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Diet-Induced Obesity in Cannabinoid-2 Receptor Knockout Mice and Cannabinoid Receptor 1/2 Double-Knockout Mice

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Abstract

Objective: Evidence suggests that cannabinoid-1 receptor activation is associated with increased food intake and body weight gain. Human epidemiological studies, however, show decreased prevalence of obesity in cannabis users. Given the overlapping and complementary functions of the cannabinoid receptors (CB1R and CB2R), mice lacking CB2R, and mice lacking both CB1R and CB2R were studied.

Methods: High-fat diet (HFD) was used to study metabolic changes in male mice lacking CB2R (CB2^{-/-}) or lacking both CB1R and CB2R (CB-DKO) compared to wild type (WT) mice.

Results: When maintained on HFD, weight gain in CB2^{-/-} mice was not different from WT mice (gaining 19 and 21 grams, respectively), whereas CB-DKO mice gained only 5 grams. There were no significant differences in food intake or locomotor activity between the three groups. Respiratory exchange rate and heat production were elevated in CB-DKO mice, with upregulation of adipose tissue thermogenic genes. Glucose tolerance test and insulin levels indicated increased insulin sensitivity in CB-DKO mice, whereas CB2^{-/-} displayed signs of impaired glucose clearance.

Conclusion: These results indicate that lacking both CB1R and CB2R protected mice from diet-induced obesity, possibly through the prominent role of CB1R in obesity or through an interactive effect of both receptors.

Keywords

Cannabinoids; Body weight; Food intake; Cannabinoid receptor knockout mice

Introduction

The appetite-stimulating effects of Delta-9-tetrahydrocannabinol (THC), the active constituent of cannabis, have been widely reported (1, 2). Evidence suggests that the metabolic effects of cannabis are mediated through the cannabinoid-1 receptor (CB1R, 3). Upregulation of CB1R in adipocyte tissue in individuals with obesity was previously

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reported (4). The administration of CB1R inverse agonist rimonabant (SR 141716) in rodents significantly decreased food intake and body weight (5, 6). Other CB1R antagonists exert similar effects (7, 8, 9). In humans, rimonabant has been shown to reduce body weight alongside improvements in other elements of the metabolic syndrome (10). Using mouse knockout models, CB1R knockout (CB1^{-/-}) was associated with leanness and resistance to diet-induced obesity (11, 12). In agreement, human population-based studies reported higher caloric intake in cannabis users when compared to non-users (13, 14). Hence, with the activation of cannabinoid receptors in cannabis users, the higher caloric intake and decreased physical activity levels (15), a positive association between cannabis use, obesity and type 2 diabetes mellitus can be expected. Interestingly, epidemiological studies with large sample sizes have found reduced prevalence of obesity, insulin resistance and diabetes mellitus among cannabis users (16, 17, 18).

Cannabinoid 2 receptors (CB2R) are expressed mainly in the immune system and mediate immunomodulatory responses (19). Human CB1R and CB2R share 44% overall amino acid identity, with greater homology (68%) at the transmembrane level (20). The CB2R recognizes the same structural groups of agonists as CB1R including THC, with differing affinities in some cases (21). Studies on CB2R and body weight have resulted in mixed findings. Administration of CB2R agonist JWH-015 in obese mice was associated with reductions in food intake and body weight (22), whereas no effect on weight was reported with the administration of the CB2R agonists AM1241 and JWH-133, or antagonist AM630 (23, 24). Using knockout models, Agudo et al. reported that CB2R knockout mice fed with high fat diet (HFD) have reduced body weight gain (25), whereas Schmitz et al. reported that CB2R knockout mice on standard diet were obese with hypertrophy of visceral fat (26). Deveaux et al. reported reduced expression of CB1R in adipose tissue and liver of HFD-fed CB2R knockout mice, suggesting that the reduction of CB1R expression might account for the reduction in body weight gain observed in CB2R knockout mice (24). This altered CB1R expression in CB2R knockout mice may suggest a critical interaction between both receptors. Indeed, efforts to study the potential anti-inflammatory effects of CB2R agonists in disease models are often complicated by the possibility of CB1R interactions and unwanted CB1R activation effects (27, 28).

Herein, we use male mice lacking CB2R and mice lacking both CB1R and CB2R to further evaluate the contribution of the cannabinoid receptors in regulating body weight and glucose metabolism.

Methods

Animals

All mice were on C57Bl/6 background as this background is susceptible to diet induced obesity and insulin resistance (29). Age-matched male mice deficient in CB2R (30) originally created by Deltagen (CB2^{-/-}, Deltagen Inc. CA, USA) and wild-type littermates were obtained from Jackson Laboratory (Jackson Laboratory, ME, USA). Eight-week old male mice deficient in both CB1R and CB2R (double knockout, hereinafter referred as CB-DKO) were generated as previously described (31), and were obtained with the permission of Dr. Andreas Zimmer (University of Bonn, Germany), and bred at Michigan State

University. Mice were housed with up to 5 animals per cage. Mice fed *ad libitum* either LFD (D12450B, Research Diets, NJ, USA), or HFD (D12451, Research Diets, NJ, USA) and water. Table S1 provides the caloric information for each diet. Rooms were maintained at 21 to 24°C and 40 to 60% humidity with a twelve-hour light-dark cycle. All procedures were performed in accordance with guidelines set forth by Michigan State University Institutional Animal Care and Use Committee, and the United States of America regulations concerning the use of animals in research.

Food intake, body weight and composition, and indirect calorimetry

Food intake and body weight were measured weekly for 12 weeks. At baseline (8 week old mice) and after 12 weeks on diet (20 week old mice), body composition (% lean tissue, fat and fluid) was measured in a subset of mice using a Time Domain-Nuclear Magnetic Resonance-based analyzer (Minispec LF50; Bruker, MA, USA). Individual mice were then placed in metabolic cages (TSE PhenoMaster/LabMaster System, MO, USA) to measure metabolic performance, activity, drinking, and feeding (32). Ambient temperature was maintained at 20 to 23°C throughout analysis and the airflow rate through the chambers was adjusted to maintain an oxygen differential of ~0.4% at resting conditions. Data were continuously collected over 7 days, with the first 24-hours used for habituation but excluded from the analysis. Food intake, oxygen consumption (the difference between oxygen input and output, VO₂) and carbon dioxide production (the difference between carbon dioxide output and input, VCO₂) in each chamber were monitored every 3-minutes, while locomotor activity (beam breaks in the x-, y-, and z-direction) was continuously recorded. Respiratory exchange rate (RER) was measured as a ratio of VCO₂ (ml/h/kg) / VO₂ (ml/h/kg), and heat production was measured as (CVO₂ * VO₂ + CVCO₂ * VCO₂) / 1000. The default values for CVO₂ and CVCO₂ were 3.941 and 1.106, respectively.

Glucose tolerance test

After 12 weeks on the specified diet, mice were fasted early in the morning for 5 hours, and then glucose (2 g/kg) was administered by intraperitoneal injection (33). Blood glucose was measured from the tail vein at 0, 30, 60, 90 and 120 min after glucose injection. Blood glucose was measured with a Freestyle Lite Glucometer (Abbott, IL, USA).

Necropsy, Tissue Collection and Preparation

Mice were deeply anaesthetized with isoflurane (3–5% for induction and approximately 2–3% for maintenance) and blood samples were collected in heparinized tubes via cardiac puncture (Becton Dickinson, NJ, USA). Blood samples were centrifuged for 10 minutes at 2000 x g at 4°C. Plasma was collected for measuring cholesterol and triglyceride levels using Beckman Coulter AU 680 analyzer (Beckman Coulter, CA, USA). Plasma insulin levels were measured using ultra-sensitive mouse insulin ELISA kit (Crystal Chem, IL, USA). Epididymal white adipose tissue (WAT), scapular brown adipose tissue (BAT), and liver were either snap frozen and stored at –80°C, or fixed in 10% formalin for further analyses.

RNA analysis

Total RNA was obtained using TRIZOL (Invitrogen, CA, USA) and its concentration was determined by NanoDrop 2000 (Thermo Scientific, IL, USA). Total RNA was reverse transcribed into complementary DNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, MA, USA). Real-time quantitative PCR (qPCR) was performed using SYBR Green Master Mix (Life Technologies, CA, USA), and mouse gene-specific primers (Table S2). Relative amounts of mRNA were calculated using the comparative cycle threshold method and normalized to the abundance of β -actin mRNA.

Immunohistochemistry

Tissue processing and Hematoxylin and Eosin (H&E) staining were performed at Michigan State University Investigative HistoPathology Laboratory. In short, formalin-fixed specimens were sectioned (4 μ), deparaffinized, and underwent heat-induced epitope retrieval in Citrate Plus buffer (ScyTek Laboratories, UT, USA). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide/methanol bath for 30 minutes. Standard micro-polymer complex staining steps were performed at room temperature on the IntelliPATH FLX® automated stainer, and all staining steps were followed by rinses in TBS automation wash buffer (Biocare Medical, CA, USA). Sections were blocked with non-specific protein with Background Punisher (Biocare Medical, CA, USA) for 10 minutes, and then incubated with specific Rabbit anti – Ucp1 (Alpha Diagnostics, TX, USA) in normal antibody diluent (Tris Buffered) for 30 minutes (ScyTek Laboratories, UT, USA). Micro-polymer reagents were subsequently applied for specified incubations followed by reaction development with Romulin AEC™ chromogen and counterstained with CAT Hematoxylin (Biocare Medical, CA, USA).

Statistical analysis—Data are presented as mean and standard error (SEM). Results were analyzed by analysis of variance. Student t-test was used to compare LFD fed mice with HFD fed mice in the same genotype group. One-way ANOVA was used to compare the three genotypes on the same diet. When outcomes were repeatedly measured over time, repeated measures ANOVA was used to compare the three genotypes on the same diet. Tukey's test for multiple comparisons was used to test all possible pairwise differences whenever ANOVA (one-way or repeated) was statistically significant at the 0.05 level. Analyses were performed using GraphPad Prism 7 (GraphPad Software, CA, USA).

Results

Impact of CBR knockout on diet induced obesity, food intake and locomotor activity

At the beginning of the experiment, 8-week old CB2^{-/-} mice were heavier ($p < 0.001$) compared to WT or CB-DKO mice (mean body weight in grams = 27 ± 0.4 , 25 ± 0.3 and 24 ± 0.3 , respectively). No differences in body weight were detected comparing 8-week old CB-DKO with WT mice ($p = 0.2$). After 12 weeks, WT mice gained an average of 21 grams on HFD, compared to 9 grams for the LFD fed group ($p < 0.001$). Similarly, CB2^{-/-} mice gained an average of 19 grams on HFD and 11 grams on LFD ($p < 0.001$). CB-DKO mice did not respond to HFD, gaining an average of 4 grams and 5 grams on LFD and HFD, respectively ($p = 0.3$).

Weight gain rate (% of starting weight) of CB2^{-/-} mice fed with LFD or HFD was not different from WT mice on the same diet, whereas CB-DKO mice gained less weight compared to CB2^{-/-} or WT mice on the same diet (Fig 1A). After 12 weeks, the final weight of CB-DKO mice was significantly less than CB2^{-/-} or WT mice on the same diet ($p < 0.001$).

Automated food intake measurements indicated that the reduced body weight and lean phenotype of CB-DKO mice fed with HFD or LFD were not associated with decreased food intake (Fig 1B, and Fig S1) irrespective of the light or dark cycle. Food intake of CB-DKO mice was lower towards the end of the study, however when expressed as a percentage of body weight, the relative food intake of CB-DKO was similar to CB2^{-/-} or WT mice. Nor was the reduced body weight and the lean phenotype due to increased locomotor activity during the light or dark cycle (Fig 1C and Fig S1).

CB-DKO mice display a lean phenotype.—*In vivo* measurement of body composition was conducted in a subset of mice prior to and after LFD or HFD feeding (Table 1). On HFD, CB-DKO mice gained less adipose tissue compared to CB2^{-/-} or WT mice. No differences were detected comparing CB2^{-/-} and WT mice when fed with HFD. On LFD, CB-DKO also gained less fat compared to the other two groups, and CB2^{-/-} mice gained more fat compared to WT mice. Changes in lean mass % were not statistically different across the groups on HFD or LFD.

Histologic examination demonstrated an increase in the size of adipocytes in WAT or BAT of CB2^{-/-} and WT mice compared to CB-DKO mice (Fig S2 and S3). Similarly, H&E staining of liver sections demonstrated HFD-induced enlarged hepatic vacuoles in CB2^{-/-} and WT mice indicating fat deposition, whereas these changes were less observed in CB-DKO mice fed with HFD (Fig S4). Blood triglyceride levels were not significantly altered by diet or by the mouse genotype, whereas plasma cholesterol levels were elevated in WT mice on HFD compared to WT fed with LFD, and compared to CB-DKO fed with HFD (Fig S5).

Energy balance measured by indirect calorimetry

Respiratory exchange rate and heat production were higher in CB-DKO mice compared to the other groups on HFD (Fig 2A and 2B, and Fig S6). No statistically significant differences in indirect calorimetry parameters were detected when comparing CB2^{-/-} and WT mice. Less pronounced but similar findings were detected when mice were fed with LFD (Fig S6).

Increased RER and heat production suggested that the CB-DKO mice had altered thermogenesis and potentially browning of WAT (34). To test this possibility, expression of thermogenic genes were measured in gonadal WAT. Levels of mRNA for Uncoupling protein 1 (Ucp1), Cell Death-Inducing DFFA-Like Effector A (CIDEA), and Cytochrome c oxidase subunit 8B (COX8B) were upregulated in CB-DKO mice fed with HFD compared to CB2^{-/-} or WT mice, whereas no differences were found when comparing CB2^{-/-} and WT mice on HFD (Fig 3A). Immunohistochemistry and staining of WAT showed an abundance of Ucp1 in CB-DKO mice when compared to the other groups on HFD (Fig 3B). Similar

trend was detected comparing the 3 genotypes on LFD (Fig 3B). Immunohistochemistry and staining of scapular BAT showed similar differences (Fig S7).

Impact of CBR knockout on glucose tolerance

All mouse groups on HFD for 12 weeks showed signs of impaired glucose tolerance when compared to their littermates on LFD (Figure 4A and 4B). Nevertheless, CB-DKO mice showed better glucose tolerance when compared to CB2^{-/-} or WT mice. Surprisingly, CB2^{-/-} mice fed with HFD showed delayed glucose clearance when compared to WT mice. There was also a similar trend for CB2^{-/-} mice to have delayed glucose clearance when compared to the other groups on LFD. Impaired glucose tolerance in HFD fed CB2^{-/-} and WT mice was associated with increased fasting plasma insulin levels (Figure 4C). This elevation in fasting insulin was also observed in CB2^{-/-} mice fed with LFD. Results were similar when plasma from mice injected with glucose for the 2-hr IPGTT were analyzed for insulin (Fig S8).

Discussion

When mice were maintained on HFD for 12 weeks, the absence of both receptors of the cannabinoid system resulted in resistance to diet-induced obesity. Differences in weight gain were less pronounced between CB2^{-/-} and WT mice (~10%, $p>0.05$). Food intake and locomotor activity could not explain the leanness of CB-DKO mice, whereas indirect calorimetry indicated that the inactivation of both receptors of the cannabinoid system was capable of counterbalancing the development of the obesity through increasing energy expenditure. Lower fasting glucose, total cholesterol and insulin levels accompanied the low adiposity in CB-DKO mice suggesting that enhanced thermogenesis improves insulin sensitivity and other metabolic parameters.

A strong anti-obesity potential has been previously demonstrated with genetic or pharmacologic downregulation of CB1R (3). In rodent models, CB1R inverse agonist rimonabant significantly decreased food intake and body weight without affecting locomotor activity (5). Verty et al. reported that the weight loss associated with rimonabant is due, at least in part, to an elevation in energy expenditure (35). In line with these findings, CB1^{-/-} mice are lean and resistant to obesity when fed a HFD (11, 12). Recently, Ruiz de Azua et al. reported that CB1R deletion promotes profound reprogramming in adipocytes, with upregulation of thermogenic genes indicating browning of WAT in CB1^{-/-} mice (36).

Studies elucidating the role of CB2R in weight gain were not as conclusive. The administration of the CB2R agonist JWH-015 in obese mice was associated with reduction in food intake and body weight (22). Mice injected with the CB2R antagonist SR 144528 for 4 weeks displayed a slight non-significant increase in body weight (24). In contrast, Jenkin et al. reported that treatment with the CB2R agonist AM1241 or antagonist AM630 did not reduce weight gain or food consumption in diet-induced obese rodents (23). Using knockout models, Schmitz et al. reported age-dependent pro-inflammatory obesity and visceral hypertrophy in CB2R deficient mice on standard diet when compared to WT mice (26), a conclusion consistent with our findings of slightly higher body weight of CB2^{-/-} mice at baseline, and after 12-weeks of LFD. Agudo et al. also reported that the lack of CB2R in

mice led to greater increases in food intake and body weight with age (25). However, when fed with HFD for 8 weeks, Agudo et al. reported less weight gain in CB2^{-/-} mice when compared to WT mice (130 and 150%, respectively). In the current study, differences in the rate of weight gain were less pronounced between CB2^{-/-} and WT mice (172% and 183%, respectively, $p>0.05$). These conflicting findings can be related to differences in the genetic background of CB2^{-/-} mice used, differences in the composition of diet used (60% energy from fat HFD in Agudo et al. vs. 45% energy from fat in the current study) or other unknown mechanisms.

Given the established role of CB1R in energy balance, the observed lean phenotype and the altered thermogenesis in CB-DKO could be driven by CB1R. This conclusion, however is limited by the inability to include CB1R knockout model in the study. The phenotype of CB-DKO could also result from a unique interactive effect of the two-receptor systems, as opposed to merely CB1R mediated effect. The study findings should be interpreted with caution. The use of knockout mice can help to elucidate the role of cannabinoid receptors in obesity and insulin resistance, but there is a possibility that the observed phenotype might be due to compensation to the developmental loss of the cannabinoid receptors, and not due to cannabinoid receptor absence (37).

CB-DKO mice displayed higher indirect calorimetry parameters with upregulation of adipose tissue thermogenic markers when compared to CB2^{-/-} or WT mice. All mice in the current study were housed at 20–24°C. Optimal housing temperatures for mice to mimic the thermal environment of humans have been widely debated (38, 39). Under cold stress, mice increase food intake and energy metabolism for maintenance of body temperature. Surprisingly, Ruiz de Azua et al. reported that HFD fed CB1^{-/-} mice housed at 30°C (lacking thermal drive for browning) showed greater body weight reduction compared to that of CB1^{-/-} mice housed under the standard animal housing temperature (22°C). In addition, upregulation of Ucp1 and other mitochondrial gene expression were observed in CB1^{-/-} mice housed at 30°C compared with control littermates at the same conditions (36) suggesting that additional receptor-related mechanisms might be involved in adipose tissue browning beyond simply generating heat in response to cold stimulation. Future directions might include testing the effect of different housing temperature on energy metabolism in CB-DKO mice, and whether it mimics findings on higher thermogenesis in CB1^{-/-} mice housed at 30°C.

Data on pharmacological manipulation of CB2R and glucose metabolism are also inconclusive. The administration of the CB2R agonist JWH-015 was associated with reduction of fasting insulin levels, whereas the administration of the CB2R agonist JWH-133 enhanced insulin resistance in HFD fed mice (22, 24). In prior studies, CB2^{-/-} mice failed to develop abnormal glucose clearance (25, 26). In the current study, CB2R deletion was associated with impaired glucose clearance. The differences in these findings might be related to the genetic background of CB2^{-/-} mice or diet used. The longer duration of HFD feeding in the current study might also have provided CB2^{-/-} mice enough time to develop obesity and show signs of insulin resistance. Schmitz et al. reported immune cell polarization toward pro-inflammatory subpopulations in fat and liver of CB2^{-/-} mice (25). This was also detected in our study through the abundance of F4/80 staining of WAT and

liver in CB2^{-/-} mice. In addition, prior studies have reported stronger inflammatory responses in CB2R deficient mice (40). Hence, impaired glucose clearance in CB2^{-/-} might be driven by inflammatory mechanisms (41).

Impairment of glucose clearance was not observed in CB-DKO mice on HFD. Surprisingly in the current study, immune cell infiltration was minimal in adipose tissue or liver of CB-DKO when fed with HFD or LFD. The absence of immune cell infiltration of liver or WAT in CB-DKO mice might be driven by CB1R weight-related effect. Antagonism of CB1R in mice fed with HFD was reported to decrease inflammation possibly through a primary effect on weight gain (42). This was associated with normal glucose clearance in HFD-fed mice (3). Because CB2^{-/-} mice showed an opposite effect, the phenotype of CB-DKO can be seen as a unique interactive effect of the two receptor systems, as opposed to merely CB1R mediated.

Conclusion

We demonstrated that mice lacking both of the cannabinoid receptors are lean and resistant to diet-induced obesity, a phenotype that is distinctive from CB2R deficient mice but similar to CB1R knockout. We speculate that the phenotype of CB-DKO is driven mainly by CB1R or through an interaction between the two receptors. Together with the epidemiological findings, downregulation of the cannabinoid receptors might be responsible for the decreased prevalence of obesity and insulin resistance in cannabis users. Selective downregulation of CB1R in the brain was reported in chronic daily cannabis users (43). In rodents, short-term THC administration attenuated weight loss (44), whereas chronic administration was associated with reduced weight gain (45). Because cannabis contains numerous compounds other than THC which may act through cannabinoid receptors or through other receptors and mechanisms (21), further studies are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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What is already known about this subject?

- Pre-clinical studies reported an increase of food intake and body weight gain by the activation of the cannabinoid-1 receptor.
- Human epidemiological studies, however, found decreased prevalence of obesity in cannabis users.

What does this study add?

- Given the overlapping and complementary functions of the cannabinoid receptors, mice lacking CB2R and mice lacking both CB1R and CB2R were studied.
- Mice lacking both receptors of the cannabinoid system were resistant to diet-induced obesity, possibly through enhanced thermogenesis.
- This metabolic profile was not observed in knockout mice for the cannabinoid-2 receptor suggesting that it is driven by cannabinoid-1 receptor or by a unique interactive effect of the two-receptor systems.

Figure 1.

Impact of CBR knockout on rate of weight gain, food intake and locomotor activity. Values presented are mean and SEM.

(A) Repeated measures ANOVA showed statistically significant effect of the mouse genotype on rate of weight gain in mice maintained on a LFD ($F(2, 46) = 16.98, p < 0.001$) or HFD ($F(2, 44) = 41.45, p < 0.001$) for 12 weeks ($n = 15-20$). Multiple comparison tests indicated no significant differences in weight gain between CB2^{-/-} and WT mice on LFD ($p = 0.4$), whereas significant differences were detected comparing CB-DKO and WT on LFD ($p < 0.001$), and CB-DKO and CB2^{-/-} on LFD ($p < 0.001$). Similarly, multiple comparison tests indicated no significant differences in weight gain between CB2^{-/-} and WT mice on HFD ($p = 0.4$), whereas significant differences were detected comparing CB-DKO and WT on HFD ($p < 0.001$), and CB-DKO and CB2^{-/-} on HFD ($p < 0.001$).

(B) No differences in food intake (one-way ANOVA 24-hr ($F(2, 12) = 2.265; p = 0.1$) and (C) locomotor activity (one-way ANOVA 24-hr ($F(2, 12) = 2.719, p = 0.1$) were detected comparing the 3 genotypes on HFD. Food intake and locomotor activity were recorded via metabolic cages, and averaged over 6 days ($n = 5/\text{group}$).

Figure 2.

Impact of CBR on respiratory exchange rate, and heat production of HFD fed male mice. Data were recorded via metabolic cages and averaged over 6 days, $n = 5/\text{group}$. Values presented are mean and SEM. * Indicates statistically significant ANOVA with multiple comparison results.

(A) One-way ANOVA (24 hr; $F(2, 12) = 11.92$, $p = 0.001$) showed statistically significant effect of the mouse genotype on respiratory exchange rate between the 3 group. Multiple comparison tests indicated higher respiratory exchange rate in CB-DKO compared to CB2^{-/-} ($p = 0.002$), and compared to WT mice ($p = 0.004$). No differences were detected comparing CB2^{-/-} and WT mice ($p = 0.9$).

(B) one-way ANOVA (24 hr; $F(2, 12) = 14.55$, $p < 0.001$) showed statistically significant effect of the mouse genotype on heat production between the 3 group. Multiple comparison tests indicated higher respiratory exchange rate in CB-DKO compared to CB2^{-/-} ($p = 0.01$), and compared to WT mice ($p < 0.001$). No differences were detected comparing CB2^{-/-} and WT mice ($p = 0.2$).

Figure 3.

Impact of CBR knockout on thermogenesis. * Indicates statistically significant ANOVA with multiple comparison results.

(A) The relative mRNA expression of thermoregulatory genes in male mice fed with HFD for 12 weeks. $n = 5-9/\text{group}$. Values presented are mean and SEM. Ucp1: One-way ANOVA ($F(2, 21) = 10.27$, $p < 0.001$). Multiple comparisons: CB-DKO vs. CB2^{-/-}, $p = 0.003$, CB-DKO vs. WT, $p = 0.001$, and CB2^{-/-} vs. WT, $p = 0.96$.

COX8B: One-way ANOVA ($F(2, 21) = 14.27, p < 0.001$). Multiple comparisons: CB-DKO vs. CB2^{-/-}, $p < 0.001$, CB-DKO vs. WT, $p < 0.001$, and CB2^{-/-} vs. WT, $p = 0.99$.

CIDEA: One-way ANOVA ($F(2, 15) = 3.728, p = 0.048$). Multiple comparisons: CB-DKO vs. CB2^{-/-}, $p = 0.4$, CB-DKO vs. WT, $p = 0.04$, and CB2^{-/-} vs. WT, $p = 0.4$.

(B) Representative Ucp1 immunohistochemical staining (20x) in gonadal WAT of male mice fed with LFD or HFD for 12 weeks. Increased unilocular Ucp1 staining was detected in CB-DKO mice with islands of Ucp1 stained multilocular cells (arrows).

Figure 4.

Impact of CBR knockout on glucose tolerance in male mice maintained on LFD or HFD for 12 weeks $n = 6-8/\text{group}$. Values presented are mean and SEM. + Indicates statistically significant Student t-test results. * Indicates statistically significant ANOVA with multiple comparison results.

(A) Intra-peritoneal glucose tolerance test.

(B) Area under the curve to quantify glucose tolerance test indicated delayed glucose clearance in WT-HFD when compared to WT-LFD (t-ratio = 3.891, DF = 12, $p = 0.002$), in CB2^{-/-}-HFD when compared to CB2^{-/-}-LFD (t-ratio = 4.977, DF = 12, $p < 0.001$), and in CB-DKO-HFD vs CB-DKO-LFD (t-ratio = 3.784, DF = 12, $p = 0.003$).

No statistically significant differences were detected comparing the 3 groups on LFD (One-way ANOVA $F(2, 18) = 2.002$, $p = 0.1$).

Statistically significant differences were detected comparing the 3 groups on HFD (One-way ANOVA $F(2, 18) = 17.54$, $p < 0.001$). Multiple comparisons: CB-DKO vs. CB2^{-/-}, $p < 0.001$, CB-DKO vs. WT, $p = 0.02$, and CB2^{-/-} vs. WT, $p = 0.02$.

(C) Fasting plasma insulin levels were higher in WT-HFD when compared to WT-LFD (t-ratio = 2.272, DF = 11, $p = 0.04$), but not in CB2^{-/-}-HFD when compared to CB2^{-/-}-LFD (t-ratio = 2.068, DF = 10, $p = 0.07$), or in CB-DKO-HFD vs CB-DKO-LFD (t-ratio = 1.457, DF = 12, $p = 0.17$).

Statistically significant differences were detected comparing the 3 groups on LFD (One-way ANOVA $F(2, 15) = 9.247$, $p = 0.002$). Multiple comparisons: CB-DKO vs. CB2^{-/-}, $p = 0.003$, CB-DKO vs. WT, $p = 0.9$, and CB2^{-/-} vs. WT, $p = 0.01$.

Statistically significant differences were detected comparing the 3 groups on HFD (One-way ANOVA $F(2, 18) = 9.346$, $p = 0.002$). Multiple comparisons: CB-DKO vs. CB2^{-/-}, $p = 0.001$, CB-DKO vs. WT, $p = 0.09$, and CB2^{-/-} vs. WT, $p = 0.08$.

Table 1.

Body composition of mice lacking the cannabinoid receptors maintained on LFD or HFD for 12 weeks

Panel A: Body fat^a				
Group	Diet	Initial %	Final %	Change %^b
WT (n = 6)	LFD	1.7 (0.8)	8.3 (1.7)	6.7 (1.7)
WT (n = 6)	HFD	2.3 (0.7)	20.5 (1.4)	18.2 (1.5)
CB2 ^{-/-} (n = 5)	LFD	1.4 (0.5)	14.4 (0.7)	13.0 (0.3)
CB2 ^{-/-} (n = 5)	HFD	1.4 (0.6)	17.3 (2.6)	16.0 (2.4)
CB-DKO (n = 5)	LFD	0.8 (0.4)	2.6 (0.9)	1.8 (1.1)
CB-DKO (n = 5)	HFD	0.3 (0.2)	5.5 (0.6)	5.1 (0.8)
Panel B: Lean mass				
Group	Diet	Initial %	Final %	Change %^b
WT (n = 6)	LFD	60.2 (1.1)	61.0 (0.9)	0.9 (1.5)
WT (n = 6)	HFD	60.2 (0.9)	63.7 (0.3)	3.6 (1.0)
CB2 ^{-/-} (n = 5)	LFD	62.7 (1.4)	66.6 (0.9)	3.9 (1.4)
CB2 ^{-/-} (n = 5)	HFD	62.7 (0.8)	64.0 (0.1)	1.3 (0.8)
CB-DKO (n = 5)	LFD	66.9 (1.4)	69.2 (1.2)	2.3 (1.9)
CB-DKO (n = 5)	HFD	64.2 (1.3)	65.4 (0.5)	2.2 (1.1)

^aValues presented are mean and SEM^bChange is calculated as the mean of the differences in the percentage of fat or lean mass before and after diet for each mouse.

Change in fat on LFD: One-way ANOVA $f(2,13) = 17.53$, $p < 0.001$, multiple comparison CB-DKO vs WT $p = 0.01$, CB-DKO vs CB2^{-/-} $p < 0.001$, and CB2^{-/-} vs WT $p = 0.04$.

Change in lean mass on LFD: One-way ANOVA $f(2,13) = 0.927$, $p = 0.4$.

Change in fat on HFD: One-way ANOVA $f(2,12) = 13.73$, $p < 0.001$, multiple comparison CB-DKO vs WT $p < 0.001$, CB-DKO vs CB2^{-/-} $p = 0.004$, and CB2^{-/-} vs WT $p = 0.6$.

Change in lean mass on HFD: One-way ANOVA $f(2,12) = 1.592$, $p = 0.2$