (BDV) encephalomyelitis, generated in PD28 male Lewis rats (Solbrig et al., 2010), demonstrated that WIN55212-2 (1 mg/kg, IP daily for 7-days starting 1 week after virus inoculation) increased OPC proliferation in striatum, decreased apoptosis of proliferating cells, skewed precursor differentiation away from astrocytes and toward oligodendrocytes, and promoted OPC maturation. In uninfected controls, WIN55212-2 increased proliferation in both PFC and striatum.

In Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD), PD28 female CJL/J mice received an intracerebral injection of the Daniel strain virus (Feliu et al., 2017). When started after symptom onset at day-75, a 10-day treatment with MAGL inhibitor UCM03025 (5 mg/kg, IP) increased the spinal cord populations of both mature oligodendrocytes and OPCs, and restored MBP level to that of sham controls (Feliu et al., 2017).

In the cuprizone oligodendrotoxic model (Bernal-Chico et al., 2015), PD56 C57BL/6 mice were fed a cuprizone-supplemented diet (0.3%) for 3 weeks. Concurrent MAGL inhibitor JZL184 (8 mg/kg, IP daily) ameliorated cuprizone-induced reduction in corpus callosum MBP staining (Bernal-Chico et al., 2015), implicating 2-AG-mediated protection.

Seizures are known to accompany demyelination in experimental models (DePaula-Silva et al., 2017; Lapato et al., 2017; Spatola and Dalmau, 2017) as well as MS (Koch et al., 2008; Anderson and Rodriguez, 2011; Sponsler and Kendrick-Adey, 2011). The ECS promotion of OPCs (Solbrig et al., 2010; Feliu et al., 2017) and mature oligodendrocytes (Ribeiro et al., 2013; Wen et al., 2015; Feliu et al., 2017) may counteract demyelination observed in patients with intractable epilepsy (Hu et al., 2016).

CBD and OPCs in Inflammation

CBD has been promoted for potential therapeutic applications (Devinsky et al., 2014; Blessing et al., 2015; Ibeas Bih et al., 2015)

including anti-inflammation (Burstein, 2015). Inflammation underlies a range of pathologies including neurodegeneration (Glass et al., 2010; Cunningham, 2013), stroke (Turner and Vink, 2007; Ahmad et al., 2014), and demyelination (Popescu and Lucchinetti, 2012; Kutzelnigg and Lassmann, 2014). To examine CBD's anti-inflammatory impact on OPC survival, cultured OPCs isolated from the forebrain of newborn Wistar rats were exposed to inflammation-related stressors (Mecha et al., 2012). Treatment with CBD (1 μM) reduced: (1) caspase-3-mediated apoptosis resulting from lipopolysaccharides (LPS) and interferon-γ (IFNγ)-mediated inflammation (48 h); (2) cell death induced by endoplasmic reticulum stress instigated by tunicamycin (1 μg/ml, 24 h); and (3) cell detachment and ROS production in response to hydrogen peroxide (2 h). CBD was unable to increase OPC proliferation in culture (Mecha et al., 2012), in contrast to its chronic administration in SVZ of adult Swiss mice (Schiavon et al., 2016). CBD did not promote apoptosis in culture, as observed in unstressed cultured oligodendrocytes (Mato et al., 2010). CBD's cellular mechanism(s) have yet to be established for OPC and oligodendrocyte function, but might counter the endocannabinoid responses at their receptors. Further, CBD may target other cell types in the neuro-immune complex, explaining differences between *in vitro* vs. *in vivo* models.

PERSPECTIVES

Although much has been learned about the impact of cannabinoid agonists on oligodendrocytes in health and disease, many questions remain unexplored, such as the cannabinoid impact on OPC local migration (Kirby et al., 2006; Hughes et al., 2013) and glutamate signaling (Spitzer et al., 2016), the oligodendrocyte's ability to produce myelin and provide metabolic support to axons (Zecca et al., 2004; Saab et al., 2013; Simons and Nave, 2015). Cannabinoid agonists also comprise structurally and functional distinct ligands (Howlett et al., 2002; Pertwee et al., 2010; Laprairie et al., 2017; Priestley et al., 2017), and as such, it is important to characterize their pharmacological profiles in cell pathologies related to oligodendrocytes and other cell types in the neuro-immune complex. Although the impact of cannabinoid extracts on MS disease progression remains inconclusive (Pertwee, 2007; Pryce et al., 2015), the outlook is optimistic (Arévalo-Martin et al., 2008; Chiurchiu et al., 2015, 2018). Evidence of cannabinoid agonist effects on oligodendrocyte survival and OPC lifecycle suggests their usefulness in CNS pathologies such as demyelination (Popescu and Lucchinetti, 2012; Kutzelnigg and Lassmann, 2014), neurodegeneration (Ettle et al., 2016; Tauheed et al., 2016), ischemia (Dewar et al., 2003; Mifsud et al., 2014), epilepsy (Friedman and Devinsky, 2015; Stockings et al., 2018), and traumatic injuries to spinal cord (Alizadeh and Abdolrezaee-Karimi, 2016; Levine, 2016; Arevalo-Martin et al., 2016) and brain (Armstrong et al., 2016; Takase et al., 2018).

AUTHOR CONTRIBUTIONS

AI conceived the idea and approach to the review, wrote and edited the manuscript. CM and EP provided the feedback and edited the manuscript. AH developed approach to the review, structured, wrote and edited the manuscript.

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CASE REPORT
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Case Report: Clinical Outcome and Image Response of Two Patients With Secondary High-Grade Glioma Treated With Chemoradiation, PCV, and Cannabidiol

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We describe two patients with a confirmed diagnosis of high-grade gliomas (grades III/IV), both presenting with O6-methylguanine-DNA methyltransferase (MGMT) methylated and isocitrate dehydrogenase (IDH-1) mutated who, after subtotal resection, were submitted to chemoradiation and followed by PCV, a multiple drug regimen (procarbazine, lomustine, and vincristine) associated with cannabidiol (CBD). Both patients presented with satisfactory clinical and imaging responses at periodic evaluations. Immediately after chemoradiation therapy, one of the patients presented with an exacerbated and precocious pseudoprogression (PSD) assessed by magnetic resonance imaging (MRI), which was resolved in a short period. The other patient presented with a marked remission of altered areas compared with the post-operative scans as assessed by MRI. Such aspects are not commonly observed in patients only treated with conventional modalities. This observation might highlight the potential effect of CBD to increase PSD or improve chemoradiation responses that impact survival. Further investigation with more patients and critical molecular analyses should be performed.

Keywords: high-grade glioma, cannabidiol, pseudoprogression, PCV, chemoradiation, cancer, cannabis, THC-tetrahydrocannabinol

BACKGROUND

Gliomas, the most common primary brain tumors, account for more than 40% of all CNS neoplasms and are highly resistant to available therapeutic approaches (1, 2). These tumors often have a poor prognosis with a median survival time of ∼1 year for patients with high-grade gliomas (grades III/IV) (3, 4). The Stupp protocol has become the standard of care treatment for primary glioblastoma (GBM), and has led to significantly improved survival (5). It consists of chemoradiation (combining chemotherapy and radiation therapy simultaneously) followed by adjuvant temozolomide (TMZ), an alkylating agent, and is associated with a medium survival rate of 15 months.

It was reported that 10% of GBM are secondary (progressing from a low-grade tumor, IDH mutated) (6) and that these patients often previously received prior chemotherapeutic treatment, there being no stand-by treatment for this condition. After progression from a low-grade tumor to a high-grade tumor, combination radiotherapy with TMZ is a good therapeutic option (5). However, patients who failed with TMZ started PCV, a multiple drug regimen (procarbazine, lomustine, and vincristine) adjuvant. Because of the adverse events (nausea and seizures) related to this treatment, we decided to use cannabidiol (CBD) from the irradiation phase onwards. A significant inflammatory response to the therapy was observed in one patient, which we considered pseudoprogression (PSD), and in the other patient, it presented with a marked remission of altered areas compared with the post-operative scans as assessed by MRI.

Tumor pseudoprogression (PSD) can occur in up to 30% of patients after chemoradiation, especially O6-methylguanine-DNA methyltransferase (MGTM) methylated cases. This corresponds to a treatment-related increase in lesion size related to inflammatory responses that simulate the progression of the disease. In almost 60% of cases, PSD occurs within the first 3–6 months after completing chemoradiation (5, 7, 8). PSD does not represent a progression of the disease, and is often a marker of longer survival, presumably because it represents a robust response to treatment (9).

Cannabidiol (CBD) is a prevalent natural cannabinoid. It is a non-intoxicating compound, instead of which may have, antipsychotic effects (10–12), and promote a wide spectrum of pharmacological effects including anti-inflammatory, anti-oxidant, anti-proliferative, anti-invasive, anti-metastatic, and pro-apoptotic activity (13–15). Recent studies have suggested CBD has immunomodulatory effects (15–18).

Cannabinoids are becoming promising anti-tumor drugs and increasing preclinical evidence reported that these compounds, including Δ^9-tetrahydrocannabinol (THC), inhibited tumor growth in animal models of cancer by targeting specific cancer-cell signaling pathways. Furthermore THC act as broad-spectrum antiemetic against different emetic stimuli. And interestingly displayed as an active agent against immediate and delayed phases of chemotherapy-induced nausea and vomiting (19–23). Unfortunately, there are very few reports of the potential anti-tumor activity of cannabinoids in cancer patients.

Here, we describe two patients with a confirmed diagnosis of high-grade gliomas (grades III/IV), both presenting with MGMT methylated and IDH-1 mutated who, after subtotal resection, were submitted for chemoradiation followed by PCV associated with CBD. Despite the impressive inflammatory imaging immediately after chemoradiation, both patients presented with satisfactory clinical and imaging responses in the following periodic evaluations.

The dose of CBD administered was based on individual tolerance and side effects (mainly drowsiness). All patients received it orally, as capsules containing 50 mg of CBD [CBDRx Functional Medicine Company, who confirmed it is made by hemp oil that contains <0.3% of tetrahydrocannabinol (THC)]. This regimen was maintained during the whole study, which lasted ∼2 years after the second surgery. It is important to mention that, in order to alleviate the symptoms of chemotherapy, both patients used vaporisers to inhale THC-rich cannabis flowers during their first year of treatment.

CASE PRESENTATION

Patient 1 was a 38 years-old male. In May 2010, this patient was diagnosed with glioma soon after an episode of seizures. MRI showed intra-axial expansive and infiltrative lesions that were cortical and subcortical, and which affected the anterior half of the right temporal lobe and extending from the pole to the Sylvian fissure superiorly and to the right parahippocampal gyrus, posteriorly, and medially. Partial surgical resection was performed in August 2010 and the first pathologic diagnosis was astrocytoma grade II. He underwent chemotherapy with TMZ at a dose of 2,000 mg with cycles every 28 days for 5 days in the years 2011–2013, with no tumor regrowth until the beginning of 2015. At this time, he underwent MRI, which was used to compare the discrete extension of the signal alteration areas, especially the subinsular regions. In March 2015, he resumed chemotherapy with TMZ at a dose of 100 mg/day and the patient then lost 12 kg of body weight, which was associated with anorexia, insomnia, and depression. In May 2015, he suffered a seizure requiring hospitalization. In June 2015, the patient resumed the old chemotherapy regimen with TMZ (2,000 mg every 28 days for 5 days), and a follow-up with MRI; however, the tumor size continued to increase. In January 2016, the neuro-oncology team decided to discontinue treatment with TMZ considering the risk/benefit and planned a surgical re-approach. This was followed by chemoradiation and lasting 6 cycles of PCV associated with CBD. The CBD dosage was ranging from 300 to 450 mg/day.

During chemoradiation, the patient had an excellent clinical performance, practiced sports and had few symptoms of fatigue and/or nausea.

At 1 month after the end of chemoradiation, control MRI (**Figure 1**) was characterized by exacerbation and the ultra-precocious phenomenon of PSD with increased edema and inflammatory disease characterized by extensive areas of contrast enhancement associated with tissue hypoperfusion (not shown). MRI controls demonstrated the progressive reduction of these findings.

The result of a pathological study after the first surgery was astrocytoma grade II with Ki67 staining of 5%. After the second surgery, he progressed to GBM grade IV (**Figure 2**), related to increased cellularity, frequent mitosis, presence of micronecrosis, microvascular proliferation/endothelial, Ki67 staining of 30%, and loss of ATRX expression. Biomolecular marker analysis indicated IDH-1 mutated and MGMT methylated.

Patient 2 was a 38 years-old male diagnosed as left temporal glial neoplasia in May 2014 after a seizure. MRI showed an expansive infiltrative lesion predominantly in the subcortical region, with poorly defined contours located in the left temporal lobe, involvement of the upper, middle, and lower temporal

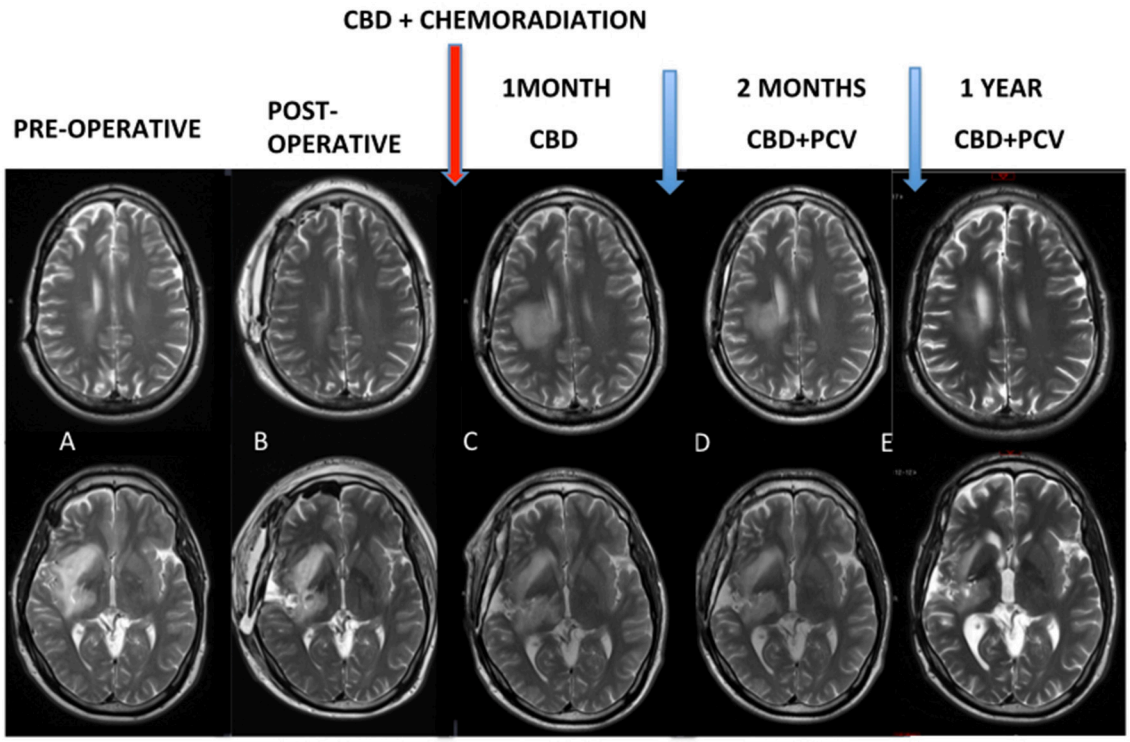


FIGURE 1 | (A,B) Pre and post-operative MRI. Red arrow: chemoradiation associated with cannabidiol in a dosage ranging from 300 to 450 mg/day. **(C)** Control MRI after 1 month of the end of the chemoradiation was characterized an exacerbated and ultra-precocious phenomenon of pseudoprogression with increased edema and inflammatory disease. **(D,E)** MRI controls demonstrated progressive reduction of these findings.

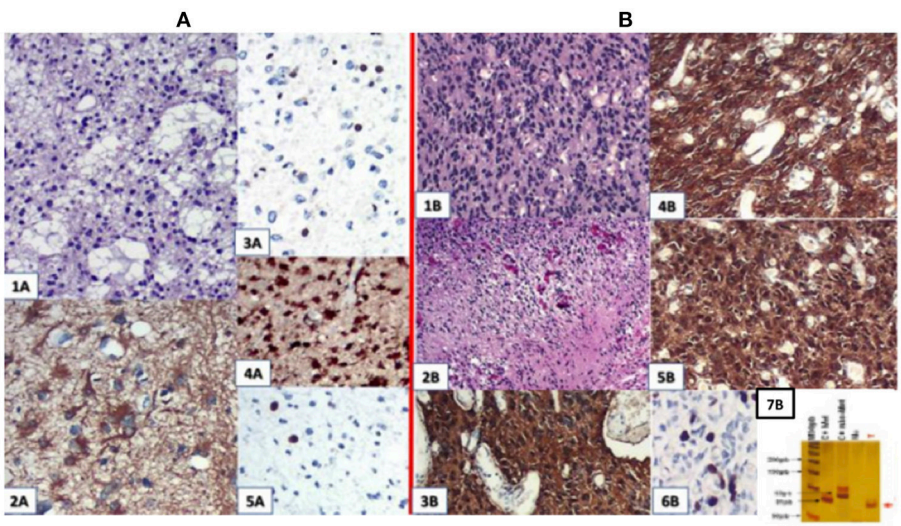


FIGURE 2 | (A) First surgery: June/2010−1A.HE; 2A. GFAP; 3A. ATRX; 4A. IDH-1 ; 5A.Ki67:4,5%. **(B)** Second surgery: May/2016−1 B and 2 B (micronecrosis). HE; 3B. GFAP; 4B. EGFR; 5B. IDH-1 ; 6B. Ki67: 30%; 7B: MGMT methylated.

gyrus, and an increase in the left temporal gyrus cortex. The lesion compromised a large part of the temporal lobe and extended to the temporal isthmus, the posterior aspect of the insula, and was deep in the trigeminal effigy of the left lateral ventricle. There was diffuse erasure of the regional cortical sulci and the Sylvian fissure, as well as a slight compression over the

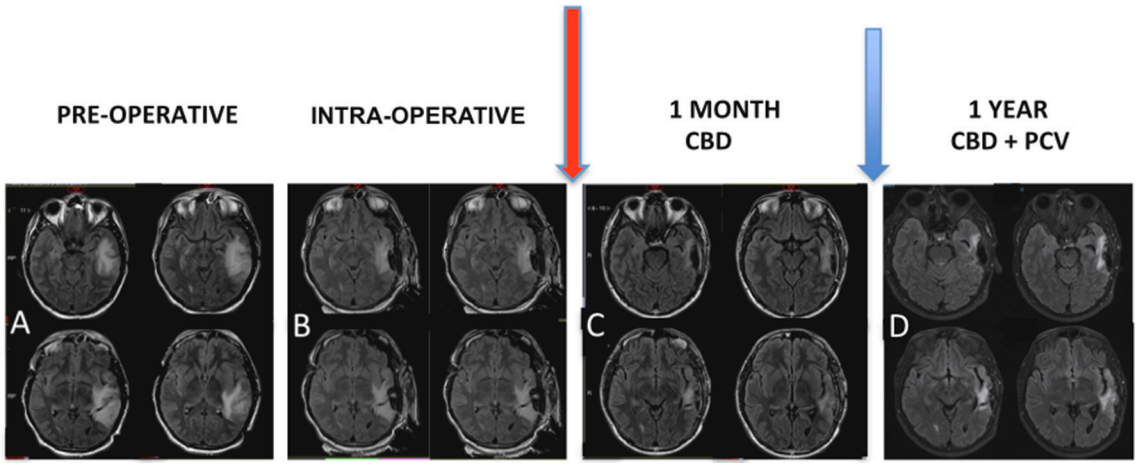


FIGURE 3 | (A) MRI showed expansive infiltrative lesion predominantly subcortical of poorly defined contours, located in the left temporal lobe, involving the upper, middle and lower temporal gyrus, with increase of the left temporal gyrus cortex. The lesion compromises a large part of the temporal lobe and extends to the temporal isthmus, posterior aspect of the insula, and deeply to the trigeminal effigy of the left lateral ventricle. There is diffuse erasure of the regional cortical sulci and the Sylvian fissure, as well as slight compression over the atrium of the left lateral ventricle. **(B)** Intraoperative MRI. Red arrow: chemoradiation associated with cannabidiol in a dosage ranging from 100 to 200 mg/day. **(C)** MRI control right after chemoradiation was characterized post-operative changes associated with a significant reduction of the infiltrative components of the tumor. **(D)** Magnetic resonance control after 1 year characterized no evidence of disease progression.

atrium of the left lateral ventricle. Stereotactic biopsy on April 2014 indicated a diagnosis of oligodendroglioma grade II. He received TMZ 1,875 mg with cycles every 23 days (during the 5 days of use he received a dose of 375 mg/day) from September 2014 to July 2015, with no tumor growth until the beginning of 2016. After checking the evolution of the tumor by MRI in February 2016, there was an increase in the dimensions of the remaining lesion, notably in the temporal isthmus, which had a similar expansive effect on the adjacent encephalic structures. The patient was submitted to a partial surgical resection followed by chemoradiation and lasting 6 cycles of PCV associated with CBD. The CBD dosage was ranging from 100 to 200 mg/day.

During the chemoradiation he had an excellent clinical performance, practiced sports, and had few symptoms of fatigue and/or nausea.

MRI control immediately after chemoradiation (**Figure 3**) was used to characterize post-operative changes and showed a significant reduction of the infiltrative components of the tumor. The result of the pathological study after the first surgery (**Figure 4**) was oligodendroglioma grade II. After the second surgery, he was diagnosed as oligodendroglioma grade III characterized by an increase in Ki67 staining of 9% and increased cellularity. Biomolecular marker analysis indicated IDH-1 mutated and MGMT methylated.

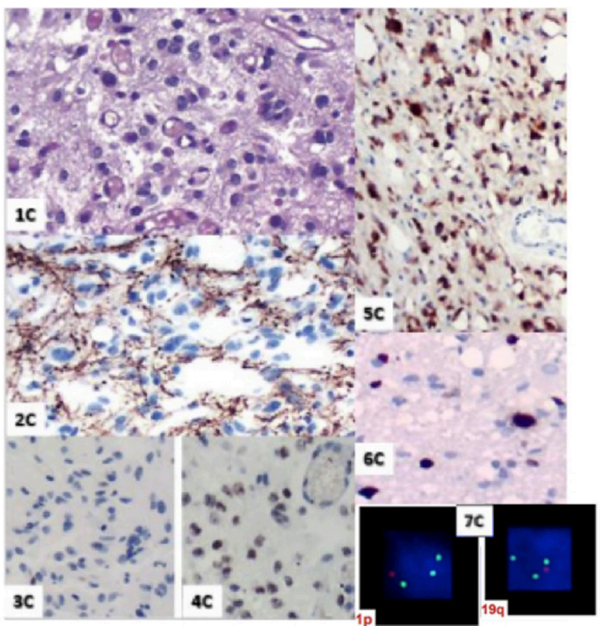


FIGURE 4 | 1 C. HE; 2C. GFAP; 3C. P53; 4C. Retained ATRX expression; 5C. IDH-mutated; 6C. Ki67: 9%; 7C. 1 p19q / Co-deleted.

DISCUSSION

Despite multimodal treatment, it is not possible to cure high-grade glioma patients. Therefore, the aim of treatment is not only to prolong life, but also to prevent deterioration of health-related quality of life as much as possible. In these two cases, we observed a significant improvement of clinical evolution. Both had a positive response to the treatment, with no evidence of disease progression for at least for 2 years and they are both alive. Unfortunately, the patient 1 presented tumor recurrence in the

brainstem after approximately two and a half years of starting the treatment with chemoradiation followed by PCV.

Even with the prolonged use of CBD, the two patients did not develop any significant alterations in blood counts/plasma biochemistry, which is in accord with other studies showing that the prolonged use of CBD did not significantly affect hepatic or cardiac functions (24, 25). In contrast, a study evaluating the effectiveness and safety of CBD as an adjunctive treatment for seizures in patients with Lennox-Gastaut syndrome using meta-analytical techniques, observed increased alanine or aspartate aminotransferases more than three times the upper normal limit (14.5% vs. 0.6%, respectively) (26). It is important to note that the patients were using high doses of CBD (20 mg/kg/day) associated with anti-convulsive drugs.

The most common side effects associated with chemoradiation are chronic fatigue, loss of appetite, and nausea (27). In addition, the use of steroids causes side effects including increased appetite, agitation, insomnia, moon facies, fatigue, and myopathy (28). Both patients in the current study did not present with any of these side effects, nor did the usual continuous use of steroids during this phase of treatment. In addition, they could maintain their usual work and sports activities.

One of the patients presented with an exacerbated inflammatory response in the first MRI control soon after chemoradiation. The anti-inflammatory and neuroprotective actions of CBD may be related to the absence of side effects associated with PSD, such as headache, or changes relevant to tumor location (29, 30).

The high toxicity associated with PCV has an important impact on the course of treatment (31). A study stated that 28.5% of patients had to stop chemotherapy because of the severe side effects (32). Another study reported a delay in the treatment in 31.3% of patients to permit the toxicity to resolve (33). PCV chemotherapy is associated with major adverse events that need to be taken into consideration. Procarbazine, lomustine, and vincristine-induced hematological toxicity is severe as previous studies reported grade 3 lymphopenia and thrombocytopenia in 75 and 64% of patients, respectively (34). Another study reported that procarbazine induced major hepatotoxicity because it is metabolized by hepatic enzymes (35). Vincristine, as well as anticonvulsants, can also induce hepatic toxicity (33). Nausea and emesis were reported in 70–80% of patients receiving PCV without anti-nausea drugs (34, 36). Neurotoxicity, mostly attributable to vincristine, was also reported (37). Finally, rash was reported as a side effect of PCV (34, 36, 38).

The most common side effects of prolonged used of CBD are somnolence, decreased appetite, gastrointestinal disorders (diarrhea and nausea) (10–13) and increased transaminases levels (39, 40). In our two case studies, treatment with PCV associated with CBD did not cause lymphopenia, thrombocytopenia, hepatic toxicity, or neurotoxicity; however, a rash was observed in one patient and despite the fact that THC was often inhaled in the course of the PCV treatment, moderate nausea, emesis and fatigue were observed in both patients. No negative side effects were reported of the use of THC, but instead an increase in appetite and a reduction of fatigue were observed. The psychoactive effect of THC was considered positive as well as mood improving.

Studies of CBD in animal models of glioma reported its anti-tumor activity (41, 42). Preclinical studies support the idea that the combined administration of TMZ and cannabinoids might be therapeutically exploited for the management of GBM (43, 44). Results showed that the oral administration of THC and CBD in combination with TMZ produced a strong antitumoral effect in both subcutaneous and intracranial glioma cell-derived tumor xenografts (44). Another study investigated the effect of THC and CBD alone and in combination with radiotherapy in a number of glioma cell lines and in an orthotopic murine model for glioma. They showed dramatic reductions in tumor volumes when both cannabinoids were used with irradiation (45).

Two clinical studies (46, 47) of cannabinoid-based therapies in gliomas have been reported. Both clearly showed that cannabinoids did not facilitate tumor growth or decrease patient survival. A phase II clinical trial of 21 patients showed that those treated with a combination of THC and CBD in addition to TMZ had an 83% 1 year survival rate compared with 44% for those who did not receive the study drug. The median survival of the treated group was >662 days compared with 369 days in the group who did not receive the study drug (47). These first results of clinical investigations are promising and indicate the importance of cannabinoid translational research leading to clinically relevant studies.

Histologically, the presence of the 2 mutations, *1p19q* and *IDH1*, have been identified as factors with a favorable prognosis (48, 49), and their impact on the clinical course of glioma patients led to a change in the World Health Organization (WHO) classification in 2007 (6). Both of our patients had IDH-1 mutated and one patient had a 1p19q co-deletion, suggesting they are more likely to respond to the treatment and have a longer life expectancy.

The study had several limitations. Previous preclinical and clinical studies evaluated the combination of THC and CBD associated with TMZ. In this case report it was not possible to use TMZ because the patients had already failed this therapy. We could not find any study that described an association between treatment with PCV and cannabinoids. Furthermore, previous studies reported that combined THC and CBD treatment had a greater anti-tumor effect and impact on survival compared with THC or CBD alone. In this report, it was not possible to make such an association for legal reasons and the absence of a medication containing high doses of THC. Both patients were under 40 years old and had molecular markers that favored a better prognosis. Of note, they remained without disease progression during the time of the study, and they did not develop major side effects between the clinical course of chemoradiation to the follow-up of 6 cycles with PCV drugs, which often prevent the completion of treatment.

Although this study only had two cases, it is interesting to note the good clinical and radiological evolution that might be related to this therapeutic association. Future randomized placebo-controlled trials with a larger number of patients are needed to confirm the study findings.

The wide use of CBD in the neuro-oncology field should be undertaken with caution. Preclinical and clinical studies are essential to demonstrate interactions with standard of care treatments, and its effects on the symptoms, quality of life, and possible immunomodulation should be determined.

These observations are of particular interest because the pharmacology of cannabinoids appears to be distinct from existing oncology medications and may offer a unique and possibly synergistic option for future glioma treatment.

ETHICS STATEMENT

Patients submitted for treatment with cannabinoids had to complete an extensive form. This form was then sent to Anvisa, Brazil's highest health regulatory agency for approval. Therefore, permission by the local ethics committee was not required. The patients signed consent forms, which are attached to the medical records.

AUTHOR CONTRIBUTIONS

PD contributed to the design and implementation of the cannabidiol in the study and to the writing of the manuscript. MM and OF were involved in planning and supervised the study. MD brilliantly interpreted the radiological images and the follow-up and with CL and PD designed the figures. CL performed the pathology's diagnostic and the complete analysis of the surgery material. Also CL encourage PD to investigate the use of cannabidiol in neuro-oncology tumours and supervised the findings of this work.

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frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
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Acute Cannabinoids Produce Robust Anxiety-Like and Locomotor Effects in Mice, but Long-Term Consequences Are Age- and Sex-Dependent

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The rise in cannabinoid legalization and decriminalization in the US has been paired with an increase in adolescents that perceive marijuana as a "no risk" drug. However, a comprehensive review of human literature indicates that cannabinoid usage may have both beneficial and detrimental effects, with adolescent exposure being a critical window for harming cognitive development. Although the cannabinoids Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD) are often used together for recreational and medical purposes, no study has previously observed the acute and long-lasting effects of THC+CBD in a battery of behavioral assays analogous to subjective human reports. The current study observed the acute and long-term effects of THC, CBD, and THC+CBD on object recognition memory, anxiety-like behavior, and activity levels in adolescent and adult mice of both sexes. Acute THC alone and in combination with CBD resulted in robust effects on anxiety-like and locomotor behavior. A history of repeated cannabinoid treatment followed by a period without drug administration resulted in minimal effects in these behavioral assays. Most notably, the strongest effects of repeated cannabinoid treatment were seen in adult females administered THC+CBD, which significantly impaired their object recognition. No effects of repeated cannabinoid history were present on hippocampal protein expression. These studies represent a detailed examination of age- and sex-effects of acute and repeated cannabinoid administration. However, the acute and long-term effects of THC with and without CBD on additional behaviors in adolescents and adults will need to be examined for a more complete picture of these drug effects.

Keywords: cannabinoids, THC, anxiety, cognition, sedation, mice, sex differences

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INTRODUCTION

Cannabinoids, such as Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD) found in marijuana (cannabis), bind to cannabinoid receptors (CBRs) and may disrupt well-maintained inhibitory signaling regulated by endogenous cannabinoids. Long-term effects of repeated use may persist even following a period of abstinence (Freund and Katona, 2007; Svizenksa et al., 2008; Chevaleyre and Piskorowski, 2014). Although cannabis usage rates have been relatively stable

since 2002, the number of young adolescents and adults that report perceiving cannabis as a "no risk" drug has doubled to more than 17% in each age group (Azofeifa et al., 2016). However, no drug is fully without risks. A recent review by the National Academies of Sciences (2017) found that cannabinoid usage may have both beneficial and detrimental effects, but that adolescent exposure may be particularly harmful for cognitive development. This may be due to the increased expression and function of CB1Rs during early adolescence, which contribute to brain development (Romero et al., 1997; Verdurand et al., 2011; Lee and Gorzalka, 2012). Human research has demonstrated impairments in learning and memory even after cannabis use has ceased, with adolescent use linked to reduced educational and employment achievement.

Conclusions based on human research are weak and relatively constrained by methodological limitations (National Academies of Sciences, 2017). Several recommendations from the National Academies of Sciences include evaluating feelings of anxiety and sedation in all studies, focusing on the developmental period of adolescence, and including the use of preclinical studies examining both acute and chronic exposure to guide clinical research. Novel object recognition (NOR) is a preclinical analog of the human visual paired-comparisons task which harnesses a "normal" rodent's preference for a novel object over a familiar one (Burbacher and Grant, 2012; Cohen and Stackman, 2015). Unconditioned anxiety-like activity may be assessed using the elevated plus maze (EPM) or quantifying the amount of total time spent in the center of an open field (Mohammad et al., 2016). Both of these measures compare an animal's drive to remain in a "safe" space versus the drive to explore an open area, and open field activity also gives a measure of locomotor activity. NOR, EPM, and open field activity are optimal behavioral tasks for assessment of adolescent exposure, as they are quick, relatively free of stress, independent of external reward and punishment, and require minimal to no training (Cohen and Stackman, 2015; Mohammad et al., 2016). Our lab has previously demonstrated that mice can be selectively bred for resilience or susceptibility to the locomotor effects of THC, thereby highlighting the importance of monitoring this behavior (Kasten et al., 2018).

Using these tasks, preclinical studies have indicated both acute and long-term effects of cannabinoid exposure. Acute THC has been demonstrated to affect object recognition memory in CD-1 mice (Barbieri et al., 2016; Busquets-Garcia et al., 2018), but not in other rodent strains (Ciccocioppo et al., 2002; Long et al., 2010; Swartzwelder et al., 2012; Kasten et al., 2017). It reliably produces anxiogenic and sedative effects at higher doses (Onaivi et al., 1990; Célérier et al., 2006; Schramm-Sapyta et al., 2007; Lee et al., 2015; Kasten et al., 2017). A history of THC during adolescence results in memory impairments in the novel object task during adulthood in rats and mice (Quinn et al., 2008; Realini et al., 2011; Zamberletti et al., 2012; Kevin et al., 2017; Murphy et al., 2017, but see O'Tuathaigh et al., 2010; Cadoni et al., 2013; Segal-Gavish et al., 2017), but studies at the adult time-point have been inconclusive (Quinn et al., 2008; Vigano et al., 2009; Kasten et al., 2017; Rodríguez et al., 2017). Of particular interest, CBD is unable to independently alter NOR, but successfully rescues NOR deficits and proinflammatory responses in models of inflammation (Fagherazzi et al., 2012; Cadoni et al., 2013; Campos et al., 2015; Gomes et al., 2015). As adolescent THC administration results in a proinflammatory shift in the CNS during adulthood (Zamberletti et al., 2015), this may indicate that co-administration of CBD with THC may inhibit the NOR impairment demonstrated following adolescent treatment. Further, systemic and site-specific CBD exerts anxiolytic effects in the EPM via action at the serotonin 5HT1a receptor (Guimarães et al., 1990; Onaivi et al., 1990; Campos and Guimarães, 2008; Gomes et al., 2011; Marinho et al., 2015; Schiavon et al., 2016), and may thereby attenuate the anxiogenic effects of THC administration.

Although THC and CBD are often used together for recreational and medical purposes, no study has observed the acute and long-lasting effects of THC+CBD on the NOR, EPM, and open field tasks. Further, these assessments have not been systematically conducted in adolescents and adults of both sexes. The current work used adolescent (PND28) or adult (PND63) male and female C57Bl/6J (B6) mice. First, a dose-response to acute THC or CBD was assessed on EPM and open field activity to inform dose choices for repeated exposure. Following the dose-response studies, the acute effects of vehicle, 10 mg/kg THC, 20 mg/kg CBD, and THC+CBD were assessed for their effects on object recognition, EPM, and open field activity. Mice from the acute assessment received a total of eight injections over a 3-week period, then were given 3 weeks of rest. Following rest, all mice were again tested for object recognition, EPM, and open field activity under no-drug conditions to assess the effects of an adolescent or adult history of cannabinoids in male and female mice. Finally, protein levels of CB1R, interleukin 1 receptor 1 (IL-1R1), and serotonin 5HT1a receptors in the hippocampus were assessed. Although cannabinoids primarily work at cannabinoid receptors, CBD is known to exert behavior effects via the 5HT1a receptor (Russo et al., 2005; Campos and Guimarães, 2008). Further, repeated THC administration results in a pro-inflammatory shift in the CNS (Zamberletti et al., 2015), which may affect hippocampal-dependent memory due to changes in interleukin-1 signaling (Goshen et al., 2007). NOR tasks utilizing long delays are dependent on hippocampal function (Cohen and Stackman, 2015) and the hippocampus displays high levels of THC metabolites following acute administration (Leishman et al., 2018) making it a target region for these analyses.

MATERIALS AND METHODS

Mice

A total of 440 male and female C57BL/6J (B6) mice were purchased from Jackson Laboratories and arrived at PND21 or PND56 and kept on a 12:12 h reverse light cycle. Mice were single-housed in Experiment 1 as in our previous work (Kasten et al., 2017). Although short-term social isolation during adolescence does not affect anxiety-like behavior in B6 mice, long-term isolation does (Lin et al., 2018). Further, object discrimination in the NOR task is not affected by social isolation

alone, but is impaired when inflammatory processes are present in the hippocampus (Hueston et al., 2017). To avoid these potential confounds, mice were pair-housed in Experiments 2 and 3 due to the long-term nature of these experiments. All procedures adhered to the protocol approved by Indiana University-Purdue University Indianapolis School of Science Institutional Animal Care and Use Committee and conform to the Guidelines for the Care and Use of Laboratory Animals (Guide for the Care Use of Laboratory Animals, 2011) and the Public Health Service Policy on Human Care and Use of Laboratory Animals (National Institutes of Health, 2015).

Drug

Both THC and CBD were generous gifts from the National Institutes of Health/National Institute on Drug Abuse (Bethesda, MD, United States). THC (1, 5, and 10 mg/kg), CBD (5, 10, and 20 mg/kg), or the combination (10 mg/kg THC + 20 mg/kg CBD) were dissolved in a vehicle solution of 5% Tween80, 5% 100 proof ethanol, and 90% saline. These doses cover the low to high ranges used throughout previous studies. Drug was administered in a pseudorandomized order, with each drug being equally represented in every cohort. Pair-housed mice received the same drug so that subordinate/dominate mates were equally represented across drug groups. All solutions were delivered via intraperitoneal injections in a volume of 0.1 mL per 10 g of body weight. To reduce the stress of multiple injections during Experiments 2 and 3 and mimic human use patterns, THC and CBD were combined in one solution. All injections were administered in the vivarium to keep the site of drug injection consistent.

Behavioral Tasks

To maximize our ability to detect anxiogenic drug effects, injections and behavioral tasks were conducted during the active dark phase under red light conditions to potentially increase exploration time in the NOR task and amount of open arm time in the EPM. Because sex-related olfactory cues contribute to aggressive and territorial-related behaviors (Ervin et al., 2015), males and females were tested at different times and housed in different rooms to minimize exposure to the opposite sex.

Elevated Plus Maze

Mice were injected in the animal vivarium to avoid disruption of behavioral tasks by ultrasonic vocalizations used to communicate stressful and aversive stimuli, such as drug injections and restraint (Ko et al., 2005; Grimsley et al., 2016). Thirty minutes following injection, mice were individually transported approximately 30 feet to the EPM testing room (Kasten et al., 2017). Mice were placed in the EPM facing an open arm and given 5 min to explore. Two separate black Plexiglas plus mazes (Med Associates, Inc., St. Albans, VT, United States) adjusted for mouse size were used (see Moore et al., 2011 for details). Each session was video recorded and scored. Time in the open arms was recorded when all four of the animal's paws crossed the center zone into the open arm. Each occurrence of four paws crossing into an open arm was counted as one open arm entry.

Open Field

Mice were individually transferred approximately 15 feet from the EPM room to the open field testing room. Each mouse was placed in a Versamax Animal Activity Monitor (Accuscan Instruments, Columbus, OH, United States) for 10 min without a habituation period. Locomotor activity was recorded by eight pairs of intersecting photocell beams (2 cm above the chamber floor) evenly spaced along the walls of the 40 × 40 cm test chamber. Sound-attenuating box chambers (inside dimensions, 53 cm across × 58 cm deep × 43 cm high) equipped with a house light and fan for ventilation and background noise encased the test chamber. The house light was off. The chambers were attached to a Dell computer which recorded activity counts every minute. Animals were immediately returned to their home cage in the vivarium following the session.

Novel Object Recognition

The NOR apparatus consists of a 40 × 40 × 40 cm wooden chamber painted light brown and sealed to block any spatial cues and allow for cleaning. The NOR task took place over 3 days, with each session being spaced 24 h apart. Sessions were recorded by a video camera and object investigation was hand-scored. On each day, the mice were individually walked into the testing room immediately prior to their session and returned to the vivarium immediately following their session. On the habituation day, animals were placed in the arena for 10 min without any objects present. On the training day, animals were placed into the arena with two identical objects and given 10 min to explore. The objects were placed approximately 10 cm out from diagonal corners. On the test day, one familiar object was replaced with a novel object in the test chamber, and mice were given 5 min to explore. Objects were optimized for each sex- and age-group in pair-housed naïve mice (**Table 1** and **Figure 1**). Exploration time is time the animal spent oriented toward the object sniffing within 2 cm or in physical contact with the object.

TABLE 1 | Indicates the objects used for the NOR task for each treatment and sex group at each time-point.

Treatment/Sex	PND28–30	PND70–72	PND111–113
Adolescent Males	Small "5 H Energy" and opaque drug vial	Small Erlenmeyer Pyrex and mini brown ceramic mug	
Adult Males		Small Erlenmeyer Pyrex and mini brown ceramic mug	Small "5 H Energy" and opaque drug vial
Adolescent Females	Small Erlenmeyer Pyrex and mini brown ceramic mug	Small conical tube and white plastic slide case	
Adult Females		Small conical tube and white plastic slide case	Small Erlenmeyer Pyrex and mini brown ceramic mug

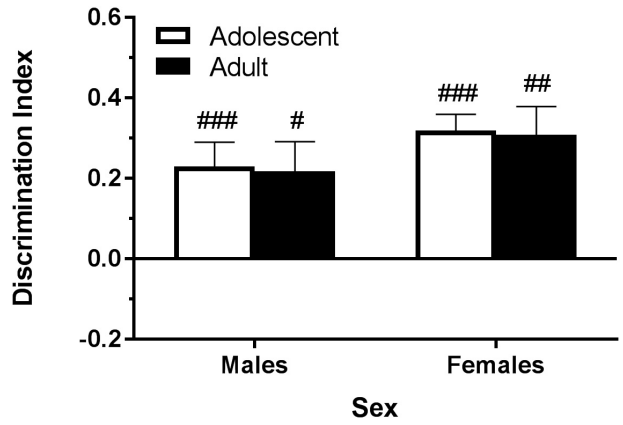


FIGURE 1 | Depicts that, using the objects described in **Table 1**, pair-housed naïve B6 mice are able to significantly discriminate the novel object during the test phase. Pound sign indicates significantly different from zero at $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$, *n*'s = 8–10.

Experiments

Experiment 1: THC and CBD Dose Responses

Experiment 1 sought to characterize dose-response relationships to acute THC (0, 1, 5, or 10 mg/kg) and CBD (0, 5, 10, or 20 mg/kg) in adolescent (PND28-30) and adult (PND70+) B6 mice on anxiety-like and sedative behaviors. Mice received a 30-min cannabinoid pretreatment (Onaivi et al., 1990) before being placed on the EPM. Following the EPM task, mice were immediately transferred to and placed in the open field.

Experiment 2: Acute Effects of THC, CBD, and THC+CBD

Following the results Experiment 1, Experiment 2 assessed the effects of acute vehicle, 10 mg/kg THC, 20 mg/kg CBD, or THC+CBD on object recognition memory, anxiety-like behavior, and locomotor activity in adolescent (PND28) and adult (PND70) B6 mice. 10 mg/kg THC was chosen for its significant drug effects across all age*sex groups, whereas 20 mg/kg CBD was chosen because it was the highest dose assessed in Aim 1. Although the ratio of THC:CBD varies greatly across both strains and laboratories, the 1:2 THC to CBD ratio is commercially available for recreational and medical use (Jikomes and Zoorob, 2018). For the NOR task, an acute injection was administered 10 min after conclusion of the training session and the test session occurred approximately 24 h later. A post-training injection time-point with a 24-h inter-trial interval has been used in previous studies observing the acute effects of THC on object recognition memory consolidation (Swartzwelder et al., 2012; Barbieri et al., 2016; Kasten et al., 2017; Busquets-Garcia et al., 2018) and avoids any cannabinoid-induced locomotor effects from interfering with object exploration. Acute effects of THC, CBD, or THC+CBD were also tested on EPM and open field. As in Experiment 1, mice were given a 30 min drug pretreatment before being placed on the EPM. Open field exploration was quantified immediately following testing on the EPM task.

Experiment 3: Aged Effects of THC, CBD, and THC+CBD

Experiment 3 assessed the effects of adolescent or adult cannabinoid history on later behavior. Mice from Experiment 2 received a total of eight injections of vehicle, 10 mg/kg THC, 20 mg/kg CBD, or THC+CBD over 3 weeks. These injections included the post-training NOR injection, the EPM pretreatment, and six additional maintenance injections. Mice then received 3 weeks of rest so that adolescent-treated mice could age to adulthood (PND70) and adult-treated mice aged to later adulthood (PND111). The NOR, EPM, and open field tasks were then run in the same manner as Experiment 2, with the exception that no drug was administered. See **Table 2** for a timeline of Experiments 2 and 3.

Hippocampal Western Blot

Approximately 24 h following completion of the aged behavioral tasks, mice were euthanized by cervical dislocation and brains were extracted (see **Table 2**). Whole brains were submerged in ice-cold autoclaved 1X PBS buffer for approximately 1 min. Brains were removed from the buffer, halved along the longitudinal fissure, and right and left whole hippocampi were removed. Samples were placed directly into 300 µl of ice-cold RIPA buffer with protease inhibitor (1 ml of RIPA buffer containing 100 µl of 10 × PI and 10 µl of 0.1 M PMSF) (Thermo Fisher) and frozen in a −80° F freezer. Details on tissue homogenization, sample denaturization, and Western Blot procedure can be found in Kasten et al. (2017). Western blots were run to identify levels of CB_1R (Anti-Cannabinoid Receptor 1, Rabbit polyclonal, Abcam), interleukin 1 Receptor 1 (IL-1R1) (Anti-IL-1R1 antibody, Goat polyclonal, Thermo Fisher), and serotonin $5HT_{1a}$ receptor (Anti-5HT1a Receptor antibody, Rabbit polyclonal, Abcam). Protein expression for each mouse was calculated as the signal strength of protein of interest expression normalized to the signal strength of β-actin expression (Beta-actin Mouse Monoclonal Antibody, Li-Cor Biosciences Inc.).

Statistical Analyses

All analyses were run separately in males and females to conserve statistical power to assess the primary question of these studies: does adolescent administration of cannabinoids differentially affect behavior compared to adult administration? Therefore, omnibus tests were Dose*Age at Treatment for

TABLE 2 | Details the timeline of Experiments 2 and 3 for each cohort.

	Monday	Tuesday	Wednesday	Thursday	Friday
Week 1		NOR habituation	NOR Training Injection 1	NOR Test	Injection 2 EPM and open field
Week 2	Injection 3		Injection 4		Injection 5
Week 3	Injection 6		Injection 7		Injection 8
Weeks 4–6			REST		
Week 7	NOR habituation	NOR Training	NOR Test	EPM and open field	Brain Extraction

each sex independently. For all statistical analysis, the omnibus significance was set at $p < 0.05$ and corrected for follow-up tests. For Experiment 1, time in open arms, open arm entries, total locomotion in the open field, and percent of time spent in the center of the open field was analyzed using a Dose*Age factorial ANOVA for THC and CBD. There was an *a priori* hypothesis that each age*sex group may have different sensitivities to THC and CBD, so a one-way ANOVA analyzing dose response to each drug were run for all groups to determine dosage for Experiments 2 and 3. Dunnett's *post hoc* tests were used to compare all drug doses to the vehicle group. Cohen's *d* (*d*) is reported as a measure of effect size for all significantly different comparisons.

For Experiments 2 and 3, a Drug*Age at Treatment factorial ANOVA was run to assess acute or prior history effects of vehicle, THC, CBD, or THC+CBD on novel object discrimination index, time in open arms, open arm entries, and activity in the open field for each sex independently. To reduce animal usage, n's were kept at 8–10 and one-way ANOVAs corrected for statistical significance were run to assess the effect of dose or drug on each age group. Planned comparisons corrected for multiple analyses were used to analyze whether drug groups were significantly different from vehicle and whether THC+CBD was significantly different than THC alone for discrimination index, time in open arms, open arm entries, and open field activities. Pearson correlations within each age*sex*drug were used to determine whether investigation during the training session influenced discrimination index, as it has been previously suggested that more investigation during the training session may increase object recognition memory (Cohen and Stackman, 2015).

The discrimination index was calculated as (time spent with novel object – time spent with familiar object)/total object investigation time. It ranges from −1 to +1, with more positive numbers indicating more time spent with the novel object and 0 indicating no preference. Significant object discrimination is defined as a group being significantly different than 0 using a one-sample *t*-test. Percent of time spent in the center of the OF, an alternative measure of anxiety (Mohammad et al., 2016), were calculated as [(center activity/total activity)*100]. This method of calculation controls for differences in total locomotion that may result from drug administration.

Western blots were analyzed using Drug*Age at Treatment factorial ANOVAs, with follow-up one-way ANOVAs within each age group as described above. However, we also ran Sex*Age at Treatment ANOVAs comparing the expression levels of the target protein in each vehicle group to discern whether there are any basal differences in expression levels.

RESULTS

Experiment 1: THC and CBD Dose Responses

Elevated Plus Maze Activity

In single-housed males, Dose*Age ANOVAs revealed a significant interaction of THC on time spent in the open arms of the EPM [$F(3,61) = 3.21$, $p < 0.05$] (**Figure 2A**) as well as number of open arm entries [$F(3,61) = 3.10$, $p < 0.05$] (data not shown). There was not a significant effect of age on either metric, but there were significant dose effects [time in open arms $F(3,61) = 7.71$, $p < 0.001$; open arm entries $F(3,61) = 6.76$, $p < 0.001$]. One-way ANOVAs for each age group revealed that the 10 mg/kg dose reduced time spent in the open arms [$t(29) = 4.13$, $p < 0.01$, $d = 2.12$] and number of open arm entries [$t(29) = 2.21$, $p < 0.01$, $d = 1.83$] only in adult male mice. Adolescent open arm entries mean (*SEM*): Vehicle – 5.00 (*0.71*); 1 mg/kg THC – 8.88 (*1.14*); 5 mg/kg THC – 9.00 (*1.08*); 10 mg/kg THC 5.56 (*1.62*). Adult open arm entries mean (*SEM*): Vehicle – 9.88 (*1.30*); 1 mg/kg THC – 11.63 (*1.44*); 5 mg/kg THC – 8.00 (*1.80*); 10 mg/kg THC 3.33 (*1.16*).

In single-housed males, Dose*Age ANOVAs revealed no interaction or main effect of dose of CBD on time in the open arms (**Figure 3A**) or number of open arm entries in males (*p*'s > 0.05) (data not shown). There was a significant effect of age for both variables, with adults spending more time in the open arms [$F(1,56) = 17.13$, $p < 0.001$] and making more open arm entries compared to adolescents [$F(1,56) = 16.31$, $p < 0.001$]. Adolescent open arm entries mean (*SEM*): Vehicle – 5.00 (*0.71*); 5 mg/kg CBD – 5.50 (*0.80*); 10 mg/kg CBD – 6.25 (*1.00*); 20 mg/kg CBD 5.22 (*0.94*). Adult open arm entries mean (*SEM*): Vehicle – 9.88 (*1.30*); 5 mg/kg CBD – 7.29 (*1.06*); 10 mg/kg CBD – 8.00 (*1.00*); 20 mg/kg CBD 5.22 (*0.94*).

In single-housed females, Dose*Age ANOVAs revealed no significant interaction or effect of age on time in the open arms (**Figure 2B**) or number of open arm entries following THC administration (*p*'s > 0.05) (data not shown). However, there was a significant effect of THC dose on both variables [time in open arms $F(3,60) = 8.08$, $p < 0.001$; open arm entries $F(3,60) = 3.35$, $p < 0.05$]. In adult mice, the 5 and 10 mg/kg doses of THC reduced time spent in the open arms [5 mg/kg $t(29) = 3.53$, $p < 0.05$, $d = 1.40$; 10 mg/kg $t(29) = 3.69$, $p < 0.01$, $d = 1.68$] as well as number of open arm entries [10 mg/kg $t(29) = 3.06$, $p < 0.05$, $d = 1.43$]. There were no significant dose effects of THC in adolescents ($p > 0.05$). Adolescent open arm entries mean (*SEM*): Vehicle – 7.44 (*0.74*); 1 mg/kg THC – 6.22 (*0.64*); 5 mg/kg THC – 7.25 (*1.58*); 10 mg/kg THC 5.11 (*1.25*). Adult open arm entries mean (*SEM*): Vehicle – 9.88 (*1.22*); 1 mg/kg THC – 9.25 (*1.74*); 5 mg/kg THC – 6.75 (*1.37*); 10 mg/kg THC 1.22 (*1.22*).

In single-housed females, Dose*Age ANOVAs revealed no interaction or main effect of dose of CBD on time in the open arms (**Figure 3B**) or number of open arm entries in females (*p*'s > 0.05) (data not shown). There was a significant effect of age for both variables, with adults spending more time in the open arms [$F(1,59) = 27.75$, $p < 0.001$] and making more open arm entries compared to adolescents [$F(1,59) = 27.62$, $p < 0.001$]. Adolescent open arm entries mean (*SEM*): Vehicle – 7.44 (*0.74*); 5 mg/kg CBD – 6.5 (*1.00*); 10 mg/kg CBD – 7 (*1.05*); 20 mg/kg CBD 5.44 (*0.80*). Adult open arm entries mean (*SEM*): Vehicle – 9.88 (*1.22*); 5 mg/kg CBD – 9.38 (*1.28*); 10 mg/kg CBD – 12.63 (*1.02*); 20 mg/kg CBD 4.70 (*1.57*).

Total Locomotion in the Open Field

In single-housed males, a Dose*Age ANOVA revealed no significant interaction or main effect of age on THC-induced

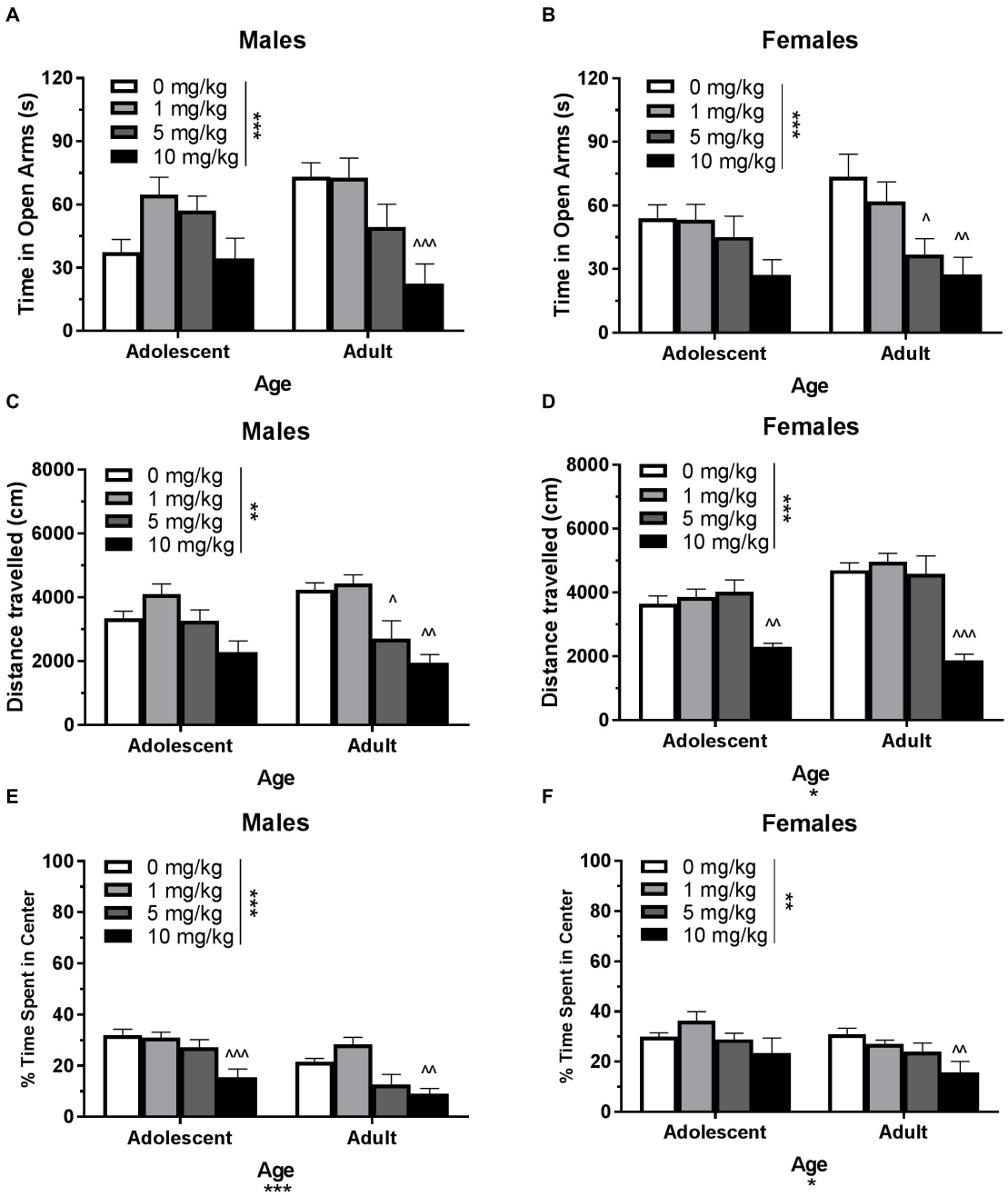


FIGURE 2 | Depicts the effects of different doses of acute THC pretreatment in single-housed adolescent and adult mice on time in the open arms of the EPM (males, **A**; females **B**), total locomotion in the open field (males, **C**; females, **D**), and percent of time spent in the center of the open field (males, **E**; females, **F**). Asterisks indicate a significant main effect at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. Carrots indicate significantly different from respective control at $\hat{}p < 0.05$, $\hat{}\hat{}p < 0.01$, and $\hat{}\hat{}\hat{}p < 0.001$, *n*'s = 8–9.

locomotor activity in the open field (*p*'s > 0.05) (**Figure 2C**). There was a significant main effect of dose [$F(3,62) = 15.50$, $p < 0.001$]. One-way ANOVAs for each age revealed that the 5 and 10 mg/kg doses reduced total locomotion, but only in adult mice [5 mg/kg $t(31) = 3.07$, $p < 0.05$, $d = 1.21$; 10 mg/kg $t(31) = 4.59$, $p < 0.001$, $d = 3.19$)]. Reduced activity in 5 mg/kg adult group was significantly correlated with reduced time in the open arms [$r(9) = 0.682$, $p < 0.05$] and percent of time spent in the center of the open field [$r(9) = 0.886$, $p < 0.01$]. Reduced activity in the 10 mg/kg adult group was

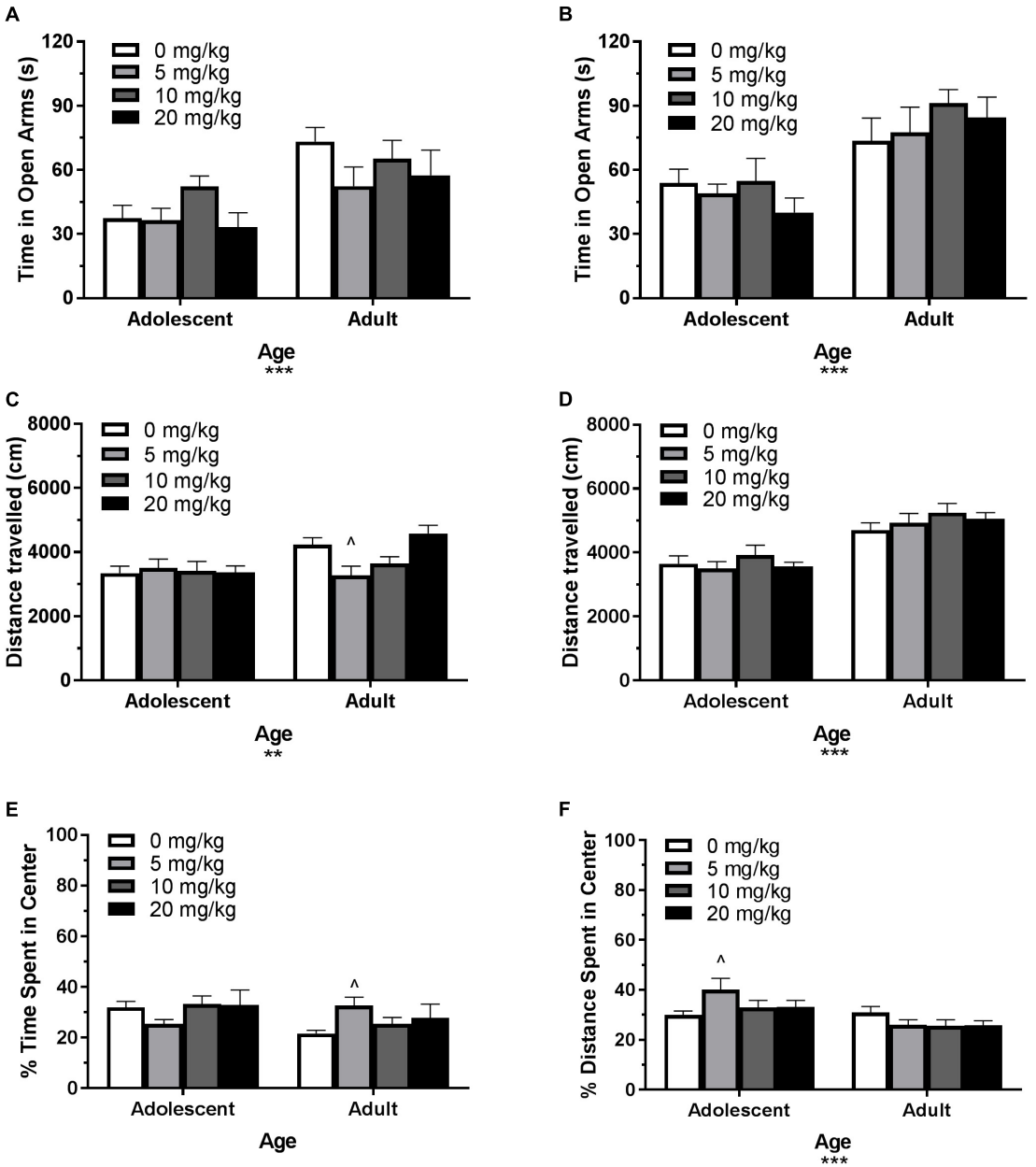


FIGURE 3 | Depicts the effects of different doses of acute CBD pretreatment in single-housed adolescent and adult mice on time in the open arms of the EPM (males, **A**; females **B**), total locomotion in the open field (males, **C**; females, **D**), and percent of time spent in the center of the open field (males, **E**; females, **F**). Asterisks indicate a significant main effect $^{**}p < 0.01$ and $^{***}p < 0.001$. Carrot (^) indicates significantly different from respective control at $p < 0.05$, *n*'s 7–9.

not significantly correlated with anxiety-like metrics (*p*'s > 0.05). For CBD, a Dose*Age ANOVA revealed a significant interaction [$F(3,61) = 3.42$, $p < 0.05$] and main effect of age [$F(1,61) = 9.20$, $p < 0.01$] (**Figure 3C**), with adults moving more. Although there was no main effect of dose, one-way ANOVAs for each age group revealed that the 5 mg/kg dose reduced total locomotion in adults [$t(31) = 2.67$, $p < 0.05$, $d = 1.31$]. Reduced activity in this group was not significantly correlated with anxiety-like metrics (*p*'s > 0.05).

In single-housed females, a Dose*Age ANOVA revealed no significant interaction on THC-induced locomotor activity in the open field ($p > 0.05$) (**Figure 2D**). There were significant effects

of age [$F(1,61) = 6.95$, $p < 0.05$] and dose [$F(3,61) = 26.85$, $p < 0.001$]. Overall, adults moved more, and the 10 mg/kg dose reduced activity in both age groups [(adolescent $t(31) = 3.86$, $p < 0.01$, $d = 2.38$; adult $t(30) = 5.78$, $p < 0.001$, $d = 4.46$]. Reduced activity was not significantly correlated with anxiety-like metrics in either age group (p's > 0.05). For CBD, a Dose*Age ANOVA revealed only a significant effect of age on total locomotion, with adults moving more [$F(1,59) = 59.23$, $p < 0.001$] (**Figure 3D**). One-way ANOVAs for each age group also revealed no significant effects of CBD dose on total locomotion.

Percent of Time Spent in the Center of the Open Field

In single-housed males, a Dose*Age ANOVA revealed no significant interaction of THC on anxiety-like activity in the open field ($p > 0.05$) (**Figure 2E**). There was a significant main effect of age [$F(1,62) = 19.77$, $p < 0.001$], with adults spending significantly less time in the center of the open field as a percent of overall time moving, indicating a more-anxious phenotype than adolescents. There was also a significant anxiogenic effect of THC dose [$F(3,62) = 16.27$, $p < 0.001$], with 10 mg/kg decreasing the percent of time spent in the center of the open field [adolescent $t(31) = 4.33$, $p < 0.001$, $d = 1.95$; adult $t(31) = 3.36$, $p < 0.01$, $d = 2.43$]. For CBD, a Dose*Age ANOVA revealed no significant interaction or main effect of dose or age on anxiety-like activity (p's > 0.05) (**Figure 3E**). One-way ANOVAs at each age revealed that 5 mg/kg CBD significantly increased percent of time spent in the center of the open field in adult males [$t(31) = 2.72$, $p < 0.05$, $d = 1.88$].

In single-housed females, a Dose*Age ANOVA revealed no significant interaction of THC on anxiety-like activity in the open field ($p > 0.05$) (**Figure 2F**). There was a significant main effect of age [$F(1,61) = 4.20$, $p < 0.05$], with adults spending significantly less time in the center of the open field as a percent of overall time moving, indicating a more-anxious phenotype than adolescents. There was a significant main effect of dose [$F(3,61) = 4.58$, $p < 0.01$] with 10 mg/kg significantly decreasing the percent of time spent in the center of the open field only in adults [$t(30) = 3.35$, $p < 0.01$, $d = 1.43$]. For CBD, a Dose*Age ANOVA revealed a significant interaction on percent of time spent in the center of the open field [$F(3,59) = 2.88$, $p < 0.05$]. There was no main effect of dose ($p > 0.05$), but there was a significant main effect of age [$F(1,59) = 14.02$, $p < 0.001$], with adults demonstrating a more-anxious phenotype than adolescents (**Figure 3F**). One-way ANOVAs at each age revealed that 5 mg/kg CBD significantly increased percent of time spent in the center of the open field in adolescent females [$t(29) = 2.43$, $p < 0.05$, $d = 1.07$].

Experiment 2: Acute Effects of THC, CBD, and THC+CBD

Novel Object Recognition

In pair-housed males, one-sample t-tests indicated that significant novel object discrimination occurred in adolescents treated with THC and CBD and adults treated with vehicle following object training (p's < 0.05) (**Figure 4A**). A Drug*Age ANOVA revealed no significant effects of drug or age (p's > 0.05), but a trend toward an interaction [$F(3,71) = 2.47$, $p = 0.069$]. One-way ANOVAs examining drug effects within each age group indicated a trend toward a significant effect in adolescents [$F(3,35) = 2.51$, $p = 0.074$] with THC trending toward increasing object discrimination compared to vehicle [$t(35) = 2.30$, $p = 0.071$, $d = 1.17$]. There were no significant effects of drug in the adult groups ($p > 0.05$). Importantly, the lack of differences in total object investigation time during the training and test sessions (data not shown) indicate that these differences in discrimination index are not due to basal or drug-induced motivational differences in investigation. Discrimination index was not significantly correlated with training investigation time within any drug group (p's > 0.05) (data not shown).

In pair-housed females, one-sample t-tests indicated that significant novel object discrimination occurred in all adolescent-treated groups, as well as in adults treated with vehicle and THC following object training (**Figure 4B**). There were no

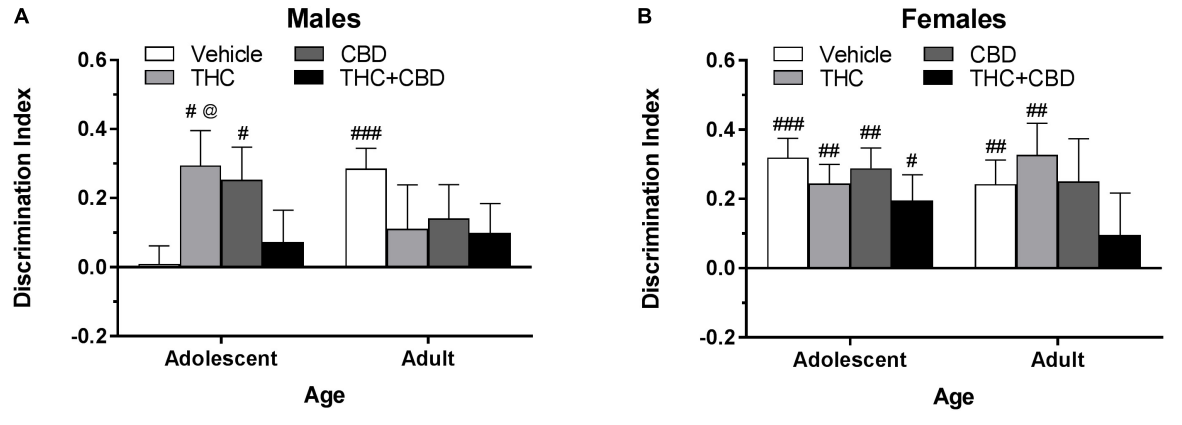


FIGURE 4 | Depicts discrimination of a novel object in the NOR task in pair-housed adolescent and adult males **(A)** and females **(B)** when cannabinoids were administered post-training. Higher positive values indicate more time spent with the novel object. Hashtag indicates significant discrimination (different from 0) at $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$. Ampersand (@) indicates a trend toward different from control at $p = 0.05 - 0.075$, n's = 9–10.

significant main effects of age or drug, nor interaction of the two variables, on discrimination index (p's > 0.05). Further, there were no significant drug effects within each age group (p's > 0.05). While all groups spent a similar amount of time investigating the objects during training, acute CBD increased object investigation during the test phase in adolescent females [$t(36) = 3.10$, $p < 0.05$] (data not shown). Discrimination index was significantly positively correlated with training investigation in adolescents treated with vehicle [$r(10) = 0.701$] and adults treated with THC+CBD [$r(10) = 0.683$] (p's < 0.05) (data not shown).

Elevated Plus Maze Activity

In pair-housed males, a Drug*Age ANOVA revealed a strong trend toward an interaction of drug treatment and age on time spent in the open arms of the EPM following acute cannabinoid pretreatment [$F(3,67) = 2.73$, $p = 0.0505$]. There was no significant effect of age ($p > 0.05$), but there was a main effect of drug ($p < 0.05$). One-way ANOVAs for each age group indicated a significant effect of drug in adults [$F(3,32) = 4.13$, $p < 0.05$], with THC+CBD reducing time in the open arms [$t(32) = 2.61$, $p < 0.05$, $d = 1.26$) (**Figure 5A**). For open arm entries, a Dose*Age ANOVA also revealed a significant interaction [$F(3,67) = 2.86$, $p < 0.05$] and effect of dose [$F(3,67) = 2.91$, $p < 0.05$], but no significant effect of age ($p > 0.05$). One-way ANOVAs for each age group revealed that THC significantly increased open arm entries in adolescent males compared to vehicle [$t(35) = 2.60$, $p < 0.05$, $d = 0.64$], but that no adult group was significantly different from control ($p > 0.05$) (data not shown).

In pair-housed females, analyses revealed no significant interaction of Dose*Age, no significant effect of age, and no effects of drug on time spent in the open arms (**Figure 5B**) or number of open arm entries (p's > 0.05) (data not shown).

Open Field Activity

In pair-housed males, a Drug*Age ANOVA revealed no significant interaction on total locomotion in the open field ($p > 0.05$). There was a significant effect of age [$F(1,72) = 5.91$, $p < 0.05$], with adolescents traveling a greater distance overall. There was also a significant effect of drug [$F(3,72) = 17.46$, $p < 0.001$], with THC and THC+CBD significantly reducing total locomotion in both age groups [adolescent THC $t(36) = 3.45$, $p < 0.01$, $d = 1.76$; adolescent THC+CBD $t(36) = 2.64$, $p < 0.05$, $d = 1.19$; adult THC $t(36) = 2.91$, $p < 0.05$, $d = 1.15$; adult THC+CBD $t(36) = 4.64$, $p < 0.001$, $d = 2.75$] (**Figure 5C**). In all groups with the exception of adults administered THC+CBD, total locomotion was significantly positive correlated with percent time in the center of the open field (p's < 0.01), but not significantly correlated with time spent in the EPM open arms (p's > 0.05). Anxiety-like activity in the open field was quantified using percent of time spent in the center of the open field. A Drug*Age ANOVA revealed no significant interaction ($p > 0.05$), but a significant effect of age [$F(1,72) = 8.52$, $p < 0.01$] and effect of drug [$F(3,72) = 6.86$, $p < 0.001$] on anxiety-like activity following acute cannabinoid administration. Adolescents demonstrated increased percentage of time spent in the center of the open field compared to their adult counterparts, indicating a less-anxious phenotype. One-way ANOVAs for each age indicated no significant effect of cannabinoid pretreatment in adolescents ($p > 0.05$), but that THC and THC+CBD significantly decreased the percentage of total locomotion in the center of the open field in adults, indicating anxiogenic effects [THC $t(36) = 4.2$, $p < 0.001$, $d = 1.51$; THC+CBD t(36) = 6.03, $p < 0.001$, $d = 2.85$] (**Figure 5E**).

In pair-housed females, a Drug*Age ANOVA revealed no significant interaction or main effect of age on total locomotion in the open field (p's > 0.05). There was a significant main effect of drug [$F(3,71) = 10.43$, $p < 0.001$], with THC reducing total locomotion in both age groups [adolescent $t(36) = 2.68$, $p < 0.05$, $d = 1.26$; adult $t(35) = 2.64$, $p < 0.05$, $d = 1.08$], and THC+CBD reducing total locomotion only in adults [$t(35) = 4.85$, $p < 0.001$, $d = 2.61$] (**Figure 5D**). Reduced activity was not correlated with anxiety-like activity in the open field and EPM (p's > 0.05). A Drug*Age ANOVA revealed a significant interaction [$F(3,71) = 4.57$, $p < 0.01$], effect of age [$F(1,71) = 26.12$, $p < 0.001$], and effect of drug [$F(3,71) = 9.12$, $p < 0.001$] on anxiety-like activity following acute cannabinoid administration. As with males, female adolescents demonstrated increased percentage of time spent in the center of the open field compared to their adult counterparts, indicating a less-anxious phenotype. Cannabinoid pretreatment did not significantly alter this behavior in adolescent females ($p > 0.05$). In adults, all cannabinoids significantly reduced percentage of time spent in the center [THC $t(35) = 7.10$, $p < 0.001$, $d = 3.11$; CBD $t(35) = 3.68$, $p < 0.01$, $d = 1.48$; THC+CBD $t(35) = 9.78$, $p < 0.001$, $d = 5.81$], and THC+CBD decreased this parameter synergistically when compared to THC-alone [$t(35) = 2.87$, $p < 0.05$, $d = 1.55$] (**Figure 5F**).

Experiment 3: Aged Effects of THC, CBD, and THC+CBD

Weight

One-way ANOVAs on weight at the first drug administration, the last drug administration, and the aged testing point indicated no significant differences in baseline weight, weight gain over injections, or long-term weight gain in any group (p's > 0.05, **Table 3**).

Novel Object Recognition

In pair-housed males, one-sample t-tests indicated that significant novel object discrimination occurred in mice with an adolescent and adult history of vehicle or THC, as well as mice with an adolescent history of CBD (p's < 0.05). Although some cannabinoid treated groups failed to demonstrate NOR, a Drug*Age at Treatment ANOVA revealed no significant main effects of or interaction of the variables on discrimination index (p's > 0.05). Further, there were no effects of drug history within either age group (p's > 0.05) (**Figure 6A**), and time spent investigating the objects during the training and test phases were not altered by drug history (data not shown). Discrimination index was not significantly correlated with training investigation time within any drug group (p's > 0.05) (data not shown).

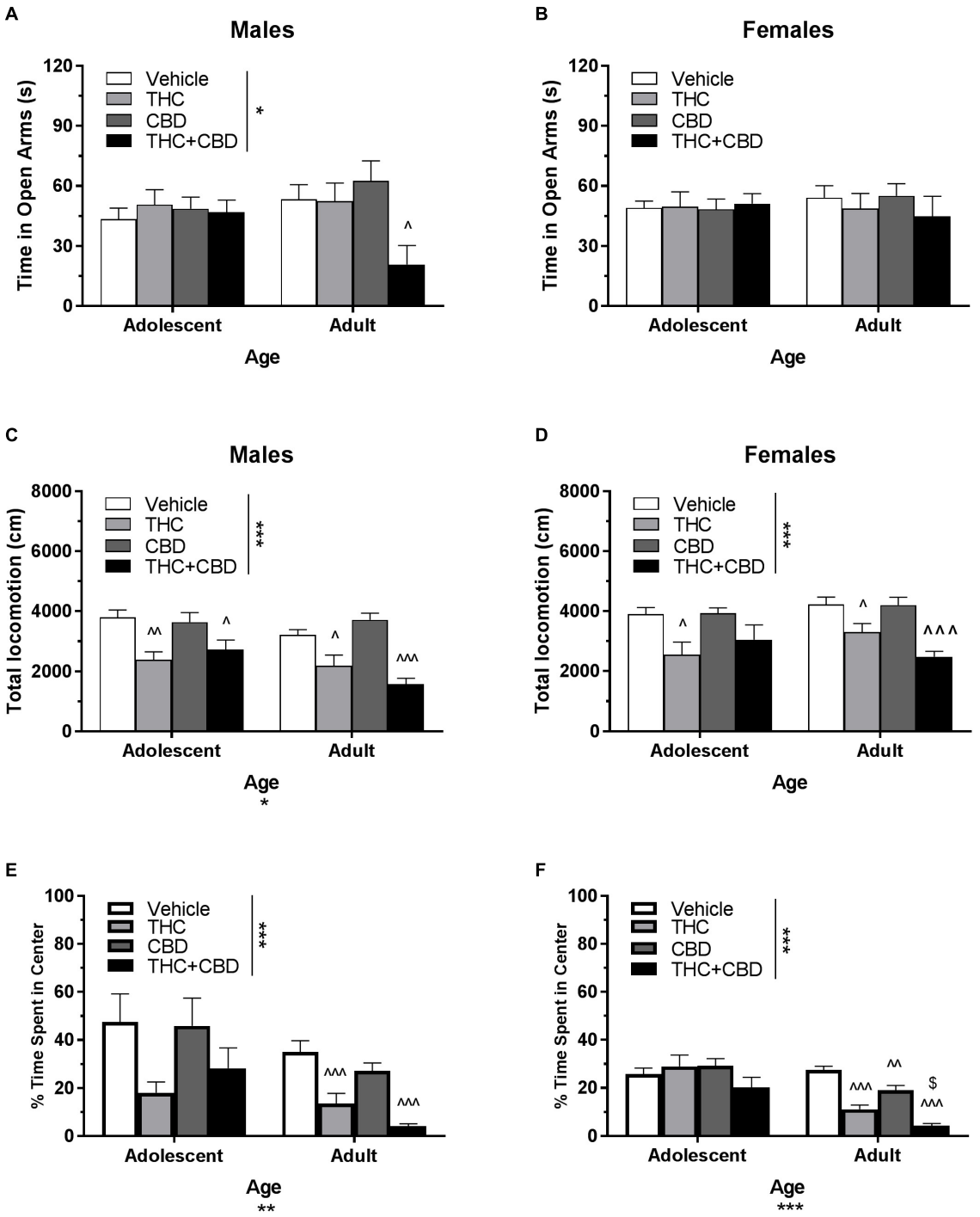


FIGURE 5 | Depicts the effects of acute cannabinoid pretreatment in pair-housed adolescent and adult mice on time in the open arms of the EPM (males, **A**; females **B**), total locomotion in the open field (males, **C**; females, **D**), and percent of time spent in the center of the open field (males, **E**; females, **F**). Asterisks indicate a significant main effect at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. Carrots indicate significantly different from respective control at $\hat{}p < 0.05$, $\hat{}\hat{}p < 0.01$, and $\hat{}\hat{}\hat{}p < 0.001$. Dollar sign ($) indicates significantly different from respective THC group at $p < 0.05$, n's = 7–10.

In pair-housed females, mice treated during adolescence with THC, CBD, and THC+CBD as well as mice treated during adulthood with vehicle and THC demonstrated object discrimination to varying levels of significance (p's < 0.05). A Drug*Age at Treatment ANOVA revealed no significant main effects or interaction on discrimination index (p's > 0.05). One-way ANOVAs for each age group indicated a weak trend of drug history in adult-treated animals [$F(3,33) = 2.46$,

TABLE 3 | Displays the mean weight ± standard error for the first day of drug treatment, the last day of drug treatment, and the aged behavior testing point for all mice in Aims 2 and 3.

	Weight (g ± SEM)			
	Vehicle	**THC**	**CBD**	**THC+CBD**
Adolescent-Treated Females				
PND29	13.93 ± *0.21*	14.04 ± *0.35*	13.98 ± *0.44*	14.36 ± *0.33*
PND45	16.55 ± *0.56*	16.04 ± *0.36*	16.25 ± *0.62*	16.92 ± *0.34*
PND73	18.85 ± *0.45*	18.54 ± *0.53*	18.54 ± *0.70*	19.69 ± *0.34*
Adult-Treated Females				
PND64	18.71 ± *0.33*	18.52 ± *0.56*	19.00 ± *0.44*	18.89 ± *0.25*
PND80	20.34 ± *0.34*	19.49 ± *0.50*	20.34 ± *0.36*	19.69 ± *0.15*
PND108	21.31 ± *0.31*	21.10 ± *0.52*	21.43 ± *0.32*	20.87 ± *0.17*
Adolescent-Treated Males				
PND29	15.42 ± *0.65*	15.28 ± *0.52*	15.29 ± *0.54*	15.66 ± *0.89*
PND45	20.69 ± *0.29*	19.53 ± *0.49*	20.48 ± *0.55*	19.20 ± *0.63*
PND73	24.61 ± *0.45*	23.85 ± *0.59*	23.88 ± *0.35*	23.09 ± *0.43*
Adult-Treated Males				
PND64	24.23 ± *0.53*	23.53 ± *0.48*	25.35 ± *0.50*	24.47 ± *0.51*
PND80	26.41 ± *0.70*	24.83 ± *0.52*	27.61 ± *0.63*	26.24 ± *0.58*
PND108	27.92 ± *0.61*	26.56 ± *0.57*	27.23 ± *1.19*	27.77 ± *0.61*

$p = 0.08$], with a THC+CBD history significantly reducing object discrimination compared to a vehicle history [$t(33) = 2.56$, $p < 0.05$, $d = 1.51$] (**Figure 6B**). Contrary to the male data, various investigative behaviors in the NOR task were altered by a history of cannabinoids. Females with an adolescent history of CBD spent more time investigating the objects during the training [$t(35) = 3.32$, $p < 0.01$] phase (data not shown). Discrimination index was significantly positively correlated with training investigation in mice treated during adulthood with THC [$r(9) = 0.740$] and THC+CBD [$r(9) = 0.726$] (p's < 0.05) (data not shown), indicating that adult females with a history of THC may require more time to create a detailed memory of the object during the training phase.

Elevated Plus Maze Activity

In pair-housed males, no significant interaction of Dose*Age at Treatment or effect of drug history was revealed on time spent in the open arms or number of open arm entries (p's > 0.05). There was a main effect of age on both variables (p's < 0.05), with adolescent-treated mice spending more time in the open arms (**Figure 7A**) and making more open arm entries (data not shown).

In pair-housed females, a Dose*Age at Treatment ANOVA revealed no significant effect of drug history or age at treatment on time spent in the open arms (p's > 0.05). However, there was a significant interaction effect [$F(3,69) = 3.36$, $p < 0.05$]. One-way ANOVAs assessing drug treatment for each group revealed no effect in adolescent-treated mice ($p > 0.05$), but that treatment with CBD during adulthood reduced time in the open arms compared to vehicle [$t(33) = 2.87$, $p < 0.05$, $d = 1.55$] (**Figure 7B**). There was no significant interaction of Drug*Age at Treatment or effect of drug treatment on number of open arm entries (p's > 0.05). However, there was a significant main effect of age [$F(1,69) = 5.17$, $p < 0.05$], with adolescent-treated mice making more entries (data not shown).

Open Field Activity

In pair-housed males, a Drug*Age at Treatment ANOVA revealed no significant interaction or effect of previous drug treatment on total locomotion in the open field (p's > 0.05). There was a significant effect of age [$F(1,72) = 23.8$, $p < 0.001$], with mice treated in adolescence traveling a greater distance. One-way ANOVAs indicated no significant effect of prior drug history in adolescent-treated mice, but a trend toward an effect in adult-treated mice ($p = 0.072$). Mice treated in adulthood with CBD traveled significantly more distance than their vehicle counterparts did [$t(36) = 2.76$, $p < 0.05$, $d = 1.16$] (**Figure 7C**). Anxiety-like activity in the open field was quantified using percent of time spent in the center of the open field. A Drug*Age at Treatment ANOVA revealed no significant interaction or main effect of drug treatment on anxiety-like activity following

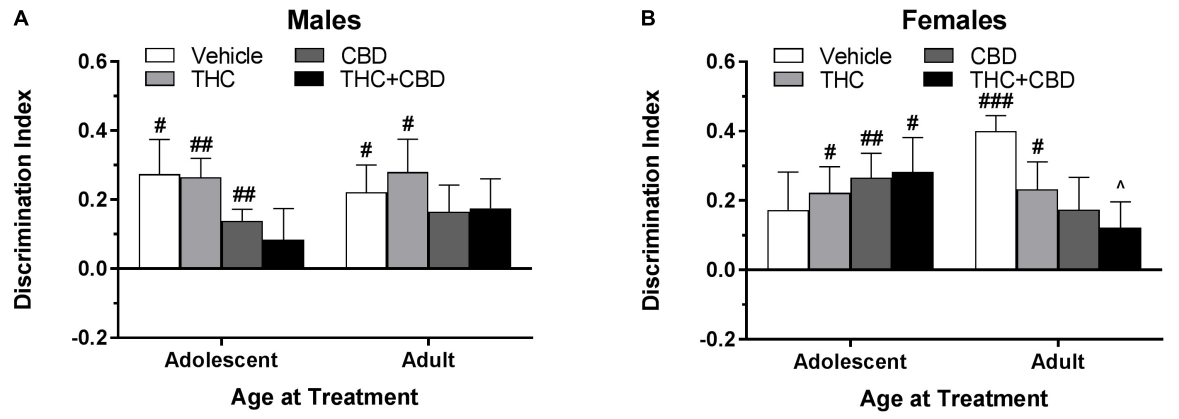


FIGURE 6 | Depicts discrimination of a novel object in the NOR task in pair-housed adolescent and adult males **(A)** and females **(B)** following a history of repeated cannabinoid treatment. Higher positive values indicate more time spent with the novel object. Hashtag indicates significant object discrimination (different from 0) at $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$. Carrot (^) indicates significantly different than respective control at $p < 0.05$, n's = 9–10.

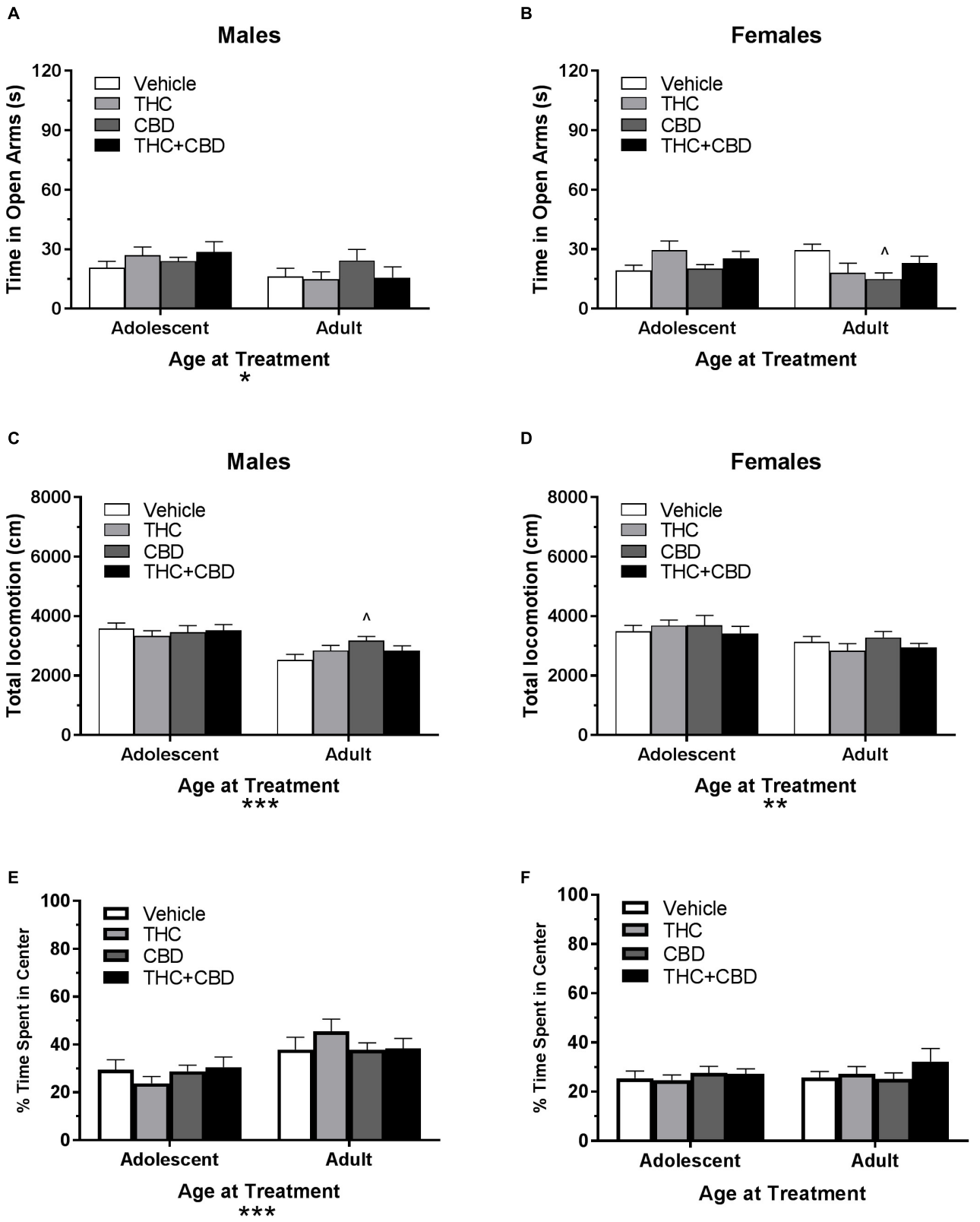


FIGURE 7 | Depicts the effects of repeated cannabinoid treatment in pair-housed adolescent and adult mice on time in the open arms of the EPM (males, **A**; females **B**), total locomotion in the open field (males, **C**; females, **D**), and percent of time spent in the center of the open field (males, **E**; females, **F**). Asterisks indicate a significant main effect at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. Carrot (^) indicate significantly different from respective control at $p < 0.05$, *n*'s = 9–10.

a history of cannabinoid administration (p's > 0.05). There was a significant effect of age at treatment [$F(1,72) = 16.96$, $p < 0.001$], with adult-treated mice spending more time in the center of the open field. One-way ANOVAs indicated no significant effect of drug in adolescent- or adult-treated mice (p's > 0.05) (**Figure 7E**).

In pair-housed females, a Drug*Age at Treatment ANOVA revealed no significant interaction or main effect of drug history on total locomotion in the open field (p's > 0.05). There was a significant main effect of age at treatment [$F(1,71) = 6.59$, $p < 0.05$], with adolescent-treated mice traveling a greater distance. One-way ANOVAs did not indicate a significant effect of drug history for either age group (p's > 0.05) (**Figure 7D**). A Drug*Age at Treatment ANOVA revealed no significant interaction or main effects on anxiety-like activity following a history of cannabinoid administration (p's > 0.05). One-way ANOVAs indicated no significant effect of drug in adolescent- or adult-treated mice (p's > 0.05) (**Figure 7F**).

Hippocampal Western Blot

CB1R Protein Expression

A Sex*Age at Treatment ANOVA revealed no significant interaction or main effect of age at treatment on CB1R, IL-1R1, or 5HT1a expression levels in vehicle groups (p's > 0.05). There were main effects of sex, with males vehicle-exposed having greater CB1R [$F(1,33) = 8.53$, $p < 0.01$] and IL-1R1 [$F(1,35) = 5.76$, $p < 0.05$] expression levels than females. However, vehicle-exposed females had greater 5HT1a expression [$F(1,33) = 43.68$, $p < 0.001$] than males.

Drug*Age at Treatment ANOVAs revealed no significant interaction or main effect of drug or age at treatment on CB1R, IL-1R1, or 5HT1a expression levels in males or females (p's < 0.05). Further, one-way ANOVAs within each age group and sex revealed that no drug history group was significantly different from their vehicle counterpart (data not shown).

DISCUSSION

The current project comprises a set of experiments examining age- and sex-effects of cannabinoid administration on acute and long-term behaviors. Although many significant acute actions of cannabinoids were demonstrated, there were minimal long-term effects associated with a history of repeated drug administration across age and sex. However, the significant effects reported are robust. The effect sizes reported herein indicate that significantly different p-values represent group differences wherein at least 73% of the treatment group is beyond the mean of the control group (Magnusson, 2014).

Experiment 1: THC and CBD Dose Responses

Based on previous studies (e.g., Guimarães et al., 1990; Onaivi et al., 1990; Kasten et al., 2017), acute THC and CBD were expected to have respective anxiogenic and anxiolytic effects in each age group. Although THC produced a strong anxiogenic effect in adults of both sexes, CBD did not produce an anxiolytic effect (**Figures 2**, **3**). The anxiogenic effect was milder in adolescents, with only significant effects seen in the open field metric. However, adolescent control mice demonstrated more anxiogenic activity in the EPM than adults, suggesting that a higher dose of THC may be required to significantly increase anxiety-like behavior in this assay. The 10 mg/kg dose of THC produced a decrease in locomotor activity in all mice, including an insignificant decrease of 31.8% in adolescent males, suggesting that it was pharmacologically active in all groups. Significant reductions in locomotor activity were only correlated with anxiety-like activity in adult males that received 5 mg/kg THC. Interestingly, this group did not show significant changes in anxiety-like activity versus their vehicle counterparts. However, there were no significant correlations between locomotor activity and the increases in anxiety-like activity seen in mice administered 10 mg/kg THC (**Figure 2**). This indicates that, in this study, activity levels did not directly contribute to changes in anxiety-like activity.

Effects of Cannabinoids on Anxiety-Like and Locomotive Behavior

Although the acute effects of THC and CBD were tested in Experiment 1 to inform drug doses for Experiments 2 and 3, mice in Experiment 2 were also tested for acute effects of cannabinoids on EPM and open field activity to include the combination of THC+CBD (**Figure 5**). Notably, the only significant anxiogenic response on the EPM in Experiment 2 was in adult males administered THC+CBD. This is in opposition to Experiment 1, in which THC alone produced an anxiogenic profile (**Figure 2**). However, the anxiogenic profile of THC was still present in adult males and females in the open field (**Figure 5**).

Two major differences exist between procedures in Experiments 1 and 2. Mice were single-housed in Experiment 1 as in our previous work (Kasten et al., 2017), but pair-housing was used in Experiment 2 to avoid potential confounds of long-term isolation housing on EPM activity and object recognition (Hueston et al., 2017; Lin et al., 2018). Secondly, Experiment 1 only observed activity in the EPM and open field following one drug injection. In Experiment 2, EPM and open field followed the NOR task. Therefore, mice received their second drug administration prior to EPM and open field in Experiment 2 (**Table 2**). Development of rapid tolerance to cannabinoids and/or influence of housing may have contributed to these differential findings. It has been previously demonstrated that strong anxiogenic responses in mice on the EPM following an initial acute injection are no longer present on the 5th day of injections (Onaivi et al., 1990). However, our previous work has demonstrated that single-housed adult male mice have a persistent anxiogenic response in the EPM following a second injection with 10 mg/kg THC (Kasten et al., 2017). Although short-term isolation housing in B6 mice does not alter activity in the EPM alone (Lin et al., 2018), the combination of isolation housing with injection stress and drug exposure may potentiate anxiety-like activity. Examining EPM behavior following the first injection of THC+CBD may have revealed an anxiogenic response in more groups, and this response may have been synergistically greater than THC alone, similar to the center

field activity in adult females. The percent of time spent in the center of the open field results more closely resemble the anxiogenic effect in adults on the EPM following one dose of THC (**Figure 2**). While it is tempting to assert that anxiogenic activity should be consistent between the EPM and center metrics in the open field, a recent meta-analysis by Mohammad et al. (2016) indicates that these two tasks do not reliably reproduce one another, and should not be interpreted as reflecting the same behavioral motivation.

Although the anxiogenic effects of THC in the EPM were attenuated following a second THC administration, locomotor effects persisted (**Figure 5**). THC reduced total locomotion in all groups, whereas THC+CBD reduced total locomotion in all groups but adolescent females. Similar to the dose-response results, overall changes in activity levels had a tenuous relationship with anxiety-like activity. Although reductions in activity were correlated with increased anxiety-like activity under some instances, this relationship was only present in males for percent of time spent in the center of the open field, but not open arm entries in the EPM. As adolescent males did not demonstrate a locomotor depressant effect following one dose of THC (**Figure 2**), this may indicate that locomotor depression may develop over repeated THC injections. These results, paired with those of the EPM, may indicate both tolerance and increased sensitivity (behavioral sensitization) to repeated THC injections in different behavioral assays in the same mice. Support for these opposing processes has been previously reviewed by Pertwee (2008). Due to the regional differences in density, location, and coupling efficiencies of CBRs, repeated cannabinoid administration may reduce CB1R density and coupling efficiency at a different rate across brain regions. Therefore, rapid tolerance may develop for some, but not all *in vivo* effects of cannabinoids (Pertwee, 2008).

Long-lasting effects of cannabinoid exposure were minimal. One primary concern is that these tests may be susceptible to one-trial tolerance, which is particularly notable in the EPM (Walf and Frye, 2007). Although there was a 6-week period between the acute and long-term tasks, the time spent in the open arms of the EPM in the vehicle groups at the long-term time point was approximately 1/3rd of their open-arm time at the acute time-point, potentially contributing to a floor effect. Total locomotion in the open field and percent of time spent in the center of the open field were relatively resilient to effects of repeated exposures (**Figures 5**, **7**). Interestingly, an adult history of CBD resulted in anxiogenic activity in females, whereas an adolescent history of THC in females increased the number of open arm entries. However, this change in arm entries did not translate to more time spent in the open arms (**Figure 7**). Our previous work in single-housed mice also demonstrated minimal long-lasting effects of THC exposure, only finding that repeated exposure in males during adulthood lead to significantly greater percentage of total locomotion in the center of the open field (Kasten et al., 2017). This anxiolytic phenotype is in direct opposition to the anxiogenic phenotype demonstrated by Demuyser et al. (2016) using B6 mice from Charles River France that were pair-housed further into adulthood. As a whole, current work in the field suggests a tenuous relationship between cannabinoids, anxiety, and single-housing (Võikar et al., 2005; Lopez and Laber, 2015; Demuyser et al., 2016; Kasten et al., 2017; Lin et al., 2018). However, the disparate findings in the current study highlight the importance of standardizing housing conditions across studies.

Effects of Cannabinoids on Object Recognition Memory

Previous studies using a range of THC doses have demonstrated an acute effect on object recognition memory in CD-1 mice (Barbieri et al., 2016; Busquets-Garcia et al., 2018), but not other rodent strains (Ciccocioppo et al., 2002; Long et al., 2010; Swartzwelder et al., 2012; Kasten et al., 2017). Acute effects of CBD or THC+CBD have not been reported. As hypothesized, all mice but adolescent males significantly discriminated the novel object when injected with vehicle post-training (**Figure 4**). These objects were specifically chosen for their ability to produce significant discrimination under naïve conditions (**Figure 1**), suggesting that a single injection produces similar deficits in object recognition as restraint stress following the object training session in adolescent males (Kim et al., 2018). Interestingly, THC administration trended toward rescuing the injection effect in adolescent male mice and the CBD group also significantly discriminated, whereas adult male mice only showed significant object discrimination following the vehicle injection (**Figure 4A**). Females did not display a similar stark age-effect of injection or cannabinoid action as the males (**Figure 4B**). A shorter inter-trial interval may have produced more consistent and significant acute cannabinoid effects in the NOR task (Barbieri et al., 2016; Busquets-Garcia et al., 2018). Although it has been suggested that more time spent with the objects during training may indicate better performance in the test session (Cohen and Stackman, 2015), we found no consistent evidence supporting this relationship when acute cannabinoids were administered following the training session.

The effects of cannabinoid history were tested 23 days following the last of eight injections. Based on prior research in rats and mice, it was hypothesized that mice with an adolescent history of THC would show impaired object recognition (Quinn et al., 2008; Realini et al., 2011; Zamberletti et al., 2012; Kasten et al., 2017; Kevin et al., 2017; Murphy et al., 2017), and that addition of CBD to THC would rescue this deficit (Fagherazzi et al., 2012; Cadoni et al., 2013; Campos et al., 2015; Gomes et al., 2015). Our hypothesis was not supported. Males and females treated with THC during adolescence significantly discriminated the novel object following a period of drug removal (**Figure 6**). Although six injections over the same age period were sufficient to impair object recognition memory in our previous study (Kasten et al., 2017), the use of pair housing may reduce susceptibility to THC's impairing effects (Võikar et al., 2005). A shorter inter-trial interval between training and testing or a more frequent or increasing dosing regimen over the same age period may have produced the previously seen deficits, such as the every-day dosing paradigm over at least 10 days (Quinn et al., 2008; Realini et al., 2011; Zamberletti et al., 2012; Murphy et al., 2017; Rodríguez et al., 2017).

A few studies have used adult controls to observe whether the effects of THC treatment on object recognition memory are specific to adolescent administration. O'Tuathaigh found no effect of THC history at either age, Quinn et al. (2008) and Murphy et al. (2017) found no effect of adult THC treatment on later object recognition memory, whereas our previous findings demonstrated that an adult history of THC rescued a significant impairment in object recognition memory seen in vehicle-treated male mice (Kasten et al., 2017). However, the current study found no major differences between treatment groups in adult-treated males (**Figure 6A**). Conversely, the adult-treated females showed a step-wise response to cannabinoid treatment, with the vehicle group showing very strong object discrimination (**Figure 6B**). The females that received THC+CBD during adulthood demonstrated significantly impaired object discrimination compared to the vehicle group. Further, the THC and THC+CBD adult-treated females had training investigation times that were significantly positively correlated with discrimination index, indicating that increased exploration during training facilitated object recognition memory in the test session and that previous THC exposure in this group may require more cognitive effort to successfully complete a task. This interpretation is supported by findings in the human visual paired-comparison task, which indicate that impaired visual recognition in high-risk infants can be bolstered by increasing the length of time to familiarize with an object (Burbacher and Grant, 2012).

Western Blots

The current study used Western blotting to identify protein expression of CB1R, IL-1R1, and 5HT1a following cannabinoid history in the hippocampus. Although the hippocampus is necessary for the current NOR design (Cohen and Stackman, 2015) and shows high levels of THC metabolism (Leishman et al., 2018), no significant effects were found in protein levels when examining homogenized whole hippocampal tissue. Due to the changes in CB1R expression over development (Rodríguez de Fonseca et al., 1993; Romero et al., 1997; Verdurand et al., 2011; Lee and Gorzalka, 2012) paired with changes in density following repeated cannabinoid administration (Pertwee, 2008), the lack of persistent change in CB1R expression levels was surprising. However, samples were taken approximately a month following the final cannabinoid treatment. Samples gathered closer to the completion of cannabinoid injections may have revealed changes in protein expression that begin to normalize at 1 month post-treatment. Further, overall protein expression may not consistently reflect changes seen regionally within the hippocampus, at the cellular level (synaptic versus extrasynaptic, receptor internalization), or functional changes in existing receptors.

5HT1aR and IL1-R1 were chosen as secondary targets due to the ability of CBD to influence behavior via these receptors (e.g., Russo et al., 2005; Campos et al., 2012) and the role of inflammatory shifts and interleukin-1 in hippocampal-dependent memory (Goshen et al., 2007; Hueston et al., 2017). Although no changes were found in western blot levels, the role of 5HT1a receptors in the NOR-impairment seen in adult-treated females is of particular interest due to the relationship between changes in estrogen and the 5HT1a receptor system that result from repeated stress exposure, such as chronic injections. Stressors reduce estrogen release in fully developed females, potentially resulting in a shift toward more hetero- and less post-synaptic 5HT1a receptors being expressed at raphe nucleus → hippocampal synapses (Toufexis et al., 2014). Increased heteroreceptor activation at the raphe nucleus results in suppression of serotonin transmission (Glikmann-Johnston et al., 2015), which is critical for object recognition (Busquets-Garcia et al., 2016). The combination of THC with CBD may increase the time of action of CBD at 5HT1a receptors (Stout and Cimino, 2014), resulting in long-term impairment of hippocampal memory development in sexually developed females that were administered THC+CBD. Conversely, the adolescent brain may be undergoing rapid developments in this system, which makes it less susceptible to long-term consequences of repeated exposure. The role of 5HT1a receptors in this phenomenon could be investigated using pharmacological or neurochemical approaches including WAY-100,135 co-administration, conditional receptor knockdown, electrophysiology, and *in situ* hybridization.

CONCLUSION

The current studies examined age- and sex-effects of cannabinoid administration on acute and long-term behaviors. Although many significant acute actions of cannabinoids were observed, there were minimal long-term effects associated with repeated drug administration across age and sex. Contrary to our initial hypotheses, acute administration of THC+CBD resulted in behavioral deficits, potentially due to the ability of administration of two or more cannabinoids to prolong metabolism and drug availability (Klein et al., 2011; Stout and Cimino, 2014; Murphy et al., 2017). THC+CBD administration also resulted in long-lasting effect of cannabinoids, wherein females repeatedly treated in adulthood demonstrated impaired object recognition memory. Although CBD is generally considered to be a safe, non-intoxicating therapeutic (e.g., Leweke et al., 2012; Englund et al., 2013, 2017), recent studies in humans have indicated that CBD alone may produce intoxicating effects and enhance psychotic symptoms dependent upon individual cannabinoid history (Morgan et al., 2018; Solowij et al., 2019). The current results indicate that females may have a different sensitivity to CBD, potentially due to its actions at 5HT1a receptors. In females, stress, hormones, and 5HT1a activation may be more likely to contribute to negative outcomes of cannabinoid usage, such as impaired cognition or increases in susceptibility for major depression (see Grigoriadis and Robinson, 2007; Martin et al., 2009).

The findings that THC+CBD resulted in increased impairment were in conflict with the hypotheses that combining THC+CBD would result in reduced impairment. Concerning medical and recreational use, this may indicate that higher concentrations of CBD with lower concentrations of THC serve to extend moderate and beneficial effects of THC administration. However, at a higher ratio, such as the 1:2 ratio used in the

current studies, CBD may enhance and prolong the negative effects of THC use. A range of THC:CBD ratios, including the commercially popular 2:1 ratio or the medically popular 1:1 ratio (Jikomes and Zoorob, 2018), should be investigated to fully understand how their pharmacological interaction affects behavior.

There were minimal long-lasting effects of cannabinoid injections, suggesting that both male and female mice demonstrate a relative robustness against cannabinoid use at both adolescent and adult time points. This study alone may indicate that cannabinoids are more suitable for long-term medical treatment and may be more appropriate as an intervention for diseases that occur during childhood. However, only eight injections were given in the current study, and the adolescent treatment regimen ended at PND45. PND45 is roughly equivalent to 18 years of age in humans (Lee and Gorzalka, 2012), which is the same period of age when self-reports of past-month cannabis use nearly triples (Azofeifa et al., 2016). Previous studies using escalating THC doses over the same age period in adolescent rats have demonstrated long-term deficits in object recognition, indicating that the dosing regimen may also play a large role in these findings.

The choice of behaviors used in the current studies must be considered. A recent review by the National Academies of Sciences (2017) reported that there is moderate evidence of cognitive impairment following acute cannabinoid use and limited evidence of long-lasting cognitive impairment following abstinence. There is also limited evidence of a relationship between development of non-social anxiety disorders and cannabis use, although anxiety-like and sedative responses should be monitored. Although the current behaviors were chosen based on previous literature and findings in our own lab which suggested that cannabinoid treatment results in deficits in object recognition memory and unconditioned anxiety, it is possible that the role cannabinoid use plays in these impairments is more limited than initially expected. The use of preclinical behavioral assays that are analogs to the conditions that the National Academies of Sciences have more strongly associated with cannabinoid use - such as development of other substance use disorders, social anxiety, depressive symptomology, and psychoses – may reveal more effects than the behavioral assays chosen herein. Therefore, the current studies may not represent the trajectory of behavioral outcomes following actual medical or recreational cannabinoid usage.

AUTHOR CONTRIBUTIONS

CK and SB were responsible for the study concept, design, and interpretation of the findings. CK and YZ were responsible for acquisition and analysis of the data. CK was responsible for the first draft of the manuscript. CK, YZ, and SB contributed to critical review and approval of the version for publication.

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The Cannabinoid CB1 Antagonist TM38837 With Limited Penetrance to the Brain Shows Reduced Fear-Promoting Effects in Mice

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Rimonabant was the first selective CB1 antagonist/inverse agonist introduced into clinical practice to treat obesity and metabolic-related disorders. It was withdrawn from market due to the notably increased rates of psychiatric side effects. We have evaluated TM38837, a novel, largely peripherally restricted CB1 antagonist, in terms of fear-promoting consequences of systemic vs. intracerebral injections. Different groups of male C57BL/6 N mice underwent auditory fear conditioning, followed by re-exposure to the tone. Mice were treated *per os* (p.o.) with TM38837 (10, 30, or 100 mg/kg), rimonabant (10 mg/kg; a brain penetrating CB1 antagonist/inverse agonist which served as a positive control), or vehicle, 2 h prior the tone presentation. Only the high dose of TM38837 (100 mg/kg) induced a significant increase in freezing behavior, similar to that induced by rimonabant (10 mg/kg) ($p < 0.001$). If injected into the brain both TM38837 (10 or 30 μg/mouse) and rimonabant (1 or 10 μg/mouse) caused a sustained fear response to the tone, which was more pronounced after rimonabant treatment. Taken together, TM38837 was at least one order of magnitude less effective in promoting fear responses than rimonabant. Given the equipotency of the two CB1 antagonists with regard to weight loss and metabolic syndrome-like symptoms in rodent obesity models, our results point to a critical dose range in which TM3887 might be beneficial for indications such as obesity and metabolic disorders with limited risk of fear-promoting effects.

Keywords: cannabinoid CB1 receptor, rimonabant, peripheral CB1 receptor antagonist, TM38837, fear conditioning

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INTRODUCTION

Based on the animal and clinical studies showing that a pathological overactivation of the endocannabinoid transmission through the cannabinoid CB1 receptor contributes to obesity (for review, see Di Marzo et al., 2011), the CB1 antagonist/inverse agonist rimonabant (SR141716A, Rinaldi-Carmona et al., 1994) was the first compound introduced into clinical practice as an antiobesity agent in several countries (Rimonabant in Obesity: RIO studies) (Christopoulou and Kiortsis, 2011). However, enthusiasm for such agent has waned as a result of the withdrawal

from the market due to the higher incidence in treated patients as compared to placebo controls of psychiatric side effects such as mood symptoms, anxiety, and suicidal tendencies (Van Gaal et al., 2005; Christensen et al., 2007). Despite this experience, there is still interest in the development of CB1 antagonism as a pharmacological tool for the treatment of metabolic disorders, however, with a better safety profile (Le Foll et al., 2009; Janero et al., 2011; Ward and Raffa, 2011; Kirilly et al., 2012). In this context, two main alternatives are currently discussed: (1) the use of CB1 neutral antagonist, such as AM4113, NESS0327, or AM6545, instead of the CB1 receptor antagonists/inverse agonists (e.g., rimonabant), which have recently shown efficacy to reduce body weight and food intake in rodents with less unwanted side effects than rimonabant (Sink et al., 2008, 2010a,b; Meye et al., 2013; Gueye et al., 2016) and (2) the use of peripherally directed CB1 inverse agonist/antagonist, which revealed promising preclinical results to reduce body weight (Tam et al., 2012, 2018; Chorvat, 2013; Sharma et al., 2018). Among them, TM38837 was shown to induce a significant weight loss in obese mice similarly to rimonabant (Noerregaard et al., 2010), with no clear central nervous system (CNS) effects and a potential favorable side effects profile (Klumpers et al., 2013), possibly because of reduced brain CB1 receptor occupancy (Takano et al., 2014). Since previous data have consistently shown that genetic or pharmacological blockade of intracerebral CB1 receptors leads to sustained conditioned fear in rodents (for reviews see Riebe et al., 2012; Micale et al., 2013), a CB1 antagonist with confined actions in the periphery is expected to preserve its beneficial functions on several aspects of the metabolic syndrome, without exerting its psychopathological side effects. Thus, the peripheral-restricted CB1 antagonist TM38837 provides a very interesting tool to answer this question.

Based on the above premises, this study was undertaken to assess the effects of systemic (*per os* – *p.o.*) and local (*intracerebroventricular* – *icv*) treatment with the cannabinoid CB1 antagonist TM38837 on expression of conditioned fear in mice. If CB1 controls fear adaptation primarily *via* cortical glutamatergic neurons (Kamprath et al., 2009), it is expected that a CB1 antagonist (such as rimonabant) used here as a positive control with free access to the brain will inhibit fear adaptation (Marsicano et al., 2002; Plendl and Wotjak, 2010; Höfelmann et al., 2013). Systemic administration of an antagonist with restricted access to the brain such as the CB1 antagonist TM38837, in contrast, is expected to leave fear adaptation largely unaffected.

MATERIALS AND METHODS

Animals

Male C57BL/6 N (B6N, 7–8 weeks old, purchased from Charles River) mice (n = 9–13 per group) were single housed in type 2 Macrolon cages and maintained in standard conditions with food and water *ad libitum* under a 12 h inversed light-dark cycle (lights off at 9 a.m.) for at least 14 days before starting the experiments. All behavioral experiments were performed between 9:30 a.m. and 5 p.m. (i.e., during the active phase of the animals). Note that the final sample size used for analysis was smaller due to heavy fighting in the home cage before separation, failures to cope with p.o. treatment by gavage (irrespective of the compounds), or escape during the experiment. All behavioral tests took place in an experimental room with the same light-dark cycle and environmental conditions (i.e., humidity, temperature) as in the housing facility. All experimental procedures were approved by the Government of Upper Bavaria (55.2.1.54-2532-44-09; 55.2.1.54-2532-141-12). All experiments were carried out according to the European Community Council Directive 2010/63/EEC, and efforts have been made to minimize animal suffering and reduce the number of animals used.

Behavioral Procedure

Fear Conditioning

The set-up has been described and displayed in detail before (Kamprath and Wotjak, 2004; Direnberger et al., 2012; Yen et al., 2012; Llorente-Berzal et al., 2015). Briefly, mice were placed in the conditioning context (chamber) (d0). Three minutes later, a tone (80 dB, 9 kHz sine wave, 10 ms rising and falling time) was presented to the animals for 20 s that coterminated with a 2-s scrambled electric foot shock of 0.7 mA. Mice were returned to their home cages 60 s later.

Tone Re-exposure

Mice were placed in test context, which differed from the conditioning context in material, shape, surface texture, and odor of the cleaning solution (cylinder; Kamprath and Wotjak, 2004). After an initial 3 min of habituation, mice were confronted with a permanent 180 s tone (80 dB, 9 kHz, sine wave). Mice were returned to their home cage 60 s after the end of the exposure protocol. Tone re-exposure was started 24 h after conditioning and performed at d1, d2, d3, and d10 after the conditioning (Plendl and Wotjak, 2010; Llorente-Berzal et al., 2015).

Behavioral Analysis

The behavior of the mice was videotaped and scored off-line by a trained observer who was blind to the animals' treatment by typing preset keys on a keyboard (EVENTLOG, Robert Henderson, 1986). Freezing was defined as the absence of all movements, except for those related to respiration.

Drugs and Experimental Design

Two different experiments were performed. In *Experiment 1*, rimonabant (Kd~0.61 nM; Rinaldi-Carmona et al., 1996) (RIM: 10 mg/kg, National Institute of Mental Health Chemical Synthesis and Drug Supply Program), TM38837 (Kd~16 nM; Noerregaard et al., 2010) (10, 30, or 100 mg/kg, 7TM PHARMA), or vehicle (VHC: 0.1% Tween 80 and 1% hydroxypropyl methylcellulose, Sigma) were administered *per os* (p.o.) in a volume of 5 ml/kg, 2 h prior the tone re-exposure (days 1–3). On day 10, all the mice were treated with vehicle (VHC) 2 h prior the exposure to a 3-min tone. The dose of rimonabant (10 mg/kg) was selected

based on a dose-response curves experiment, where an additional group of mice was treated with rimonabant (RIM 3 mg/kg, s.c.) as a positive control (Plendl and Wotjak, 2010; Terzian et al., 2014), 1 h prior to exposure to the 3-min tone. On day 11, four groups of mice (n = 5–6 per group) were treated with TM38837 (TM: 10, 30 or 100 mg/kg, p.o.) or rimonabant (RIM: 10 mg/kg, p.o.) and, 2 h later, were decapitated after short isoflurane anesthesia, and trunk blood was collected in pre-chilled EDTA tubes (KABE, Nümbrecht-Elsenroth, Germany). The samples were centrifuged at 1500 g for 10 min at 4°C. The entire resultant plasma obtained was transferred to suitably labeled polypropylene tubes and stored upright at −20°C for subsequent measurement of plasma levels.

In ***Experiment 2***, rimonabant and TM38837 were dissolved in vehicle solution (10% DMSO and 10% Cremophor EL in saline) (Sigma). The compounds were administered intracerebroventricularly (icv) in a volume of 2.0 µl/mouse. Different groups of mice were treated icv with TM38837 (TM: 10 or 30 µg/mouse), rimonabant (RIM: 1 µg/mouse), or vehicle (VHC) 30 min prior to re-exposure to the tone (days 1–3). On day 10, all mice were treated with vehicle (VHC) 30 min prior the tone re-exposure. The dose of rimonabant (1 µg/mouse) was selected on the basis of a dose-response experiment. For all groups, injections were given under light isoflurane (Forene®; Abbott, Wiesbaden, Germany) anesthesia to avoid differences in coping with the stressful injection procedure. The injection cannula protruded the guide cannula by 1 mm.

Surgery

Following preoperative analgesia with Meloxicam (Metacam®, Boehringer-Ingelheim, Ingelheim, Germany; 0.5 mg/kg in 0.9% saline, s.c.), mice were deeply anesthetized with isoflurane and fixed to a stereotaxic frame (TSE-Systems, Heidelberg, Germany). Body temperature was kept constant at 36°C by a feedback-controlled heating pad. Two holes were drilled into the skull in order to insert an anchoring screw and a guide cannulae (manufactured from injection cannulae, 23 G; Braun-Melsungen, Melsungen, Germany). The guide cannula was implanted as follows (0.3 mm posterior to Bregma, 1.0 mm laterally from midline, 1.2 mm beneath the surface of the skull). Fixation was achieved with dental cement (Dual Cement; Ivoclar, Schaan, Liechtenstein). The wound was disinfected with Braunoderm® and closed with sutures. Post surgery, mice received Meloxicam (0.5 mg/kg i.p.) for 3 days and were allowed to recover for 10–14 days before the experiment. The recovery process was monitored daily by visual inspection. The injection cannula extended the guide cannula by 1 mm, thus reaching into the lateral ventricle.

Analysis of Cannula Placement

At completion of behavioral testing, all mice of Experiment 2 were anesthetized with a ketamine/xylazine mixture and injected with 1.0 µl of Cresyl Violet icv in order to verify the injection sites. Brains were removed 20 min later. Histological examinations revealed particles of the ink in the lateral and third ventricles, but not in the brain parenchyma. We have used only data obtained from mice exhibiting a correct insertion at histological examination.

Statistical Analysis

Freezing behavior was analyzed in 20-s interval or averaged over the entire tone presentation (180 s) and expressed as a percentage of the respective analysis interval. Data were analyzed using one-way ANOVA (total freezing) or 2-way ANOVA for repeated measures (development of the freezing response over the course of tone presentation) by means of SPSS 17.0 (Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). Newman-Keuls test was used as post-hoc test, if appropriate. Data are presented as mean ± SEM. Statistical significance was accepted if $p < 0.05$.

RESULTS

This study was based on the comparison of fear-promoting effects of CB1 receptor antagonists with limited (TM38837 – TM) vs. unrestricted (rimonabant – RIM) penetrance into the brain.

Experiment 1: Systemic Antagonist Treatment

Before starting with TM38837 (TM) treatment, we defined the dose of rimonabant (RIM), which causes sustained fear upon *per os* (*p.o.*) treatment compared to subcutaneous (s.c.) treatment as positive control (Experiment 1). Mice underwent auditory fear conditioning (day 0) followed by random assignment to one out of four groups (RIM 3 mg/kg s.c., VHC p.o., RIM 3 mg/kg p.o., or RIM 10 mg/kg p.o.). Mice were treated on three consecutive days 2 h (p.o.) or 1 h (s.c.) before re-exposure to the conditioned tone for 3 min. Analysis of the total freezing responses to the tone shown at the three consecutive days revealed that 10 mg/kg p.o. and 3 mg/kg s.c., but not 3 mg/kg RIM p.o., caused increased fear, compared to vehicle-treated controls, with 10 mg/kg being most effective [Treatment: $F_{3,43} = 22.08$, $p < 0.0001$; Treatment × Day: $F_{6,86} = 1.845$, $p = 0.099$; 2-way ANOVA (treatment, day) for repeated measures (day); **Figure 1**]. If analyzed in 20-s interval, all mice showed the same initial freezing response at day 1. However, whereas mice treated with vehicle or 3 mg/kg RIM p.o. showed a rapidly waning freezing response, treatment with 3 mg/kg s.c. (= positive control) and 10 mg/kg p.o. led to sustained fear (Treatment: $F_{3,43} = 15.55$, $p < 0.0001$; Treatment × Time: $F_{24,344} = 3.533$, $p < 0.0001$). Treatment at days 2 (Treatment: $F_{3,43} = 20.92$, $p < 0.0001$; Treatment × Time: $F_{24,344} = 1.795$, $p < 0.05$) and 3 (Treatment: $F_{3,43} = 8.584$, $p < 0.0005$; Treatment × Time: $F_{24,344} = 1.502$, $p = 0.06$) revealed essentially the same effects except for the increase in initial freezing observed in mice treated with RIM 10 mg/kg p.o. (**Figure 1**).

On basis of this dose-response study, we selected 10 mg/kg RIM p.o. as the reference dose for Experiment 2. New cohorts of mice underwent auditory fear conditioning (day 0) followed

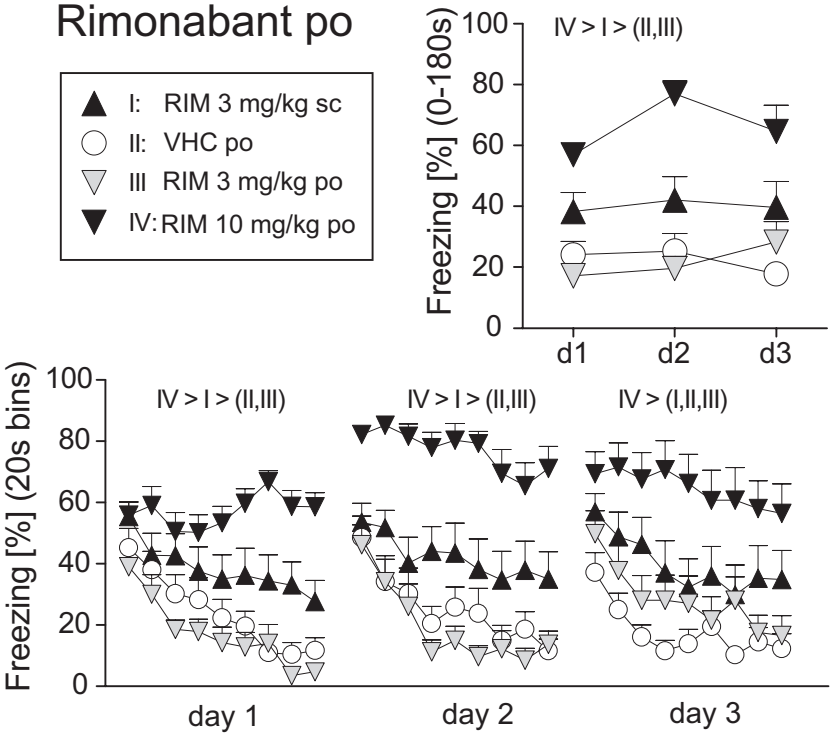


FIGURE 1 | Effects of systemic rimonabant treatment on the expression of auditory-cued fear memory. Freezing responses (mean ± S.E.M.) of the animals after *per os* (RIM: 0, 3, or 10 mg/kg) or *subcutaneous* rimonabant (RIM: 3 mg/kg) treatment before re-exposure to the 3 min tone at days 1–3 after conditioning (day 0) averaged over the entire 180 s tone presentation (upper right) or in 20-s interval (lower panel). *n* = 11–13 mice per group. Data were analyzed by two-way ANOVAs for repeated measurements. Newman-Keuls post-hoc comparisons for the main effects *Treatment* are indicated above the graphs. For the matter of clarity, we do not report post-hoc results in case of factorial interactions.

by re-exposure to the conditioned tone at days 1, 2, 3, and 10. At days 1–3, mice were treated with VHC, RIM 10 mg/kg (positive control), or TM 10, 30, or 100 mg/kg p.o. 2 h before tone presentation; at day 10, all mice received vehicle. Analysis of the total freezing responses revealed that 10 mg/kg RIM and 100 mg/kg TM caused a significant increase in conditioned fear, whereas 30 and 10 mg/kg TM were indistinguishable from vehicle treatment. These treatment effects were abolished at day 10 when all mice received vehicle (Treatment × Day: $F_{12,120} = 8.726$; $p < 0.0001$; **Figure 2**). Analysis in 20 s bins separately for days 1–3 confirmed the sustained freezing responses in mice treated with 100 mg/kg TM p.o. or 10 mg/kg RIM p.o., as compared to the other groups (Treatment: $F_{4,40} > 6.454$, $p < 0.0005$; Treatment × Time: $F_{32,320} = 1.649$, $p < 0.05$; **Figure 2**). Importantly, in no case, there were significant differences between mice treated with vehicle and TM 10 or TM 30 mg/kg. Also, drug treatment had no general effects on exploratory behavior, as exemplarily assessed by measuring freezing/immobility during the 20 s preceding the first tone presentation at day 1 (VHC: 18.1 ± 4.1%, RIM10: 17.8 ± 3.2%, TM10: 20.3 ± 3.8%, TM30: 20.3 ± 4.2%, TM100: 17.5 ± 3.8%). The plasma drug concentrations (mean ± SEM) 2 h post treatment were as follows: RIM 10 mg/kg = 139 ± 12 nM; TM 10mg/kg = 9,955 ± 1,325 nM; TM 30 mg/kg 113,574 ± 14,129 nM; TM 100 mg/kg = 178,479 ± 11,977 nM.

Experiment 2: Intracerebral Antagonist Treatment

In order to include RIM as a positive control for intracerebral TM treatment, we first assessed the efficacy of different doses of RIM (1 vs. 10 μg) administered intracerebroventricularly (icv) on the expression of auditory-cued fear memory at days 1–3 after conditioning (day 0). Analysis of the total freezing responses revealed increased freezing following treatment with 1 and 10 μg RIM compared to vehicle controls, with no differences between the two doses (Treatment: $F_{2,31} = 19.98$, $p < 0.0001$; Treatment × Day: $F_{4,62} = 1.10$, $p = 0.3625$; **Figure 3**). If analyzed in 20-s interval, mice treated with vehicle showed a rapidly waning freezing response, whereas icv treatment with 1 or 10 μg lead to sustained fear. This became evident at all three experimental days (Treatment × Time: $F_{16,248} > 1.8350$, $p < 0.05$; **Figure 3**).

On basis of this dose-response study, we selected 1 μg RIM as the reference dose for comparisons with icv TM (10 or 30 μg). New groups of mice underwent surgery and fear conditioning and were treated before expression of auditory-cued fear memory at days 1–3 after conditioning (day 0); at day 10, all mice received vehicle. Analysis of the total freezing responses revealed a significant Treatment × Day interaction ($F_{9,108} = 4.086$, $p < 0.0005$). Post-hoc analyses confirmed that vehicle-treated controls froze significantly less than all other treatment groups at days 1–3, but not day 10. In addition, mice treated with 10

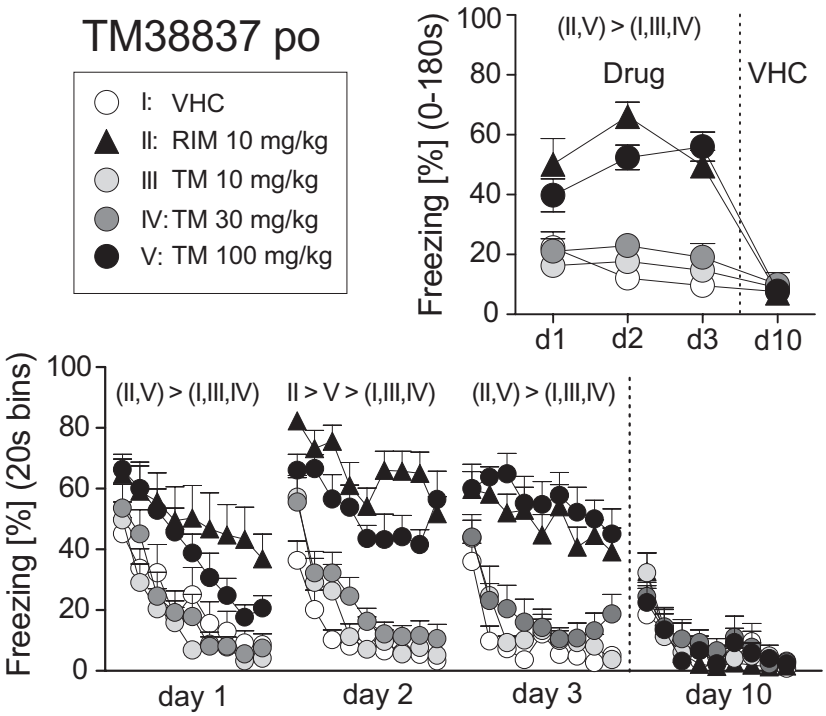


FIGURE 2 | Effects of systemic treatment with the peripheral-restricted CB1 antagonist TM38837 on the expression of auditory-cued fear memory. Freezing responses (mean ± S.E.M.) of the animals after *per os* treatment with TM38837 (TM: 10, 30 or 100 mg/kg), rimonabant (RIM: 10 mg/kg), or vehicle (VHC) before re-exposure to the 3 min tone at days 1–3 after conditioning (day 0) averaged over the entire 180 s tone presentation (upper right) or in 20-s interval (lower panel). *n* = 9 mice per group. For further details, see **Figure 1**.

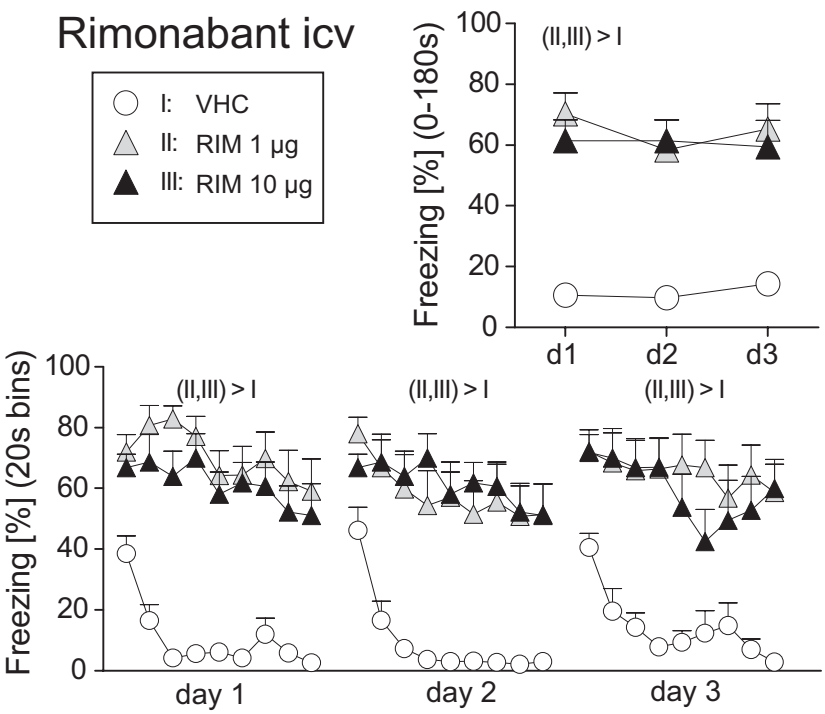


FIGURE 3 | Effects of intracerebroventricular rimonabant treatment on fear memory in mice. Freezing responses (mean ± S.E.M.) of the animals after intracerebroventricular (icv) rimonabant (RIM: 1 or 10 µg/mouse) or vehicle (VHC) treatment averaged over the entire 180s tone presentation (upper right) or in 20s intervals (lower panel). *n* = 11-12 mice per group. For further details see **Figure 1**.

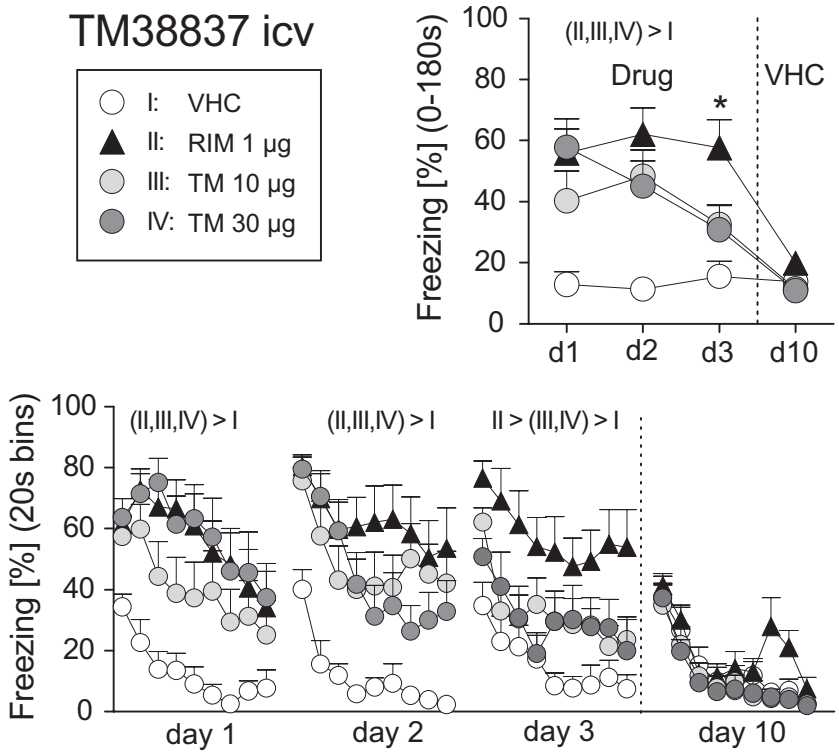


FIGURE 4 | Effects of intracerebroventricular treatment with the peripheral restricted CB1 antagonist TM38837 on the expression of auditory-cued fear memory. Freezing responses (mean ± S.E.M.) of the animals after *intracerebroventricular* treatment with TM38837 (TM: 10 or 30 µg/mouse), rimonabant (RIM: 1 µg/mouse) or vehicle (VHC) averaged over the entire 180s tone presentation (upper right) or in 20s intervals (lower panel). n = 10 mice per group. *p < 0.05 vs. I, III and IV. For further details see **Figure 1**.

or 30 µg TM showed significantly less freezing than mice treated with 1 µg RIM at day 3 (**Figure 4**). These findings were confirmed if we compared the development of freezing over the course of the tone presentation separately for days 1–3 (Treatment: $F_{3,36} > 5.588$, $p < 0.005$; **Figure 4**).

DISCUSSION

In the present study, we demonstrate that the peripherally restricted CB1 antagonist TM38837 elicited fear-promoting effects following systemic treatment only at a dose 10 times higher than rimonabant. More specifically, the dose of 100 mg/kg p.o. appeared to be as potent as rimonabant (10 mg/kg, p.o.) to induce a sustained fear response upon recall of auditory-cued fear memory. Mice treated with lower doses of TM38837 (10 and 30 mg/kg, p.o.) were indistinguishable from the vehicle-treated control group. This observation is in accordance with the negligible access of TM38837 to the brain at therapeutic effective doses in mice (Noerregaard et al., 2010), the low brain CB1 receptor occupancy in nonhuman primates (Takano et al., 2014), and the approx. 10 times stronger rimonabant affinity to the CB1 receptors as compared to TM38837. Interestingly, TM38837 exerts its beneficial effects in animal models of metabolic diseases at similar doses as rimonabant (Noerregaard et al., 2010). Furthermore, the higher plasma concentration of TM38837 as compared to rimonabant level could be due as recently described to the low clearance and long terminal half-life of the peripherally restricted CB1 receptor antagonist (Klumpers et al., 2013) and its limited penetrance through biological membranes into different tissues.

TM38837 is belonging to the subclass of peripherally restricted CB1 antagonists. It was developed as a neutral antagonist with highly limited penetrance to the brain in order to minimize or prevent CNS adverse reactions while preserving potential antiobesity effects (Ward and Raffa, 2011; Chorvat, 2013). With the discovery of the endocannabinoid system, blockade of CB1 receptors became a preferred drug target. In line with this strategy, a particular emphasis has been on the antiobesity potential of prototypical selective CB1 receptor antagonist/inverse agonist rimonabant (Rinaldi-Carmona et al., 1994), which was discontinued, however, once its use was associated with psychiatric side effects (Van Gaal et al., 2005; Christensen et al., 2007; Moreira and Crippa, 2009; Christopoulou and Kiortsis, 2011; Micale et al., 2015). Thus, orally bioavailable CB1 receptor antagonists with molecular properties that limit their penetration across the blood-brain barrier and restrict their CNS access may reduce obesity-associated cardiometabolic risk with improved safety over rimonabant (Shrestha et al., 2018). This concept is based on the fact that CB1 receptors at peripheral sites (e.g.,

adipocytes or hepatocytes) could decisively influence energy expenditure and body fat storage/disposition, since visceral fat accumulation has been correlated with peripheral endocannabinoid system hyperactivity in human obesity (Cota et al., 2003; Silvestri et al., 2011; Bellocchio et al., 2013).

Our results confirm that the CB1 receptor antagonist/inverse agonist rimonabant following systemic administration is able to inhibit fear adaptation, further supporting the concept that pharmacological (Marsicano et al., 2002; Kamprath et al., 2006, 2009; Plendl and Wotjak, 2010; Höfelmann et al., 2013; Llorente-Berzal et al., 2015) as well as genetic (Haller et al., 2002; Marsicano et al., 2002; Jacob et al., 2009, 2012; Terzian et al., 2011; Metna-Laurent et al., 2012; Rey et al., 2012; Micale et al., 2017) inactivation of CB1 signaling exerts fear promoting/anxiogenic effects (for review, see Riebe et al., 2012). However, in our study, only the highest dose of TM38837 increased fear response, which was far more than the dose used to ameliorate metabolic symptoms (10 mg/kg; Noerregaard et al., 2010).

To analyze whether the difference in fear expression after systemic treatment could be attributed to the negligible access of TM38837 to the brain as compared to the good brain penetration of rimonabant, we injected the two compounds directly into the brain. Intracerebroventricular administration of both CB1 antagonists increased the fear response, even though rimonabant elicited a more pronounced and prolonged fear response as compared to TM38837. In fact, the effects of TM38837 were observed at higher doses and started to wane upon repeated treatment. This might be ascribed, at least in part, to the lower affinity to CB1 receptors compared to rimonabant (Takano et al., 2014).

In conclusion, TM38837 and rimonabant showed great similarity in their potential therapeutic effects against obesity and metabolic disorders (Noerregaard et al., 2010; Ward and Raffa, 2011; Kirilly et al., 2012; Shrestha et al., 2018). Our findings suggest that they could differ on potentially harmful effects, supporting a favorable prognosis for the absence of adverse side effects in case of chronic systemic treatment with the peripheral CB1 antagonist. Although further preclinical studies and controlled clinical studies are necessary to assess the efficacy and the safety profile of TM38837, these findings correspond well with the alternative approach in the treatment of obesity, which could be represented by the use of peripheral or neutral CB1 antagonists, lacking many of the adverse events associated with CB1 inverse agonist (Shrestha et al., 2018; Tam et al., 2018). Nevertheless, TM3887 is not devoid of fear-promoting effects, even though at 10 times higher concentrations than rimonabant, both after systemic and intracerebral injection.

Limitations of the study: Our study has a number of limitations which have to be considered. First, given the differences in receptor affinity, fear-promoting effects of TM38837 may become evident at higher concentrations (as shown in the present manuscript). In this context, it is of importance to define a low dose of TM38837 treatment, which still exerts its beneficial effects on metabolic syndrome while avoiding adverse effects on fear expression. Interestingly, plasma concentrations of TM38837 were orders of magnitude higher than that of the lipophilic rimonabant, which may favor peripheral effects. Second, our conclusions rely on a single behavioral readout (i.e., expression of conditioned fear), which might be additionally "contaminated" by unspecific effects in particular of rimonabant, even at lower doses (3 mg/kg), on locomotor activity (e.g., Llorente-Berzal et al., 2015). Therefore, future studies have to significantly broaden the number of behavioral measures of "discomfort," including anxiety-related behavior and hormonal stress responses to unequivocally demonstrate the superiority of TM38837 compared to rimonabant in terms of potential side effects on emotional regulation.

AUTHOR CONTRIBUTIONS

VM has designed the study, performed the experiments, analyzed the data, and written the draft of the manuscript. FD has participated and contributed to the experimental design and data interpretation. CE has provided the new compound and discussed the data. CW has designed the study, supervised the experiments, contributed to data analysis, and participated in manuscript preparation. PN has participated in developing the concept and designing parts of the experiments. In addition, she has contributed data about pharmacokinetics and locomotor activity.

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The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Effects of CBD-Enriched *Cannabis sativa* Extract on Autism Spectrum Disorder Symptoms: An Observational Study of 18 Participants Undergoing Compassionate Use

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Autism Spectrum Disorders comprise conditions that may affect cognitive development, motor skills, social interaction, communication, and behavior. This set of functional deficits often results in lack of independence for the diagnosed individuals, and severe distress for patients, families, and caregivers. There is a mounting body of evidence indicating the effectiveness of pure cannabidiol (CBD) and CBD-enriched *Cannabis sativa* extract (CE) for the treatment of autistic symptoms in refractory epilepsy patients. There is also increasing data support for the hypothesis that non-epileptic autism shares underlying etiological mechanisms with epilepsy. Here we report an observational study with a cohort of 18 autistic patients undergoing treatment with compassionate use of standardized CBD-enriched CE (with a CBD to THC ratio of 75/1). Among the 15 patients who adhered to the treatment (10 non-epileptic and five epileptic) only one patient showed lack of improvement in autistic symptoms. Due to adverse effects, three patients discontinued CE use before 1 month. After 6–9 months of treatment, most patients, including epileptic and non-epileptic, showed some level of improvement in more than one of the eight symptom categories evaluated: Attention Deficit/Hyperactivity Disorder; Behavioral Disorders; Motor Deficits; Autonomy Deficits; Communication and Social Interaction Deficits; Cognitive Deficits; Sleep Disorders and Seizures, with very infrequent and mild adverse effects. The strongest improvements were reported for Seizures, Attention Deficit/Hyperactivity Disorder, Sleep Disorders, and Communication and Social Interaction Deficits. This was especially true for the 10 non-epileptic patients, nine of which presented improvement equal to or above 30% in at least one of the eight categories, six presented improvement of 30% or more in at least two categories and four presented improvement equal to or above 30% in at least four symptom categories. Ten out of the 15 patients were using other medicines, and nine of these were

able to keep the improvements even after reducing or withdrawing other medications. The results reported here are very promising and indicate that CBD-enriched CE may ameliorate multiple ASD symptoms even in non-epileptic patients, with substantial increase in life quality for both ASD patients and caretakers.

Keywords: autism spectrum disorders, cannabidiol, epilepsy, *Cannabis sativa*, endocannabinoid system

INTRODUCTION

According to the DSM 5 (2013), Autism Spectrum Disorder (ASD) is characterized by functional deficits in three areas: mental development, social interaction, and behavior (1). In a multicenter epidemiological study done in 2012, involving nine countries, the median estimate of prevalence of ASD was 62/10.000 inhabitants (2). In clinical practice, the term ASD comprises a broad group of syndromes, diseases, and disorders (3, 4), that can affect cognitive development, motor skills, social interaction, communication, and behavior (frequently including auto and hetero-aggressiveness) (5–15). Often, this set of functional deficits results in incapacitation, lack of independence and severe distress for patients, families, and caregivers. For a recent review on this topic, refer to (16).

It is believed that ASD has multifactorial causes, generally associated with chromosomal or epigenetic changes in many different genes, which are often associated with neuronal function (17–24). Currently, there are no drugs or psychotherapeutic approaches capable of comprehensively improving life quality, social skills, and cognitive development of the most severe ASD patients (25–31). Currently available drugs may mitigate some specific symptoms, but generally speaking, they do so with a narrow range of effectiveness, and are often associated with important side effects (32, 33). Antipsychotic, antidepressant, or anxiolytic drugs, for example, may soothe autistic patients who display self-aggressive behavior (33–36). Antiepileptic drugs may be effective for seizure control and may even improve sleep quality and behavioral aspects (37). However, these drugs are known to cause major side effects (38–46). Moreover, none of these drus has been shown to significantly improve the lack of social interaction and communication skills that characterize and impose great impact on the lives of patients with ASD and their families.

Recent observational studies and trials reporting the use of pure CBD or CBD-enriched cannabis extracts for the treatment of syndromes characterized by refractory epilepsy and regressive autism suggest therapeutic potential of cannabinoids for autistic symptoms (47–60). These studies, which include extracts with a CBD/THC ratio of up to 20/1, showed that, even in children and adolescents, the side effects of these extracts are infrequent and less damaging than those reported for drugs traditionally used either for ASD, ADHD, sleep disorders, or epilepsy.

Changes in the expression of peripheral cannabinoid receptors were verified in autistic patients, suggesting possible deficiencies in the production and regulation of endogenous cannabinoids in ASD (61). This hypothesis has been recently confirmed specifically for anandamide, a major endocannabinoid, which is reduced in ASD patients (62). The understanding of the possible mechanisms involving the endocannabinoid system in the etiology of ASD has been derived from basic research in animal models. Special attention has been given to the neuronal hyperexcitability hypothesis and its possible relationship with the endocannabinoid system, which may also explain the higher incidence of epilepsy among ASD patients (63–68). Significant epileptform EEG activity has been recorded even in the central nervous system of non-epileptic autistic children (69), which is consistent with the "intense world hypothesis," that relates autistic symptoms to excessive neuronal activity and connectivity (70). Together, these findings strongly support the need for testing *Cannabis sativa* extracts (CEs) and isolated phytocannabinoids as pharmacological approaches to control severe symptoms in both epileptic and non-epileptic ASD patients (68). Furthermore, CBD has been shown to have anxiolytic (71–75) and antipsychotic effects (76–79) in humans. It is plausible to assume that such effects are, at least in part, mediated by CBD-induced accumulation of the endocannabinoid anandamide (80). Although the mechanisms underlying CBD-induced antiepileptic effects are not entirely clear, anandamide modulation is likely to play an important role (68). In this context, it is interesting to note that anandamide accumulation, caused by inhibitors of its metabolic degradation, leads to reduction of social interaction deficits in the valproate-treated animal model of autism (81).

Here we report an observational study analyzing the effects of the compassionate use of *Cannabis sativa* extract (CE) containing a 75/1 CBD/THC ratio, which was given to a group of 18 ASD patients. The participant group includes 11 patients with no history of epilepsy, two previously diagnosed with epilepsy but seizure-free for over a year, and 5 currently diagnosed with epilepsy who had seizures during the month preceding treatment with CE. Treatment results were assessed by means of monthly questionnaires and clinical evaluation. The results after 6–9 months of treatment were extremely promising for both epileptic and non-epileptic patients. For the latter, observed improvements were much more comprehensive with fewer adverse effects than it would have been expected from currently available therapies. These preliminary results indicate, therefore, the urgent need for more extensive and detailed clinical studies to further validate the use of ECs and cannabinoids for the treatment of severe ASD symptoms.

TABLE 1 | Cohort description and individual dosage* of phytocannabinoids prescribed.

Case #	Age (years)	Weight (kg)	CBD (mg/kg/day)	THC (mg/kg/day)	CBD (mg/day)	THC (mg /day)
01[f]	07	25.0	4.00	0.05	100.00	1.33
02[m]	12	45.0	3.89	0.05	175.00	2.33
03[m]	09	33.0	3.79	0.05	125.00	1.67
04[m]	12	80.0	4.38	0.06	350.00	4.67
05[f]	11	34.0	5.88	0.08	200.00	2.67
06[m]	10	26.0	3.85	0.05	100.00	1.33
07[m]	09	32.0	3.91	0.05	125.00	1.67
08[f]	08	35.0	4.29	0.06	150.00	2.00
09[m]	14	49.0	4.08	0.05	200.00	2.67
10[m]	12	32.0	4.69	0.06	150.00	2.00
11[m]	18	89.5	3.35	0.04	300.00	4.00
12[m]	07	15.5	6.45	0.09	100.00	1.33
13[f]	15	46.0	5.43	0.07	250.00	3.33
14[m]	09	25.0	6.00	0.08	150.00	2.00
15[m]	11	35.0	4.29	0.06	150.00	2.00
Average	10.9	40.1	4.6	0.06	175.0	2.33
STD	3.06	20.18	0.94	0.01	74.40	0.99

*The administration schedule was of two daily doses, one in the morning and one in the evening.
[f] Female patients.
[m] Male patients.

MATERIALS AND METHODS

Participants

The initial cohort included 18 ASD patients (ICD 10 = F84), aged 06–17 years (average 10), including five (28%) females and 13 (72%) males. Treatment with CE was spontaneously pursued by the patient's parents, who obtained legal authorization from the National Sanitary Surveillance Agency of Brazil (ANVISA) for the compassionate use of CE with all clinical assistance and treatment follow-up supervised by one of the authors of this article (P. F). Out of the 18 patients who had initiated treatment with standardized CE, three abandoned the treatment in the first month. Among the 15 patients who remained in the study, 05 had a diagnosis of epilepsy and had had seizures in the month preceding CE treatment, while the remaining 10 had never been diagnosed with epilepsy or had not had any clinical seizures for more than 12 months before treatment with CE. Among the five epileptic patients, one was diagnosed with Dravet's syndrome, two had epilepsy associated with cerebral palsy, and two had refractory epilepsy of undetermined etiology. Non-epileptic cases were randomly numbered 1–10, while epileptic cases were randomly numbered from 11 to 15. Demographic data are detailed in **Table 1**, while the individual patient's symptom profiles are detailed in **Table 2**.

Treatment

In August 2016, all patients started receiving standardized CE, with the same composition and origin, manufactured by CBDRx® (Colorado, USA). The standardized CE contained a proportion of ∼75/1 CBD/THC and was administered orally in capsules containing 25 or 50 mg of CBD and ∼0.34 or 0.68 mg of THC, respectively (according to data provided by the manufacturer).

From the 18 patients who started standardized CE treatment, 15 had never used any CE previously, while three had already used CEs for periods ranging from 5 to 24 months. The standardized CE doses were established individually by a titration process within a dose range based on CBD doses previously reported for use of CBD-enriched CEs for treatment of refractory epilepsy associated with regressive autism (53, 54, 57, 58, 60). Thus, the average initial dose of CBD was ∼2.90 mg/kg/day, varying according to individual case severity at the beginning of treatment (minimum: 2.30 mg/kg/day and maximum: 3.60 mg/kg/day). Dosage adjustment was done intensively during the first 30 days and more sparsely over the following 150 days. The average dose of CBD administered from then until the end of the study was 4.55 mg/kg/day, with a minimum of 3.75 and a maximum of 6.45 mg/kg/day (**Table 1**). The average dose of THC in the same period was 0.06 mg/kg/day, with a minimum of 0.05 and a maximum of 0.09 mg/kg/day. Individual maintenance doses used by patients after the adjustment period are shown in **Table 1**, which does not include patients who abandoned the standardized CE treatment during the first month. The administration schedule was of two daily doses, one in the morning and one in the evening.

Cannabinoid Extract Acquisition

By means of a non-commercial collaboration between the *Brazilian Association of Medicinal Cannabis Patients* (also known as AMAME) and the manufacturer CBDRx®, the standardized CE was donated by the company CBDRx LLC at no charge to the patients.

Data Acquisition

The patient's parents and/or caregiver received a standardized form by e-mail (**Supplementary Material**), which should be answered once before the beginning of the study (baseline), and monthly throughout the duration of the CE treatment. In these forms the parents/caregivers were asked to estimate the severity of each of the eight symptom categories evaluated (see **Supplementary Material**). They should inform a score between 0 and 100, in which 0 means the lowest level of performance (or the maximum level of deficit and impairment associated to the symptom), and 100 means maximum performance (or complete absence of deficit and impairment associated to that symptom). The data presented here correspond to the difference observed between baseline results and results reported in the final month of treatment.

To ensure that the parents/caregiver properly understood the meaning of each category and that they were using the numeric scores in a consistent way throughout the study, the forms also contained two accessory questions (see **Supplementary Material**). In the first of these accessory questions the caregivers were asked to freely describe, in their own words, what changes they had observed since the last month.

TABLE 2 | Caretakers' perception of improvement in each symptom category.

Case #	Months of CE treatment[#]	Perception of improvement of symptoms (%)*							
		ADHD	BD	MD	AD	CSID	CD	SD	SZ**
01[f]	09	15	15	-	10	15	15	50	-
02[m]	06	40	10	20	30	60	40	40	-
03[m]	09	40	30	40	20	40	30	50	-
04[m]	15	30	20	20	10	40	30	30	-
05[f]	27	50	25	35	20	25	35	40	-
06[m]	09	30	00	-	00	00	20	-	-
07[m]	09	15	15	15	15	15	15	50	-
08[f]	09	20	20	-	10	60	20	60	-
09[m]	09	00	−10	20	00	00	20	-	-
10[m]	09	30	25	20	10	30	15	-	-
11[m]	09	85	85	10	25	30	50	60	100
12[m]	09	50	00	55	00	40	10	25	≥50
13[f]	09	20	20	00	00	00	00	20	≥50
14[m]	39	35	40	20	15	25	30	85	≥50
15[m]	09	00	00	00	00	00	00	10	100
n[##]		15	15	12	15	15	15	12	05
Median		30	20	20	10	25	20	40	NA

*ADHD, Attention Deficit/Hyperactivity Disorder; BD, Behavioral Disorders; MD, Motor Deficits; AD, Autonomy Deficits; CSID, Communication and Social Interaction Deficits; CD, Cognitive Deficits; SD, Sleep Disorders; SZ, Seizures. [f] female patients. [m] male patients. *Lack of improvement is computed as 00% and worsening of symptoms are recorded as negative values. [#] Total time of CE use, including before the onset of standardized CE. [##] Number of patients presenting each symptom. A dash (-) indicate lack of the symptom before treatment onset. NA, Not applicable. [**] Scores for seizures are: 00, for lack of improvement, <50%, for reduction of <50% in the occurrence of SZ, ≥50%, for reduction of more than 50% in the occurrence of SZ; or 100% for cases of complete cessation of SZ.*

In the second accessory question parents/caregivers were asked to inform the degree of change in a 5-level Likert-like scale, for each group of symptoms, in relation to the previous month. The three different responses allowed the detection of inconsistencies. Every month the patient's physician (P. F.) checked the numeric evaluation for consistency, and whenever an inconsistency was observed the physician would contact the parent/caregiver, either in person or by phone, and ask them to consider adjusting the response.

Evaluation of the Results

Patients were followed by means of periodic clinical evaluations made by the physician in charge. A monthly questionnaire was used to record treatment effects based on the answers given by the parents. Monthly standardized forms were filled out and contained questions covering the following symptom categories (see **Supplementary Material** for a detailed description of each category):

1. Attention Deficit/Hyperactivity Disorder (ADHD);
2. Behavioral Disorders (BD);
3. Motor Deficits (MD);
4. Autonomy Deficits (AD);
5. Communication and Social Interaction Deficits (CSID);
6. Cognitive Deficits (CD);
7. Sleep Disorders (SD);
8. Seizures (SZ).

Parents answered the initial questionnaires in August 2016 to assess the presence or absence of these symptoms before the onset of CE treatment. In the monthly questionnaires that followed for the next 9 months, until April 2017, the perceived percentage change for each symptom category was assessed. Clinical assessments and monthly records also included information regarding side effects and changes, maintenance, reduction, or withdrawal of neuropsychiatric drugs that were already in use (**Table 2**).

The descriptive statistics in **Figures 1A,B** were plotted in MATLAB 2017a using the default settings of the *boxplot* function from the "Statistics and Machine Learning Toolbox."

RESULTS

General Results

Three patients (one female and two males, or 17% out of the cohort of 18 patients) chose to suspend treatment before the end of the first month due to the occurrence of adverse effects. In two of these patients a worsening of symptoms may have been due to the concomitant and unsupervised attempt to remove or reduce the dosage of antipsychotics. The third patient may have suffered adverse effects of the interaction of the prescribed cannabinoids with two other psychiatric medications that were being used simultaneously. For the remaining 15 patients that adhered to the standardized CE treatment, the consolidated results recorded during the final month of treatment are presented in **Table 2** and graphically depicted in **Figure 1A**. Results for all non-epileptic patients are presented in **Figure 1B**. No differences were observed between genders, and for that reason results for both genders are shown together.

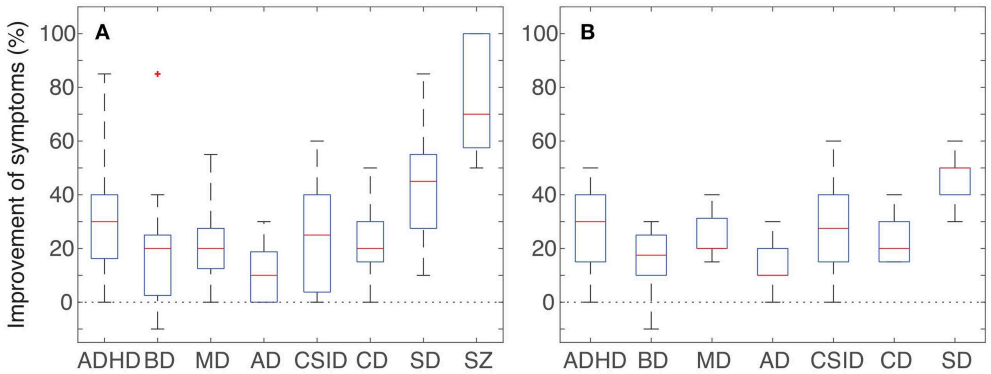


FIGURE 1 | Improvement of symptoms observed in the patients that underwent CE treatment for at least 6 months. Data was collected from the caretakers' monthly follow-up questionnaires. **(A)** Pooled data from all 15 patients in the following categories: attention deficit/hyperactivity disorder (ADHD, $n = 15$); behavioral disorders (BD, $n = 15$); motor deficits (MD, $n = 12$); autonomy deficits (AD, $n = 15$); communication and social interaction deficits (CSID, $n = 15$); cognitive deficits (CD, $n = 15$); sleep disorders (SD, $n = 12$); convulsive seizures (SZ, $n = 5$). **(B)** Subset including only the 10 non-epileptic patients that underwent CE treatment for at least 6 months. Attention deficit/hyperactivity disorder (ADHD, $n = 10$); behavioral disorders (BD, $n = 10$); motor deficits (MD, $n = 7$); autonomy deficits (AD, $n = 10$); communication and social interaction deficits (CSID, $n = 10$); cognitive deficits (CD, $n = 10$); sleep disorders (SD, $n = 7$). Red lines denote medians, blue boxes the interquartile range, black whiskers the minimum and maximum values. Note that all categories present improvements that range from modest to robust, in spite of a very short period of treatment with CE.

Overall, mostly positive outcomes were reported for the 15 patients that adhered to the standardized CE treatment (one case for 6 months and 14 cases for 9 months), especially regarding improvements in sleep disorders, seizures, and behavioral crisis. Also, signs of improvement were reported for motor development, communication and social interaction, and cognitive performance (**Table 2**). We highlight that 14 out of these 15 patients (93%) showed improvements equal to or above 30% in at least one symptom category. Most patients that adhered to the treatment had improvements in more than one symptom category: seven patients (47%) had improvements equal to or above 30% in four or more symptom categories; two patients (13%) presented improvements equal to or above 30% in two symptom categories, and five patients (33%) presented improvements equal to or above 30% in one symptom category. Only one patient, referred to as Case 9, who was receiving multiple neuropsychiatric medications throughout the study, presented overall maintenance or worsening of symptoms.

Results Grouped by Symptom Categories

Clinical assessment and records of patients' evolution, which were filled in monthly by the patients' guardians/caretakers, targeted the main symptom categories associated with autism. Possible side effects of CE administration and modifications in the dosage of other neuropsychiatric drugs that were prescribed were also evaluated and are presented in **Table 3**. From the 15 patients who adhered to the treatment with standardized EC, 15 had symptoms of ADHD; 15 of BD; 12 of MD; 15 of AD; 15 of CSID; 15 of CD; 12 of SD; and 5 of SZ. Also, as shown in **Table 3**, 10 of these patients were also concomitantly taking other prescribed neuropsychiatric medications (OM).

At least 60% of patients showed improvements of 20% or more in ADHD, MD, CSID, BD, SD, and SZ. From the 15 patients who presented BD, eight (53.3%) had improvements equal to or above 20% in this symptom category. In AD, only four (26.7%) had improvements equal to or above 20%. The most robust results were found for ADHD, SD, and SZ, with more than 80% of patients presenting improvements equal to or above 30%. The results were particularly impressive for the control of seizures in the five epileptic patients, with seizure reduction of 50% in three cases and 100% in the other two cases. It is also worth noting that CE treatment made it possible to achieve a decrease in the dosage or to discontinue other neuropsychiatric medications in eight out of 10 patients that were receiving OM (**Table 2**).

Untoward Effects

The following adverse effects were reported among the 15 patients who adhered to CE treatment: sleepiness, moderate irritability (three cases each); diarrhea, increased appetite, conjunctival hyperemia, and increased body temperature (one case each). All these side effects were mild and/or transient. Two patients presented nocturia, which in one case appeared concomitantly to an improvement in sleep quality.

As stated previously, three patients interrupted the treatment before the end of the first month of CE treatment due to adverse effects such as insomnia, irritability, increased heart rate, and worsening of psycho-behavioral crisis. Additionally, there was one patient (Case 2) who adhered to the treatment until the sixth month and, in spite of improvement in some respects, showed significant worsening in psycho-behavioral aspects. The patients who experienced relevant side effects were all receiving several drugs (Patient 1: Clomipramine + Pericyazine; Patient 2: Risperidone + Prometazine + Sodium Valproate; Patient 3: Risperidone + Prometazine), including at least one antipsychotic, and in two cases there was an abrupt cessation of the antipsychotic.

TABLE 3 | Neuropsychiatric drugs taken by each patient during the study.

Case #	OM in use before CE treatment	OM in use after CE treatment	Summary of changes in OM	CE side effects
01[f]	None	None	No OM	None
02[m]	Risperidone + Melatonin	None	Complete withdrawal	None
03[m]	None (used oxcarbazepine before the study)	None	No OM	None
04[m]	None	None	No OM	Nocturia and polyuria
05[f]	None (used several OM before the study)	None	Complete withdrawal	None
06[m]	None	None	No OM	Hyperaemia, sleepiness, and transient increase in core temperature
07[m]	Promethazine + Risperidone	Risperidone	Partial withdrawal	Transient sleepiness
08[f]	Melatonin + Risperidone	Risperidone	Partial withdrawal + dosage reduction	Slight increase in appetite
09[m]	Oxcarbazepine + Risperidone + Levomepromazine	Oxcarbazepine + Risperidone + Levomepromazine	None	None
10[m]	None	None	No OM	None
11[m]	Carbamazepine + Risperidone	Risperidone	Partial withdrawal + dosage reduction	Transient diarrhea at treatment onset
12[m]	Phenobarbital	Phenobarbital	Complete withdrawal	Nocturia
13[f]	Lamotrigine + Topiramate	Lamotrigine + Topiramate	Dosage reduction	Sleepiness and mild irritation
14[m]	Oxcarbazepine + Levetiracetam + Topiramate + Valproate semisodium + Risperidone	Topiramate + Risperidone	Partial withdrawal + dosage reduction	Transient sleepiness and mild irritation when waking up
15[m]	Risperidone + Oxcarbazepine	Risperidone + Oxcarbazepine	Dosage reduction	None

OM, alterations in other prescribed medication after introduction of CE (unaltered: no changes in the use of other medication was made; reduction, reduced the dosage of one or more medication; partial withdrawal, stopped completely the use of one of the medications; withdrawal, stopped completely the use of all other medication with the exception of CE. [f] female patients; [m] male patients.

DISCUSSION

Here we report an observational study, which collected information provided by the clinician and the patients' parents during treatment of autistic patients with a CBD-enriched CE containing a rate of ∼75:1 CBD to THC. Treatment duration ranged from 6 to 9 months. The initial cohort included 18 patients aged between 7 and 18. Three participants suspended CE use in the first 30 days of treatment, while 15 continued the use of standardized CE for six (01 patient) or nine (14 patients) months. All patients received the equivalent to an average CBD dose of 4.6 mg/kg/day and an average THC dose of 0.06 mg/kg/day. The prescribed THC dose is considered to be substantially below its safety margin (54). On the other hand, even low doses of pure THC, ranging from 0.04 to 0.12 mg/kg/day, have been previously shown to cause spasticity reduction, increased interest and connection with the environment, increased demonstration of initiative, reduction of seizure frequency, and improvement in dystonia of children with severe epileptic syndromes (82).

Previous studies have shown reliable efficacy and safety of CE containing a 20:1 CBD to THC proportion for the treatment of syndromes characterized by refractory epilepsy and regressive autism (54). Our positive results obtained from five epileptic patients (**Table 1** and **Figure 1A**) corroborate the existing data regarding the effectiveness of CBD-enriched CE in the control of refractory seizures (47–60). Moreover, to the best of our knowledge, this is the first report of a marked improvement in autistic symptoms of non-epileptic patients with the use of CE (**Figure 1B**).

Not all patients benefited equally from the treatment. From the initial cohort of 18 patients, four patients reported negative results. All of these participants were receiving multiple drugs, including at least one antipsychotic, which suggests the occurrence of undesirable drug interactions. In one of these cases, we suspect that the worsening of symptoms may have been due to an abrupt and unsupervised withdrawal of an antipsychotic drug. These observations point to a potential risk of paradoxical effects when introducing CE in a drug combination that includes antipsychotic drugs. This underscores the need for extra vigilance and of a gradual increase in the dosage of EC in patients receiving many drugs, and also to evaluate with caution the possibility of either partial or complete withdrawal of previously prescribed drugs.

Among the 15 patients who adhered to treatment for at least 6 months, 10 were non-epileptic or had not experienced seizures for at least 1 year (**Table 2** and **Figure 1B**). These patients showed positive effects in almost all evaluated categories, namely: ADHD, MD, AD, CSID, CD, and SD. Particularly among non-epileptic, nine (90%) presented improvement equal to or above 30% in at least one of these categories, six (60%) presented improvement of 30% or more in at least two categories, and four (40%) presented

improvement equal to or above 30% in at least four symptom categories (**Table 2**). Therefore, the present observational study corroborates the notion that the range of therapeutic benefits of CBD-enriched CE extends to several distinct autistic symptoms, even in non-epileptic patients.

We note that due to the fact that the behavior/symptoms were annotated by caregivers, results on behavior improvement contain a significant degree of subjectivity. We also note that the reported results are subjectively quantitative, and that the degree of improvement may be non-linear (so that 60% improvement does not necessarily mean twice as much improvement as 30%).

Conspicuous positive effects, in both epileptic and non-epileptic patients, were observed in ADHD, SD, and CSID categories. It is evident that sleep quality improvement and hyperactivity reduction tend to have major positive impacts on mood and general health, as well as on the efficacy of psycho-pedagogic therapeutic interventions. Furthermore, in a long-term perspective, psycho-pedagogic therapy may potentiate the social, cognitive, and behavioral benefits observed after CE treatment. The least pronounced effects were seen on improvement of autonomy deficits (AD). This may indicate a need for a larger time interval to allow for consolidated routines and behavioral patterns, both from patients and from caretakers, to be remodeled before any benefit can be obtained from CE treatment.

The findings presented here, taken together, support the notion that many autism symptoms are associated to neuronal hyperexcitability, and indicate that CBD-enriched CE yields positive effects in multiple autistic symptoms, without causing the typical side effects found in medicated ASD patients. Most patients in this study had improved symptoms even after supervised weaning of other neuropsychiatric drugs. The intrinsic limitations of the present study, due to its observational nature, are the lack of control groups, the small cohort size, and potentially significant placebo effects (83). Further clinical trials are warranted to confirm these initial findings.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee on Human Research of the Health Sciences College of the University of Brasilia (Universidade de Brasília- UnB), under the protocol number CAAE 16308719.3.0000.0030. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

PF-T: concept, methods design, patient care, clinical supervision, writing contributions to the manuscript introduction, methods, and discussion. FC: data analysis, critical review of the manuscript, submission. LR: concept, methods design, writing contributions to the manuscript introduction. JB-N: critical review of the manuscript. RM-L: concept, methods design, scientific supervision, bibliographic review, writing contributions to the manuscript introduction, methods, and discussion.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2019.01145/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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