Supporting Information

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SI Materials and Methods

Tissue Collection. After treatment, animals were killed by rapid decapitation. Trunk blood was collected in BD Vacutainer K3 EDTA-coated glass tubes (VWR), placed on ice, and centrifuged at 1,200 × *g* for 15 min at 4 °C. Plasma was removed and stored at −80 °C until used for analyses. Spleen, adrenals, liver, and gonadal fat pads from each animal were collected and weighed. A portion of the liver and fat pad along with the entire brain were flash-frozen for additional analysis. Remaining fat pad, liver, and pancreas were fixed with 4% paraformaldehyde (PFA)/PBS overnight for histology.

Oil Red O. Frozen sections of the liver (10 μm) were mounted on Superfrost Plus microscope slides (Fisherbrand), air-dried, and fixed in 10% formalin. Mounted sections were stained with warmed Oil Red O solution for 10 min in a 60 °C oven, differentiated with 85% proplylene glycol for 5 min, and counterstained with Gill no. 2 hematoxylin for 30 s. Slides were coverslipped with Aqua Poly/Mount (Polysciences). Staining was done on five samples per experimental group.

H&E Staining. Paraffin-embedded samples were sectioned at 8-μm thickness with a microtome (Microtom). Sections were floated in a water bath at 42 °C, placed on poly-L-lysine–coated Polysine microscope slides (Erie Scientific Company), and allowed to air dry. For H&E staining, sections were dewaxed in xylenes and rehydrated in ethanol baths. Nuclei were stained with Gill no. 2 hematoxylin stain for 8 min and eosin for 10 dips. Sections were then covered with permount and coverslipped. H&E staining was done on five samples per experimental group; 20 randomly selected droplets were measured using Nis-Elements AR software and averaged per animal.

Measurements of Circulating Factors. Plasma leptin and insulin levels were assayed using ELISAs (Millipore), and plasma glucose and triglyceride levels were assayed using colormetric assays (Cayman Chemicals; all using the manufacturers' instructions). Plasma CORT concentrations were measured using a commercially available RIA (MP Biomedicals, Inc.). Samples were analyzed in duplicate, and results are reported as nanograms per milliliter.

Pair Feeding. The amount of food eaten and body weight were recorded daily. For pair feeding, mice receiving CORT were fed the same amount of regular chow as their age-matched vehicle group, which was determined to be ∼5 g chow/d through pilot studies.

Quantitative RT-PCR. mRNA from liver was extracted using Qiagen's RNeasy Mini Kit. mRNA from WAT and brain was separately homogenized in 1 mL QIAzol (Qiagen). For the latter, total RNA was extracted using Qiagen's RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacturer's recommendations. For all samples, DNase digestion was used (Qiagen) to remove contaminating genomic DNA following the manufacturer's protocol. After extraction and digestion, the purity and concentration of RNA were determined by NanoDrop 260/280 ratios (NanoDrop Technologies). All samples tested had a purity of 1.7–2.0; 2 μg total RNA was reverse-transcribed using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems) using the manufacturer's recommendations. Target gene expression was quantified with gene-specific primers and Power SYBR Green Master Mix (ABI) using the Applied Biosystems 7900HT Sequence Detection System at 95 °C for 10 min followed by 35 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s. Each sample was done in triplicate, and each reaction was repeated at least one time to ensure reproducibility. Raw threshold cycle (Ct) values were obtained from the Sequence Detection Systems 2.0 software (Applied Biosystems). An average Ct value was calculated from triplicate results for each gene. Threshold values were normalized to the housekeeping gene GAPDH or β-actin to provide ΔCt values. Fold change for each gene was then calculated using the equation $2^{-\Delta Ct}$. Forward and reverse primers were as described in Table S1.

Metabolic Phenotyping of CB1R$^{-/-}$ Mice on Chronic CORT. To determine energy expenditure, mice were adapted to individual metabolic chambers. Metabolic measurements (oxygen consumption, carbon dioxide production, and food intake) were obtained continuously using a CLAMS (Columbus Instruments) open-circuit indirect calorimetry system for 5 consecutive d. RQ was calculated as CO_2 (VCO_2) production/O_2 (VO_2) consumption, with the values of 1 and 0.7 indicating 100% carbohydrate oxidation and 100% fatty acid oxidation, respectively. Carbohydrate oxidation and fatty acid oxidation were separately calculated as previously described (1).

Fasting blood glucose was analyzed using the OneTouch Ultra Blood Glucose Monitoring System (LifeScan, Inc.). Circulating leptin, insulin, triglycerides, and adiponectin were measured as described above. Additional blood parameters, including cholesterol and ALT levels, were measured and carried out by the Tri-Institutional Core Facility.

Measurement of Hepatic Triglycerides and Very LDL Triglyceride Production. In both WT and $CB_1R^{-/-}$ mice on chronic CORT, total liver lipids were extracted with Folch extractions. Briefly, snap-frozen liver tissues (∼100 mg) were homogenized and extracted two times with 2:1 chloroform:methanol (vol/vol) solution. The organic layer was dried under nitrogen gas and resolubilized in chloroform containing 2% Triton X-100. This extract was dried again and resuspended in water, and then, it was assayed for triglyceride concentration using the commercial kits as described above.

Very LDL triglyceride production rates were determined in mice fasted for 4 h and i.p. injected with 400 μL Pluronic-407 (1 mg/g body weight) resuspended in PBS. Blood was collected in silastic tubes pretreated with heparin before injection and at indicated time points (1, 2, and 4 h after injection). Plasma triglyceride levels were measured enzymatically as described above. The triglyceride production rate (micromoles per kilogram per hour) was calculated from the difference in plasma triglyceride levels over a given interval after detergent injection. Triglyceride production rates were then expressed as relative changes compared with respective controls.

eCB Analysis of Adipose Tissue. Mixtures of the evaporated eCBs and their deuterated analogs that had been stored at −80 °C were reconstituted in ethanol for additional dilution in a 20 mg/mL solution of fatty acid-free BSA to simulate analyte-free tissue and make the calibration standards and quality control samples as previously described (2). The calibration curves were constructed from the ratios of the peak areas of the analytes vs. the internal standard.

The extraction procedure for the calibration standards and quality control started with protein precipitation with ice cold PBS (pH 7.4), acetonitrile, and the internal standard mixture

followed by centrifugation at 14,000 × *g* for 5 min at 4 °C. Tissue samples were weighed and homogenized in the same solvents before centrifugation. The resulting supernatants were diluted in 5% phosphoric acid in water followed by solid-phase extraction. OASIS HLB cartridges (30-mg, 1-mL cartridges) were rinsed with methanol and water before loading the diluted samples. The loaded samples were washed with 40% methanol in water, eluted with acetonitrile, and evaporated to dryness under nitrogen. Samples were reconstituted in ethanol, vortexed, sonicated briefly, and centrifuged before immediate analysis for the eCBs.

Chromatographic separation was achieved using an Agilent Zorbax SB-CN column (2.1 × 50 mm, 5 mm) on a Finnigan TSQ Quantum Ultra Triple Quad Mass Spectrometer (Thermo Electron) with an Agilent 1100 HPLC on the front end (Agilent Technologies) as previously described (2). The mobile phase consisted of 10 mM ammonium acetate (pH 7.3) and methanol in a flow rate of 0.5 mL/min; the autosampler was kept at 4 °C to prevent analyte degradation. Eluted peaks were ionized by atmospheric pressure chemical ionization in multiple reaction monitoring mode. Deuterated internal standards were used for each analyte's standard curves, and their levels per 1 g tissue were determined.

Statistics. Analysis of total body, WAT, and liver weight in $CB_1R^{-/-}$ mice revealed a significant interaction between genotype and CORT treatment [$F(1, 16) = 14.08$, $P < 0.01$ (Fig. 1*A*); $F(1, 16) = 7.052$, $P < 0.05$ (Fig. 1*B*); and $F(1, 12) = 5.644$, $P < 0.05$ (Fig. 1*G*), respectively.] A priori Bonferroni analysis revealed that all of these markers were elevated in CORT-exposed WT mice relative to all other experimental conditions, including $CB_1R^{-/-}$ mice exposed to CORT ($P < 0.01$ for all variables). Metabolic markers in the blood also showed a significant interaction on leptin [$F(1, 16) = 22.88$, $P < 0.001$] (Fig. 1*D*), insulin [$F(1, 16) = 39.17$, $P < 0.001$] (Fig. 1*E*), cholesterol [$F(1, 12) = 10.58$, $P < 0.01$] (Fig. 1*H*), ALT [$F(1, 12) = 10.51$, $P < 0.01$] (Fig. 1*I*), triglycerides [$F(1, 16) = 12.49$, $P < 0.01$] (Fig. 1*J*), and glucose [$F(1, 11) = 8.791$, $P < 0.05$] (Fig. S3*D*). Hepatic triglycerides showed an interaction [$F(1, 12) = 4.983$, $P < 0.05$] (Fig. 1*K*) with a main effect of treatment ($F = 65.74$, $P < 0.0001$). Of these markers, post hoc analysis showed that cholesterol, ALT, and glucose levels were elevated in WT CORT-treated mice exclusively. Food intake for $CB_1R^{-/-}$ mice also revealed an interaction [$F(1, 20) = 29.34$, $P < 0.0001$] (Fig. 1*F*), with the post hoc test showing increased intake in WT CORT-treated mice only. Clearance of triglycerides was increased in WT CORT-treated mice 4 h after treatment with Pluronic-407 [$F(3, 10) = 6.324$, $P < 0.05$] (Fig. S3*F*). Levels of plasma CORT and spleen weights in $CB_1R^{-/-}$ mice both showed a main effect of CORT treatment [$F(1, 16) = 46.95$, $P < 0.01$ (Fig. S4*A*) and $F(1, 16) = 61.63$, $P < 0.0001$ (Fig. S4*B*), respectively].

In drug-treated mice, analysis of total body weight showed an interaction [$F(2, 35) = 6.219$, $P < 0.01$] (Fig. 2*A*). Bonferroni post hoc analysis revealed saline-injected CORT mice to have the only significant weight gain. The WAT weight also showed an interaction in drug treatments [$F(2, 35) = 5.559$, $P < 0.01$] (Fig. 2*B*); post hoc analysis showed a significant decrease in WAT of CORT mice treated with AM251 and AM6545. Patterns of circulating hormones in the blood exhibited an interaction in plasma leptin [$F(2, 33) = 22.57$, $P < 0.0001$] (Fig. 2*C*), insulin [$F(2, 29) = 4.318$, $P < 0.05$] (Fig. 2*D*), and triglycerides [$F(2, 29) = 3.375$, $P < 0.05$] (Fig. 2*E*). Post hoc analysis showed AM251 and AM6545 to significantly reduce insulin and leptin levels in CORT-treated mice, and only AM6545 reduced triglyceride levels.

$LCB_1R^{-/-}$ did not alter the main effect of CORT for body weight [$F(1, 19) = 58.14$, $P < 0.001$] (Fig. 3*A*), WAT weight [$F(1, 19) = 92.00$, $P < 0.001$] (Fig. 3*B*), plasma leptin [$F(1, 19) = 244.0$, $P < 0.0001$] (Fig. 2*C*), or plasma insulin [$F(1, 19) = 383.7$, $P < 0.0001$] (Fig. 3*D*). Analysis of plasma triglycerides and ALT levels and liver weight showed an interaction between groups [$F(1, 19) = 16.25$, $P < 0.001$ (Fig. 3*E*); $F(1, 19) = 10.56$, $P < 0.01$ (Fig. 3*F*); and $F(1,19) = 4.755$, $P < 0.05$ (Fig. 3*G*), respectively]. Post hoc analysis showed $LCB_1R^{-/-}$ CORT-treated mice to have lower triglycerides, ALTs, and liver weights compared with WT CORT mice.

eCB measures in the hypothalamus revealed significant changes in 2-AG [$F(7) = 2.457$, $P < 0.05$] and OEA [$F(7) = 0.0313$, $P < 0.05$]. Measures in blood showed significant increases of AEA [$t(15) = 2.297$, $P < 0.05$], PEA [$t(15) = 2.458$, $P < 0.05$], and OEA [$t(15) = 2.159$, $P < 0.05$]. In WAT, only AEA was significant [$t(14) = 3.563$, $P < 0.01$] in CORT-treated mice, whereas AEA [$t(15) = 3.267$, $P < 0.01$], 2-AG [$t(15) = 7.019$, $P < 0.0001$], PEA [$t(15) = 2.422$, $P < 0.05$], and OEA [$t(15) = 3.557$, $P < 0.01$] were significantly altered in the livers of mice with CORT treatment. All data are presented in Table 1.

WAT mRNA showed a significant decrease of CB_1R [$t(6) = 3.9$, $P < 0.05$] and fatty acid amide hydrolase [$t(6) = 2.523$, $P < 0.05$] in CORT mice. In the liver, CB_1R [$t(6) = 3.05$, $P < 0.05$], fatty acid amide hydrolase [$t(6) = 3.756$, $P < 0.01$], and *N*-acylphosphatidylethanolamine–specific phospholipase D [$t(8) = 5.711$, $P < 0.001$] mRNA were significantly altered in CORT-treated mice. Data are presented in Table 2.

1. Tam J, et al. (2010) Peripheral CB1 cannabinoid receptor blockade improves cardiometabolic risk in mouse models of obesity. *J Clin Invest* 120(8):2953–2966.

2. Williams J, et al. (2007) Quantitative method for the profiling of the endocannabinoid metabolome by LC-atmospheric pressure chemical ionization-MS. *Anal Chem* 79(15): 5582–5593.

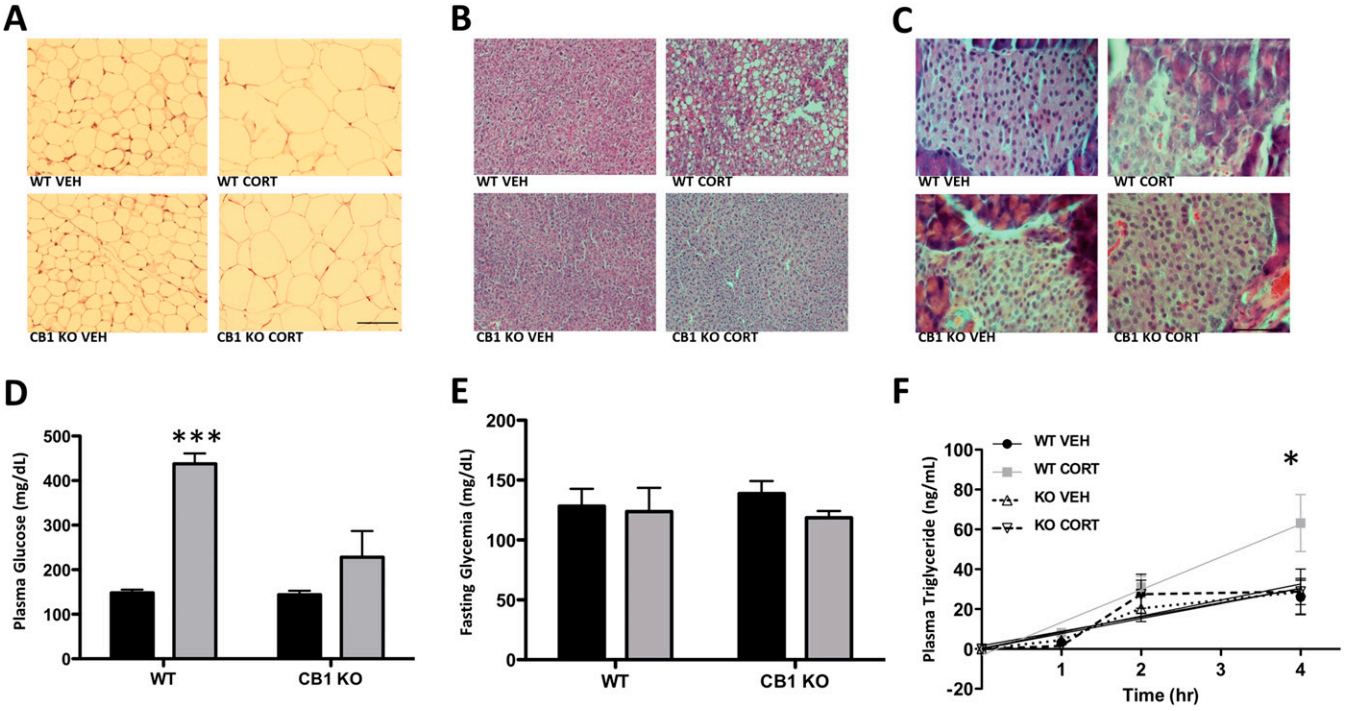

Fig. S1. Extended metabolic profile of CORT-treated $CB_1R^{-/-}$ mice. H&E stain in the (*A*) WAT, (*B*) liver, and (*C*) pancreas; genotype and treatment are as indicated. (*D*) $CB_1R^{-/-}$ results in decreased circulating plasma glucose but (*E*) does not alter fasting glucose levels. (*F*) Triglycerides were determined in plasma 1, 2, 3, and 4 h after a challenge with 400 μL Pluronic-407 (1 mg/g body weight) resuspended in PBS and show increased production in WT CORT-treated mice. Data are expressed as means ± SEMs. Asterisks indicate statistically significant differences ($n = 3$–5 per group). VEH, vehicle. *$P < 0.05$; ***$P < 0.001$. (Scale bar: *B*, 100 μm; *C*, 50 μm.)

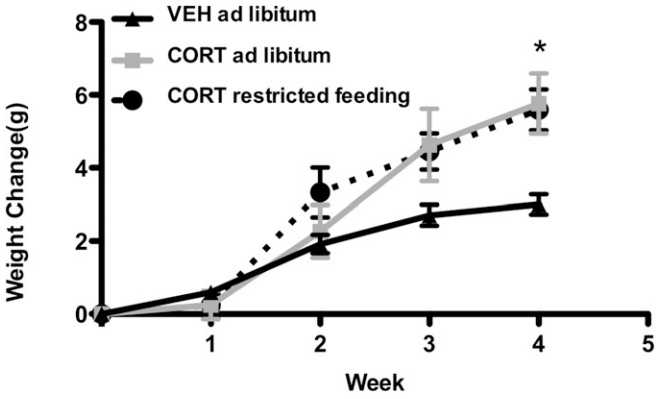

Fig. S2. CORT-induced metabolic syndrome is not dependent on food intake. Pair feeding in chronically CORT-treated (28 d) mice (mice on CORT received the same levels of food as VEH mice; 5 g chow/d). Mice continued to gain weight at the same degree as ad libitum mice. Data are expressed as means ± SEMs. VEH, vehicle. *Statistically significant difference ($n = 5$) at $P < 0.05$.

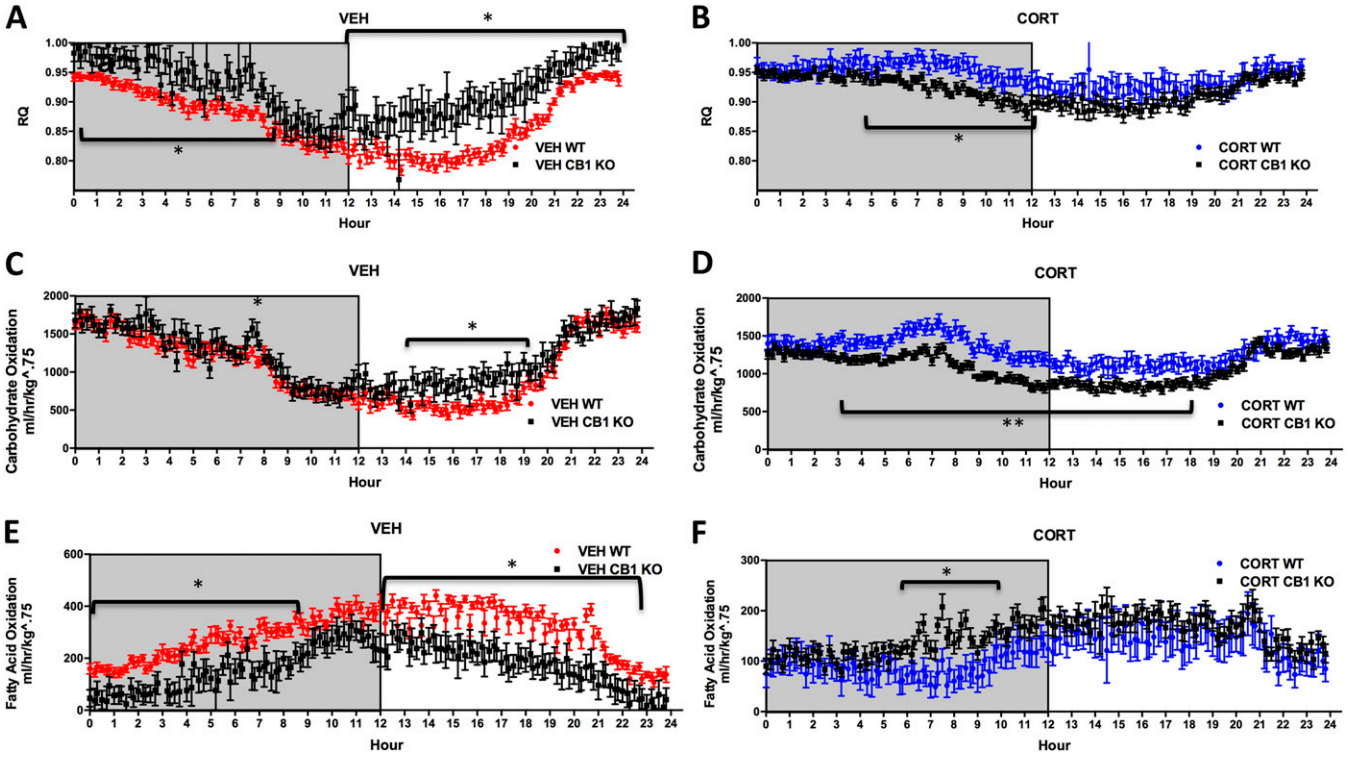

Fig. S3. (*A*) $CB_1R^{-/-}$ mice have an increased RQ throughout the daylight cycle (gray box indicates nonactive light phase) in response to VEH treatment compared with WT mice resulting from (*C*) an increased carbohydrate oxidation at hours 8 and 14–19 and (*E*) decreased fatty acid oxidation compared with VEH WT mice. (*B*) Chronic CORT treatment in the drinking water increases RQ across the day with decreased fluctuation between carbohydrate and fatty acid oxidation in both WT and $CB_1R^{-/-}$ mice. RQ in CORT-treated $CB_1R^{-/-}$ mice is decreased from the hours 5–12 as a result of (*D*) a decreased preference for carbohydrate oxidation and (*F*) an increased preference for fatty acid oxidation. Data are expressed as means ± SEMs. Asterisks indicate statistically significant differences. VEH, vehicle. $*P < 0.05$; $**P < 0.01$.

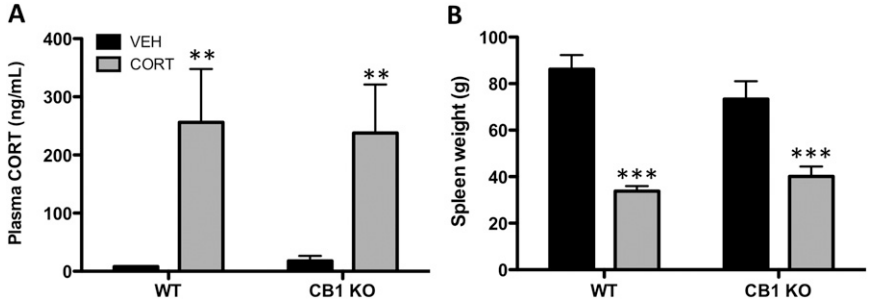

Fig. S4. WT and $CB_1R^{-/-}$ mice have similar levels of circulating CORT and splenic atrophy from treatment. (*A*) Circulating CORT levels reached equal levels in WT and $CB_1R^{-/-}$ CORT-treated mice. (*B*) The treatment results in decreased spleen weight. Data are expressed as means ± SEMs. Asterisks indicate statistically significant differences ($n = 5$ per group). VEH, vehicle. $**P < 0.01$; $***P < 0.001$.

Table S1. Forward and reverse primers for quantitative RT-PCR

Primer	Direction	Sequence (5′–3′)
β-Actin	Forward	TGTTCCCTTCCACAGGGTGT
β-Actin	Reverse	TCCCAGTTGGTAACAATGCCA
GAPDH	Forward	ATGACATCAAGAAGGTGGTG
GAPDH	Reverse	CATACCAGGAAATGAGCTTG
CB1R	Forward	GGTTCTGATCCTGGTGGTGTTGAT
CB1R	Reverse	CCGATGAGACAACAGACTTCT
FAAH	Forward	TGTGTGGTGGTGCAGGTACT
FAAH	Reverse	CTGCACTGCTGTCTGTCCAT
MAGL	Forward	CCTGTGTGGCGTGCCGATGAC
MAGL	Reverse	GCTGGAGTCAATGCGCCCCAA
DAGLα	Forward	TGGAAACCCCCGCCCATTGC
DAGLα	Reverse	CTGCTTGCCTGCACACCCCA
NAPE-PLD	Forward	GCTGGGACATGCGACGCTGA
NAPE-PLD	Reverse	GCGAAACCGCTTCGGACCCA

DAGLα, diacylglycerol lipase-α; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; NAPE-PLD, *N*-acyl-phosphatidylethanolamine–specific phospholipase D.