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UNIVERSITY OF CALIFORNIA
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Endogenous and Exogenous Cannabinoids Regulate Energy Homeostasis
During Obesity

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Biomedical Sciences

by

Bryant Avalos Leyva

September 2023

Dissertation Committee:

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2023

The Dissertation of Bryant Avalos Leyva is approved:

Committee Chairperson

University of California, Riverside

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1. Assisted with conceptualization, methodology for investigations, data analysis, and writing (review and editing)
2. Assisted with methodology for investigations, data analysis and curation, and writing (review and editing)
3. Assisted with methodology for investigations, data analysis

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Dedication

I dedicate this body of work to:

1. The countless first-generation students who struggle with self-doubt
2. The city of Palmdale, CA
3. The late great Kobe Bryant

ABSTRACT OF THE DISSERTATION

Endogenous and Exogenous Cannabinoids
Regulate Energy Homeostasis During Obesity

by

Bryant Avalos Leyva

Doctor of Philosophy, Graduate Program in Biomedical Sciences
University of California, Riverside, September 2023
Dr. Nicholas V. DiPatrizio, Chairperson

The peripheral endocannabinoid (eCB) system is a key determinant of energy homeostasis in various biological tissues. Studies indicate eCB dysregulation in multiple peripheral organs, including the small intestine and adipose tissue, is strongly associated with diet-induced obesity (DIO). Moreover, elevated levels of peripheral endogenous cannabinoids can perpetuate the obesity phenotype by promoting appetite. To assess the impact of intestinal eCB activity on food intake, these studies first examined the role of cannabinoid type-1 receptor (CB₁R) in modulating dietary preferences. CB₁R, a key component of the ECS, has been extensively studied for its involvement in the modulation of appetite, adipogenesis, and adipokine secretion. Cannabinoid-mediated influence on appetite is well-established with certain exogenous cannabinoids found in *Cannabis sativa*, such as Δ^9 -tetrahydrocannabinol (THC), which stimulates appetite by activating the eCB system. Paradoxically, however, recent evidence from retrospective human observational studies reports lower prevalence of

obesity and decreased incidence of diabetes in cannabis users compared to non-users. It remains unclear if cannabinoids like THC are responsible for any metabolic improvements associated with long-term cannabis use. Thus, these studies also assessed the impact of chronic cannabinoid exposure on the development of DIO. By utilizing ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC/MS/MS), we compare the metabolic outcomes of chronic exposure to pure THC versus whole cannabis extract with dose-matched THC content. Furthermore, we examined the role of adipocyte CB₁R in regulating energy homeostasis within adipose tissue in response to chronic cannabinoid exposure *in vivo* and *in vitro*. Collectively, these studies use a combination of genetic and pharmacological tools to understand the role of peripheral CB₁R in facilitating shifts in energy homeostasis. Findings from these studies indicate a substantial role for intestinal CB₁R in modulating food intake and dietary preference. This body of work also provides novel insight into the role of adipocyte CB₁R in mitigating the differential metabolic improvements following chronic cannabinoid exposure.

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Introduction

Obesity

Obesity is defined as an abnormal or excessive accumulation of adipose tissue, which is the primary factor dictating the rising trend of body mass index (BMI) measurements throughout the world. Chronic consumption of high-fat, high-sugar foods (i.e. the westernized diet (WD)) contributes immeasurably to the development of diet-induced obesity (DIO) in the United States¹⁻³. Current projections estimate that 51% of America adults will suffer from obesity by the year 2030⁴, further increasing the current \$147-342 billion spent in healthcare costs related to being severely overweight^{5,6}. In 2013, the American Medical Association first classified obesity as a disease as accumulating evidence showed that the disease can significantly increase the risk of developing other major health concerns such as diabetes. Obesity and type 2 diabetes mellitus (T2DM) are intricately linked through the common denominator of insulin resistance⁷. While obesity often precedes and exacerbates T2DM, diabetes can contribute to weight gain and complications. Understanding the multifaceted mechanisms connecting these conditions is crucial for developing effective prevention and treatment strategies.

The Peripheral Endocannabinoid System

The peripheral endocannabinoid (eCB) system, a key determinant of energy homeostasis that becomes dysregulated in obesity, has recently become a target for therapeutic treatment of DIO. Anandamide (AEA) and 2-arachidonoylglycerol (2-AG), the endogenous cannabinoids that activate the cannabinoid type-1 (CB₁) and type-2 (CB₂) receptors, contribute pivotal roles to the development of obesity. In DIO, elevated levels of AEA and 2-AG cause overactivation of peripheral CB₁ which drives increased food intake⁸. Conversely, pharmacological inactivation of CB₁ reduces food intake, adipose tissue mass, and insulin hypersecretion^{9,10}. Targeting the eCB system has been considered as a pharmacological approach to tackling the obesity epidemic. In 2006, Rimonabant became the first approved drug in Europe to be used as an anti-obesity medication behaving as an inverse agonist of the CB₁ receptor. However, the restoration of adipoinular function linked with eCB system inhibition is heavily outweighed by severe psychiatric side effects resulting from non-specific CB₁R antagonism within the central nervous system¹¹⁻¹⁴. The drug was subsequently removed from the market after its serious adverse effects and was never approved in the United States.

Antagonizing the ECS has been pursued with an array of cannabinoid receptor antagonists. The peripherally restricted CB₁R antagonist AM6545 has been shown to ameliorate the dysregulation of triglycerides, cholesterol, and fat phospholipids, in monosodium glutamate-induced obese mice^{15,16}. In mice that

become diet-induced obese from a high fat/high sugar diet, AM6545 blocked hyperphagia associated with the calorically dense and innately palatable diet¹⁷. This compound, which is unable to cross the blood brain barrier, maintains its receptor-blocking activity in the periphery however this effect has only been studied in animal models and has yet to display clinical efficacy in humans. Other peripherally restricted compounds, such as URB447 have also led to lower food intake and reduced weight gain in mice closely mimicking the effects of Rimonabant^{18,19}. Conversely, agonists of the ECS such as WIN 55,212-2 have been shown to increase food intake by up to 80% in mice²⁰. This effect appears to be altered in rats, in which exposure to THC during adolescence caused an increase propensity for the rats to self-administer WIN 55,212-2 during adulthood²¹⁻²³. Furthermore, studies have shown that co-administration of WIN 55,212-2 with a CB₁R antagonist, such as AM251, results in a reduction of hyperphagic behavior illustrating a CB₁R-dependent hunger signaling event occurring within the central nervous system or periphery^{24,25}. These findings encapsulate the homeostatic nature of a system that is capable of being pharmacologically manipulated to produce opposing physiological effects depending on the compound, while also depicting the developmental dependency the system holds that ultimately affects a healthy metabolism.

The Adipoinsular Axis

Adipose tissue is no longer considered solely a passive energy storage depot; it is now recognized as an active endocrine organ capable of secreting numerous bioactive molecules, collectively termed adipokines²⁶. These adipokines have emerged as key regulators of energy metabolism, inflammation, and insulin sensitivity²⁷. The interaction between adipokines and cannabinoids contributes to the intricate regulatory network governing obesity and related metabolic disorders. Leptin and adiponectin are crucial adipokines that modulate energy balance. Leptin, known as the "satiety hormone," suppresses appetite and stimulates energy expenditure²⁸. In obesity, leptin resistance often develops, contributing to increased food intake. Adiponectin levels are inversely correlated with adiposity; as obesity progresses, adiponectin levels tend to decrease²⁶. Reduced adiponectin availability contributes to insulin resistance, as this hormone is known to enhance insulin sensitivity by stimulating fatty acid oxidation and glucose uptake in skeletal muscles and liver²⁹. Dysregulated adiponectin levels have been associated with obesity and metabolic dysfunction, making it a potential link between CB₁ receptors, adipose tissue, and obesity-related complications³⁰.

Shifts in metabolic activity within adipose tissue depots are not exclusively pathological. Unlike white adipose tissue (WAT), brown adipose tissue (BAT) serves a specialized role in thermogenesis, utilizing its dense mitochondrial reserve high in uncoupling protein 1 (UCP1) activity to oxidize fat and release energy in the form of heat^{31,32}. White and brown adipocytes share characteristics

with a subset of fat cells referred to as “beige” or “brite” adipocytes³³. Certain WAT depots like inguinal fat maintain the ability to undergo “browning” and produce an intermediate beige phenotype CB₁R signaling. WAT depots capable of browning maintain a different thermogenic gene profile compared to BAT until stimulated to express high levels of the UCP1, a mitochondrial membranous protein committed to adaptive thermogenesis³⁴⁻³⁶. This transdifferentiation potential can shift the energy balance in adipocytes by reducing the storage of triglycerides, increasing uptake of glucose, and decreasing the release of appetite-suppressing hormones^{37,38}. Given that CB₁R blockade can induce this phenotypic change, the therapeutic relevance of targeting the ECS is further signified.

Cannabis & Metabolic Health

Archaeological artifacts containing residues of the *Cannabis sativa* plant indicate its historical use originated in the continent of Asia, more specifically the steppes of Central Asia^{39,40}. Having served a medicinal, spiritual, and recreational use throughout its history, cannabis’ complex molecular makeup has been shown to provide more than just a psychoactive effect to its users. The natural compounds found in cannabis, known as phytocannabinoids, are abundant in nature and up until the end of the 19th century, were unidentified. Purification and extraction of oil via fractional distillation of cannabis marked the first step in elucidating the physiological and biochemical relevance of the plant⁴¹. Soon after, isolation of the first two inactive natural cannabinoids, cannabitol (CBN) and cannabidiol (CBD),

paved way to uncovering the constituents of cannabis that promote psychoactivity⁴². Successful isolation and structural characterization of the primary psychoactive constituent of the plant, Δ 9-tetrahydrocannabinol (THC), was reported in 1964 and further prompted investigation into the stereochemistry of the two previously discovered cannabinoids^{43,44}.

Also known as marijuana, the psychoactive herb and its pharmacodynamics remained unearthed for several decades following the discovery of the early cannabinoids. Towards the end of the century, the discoveries of the receptor binding sites marked the start of the elucidation of cannabis' mechanism of action within the mammalian body. Cannabinoid-mediated influence on appetite is well-established with certain phytocannabinoids found in *Cannabis sativa*, like Δ 9-tetrahydrocannabinol (THC), which stimulates appetite via CB₁R activation^{41,43,45}. Dronabinol (Marinol), a synthetic analog of THC, was first approved by the Food and Drug Administration in 1985 as an antiemetic agent prescribed to combat nausea and stimulate appetite during chemotherapy or in acquired immune deficiency syndrome (AIDS) patients. Paradoxically, however, several prospective studies have shown positive metabolic benefits associated with cannabis use among adults. The National Epidemiological Survey on Alcohol and Related Conditions (NESARC) reports an inverse association between cannabis and body mass index (BMI) in a study consisting of adult cannabis users, age 18+, within the United States when compared to never-users^{46,47}. This attenuated BMI response is recapitulated in the data from the National Health and Nutrition

Examination Survey (NHANES) III, where current cannabis users reported slightly lower BMI values compared to non-current cannabis users despite higher caloric intake⁴⁸⁻⁵⁰. The endocannabinoid system's role in regulating glucose and insulin homeostasis is further restated in the findings illustrating cannabis users maintain a lower HOMA-IR index, a Homeostasis Model Assessment of Insulin Resistance, when compared to now users⁵¹⁻⁵³.

Together, these studies provide accumulating evidence elucidating cannabis' potential in altering the phenotype associated with obesity and marijuana consumption. Additionally, habitual use of cannabis has been associated with a down regulation of CB₁ receptors serving as a plausible explanation for effects seen in both the central and peripheral ECS. It is important to note that data from the mentioned prospective studies are dependent on self-reporting which may cause a response bias. Regardless, the endocannabinoid system's ability to be manipulated by cannabis which correlates with several beneficial metabolic effects should be explored further to avoid heavily relying on correlational information concerning acute and chronic cannabis use. Understanding the complex interplay between cannabinoids and adipokines is essential for developing targeted interventions that address the global health burden of obesity.

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Chapter 1: Cannabinoid CB₁ receptors in the intestinal epithelium are required
for acute western-diet preferences in mice

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Abstract

The endocannabinoid system plays an important role in controlling intake of palatable food. Endocannabinoid signaling in the upper small-intestinal epithelium is increased (i) in rats after tasting dietary fats, which controls intake of fats, and (ii) in a mouse model of diet-induced obesity, which promotes overeating via impaired nutrient-induced gut-brain satiation signaling. We now utilized a combination of genetic, pharmacological, and behavioral approaches to identify roles for cannabinoid CB₁Rs in upper small-intestinal epithelium in preferences for a western-style diet (WD, high-fat/sucrose) versus a standard rodent diet (SD, low-fat/no sucrose). Mice were maintained on SD in automated feeding chambers. During testing, mice were given simultaneous access to SD and WD, and intakes were recorded. Mice displayed large preferences for WD, which were inhibited by systemic pretreatment with the cannabinoid CB₁R antagonist/inverse agonist, AM251, for up to 12 h. We next used our novel intestinal epithelium-specific conditional cannabinoid CB₁R-deficient mice (IntCB₁^{-/-}) to investigate the necessity of intestinal CB₁Rs in preferences for WD. Similar to AM251 treatment, preferences for WD were largely absent in IntCB₁^{-/-} mice when compared to control mice for up to 12 h. Together, these data suggest that CB₁Rs in the murine intestinal epithelium are required for acute WD preferences.

Introduction

Humans and other mammals, when given a choice, generally prefer food that contains fats, sugars, or a combination of both [1]. Homeostatic and hedonic

feeding are controlled by diverse, albeit overlapping, neural and molecular signaling pathways throughout the brain, including those regulated by the endocannabinoid (eCB) system [see for review 2-5]. Recent studies, however, suggest important roles for the peripheral eCB system in energy homeostasis and intake of palatable food [6-29]. For example, we reported that tasting dietary lipids was sufficient to increase levels of eCBs in the rat upper small-intestinal epithelium, which required an intact vagus nerve, and pharmacological inhibition of cannabinoid subtype-1 receptors (CB₁Rs) in the periphery blocked consumption of lipids [14,15]. Moreover, levels of eCBs in the upper small-intestinal epithelium were increased in mice maintained for eight weeks on a western-style diet high in fat and sugar (WD) when compared to mice fed a standard diet low in fat and sugar, and pharmacological inhibition of CB₁Rs in the periphery blocked overeating associated with WD-induced obesity [17].

Nutrients are sensed by gustatory mechanisms in the oral cavity and enteroendocrine cells in the intestinal epithelium. In response, these cells release several satiation- and satiety-related molecules that communicate with the brain via the afferent vagus nerve [30-38]. We recently reported that eCB signaling in the gut controls nutrient-induced release of satiation peptides [16]. Gene transcripts for CB₁Rs were enriched in a subpopulation of enteroendocrine cells in the upper small-intestinal epithelium that secrete the satiation peptide, cholecystokinin [16,39]. Notably, the ability for nutrients to stimulate an increase in levels of circulating cholecystokinin was impaired in mice fed WD for eight weeks

when compared to lean control mice, and pharmacological inhibition of overactive eCB signaling at peripheral CB₁R_s in mice fed WD restored the ability for nutrients to induce release of cholecystokinin [16]. Furthermore, the appetite-suppressing effects of peripheral CB₁R inhibition in mice maintained on WD were attenuated by co-treatment with an antagonist for cholecystokinin-A receptors [16], which are expressed by sensory vagal neurons and other organs [40]. Collectively, these studies suggest that eCB signaling in upper small-intestinal epithelium is dysregulated in WD-induced obese mice and promotes overeating by a mechanism that includes blocking nutrient-induced gut-brain satiation signaling.

In the current study, we used a novel conditional intestinal epithelium-specific CB₁R-deficient mouse model to investigate the necessity for intestinal CB₁R_s in preferences for WD.

Materials & Methods

Animals

C57BL/6 male mice (Taconic, Oxnard, CA, USA) or transgenic mice (described below in Transgenic Mouse Generation) 8-10 weeks of age were group-housed with ad-libitum access to standard rodent laboratory diet (SD; Teklad 2020x, Envigo, Huntingdon, UK; 16% kcal from fat, 24% kcal from protein, 60% kcal from carbohydrates) and water throughout all experiments. Mice were maintained on a 12-h dark/light cycle beginning at 1800 h. All procedures met the U.S. National Institute of Health guidelines for care and use of laboratory animals

and were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, Riverside.

Transgenic Mouse Generation

Conditional intestinal epithelium-specific CB₁R-deficient mice (Cnr1^{tm1.1} mrl/vil-cre ERT2) were generated by crossing Cnr1-floxed mice (Cnr1^{tm1.1} mrl; Taconic, Oxnard, CA, USA; Model # 7599) with Vil-CRE ERT2 mice donated by Dr. Randy Seeley (University of Michigan, Ann Arbor, MI, USA) with permission from Dr. Sylvie Robin (Curie Institute, Paris, France). Cre recombinase expression in the intestinal epithelium is driven by the villin promotor, which allows for conditional tamoxifen-dependent Cre recombinase action to remove the Cnr1 gene from these cells, as described by el Marjou et al., [41]. Cnr1^{tm1.1} mrl/vil-cre ERT2 mice used in these experiments are referred to as IntCB₁^{-/-}, and Cnr1^{tm1.1} mrl control mice (lacking Cre recombinase) are referred to as IntCB₁^{+/+}. Tail snips were collected from pups at weaning and DNA was extracted and analyzed by conventional PCR using the following primers (5'-3'):

GCAGGGATTATGTCCCTAGC (CNR1-ALT), CTGTTACCAGGAGTCTTAGC (1415-35), GGCTCAAGGAATACACTTATACC (1415-37), GAACCTGATGGACATGTTTCAGG (vilcre, AA), AGTGCGTTTCGAACGCTAGAGCCTGT (vilcre, SS), TTACGTCCATCGTGGACAGC (vilcre, MYO F), TGGGCTGGGTGTTAGCCTTA (vilcre, MYO R).

Western Diet Preference Test

Mice were single-housed in two-hopper feeding chambers (TSE Systems, Chesterfield, MO, USA) for five days to acclimate, and received ad-libitum access to SD and water throughout behavioral testing. At the time of testing, mice were given access for the first time to the hopper containing Western Diet (WD; Research Diets D12709B, New Brunswick, NJ, USA; 40% kcal from fat, 17% kcal from protein, 43% kcal from carbohydrates as mostly sucrose). Preferences for WD versus SD (% total kcals from WD), total caloric intake of each diet (kcals), and average meal size of each diet (kcals) were calculated every minute across the testing period, beginning one hour before dark cycle (1700 h).

Chemical Preparation and Administration

IntCB₁^{-/-} and IntCB₁^{+/+} mice were administered tamoxifen (IP, 40 mg per kg) every 24 h for five consecutive days. Tamoxifen (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in corn oil at a concentration of 10 mg per mL then stored at 37°C protected from light until administration. Tamoxifen in corn oil was placed in a bath sonicator for 10 minutes prior to administration. Mice were group housed in disposable cages throughout the injection window and for a 3-day post-injection period. The CB₁R antagonist/inverse agonist, AM251 (Tocris, Minneapolis, MN, USA), was administered (IP 3 mg per kg per 2 mL) 30 minutes prior to testing. Vehicle consisted of 7.5% dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA), 7.5% Tween 80 (Chem Implex Intl Inc., Wood Dale, IL, USA), and 85% sterile saline.

Immunohistochemistry

Proximal small intestinal tissue was collected from IntCB₁^{-/-} and IntCB₁^{+/+} control mice 7 days after the completion of tamoxifen schedule. Tissue was flushed with ice-cold 4% paraformaldehyde/PBS, then fixed for 4 hours at 4°C. Cross sections of the upper small intestine were cut and frozen in OCT (Fisher Healthcare, Chino, CA, USA) on dry ice. Approximately 16 µm sections were obtained using a cryostat (Leica, Wetzlar, Germany) then mounted onto charged glass slides. Sections were permeabilized with 0.5% Tween20/PBS and then blocked with 0.1% Tween20 in casein solution (Thermo Fisher, Waltham, MA, USA). Primary antibodies for CB₁Rs (kindly provided by Dr. Ken Mackie, Indiana University, USA) raised in rabbit were diluted 1:500 in blocking buffer, slides were incubated for 1 h at room temperature. Sections were washed three times with 0.1% Tween20/PBS solution then incubated for 1 h at room temperature with goat anti-rabbit secondary antibodies conjugated with alexafluor 647. Following repeated washes, coverslips were mounted with Prolong Gold Antifade reagent with DAPI (Thermo Fisher) for nuclear counterstaining. Images were obtained at room temperature using an Axio Observer Z1 Inverted Microscope (Zeiss, Oberkochen, Germany) as previously described [16].

Gene Expression

Total RNA from intestinal epithelium tissue was extracted using RNeasy kit (Qiagen, Valencia, CA, USA) and first-strand cDNA was generated using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Areas used for tissue

collection and processing were sanitized with 70% ethanol solution then treated with RNase inhibitor (RNase out, G-Biosciences, St. Louis, MO, USA). Reverse transcription of total RNA was performed as previously described [16]. Quantitative RT-PCR was performed using PrimePCR Assays (Biorad, Irvine, CA, USA) with primers for CB₁R (Cnr1), CB₂R (Cnr2), G-protein coupled receptor 55 (Gpr55), diacylglycerol lipase alpha (Dagla), diacylglycerol lipase beta (Daglb), monoacylglycerol lipase (Mgll), alpha beta hydrolase domain containing 6 (Abhd6), N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D (Napepld), and fatty acid amide hydrolase (Faah) gene transcripts under preconfigured SYBR Green assays (Biorad, Irvine, CA, USA). Hprt was used as a housekeeping gene for stomach, duodenum intestinal epithelium, jejunum intestinal epithelium, ileum intestinal epithelium, small-intestinal submucosa/muscle/serosal layer, large intestinal epithelium, and liver; β -actin (Actb) as housekeeping gene for pancreas; and β 2-microglobulin (B2m) as housekeeping gene for epididymal fat. Reactions were run in triplicates and values are expressed as relative mRNA expression.

Statistical Analysis

Data were analyzed by GraphPad Prism 8 software using unpaired Student's t-tests (two-tailed) or two-way ANOVA with Holm-Sidak's multiple comparisons post-hoc test when appropriate. Results are expressed as means \pm S.E.M. and significance was determined at $p < 0.05$.

Results

Systemic Pharmacological Blockade of CB₁Rs Reduces Acute Preferences for Western Diet in Mice

We investigated roles for cannabinoid CB₁ receptors in preferences for Western Diet (WD). Naïve mice maintained on ad-libitum standard laboratory chow diet (SD) were administered vehicle or the cannabinoid CB₁R antagonist/inverse agonist, AM251, and subjected to a 24 h preference test for WD versus SD. Vehicle-treated mice displayed robust preferences for WD when compared to SD, an effect inhibited by AM251 by 3 h (Fig 1a, from 84.7 ± 7.1% total kcals from WD in vehicle-treated mice to 58.7 ± 8.3% in AM251-treated mice; $p = 0.042$), 6 h (Fig 1b, from 89.1 ± 3.7% total kcals from WD in vehicle-treated mice to 64.0 ± 7.4% in AM251-treated mice; $p = 0.012$), and 12 h (Fig 1c from 92.4 ± 2.8% total kcals from WD in vehicle-treated mice to 72.2 ± 7.2% in AM251-treated mice; $p = 0.028$) after initiation of the preference test. AM251 had no significant effect on WD preferences by 24 h after administration (Fig 1d, from 86.9 ± 3.1% total kcals from WD in vehicle-treated mice to 75.0 ± 6.7% in AM251-treated mice; $p = 0.176$).

Consistent with these data, vehicle-treated mice ate significantly more kcals from WD than from SD by 3 h (Fig 1e, $p = 0.046$), 6 h (Fig 1f, $p = 0.014$), 12 h (Fig 1g, $p = 0.002$), and 24 h (Fig 1h, $p < 0.001$) after initiation of the preference test. These effects were absent in mice treated with AM251 by 3 h (Fig 1e, $p = 0.951$), 6 h (Fig 1f, $p = 0.679$), and 12 h (Fig 1g, $p = 0.244$); however, mice consumed

more WD than SD by 24 h (Fig 1g, $p = 0.021$), regardless of treatment. Moreover, vehicle-treated mice displayed larger meal sizes of WD versus SD by 3 h (Fig 1i, $p = 0.028$), 6 h (Fig 1j, $p = 0.018$), 12 h (Fig 1k, $p = 0.009$), and 24 h after initiation of the preference test (Fig 1l, $p = 0.006$). These effects were absent in mice treated with AM251 by 3 h (Fig 1i, $p = 0.816$), 6 h (Fig 1j, $p = 0.827$), 12 h (Fig 1k, $p = 0.803$), and 24 h (Fig 1l, $p = 0.939$). In addition, there were no significant differences in total cumulative caloric intake (i.e., total kcals from WD+SD) between treatment groups by 3 h (Fig 1m, $p = 0.501$), 6 h (Fig 1n, $p = 0.444$), 12 h (Fig 1o, $p = 0.734$), and 24 h after initiation of the preference test (Fig 1p, $p = 0.151$). Collectively, these results suggest that cannabinoid CB₁Rs control acute preferences for WD in mice.

Acute Preferences for Western Diet are Absent in Mice with CB₁R Deletion in the Intestinal Epithelium

Endocannabinoid signaling in the rodent upper small-intestinal epithelium is important for consumption of dietary fats based on their taste properties [14,15], re-feeding after a fast [10], and hyperphagia in a mouse model of WD-induced obesity via a mechanism that includes blocking nutrient-induced gut-brain satiation signaling [16,17]. We used our novel intestinal epithelium-specific conditional CB₁R-deficient mice (IntCB₁^{-/-}) to probe the necessity for CB₁Rs in the intestinal epithelium in preferences for WD. Moreover, AM251 is reported to have some off-target effects [42,43]; therefore, this mouse model allows for direct evaluation of roles for CB₁Rs in the intestinal epithelium in these processes. CB₁R deficiency in

the intestinal epithelium of IntCB₁^{-/-} mice was verified by immunohistochemistry (Figure 2a-d).

CB₁R deficiency in the intestinal epithelium of IntCB₁^{-/-} mice was further confirmed by qRT-PCR (Fig 3a, b). IntCB₁^{-/-} mice, when compared to IntCB₁^{+/+} controls, were deficient in expression of mRNA for CB₁Rs (Cnr1) in the jejunum epithelium (Fig 3a, p = 0.031). Expression of mRNA for other components of the endocannabinoid system in the jejunal epithelium were unaffected, including cannabinoid CB₂Rs (Cnr2; Fig 3a, p = 0.892), G-protein coupled receptor 55 (Gpr55; Fig 3a, p = 0.736), diacylglycerol lipase alpha (Dagla; Fig 3a, p = 0.825), diacylglycerol lipase beta (Daglb; Fig 3a, p = 0.798), monoacylglycerol lipase (Mgll; Fig 3a, p = 0.872), alpha beta hydrolase domain containing 6 (Abhd6; Fig 3a, p = 0.314), N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D (Napepld; Fig 3a, p = 0.217), and fatty acid amide hydrolase (Faah; Fig 3a, p = 0.986). In addition to the jejunum epithelium, IntCB₁^{-/-} mice were deficient in expression of mRNA for CB₁Rs (Cnr1) in the duodenum epithelium (Fig 3b, p = 0.009), ileum epithelium (Fig 3b, p = 0.038), large intestine epithelium (Fig 3b, p = 0.039), but not in the small-intestinal submucosa/muscle/serosal layers (Fig 3b, p = 0.633), stomach (Fig 3b, p = 0.602), liver (Fig 3b, p = 0.593), pancreas (Fig 3b, p = 0.9), and epididymal fat (Fig 3b, p = 0.14).

IntCB₁^{-/-} and IntCB₁^{+/+} control mice displayed similar body weights (Fig 4a, p = 0.404), and baseline 24-h caloric intake (Fig 4b, p = 0.52), 24-h water intake (Fig 4c, p = 0.487), average meal size (Fig 4d, p = 0.653), ambulation (Fig 4e, p =

0.741), and glucose clearance during an oral glucose tolerance test (Fig 4f-g; 15, 30, 60, 120 min, ns; total area under curve, $p = 0.847$). Control IntCB₁^{+/+} mice displayed robust preferences for WD when compared to SD, an effect largely absent in IntCB₁^{-/-} mice by 3 h (Fig 5a, from $92.8 \pm 2.8\%$ total kcals from WD in IntCB₁^{+/+} mice to $46.5 \pm 12.5\%$ in IntCB₁^{-/-} mice; $p = 0.029$), 6 h (Fig 5b, from $95.0 \pm 2.0\%$ total kcals from WD in IntCB₁^{+/+} mice to $49.9 \pm 13.8\%$ in IntCB₁^{-/-} mice; $p = 0.048$), and 12 h (Fig 5c, from $94.3 \pm 0.2\%$ total kcals from WD in IntCB₁^{+/+} mice to $72.2 \pm 6.8\%$ in IntCB₁^{-/-} mice; $p = 0.049$) after initiation of the preference test. Preferences for WD in IntCB₁^{-/-} mice were not significantly different from controls by 24 h (Fig 5d, from $94.9 \pm 1.1\%$ total kcals from WD in IntCB₁^{+/+} mice to $76.3 \pm 6.3\%$ in IntCB₁^{-/-} mice; $p = 0.069$). Congruent with these data, control IntCB₁^{+/+} mice ate significantly more kcals from WD when compared to SD by 3 h (Fig 5e, $p = 0.02$), 6 h (Fig 5f, $p = 0.002$), 12 h (Fig 5g, $p < 0.001$), and 24 h (Fig 5h, $p < 0.001$) after initiation of the preference test. These effects were absent in IntCB₁^{-/-} mice by 3 h (Fig 5e, $p = 0.732$, ns), 6 h (Fig 5f, $p = 0.92$), and 12 h (Fig 5g, $p = 0.06$); however, IntCB₁^{-/-} mice consumed more WD than SD by 24 h (Fig 5g, $p = 0.003$). Moreover, IntCB₁^{+/+} mice consumed larger meal sizes of WD versus SD by 3 h (Fig 5i, $p = 0.031$), 6 h (Fig 5j, $p = 0.009$), 12 h (Fig 5k, $p < 0.001$), and 24 h after initiation of the preference test (Fig 5l, $p < 0.001$). These effects were absent in IntCB₁^{-/-} mice by 3 h (Fig 5i, $p = 0.404$), 6 h (Fig 5j, $p = 0.387$), 12 h (Fig 5k, $p = 0.076$), and 24 h (Fig 5l, $p = 0.149$). In addition, there were no significant changes in total cumulative caloric intake (i.e., total kcals from WD+SD) for IntCB₁^{-/-} mice

when compared to IntCB₁^{+/+} controls by 3 h (Fig 5m, $p = 0.196$), 6 h (Fig 5n, $p = 0.105$), 12 h (Fig 5o, $p = 0.111$), and 24 h after initiation of the preference test (Fig 5p, $p = 0.051$).

Discussion

We report that acute preferences for WD (i) were inhibited by global pharmacological blockade of CB₁Rs, and (ii) were largely absent in mice conditionally deficient in CB₁Rs selectively in the intestinal epithelium. These results suggest that CB₁Rs in the intestinal epithelium are required for acute WD preferences in mice. Moreover, these studies expand our understanding of critical pathways for gut-brain communication in the control of preferences for palatable foods.

Dietary components are detected by receptors located throughout the oral cavity [36] and intestinal epithelium [44], which provide feedback associated with the nutritional content of food and contribute to determination of food preferences. For example, we reported that tasting dietary unsaturated lipids – but not sugar or protein – triggered production of endocannabinoids in the rat upper small-intestinal epithelium, and pharmacological inhibition of endocannabinoid signaling at CB₁Rs in the periphery blocked intake and preferences for fats in a sham-feeding model [14,15]. These studies suggest that endocannabinoid signaling in the gut contributes to the positive feedback control of fat intake based on its unique taste properties. Despite localized increases of endocannabinoids selectively in the upper small-intestinal epithelium and blockade of intake following pharmacological

treatment with a peripherally-restricted neutral CB₁R antagonist, these studies were limited in ability to identify necessity for CB₁R_s in the intestinal epithelium in food intake and dietary preferences. To overcome these challenges and examine whether CB₁R_s in the small-intestinal epithelium were required for WD preferences, we generated a novel conditional intestinal epithelium-specific CB₁R-deficient mouse. Preferences for WD compared to a low-fat, no-sucrose, chow were largely absent in IntCB₁^{-/-} mice when compared to IntCB₁^{+/+} control mice during the first 12 h of preference testing. Moreover, these effects were mimicked by systemic treatment with the globally acting CB₁R antagonist/inverse agonist, AM251, in wild-type mice. Collectively, these results provide evidence of a critical role for CB₁R_s in the rodent intestinal epithelium in acute preferences for food containing high levels of fats and sugars.

The specific mechanism(s) underlying intestinal epithelium CB₁R-mediated preferences for WD are unknown, but may include CB₁R control of gut-brain signaling. We reported that hyperphagia and increased meal size associated with WD-induced obesity in mice are dependent on (i) elevated levels of endocannabinoids in the upper small-intestinal epithelium; and (ii) CB₁R-mediated inhibition of nutrient-induced signaling of the satiation peptide, cholecystokinin [16]. Cholecystokinin is secreted from enteroendocrine cells in the upper small-intestinal epithelium when nutrients arrive in the lumen, and transmits satiation signals to the brain by interacting with cholecystokinin A receptors on the afferent vagus nerve [30,40,45-48] and possibly the brain [49,50]. Bohorquez and

colleagues recently characterized enteroendocrine cells (i.e., neuropods) in the mouse intestinal epithelium that form functional synapses with afferent vagal fibers [33]. Neuropods sense nutrients in the lumen and in response, release glutamate and cholecystinin, which activate afferent vagal neurons in a coordinated manner [51]. Moreover, afferent vagal neurons participate in reward-related behaviors – including flavor and place preferences – and control dopamine outflow in the mouse striatum [48]. Notably, however, studies suggest that the afferent vagus nerve is required for nutrient-induced negative feedback from the gut associated with satiation and satiety, but is dispensable for positive feedback [i.e., appetite [52]] associated with nutrient reinforcement and flavor-nutrient preference conditioning [53]. Nonetheless, it is possible that CB₁Rs in the intestinal epithelium participate in preferences for WD by a mechanism that includes control of nutrient-induced, neuropod-mediated, afferent vagal activity and recruitment of brain reward circuits. A direct test of this hypothesis and evaluation of distinct roles for intestinal CB₁Rs in satiation versus appetite remain for future studies.

We propose that CB₁Rs indirectly regulate afferent vagal activity by controlling nutrient sensing and release of satiation peptides from enteroendocrine cells in the small-intestinal epithelium that directly interact with the afferent vagus nerve [16,39]. Recent studies also suggest that CB₁Rs in the mouse stomach participate in alcohol intake by controlling formation of the appetite-stimulating hormone, ghrelin, which interacts with ghrelin receptors on afferent vagal fibers [54]. In addition to these indirect mechanisms, CB₁Rs may also directly control

afferent vagal neurotransmission and food intake [55]. For example, Burdyga and colleagues reported that fasting was associated with increased expression of CB₁Rs in the rat vagal afferent neurons [56]. Refeeding or administration of cholecystokinin rapidly reversed fasting-induced expression of CB₁Rs [56], which was also blunted in rats maintained on a high-fat diet [57]. In addition, administration of ghrelin blocked the effects of refeeding on CB₁R expression [58]. Moreover, Christie and colleagues reported that low and high concentrations of methanandamide – a stable analog of anandamide – differentially modified mechanosensitivity of mouse gastric vagal afferents *in vitro* via a mechanism that included CB₁Rs, TRPV1, and ghrelin receptors [59], and these effects were dysregulated in mice fed a high-fat diet for 12 weeks [60]. These studies suggest that CB₁Rs on the afferent vagus nerve may participate in gut-brain signaling important for food intake and energy balance. Interestingly, mice with genetic deletion of CB₁Rs on afferent vagal neurons displayed no changes in body weight or food intake, irrespective of test diet (i.e., standard versus high-fat), which suggests that vagal CB₁Rs may not be necessary for long-term maintenance of body weight and feeding [61]. Further investigations are necessary to expand our understanding of physiological roles for the endocannabinoid system in vagal afferent neurons.

It is noteworthy that attenuation of preferences for WD were limited to the first 12 h in, both, AM251-treated wild-type mice and IntCB₁^{-/-} mice when compared to vehicle and IntCB₁^{+/+} mice, respectively. It is plausible that restricted

temporal effects of AM251 reflect the pharmacokinetic properties of this compound, which displays a half-life of 22 h in rats [62]. IntCB₁^{-/-} mice, however, displayed a similar restriction of preferences for WD to the first 12 h of the test. The mechanism(s) in this restricted response remains unknown. Post-prandial cues at later time-points, however, may provide compensatory feedback and reinforcement, and restore preferences for WD in the absence of CB₁R in the intestinal epithelium. One candidate in this proposed mechanism is the satiety factor oleoylethanolamide, which is synthesized in the intestinal epithelium from dietary fats and controls food intake and possibly reward through a mechanism that requires peroxisome proliferator-activated receptor (PPAR α) and the afferent vagus [63-65]. Studies examining interactions between orexigenic endocannabinoid and anorexic oleoylethanolamide signaling pathways in acute and long-term dietary preferences remain for future inquiry.

Conclusion

In summary, these studies extend our understanding beyond central roles for the endocannabinoid system in intake and reward value of palatable food [66-82], and provide evidence that CB₁R in the intestinal epithelium are an integral component of a gut-brain axis that controls dietary preferences. Future studies will be important to elucidate (i) specific mechanism(s) of intestinal CB₁R-mediated preferences for palatable food, (ii) roles for CB₁R in the intestinal epithelium in recruitment of brain reward circuits and the “wanting” or “liking” of palatable food [83], (iii) roles for intestinal CB₁R in satiation versus appetite, (iv) interactions

between CB₁R and PPAR α signaling pathways in preferences for palatable food, (v) roles CB₁Rs in the intestinal epithelium in development and maintenance of diet-induced obesity, and (vi) physiological roles for CB₁Rs on vagal neurons.

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Figures

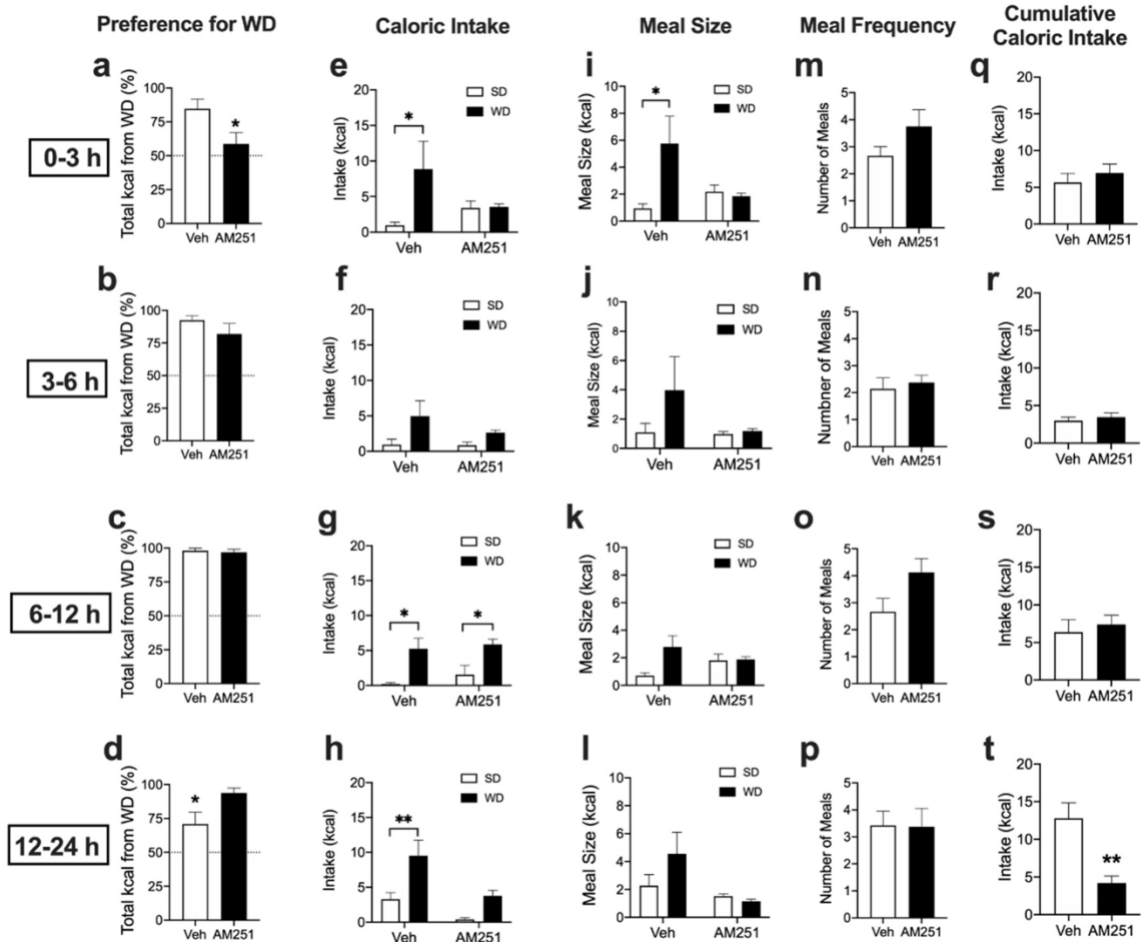


Figure 1.1. Cannabinoid CB₁Rs control acute preferences for Western Diet.

When compared to vehicle treatment (Veh), preferences for western diet (WD) are reduced in AM251-treated animals for up to 12 hours (a-c), an effect absent after 24 hours (d). Similarly, cumulative intake of WD is increased versus SD in vehicle-treated mice, an effect absent in AM251-treated mice (e-g) that is regained in AM251-treated mice after 24 hours (h). Average meal size for WD, when compared to SD, is increased throughout the 24 hour preference test in Veh mice, an effect absent in AM251-treated mice (i-l). Total cumulative intakes were similar between treatment groups throughout all time points (m-p). Unpaired Student's t-test, two-tailed (a-d, m-p); Two-way ANOVA with Holm-Sidak's multiple comparison tests (e-l); * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Results are expressed as means \pm S.E.M; $n = 7-8$ per condition.

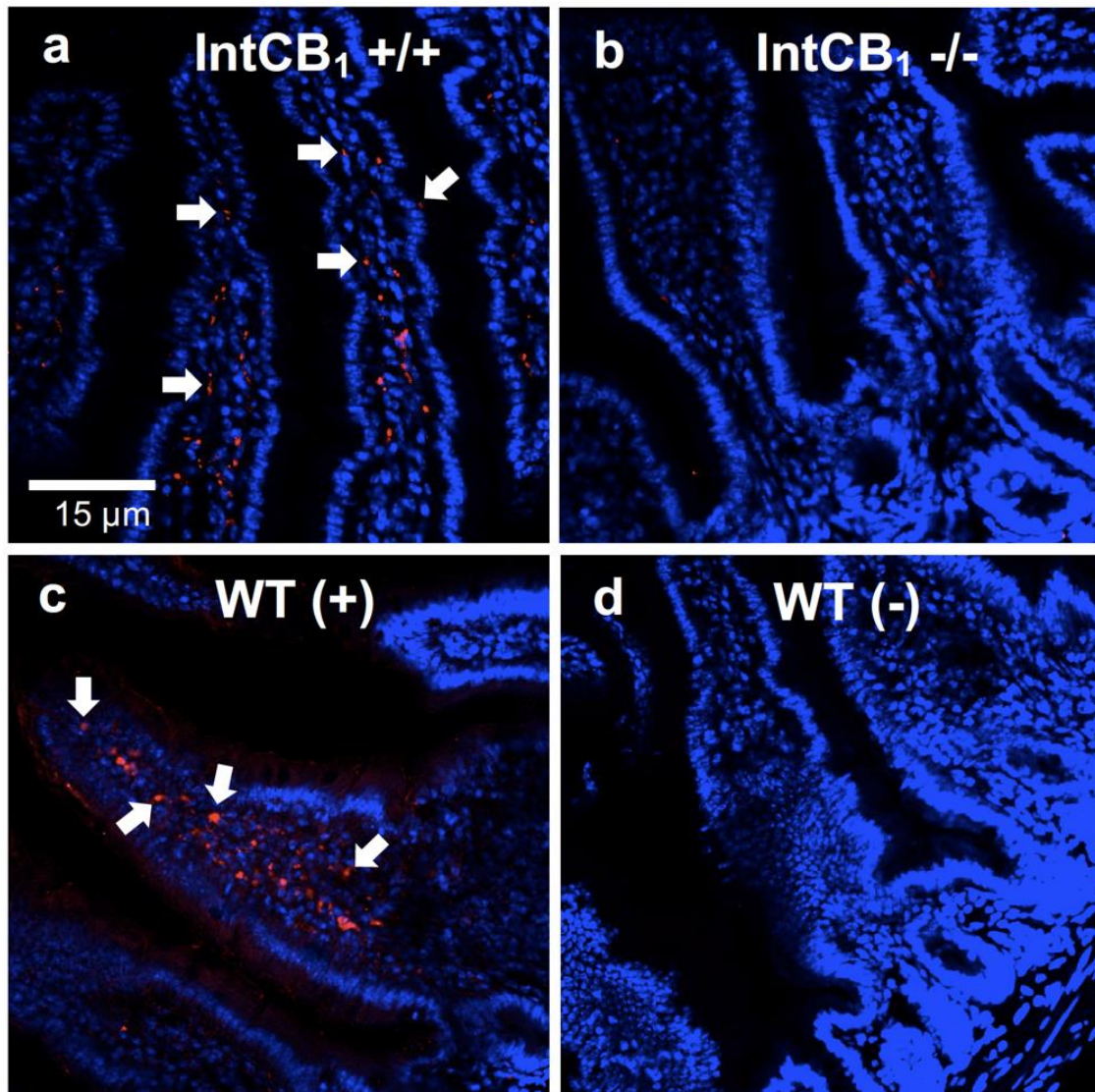


Figure 1.2. CB₁R immunoreactivity is absent in the upper small-intestinal epithelium of conditional intestinal epithelium-specific CB₁R-deficient mice.

When compared to control mice (a, IntCB^{+/+}), conditional intestinal epithelium-specific CB₁R-null mice (b, IntCB₁^{-/-}) are deficient in immunoreactivity for CB₁Rs in the upper small-intestinal epithelium. Wild-type C57BL/6Tac mice display immunoreactivity for CB₁Rs in the upper small-intestinal epithelium [c, WT (+)], which is absent when the primary CB₁R antibody is not included [d, WT(-)]. White arrows point to representative red immunoreactivity for CB₁Rs. Red = CB₁R immunoreactivity; blue = DAPI. WT = wild-type mice. (+) = with CB₁R primary antibody; (-) = without CB₁R primary antibody.

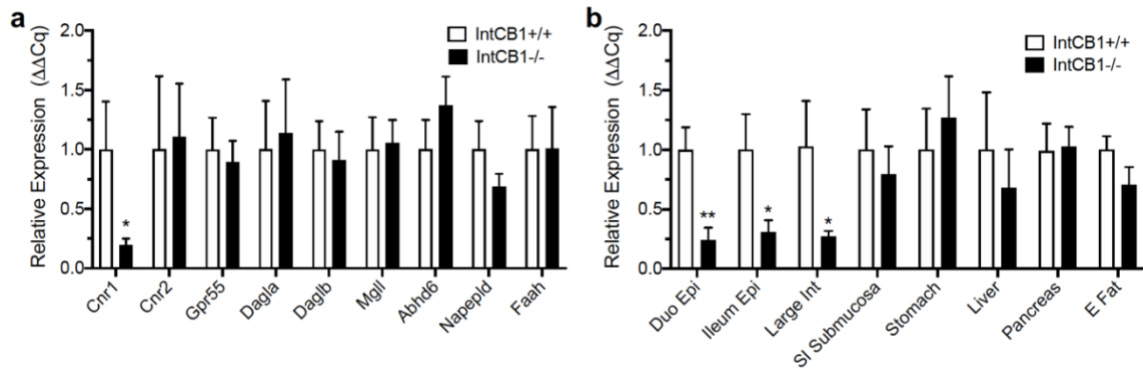


Figure 1.3. Expression of endocannabinoid system genes in conditional intestinal epithelium-specific CB₁R-deficient mice and controls.

Expression of cannabinoid CB₁Rs (Cnr1) was reduced in the jejunum epithelium of conditional intestinal epithelium-specific CB₁R deficient mice (IntCB₁^{-/-}) when compared to control mice (IntCB₁^{+/+}), and expression of mRNA for other components of the endocannabinoid system were unaffected, including cannabinoid CB₂Rs (Cnr2), g-protein coupled receptor 55 (Gpr55), diacylglycerol lipase alpha (Dagla), diacylglycerol lipase beta (Daglb), monoacylglycerol lipase (Mgll), alpha beta hydrolase domain containing 6 (Abhd6), N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D (Napepld), and fatty acid amide hydrolase (Faah)(a). IntCB₁^{-/-} mice, when compared to IntCB₁^{+/+} controls, were deficient in expression of mRNA for CB₁Rs (Cnr1) in the duodenum epithelium (Duo Epi), ileum epithelium (Ileum Epi), large intestine (Large Int), but not in the small-intestinal submucosa/muscle/serosal layers (SI Submucosa), stomach, liver, pancreas, and epididymal fat (E Fat). Unpaired Student's t-tests, two-tailed; * = p<0.05, ** = p<0.01. Results are expressed as means ± S.E.M; n = 5-8 per condition.

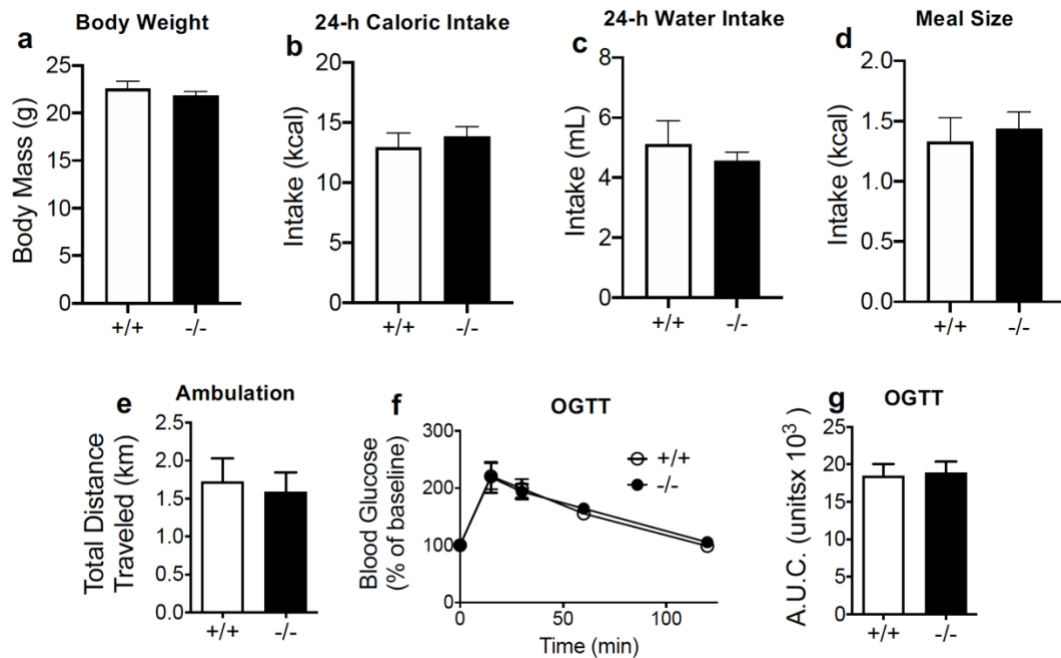


Figure 1.4. Conditional intestinal epithelium CB_1R -deficient mice display no changes in baseline feeding parameters, motor activity, or glucose clearance. Unpaired Student's t-test, two-tailed (a-e, g; $p > 0.05$); Two-way Repeated Measures ANOVA with Holm-Sidak's multiple comparison tests (f; not significant). Results are expressed as means \pm S.E.M; $n = 7-8$ per condition (a-e), $n = 3-4$ (f-g). +/+ = Int CB_1 +/+ mice, -/- = Int CB_1 -/- mice; OGTT = oral glucose tolerance test; AUC = area under the curve.

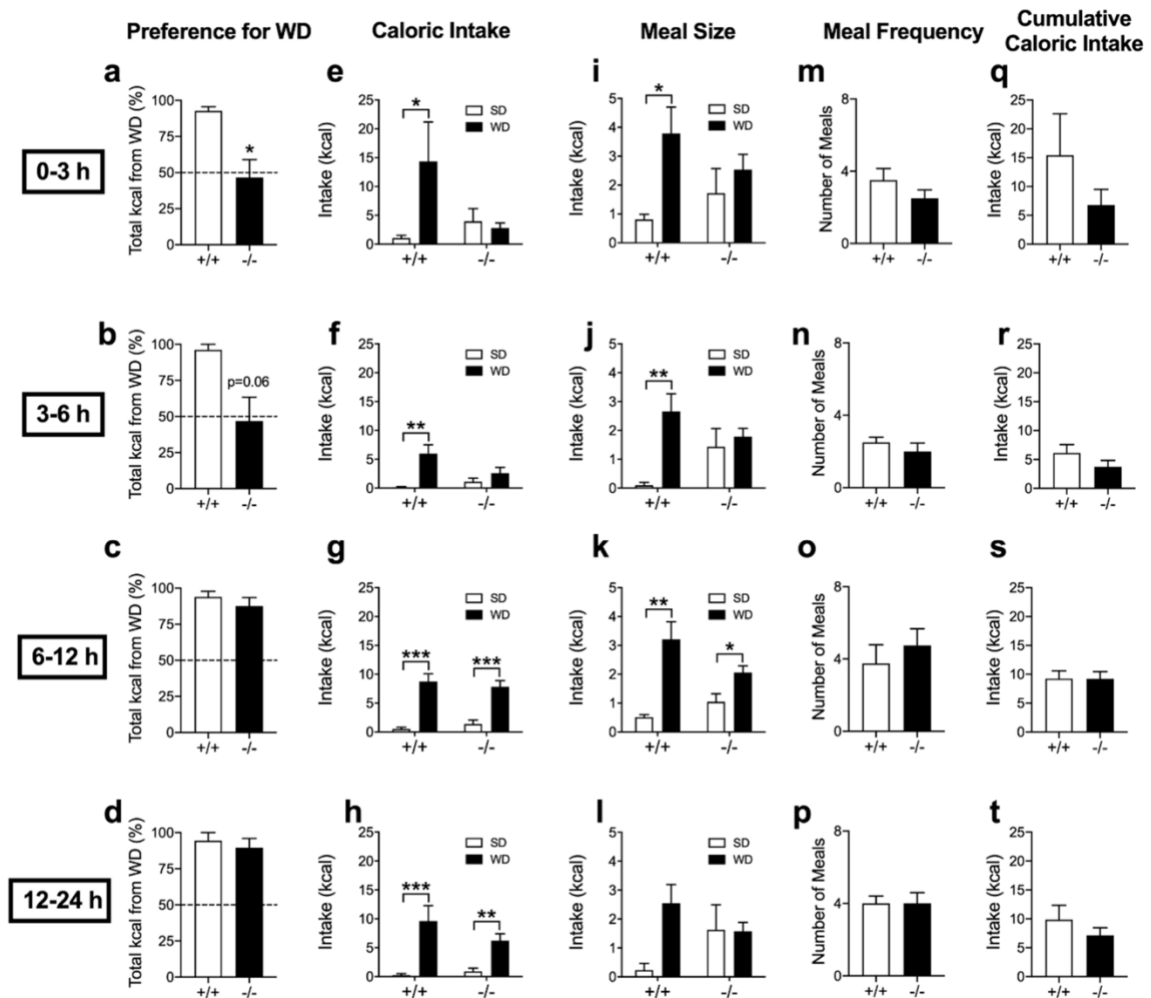


Figure 1.5. Acute preferences for western diet are absent in conditional intestinal epithelium-specific CB₁R-deficient mice.

When compared to IntCB₁^{+/+} control mice, preferences for western diet (WD) are reduced in IntCB₁^{-/-} mice for up to 12 hours (a-c), an effect absent after 24 hours (d). Similarly, cumulative intake of WD is increased versus SD in IntCB₁^{+/+} control mice, an effect absent in IntCB₁^{-/-} mice (e-g) that is regained after 24 hours (h). Average meal size for WD, when compared to SD, is increased throughout the 24 hour preference test in IntCB₁^{+/+} control mice, an effect absent in IntCB₁^{-/-} mice (i-l). Total cumulative intakes were similar between groups throughout all time points (m-p). Unpaired Student's t-test, two-tailed (a-d, m-p); Two-way ANOVA with Holm-Sidak's multiple comparison tests (e-l); * = p<0.05, ** = p<0.01, *** =p<0.001. Results are expressed as means ± S.E.M; n = 4-8 per condition.

Chapter 2: Chronic Phytocannabinoid Exposure Reduces Adipoinsular Dysfunction During Diet-Induced Obesity

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Abstract

Chronically elevated endocannabinoid (eCB) system activity is associated with caloric overconsumption during obesity. Emerging evidence reports inverse associations between cannabis use and prevalence of obesity, type 2 diabetes, and metabolic syndrome in humans. In this study, we investigated the impact of chronic cannabinoid exposure on the development of DIO and examined the potential mechanisms involved with the amelioration of metabolic dysfunction following cannabinoid exposure. Adipoinsular function was assessed following chronic exposure to pure THC or THC dose-match whole cannabis in an adult mouse model of obesity. Body weights and food intake were measured daily throughout the 30d exposure period before assessing glucose tolerance and insulin sensitivity. Changes in adipose tissue biology were assessed by histological

and genetic analysis. Additional in vivo and in vitro exposure studies in adipose-specific CB₁R knockout mice and 3T3-L1 adipocytes, respectively, further examined the role of the eCB system in mediating the pro-metabolic effects of cannabinoid exposure. Both THC and extract led to decreases in body weight and white adipose tissue mass in DIO mice, which was accompanied by transient reductions in food intake. Drug treatments also reversed DIO-associated changes in expression of genes that regulate the adipoinular axis. Specifically, extract more potently restored levels of adipokine levels found in lean control mice fed a low-fat/no sucrose standard lab chow. Moreover, chronic exposure to extract, but not THC, was associated with improved glucose clearance in DIO mice. Similar outcomes were seen in adipose-specific CB₁R knockout mice whereas in vitro studies revealed contrasting effects in adipocyte biology. The relationship between cannabis, THC, and obesity is complex and multifaceted, involving adipose tissue dysfunction and altered adipokine secretion. Altogether, these data suggest chronic cannabinoid exposure influences production of fat-derived adipokines that control glucose homeostasis. Moreover, results highlight an ability for cannabis extract to affect the adipoinular axis and ameliorate dysfunctional energy metabolism associated with obesity.

Introduction

Adipose tissue overabundance is a hallmark of diet-induced obesity (DIO) and a strong risk factor for development of type 2 diabetes mellitus (T2DM) [1, 2].

DIO is primarily driven by an excessive intake of high-calorie foods which are typically high in fat and sugar content. The caloric surplus from a Westernized Diet (WD) leads to hypertrophy and hyperplasia of white adipose tissue (WAT). As the primary storage units for excess energy, adipocytes composing WAT also secrete adipokines involved with various autocrine, paracrine, and endocrine processes. Adipokines influence insulin secretion and insulin reciprocally regulates adipokine production, thus forming a dynamic feedback loop termed the adipoinsular axis[3]. Adipoinsular dysfunction in DIO can be characterized by the dysregulation of prominent adipokines which modulate insulin sensitivity, lipid metabolism, and appetite. Adiponectin, adipisin (also known as complement factor D; Cfd), leptin, fibroblast growth factor 21 (FGF21), and secreted frizzled-related protein 5 (SFRP5) are among the dysregulated adipokines implicated in DIO-associated insulin resistance. Thus, factors influencing adiposity can directly impact the dysregulated production of adipokines involved with glucose homeostasis.

While dietary habits and sedentary lifestyles play pivotal roles in the development of obesity, emerging research suggests that the endocannabinoid (eCB) system, primarily influenced by compounds in cannabis, may contribute to its etiology. One of the well-known effects of THC is its impact on appetite stimulation, which is often referred to as the "munchies." This phenomenon is primarily mediated through CB₁ receptor activation as THC is a partial agonist of CB₁. Despite these appetite-inducing effects, epidemiological studies on the association between cannabis use and obesity have reported paradoxical findings.

Chronic cannabis use has been associated with lower rates of obesity and T2DM, which suggests a more complex interaction between cannabinoids, adipokines, and obesity. Moreover, peripheral eCB signaling is a key determinant of energy homeostasis that becomes dysregulated in DIO. Anandamide (AEA) and 2-arachidonoylglycerol (2-AG), the endogenous cannabinoids that activate the cannabinoid type-1 (CB₁) and type-2 (CB₂) receptors, contribute pivotal roles to the development of obesity by driving increased food intake [4]. In adipose tissue, CB₁ activation has been linked to significant alterations in the secretion of several adipokines including adiponectin, adipisin, and leptin. Additional research is required to understand cannabis' impact on adipokine and endocannabinoid dysregulation. These studies could offer novel insight for the development of therapeutic strategies to improve insulin sensitivity, manage inflammation, and mitigate the impact of adipose dysfunction on the development of T2DM.

In this study, we evaluated the metabolic impact of chronic THC exposure in a DIO mouse model. Specifically, we aimed to investigate the effects of chronic (30d) daily exposure to pure THC or whole cannabis extract matched for THC content quantified via Ultrahigh Performance Liquid Chromatography and Tandem Mass Spectrometry (UPLC/MS/MS). Utilization of UPLC/MS/MS technology also allowed for the detection of discrete differences in endocannabinoids (eCBs) levels within WAT and in circulation. Generation of conditional genetic knockout mice lacking adipose-specific CB₁R (AdiCB₁^{-/-}) via CreLox recombination was used to further explore the role of adipocyte CB₁R in response to chronic THC exposure.

Additional in vitro experimentation in cultured 3T3-L1 adipocytes was also performed to assess ECS function and adipocyte biology during chronic (14d) cannabinoid exposure. Altogether, data from these investigations recapitulate many of the pro-metabolic improvements observed in cannabis users and overall demonstrates differences in energy metabolism that arise in response to pure THC versus whole cannabis extract.

Materials & Methods

Mouse models & diets

Male C57BL/6 mice (Taconic, Oxnard, CA, USA) were group-housed and maintained on a 12h:12h light:dark cycle with ad libitum access to food and water, unless indicated otherwise. Conditional adipose-specific CB₁R knockout mice (Ati-CB₁R-KO) were generated by breeding Cnr1loxP/loxP mice (Cnr1tm1.1 mrl; Taconic, Oxnard, CA; Model #7599) with AdipoqCreERT2 mice, both of C57BL/6 background. The resulting Cnr1fl/flAdipoqCreERT2 transgenic mice expressed excisable loxP genetic sequences flanking the CB₁ gene (Cnr1) with Cre-recombinase expression under the control of the adipose-specific adiponectin (adipoq) promoter. Gene deletion was induced in 4- to 6-week-old Cnr1fl/flAdipoqCreERT2 mice treated with tamoxifen (40 mg/kg per 4 mL corn oil), which was intraperitoneally (IP) administered daily for five consecutive days. Cre-negative Cnr1loxP/loxP mice served as wildtype controls following tamoxifen treatment. After 72h following the final tamoxifen injection, mice were transferred

back to home cages for a one week acclimation period. All mice were genotyped for transgenes via PCR using DNA extracted from tail clippings using the following primers (5'-3'): GCAGGGATTATGTCCCTAGC (CNR1-ALT), CTGTTACCAGGAGTCTTAGC (1415-35), GGCTCAAGGAATACACTTATAACC (1415-37), GAGTCTGCCTTTCCCATGAC (22925), and TCCCTCACATCCTCAGGTTC (22926).

Adult mice, 7-8 weeks-of-age, were placed on a high-fat, high-sugar western-style diet (WD; Research Diets D12709B, New Brunswick, NJ, USA; 40% kcal from fat, 17% kcal from protein, 43% kcal from carbohydrates as mostly sucrose) or maintained on a standard rodent chow diet (SD; Teklad 2020x, Envigo, Huntingdon, UK; 16% kcal from fat, 24% kcal from protein, 60% kcal from carbohydrates) for a total of 60 days. Mice were age- and weight-matched to begin experiments with body weight and food intake measurements taken daily. Motor activity was measured in mice that were single-housed in two-hopper feeding chambers (TSE Systems, Chesterfield, MO, USA) after being given 3 days to acclimate before experiments. All procedures met the U.S. National Institute of Health guidelines for care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California Riverside.

Cannabis extraction and LCMS quantitation

Whole cannabis flower was weighed and submerged in ice-cold 100% ethanol (KOPTEC, Decon Labs, PA, USA) before being placed on a shaker for 15

minutes at 125 rpm. The extract was filtered and dried under N₂ stream 37C (ultrapure 99.9%) and then heated for 1h at 120°C for complete decarboxylation of cannabis oil concentrate. Chemical analysis of phytocannabinoid content in extracts, including acidic precursors such as THCA and CBDA, was performed using Acquity Class I UPLC coupled with Xevo TQ-S Micro Mass Spectrometry (Waters Corporation, MA, USA). Chromatographic separation of cannabinoids using an Acquity UPLC BEH C18 column was performed using previously established methods for lipid quantification[5]. Multiple Reaction Monitoring (MRM) under positive ion mode (ES⁺) conditions for analyte detection used the following mass-to-charge ratios (m/z), cone voltages, and collision energy values, respectively: Δ⁹-THC (315.2>193.0) = 8v, 18v; CBD (315.2>122.9) = 8v, 30v; THCa (359.3>94.9) = 2v, 26v; CBDa (359.2>219.0) = 26v, 30v; Δ⁹-THC-D₃ (318.3>196.1) and CBD-D₃ (318.3>196.1) = 18v, 18v. Generation of standard calibration curves included deuterated standards used for cannabinoid quantification. Extracts were prepared in ethanol and stored at -20°C until day-of-use.

Drug preparation and administration

After 30 days on respective diets, mice began the chronic exposure to pure THC (5 mg/kg) or whole cannabis oil extract matched for THC content. Vehicle consisted of 5% dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA), 5% Tween-80 (Chem Implex Intl Inc., Wood Dale, IL, USA), and 90% sterile saline (0.9% w/v; LabChem Inc., PA, USA). Compounds were prepared freshly each day

by adding Tween-80 to drug aliquots followed by heated-bath sonication for 30 minutes. Saline was added drop-wise before additional sonication for 10 minutes. Daily IP administration, using 25G needles, occurred 1h before the dark cycle (6pm). Injection sites alternated between the left and right peritoneal area to minimize tissue scarring.

Assessment of glucose homeostasis

Metabolic tests were performed in separate cohorts of mice after 3 weeks (21d) of daily cannabinoid exposure. Mice were fasted overnight (16h) or 6h while housed in wire-bottom cages to prevent coprophagia leading up to glucose tolerance tests (IP GTT) and insulin tolerance tests (IP ITT), respectively. Blood-glucose measurements were taken with an Accu-Chek Aviva Plus glucometer (Roche, IN, USA) after administering either glucose (2g/kg) or insulin (1 IU/kg) solution. Tail-snip blood samples were collected at baseline (0 minutes), 15, 30, 60, and 120 minutes post-injection for both tolerance tests. After metabolic tests, mice were returned to home cages with respective diets for the remaining duration of the daily exposure period.

Tissue processing and histology

Animals were euthanized the morning following the final injection and tissues were harvested, rinsed in phosphate-buffered saline (PBS; pH 7.4 at 1X, Alpha Teknova, CA, USA), flash-frozen in liquid nitrogen, and stored at -80°C for future analysis. Epididymal fat (eFat) was weighed prior to being flash-frozen and subcutaneous, or inguinal fat (iFat), was collected from beneath the hind legs.

Blood samples collected in EDTA-coated tubes (BD and Co., NJ, USA) via cardiac puncture were centrifuged at 1,500g for 10 minutes at 4 °C before storing plasma at -80°C. Plasma collected from mice that completed the 30d exposure without any metabolic testing was used for enzyme-linked immunoassays (ELISA) measuring circulating levels of leptin, insulin (Invitrogen, Thermo Fisher Scientific, USA), adipisin (Boster Biological Technology, Pleasanton, CA, USA), and triglycerides (Cayman Chemical, Ann Arbor, MI, USA). For histological analyses, 1-2 gram(s) of eFat was fixed in 10 mL of 10% w/v buffered formalin (4% paraformaldehyde) for 24h at 4°C before embedding in tissue freezing medium (Electron Microscopy Sciences, PA, USA). Using a cryostat set at -35°C (Leica CM1950, Leica Biosystems, IL, USA), samples were sectioned at 10 µM-thickness then mounted on charged glass slides (Fisher Scientific, PA, USA) before drying overnight. Sections were stained with hematoxylin (Abcam, MA, USA) and then rinsed with xylene (Sigma-Aldrich Co., MO, USA) before imaging using an Olympus EP50 bright-field microscope at 10x magnification. Image J software was used to quantify average adipocyte area.

Gene expression analyses

Areas used for RNA isolation and processing were sanitized with 70% ethanol solution and treated with RNase inhibitor (RNase out, G-Biosciences, St. Louis, MO, USA). Total RNA from eFat and iFat was extracted by method of RNeasy kit (Qiagen, Valencia, CA, USA) and first-strand cDNA was generated from normalized RNA using M-MLV reverse transcriptase (Invitrogen, Carlsbad,

CA, USA). Quantitative RT-PCR was performed with preconfigured SYBR Green assays (Biorad, Irvine, CA, USA) and PrimePCR gene-specific primers. Reactions were run in duplicates and expression was measured using $2^{-\Delta\Delta Ct}$. Normalized RNA from both fat depots was used for NanoString CodeSet (NanoString Technologies, Seattle, WA, USA) analysis designed with probe sequences of genes related to adipose tissue maintenance, metabolism, and differentiation. NanoString gene expression was normalized to lean controls and values are expressed as relative fold-change.

Endocannabinoid measurement

Lipid extractions and subsequent UPLC/MS/MS analyses were performed on collected tissues, as previously described [5]. Briefly, 10-20 mg of tissue (100 μ l plasma) was homogenized in methanol containing internal standards for 2-AG and AEA (Caymen Chemicals, Ann Arbor, MI). Tissue lipids were extracted using chloroform and water washes prior to organic phase collection and separation via silica gel column chromatography. Collected eluates were dried under a gentle N₂ stream at 37°C before immediate resuspension in 100 μ l of 1:1 UPLC-grade chloroform:methanol, then analyzed via ultra-performance liquid chromatography couple to tandem mass spectrometry (UPLC/MS²). Multiple Reaction Monitoring (MRM) under positive ion mode (ES⁺) conditions for analyte detection used the following mass-to-charge ratios (m/z), cone voltages, and collision energy values, respectively: AEA (348.3>62.0) = 30v, 14v; 2-AG (379.3>287.3) = 30v, 12v; [2H₄] AEA (352.3>66.1) = 26v, 16v and [2H₅] 2-AG (384.3>93.4) = 25v, 44v. Generation

of standard calibration curves included deuterated standards used for cannabinoid quantification.

3T3-L1 cell culture differentiation & exposure

3T3-L1 pre-adipocytes (C-173, ATCC, USA) were cultured and maintained at 37°C in 5% CO₂ (HeraCell Vios 160i CO₂ incubator, Thermo Scientific). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2mM L-glutamine, 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin (Gibco, Grand Island, NY, USA). Pre-adipocytes were passaged upon reaching 80% confluency in 25 or 75 cm² polystyrene tissue culture flasks (CC7682-4825, CytoOne). Cultures were split by incubating in 0.25% trypsin-EDTA (Sigma-Aldrich Co., MO, USA) for 5 minutes at 37°C. Cells were seeded in 12- or 24-well polystyrene cell culture plates (Costar REF-3513, Corning Inc., USA) at a density of 4.0x10⁵ cells per well. After 48-72h, confluent cells were washed twice with DPBS before inducing differentiation by adding differentiation media (DM), which was prepared day-of-use with supplemented DMEM containing 3-isobutyl-1-methylxanthine (IBMX; 0.5 mM; Sigma), dexamethasone (DEX; 1 µM; Sigma), and insulin (INS; 20 µg/mL; Sigma). After 48-72h, DM was replaced with post-differentiation maintenance media (MM) consisting of supplemented DMEM with insulin (20 µg/mL). MM was renewed every 48-72h throughout the 14-day differentiation period.

Fully differentiated adipocytes were washed twice with DPBS before beginning the cannabinoid exposure period. THC (1-10 µM) or THC-dose matched

whole cannabis extract was added to wells with MM and renewed with each media change over 14 days, with 100% ethanol serving as the vehicle control. Following exposure periods, media was collected and stored at -80°C for future ELISA analyses. Cells were washed twice with phosphate-buffered saline (DPBS 1x; Thermo Scientific) then incubated with trypsin for 5 minutes before adding Qiazol for RNA extraction (Qiagen, USA). Isolated RNA from cells was processed according to the manufacture's protocol and was used for gene expression analysis via RT-qPCR.

Oil Red O staining

Mature 3T3-L1 adipocytes were fixed with 4% paraformaldehyde (10% formalin) for 30 minutes at room temperature. Cells were then washed with 60% isopropanol and wells were allowed to completely dry before adding 500 µL of Oil Red O (ORO) solution for 10 minutes at room temperature. ORO was subsequently aspirated from wells before washing with 1 mL dH₂O. Plates were then washed gently 4 times under running tap water and additional dH₂O was added to wells until live imaging analysis (Incucyte S3, Sartorius) was completed. Plates were dried overnight before 500 µL of 100% isopropanol was added to each well with gentle shaking for 10 minutes at room temperature. ORO was eluted from cells and absorbance was measured at 490nm (BioRad iMark Plate Reader).

Seahorse analysis

Cellular bioenergetic profiling of 3T3-L1 adipocytes following chronic cannabinoid exposure was performed via Seahorse ATP XF Real-Time ATP Rate

Assay (Agilent Technologies, Santa Clara, CA). 3T3-L1 cells were seeded in Seahorse XFe96 plates at a density of 4.0×10^4 cells per well. After 48-72h, pre-adipocytes began the respective 14d differentiation and exposure periods, as detailed above. One day prior to the assay, the XFe96 cartridge was filled with Seahorse XF calibrant (200 μ L per well) and incubated overnight in a CO₂-free incubator at 37°C. On the day of assay, 3T3-L1 cells were primed with Seahorse XF DMEM (pH 7.4, 10mM glucose, 2nM L-glutamine, 1 mM sodium pyruvate) and were kept in a 37°C humidified incubator at 5% CO₂ for one hour prior to beginning the Seahorse XF Real-Time ATP Rate assay.

Statistical analysis

Data were analyzed by GraphPad Prism 8 software using either unpaired Student's t-tests (two-tailed), ordinary one-way ANOVA, or two-way ANOVA with Holm-Sidak's multiple comparisons post-hoc test when appropriate. Results are expressed as means \pm S.E.M. and significance was determined at $p < 0.05$.

Results

Body weight reductions are concurrent with transient differences in food intake following chronic cannabinoid exposure

To assess the impact of chronic cannabinoid exposure on DIO, measurements for body weight, motor activity, food intake were recorded daily during the 30d drug exposure period (Figure 1A-C). Prior to the initial exposure, DIO animals (obese controls) weighed significantly more than lean control

counterparts fed a standard rodent chow (Figure 1D, $p < 0.001$). Overall change in body weight indicates significant reductions in both THC and extract cohorts, while vehicle controls gained weight throughout the exposure (Figure 1E, $p < 0.001$). Final body weights for THC- and extract-treated animals were significantly reduced compared to obese controls after the 30d exposure period (Figure 1F, $p < 0.05$, $p < 0.01$). Daily motor activity, represented as daily distance traveled, showed no significant differences across groups (Figure 1G). Despite maintaining significantly higher cumulative intake compared to lean controls (Figure 1H, $p < 0.001$), THC- and extract-treated mice initially displayed transient reductions in food intake that normalized by day 10 (Figure 1I). Collectively, these data suggest that the time-dependent gain in body weight seen in DIO mice fed WD is ameliorated following chronic exposure to THC or cannabis extract.

Amelioration of glucose dyshomeostasis is associated with reduced adiposity in cannabinoid-treated mice.

To determine if THC and extract exposure differentially affect glucose homeostasis, metabolic testing was performed during the exposure period. Glucose tolerance testing revealed impaired glucose clearance capacity in DIO mice that was significantly improved with extract treatment, an effect that was absent in THC-treated animals (Figure 2A, $p < 0.05$). To further investigate mechanisms of this response, an insulin tolerance test was performed in a separate cohort of mice. Although not statistically significant, extract treatment

improved insulin sensitivity (Figure 2B, $p < 0.054$). Furthermore, both THC and extract treatment resulted in significantly reduced fasting blood glucose levels comparable to lean controls (Figure 2C, $p < 0.05$, $p < 0.01$). Similarly, elevated levels of circulating insulin found in DIO mice were reversed following chronic exposure to THC or extract (Figure 2D, $p < 0.05$). To assess if reduced adiposity was associated with improvements in glucose homeostasis, we also investigated the impact of chronic cannabinoid exposure on circulating adipokines. Circulating levels of leptin (Figure 2E, $p < 0.05$, $p < 0.01$) and adiponin (Figure 2F, $p < 0.01$) were comparable to lean controls and overall more potently restored by extract treatment. Moreover, significant reductions in visceral epididymal fat mass were observed in both THC- and extract-treated mice (Figure 2G, $p < 0.01$). Histological analysis of epididymal fat tissue revealed differences in adipocyte morphology (Figure 2H), with significantly reduced average adipocyte area seen in THC and extract treatment groups.

Visceral WAT gene expression is differentially impacted by THC and extract

Gene Ontology (GO) and enrichment analyses revealed altered expression of genes involved with energy metabolism in visceral WAT of THC- and extract-treated mice (Figure 3A). When compared to vehicle, dysregulated adipokine gene expression is normalized following extract treatment and, to a lesser degree, in mice treated with THC (Figure 3B-F, $p < 0.05$). GLUT4 expression within adipose tissue of DIO mice remained significantly downregulated relative to lean controls,

regardless of treatment (Figure 3G, $p < 0.05$). Minimal changes occurred in gene expression related to fatty acid metabolism, as compared to DIO controls (Figure 3H-I, $p < 0.05$). Further normalization of dysregulated gene expression associated with DIO, but restored with THC or extract treatment, includes *Insr* (Figure 3J, $p < 0.05$), *Ppara* (Figure 3K), *Ppar γ* (Figure 3L), and *Sirt1* (Figure 3M, $p < 0.01$). Collectively, restoration of dysregulated adipokines levels is recapitulated by normalized gene expression in WAT of mice treated with THC or extract.

Endocannabinoid tone associated with DIO becomes normalized following chronic THC and extract exposure

Heat map depicting DIO-associated changes in epididymal fat depot endocannabinoid system gene expression following chronic cannabinoid treatment (Figure 4A). Predominant changes were seen in *Cnr1* (Figure 4B, $p < 0.05$) and *Faah* (Figure 4E, $p < 0.05$), but no differences in *Cnr2*, *Dagla*, *Mgll*, or *Nape-pld* expression (Figure 4C, 4D, 4F, 4G, respectively). DIO-induced changes in expression of the ECS were reversed following chronic cannabinoid exposure. Specifically, levels of 2-AG (Figure 4H) and AEA (Figure 4I) in epididymal fat were lowered with THC or extract exposure. While circulating levels of endocannabinoids were also impacted by chronic cannabinoid exposure, extract treatment significantly restored plasma levels of 2-AG (Figure 4J, $p < 0.05$) and AEA (Figure 4K, $p < 0.05$). These alterations to the ECS suggest that the adipocyte

CB₁R may play an important role in restoring adipoinsular axis function following chronic cannabinoid exposure.

Adipocyte CB₁R deletion impacts glucose homeostasis but does not affect body weight reductions caused by chronic cannabinoid exposure

To assess a role of adipocyte CB₁R in adipoinsular function, transgenic mice with conditional deletion of CB₁R adipose-specific were generated. Overall 30d body weight trends in AdiCB₁^{+/+} (Figure 5A) and AdiCB₁^{-/-} (Figure 5B) mice following chronic cannabinoid exposure show similar reductions in body weight (Figure 5C, 5E, $p < 0.001$). EchoMRI analyses revealed differences in body mass composition with no changes in lean mass (Figure 5D, 5F). Extract treated AdiCB₁^{+/+} animals displayed moderate improvement in glucose tolerance (Figure 5G-H, $p < 0.05$) whereas no differences in glucose clearance capacity were seen in AdiCB₁^{-/-} animals chronically treated with THC or extract (Figure 5I-J). These data suggest adipocyte CB₁R plays an important role in regulating glucose homeostasis, but is not necessary to observe the reduced adiposity phenotype following THC or extract exposure.

Chronic cannabinoid exposure in 3T3-L1 cultured adipocytes results in reduced lipid accumulation

To further investigate the impact of chronic cannabinoid exposure on adipocyte physiology, in vitro experimentation was performed in 3T3-L1

adipocytes. Cultured 3T3-L1 pre-adipocytes were differentiated for 14d before beginning a 14d exposure to THC or extract (Figure 6A). Oil Red O staining revealed reduced lipid accumulation in adipocytes exposed to 1-10 μ M THC and THC-dose matched extract (Figure 6B, 6D). Corresponding optical density of eluted ORO measured at 490 nm revealed significant reductions at high THC concentrations, however, both extract concentrations produced similar reduced lipid accumulation (Figure 6C, 6E, $p < 0.01$). Extract treatment led to more potent downregulation of adipokine genes including *Adipoq*, *Cfd*, and *Lep* (Figure 6F, 6G, $P < 0.01$, $p < 0.001$). Gene expression in 3T3-L1 adipocytes treated with 10 μ M THC shows a downregulation in the *Cnr1* gene (Figure 6G, $p < 0.05$). In contrast, low concentrations of THC in extract caused downregulation of *Insr* (Figure 6F, $p < 0.01$) whereas high THC concentrations led to upregulation of *Insr* (Figure 6G, $p < 0.05$, $p < 0.01$). Minimal changes were seen in *Pparg* expression (Figure 6G, $p < 0.05$) and no differences in *Ucp1* expression were observed (Figure 6F, 6G). Only high THC concentrations caused significant reductions in adipsin (Figure 6H, $p < 0.01$) and leptin (Figure 6I, $p < 0.001$) levels found in media from 3T3-L1 adipocytes. In the presence of the CB₁R antagonist AM251 or the CB₂R antagonist AM630, extract was still capable of reducing lipid content in cultured adipocytes at low concentrations whereas reductions were more prominent at high THC concentrations (Figure 6J, $p < 0.05$). In the absence of THC or extract, cannabinoid receptor antagonists displayed reductions in lipid accumulation, with CB₁R antagonism causing greater reductions than CB₂R antagonism; no synergistic

effects in dual-antagonist treatment were observed (Figure 6K, $p < 0.01$, $p < 0.001$). The dose-dependent differences observed here prompted further investigation into possible dose-dependent shift in energy homeostasis in 3T3-L1 adipocytes.

Chronic THC exposure produces dose-dependent changes in cellular bioenergetic profile of 3T3-L1 adipocytes

We next investigated the impact of THC and extract on cellular bioenergetics. A Seahorse assay was performed following 14d exposure in mature adipocytes. Extracellular acidification rate (ECAR), oxygen consumption rate (OCR), and proton efflux rate (PER) measurements in 3T3-L1 cells revealed dose dependent differences in basal rates following low and high THC and THC-dose matched extract treatment (Figure 7A-F, $p < 0.01$, $p < 0.001$). A shift in the bioenergetic profile depicts low THC and extract concentrations are associated with an energetic phenotype, whereas high THC and extract concentrations are associated with a quiescent phenotype (Figure 7G). Glycolytic and mitochondrial respiration ATP production rates are both reduced at high THC and extract concentrations (Figure 7H, $p < 0.05$, $p < 0.01$). As an overall percentage, only extract treatment at both low and high THC concentrations displayed a shift towards more ATP production from glycolysis than from oxidative phosphorylation (Figure 7I, $p < 0.01$, $p < 0.05$). Together, these results indicate dose-dependent

differences in adipocyte bioenergetics for 3T3-L1 cells treated with low and high THC and extract concentrations.

Discussion

DIO is a multifactorial disorder influenced by various molecular mechanisms, including the intricate interplay between adipokines and cannabinoids. CB₁R activation in adipose tissue contributes to insulin resistance through various mechanisms, including impaired insulin signaling, dysregulated adipokine secretion, and altered glucose uptake⁵⁴⁻⁵⁷. Despite the dysregulation of appetite and glucose homeostasis associated with eCB system hyperactivity, results from these studies suggest that chronic cannabinoid exposure improves adipoinular dysfunction associated with DIO. Specifically, chronic exposure to THC and extract reduced adiposity following initial transient reductions in food intake during the first week daily exposure. Regarding glycemic improvements, we postulated that cannabis extract differs from THC in part by differentially impacting the production of multiple adipokines involved with glucose homeostasis. Fat cell enlargement is a major contributor to the development of insulin and leptin resistance⁵⁸, thus morphological analyses showing reductions in average adipocyte area served as a strong indicator of normalized adipokine production. Hyperleptinemia, a key indicator of leptin resistance commonly observed in obesity^{7,59}, was attenuated following chronic exposure to THC and especially cannabis extract. Extract exposure resulted in more potent restoration of additional

circulating adipokines like adiponin. These data align with the effects observed in adipokine gene expression within WAT, wherein THC and extract caused normalization of these and other adipokines.

Chronic cannabinoid exposure reversed many of the typical eCB characteristics associated with DIO. Levels of AEA and 2-AG in epididymal adipose tissue and in circulation more closely resembled levels of the lean phenotype following chronic THC and extract treatment. Downregulation of *Cnr1*, the gene encoding CB₁, within the WAT of cannabinoid-treated mice suggested an altered role for CB₁R in DIO during chronic cannabinoid exposure. By generating a transgenic mouse line with inducible genetic deletion of adipocyte-CB₁R, we observed similar changes in adiposity seen in control and wildtype mice. EchoMRI analyses of body mass composition revealed similar reductions in body fat mass with no changes in lean mass, further indicative of CB₁R-independent alterations to adipose tissue function. Despite its impact shown in glucose tolerance tests, adipocyte-CB₁R appears to be only partially responsible for THC-mediated reductions in adipose dysfunction during DIO. To further study the impact of CB₁R on adipose tissue, we utilized the 3T3-L1 pre-adipocyte cell line, a widely used cell culture model of adipogenesis^{60,61}. These cells can differentiate into mature adipocytes and are responsive to cannabinoid ligands, providing novel insights into the molecular mechanisms underlying CB₁R-mediated effects on adipocyte differentiation, lipid metabolism, and adipokine secretion.^{62,63} Activation of CB₁R in cultured 3T3-L1 adipocytes can mimic the energy-conserving effects observed in

vivo, including enhanced adipogenesis and lipid accumulation. Furthermore, treatment of differentiating adipocyte cells with the antidiabetic drug rosiglitazone, a PPAR γ ligand, enhances thermogenesis in adipocytes as well as causes a reduced expression of CB $_1$ R and increased expression of FAAH⁶⁴. These data are in conjunction with the data on eCB system gene expression in WAT of DIO mice.

Interestingly, chronic exposure to THC and extract caused reductions in lipid content of 3T3-L1 adipocytes. Furthermore, we observed concentration-dependent effects present in cultured adipocytes treated with low (1 μ M) and high (10 μ M) concentrations of pure THC and THC content-matched extract. This range represents the theoretical concentration of THC in blood circulation following consumption of one cannabis cigarette, approximately 3-5 μ M. In addition to reduced lipid accumulation, low and high THC concentrations in cannabis extract resulted in changes in gene expression in many DIO-associated genes. *Lep* and *Cfd*, the genes encoding leptin and adiponectin, respectively, were subject to downregulation most potently by extract. Measuring leptin in media following cannabinoid exposure indicates similar reductions in leptin seen in vivo. Adiponectin, however, was also decreased in the media of adipocytes cultured in high THC conditions, unlike the increased expression and circulation of adiponectin seen in vivo. Notably, THC activation of the adipocyte-CB $_1$ R produces a concentration-dependent decrease in lipid accumulation, however, this effect was not attenuated by CB $_1$ R and/or CB $_2$ R antagonism suggesting a possible receptor-independent mechanism^{65,66}. Nevertheless, differences between THC and extract were further

apparent following a Seahorse assay of cellular bioenergetics, which showed concentration-dependent shifts in ECAR and OCR in fully differentiated 3T3-L1 cells. Only extract, however, caused an overall shift in ATP production which favored glycolysis over oxidative phosphorylation at low and high THC concentrations. We recognize the limitations of in vitro assessment of adipocyte biology in response to chronic cannabinoid exposure given the absence of metabolic challenges presented in DIO, such as chronic low-grade inflammation. Recent evidence suggests a role for alternatively activated macrophages in adipocyte-CB₁R mediated regulation of energy homeostasis⁶⁷. Nevertheless, cannabis extract collectively showed a pronounced impact on adipokine production in vivo and in vitro.

Overall, these studies indicate that in addition to reducing adipose tissue accumulation, THC and extract exposure positively impacted adipoinular function by ameliorating hyperglycemia, hyperinsulinemia, and dysadipokinemia associated with DIO. The modulation of energy homeostasis governed by dynamic cannabinoid and adipokine signaling evidently highlights the therapeutic potential targeting the eCB system for obesity treatment. Cannabis' therapeutic potential in the context of T2DM remains unclear; however, other phytocannabinoids may be affecting the hormonal feedback loop between adipose tissue and the pancreas. In addition to THC, cannabis contains several cannabinoids that influence energy metabolism including cannabidiol (CBD), tetrahydrocannabivarin (THCV), cannabinol (CBN), and cannabigerol (CBG). Lipophilic metabolites of THC, such

as 11-hydroxy-tetrahydrocannabinol (11-OH-THC) and 11-nor-9-carboxy-tetrahydrocannabinol (THC-COOH), are prone to long-term storage in adipose tissue⁶⁸⁻⁷⁰. Other cannabis-derived compounds, such as limonene, also accumulate in adipose tissue and can modify the bioenergetic profile of adipocytes⁷¹⁻⁷³. Understanding the impact of cannabis metabolites on energy homeostasis is crucial given the increasing prevalence of cannabis use, and further research is needed to elucidate the specific molecular mechanisms through which cannabis-derived metabolites stored in adipose tissue may affect glucose homeostasis. Future studies will aim to further elucidate specific mechanisms in the pro-metabolic effects of cannabis exposure and will focus on identifying differential effects on energy homeostasis for THC and other chemical constituents in cannabis.

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Figures

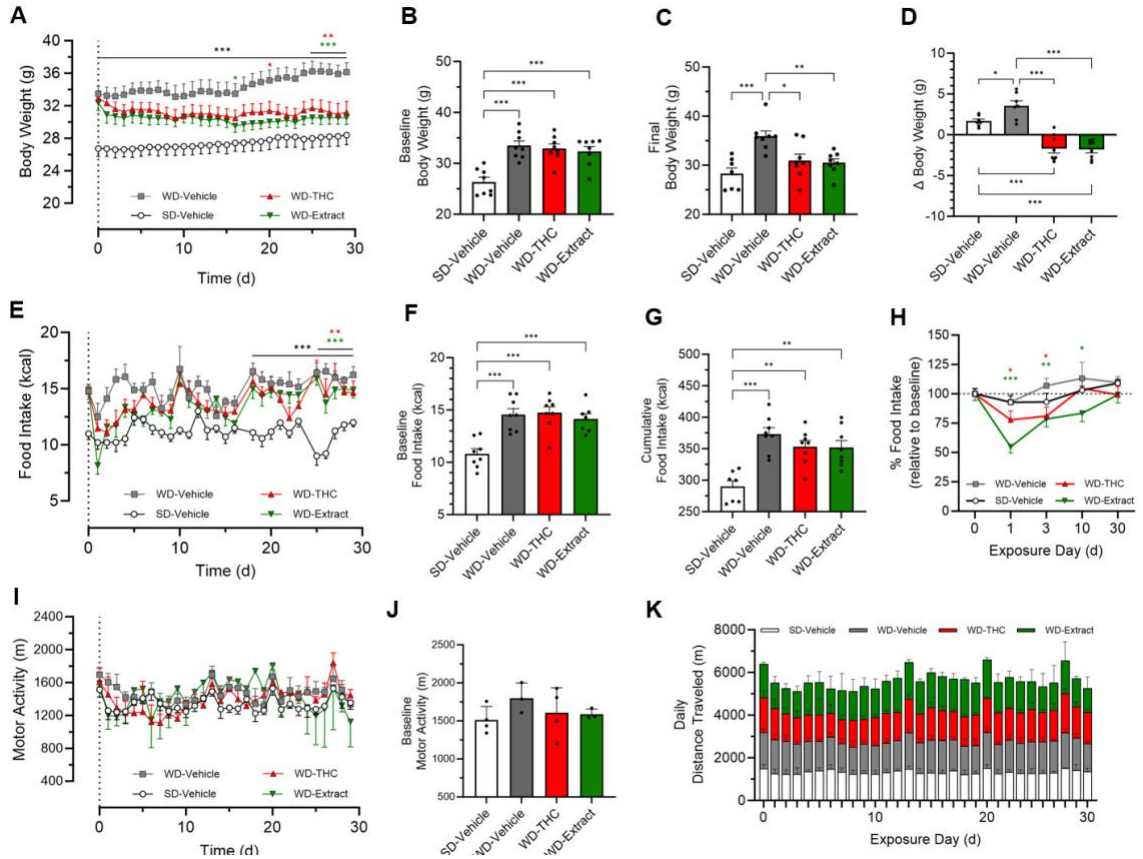


Figure 2.1. Changes in body weight, motor activity, and food intake in mice chronically treated with THC or extract.

Complete 30d trends for (A) body weight, (B) motor activity, and (C) food intake. (D) Baseline body weights after 30d of respective diets. (E) Final body weights and (F) overall change in body weights after 30d of drug exposure. (G) Average daily distance traveled throughout 30d exposure. (H) Cumulative and (I) transient food intake measured during 30d exposure. Two-way ANOVA (A-C, I) and one-way ANOVA (D-H) with Holm-Sidak's multiple comparisons post-hoc tests; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Data are expressed as means \pm S.E.M; $n = 6-8$ per condition; $n = 4$ for motor activity.

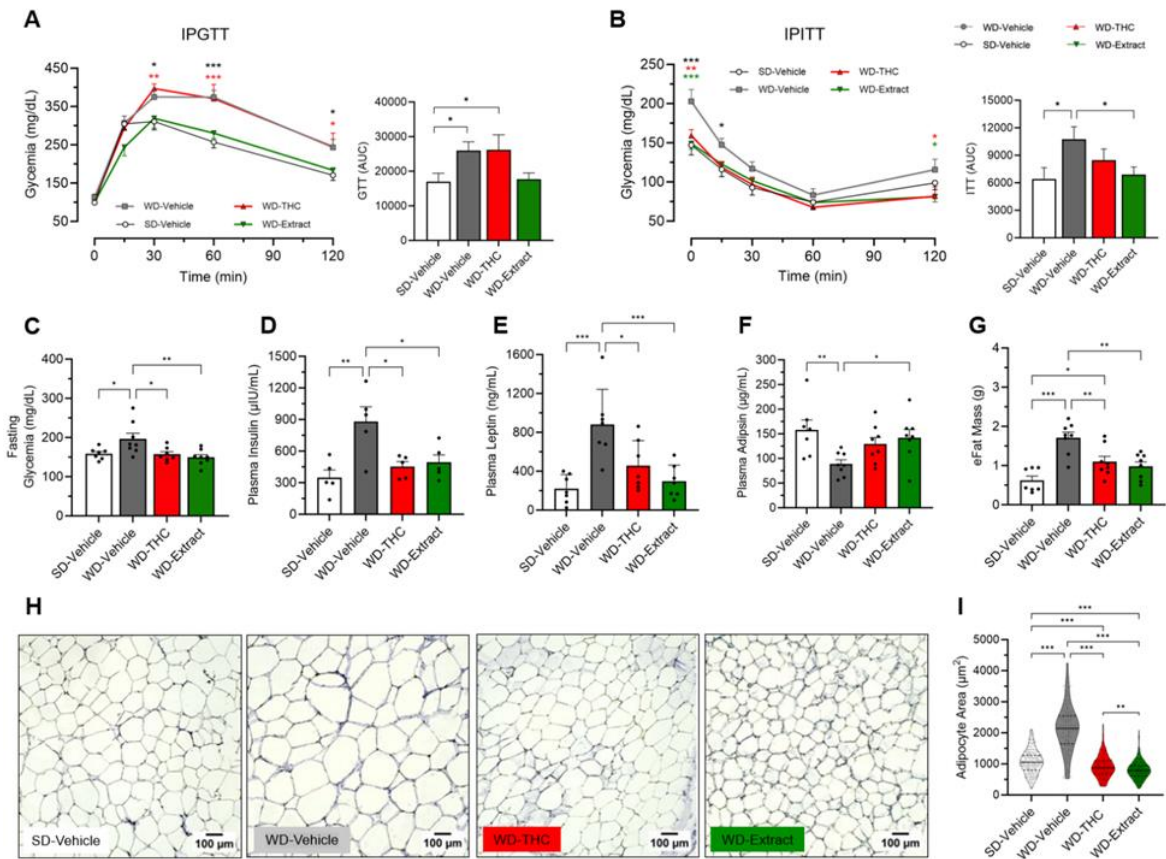


Figure 2.2. Amelioration of glucose dyshomeostasis is associated with reduced adiposity in cannabinoid-treated mice.

Glucose tolerance test curve with GTT AUC (A) following glucose administration (2 g/kg BW) in mice fasted overnight. Insulin tolerance test curve with ITT AUC (B) following insulin administration (1 IU/kg) in mice fasted for 6h. (C) Fasting blood-glucose levels and (D) non-fasted circulating insulin levels following 30d chronic exposure in mice. Circulating adipokine levels for (E) leptin and (F) adipsin measured via ELISA. (G) Overall eWAT mass after 30d exposure. (H) Representative histological sections of hematoxylin-stained eWAT; 10x magnification at 10 μm-thickness; 100 μm scale bar. (I) Average adipocyte area from eWAT of chronically treated mice. Two-way ANOVA (A, B) and one-way ANOVA (C-G, I) with Holm-Sidak's multiple comparisons post-hoc test; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Data are expressed as means \pm S.E.M; $n = 5-8$ per condition; $n = 3$ for histological sections.

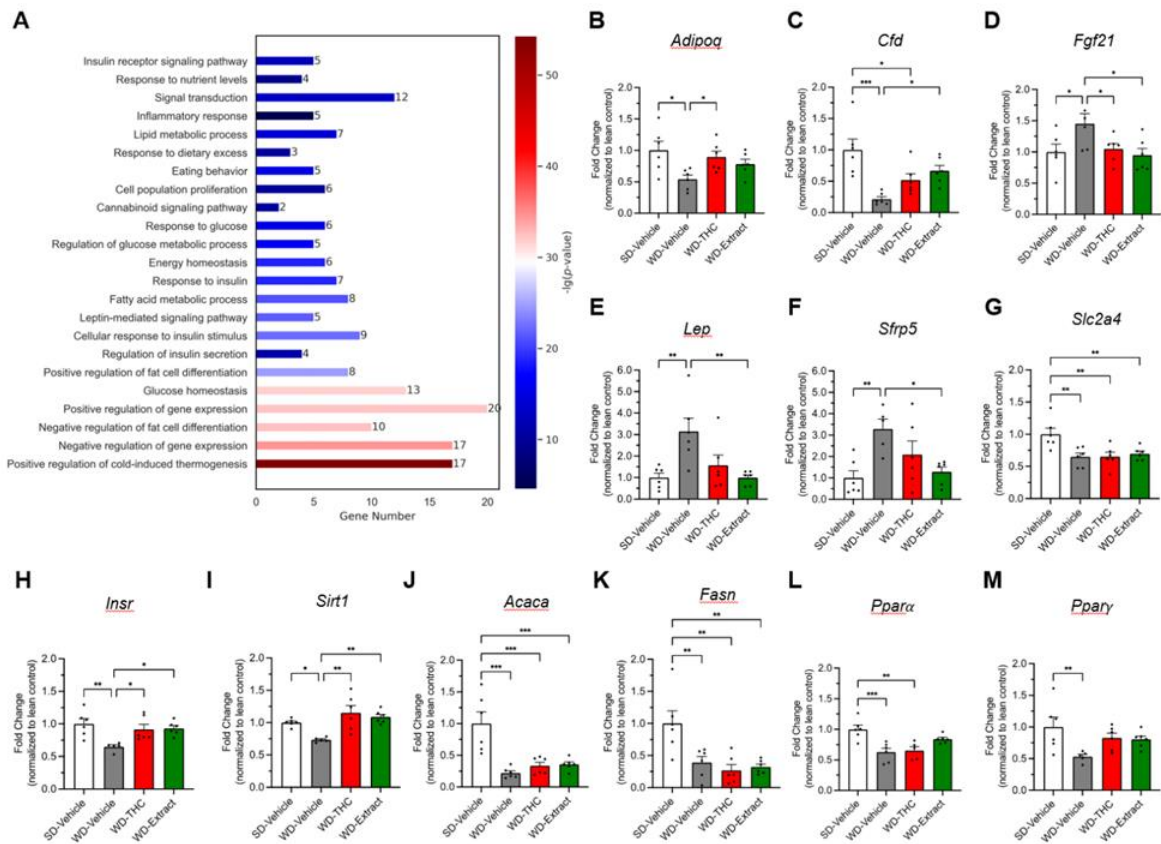


Figure 2.3. Visceral fat gene expression is differentially impacted by 30d exposure to THC and extract.

Gene Ontology (GO) with enrichment analyses (A) for adipose tissue genes impacted by chronic cannabinoid exposure. Dysregulated expression for genes related to the adipoinular axis (B-I), fatty acid synthesis (J-K), and regulation of transcription and differentiation (L-M). *Adipoq*: adiponectin, *Cfd*: adipisin, *Fgf21*: fibroblast growth factor 21, *Lep*: leptin, *Sfrp5*: secreted frizzled related protein 5, *Slc2a4*: glucose transporter 4 (GLUT4), *Insr*: insulin receptor, *Acaca*: acetyl-CoA carboxylase, *Fasn*: fatty acid synthase, *Ppara*/ γ : peroxisome proliferator activated receptor alpha/gamma. NanoString gene expression is normalized to lean controls. One-way ANOVA with Holm-Sidak's multiple comparisons post-hoc test (B, D). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Results are expressed as means \pm S.E.M; $n = 5-8$ per condition.

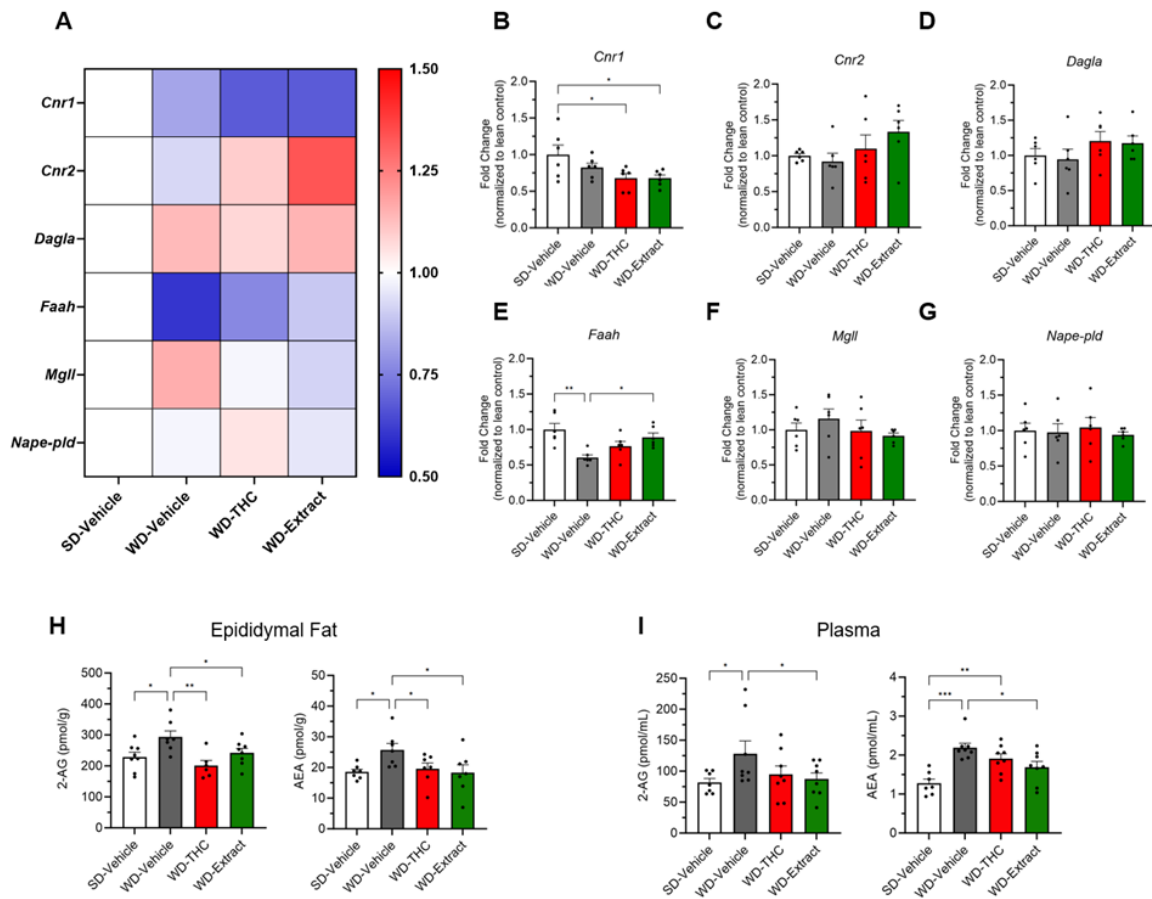


Figure 2.4. Endocannabinoid tone associated with DIO becomes normalized following chronic THC and extract exposure.

Heat map (A) depicting DIO-associated changes in epididymal fat depot endocannabinoid system gene expression following chronic cannabinoid treatment. (B-G) ECS gene expression relative to lean controls. Endocannabinoid levels in eWAT (H-I) and in plasma (J-K). One-way ANOVA with Holm-Sidak's multiple comparisons post-hoc test (B-K); * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Results are expressed as means \pm S.E.M; $n = 5-8$ per condition.

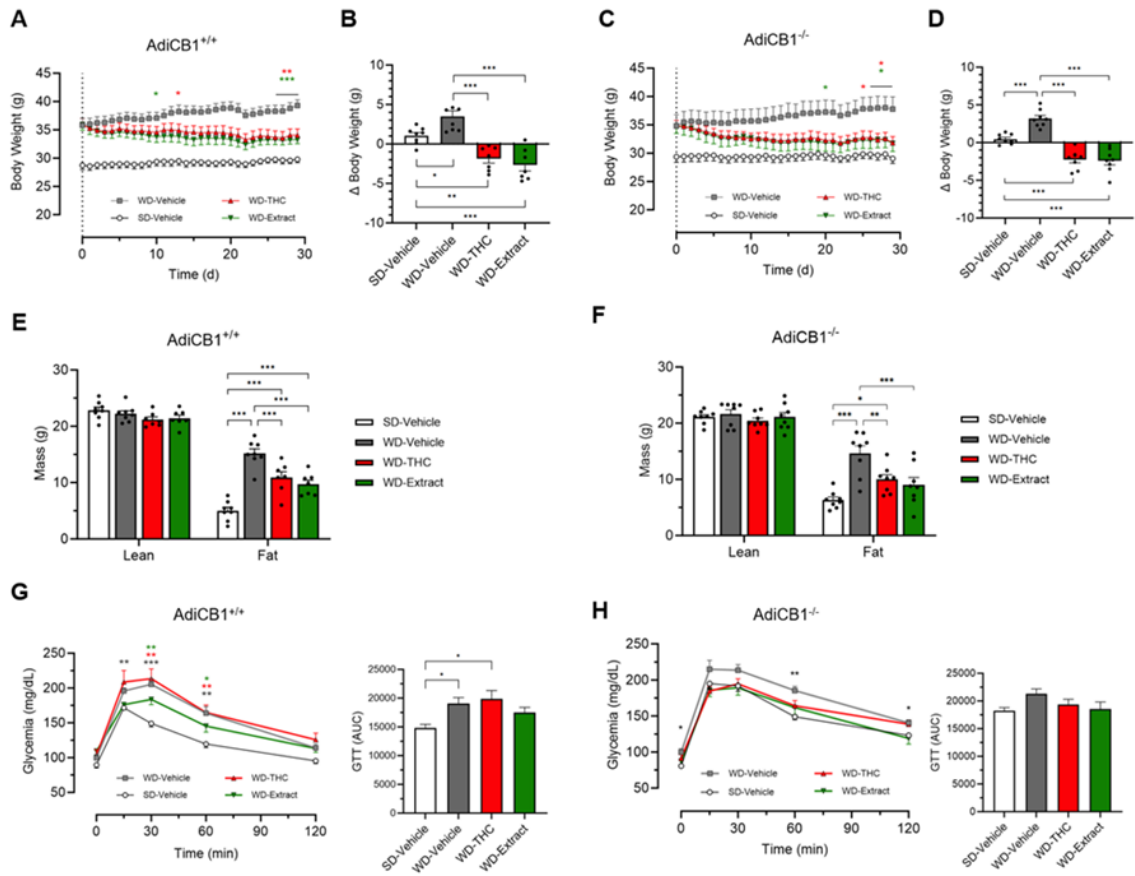


Figure 2.5. Adipocyte CB₁R deletion impacts glucose homeostasis but does not affect body weight reductions caused by chronic cannabinoid exposure. Complete 30d body weight measurements for *Adicb1*^{+/+} (A) and *Adicb1*^{-/-} (C) mice. Overall change in body weight (B, D) and body mass composition (E, F) in *Adicb1*^{+/+} and *Adicb1*^{-/-} mice, respectively. Glucose tolerance test curve with GTT AUC (G) for *Adicb1*^{+/+} mice. Glucose tolerance test curve with GTT AUC (H) for *Adicb1*^{-/-} mice. Two-way ANOVA (A, C, E, F, G, H) and one-way ANOVA (B, D; G, H AUC data) with Holm Sidak multiple comparisons post hoc test, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Results are expressed as means \pm S.E.M; $n = 5-8$ per condition.

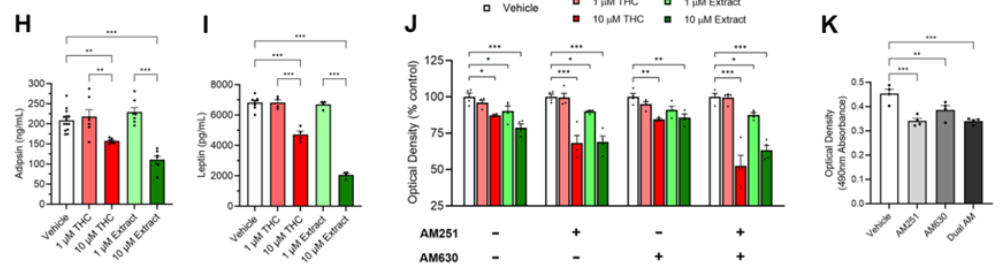
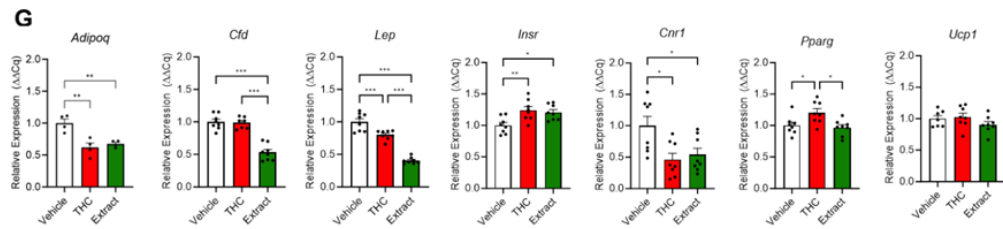
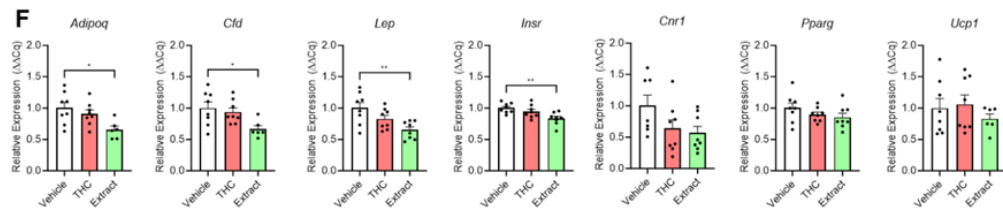
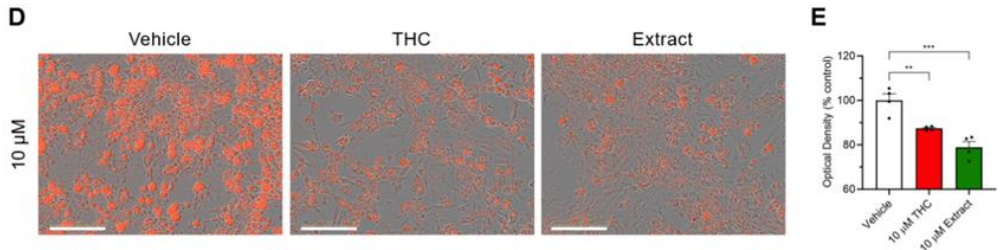
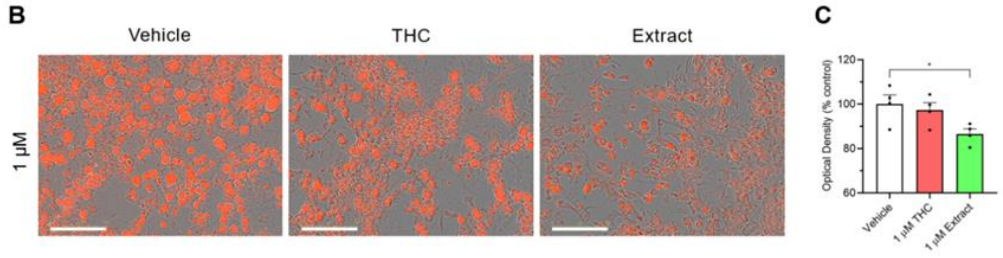
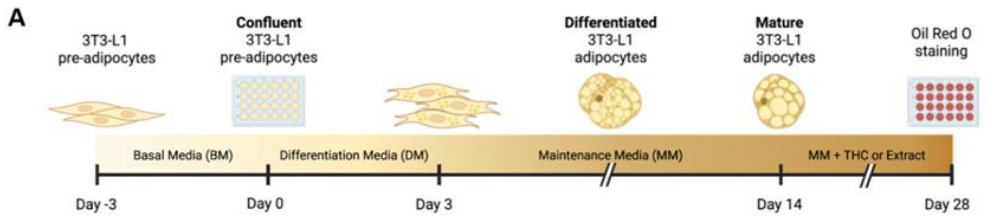


Figure 2.6. Chronic cannabinoid exposure in 3T3-L1 cultured adipocytes results in reduced lipid accumulation.

(A) Differentiation and exposure paradigm in 3T3-L1 cells. (B, D) Oil Red O stained adipocytes exposed to 1 or 10 μM THC and THC-dose matched extract with (C, E) corresponding optical density of eluted ORO measured at 490 nm. (F, G) Gene expression in 3T3-L1 adipocytes treated with 1 or 10 μM THC. (H) Adipsin and (I) ELISA measurements of adipokines in media of cannabinoid-treated adipocytes. (J) Normalized optical density of low and high THC treatment in the presence of CB₁R (AM251) and/or CB₂R (AM630) antagonists. (K) Raw optical density values of control wells treated with only cannabinoid antagonists. One-way ANOVA with Holm-Sidak multiple comparisons post hoc test, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Results are expressed as means \pm S.E.M; n=4-8 per condition.

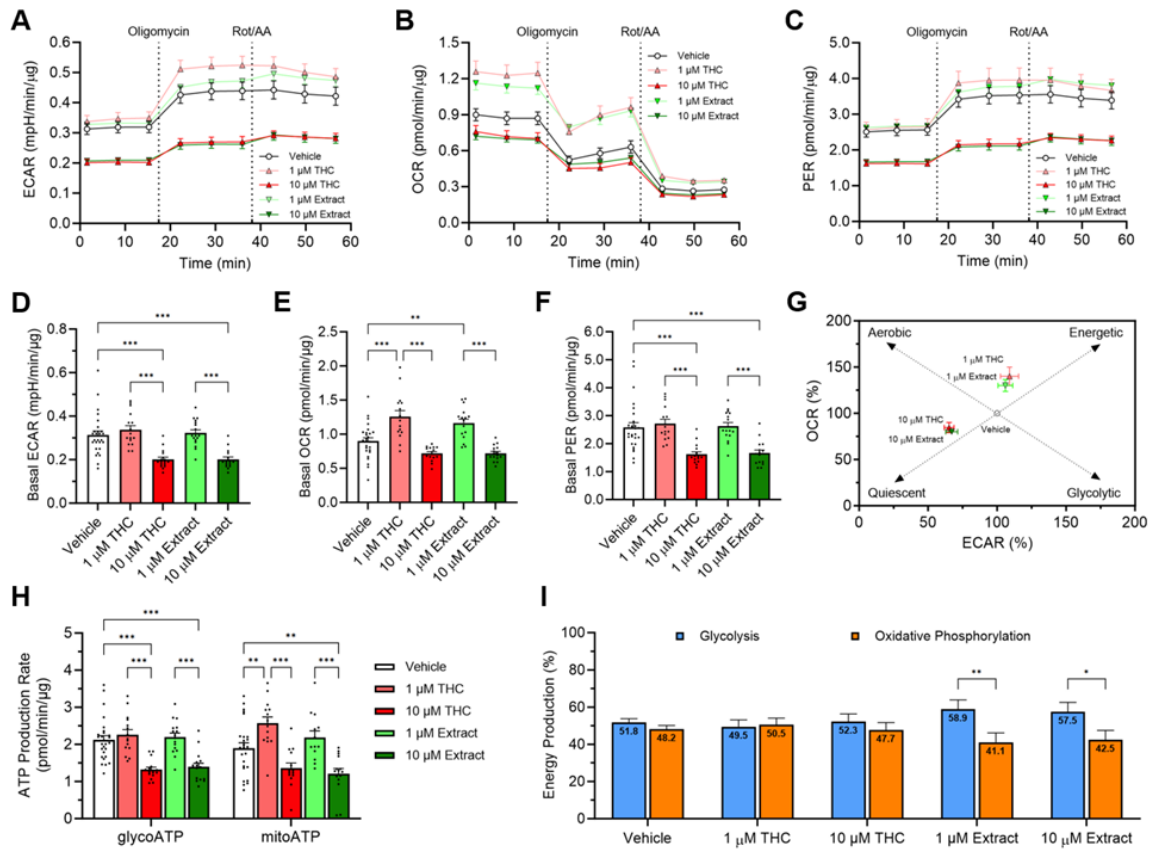


Figure 2.7. Chronic THC exposure produces dose-dependent changes in cellular bioenergetic profile of 3T3-L1 adipocytes. (A) Extracellular acidification rate (ECAR), (B) oxygen consumption rate (OCR), and (C) proton efflux rate (PER) measuring real-time ATP production in 3T3-L1 cells treated with low (1 μ M) and high (10 μ M) THC and THC-dose matched extract concentrations. (D-F) Basal rates for ECAR, OCR, and PER indicating differences in cellular phenotypes based on drug concentration. (G) Energy map of THC- and extract-treated adipocytes based on OCR and ECAR. (H) ATP production rates from glycolysis and mitochondrial respiration. (I) Overall ATP produced from glycolysis and oxidative phosphorylation represented as a percentage. Two-way ANOVA (A-C, H), one-way ANOVA (D-F), and multiple unpaired t-test (I) with Holm-Sidak multiple comparisons post hoc test, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. All data are expressed as means \pm S.E.M; $n = 16$ per condition.

Conclusion

The eCB system plays a multifaceted role in adipose tissue biology, influencing appetite regulation, adipogenesis, and the secretion of adipokines. The interaction between CB₁ receptors and cannabinoids, particularly THC, has profound effects on energy balance and metabolic function. Understanding the complex interplay between CB₁ receptors, adipose tissue, and adipokines is essential for developing targeted interventions that address the global health burden of obesity. DIO is primarily driven by an excessive intake of high-calorie, energy-dense foods coupled with sedentary lifestyles. This caloric surplus leads to the expansion of adipose tissue mass and the subsequent dysregulation of adipokines. As obesity progresses, adipose tissue undergoes changes in its cellular composition and function. Adipogenesis, the process of adipocyte differentiation, is a critical determinant of adipose tissue expansion. Studies using the 3T3-L1 adipocyte cell line have demonstrated that CB₁ receptor activation enhances adipogenesis by promoting preadipocyte differentiation into mature adipocytes^{61,62,74,75}. This effect is mediated by the activation of intracellular signaling pathways involved in adipogenic gene expression¹⁰. Consequently, CB₁ receptor blockade has been proposed as a potential strategy to mitigate excessive adipocyte differentiation, thereby limiting adipose tissue expansion.

Adipokines are central players in the intricate web of interactions between adipose tissue, energy metabolism, glucose homeostasis, and insulin resistance. Their multifaceted roles underscore the importance of adipose tissue as a dynamic

endocrine organ with far-reaching systemic effects. By elucidating the mechanisms by which cannabinoids and adipokines influence metabolic health, researchers and clinicians can develop targeted strategies for preventing and treating obesity-related metabolic disorders, ultimately improving the quality of life for individuals affected by these conditions. The CB₁ receptor's presence in adipose tissue underscores its importance in adipocyte function, adipokine secretion, and metabolic regulation. Dysregulated CB₁ signaling contributes to adipose tissue dysfunction, altered adipokine profiles, appetite dysregulation, and insulin resistance, all of which are key components of obesity-related metabolic complications. A thorough understanding of CB₁ receptor-mediated effects on adipose tissue could pave the way for novel therapeutic strategies targeting this pathway to effectively manage obesity and its associated metabolic consequences. Thus, targeting peripheral cannabinoid receptors, particularly CB₁ in the small intestine and in adipose tissue, with selective antagonists or modulators could help mitigate obesity-related metabolic disturbances.

As a homeostatic system of endogenous lipid signaling molecules, the eCB system is also a target of exogenous cannabinoids, such as those found in cannabis. The primary psychoactive constituent of cannabis is Δ^9 -tetrahydrocannabinol (THC), which is among more than 100 phytocannabinoids found within the plant. These phytocannabinoids, especially THC, are capable of binding to the cannabinoid receptors found both in the central nervous system and the periphery ultimately altering appetite and energy homeostasis^{43,76,77}.

Furthermore, the consequences associated with the dysregulation of energy homeostasis caused by obesity extend to the small intestine and pancreas. Upon introduction of dietary nutrients to the small intestine, the peripheral endocannabinoid system found here affects gut-derived hormone release of GIP, GLP, and CCK, which collectively can control up to 70% of insulin release from the pancreas⁷⁸⁻⁸⁰. In this regard, dysfunction of the eCS may influence the unbalanced glucose homeostasis seen in type 2 diabetes, one of obesity's most common comorbidities. Currently, the consequences on glucose homeostasis and pancreatic function associated with cannabis exposure remain unclear.

Overall, this work highlights the pro-metabolic effects of chronic cannabinoid exposure in DIO mice, which promoted a metabolic profile comparable to lean control mice fed a low-fat/no sucrose standard diet. Together, these results highlight cannabis' therapeutic potential to target the molecular mechanism(s) linking obesity and T2D. Whole cannabis extract contains several non-psychoactive compounds such as tetrahydrocannabivarin (THCV) that can affect adipocyte biology, however the precise biological underpinnings remain unclear^{81,82}. Moreover, the potential anti-inflammatory and metabolic-enhancing effects of CBD warrant further investigation⁸³⁻⁸⁵. With an increase in popularity and overall societal acceptance, it is imperative to further investigate the role of both acute and chronic cannabis use and how the endocannabinoid system may adapt to the increasingly potent marijuana consumed across the world.

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