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Peripheral Endocannabinoid Control of Feeding Behavior and Obesity

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Peripheral Endocannabinoid Control of Feeding Behavior and Obesity

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

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in

Bioengineering

by

Donovan Alexander Argueta

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western diet-induced obesity” Physiology & behavior, 2017 (Chapter 2), “Peripheral endocannabinoid signaling controls hyperphagia in western diet-induced obesity” Frontiers in Physiology, 2019 (Chapter 3), and “Host and helminth-derived endocannabinoids are generated during infection with effects on host immunity” Infection and Immunity, 2018 (Chapter 4). The co-author Nicholas V. DiPatrizio listed in all publications directed and supervised the research, which forms the basis for this dissertation.

Additional co-authors include a) R Angelini, b) D Piomelli, c) PA Perez, d) A Makriyonnis e) HM Batugedara, f) JC Jang, g) D Lu, h) M Macchietto, i) J Kaur, j) S Ge, k) AR Dillman, and l) MG Nair. Respective contributions are as follows:

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b) Co-directed experiments for Chapter 1
c) Provided technical support for Chapter 3
d) Provided intellectual support and materials for Chapter 3
e) Assisted with design and implementation for Chapter 4
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i) Assisted with sample processing for Chapter 4
j) Assisted with statistical analysis for Chapter 4
k) Assisted with design for Chapter 4
l) Co-directed experiments for Chapter 4
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Dedication

This work would not have been possible without the support and encouragement provided to me by my mother. This is for you.
The endocannabinoid (eCB) system is a key modulator of central brain processes that control feeding, however recent evidence points to peripheral mechanisms by which eCB signaling may modulate feeding in various disease states. This body of work provides novel mass spectrometric analyses for components of the eCB system, while describing the pitfalls of currently used techniques. Using the described methods, a role for increased small intestinal eCB ligands signaling via peripheral cannabinoid type 1 receptor (CB1R) in the control of hyperphagia is discussed in the context of obesity. These findings were enhanced by further scrutiny of the intestinal eCB system, which revealed that corn-oil induced secretion of an intestine-derived satiation peptide, cholecystokinin (CCK), is blunted by CB1R activation; activation occurred via pharmacological agonists or enhanced eCB levels following diet induced obesity. Further, administration of a CCKA receptor antagonist, devazepide, blocked the anorexigenic effects of peripheral CB1R restriction, suggesting that peripheral
CB₁R act via intestine derived CCK to block satiation by impaired gut-brain signaling. Additionally, helminth infection was used to evaluate intestinal and lung eCB levels in a model of hookworm infection. For the first time, it was observed that the helminth *Nippostrongylus brasiliensis* produces endocannabinoids. Together, this body of work provides novel insight to the varied roles that intestinal and peripheral eCBs have during obesity in the modulation of feeding behaviors and immune responses.
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Introduction

The Endocannabinoid System

The endocannabinoid (eCB) system plays a large role in regulating feeding behaviors by centrally- and peripherally-mediated biochemical processes in humans and rodents (1-4). Named for the bioactive constituents of the plants Cannabis indica and Cannabis sativa, the eCB system comprises the cannabinoid receptor types 1 (CB₁R) and 2 (CB₂R), their endogenous ligands 2-arachidonoyl-sn-glycerol (2-AG) and N-arachidonoyl ethanolamide (AEA; Anandamide)(5-10) and corresponding synthetic and degradative enzymes. CB₁R is a Gᵢₒ protein-coupled receptor that modulates neurotransmitter release in glutamatergic synapses by cAMP-dependent inhibition of voltage-gated calcium channels, thereby reducing the probability of glutamate release (11-13). Peripheral CB₁R demonstrates control over energy homeostasis and feeding through yet-unknown mechanisms, but positron emission tomography (PET) imaging has identified large amounts of human CB₁R expression in the small intestine (1, 14-19). CB₂R has been found primarily in immune cells and regulates immune response (20). Recent evidence suggests that CB₂R is involved in feeding and a large expression profile in the small intestine (2, 21). Mixed agonism of CB₁/₂R with the psychoactive -Δ⁹-tetrahydrocannabinol (THC) displays a hyperphagic, or “munchies”, phenotype in rodents and
pharmacologically increasing 2-AG and AEA contents has a similar effect (22, 23).

*De novo* synthesis of the monoacylglycerol 2-AG occurs via hydrolysis of 1-stearoyl-2-arachidonoyl-sn-glycerol by diacylglycerol lipase alpha (DGL-α) in neuronal tissue (11). 2-AG is subsequently broken down by its main and minor degradative enzymes – monoacylglycerol lipase (MGL) and alpha beta hydrolyzing domain type 6 (ABHD6), respectively (24). Diacylglycerol lipase exists in a beta isoform (DGL-β), which is dispensable for 2-AG signaling in neuronal cells, that may regulate 2-AG production in peripheral tissue (25). Despite sharing an arachidonic acid acyl component with 2-AG, anandamide has a distinct synthetic and degradative pathway; anandamide is synthesized by N-acyl phosphatidyl ethanolamine specific phospholipase D (NAPE-PLD) and degraded by fatty acid amide hydrolase (FAAH)(26-30). The DGL-α/β, MGL, NAPE-PLD, and FAAH pathways are not specific to 2-AG and AEA; other monoacylglycerols (MAGs) and fatty acid ethanolamines (FAEs) share these pathways (27, 29). MAG and FAE species containing the omega-3 docohosahexaenoic acid are suggested CBR agonists, but physiological effects of large amounts of omega-3 derivatives are still under investigation.

Current tools for evaluating amounts of the endocannabinoids in biological tissues typically rely on mass spectrometry as their detection method. Mass spectrometry is then coupled to either gas chromatography or liquid chromatography, each having strengths that are chosen by application (31).
Ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) offers a high-throughput, highly sensitive, highly resolved, and highly specific readout, while also maintaining a relatively low cost of sample preparation (32). Notwithstanding, the potential for contamination and subsequent type I (false positive) errors exist, and adequate sample preparation and method development are necessary for robust detection and quantification of biological analytes (see Chapter 1).

**Obesity and Feeding Behaviors**

Obesity is the most common preventable disease in the United States; currently, more than sixty percent of adults are overweight and approximately forty percent are obese (33). Diet greatly contributes to the obesity epidemic, especially with the ease of access to calorically-dense foods (i.e. high amounts of fats and sugar)(34, 35). Obesity drives the dysregulation of biochemical signals involved with satiation and regulating meal patterns (36, 37), where efforts to uncover novel therapeutics have been largely ineffective (38, 39). Due to the hyperphagia associated with obesity, modulation of feeding behavior has become a target for therapy.

Humans and rodents detect dietary fats (40-44) and sugars (45) with receptors inside the oral cavity and along the alimentary tract, and these receptors are critical in mediating fat and sugar preference (43, 46, 47). Many biological pathways play an important role in controlling food intake, energy homeostasis, and hedonic reward (46, 48), including the endocannabinoid (eCB)
system, signaling through central and peripheral CB₁Rs (1, 19, 22, 23, 49-67). Targeting central endocannabinoids has proven deleterious in the clinic, especially in the cases of patients with existing psychiatric disorders (68); however, peripheral endocannabinoid control of feeding is a suggested safe alternative (19, 59). Additionally, intestinal endocannabinoid mobilization has been observed in response to taste of dietary fats, obesity, and fasting (61-63, 69). Taken together, the body of research is suggestive of a peripheral mechanism for hyperphagia that may be modulated by intestine-derived endocannabinoids (see Chapter 2).

**The Gut-Brain Axis**

The gut-brain axis is a dynamic circuit that comprises biochemical messages to the gastrointestinal tract via vagal efferent neurons and back to the brain via vagal afferent neurons and intestine-derived hormones. Due to its role in energy balance, the gut-brain axis is a target for diseases and conditions that impact feeding and energy balance. Gut-brain signaling controls food intake and energy homeostasis via direct neurotransmission and control of neuropeptide secretion and signaling (70). Impaired signaling of efferent vagal fibers has been associated with over-secretion of neuropeptides, such as the stomach-derived peptide ghrelin, which is observed in the case of bulimia nervosa (71). Efferent vagal signaling controls 2-AG biosynthesis in response to fat taste and fasting, which is mediated by subtype 3 muscarinic acetylcholine receptors (62, 72). Vagal signaling to the brainstem and hypothalamus reduce food intake via
neurotransmission initiated by the gut neuropeptides cholecystokinin (CCK), peptide YY (PYY), glucagon-like peptide 1 (GLP-1), and the adipocyte-derived leptin (37, 73-82). CCK, PYY, and GLP-1 are produced in enteroendocrine cells found along the gastrointestinal tract and release in response to sensing dietary nutrients (83-89). Conversely, ghrelin stimulates appetite by vagal afferent signaling (90, 91). These compounds and their homeostatic regulation have been extensively reviewed by Steinert et al (92). Vagal signaling has been widely investigated for its potential to treat feeding disorders, and the endocannabinoid system has been identified as a potential target due to its vast interactions with the various components of gut-brain communication. For example, THC administration in human adults attenuated the nutrient-induced release of GLP-1 and enhanced preference scores for palatable foods (93). Endocannabinoid control of gut-derived GIP and GLP-1 has been demonstrated (88), but the role for cholecystokinin in intestinal endocannabinoid signaling has not been investigated. Recent evidence has indicated a presence of mRNA encoding CB₁R on CCK-producing enteroendocrine I-cells (94), and new evidence suggests a functional link between CB₁R activation and decreased CCK secretion in DIO (see Chapter 3).

**Hookworm Infection**

Another field of research that is heavily involved in impaired feeding is the investigation of hookworm infections. Helminth parasite infections affect approximately two billion individuals worldwide (95). Although not typically fatal,
helminth infection presents with an array of comorbidities, including malnutrition and growth retardation. Most soil-transmitted helminths occupy the gastrointestinal tract of their host, where they can hinder the host’s nutritional status by stealing nutrients or preventing nutrient absorption via intestinal tissue damage and/or inflammation (96). Additionally, new mechanisms by which helminths impact host feeding and metabolism have been identified in the gastrointestinal tract (97). Gastrointestinal helminth infection has been shown to decrease food intake (98), and proved to be beneficial in mice fed a high-fat diet; infection improved glucose metabolism and reduced adiposity (99, 100). The beneficial effect is partly mediated through T helper type 2 (Th2) cytokine-activated M2 macrophages in the adipose tissue, which exert beneficial effects in metabolic homeostasis (101). Helminth infection in the intestine induces a Th2 cytokine-dependent expansion of cholecystokinin (CCK)-positive enteroendocrine cells, which secrete satiation hormones that regulate meal size (102). Identification of novel helminth or host-derived mechanisms that regulate feeding may provide greater insight to the pathologic or beneficial outcomes of helminth infection, which may be leveraged therapeutically.

Among the many host-derived molecules that affect feeding and metabolism pre- and post-infection, endocannabinoids are important research targets that control these physiologic processes (46, 103); endocannabinoids promote neural-mediated behaviors such as food intake and reward (46). Endocannabinoids, however, are generated ubiquitously in the mammalian body,
and cannabinoid receptors are expressed in extraneuronal cells, which include intestinal epithelial and immune cells (6, 7, 62). Signaling by the endocannabinoids, 2-AG or AEA, via cannabinoid receptors on intestinal cells modulates feeding behavior (36, 61), while their immune cell signaling promotes anti-inflammatory pathways (104). Despite functional outcomes on intestinal physiology and immune response, no studies in the current literature have evaluated the role of endocannabinoids in intestinal parasite infection. The host-pathogen interaction of helminths and mammals with emphasis on the endocannabinoid system may provide a useful route for investigating potential modulation or treatment of infections (see Chapter 4).
References


Chapter 1: Identification of a Widespread Palmitoylethanolamide Contamination in Standard Laboratory Glassware

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Abbreviations: 2-AG, 2-arachidonoylglycerol; AEA, arachidonoylethanolamide, anandamide; CBR, cannabinoid receptor; ECBs, endocannabinoids; ESI, electrospray ionization; FAEs, fatty-acid ethanolamides; GC/MS, gas chromatography mass spectrometry; ISTDs, deuterated internal standards; IT-TOF, ion trap – time-of-flight; LC/MS, liquid chromatography mass spectrometry; MRM, multiple reaction-monitoring; MS/MS, tandem mass spectrometry; OEA, oleoylethanolamide; PEA, palmitoylethanolamide; PPAR-α, peroxisome proliferator-activated receptor-α; QQQ, triple quadrupole

Material as previously published in Cannabis and Cannabinoid Research.
Abstract

Fatty-acid ethanolamides (FAEs) are a family of lipid mediators that participate in a host of biological functions. Procedures for the quantitative analysis of FAEs include organic-solvent extraction from biological matrices (e.g., blood), followed by purification and subsequent quantitation by LC/MS or GC/MS. During the validation process of a new method for LC/MS analysis of FAEs in biological samples, we observed unusually high levels of the FAE, palmitoylethanolamide (PEA), in blank samples that did not contain any biological material. We investigated a possible source of this artifact and found that high levels of a contaminant indistinguishable from PEA is present in new 5¾” glass Pasteur pipets, which are routinely used by laboratories to carry out lipid extractions. This artifact might account for discrepancies found in the literature regarding PEA levels in human blood serum and other tissues. It is recommended to take into account this pitfall by analyzing potential contamination of the disposable glassware during the validation process of any method used for analysis of FAEs.

Introduction

Fatty-acid ethanolamides (FAEs) are a family of endogenous lipid mediators whose chemical structures consist of a fatty acid moiety bound to
ethanolamine by an amide linkage. These compounds are synthesized by cells throughout the body and control inflammation, appetite and food intake, learning and memory, and pain among other functions (1). PEA and oleylethanolamide (OEA) suppress inflammation by activating the ligand-operated transcription factor, peroxisome proliferator-activated receptor-α (PPAR-α) (2). Anandamide, i.e. arachidonoylethanolamide (AEA), acts as a partial agonist at cannabinoid receptor (CBR) type 1 and 2, and therefore belongs to the diverse family of lipid signaling molecules called endocannabinoids (ECBs) (3, 4). Due to their similar physicochemical properties, FAEs and other ECBs such as 2-arachidonoyl-snglycerol (2AG) are usually co-extracted from biological samples (5). The procedure for their analysis includes extraction with organic solvents (e.g., methanol and chloroform) followed by purification through solid-phase extraction (e.g., open-bed silica gel) and subsequent quantitation by LC/MS or GC/MS. FAEs are present in blood serum or plasma in the pmol per mL scale and in biological tissues in concentration ranging from the pmol to nmol per gram scale. A review of the literature, however, reveals that data from different laboratories reporting concentration of FAEs in human serum from healthy subjects often do not corroborate one another. In particular, reported levels of PEA and OEA in serum or plasma of healthy human subjects differ by up to two orders of magnitude, from 5-30 pmol per mL (6-17) up to 200 pmol per mL of serum or plasma (18, 19).
During the validation process of a new method for LC/MS analysis of FAEs and ECBs in human serum extracts, we observed unexpectedly high levels of PEA, as compared to data previously obtained in our laboratory (6). We suspected that these abnormal levels could be due to a recurrent contamination. We found that 5¾" Pasteur pipets of most, if not all, commercial brands, contain multiple contaminants detectable by LC/MS, including readily detectable quantities of a compound indistinguishable from PEA.

Materials and Methods

Materials

All solvents including chloroform, methanol and acetone, were of the highest purity commercially available, suggested for pesticide residue analysis, residue 1mg/L max, from Burdick & Jackson (Honeywell International Inc., Morris Plains, NJ). Water was HPLC grade from Fisher Scientific (Thermo Fisher Scientific Inc., Waltham, MA). Synthetic standards of FAEs including deuterium-containing internal standards (ISTDs) for isotope dilution ([\(^2\)H\(_4\)]PEA, [\(^2\)H\(_4\)]OEA, [\(^2\)H\(_4\)]AEA and [\(^2\)H\(_5\)]2AG) were purchased from Cayman Chemical (Ann Arbor, MI). Ammonium acetate and acetic acid were Optima grade from Fisher Chemical (Thermo Fisher Scientific Inc., Waltham, MA). Disposable glass Pasteur pipets (5¾” and 9" corresponding to 150 and 230 mm, respectively) were from the following vendors: Fisher Scientific (Thermo Fisher Scientific Inc., Waltham, MA), VWR (VWR International LLC, Radnor, PA), Corning (Corning, NY), and Wheaton (Wheaton Industries Inc., Millville, NJ). Eight-mL vials were
from Thermo Scientific (Thermo Fisher Scientific Inc., Waltham, MA). LC amber
vials with inserts were from Agilent Technologies (Santa Clara, CA). Beakers and
cylinders were Fisher Scientific (Thermo Fisher Scientific Inc., Waltham, MA).
Glass syringes were from Hamilton (Reno, NV). Silica Gel was 60Å 230-400
Mesh ASTM from Whatman Inc. (Sigma-Aldrich Corp., St. Louis, MO). Untreated
glass wool was from Grace (Columbia, MD).

Extraction of FAEs from blood serum

Samples of human serum from healthy subjects were kindly provided by Dr. Hamid Moradi. The study protocol was approved by the institutional review
board of the University of California-Irvine and was completed with the assistance of the University of California-Irvine General Clinical Research Center. Written informed consent was obtained from all subjects. Blood samples were collected in red top blood collection tubes (vacutainers, silicon-coated, no additives) which were left sitting upright at room temperature for a minimum of 30 to a maximum of 60 minutes to allow clotting. The blood sample was centrifuged immediately at the end of the clotting time in a horizontal rotor (swing-out head) for 20 minutes at 1100-1300 g at room temperature. The serum was transferred into labeled cryogenic vials, which were immediately stored upright in a rack at -80°C. Aliquot volume was 1mL. This process was completed within 1 hour of centrifugation. Aliquots were transported in dry ice, then slowly defrosted at 4°C and stirred for 30 sec prior use. A volume of serum of 0.6 mL was transferred in an 8 mL glass vial. Protein precipitation was performed by adding 1mL of cold
acetone containing deuterium labeled ISTDs as follows: 1.2 pmol of $[^2\text{H}_4]\text{PEA}$, 1.2 pmol of $[^4\text{H}_4]\text{OEA}$, 0.6 pmol of $[^2\text{H}_4]\text{AEA}$ and 30 pmol of $[^2\text{H}_5]2\text{AG}$. Samples were stirred for 60 sec then left in a cold room (4°C) for 20 min, then centrifuged at 2000 rpm at 4°C for 20 min. The supernatants were transferred with a Pasteur pipet in another 8 mL glass vial before undergoing lipid extraction procedure. Lipid extraction was performed using a modified Folch procedure (20), as follows. The excess of acetone was evaporated under $\mathrm{N}_2$. Water was added up to 1 mL of total volume and the sample was vortexed for 60 sec. Then 1 mL of methanol was added and the sample vortexed for 60 sec. Finally, 2 mL of chloroform were added and the sample vortexed for 60 sec before centrifugation for 15 min at 3500 rpm at 4°C. The lower chloroform phase was collected with a Pasteur pipet and transferred in another glass vial, evaporated under $\mathrm{N}_2$ stream and reconstituted in 2 mL of chloroform. Afterwards, fractionation of the lipid extract through open bed chromatography was performed as previously described (5). Briefly, the standardized extraction procedure employed in our lab is an open-bed silica-gel column chromatography (Silica gel 60 230–400 mesh) that precedes LC/MS analyses (i.e. a custom-made solid phase extraction procedure). Silica gel columns were prepared as follows: a 5” ¾ glass Pasteur pipette was plugged with glass wool at the beginning of the thin tip; 1 mL of a slurry of silica gel (60Å 230–400 Mesh ASTM) in chloroform (1:1, v/v) was poured into the pipet held in a rack with a metal tray underneath; the column was washed with 2 mL of chloroform. The reconstituted 2 mL of lipid extract were
loaded onto the column. FAEs were eluted with 2 mL of chloroform/methanol (9:1, v/v). The eluate was collected in another 8 mL glass vial, evaporated under N₂, reconstituted in 60 μL of methanol and transferred in a 250-μL insert of a 2 mL LC vial before LC/MS analysis. The final concentrations of ISTD were therefore the following: 20 nM [²H₄]PEA and [²H₄]OEA, 10 nM [²H₄]AEA and 500 nM [²H₅]2AG. The same concentrations were used for the calibration curves described in the LC/MS section below.

**Pipet extraction**

Disposable glass Pasteur pipets were washed with 8 mL of chloroform. The chloroform was collected in an 8 mL vial, spiked with 20 moles of ISTD [²H₄]PEA, evaporated under a stream of N₂, reconstituted in 100 μL of methanol and transferred with a glass syringe in a 250-μL insert of a 2 mL LC vial before LC/MS analysis. The final concentration of [²H₄]PEA ISTD was therefore 200 nM.

**Foam extraction**

Polyurethane foam is used by vendors to wrap the glass Pasteur pipets to protect them from breakage. Small pieces of this plastic material were cut and weighed. Pieces of 1, 2.5, 5 and 10 mg were deposited in 8 mL glass vials that were previously washed with chloroform twice. Each vial was filled with 5 mL of chloroform containing 20 pmol of ISTD [²H₄]PEA. Vials were stirred thoroughly. The chloroform was evaporated under N₂ and the extracts were suspended in
100 µL of methanol and transferred with a glass syringe in a 250-µL insert of a 2-mL LC vial.

**Transferring PEA from the foam to the glass**

A PEA-free 9” glass Pasteur pipet was further washed with chloroform. The pipet was warmed up at 60°C and wrapped in polyurethane foam used by vendors for packaging. Subsequently, a pipet extraction was performed as described above to check whether PEA could be transferred from the foam to the glass.

**LC/MS**

LC/MS analyses of samples were performed using an Agilent 1200 LC system coupled to an Agilent G6410A triple quadrupole (QQQ) MS detector (Agilent Technologies, Inc., Santa Clara, CA) equipped with an electrospray ionization (ESI) interface. FAEs were separated using a XDB Eclipse C18 column (2.1 x 50 mm i.d., 1.8 µm.), eluted with an isocratic method of methanol in water (A: 20% Water + 0.25% Acetic acid + 5 mM ammonium acetate and B: 80% methanol + 0.25% acetic acid + 5 mM ammonium acetate in 8 min) at a flow rate of 0.4 mL/min. Column temperature was kept at 40°C. MS detection was in the positive mode, capillary voltage is set at 4 kV, fragmentor voltage is varied from 120 to 140 V and collision energy was 20 eV. Helium was used as collision gas while nitrogen was used as drying gas at a flow rate of 12 liters/min at 350°C.
Nebulizer pressure is set at 50 PSI. In parallel, LC/MS and MS/MS data were also acquired on an Acquity I Class UPLC system coupled to a Xevo TQ-S Micro Mass Spectrometer (Waters, Milford, MA, USA) with accompanying electrospray ionization (ESI) interface (data presented in Figures 3B, S5, S7). On both set-ups we quantified FAEs with an isotope-dilution method, monitoring protonated adducts of the molecular ions [M+H]^+ in multiple reaction-monitoring (MRM) mode. The MRM transitions monitored for FAEs detection and quantitation were the following: PEA (m/z = 300.3>62.1); OEA (m/z = 326.3>62.1); AEA (m/z = 348.3>62.1); 2AG (m/z = 379.3>287.2); [2H₄]PEA (m/z = 304.3>66.1); [2H₄]OEA (m/z = 330.3>66.1); [2H₄]AEA (m/z = 352.3>66.1); [2H₅]2AG (m/z = 384.3>287.2). Additional analytes were investigated on the Waters UPLC/MS/MS as possible contaminants in foam: docosahexaenoyl ethanolamide (DHEA, Fig S5G, H) (m/z = 372.3>62.1), and docosahexaenoyl glycerol (DHG, Fig S5K, L) (m/z = 403.4>311.2). We used two different sets of calibration curves, one for the serum analysis with low ISTD and one for pipets and foam extracts with higher ISTD. In particular, we prepared standard calibration curves by adding a constant amount of deuterium-labeled standards to increasing amount of the corresponding unlabeled FAEs, followed by MS analysis as described above. The relative concentrations of unlabeled versus labeled ions were plotted against their relative response (i.e. peak area) and the calibration curves were constructed using linear regression. \( R^2 \) was 0.998 for all analytes, indicating linear response. In the calibration curve for serum extracts analysis the ISTDs
were kept as follows: \([\text{H}_2\text{H}_4]\text{PEA}\) and \([\text{H}_2\text{H}_4]\text{OEA}\) at 20 nM, \([\text{H}_2\text{H}_4]\text{AEA}\) at 10 nM and \([\text{H}_4\text{H}_5]\text{2AG}\) at 500 nM. In the calibration curve for foam and pipets extracts analysis, the ISTD \([\text{H}_2\text{H}_4]\text{PEA}\) was kept at 200 nM. In both cases the non-deuterated reference standard of PEA, OEA, AEA and 2AG were at concentrations ranging from 1 nM to 2 µM, for a total of 11 points of calibration.

Highly accurate mass data were obtained on a Shimadzu IT-TOF (ion trap – time-of-flight) coupled to Shimadzu Nexera HPLC system following a similar LC/MS method. A reverse phased endcapped C18 column from Knauer was employed (Eurospher II 100 C18: 2 x 100 mm i.d., 3µm, 100 Å). The solvent system and oven temperature were the same as described above for the Agilent instrument. The LC method was 13 minutes long and proceeded as follows: from 85 to 98%B in 5 min, then kept at 98%B for 5 min followed by a re-equilibration step at 85%B for 3 min. Source temperature was kept at 250°C, drying gas pressure was 103 kPa (10 L/min), detector voltage was 1.55 kV and probe voltages were 4.5 and -3.5 kV. Data were acquired and analyzed with the Shimadzu software LCMS Solution (Acquisition and Analysis) and Formula Predictor. All LC/MS data showed in this manuscript were obtained in the positive ion mode.

**Results**

**Quantitation of FAEs and 2-AG in human serum samples**

We quantified PEA, OEA, AEA and 2AG levels in serum samples from three healthy subjects. Table 1 contains a comparison of average FAEs and 2-
AG levels, measured in this work and by independent laboratories, in human serum/plasma from healthy subjects, by LC/MS analysis. For most compounds, concentrations measured in the present study were in close agreement with those previously reported by our group (6) and others (7). PEA levels were similar to those reported by Bilgin et al. (18) and by Sipe et al. (19), but 10 to 20 times higher than those reported by other groups (7-17) and previously by us (6). This suggested that a contamination might be present.

**Identification of PEA in glass Pasteur pipets and plastic foam**

First, all solvents were carefully checked as a possible source of contamination. Skonberg and coworkers (21) have found that chloroform from certain vendors contained small amounts of PEA. We could not confirm those findings: all solvents used during our procedure for extraction of FAEs and ECBs from serum were found to be FAEs-free. This prompted us to carefully check all the glassware, disposable and not, used to carry out the extraction procedure. This included: 2mL LC vials, vial inserts, 8mL disposable glass vials, glass beakers, glass solvents bottles, disposable glass Pasteur pipets, and the polyurethane foam used to wrap them. LC-MS and MS/MS traces of authentic PEA standard and chloroform-methanol washout of glass pipets and extracts of polyurethane foam were recorded in the positive ion mode. LC-MS data were acquired on two separate triple quadrupole (QQQ) instruments (Agilent G6410A and Waters Xevo TQ-S Mass Spectrometer) and on a high accuracy ion-trap time-of-flight (IT-TOF) mass spectrometer (Shimadzu IT-TOF) to confirm
identification by accurate mass measurement. Fig. 1 shows the comparative LC-MS (panel A) and LC-MS/MS (panel B) analysis of PEA standard (traces a), pipet washout (traces b) and foam extract (traces c) carried out on the Agilent QQQ. The MS1 analysis in panel A shows the Extracted Ion Chromatograms (EIC) for the m/z 300.3 belonging to the PEA positive pseudomolecular ion [M+H]^+. These three chromatograms show co-eluting peaks at RT 5.7 min for the m/z 300.3. The MS2 analysis in Fig 1B shows the fragmentation pattern obtained by Collision Induced Dissociation (Fragmentor 135V, CID 20eV) of the peaks at 300.3 in each chromatogram. The obtained MS/MS spectra are superimposable and show peaks of: the positive pseudomolecular ion of PEA [M+H]^+ at 300.3 Th, the fragment deriving from the water loss [M+H-H_2O]^+ at 282.3 Th and the diagnostic fragment of ethanolamine [Ethanolamine+H]^+ at 62.1 Th. The signal belonging to the ammonia loss (-17) at 283.3 Th is also visible except in trace a and b, but not c. Collectively, results in Fig. 1 demonstrate that one of the contaminants, present in the pipet and in the foam extracts, gives rise to a signal detected by LC/MS analyses that is attributable to PEA, having the same RT and MS/MS fragmentation pattern of authentic standard.

To confirm the presence of PEA as contaminant in the pipets and foam extracts we also analyzed the pure standard of PEA and the pipet extract on a different instrument capable of providing accurate mass measurement up to the fourth decimal and with a ppm deviation lower than 5 (Shimadzu IT-TOF). The EIC for the high accurate exact mass of the positive pseudomolecular ion of PEA
[M+H]^+ at 300.2897 Th is shown in Fig. 2. The spectra show co-eluting peaks at RT 6.3 min for the m/z 300.2897 with ppm deviation < 5. The brute formula of this compound was predicted by the Formula Predictor software as C_{18}H_{37}NO_2 both for the PEA standard and the contaminant in the pipet with a ppm deviation of 2.7 and 3.7 respectively (Fig S1 and S2). This experiment unambiguously demonstrates the presence of PEA as a contaminant in the glass Pasteur pipet extract.

Other possible contaminants

To determine whether PEA was the only contaminant present in glass pipets we analyzed the TIC of an MS1 scan of the pipet washout. We performed this analysis in the positive ion mode on the QQQ and in both positive and negative ion mode on the IT-TOF machine (Fig S3 and S4, respectively). The analysis in the negative ion mode (Fig S4, blue trace) clearly identified two peaks attributable to palmitic acid and stearic acid by RT and accurate exact mass (data not shown). Analysis in the positive ion mode showed a number of peaks on both instruments among which PEA is a contaminant. It is outside the scope of this manuscript to provide a comprehensive identification of all contaminants, which would require extensive structure elucidation work by MS analysis. Importantly, however, analysis of other FAES and monoacylglycerols commonly studied in the ECB field resulted in no evidence of their presence in foam (5 mg). These analytes included: PEA for reference (Fig S5A, 100pmol; S5B, foam), OEA (Fig S5C, 100pmol; Fig S5D, foam), anandamide [AEA (Fig S5E, 100pmol;
Fig S5F, foam), docosahexaenylethanolamide [DHEA (Fig S5G, 100pmol; Fig S5H, foam)], 2-AG [2-AG (Fig S5I, 100pmol; Fig S5J, foam)], docosahexaenoyl glycerol [DHG (Fig S5K, 100pmol; Fig S5L, foam)].

Quantitation of PEA in glass Pasteur pipets and in plastic foam

We quantified the amount of PEA that can be extracted from a single glass pipet when washed with 8 mL of chloroform. Figure 3A shows proportional increase in concentrated extracts of 1, 2 and 3 pipets. We also tested 9” pipets, where PEA was present in negligible trace amount or absent. In order to quantify the PEA content in each pipet we used an isotope dilution LC/MS method with acquisition in MRM mode as described in the experimental procedures. PEA was quantified in 100 µL of concentrated washout of each pipet and results are expressed as pmol of PEA in the reconstituted extract (Fig. 3A). We also quantified PEA content in pipets from different vendors (Fig. S5). Although the concentration of PEA increases with the number of pipets used in the extraction procedure, the multiple experiments show a range of standard deviations (SD) in triplicate experiments. This is observed also in pipets produced by the same manufacturer and having the same lot number but taken from different boxes (Fig. 1 as compared to groups 1-2 in Fig. S6, showing independent experiments in triplicate). The average amount of PEA present in one pipet, calculated considering all 9 experiments shown in Fig. 3 and Fig. S6, is 33.4 ± 4.02 pmol (mean ± SEM, n = 9). Altogether, these results show that one wash with 8 mL of chloroform can extract an average amount of PEA of 33.4 pmol per pipet.
Notably, the SD within all pipet extraction experiments ranges from 10 to 75% (average SD is around 30% in triplicate experiments). This considerable data scattering indicates that an extraction from a single pipet produced by the same manufacturer can give rise to PEA contaminations of very different extent. Finally, we employed our LC/MS method to quantify the amount of contaminant PEA present in the polyurethane foam used to wrap the glass pipets. Fig. 3B shows a strong linear relationship between mass of foam insert and calculated PEA values.

Transfer of PEA from the foam to the glass

A washed clean glass Pasteur pipet was incubated in contact with the foam as described in experimental procedures. Pipet extractions with subsequent LC/MS analysis were carried out before and after the washing step as well as after exposure to the foam. For reference, the intensity (ion counts) of 100 pmol PEA standard was 1.45e8 (Fig S7A). The PEA signal was present in the unwashed pipet at 2.19e7 (Fig S7B), absent in the washed pipet extract (Fig S7C) and present again in the pipet exposed to 5 mg foam at 1.56e6 (Fig S7D).

Discussion

A contaminant that is undistinguishable from PEA is present in glass Pasteur pipets in amounts that are sufficient to interfere with analysis of biological samples. The contaminant was identified based on its LC retention time, accurate mass, and tandem mass spectrometric (MS/MS) fragmentation
pattern, which were identical to those of authentic PEA. By contrast, only a negligible PEA contamination was found in 9” Pasteur pipets. Furthermore, we isolated the PEA contamination to the polyurethane foam used to package the pipets, which is transferred to glass pipets by contact. In line with this finding, Oddi et al. (22) recently reported that FAEs can be absorbed by plastic materials during laboratory assays. It is therefore conceivable that FAEs incidentally absorbed by plastics during industrial processes can be released later in organic solvents. Lastly, no other commonly analyzed FAEs or monoacylglycerols were found to be present in the pipets.

We published GC/MS (23) and LC/MS (5) analytical methods for the quantitation of ECBs and other related FAEs and monoacylglycerols in biological samples, including human serum (6). Prompted by the need for a novel quantitative LC/MS method to analyze ECBs in blood, we reviewed the literature and noticed discrepancies in the reported concentrations of FAEs and ECBs in human blood serum and plasma (Table 1). The EC\textsubscript{50} for anandamide and 2-AG vary depending upon assay and tissue; however, it is important to note that levels reported in Table 1 for both compounds in plasma/serum are below the apparent biologically active concentrations required to activate CB receptors [see for comprehensive review of specific assays used by a variety of research groups (24)]. Regarding relative levels of PEA and OEA, a number of studies reported very similar concentrations for both compounds (8-11, 15), whereas others reported PEA approximately twice higher than OEA (6, 7, 12, 13, 16, 17).
Regarding absolute values, two separate laboratories reported levels of PEA and OEA in plasma that were excessively high (18, 19), which reached or exceeded the concentrations needed by these ligands to engage PPAR-α as agonists. PEA and OEA are, in fact, considered high potency ligands of PPAR-α; in heterologous expression systems, these FAEs engage the receptor with median effective concentration (EC$_{50}$) values of 0.12 µM for OEA and 3 µM for PEA (25, 26). In the above-mentioned reports (18, 19), although PEA levels did not exceed the EC$_{50}$, levels of PEA were high relative to other reports (6-17). The steady-state concentrations of FAEs (and ECBs) in plasma/serum of healthy individuals possibly reflect an equilibrium of ECBs released by peripheral tissues and their enzymatic degradation in the blood stream. In animal tissues (e.g., brain, upper small intestine), levels of PEA and OEA are present in the same order of magnitude (27, 28); therefore, it was predictable to find a similar pattern in human serum or plasma, as also shown by the literature reports in Table 1.

Surprisingly, in our preliminary experiments, the measured level of OEA was in agreement with most literature reports whereas PEA was one order of magnitude higher than expected (Tab. 1). This finding prompted us to carefully screen all possible sources of contamination including solvents, reagents, and glassware used for lipid extraction and quantitative analysis.

In the present manuscript, we identify glass Pasteur pipets (5”¾) used to transfer solvents and lipid extracts as the source of PEA contamination. The contaminant was identified as PEA by its exact mass and RT in three similar but
different chromatographic systems, as well as by its MS2 fragmentation pattern, which were identical to those of standard PEA. Further, we show that PEA is present in the polyurethane foam that manufacturers use to wrap the pipets before packing, from which it leaks onto the glass pipets. Moreover, accurate exact mass measurements with ppm deviation lower than 5 unambiguously confirmed the identity of the contaminant as PEA. Quantitative assessment showed that the content of PEA is $33.4 \pm 4.02$ pmol per pipet. Unfortunately, none of the various manufacturers whose pipets were tested provides 5¾” glass Pasteur pipet that are contaminant-free (Fig. S6). Only 9” pipets from one vendor were free of PEA traces (Fig. 3A), allowing the use of these consumables in the overall procedure.

The field of lipidomics is rapidly developing; however, reproducible standard procedures across laboratories are not established. Therefore, it is not uncommon for lipidomics data to differ among from independent laboratories (29). It is generally thought that these discrepancies are a result of the use of different instruments for lipid analysis, as well as differing extraction and separation protocols. In the present manuscript, however, all results were confirmed by two independent laboratories using different LC systems and QQQ mass spectrometers (Agilent 1200 LC system coupled to an Agilent G6410A QQQ Mass Spectrometer in the Piomelli laboratory, and Waters Acquity I Class UPLC system coupled to a Xevo TQ-S QQQ Mass Spectrometer in the DiPatrizio laboratory). Furthermore, accurate mass data were acquired on a third Shimadzu
IT-TOF High Resolution Mass Spectrometer for definitive confirmation that the contaminant was indeed PEA. Another well-known issue in lipidomics is that various sources of contamination can originate artifacts. Lipids, especially fatty acids, are common contaminants in detergents, mineral oils, greases and plasticizers; hence, they are often present in laboratory equipment including glassware and solvents. As shown in the present manuscript, assessment of FAEs, a group of lipids with diverse signaling properties, is not sheltered from this pitfall. We have shown that glass Pasteur pipets, commonly used in lipidomic laboratories to transfer lipid extracts and organic solvents, can contain PEA as contaminant. This contamination gives rise to artifacts in the measurement of PEA in biological samples, especially when the procedure for sample preparation includes fractionation of the lipid extract, which concentrates the contaminant.

The scope of the present manuscript is an alert to the ECB and FAE scientific community about possible PEA analytical artifacts and thus, great care is needed to exclude the possibility of contaminants when analyzing endogenous PEA levels in biological tissues.

**Conclusion:** In the present manuscript, we identified PEA as a quantitatively relevant contaminant present in glass pipets as well as in the polyurethane foam used to wrap pipets in the packaging. We strongly recommend examining glassware used to carry out the procedure by including in analysis a “blank” extraction, whereby one additional extract is subjected to the exact protocol of lipid extraction and processing, but with no biological sample present. In
particular, we warn readers to be cautious with all plastic (foam) and glass materials that can release FAE-like compounds leading to an overestimation of concentrations in biological samples. To avoid these pitfalls it would be convenient to indicate vendors in the material section for all consumables including glassware. High quality certified disposable glassware for LC/MS analysis is already on the market but limited to vials and inserts. The present paper shows that there is an urgent need to expand the variety of high-quality disposable glassware, including glass pipets, optimized for lipid extraction. In the meantime, we additionally recommend including a washing step of the glassware, with either sulpho-cromic acid mixture or chloroform, in the laboratory workflow.

**Author Disclosure Statement:** All authors declare no conflict of interest in connection with the submitted manuscript; hence, no competing financial interests exist.
References


### Tables

**Table 1. Comparison of serum/plasma average concentrations of FAEs and 2AG determined in this study with other reports.**

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<td>3.7</td>
<td>9.9</td>
<td>4.2</td>
<td>6.1</td>
<td>8.6</td>
<td>4.9</td>
<td>162.5</td>
<td>107.9</td>
<td>4.9</td>
<td>4.6</td>
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<td>4.0</td>
<td>12.6</td>
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<td>9.6</td>
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<tr>
<td>PEA</td>
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<td>28.4</td>
<td>23</td>
<td>4.7</td>
<td>4.9</td>
<td>8.0</td>
<td>5.2</td>
<td>203.6</td>
<td>213.3</td>
<td>24.8</td>
<td>16.9</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>32.8</td>
<td>1.7</td>
<td>39.0</td>
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Values are expressed in pmol/mL of serum. The PEA value obtained in this work is marked with an asterisk, indicating that is an artifact due to the unveiled contamination. In the selected reports, when only plotted and not reported in the text, values were back calculated from digitized images. S = serum; P = plasma.
Figure 1. Comparative LC/MS (panel A) and LC-MS/MS (panel B) analysis of PEA standard (traces a), pipet extract (traces b) and foam extract (traces c) by triple quad mass spectrometer. A) Positive EIC chromatogram for the \( m/z \) 300.3 Th generated from a 5 µL injection of a 1 µM solution of PEA, 5 pmoles on column (trace a), of the pipette extract (trace b) and of the foam extract (trace c); B) MS/MS analysis in the positive ion mode of the peaks in panel A at RT=5.7 min having \( m/z \) 300.3 Th. Panel A shows coeluting peaks at RT=5.7. In panel B the MS/MS spectra show peaks of the protonated PEA molecular ion \([M+H]^+\) at \( m/z = 300.3 \) Th and of its two fragment: the molecular ion arising from the water loss \([M+H-H_2O]^+\) at 282.3 Th and the ethanolamine positive pseudomolecular ion \([\text{Ethanolamine}+H]^+\) at 62.1 Th.
Figure 2. Comparative LC/MS analysis by high-resolution accurate mass spectrometer. Positive EIC chromatogram for the m/z 300.2897 Th (ppm dev < 5) generated from a 2 µL injection of a 1 µM solution of PEA, 2 pmoles on column (trace a) and of the pipet extract (trace b). The two chromatograms show coeluting peaks for the interrogated m/z.
Figure 3. Concentrations of PEA in extracts of pipettes. Each pipette was washed once with 8 mL of CHCl₃. Number of pipettes washed in each experiment is indicated in brackets. Error bars represent SD, n=3. The Fisher Pasteur pipettes, 5¾”, were from LOT n. 16168998 (Box 1).
Supplementary Figure 1.
Supplementary Figure 2.
Supplementary Figure 3. A) BPC chromatogram generated from a 5 µL injection of a 1 µM solution of PEA, 5 pmol on column; B) BPC chromatogram generated from the injection of the concentrated pipette extract; C) MS2 scan in positive ion mode at RT=5.7 min of the pipette extract showing peaks of the protonated PEA molecular ion at \( m/z = 300 \) Th, of the sodium adduct at 322 Th and of the molecular ion arising from the water loss at 282 Th. MS2 spectra of both PEA standard and pipette extract at 5.7 min are identical (only trace of standard is shown).
Supplementary Figure 4. MS1 LC profile of the pipet extract. Negative TIC is shown in blue; positive TIC is in violet and summed TIC is in black. In brown EIC of PEA accurate mass at 300.2897 Th, RT 6.3.
Supplementary Figure 5.
1) Fisher pasteur pipettes, 5 ¼ "", LOT n. 16168998, BOX 1
2) Fisher pasteur pipettes, 5 ¾ "", LOT n. 16168998, BOX 2
3) VWR pasteur pipettes, 5 ¼ "", LOT n. 16140309
4) Fisher pasteur pipette, 9 "", LOT n. 11004660

A) 1 pp, rinsed once with 8ml CHCl3, "extracted/washed" with 8mL CHCl3, dried and reconstituted in 100μL
B) 2 pp, no rinse, "extracted/washed" with 16mL CHCl3 (8mL each), dried and reconstituted in 100μL
C) 3 pp, no rinse, "extracted/washed" with 24mL CHCl3 (8mL each), dried and reconstituted in 100μL

n=3
SD is calculated on triplicates exp.

Supplementary Figure 6.
Supplementary Figure 7.
Chapter 2: Peripheral Endocannabinoid Signaling Controls Hyperphagia in Western Diet-Induced Obesity

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Abbreviations: 2-AG, 2-arachidonoylglycerol; AEA, arachidonylethanolamide, anandamide; CBR, cannabinoid receptor; ECBs, endocannabinoids; ESI, electrospray ionization; FAEs, fatty-acid ethanolamides; ISTDs, deuterated internal standards; LLOQ, lower limit of quantitation; MRM, multiple reaction-monitoring; UPLC/MS/MS ultra-performance liquid chromatography coupled to tandem mass spectrometry.

Material as previously published in Physiology and Behavior.
Abstract

The endocannabinoid system in the brain and periphery plays a major role in controlling food intake and energy balance. We reported that tasting dietary fats was met with increased levels of the endocannabinoids, 2-arachidonoyl-sn-glycerol (2-AG) and anandamide, in the rat upper small intestine, and pharmacological inhibition of this local signaling event dose-dependently blocked sham feeding of fats. We now investigated the contribution of peripheral endocannabinoid signaling in hyperphagia associated with chronic consumption of a western-style diet in mice ([WD] i.e., high fat and sucrose). Feeding patterns were assessed in male C57BL/6Tac mice maintained for 60 days on WD or a standard rodent chow (SD), and the role for peripheral endocannabinoid signaling at CB1Rs in controlling food intake was investigated via pharmacological interventions. In addition, levels of the endocannabinoids, 2-AG and anandamide, in the upper small intestine and circulation of mice were analyzed via liquid chromatography coupled to tandem mass spectrometry to evaluate diet-related changes in endocannabinoid signaling and the potential impact on food intake. Mice fed WD for 60 days exhibited large increases in body weight, daily caloric intake, average meal size, and rate of feeding when compared to control mice fed SD. Inhibiting peripheral CB1Rs with the peripherally-restricted neutral cannabinoid CB1 receptor antagonist, AM6545 (10 mg/kg), significantly reduced intake of WD during a 6 h test, but failed to modify
intake of SD in mice. AM6545 normalized intake of WD, average meal size, and rate of feeding to levels found in SD control mice. These results suggest that endogenous activity at peripheral CB1Rs in WD mice is critical for driving hyperphagia. In support of this hypothesis, levels of 2-AG and anandamide in both, jejunum mucosa and plasma, of ad-libitum fed WD mice increased when compared to SD mice. Furthermore, expression of genes for primary components of the endocannabinoid system (i.e., cannabinoid receptors, and endocannabinoid biosynthetic and degradative enzymes) was dysregulated in WD mice when compared to SD mice. Our results suggest that hyperphagia associated with WD-induced obesity is driven by enhanced endocannabinoid signaling at peripheral CB1Rs.

**Introduction**

Significant scientific and clinical evidence suggests one major driver of obesity is chronic consumption of foods that contain large quantities of fats and sugars (i.e., the western diet) (1, 2). Humans, rodents, and possibly other mammals detect dietary fats (3-7) and sugars (8) via receptors in the oral cavity and alimentary tract, which are critical in mediating preferences displayed for these high-energy foods (6, 9, 10). Numerous signaling pathways play important roles in the control of food intake, energy balance, and reward (9, 11), including endocannabinoid (eCB) signaling at cannabinoid CB1Rs in the brain (12-23) and periphery (12, 24-34).
Recent evidence suggests that the intake of palatable foods may be controlled by peripheral eCB signaling (9). For example, tasting emulsions containing mono- (i.e., oleic acid) or di-unsaturated fats (i.e., linoleic acid) – but not carbohydrate (i.e., sucrose) or protein – was met with large increases in eCB levels in the rat upper small intestine (27, 29). Pharmacological inhibition of eCB signaling at peripheral CB$_1$Rs blocked (i) the intake of dietary fats in sham feeding rats (27) and (ii) robust preferences for di-unsaturated fats in a sham feeding two-bottle choice test (29) [see (6, 35) for description of the sham feeding paradigm in rat, which isolates the cephalic phase of feeding from post-ingestive influence].

In addition to tasting dietary fats, we reported that fasting for up to 24 h is associated with increases in production of the eCB, 2-arachidonoyl-sn-glycerol (2-AG), in the upper small intestine of rats through a cholinergic-dependent mechanism that possibly involves the vagus nerve (28). For these experiments, fasting-induced 2-AG biosynthesis in the jejunum mucosa was blunted in rats that received either full subdiaphragmatic vagotomy or local intraduodenal infusion of the subtype 3 muscarinic acetylcholine receptor (m$_3$ mAChR) antagonist, DAU5884 (28). Furthermore, pharmacological inhibition of small intestinal m$_3$ mAChRs or CB$_1$Rs blocked fasting-induced refeeding (28). Thus, gut-brain eCB signaling is a proposed orexigenic signal that may promote feeding under several distinct behavioral and metabolic conditions.
Several studies in humans and rodents suggest that peripheral eCB levels are increased under conditions of obesity (36-42); however, a role for peripheral eCB signaling in driving hyperphagia associated with a western-style diet (WD) is unknown. In the current study, we investigated the impact of chronic consumption of WD on eCB levels in circulation and the upper small intestinal epithelium of mice, the contribution of WD-induced enhancements in eCB signaling at peripheral CB₁Rs in promoting hyperphagia associated with a WD, and expression of genes encoding key eCB system components in the small intestine.

Materials and Methods

Animals

Eight-week old male C57BL/6 mice (Taconic, Oxnard, CA, USA) were group-housed with free access to water and food, unless otherwise noted for food deprivation studies, and maintained on a 12 h light/dark cycle (lights off at 1800 h). Test diets consisted of standard lab rodent chow [(SD) Lab Diet 5001, St. Louis, MO, USA; 13.4% kcal as fat, 56% kcal from carbohydrates, mostly starch], or western-style diet [(WD) Research Diets D12709B, New Brunswick, NJ, USA; 40% kcal as fat, 43% kcal fromcarbohydrates,mostly sucrose]. Five days prior to experimentation, animals were single-housed in cages with wire mesh inserts to prevent coprophagia during 24 h food deprivation experiments. For studies analyzing feeding behaviors, mice were single-housed in feeding
chambers (TSE, Chesterfield, MO, USA) with free access to water and SD or WD (described further below in Feeding behavior). 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 of the University of California, Riverside.

Feeding behavior

Animals were acclimated to feeding chambers for five days prior to experimentation, and tested following 60 days on their respective test diet. Feeding behavior was monitored for the subsequent 24 h to assess daily intake patterns, or for 6 h following drug treatments. Feeding parameters included total caloric intake, average meal size, average rate of intake (kcals from food per minute), average number of meals, average meal duration, and average post-meal interval.

Chemicals and administration schedule

The peripherally restricted Cannabinoid Receptor Type 1 (CB₁R) antagonist, AM6545 (Sigma, St. Louis, MO, USA), was administered by IP injection at 10 mg/kg in 2 mL/kg. Vehicle consisted of 7.5% dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA), 7.5% tween 80 (Chem Impex Intl Inc., Wood Dale, IL, USA), 85% sterile saline (warmed in a bath sonicator for 30 min) 30 min prior (16:30 h) to the onset of behavioral analysis in feeding chambers. All control conditions were identical, except without drug present in the vehicle. The pharmacokinetics and half-life of AM6545 are not well-established, thus we
chose to evaluate intakes over a period of 6 h from time of administration after the onset of the dark phase. Mice maintained on SD or WD were split into two groups and analyzed across two sessions of behavioral testing such that each subgroup received either vehicle or drug with 72 h between administration.

**Tissue processing**

**Lipid extraction**

Isofluorane was used to anesthetize animals at time of tissue harvest (0900 to 1100 h), following 24 h food deprivation or ad-libitum feeding. Blood was collected by cardiac puncture and stored in EDTA-lined tubes on ice, then plasma was obtained by centrifugation (1500 g for 10 min, maintained at 4 °C). Jejunum was rapidly collected, washed with phosphate-buffered saline (PBS) on ice, sliced longitudinally on a stainless steel plate on ice, scraped with a glass slide to obtain mucosa, then snap-frozen in liquid N\textsubscript{2}. All samples were stored at \textasciitilde 80 °C until processing. Frozen tissues were weighed and subsequently homogenized in 1.0 mL of methanol solution containing the internal standard, \[^2\text{H}_5\] 2-AG and \[^2\text{H}_4\]-AEA (Cayman Chemical, Ann Arbor, MI, USA). Lipids were extracted with chloroform (2 mL) and washed with water (1 mL). Lipids were similarly extracted from plasma samples, with the exception of a 0.9% saline wash replacing water (0.1 mL plasma at the expense of saline). Organic phases were collected and separated by open-bed silica gel column chromatography as previously described (28). Eluate was gently dried under N\textsubscript{2} stream (99.998% pure) and resuspended in 0.1 mL of methanol:chloroform (9:1), with 1 μL.
injection for analysis by ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC/MS/MS).

**Measurement of 2-AG and anandamide**

Data was collected using an Acquity I Class UPLC system coupled to a Xevo TQ-S Mass Spectrometer (Waters, Milford, MA, USA) with accompanying electrospray ionization (ESI) interface. Lipids were separated on an Acquity UPLC BEH C\textsubscript{18} column (2.1 × 50 mm i.d., 1.7 μm, Waters) with inline Acquity guard column (UPLC BEH C\textsubscript{18} VanGuard Pre-column; 2.1 × 5 mm i.d., 1.7 μm, Waters), and eluted by a gradient of methanol in water (0.25% acetic acid, 5 mM ammonium acetate) according to the following gradient at a flow rate of 0.4 mL per min: 80% methanol 0.5 min, 80% to 100% methanol 0.5–2.5 min, 100% methanol 2.5–3 min, 100% - 80% methanol 3– 3.1 min). Column temperature was maintained at 40 °C, and samples were maintained in the sample manager at 10 °C. Argon (99.998%) was used as collision gas. MS detection was in positive ion mode and capillary voltage set at 0.1 kV. Cone voltage and collision energy as follows, respectively: 2-AG = 30v, 12v; [\textsuperscript{2}H\textsubscript{5}] 2-AG = 25v, 44v; anandamide =30v, 14v; [2H4] anandamide =26v, 16v. Lipids were quantified using a stable isotope dilution method detecting protonated adducts of the molecular ions [M+H]\textsuperscript{+} in the multiple reaction monitoring (MRM) mode. Acyl migration from 2-AG to 1-AG is known to occur (43), thus all reported values for 2-AG represent the sum of 2-AG and 1-AG. Tissue processing and LCMS analysis from an individual experiment occurred independently of other
experiments. Extracted ion chromatograms were used to quantify 2-AG (m/z = 379.3 > 287.3) and anandamide (m/z = 348.3 > 62.0), and [²H₅] 2-AG (m/z = 384.3 > 93.4) and [²H₄] anandamide (m/z = 352.3 > 66.1), which were used as internal standards. Our established lower limit of quantitation (LLOQ; signal-to-noise ratio of > 10) of analytes using our optimized UPLC/MS/MS methods are as follows: 2-AG, 0.5 pmol; AEA, 0.008 pmol.

Gene expression analysis

Total RNA was extracted from jejunal mucosa using a Trizol (Invitrogen, Carlsbad, CA) and RNeasy (Qiagen, Valencia, CA) combined method, and generated first-strand complementary DNA using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA). All surfaces for tissue collection and processing were sanitized using 70% ethanol and then treated with an RNAse inhibitor (RNAse out, G-Biosciences, St. Louis, MO, USA) to maintain integrity of isolated RNA. Reverse transcription of total RNA (9.5 μg) was performed with random hexamers (Invitrogen, Carlsbad, CA) for 50 min at 37 °C. qRT-PCR was carried out using PrimePCR Assays (Biorad, Irvine, CA, USA) with primers for cannabinoid receptor 1 (CNR1), cannabinoid receptor 2 (CNR2), diacylglycerol alpha and beta (DAGLA and DAGLB), monoacylglycerol lipase (MGLL), and fatty acid amide hydrolase (FAAH) using the preconfigured Sybr green assay (Biorad, Irvine, CA). Reactions were run in triplicate. Hprt was selected as a housekeeping gene for experimental conditions; no changes in its expression were found across conditions included in our analysis (Cq values for conditions,
n=4, two replicates: ad-libitum fed standard diet, 24.09 ± 0.10; 24 h fasted standard diet, 24.29 ± 0.13; ad-libitum fed western diet, 24.09 ± 0.44; 24 h fasted western diet, 24.21 ± 0.23; not significant).

**Statistical analyses**

Data was analyzed using Graphpad Prism6 software. Results are expressed as the mean ± S.E.M. Significant differences between groups were assessed using Student's two-tailed t-test, and regular or repeated measures two-way analysis of variance (ANOVA) with Student-Newman-Keuls or Sidak post hoc test, respectively, for comparison of means. Differences were considered significant if \( P < 0.05 \).

**Results**

**Western diet-induced obese mice are hyperphagic**

Mice fed WD ad-libitum for 60 days, when compared to mice maintained on standard diet (SD), rapidly gained body weight [Fig. 1a, diet effect on cumulative change in body weight \( F(1,30) = 125.6 \), \( p < 0.001 \), and interaction between diet and time \( F(29,870) = 157.3 \), \( p < 0.001 \); Fig. 1b, diet effect on cumulative gross body weight \( F(1,30) = 91.74 \), \( p < 0.001 \), and interaction between diet and time \( F(29,870) = 157.3 \), \( p < 0.001 \)], and consumed substantially higher calories over a 24 h period (Fig. 1c: \( t=3.89 \), \( p < 0.001 \)). This effect was met with increased average meal size (Fig. 1d: \( t=4.75 \), \( p < 0.001 \)) and rate of food consumption (Fig. 1e: \( t = 3.77 \), \( p < 0.001 \)), and remained irrespective of body weight (SD versus WD: total calories, from 241.6 ± 32.8 to 405 ± 44.7 kcal per kg...
bodyweight, $t = 2.96$, $p = 0.01$; average meal size, from $23.4 \pm 1.5$ to $33.0 \pm 2.7$ kcal per kg body weight, $t = 2.78$, $p = 0.01$; average rate of intake, from $11.0 \pm 0.8$ to $14.3 \pm 1.2$ kcal per kg per minute, $t = 2.08$, $p = 0.04$; data from 8 animals per condition). Other feeding parameters including meal duration (Fig. 1f: $t = 1.03$, $p = 0.31$), frequency (Fig. 1g: $t = 1.69$, $p = 0.11$), and post-meal interval (Fig. 1h: $t = 0.31$, $p = 0.76$) remained unchanged in WD mice when compared to SD mice. These results indicate that mice fed WD for 60 days exhibit hyperphagia due to larger meal size and rate of consumption.

**Western diet intake is associated with increases in levels of endocannabinoids in jejum epithelium and circulation**

We previously reported that tasting dietary fats was met with increased levels of 2-AG and anandamide in the jejunum of rats, but in no other peripheral organ tested (27, 29). Importantly, direct infusion of low doses of the CB$_1$R antagonist, rimonabant (0.3mg/kg), into the jejunum, or peripheral administration of peripherally-restricted CB$_1$R inhibitors that do not reach the brain (see for AM6545 (24, 30) and URB447 (26)) blocked fat sham feeding (27) and preferences for linoleic acid in a two-bottle choice sham feeding test (29). These results suggest that tasting dietary fats drives eCB signaling in the upper small intestine, which in turn, generates positive feedback that promotes dietary fat intake (27, 29).

In order to elucidate the molecular underpinnings of WD-induced hyperphagia in the current study and evaluate the contribution of peripheral eCB
signaling in these processes, we quantified small intestinal and circulating levels of the eCBs, 2-AG and anandamide, in mice maintained on WD or SD under free-feeding and 24 h fasting conditions. Free-feeding mice fed WD for 60 days (Fig 2a), when compared to free-feeding mice maintained on SD, displayed higher levels of 2-AG in jejunum mucosa that reached concentrations indistinguishable from fasted SD or fasted WD mice [Fig 2a, diet effect on 2-AG levels $F(1,24)=4.52$ $p=0.04$, and interaction between diet and feeding condition $F(1,24)=4.98$ $p=0.03$; multiple comparisons test of free-feeding SD versus fasted SD and both free-feeding and fasted WD $p<0.05$, multiple comparisons test of free-feeding WD versus both fasted SD and WD $p=ns$]. In addition, anandamide levels in jejunum of WD mice were elevated when compared to SD mice irrespective of feeding status [Fig 2b, diet effect on anandamide levels $F(1,24)=25.88$ $p<0.001$, and no interaction between diet and feeding condition $F(1,24)=0.53$ $p=0.47$; multiple comparisons test of FF SD versus FF WD, $p<0.05$; FD SD versus both FF and FD WD, $p<0.001$]. Furthermore, levels of 2-AG were greatly elevated in plasma of both free-feeding and 24 h fasted WD mice when compared to SD mice [Fig 2c; diet effect on 2-AG levels $F(1,33)=141.3$ $p<0.001$, and interaction between diet and feeding condition $F(1,33)=6.62$ $p=0.02$; multiple comparisons test of all SD feeding conditions versus WD, $P<0.001$], an effect also found for anandamide [Fig 2d, diet effect on anandamide levels $F(1,33)=168.5$ $p<0.001$, and no interaction between diet and feeding condition $F(1,33)=0.01$ $p=0.94$; multiple comparisons test of all SD feeding conditions...
versus WD, p<0.001]. Collectively, these data suggest that chronic ad-libitum consumption of a WD is associated with elevated levels of eCBs in mouse jejunal epithelium and plasma, which may in turn, activate peripheral CB1Rs and promote hyperphagic responses to WD.

Inhibiting peripheral CB1Rs blocks hyperphagia in mice maintained on a western diet

We next evaluated the contribution of heightened eCB signaling at peripheral CB1Rs (see Fig 2) in hyperphagia associated with WD-induced obesity. Mice fed ad-libitum WD or SD for 60 days were treated with the peripherally-restricted neutral CB1R antagonist, AM6545 ((25, 30) 10 mg per kg IP) or vehicle prior to a 6 h feeding test. When compared to vehicle treatment, AM6545 inhibited intake of WD [Fig 3a, drug effect on WD intake $F(1,12)=7.45$ p=0.018, and interaction between drug effect and time ($F(5,60)=11.39$ p<0.001; multiple comparisons test of AM6545 treatment versus vehicle, 4hr p<0.05, 5hr p<0.01, 6hr p<0.001)]. In contrast to WD, AM6545 failed to affect intake of SD at all time points [Fig 3b, no drug effect on WD intake $F(1,14)=0.06$ p=0.82, and no interaction between drug effect and time $F(5,70=1.01$ p=0.42]. Similar to results found for 24 h intakes of WD versus SD (Fig 1b-g), vehicle-treated WD mice, when compared to vehicle-treated SD mice, displayed increased 6 h caloric intake [Fig 3c, diet effect on intake $F(1,26)=4.23$ p=0.05; multiple comparisons test of SD versus WD p<0.01], average meal size [Fig 3d, diet effect on intake $F(1,26)=5.41$ p=0.02; multiple comparisons test of SD versus WD, p<0.01], and
rate of feeding [Fig 3e, diet effect on intake $F(1,26)=5.52$ $p=0.02$; multiple comparisons test of SD versus WD, $p<0.05$]. Treatment with AM6545, when compared to vehicle, significantly decreased WD intake [Fig 3c, drug effect on intake $F(1,26)=9.83$ $p=0.01$, and interaction between drug and feeding condition $F(1,26)=13.96$ $p=0.001$; multiple comparisons test of AM6545 WD versus vehicle WD, $p<0.01$], and normalized intake to levels found for SC (Fig 3c; multiple comparisons test of AM6545 WD versus both AM6545 SD and vehicle SD, $p=ns$). Treatment with AM6545, when compared to vehicle, similarly decreased WD meal size [Fig 3d, drug effect on meal size $F(1,26)=10.68$ $p=0.01$, and interaction between drug and feeding condition $F(1,26)=5.52$ $p=0.02$; multiple comparisons test of AM6545 WD versus vehicle WD, $p<0.01$], and normalized intake to levels found for SC (Fig 3d; multiple comparisons test of AM6545 WD versus both AM6545 SD and vehicle SD, $p=ns$). Increased rate of intake found for vehicle WD versus vehicle SD was absent in AM6545-treated animals (Fig 3e; multiple comparisons test of WD versus SD in AM6545-treated mice, $p=ns$); however, there was high variability and a lack of group effect of drug [Fig 3e, drug effect on rate of intake $F(1,26)=0.598$ $p=0.44$, and no interaction between drug and feeding condition $F(1,26)=2.25$ $p=0.14$]. Treatment with AM6545 had no effect on any meal parameters included in our analysis in SD mice (Fig 3c-e; multiple comparisons test of AM6545 SD versus vehicle SD, $p=ns$). Our results suggest that elevated levels of endogenous eCB signaling at peripheral CB$_1$Rs drives hyperphagia in WD-induced obesity.
Western diet affects gene expression of eCB system components in jejunum

Expression of gene transcripts were increased in fasted SD mice when compared to free-feeding controls for cannabinoid receptor 1 [(CNR1) Fig 4a, feeding condition effect on expression \(F(1,41)=4.63, p=0.04\); multiple comparisons test of free-feeding SD versus fasted SD, \(p<0.01\)], cannabinoid receptor 2 [(CNR2) Fig 4b, feeding condition effect on expression \(F(1,41)=6.83, p=0.01\); multiple comparisons test of free-feeding SD versus fasted SD, \(p<0.05\)], and diacylglycerol lipase α [(DAGLA, 2-AG biosynthetic enzyme) Fig 4c, feeding condition effect on expression \(F(1,44)=13.21, p<0.001\), multiple comparisons test of free-feeding SD versus fasted SD, \(p<0.05\)]. In addition, gene expression was significantly modified in WD-fed mice, when compared to SD-fed mice, such that fasting effects were absent for CNR1 [Fig 4a, diet effect on expression \(F(1,41)=5.39\) \(p=0.03\), and interaction between diet and feeding condition \(F(1,41)=4.698\) \(p=0.04\); multiple comparisons test of free-feeding WD versus fasted WD, ns; multiple comparisons test of both free-feeding and fasted WD versus fasted SD, \(p<0.01\)] and DAGLA [Fig 4c, diet effect on expression \(F(1,44)=6.42\) \(p=0.02\), and no interaction between diet and feeding condition \(F(1,44)=0.2481\) \(p=0.63\); multiple comparisons test of free-feeding WD versus fasted WD, \(p=ns\); multiple comparisons test of both free-feeding and fasted WD versus fasted SD \(p<0.05\)]; however, a group diet effect was not found for CNR2
Feeding status was not met with any significant changes in gene expression in SD and WD mice for diacylglycerol lipase β [DAGLB, 2-AG biosynthetic enzyme; Fig 4d, feeding condition effect on expression $F(1,44)=2.1$ p=0.154], monoacylglycerol lipase [(MGLL, 2-AG degradative enzyme) Fig 4e, feeding condition effect on expression $F(1,44)=0.07$ p=0.79], and fatty acid amide hydrolase [(FAAH, anandamide degradative enzyme) Fig 4f, feeding condition effect on expression $F(1,41)=1.33$ p=0.26]. There was, however, a significant group diet effect for expression of MGLL in WD-fed mice versus SD-fed mice for [Fig 4e, diet effect on expression $F(1,44)=11.95$ p=0.001, and interaction between diet and feeding condition $F(1,44)=5.25$ p=0.03; multiple comparisons test of both free-feeding and fasted WD mice versus free-feeding SD mice] and FAAH [Fig 4f, diet effect on expression $F(1,41)=4.1$ p=0.05, and interaction between diet and feeding status $F(1,41)=4.81$ p=0.03 ; multiple comparisons test of both free-feeding and fasted WD mice versus free-feeding SD mice].

Collectively, the results suggest a dysregulation in the expression of eCB components in WD mice; however, elevated levels of eCBS in jejunum and plasma are not entirely explained by these effects.
Discussion

In 2013, the American Medical Association announced that obesity is a disease, which affects nearly one-third of American adults with significant negative impact on life expectancy (44). Mounting evidence suggests that a primary contributing factor of these obesity rates is overconsumption of high-energy foods containing large quantities of fats and sugars, known as the “western diet” (WD (1, 2)). We utilized a mouse model of WD-induced obesity in the present study to investigate the role for peripheral eCB signaling in hyperphagia associated with consumption of western diet. Collectively, our results suggest that chronic consumption of a WD is associated with elevated levels of 2-AG and anandamide in jejunum mucosa and plasma of mice, which may in turn, activate peripheral CB1Rs and promote hyperphagic responses to WD.

Mice fed ad-libitum WD for 60 days displayed robust increases in caloric intake as a result of increased meal size, and rate of feeding (i.e., consummatory behaviors) when compared to control mice maintained on SD that contained low levels of fats and sugar (Fig 1). These data suggest that hyperphagia associated with WD-induced obesity may be driven by hedonically-positive feedback from specific constituents of the diet. Indeed, merely tasting dietary fats (i.e., corn oil) or carbohydrates (i.e., sucrose) induced dopamine outflow in the ventral striatum in rats (45, 46), which suggests that palatable food taste may promote intake by a mechanism that includes activating regions of the brain associated with food
reward. Furthermore, we reported that tasting liquid diets containing dietary fats increased levels of the eCBs in the upper small intestine of rats, but no other organ tested, and pharmacological inhibition of this local signaling event with selective CB₁R antagonists blocked intake and preferences for fat (27, 29). These studies suggest a gut-brain eCB signaling axis that promotes palatable food intake based on its unique gustatory properties (6).

In the current study, hyperphagia observed in WD-induced obese mice was blocked by peripheral administration of the peripherally-restricted neutral CB₁R antagonist, AM6545; however, AM6545 exerted no effect on the intake of SD during a 6 h test (Fig 2). The pharmacokinetics and half-life of AM6545 are not well-established, thus we evaluated intakes over a period of 6 h from the onset of the dark phase. Importantly, inhibiting peripheral eCB signaling at CB₁Rs in WD mice normalized enhancements in meal size to levels found in SD mice and blocked increases in rate of feeding. These results suggest that heightened endogenous activity at peripheral CB₁Rs is responsible for driving hyperphagia associated with chronic consumption of WD, an effect not present in mice fed SD, which might not display active eCB signaling during ad-libitum feeding conditions. Based on these results and our previous studies in rats that revealed increases in eCB signaling exclusively in the small intestine in response to tasting fats (27, 29) and fasting for 24 h (28), we asked whether eCB levels in the epithelium of the jejunum may also be elevated in WD mice and drive hyperphagia. Indeed, levels of 2-AG were significantly elevated in the epithelium
of the jejunum in free-feeding mice fed WD for 60 days when compared to ad-libitum fed mice maintained on SD, and reached levels comparable to those in fasted mice maintained on SD (Fig 3a). This result is consistent with reports from other groups that show an enhancement of eCB levels in peripheral organs, namely in the upper small intestine (39, 47) and liver (40, 48), of mice maintained on high-fat diets for eight and 14 to 16 weeks, respectively. In contrast to fasting-induced increases in levels of jejunal 2-AG displayed by SD mice – which is an effect similar to results we previously reported in rats (28) – WD mice failed to display any further increases in 2-AG after a 24 h fast when compared to free-feeding conditions (Fig 3a). This result suggests a maximal level of 2-AG production in small intestinal epithelium, possibly due to a limited availability of 2-AG precursors (e.g., 1, stearoyl, 2-arachidonoyl-sn-glycerol (28, 49)).

Furthermore, in contrast to reports in rats (31, 39), we found no significant changes in anandamide levels in jejunum mucosa in response to fasting in SD mice, which might indicate species and/or strain differences among rodents. Similar to 2-AG, levels of anandamide were not affected by fasting in WD mice. Collectively, the results suggest that enhanced eCB activity at CB₁Rs in the jejunum of WD mice may promote hyperphagia by increasing meal size and rate of feeding. Further studies that investigate discrete components of WD in these responses, however, are warranted.

Specific downstream pathways that communicate peripherally-derived eCB signals to the brain and promote feeding of WD remain to be determined;
however, neural and endocrine mechanisms may play a prominent role. One potential downstream candidate mechanism includes enhanced eCB-mediated inhibition of cholecystokinin release from the upper small intestine in WD-induced obesity, which may in turn, act to increase meal size and rate of feeding of WD by delaying the activation of cholecystokinin-mediated satiation signaling to the brain carried by the afferent vagus nerve (50). In support of this hypothesis, gene transcripts for CB1Rs have been identified in enteroendocrine I cells within the duodenum of rodents, which may act to inhibit cholecystokinin release (51). In addition to enhanced local signaling in the upper small intestine of WD mice, elevated levels of circulating eCBs in WD mice may also act as an endocrine signal that directly interacts with feeding- and reward-related pathways in the brain, and thereby contribute to hyperphagia and WD-induced obesity found in our studies. Indeed, studies in normal weight and obese humans suggest that consumption of highly palatable foods (i.e., hedonic eating) is associated with elevated levels of 2-AG in plasma (52, 53). Furthermore, when compared to normal weight human subjects, plasma levels of 2-AG were elevated in obese women (38), and both 2-AG and anandamide were elevated in saliva of obese male and female subjects (36). It remains to be determined, however, if circulating eCBs may also directly interact with feeding- and reward-related pathways in the brain.

Analysis of gene expression of primary constituents of the eCB system suggests a dysregulation in the expression of key components of the eCB
system following chronic consumption of WD; however, heightened eCB tone in WD obesity is not entirely explained by these results. We found that fasting for 24 h was met with increased expression of mRNA for CB1Rs, CB2Rs, and DAGLα in the jejunum mucosa of mice maintained on SD; however, WD mice failed to display a similar response (Fig 4a,b,c). Interestingly, there was group effect on the expression of DAGLα such that WD mice had significantly lower expression when compared to SD. In light of elevated levels of 2-AG in the jejunum mucosa and plasma of mice fed a WD versus SD, this result is counter to what we expected for expression of this biosynthetic enzyme for 2-AG. Furthermore we found increases in the expression of mRNA for the 2-AG and anandamide degradative enzymes, MAGL and FAAH respectively, in mucosa of WD mice when compared to levels in SD mice (Fig 4e), which is counter to our expectation that eCB degradative enzyme expression would decrease given elevations in 2-AG and anandamide. This result may reflect an adaptive response to elevated levels of 2-AG and anandamide in WD mice. No changes were found in DAGLβ expression under all conditions (Fig 4d), which suggests that similar to results found in brain (49), the DAGLα isoform rather than DAGLβ, may be primarily involved in the biosynthesis of 2-AG in jejunum mucosa. Collectively, our gene expression results do not provide clear evidence that explains elevations in eCB levels in WD mice when compared to SD mice.

An analysis of levels of protein for all components of the eCB system is warranted, as well as analysis of the function of eCB biosynthetic and
degradative enzymes via functional enzyme assays, which will help to address the underpinnings of eCB elevations in WD mice. Furthermore, it is plausible that cholinergic signaling is enhanced under conditions of ad-libitum feeding of WD rather than any specific changes in the expression of eCB machinery. A test of these hypotheses remain. The latter scenario is supported by previous evidence from our group that suggests a key role for cholinergic signaling at small intestinal muscarinic m3 receptors, and likely the efferent vagus nerve, in driving 2-AG production and re-feeding after a fast in rats (28). In further support of this hypothesis, we reported that tasting dietary fats drives production of the eCBs in the upper small intestine of rats, an effect that is absent in animals that received complete subdiaphragmatic vagotomy. This result suggests that critical signals from the oral cavity related to fat taste are transmitted to the small intestine via the vagus nerve (27, 29). Future studies will be important to evaluate the contribution of cholinergic signaling in driving eCB production in the periphery that promotes hyperphagia associated with consumption of WD.

**Conclusion:** Our results suggest that mice fed a WD for 60 days are hyperphagic due to increases in meal size and rate of consumption, and that feeding responses are driven – at least in part – by increases in peripheral eCB tone in the upper small intestinal epithelium and circulation. Future studies will be critical to evaluate the specific downstream pathways that peripheral eCBs interact with to communicate with the brain in the control of feeding, including possible neural (e.g., vagal) and endocrine (i.e., circulation) mechanisms. In
addition, it will be important to evaluate the interaction between a variety of species of lipids (e.g., high in mono- or di-unsaturated fats versus saturated and polyunsaturated fats) and carbohydrates (e.g., fructose, sucrose) on eCB signaling in the gut and brain, and their impact on hedonic feeding behaviors that can lead to obesity.

Together, our work suggests peripheral CB₁Rs may be an effective therapeutic target for the treatment of western diet-induced obesity and eating disorders. This therapeutic approach has substantial advantage over traditional CB₁Rs antagonists/inverse agonists (e.g., rimonabant) that cross the blood-brain barrier and interact with central brain mechanisms. For example, rimonabant was found to be effective for the treatment of obesity and metabolic syndrome in humans; however, benefits were met with severe psychiatric side effects, including depression and in some instances, suicide (54). These outcomes precluded rimonabant from gaining FDA approval. On the other hand, the work in the present report as well as others (12, 24-34) suggests that targeting peripheral CB₁Rs with antagonists (e.g., AM6545) that do not reach the brain may be an effective treatment strategy for metabolic syndrome and possibly eating disorders, without deleterious psychiatric side-effects inherent to brain-penetrant CB₁R inhibitors.

**Author Disclosure Statement:** All authors declare no conflict of interest in connection with the submitted manuscript; hence, no competing financial interests exist.
References


Figure 1. Chronic consumption of a western diet is associated with hyperphagia. Male mice maintained for 60 days on a western diet (Western) become obese (a, cumulative change in body weight; b, gross body weight) and display increases in 24 h caloric intake, meal size, and rate of intake (c-e) when compared to mice maintained on a standard chow diet (Stand). Meal duration, frequency, and post meal interval do not significantly differ between diets (f-h). Repeated measures two-way ANOVA, with Sidak's multiple comparison post hoc test, *** = p < 0.001 (a); unpaired Student's t-test (two-tailed), *** = p < 0.001 between Stand and Western. Results are expressed as means ± SEM; n=16/condition (a,b), n=8/condition (c-h).
Figure 2. Mice fed a western diet display increases in levels of 2-AG and anandamide in jejunum mucosa and plasma. Mice maintained on a standard diet (Stand) that were fasted for 24 h (FD) display increases in 2-AG levels in jejunum mucosa, when compared to free feeding controls [FF (a)], and FF male mice maintained on western diet (Western) display increases in levels of 2-AG in jejunum mucosa to levels found in fasted Stand mice (a). Western mice display elevated anandamide in jejunum mucosa when compared to Stand mice, irrespective of feeding condition (b). Western mice display increases in plasma levels of 2-AG (c) and anandamide (d) when compared to Stand mice. Two-way ANOVA with Student-Newman-Keuls multiple comparison post hoc test. * = p < 0.05, *** = p < 0.001, ns = not significant. Results are expressed as means ± SEM; n = 7/condition for jejunum, n=9–10/condition for plasma.
Figure 3. Inhibiting peripheral CB1Rs reduces food intake in mice fed a western diet, but not a standard diet, and normalizes intake and meal patterns in western diet fed mice. Pharmacological blockade of peripheral CB1Rs with AM6545, when compared to vehicle treatment (Veh), inhibits the caloric intake of male mice maintained for 60 days on western diet [WD (a)] during a 6 h test, but has no effect on caloric intake in mice maintained on standard chow diet [b (SD)]. Vehicle-treated WD mice display an increase in caloric intake (c), average meal size (d), and rate of feeding [e (kcal per minute of feeding)], when compared to Veh-treated SD mice during a 6 h test. Pharmacological blockade of peripheral CB1Rs with AM6545 reduces total caloric intake (c), meal size (d), and rate of intake (e) in WD mice to levels indistinguishable from SD control mice, and has no effect on meal parameters in SD mice (c-e). Repeated measures (a,b) or regular (c-e) two-way ANOVA, with Sidak’s or Student-Newman-Keuls, respectively, multiple comparison post hoc test, * = p < 0.05, ** = p < 0.01, *** = p < 0.001. Results are expressed as means ± SEM; n=7–8/condition.
Figure 4. Expression of genes for components of the endocannabinoid system is modified in mice fed a western diet. Expression of mRNA encoding CB₁R [a (CNR1)], CB₂R [b (CNR2)], DAGL-α [c (DAGLA)], and FAAH (f) are elevated in standard chow-fed (Stand) 24 h fasted (FD) male mice, an effect absent in mice fed a western diet (Western). No changes are found under all conditions for DAGL-β [d (DAGLB)]. Expression of MAGL and FAAH genes are elevated in Western mice when compared to Stand (e,f). Two-Way ANOVA with Student-Newman-Keuls multiple comparison post hoc test. ** = p < 0.01, * = p < 0.05, ns = not significant. Results are expressed as means ± SEM; n = 3–4/condition in triplicate.
Chapter 3: Cannabinoid CB\(_1\) Receptors Inhibit Gut-Brain Satiation Signaling in Diet-Induced Obesity

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Abbreviations: 2-AG, 2-Arachidonoyl-sn-glycerol; Abhd6, alpha-beta-hydrolyzing domain 6; AEA, Anandamide; AM, AM6545; CB\(_1\)R, Cannabinoid receptor subtype 1; CB\(_2\)R, Cannabinoid receptor subtype 2; CCK, Cholecystokinin; CO, Corn oil; DAG, Diacylglycerol; Dev, Devazepide; DGL, Diacylglycerol lipase; DIO, Diet-induced obesity; eCB, Endocannabinoid; eGFP, Enhanced green fluorescent protein; FAAH, Fatty acid amide hydrolase; FACS, Fluorescence activated cell sorting; FAE, Fatty acid ethanolamide; MAG, Monoacylglycerol; MGL, Monoacylglycerol lipase; NAPE-PLD, N-acyl phosphatidylethanolamine-specific phospholipase D; SD, Standard diet; WD, Western diet; WIN, WIN 55,212-2

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Abstract

Gut-brain signaling controls feeding behavior and energy homeostasis; however, the underlying molecular mechanisms and impact of diet-induced obesity (DIO) on these pathways are poorly defined. We tested the hypothesis that elevated endocannabinoid activity at cannabinoid CB1 receptor (CB1Rs) in the gut of mice rendered DIO by chronic access to a high-fat and sucrose diet inhibits nutrient-induced release of satiation peptides and promotes overeating. Immunoreactivity for CB1Rs was present in enteroendocrine cells in the mouse upper small-intestinal epithelium that produce and secrete the satiation peptide, cholecystokinin (CCK), and expression of mRNA for CB1Rs was greater in these cells when compared to non-CCK producing cells. Oral gavage of corn oil increased levels of bioactive CCK (CCK-8) in plasma from mice fed a low-fat no-sucrose diet. Pretreatment with the cannabinoid receptor agonist, WIN55,212-2, blocked this response, which was reversed by co-administration with the peripherally-restricted CB1R neutral antagonist, AM6545. Furthermore, monoacylglycerol metabolic enzyme function was dysregulated in the upper small-intestinal epithelium from mice fed a high-fat and sucrose diet, which was met with increased levels of a variety of monoacylglycerols including the endocannabinoid, 2-arachidonoyl-sn-glycerol. Corn oil failed to affect levels of CCK in DIO mouse plasma; however, pretreatment with AM6545 restored the ability for corn oil to stimulate increases in levels of CCK, which suggests that elevated endocannabinoid signaling at small-intestinal CB1Rs in DIO mice
inhibits nutrient-induced CCK release. Moreover, the hypophagic effect of AM6545 in DIO mice was reversed by co-administration with the CCKA receptor antagonist, devazepide. Collectively, these results provide evidence that hyperphagia associated with DIO is driven by a mechanism that includes CB1R-mediated inhibition of gut-brain satiation signaling.

Introduction

Food intake and energy homeostasis are controlled by a dynamic interplay of gut-brain signaling pathways that are not well-defined but are thought to become dysregulated in obesity (1). Recent studies in humans and rodents suggest a critical role for the endocannabinoid (eCB) system in these processes (2-4). The eCB system is located in cells throughout the body and is comprised of the eCBs, 2-arachidonoyl-sn-glycerol (2-AG) and anandamide (AEA), their biosynthetic and degradative enzymes, and the cannabinoid receptor subtypes 1 and 2 [CB1R and CB2R, respectively (5, 6)]. CB1Rs in the brain control food intake and energy homeostasis (3, 7); however, targeting central CB1Rs with antagonists for the treatment of human obesity led to psychiatric side-effects that preclude their use as safe and effective anti-obesity therapeutics (8). In contrast, CB1Rs antagonists that are restricted to the periphery and do not readily cross the blood-brain barrier are associated with improvements in a variety of metabolic parameters in rodents and may be an effective anti-obesity strategy that is devoid of psychiatric side-effects inherent to brain-permeable drugs (9-17). Nonetheless, peripheral mechanisms influence brain function [e.g., signals from
the gut microbiome (18)]; thus, the impact of disrupting endocannabinoid signaling at peripheral CB1Rs on these functions is largely unknown and warrants future investigation.

Studies from our lab and others suggest key roles for the peripheral eCB system in controlling feeding behavior and energy homeostasis (2, 7, 19). Indeed, eCB levels are increased in the small-intestinal epithelium of rodents (i) during a fast (11, 20-22), (ii) after oral exposure to dietary fats (9, 10), and (iii) in a mouse model of western diet-induced obesity (DIO) (22). Pharmacological inhibition of elevated eCB signaling at small-intestinal CB1Rs with peripherally-restricted CB1R antagonists blocks (i) re-feeding after a fast (11), (ii) intake of dietary fats based on their orosensory properties (9, 10), and (iii) overeating (i.e., increased meal size and caloric intake) associated with DIO, (22). These studies suggest that the eCB system in the small-intestinal epithelium plays a key role in feeding behavior and energy balance, and becomes dysregulated in DIO.

The mechanism(s) underlying eCB control of gut-brain signaling and its dysregulation in DIO is largely unknown. Nonetheless, CB1Rs are expressed on the afferent vagus nerve and suggested to control feeding behavior and energy balance by directly modifying gut-brain vagal signaling important for food intake (23-25). For example, expression of CB1Rs in the rat nodose ganglion is upregulated after fasting, and refeeding or administration of the gut-derived satiation peptide, cholecystokinin (CCK), reversed this effect (23, 25). Moreover, both, fasting-induced increases in CB1R expression in the nodose ganglion and
the ability for CCK to decrease this response were blunted in rats fed a high-fat diet (26). Elmquist and colleagues, however, reported that select deletion of CB1Rs on the afferent and efferent vagus nerve had no effect on food intake or body weight in mice fed a standard rodent chow or high-fat diet (27). These findings suggest that CB1Rs expressed on the vagus nerve may be dispensable for feeding behavior and maintenance of body weight.

Dietary fats and other macronutrients are sensed by enteroendocrine cells in the small-intestinal epithelium and stimulate release of satiation peptides including CCK (1, 28-30), which controls meal size and satiation by activating CCKA receptors on the afferent vagus nerve (31-37) and possibly in the brain (38, 39). Furthermore, CCK-containing L-cells in the upper small-intestinal epithelium of mice express genes for CB1Rs (40). Thus, CB1Rs in the small-intestinal epithelium may control feeding behavior by an indirect mechanism that includes controlling release of gut-derived satiation peptides. We investigated this possibility by testing the hypothesis that elevated endocannabinoid activity at CB1Rs in the gut of mice rendered DIO by chronic access to a high-fat and sucrose diet inhibits nutrient-induced release of satiation peptides, which in turn, leads to overeating by delaying satiation.

Materials and Methods

Animals

Eight-week old C57BL/6 mice (Taconic, Oxnard, CA, USA) were group-housed with ad libitum food and water access and maintained on a 12 h
dark/light cycle. C57BL/6-Tg(Cck-EGFP)2Mirm/J mice with enhanced green fluorescent protein on the promoter for cholecystokinin were used for immunohistochemistry and fluorescence-activated cell sorting (FACS) of small-intestinal CCK-containing cells (Jackson Laboratories, Bar Harbor, ME, USA). Test diets included Teklad 2020x soy-purified Standard Rodent Chow (SD; Envigo, Huntingdon, UK) or Western-style diet (WD; Research Diets D12709B, New Brunswick, NJ, USA; 40% kcal as fat, 43% kcal as carbohydrates, mainly sucrose). Body weights were recorded every other day at noon. To assess feeding behaviors, mice were single-housed in behavior chambers (TSE Systems, Chesterfield, MO, USA). 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 of the University of California, Riverside.

Feeding behaviors

Animals were placed into feeding chambers five days prior to recording for acclimation, and testing began at 60 days after being placed on their respective experimental diets. Feeding behaviors were assessed starting 1 h prior to dark cycle (1700 h) over a 24 h period for acclimation and for 12 h following drug administrations. Behavioral parameters include total caloric intake, average meal size, average rate of intake, average number of meals, first meal size, average meal duration, and average post meal interval. Data were processed using TSE Phenomaster software.
Chemicals and administration schedule

AM6545, a peripherally-restricted CB1R neutral antagonist, was given by IP injection at 10 mg per kg (Northeastern University Center for Drug Discovery, Boston, MA, USA). Devazepide (Tocris, Bristol, UK), a CCKA receptor antagonist, was given IP at 0.3 mg per kg. Both drugs were dissolved in vehicle consisting of 7.5% DMSO, 7.5% Tween80, and 85% sterile saline, and warmed in a water bath to ensure solubility. All control conditions were matched, using vehicle in place of drugs and injections occurred 1 h prior to behavior recording (1600 h). A 72-h washout period was allowed between drug treatments. JZL184 (Tocris, Bristol, UK), a potent inhibitor of monoacylglycerol lipase (MGL), was used to prevent monoacylglycerol hydrolysis in the diacylglycerol lipase (DGL) assay and to validate our MGL assay (described below). Tetrahydrolipstatin (Tocris, Bristol, UK), a lipase inhibitor used routinely to study DGL activity (41, 42), was used to validate our DGL assay.

Measurement of intestinal lipids

Tissue harvest and lipid extraction

Animals were anesthetized with isofluorane at time of tissue harvest (1500-1700 h) following ad libitum food and water access. Blood was collected by cardiac puncture and deposited into vacutainers containing EDTA; plasma was collected as supernatant following 10 min centrifugation at 1500 G (kept at 4°C). Jejunum was quickly removed and washed in phosphate-buffered saline (PBS), opened longitudinally on a stainless-steel tray on ice, and contents
removed. Jejunum mucosa was isolated using glass slides to scrape the epithelial layer and was snap-frozen in liquid N\textsubscript{2}. Samples were stored at -80°C pending analysis. Frozen tissues were weighed and then homogenized in 1 mL methanol solution containing 500 pmol \[^{2}H_5\]-2-AG (Cayman Chemicals, Ann Arbor, MI) as an internal standard. Lipids were extracted as previously described (22) and resuspended in 0.1 mL methanol:chloroform (9:1) and analyzed via ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS).

\textit{LCMS detection of 2-arachidonoyl-sn-glycerol and other monoacylglycerols}

Data were acquired using an Acquity I Class UPLC with direct connection to a Xevo TQ-S Micro Mass Spectrometer (Waters Corporation, Milford, MA, USA) with electrospray ionization (ESI) sample delivery. Lipids were separated using an Acquity UPLC BEH C\textsubscript{18} column (2.1 x 50 mm i.d., 1.7 µm, Waters Corporation) and inline Acquity guard column (UPLC BEH C\textsubscript{18} VanGuard PreColumn; 2.1 x 5 mm i.d.; 1.7 µm, Waters Corporation), and eluted by a gradient of water and methanol (containing 0.25% acetic acid, 5 mM ammonium acetate) at a flow rate of 0.4 mL per min and gradient: 80% methanol 0.5 min, 80% to 100% methanol 0.5 – 2.5 min, 100% methanol 2.5 – 4.5 min, 100% to 80% methanol 4.5 – 4.6 min, and 80% methanol 4.6 – 5.5 min. The column was maintained at 40°C, and samples were kept at 10°C in accompanying sample manager. MS/MS detection was in positive ion mode with capillary voltage maintained at 1.10 kV, and argon (99.998%) was used as collision gas. Cone
voltages and collision energies for respective analytes: 2-AG (20:4) = 30v, 12v; 2-DG (22:6) = 34v, 14v; 2-PG (16:0) = 18v, 10v; 2-OG (18:1) = 42v, 10v; 2-LG (18:2) = 30v, 10v; monononadecadienoin (19:2 monoacylglycerol; product of DGL assay, see below) = 18v, 10v; and [\textsuperscript{2}H_5]-2-AG = 25v, 44v. Lipids were quantified using a stable isotope dilution method detecting H\(^+\) or Na\(^+\) adducts of the molecular ions [M + H/Na]\(^+\) in multiple reaction monitoring (MRM) mode. Acyl migration occurs in monoacylglycerols; thus, the sum of 2-AG and 1-AG is presented. Tissue processing and LCMS analyses for experiments occurred independently of other experiments. Extracted ion chromatograms for MRM transitions were used to quantify analytes: 2-AG (m/z = 379.3 > 287.3), 2-DG (m/z = 403.3 > 311.1), 2-PG (m/z = 331.3 > 239.3), 2-OG (m/z = 357.4 > 265.2), 2-LG (m/z = 355.3 > 263.3), 19:2 monoacylglycerol (m/z = 386.4 > 277.2), and [\textsuperscript{2}H_5]-2-AG (m/z = 384.3 > 93.4), which was used as an internal standard for quantitation of monoacylglycerols.

**ELISA analysis of CCK-8 octapeptide**

Mice were fasted for 12 h in order to ensure an empty stomach. Mice were pretreated with CB1R ligands, then administered corn oil (0.5 mL) by oral gavage 30 min later. Levels of CCK-8 were assessed in blood plasma 30 min following gavage. Blood was placed in BD vacutainer lavender-top EDTA blood collection tubes on ice and plasma obtained by centrifugation of tubes at 1500 G for 10 min at 4°C) by a sensitive and selective CCK-8 ELISA (Cloud Clone Corp; Katy, TX, USA). Mice were maintained for 60 days on standard diet (SD) and given IP
injection of vehicle or the general cannabinoid receptor agonist, WIN55,212-2 (3 mg per kg), or WIN 55,212-2 in combination with the peripherally-restricted CB1R antagonist, AM6545 (10 mg per kg). In addition, mice maintained for 60 d on Western diet (WD) were given IP injection of vehicle or AM6545 (10 mg per kg). ELISA reaction was measured using iMark microplate reader (BioRad, Hercules, CA, USA).

Immunohistochemistry

Intact proximal small intestine was removed, and contents were flushed with ice-cold 4% paraformaldehyde (PFA)/PBS, then fixed in 4% PFA for 4 hours at 4°C. Samples were transferred to 20% sucrose/PBS and incubated 1 d at 4°C for cryopreservation. Cross sections of upper small intestine were cut and frozen in OCT (Fisher Healthcare, Chino, CA, USA) on dry ice. 16 µm sections were taken on a cryostat (Leica) and mounted onto charged glass slides. Sections were permeabilized with 0.5% Tween-20/PBS and then blocked with 0.1% Tween in casein solution (Thermo Fisher). Primary antibodies from rabbit for Cholecystokinin (CCK; ABcam, Cambridge, UK) and Cannabinoid Receptor 1 (Generously provided by Dr. Ken Mackie, Indiana University) were diluted 1:500 in blocking buffer and separately added to slides. Slides were washed three times with 0.1% Tween/PBS solution before being incubated with AlexaFluor 647 (Donkey anti-rabbit, Thermo Fisher). Tissue was washed again and 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) at 63x magnification with a CSU-X1 Confocal Scanner Unit (Yokogawa, Tokyo, Japan), and images were captured using a Prime 95B Scientific CMOS Camera (Photometrics, Huntington Beach, CA, USA). Micro-Manager open source software was used for image capture, and final images were optimized using ImageJ 1.51n (NIH, Bethesda, MD, USA).

**Fluorescence-activated cell sorting**

*Isolation of intestinal epithelial cells*

Approximately 4 cm of proximal small intestine was inverted and mechanically disrupted with frosted glass slides into ice-cold buffer containing 5% BSA, 0.6 mM dithiothreitol (DTT) and 1 mM EDTA in PBS to disrupt mucosal cell layer. Live cells were counted following trypan blue staining and 20 x 10^6 cells were pelleted at 200 G for 5 mins and resuspended in 1 mL of 3% BSA containing 1 mM EDTA in PBS. Cell suspension was filtered through 30-micron mesh and subsequently processed by fluorescence-activated cell sorting (FACS).

*FACS sorting of eGFP (+) and eGFP (-) cells*

Isolated cells were sorted and analyzed on a MoFlo Astrios (Beckman Coulter, Brea, CA, USA). Debris was detected and excluded using forward and side scatter profiles generated with a 488 nm laser. eGFP positive (+) cells were detected by fluorescence intensity, using excitation and emission spectra of 488 and 513/26, respectively. A wild-type mouse from C57Bl/6J background was
used to establish autofluorescence, and gating for eGFP was used for final sorting (See Figure 1D,E). Samples were sorted into fresh resuspension buffer prior to qPCR analysis of gene expression. Mice were fasted for 10 h prior to acquisition of cells.

**Enzyme activity assays**

**Tissue preparation**

Intestinal epithelium was collected as described above and approximately 100 mg of frozen tissue was homogenized in 2 mL of ice-cold 50 mM Tris-HCl, 320 mM sucrose (pH 7.5) buffer. Homogenates were centrifuged at 800 G for 10 minutes at 4°C and supernatant was collected. Protein supernatants were sonicated twice for 10 s and then freeze-thawed in liquid nitrogen twice. Samples were spun again, and supernatant protein content was quantified using BCA assay and diluted to working concentration with Tris-HCl/sucrose buffer.

**DGL activity assay**

Small-intestinal epithelial tissue homogenates (25 µg, room temperature) were incubated with the MGL inhibitor, JZL184 (0.3 µM), for 10 minutes in order to block MGL activity during the assay. Homogenates were then incubated in 0.2 mL solution of Tris-HCl with 0.2% Triton X-100 (pH 7.0) containing 20 nmol dinonadecadienoin (19:2 DAG) at 37°C for 30 min. Reactions were stopped by adding 1 mL of ice-cold MeOH containing 25 pmol [²H₅]-2-AG as internal standard. Lipids were extracted and the product of the reaction,
mononadecadienoin (19:2 monoacylglycerol), was analyzed via UPLC/MS/MS as described above for 19:2 monoacylglycerol.

**MGL activity assay**

Small-intestinal epithelial tissue (10 µg) was incubated with 0.4 mL solution of Tris-HCL with 0.1% BSA (pH 8.0) containing 50 nmol nonadecadienoin (19:2 monoacylglycerol; Nu-Chek Prep, Waterville, MN, USA; final volume 0.5 mL per reaction) at 37°C for 10 min. Reactions were stopped by adding 1 mL of MeOH containing 10 nmol heptadecanoic acid (17:1 FFA; Nu-Chek Prep) as internal standard. Lipids were extracted and the product of the reaction (19:2 free fatty acid) was analyzed via UPLC/MS/MS according to the following protocol. Data were acquired using equipment described above and eluted by a gradient of water and methanol (containing 0.25% acetic acid, 5 mM ammonium acetate) at a flow rate of 0.4 mL per min and gradient: 90% methanol 0.1 min, 90% to 100% methanol 0.1 – 2.0 min, 100% methanol 2.0 – 2.1 min, 100% to 90% methanol 2.1 – 2.2 min, and 90% methanol 2.2 – 2.5 min. Column was maintained at 40°C and samples were kept at 10°C in sample manager. MS detection was in negative ion mode with capillary voltage maintained at 3.00 kV. Cone voltages for nonadecadienoic acid (19:2 FFA) = 48v and heptadecanoic acid (17:1 FFA) = 64v. Lipids were quantified using a stable isotope dilution method of proton adducts of the molecular ions [M - H] in selected ion recording (SIR) mode. Tissue processing and LCMS analyses for experiments occurred.
independently of other experiments. Extracted ion chromatograms for SIR masses were used to quantify analytes: 19:2 FFA ($m/z = 293.2$) product of MGL enzyme assay and 17:1 FFA ($m/z = 267.2$) as internal standard.

**Gastric emptying**

To evaluate drug or endogenous endocannabinoid effects on gastric emptying, corn oil was spiked with 1.0 nmol 19:2 FFA and administered by oral gavage (500uL), then quantities of 19:2 FFA remaining in the stomach were evaluated at the time of blood collection 30 minutes after gavage. The stomach was removed and immediately placed into methanol containing 17:1 FFA as internal standard. Lipids were extracted and 19:2 FFA was detected and quantified as above.

**Gene expression analysis**

*RNA isolation from intestinal epithelium*

Total RNA was extracted from intestinal epithelium using RNeasy kit (Qiagen, Valencia, CA, USA) method, and first-strand complementary DNA was generated using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). All surfaces for tissue collection and processing were sanitized using 70% ethanol and then treated with an RNase inhibitor (RNase out, G-Biosciences, St. Louis, MO, USA) to maintain integrity of isolated RNA. Reverse transcription of total RNA (1 µg epithelium) was performed as previously described (22).

*RNA isolation from sorted cells*
Sorted cell suspensions were pelleted at 3000 G for 10 mins and resuspended in 0.5 mL of Qiazol (Qiagen, Valencia, CA) and subsequently processed using RNeasy kit to isolate total RNA. Reverse transcription was performed as described above using 50 ng total RNA.

Quantitative polymerase chain reaction analysis

RT-qPCR was carried out using PrimePCR Sybr Green Assays (Biorad, Hercules, CA, USA) with the following primers for mouse genes: CB1R (Cnr1), CB2R (Cnr2), cholecystokinin (Cck), fatty-acid amide hydrolase (Faah), n-acyl phosphatidyl ethanolamine-specific phospholipase D (Napepld), diacylglycerol lipase alpha (Dagla) and beta (Daglb), monoacylglycerol lipase (Mgll), alpha-beta-hydrolyzing domain 6 (Abhd6) with Hprt and Actb as housekeeping genes for epithelium and sorted cells, respectively. Values are expressed as relative mRNA expression based on widely used methods [i.e., delta-delta cq; see (43)]. Reactions were run in triplicate for each animal.

Statistical analysis

Values are expressed as means ± SEM. Unpaired Student’s two-tailed t-test was used to compare data for standard diet- and western diet-fed groups. Repeated measures two-way ANOVA was used for groups measured over time. Additionally, regular one-way and two-way ANOVA were used to determine differences in multiple groups with post-hoc Sidak’s multiple comparisons tests or Newman-Keul’s, as appropriate. Data were analyzed using GraphPad Prism6.
software. Significance was determined as $P < 0.05$. Statistical outliers were determined using Grubb’s test in all datasets.

**Results**

**CB\(_1\)Rs are expressed in CCK-containing cells in the upper small-intestinal epithelium.**

We reported that eCB levels are increased in the upper small-intestinal epithelium from mice maintained on a Western Diet (WD; high-fat and sucrose diet) for 60 days when compared to lean controls maintained on a low-fat and low-sugar diet, and inhibiting peripheral CB\(_1\)Rs blocked overeating associated with consumption of WD (i.e., increased meal size, rate of food intake, and total caloric intake) (22). To identify the molecular underpinnings of gut-brain eCB signaling important for feeding behavior and its dysregulation in DIO, we first evaluated whether CB\(_1\)Rs are expressed in cells that produce and secrete the satiation peptide, CCK. CCK controls meal size and induces satiation by activating CCKA receptors on the afferent vagus nerve (31-35, 38, 39). CB\(_1\)R immunoreactivity was found in CCK-eGFP-positive cells from the upper small-intestinal epithelium (Figure 1) in a mouse line that expresses eGFP selectively in CCK-expressing cells [C57BL/6-Tg(Cck-EGFP)2Mirm/J] (44). Furthermore, immunoreactivity for CCK was co-localized with eGFP in the upper small-intestinal epithelium, which confirms expression of CCK in eGFP-containing cells from this mouse line (Supplementary Figure 1). We next isolated eGFP-positive
and eGFP-negative cells from the upper small-intestinal epithelium by fluorescence-activated cell sorting (FACS). Messenger RNA (mRNA) for CB₁Rs (Cnr1) was enriched in CCK-eGFP-positive cells when compared to CCK-eGFP-negative cells (Figure 2A; eGFP-positive = 1.00 ± 0.24, eGFP-negative = 0.04 ± 0.04; P = 0.016; data from three mice). Moreover, mRNA for CCK was present in CCK-eGFP-positive cells isolated by FACS but was not present in CCK-eGFP-negative cells, which highlights the specificity of our FACS gating strategy for isolating CCK-eGFP cells and further confirms expression of CCK in these cells (Figure 2B). Our gating strategy was optimized for sorting of eGFP-positive and eGFP-negative events from cells isolated from the upper small-intestinal epithelium of CCK-eGFP mice (see Figure 2C). Cells from wild-type mice (see Figure 2D) show minimal fluorescence at less than 10% of levels found in CCK-eGFP cells: eGFP-positive cells comprise 0.63% of total cells analyzed from CCK-eGFP mice, and wild-type show 0.06%, likely due to autofluorescence (see Supplementary Figure 2 for detailed FACS report). These results suggest that CCK-containing I-cells in the mouse upper small-intestinal epithelium are enriched in expression of CB₁Rs.

**Peripheral CB₁Rs control fat-induced CCK secretion.**

The arrival of fat and other macronutrients into the duodenum stimulates release of a variety of signaling molecules that include CCK, which is produced and secreted by enteroendocrine I-cells lining the upper small-intestinal epithelium (1, 28, 30, 45, 46). We next tested the hypothesis that CB₁Rs control
nutrient-induced release of CCK from the upper small-intestinal epithelium. Oral gavage of corn oil (CO) in lean mice maintained on a standard rodent diet (SD; low-fat no-sucrose chow) increased plasma levels of bioactive CCK, CCK-8 (octapeptide), when compared to control mice that received oral gavage of saline [Figure 3A; CO = 0.69 ± 0.11 ng per mL, saline control = 0.28 ± 0.02 ng per mL; P < 0.05 CO versus saline control, n=3-5). Peripheral administration of the general cannabinoid receptor agonist, WIN55,212-2 (WIN, 3 mg per kg), blocked CO-induced secretion of CCK-8 (Figure 3A; CO+WIN = 0.36 ± 0.04 ng per mL; P < 0.05 CO+WIN versus CO alone, n=5). Furthermore, the effect of WIN administration on CO-induced secretion of CCK-8 was reversed by co-treatment with the peripherally-restricted neutral CB₁R-selective antagonist, AM6545 (Figure 3A; CO+WIN+AM6545 = 0.75 ± 0.14 ng per mL; P < 0.05 CO+WIN versus CO+WIN+AM, n=5; AM6545 10 mg per kg). These results suggest that exogenous activation of CB₁Rs inhibits nutrient-induced CCK release from the upper gut.

We next tested the hypothesis that elevated endogenous activity (e.g., increased 2-AG levels) at upper small-intestinal CB₁Rs in mice maintained on Western Diet (WD; high-fat and sucrose diet) for 60 days inhibits CO-induced increases in circulating levels of CCK-8. We first confirmed that levels of 2-AG – among other monoacylglycerols – were increased in the upper small-intestinal epithelium of WD mice when compared to lean mice fed SD for 60 days (see Table 1). Next, we tested the ability for oral gavage of CO to increase CCK-8
levels in plasma of WD mice. CO failed to affect levels of CCK-8 in WD mice when compared to mice fed a standard diet (SD) that is low in fat and absent of sucrose (Figure 3B; CO+WD = 0.33 ± 0.04 ng per mL, CO+SD = 0.8 ± 0.03 ng per mL; P < 0.01, n=5). Furthermore, AM6545 treatment in WD mice that received oral gavage of CO increased levels of CCK-8 to those comparable to SD mice under the same conditions (Figure 3B; CO+WD+AM = 0.7 ± 0.1 ng per mL; P < 0.01 CO+WD versus CO+WD+AM, n=6). Collectively, these results suggest that exogenous or endogenous activation of CB1Rs in the upper small-intestinal epithelium inhibits nutrient-induced CCK secretion.

All levels of CCK-8 in these experiments fell within the range of the standard curve for CCK-8 quantitation by a sensitive and selective CCK-8 ELISA, which shows no cross-reactivity for gastrin (see Supplementary Figure 3), another gut-derived peptide that shares some common molecular features with CCK-8 (47-50). Furthermore, the range of CCK-8 levels in our studies (from 0.27 ± 0.02 to 0.8 ± 0.03 ng per mL or 0.23 ± 0.02 to 0.7 ± 0.03 nM) aligns with reported Ki and EC50 values of sulfated CCK-8 in several binding and in vitro bioassays (e.g., amylase release from pancreatic acini and ileum contractions in guinea pig) (51).

CB1R activation is reported to decrease gastric emptying, an effect also found in mice fed a high-fat diet for 14 weeks (52, 53). To identify if altered gastric emptying occurs under our conditions and may contribute in part to inhibited corn oil-induced CCK release, we developed a novel UPLC/MS/MS-
based method to evaluate if CB₁R activation with WIN 55,212-2 or exposure to WD for 60 days impacts gastric emptying following oral gavage of corn oil in SD and WD mice, respectively. Thirty min after administration of drugs, we administered by oral gavage corn oil (500µL) that contained 19:2 free-fatty acid (1nmol) as a tracer and measured by UPLC/MS/MS the remaining quantities of 19:2 free-fatty acid in the stomach 30 min after gavage. WIN 55,212-2 (3 mg per kg) alone or in combination with AM6545 (10 mg per kg) had no effect on gastric emptying of corn oil in SD mice (see Supplementary Figure 4A). Similarly, WD mice displayed no changes in gastric emptying of corn oil when compared to SD mice (see Supplementary Figure 4B). These data suggest that exogenous activation (WIN in SD mice) or endogenous activation (elevated small-intestinal epithelial eCB levels in WD mice) of CB₁Rs does not affect gastric emptying of corn oil under our conditions and does not likely impact CCK release by a mechanism that includes alterations in gastric emptying in mice.

CB₁Rs in pancreatic beta cells control insulin release and glucose homeostasis (54-59). Thus, we tested if drug treatment impacted glucose levels in response to corn oil gavage in SD mice, which in turn, could affect gastric emptying, motility, or enteroendocrine release from small-intestinal enteroendocrine cells. Glucose levels in blood were collected from tail vein and monitored via hand-held glucose monitor at (i) time of drug administration, (ii) 30 min later just prior to corn oil gavage, and (iii) 30 min later at time of kill (See Supplementary Figure 5). Drug treatment had no significant impact on blood
glucose levels at any time point prior to or after gavage of corn oil (See Supplementary Figure 5). These data suggest that, under our conditions, activating CB₁Rs does not impact blood glucose levels following oral gavage of corn oil in mice.

**Activity of enzymes responsible for metabolism of 2-AG and other monoacylglycerols is dysregulated in the upper small-intestinal epithelium in DIO.**

We next aimed to identify the mechanism(s) of increased 2-AG and related monoacylglycerol levels (see Table 1) in WD mice by analyzing activity of their biosynthetic (diacylglycerol lipase, DGL) and degradative enzymes (monoacylglycerol lipase, MGL) using our lab’s UPLC/MS/MS-based functional enzyme assay methods (see Supplementary Figure 6 for validation of enzyme assays). When compared to SD mice, WD mice displayed an increase in activity of DGL (Figure 4A; SD = 0.12 ± 0.02, WD = 0.22 ± 0.03 nmol per mg protein per minute; P = 0.016, reactions from 6 mice per diet group), and MGL (Figure 4B; SD = 36.32 ± 3.82, WD = 51.60 ± 4.95 nmol per mg protein per minute; P = 0.035, reactions from 6 mice per diet group) in isolated tissue from the upper small-intestinal epithelium. Congruent with data in Table 1 and (22), these effects were met with increased levels of 2-AG in upper small-intestinal epithelium of separate mice (Figure 4C; SD = 45.71 ± 6.93, WD = 92.57 ± 16.41 nmol per g; P = 0.014, n = 9-10 per diet group). See Figure 4D for diagram of the 2-AG metabolic pathways. Together, these results suggest that monoacylglycerol metabolic pathways are dysregulated after chronic exposure to WD, which leads
to a net increase in monoacylglycerols, including 2-AG, in the upper small-intestinal epithelium.

Expression of select eCB system components in the upper small-intestinal epithelium is dysregulated in DIO and partially conserved in CCK-positive cells.

Relative expression of mRNA for intestinal CCK, CB₁Rs, and CB₂Rs (Cnr2) was unchanged in whole upper small-intestinal epithelial scrapings from mice fed WD versus SD mice (Figure 5A: CCK, SD = 1.00 ± 0.76, WD = 0.56 ± 0.45, p = 0.64; Cnr1, SD = 1.00 ± 0.36, WD = 0.79 ± 0.31, P = 0.67; Cnr2, SD = 1.00 ± 0.31, WD = 0.83 ± 0.188, P = 0.65; data from 4 mice per diet group).

Expression of mRNA for the alpha isoform of DGL (Dagla) was also unaffected by diet (Figure 5A; SD = 1.00 ± 0.25, WD = 0.90 ± 0.29, P=0.80); however, expression of mRNA for the beta isoform of DGL (Daglb) was reduced in WD versus SD mice (Figure 5A; SD = 1.00 ± 0.15, WD = 0.35 ± 0.03, P = 0.005), while mRNA for MGL (Mgll) and the serine hydrolase alpha/beta hydrolase domain 6 (Abhd6) were increased in small-intestinal epithelium under the same conditions (Figure 5A: Mgll, SD = 1.00 ± 0.17, WD = 2.71 ± 0.46, P = 0.013; Abhd6, SD = 1.00 ± 0.16, WD = 1.54 ± 0.048, P = 0.02). No changes were found for the fatty acid ethanolamide biosynthetic enzyme, NAPE-PLD, or the fatty acid ethanolamide degradative enzyme, FAAH (Figure 5A: NAPE-PLD, SD = 1.00 ± 0.18, WD = 0.89 ± 0.08, P = 0.6; FAAH, SD = 1.00 ± 0.17, WD = 1.00 ± 0.07, P = 0.99). Furthermore, the upper small-intestinal epithelium is enriched in
expression of mRNA for Daglb when compared to Dagla (Figure 5A inset; Dagla = 1.00 ± 0.19, Daglb = 29.73 ± 4.3; \( P = 0.001 \); data from 4 mice fed SD).

It is important to note, in contrast to our previous report that included analysis of eCB system expression in the upper small-intestinal epithelium of mice maintained on WD and SD [Lab Diet 5001 used in (22)], in this study we used a soy protein-free Teklad 2020x as a control SD in order to eliminate any potential effects of phytoestrogen-containing soy protein on eCB metabolism or behavior [see (60-63)]. We found two differences in results when comparing use of the two control diets versus WD. We reported no changes in expression of mRNA for the beta isoform of DGL and increases in expression of mRNA for FAAH in WD mice when compared to control SD mice (22); however, in this study, we found decreased expression of mRNA for the beta isoform of DGL and no changes in expression of mRNA for FAAH in WD mice when compared to SD mice. These differences highlight possible effects of diets that utilize soy protein on expression of eCB metabolic enzymes and eCB metabolic function. A direct comparison of the impact of specific control diets on expression of eCB system components, however, remains to be evaluated.

CCK-eGFP-positive cells isolated from mice fed SD or WD mice displayed no differences between diet condition in expression of mRNA for CCK and components of the eCB system that include Cnr1, Cnr2, Daglb, Abhd6, and FAAH (Figure 5B; \( P > 0.05 \) not significant, data from 3 mice per diet group).
Dagla, Mgl, and Napepld mRNA were below detectable levels, which suggests a lack of expression of these eCB system components in CCK-containing cells.

Collectively, these results identify select eCB system gene transcripts in CCK-containing cells, and changes in expression of biosynthetic and degradative enzyme gene transcripts in whole epithelium of WD mice that do not fully correspond to changes in activity of their proteins, including DGL and MGL (see Figure 4). The latter suggests possible post-transcriptional and/or post-translational changes in expression of these enzymes in the upper small-intestinal epithelium in WD mice when compared to lean SD mice, although this hypothesis remains to be directly tested. Furthermore, a lack of expression of the fatty acid ethanolamide (FAE) biosynthetic enzyme, NAPE-PLD, in CCK-containing cells suggests that FAEs including anandamide – which is also found in small-intestinal epithelium of rodents (9-11, 21, 22, 64) – is generated in neighboring cells and therefore may act in a paracrine manner with I-cells that contain CB₁Rs. In contrast, expression of mRNA for the beta isoform of the monoacylglycerol biosynthetic enzyme, DGL, is abundantly expressed in CCK-containing cells, which suggests that 2-AG may signal at CB₁Rs in an autocrine manner at these cells. Expression of the primary 2-AG degradative enzyme, MGL, is absent in CCK-containing I-cells, which suggests that 2-AG is degraded at adjacent cells and therefore may additionally signal CB₁Rs on adjacent cells in a paracrine manner. A comprehensive analysis of eCB system architecture and
its cell-specific expression in the upper small-intestinal epithelium of mice remains for future studies.

Western diet exposure for 60 days is associated with obesity and hyperphagia in male mice.

Consistent with our previous studies (22), exposure to WD for 60 days, when compared to lean mice fed SD for 60 days, was associated with (i) a rapid and sustained increase in body mass when compared to control mice fed SD for 60 days, (ii) increased 24 h meal size, (iii) rate of food intake, and (iv) total 24 h caloric intake (see Supplementary Figure 7 and Table 2 for details and data). No significant changes were found for other feeding behaviors including (i) first meal size, (ii) meal frequency, (iii) meal duration, and (iv) post-meal interval. As discussed above, in contrast to our previous study (22), in this study we used a soy-protein-free lab chow. Irrespective of control diet, however, WD intake was consistently associated with increased 2-AG levels (Table 1) and hyperphagia across relevant parameters in both studies [Table 2 and Figure 6 and (22)]. Together, these data suggest that exposure to a WD rapidly induces body weight gain that is met with increased meal size, rate of intake, and daily caloric intake, when compared to lean controls.

Pharmacological inhibition of CCK\textsubscript{A} receptors blocks the anorexic effect of AM6545 in DIO.

We next tested the hypothesis that peripheral CB\textsubscript{1}Rs control feeding behavior by a mechanism that includes control of CCK-mediated satiation
signaling. When compared to vehicle treatment in mice fed WD for 60 days, AM6545 treatment (10 mg per kg) in WD mice reduced meal size (Figure 6A; vehicle = 1.47 ± 0.15 kcal, AM6545 = 1.13 ± 0.67 kcal; P<0.05, n=12), rate of intake (Figure 6B; vehicle = 0.76 ± 0.12 kcal per minute, AM6545 = 0.46 ± 0.05 kcal per minute; P < 0.01), and total caloric intake (Figure 6C; vehicle = 9.11 ± 0.67 kcal per minute, AM6545 = 6.62 ± 0.69 kcal per minute; P<0.01) during a 12 h test, which is consistent with our previous findings (21). Furthermore, AM6545 treatment had no significant effect in mice fed SD for 60 days on meal size (Figure 6A; vehicle = 0.74 ± 0.05 kcal, AM6545 = 0.71 ± 0.04 kcal; P > 0.05, n=12), rate of intake (Figure 6B; vehicle = 0.30 ± 0.03 kcal per minute, AM6545 = 0.32 ± 0.03 kcal per minute; P > 0.05), and total caloric intake (Figure 6C; vehicle = 5.51 ± 0.42 kcal per minute, AM6545 = 5.45 ± 0.28 kcal per minute; P > 0.05) during a 12 h test. Importantly, co-administration of a low dose of the CCKA receptor antagonist, devazepide (Dev; 0.1 mg per kg), in WD mice blocked the effects of AM6545 on reducing meal size (Figure 6A; vehicle = 1.47 ± 0.15 kcal, AM6545+devazepide = 1.49 ± 0.16 kcal; P > 0.05), rate of intake (Figure 6B; vehicle = 0.76 ± 0.12 kcal per minute, AM6545+devazepide = 0.62 ± 0.06 kcal per minute; P > 0.05), and total caloric intake (Figure 6C; vehicle = 9.11 ± 0.67 kcal per minute, AM6545+devazepide = 8.98 ± 0.67 kcal per minute; P > 0.05). Administration of devazepide alone affected only on total 12-h caloric intake in SD mice (Figure 6C; vehicle = 5.51 ± 0.42 kcal per minute, devazepide = 7.61 ±
0.33 kcal per minute; \( P<0.05 \). Neither AM6545 or devazepide affected other meal parameters including meal frequency (Figure 6D), meal duration, (Figure 6E), post-meal interval (Figure 6F), or first-meal size (Figure 6G) in SD or WD mice. These data suggest that the acute anorexic effects of AM6545 in DIO mice are dependent on a mechanism that includes activation of CCK\textsubscript{A} receptors and inhibition of gut-brain satiation signaling.

**Discussion**

The molecular underpinnings of gut-brain signaling and their dysregulation in DIO are poorly defined. Our studies suggest that eCB activity at CB\textsubscript{1}Rs in the upper small-intestinal epithelium is upregulated in mice chronically fed a WD, which in turn, promotes overeating by a mechanism that includes inhibiting nutrient-induced gut-brain satiation signaling (see Figure 7 for model). Six primary findings support this conclusion: (i) CB\textsubscript{1}Rs are enriched in CCK-containing cells in the mouse upper small-intestinal epithelium; (ii) oral gavage of corn oil increased circulating levels of CCK-8 in lean mice, and pharmacological activation of CB\textsubscript{1}Rs blocked this effect, which was reversed by inhibition of peripheral CB\textsubscript{1}Rs with a peripherally-restricted CB\textsubscript{1}R neutral antagonist; (iii) levels of 2-AG and other monoacylglycerols were increased in the upper-small intestinal epithelium of WD mice when compared to lean mice, and this effect was associated with dysregulated monoacylglycerol metabolism; (iv) oral gavage of corn oil failed to affect circulating levels of CCK-8 in WD mice, and inhibition of
peripheral CB\(_1\)Rs in WD mice restored the ability for corn oil to increase CCK levels; (v) pharmacological inhibition of peripheral CB\(_1\)Rs in WD mice blocked overeating associated with increased meal size, rate of feeding, and total caloric intake; and (vi) the hypophagic effects of peripheral CB\(_1\)R antagonism in WD mice were reversed by pretreatment with a low-dose CCK\(_A\) receptor antagonist. Collectively, our studies identify a previously unknown role for the eCB system at the interface of nutrient-sensing and gut-brain satiation signaling that becomes dysregulated in DIO and promotes overeating by delaying satiation.

Our studies suggest that the eCB system in the small-intestinal epithelium controls feeding behavior by a mechanism that includes inhibiting nutrient-induced release of the gut-derived satiation peptide, CCK, which in turn increases meal size and caloric intake. CCK is secreted from enteroendocrine I-cells in the upper small-intestinal epithelium after nutrients arrive in the lumen (1, 28, 30, 37) and controls meal size and induces satiation by activating CCK\(_A\) receptors on afferent vagal fibers (31-34, 36, 37) and possibly the brain (38, 39). Indeed, polymorphisms in CCK\(_A\) receptor genes in humans is associated with increased meal size and food intake, and obesity (65-67). Furthermore, CCK in a stabilized form resistant to degradation in the GI tract is effective at reducing food intake and body weight in DIO rodents (68-70) and activating CCK\(_A\) receptors enhances the anti-obesity properties of GLP-1 agonists, amylin, and leptin (71-74).
Gene transcripts and immunoreactivity for CB₁Rs were found in CCK-containing I-cells in the upper small-intestinal epithelium of mice [see Figures 1 and 2, and (40)]. Furthermore, the hypophagic effects of AM6545 were blocked by co-administration of the CCKₐ receptor antagonist, devazepide. These results suggest that when eCB activity is elevated at local CB₁Rs in the upper small-intestinal epithelium in DIO, increased CB₁R activation may inhibit nutrient-induced release of satiation peptides from small-intestinal enteroendocrine cells and lead to increased meal size and caloric intake. In support of this hypothesis, oral gavage of corn oil – which potently increases circulating levels of bioactive CCK-8 in lean mice that have low levels of small-intestinal eCB levels – failed to affect circulating levels of CCK-8 in mice chronically fed WD that have elevated eCB levels in the small-intestinal epithelium. Moreover, inhibiting elevated eCB signaling at peripheral CB₁Rs with AM6545 in WD mice – at a dose that blocked overeating – restored the ability for corn oil to increase circulating levels of CCK-8.

The mechanisms of CB₁R control of nutrient-induced release of CCK from enteroendocrine I-cells in the upper small-intestinal epithelium are unknown. Nonetheless, a primary mechanism by which CB₁Rs block neurotransmitter release is by inhibiting calcium influx or mobilization (6, 75), and nutrient-induced CCK release is calcium-dependent (29, 76-79). Thus, CB₁R activity may inhibit release of gut peptides by a mechanism that includes inhibiting calcium influx or
mobilization; however, a direct test of this hypothesis remains to be performed (see Figure 7 for proposed mechanism).

It is controversial if obesity impacts CCK secretion [see for review (1)]. In line with our present findings in mice, several studies suggest that CCK secretion is reduced in obese humans: fasting CCK levels were lower than non-obese (80) and a trend towards lower CCK release after intra-duodenal infusions of oleic acid in overweight or obese subjects (81). Fat-induced CCK secretion and satiation induced by CCK administration were also reduced in rats fed a high-fat diet (82). Other studies, however, reported no differences in CCK levels between obese or lean humans following a meal (83), and increases in CCK after a high-fat meal (84). Furthermore, several preclinical studies in rodents suggest that sensitivity of vagal afferent neurons to the satiating effects of CCK may be decreased in DIO (82, 85-87). This phenomenon may be due, in part, to changes in membrane properties of neurons in the nodose ganglion. The satiating actions of a physiological dose of CCK, however, was equally effective in suppressing food intake in obese and lean human subjects (88). Moreover, a variety of studies conducted over the past several decades show that CCK-induced satiation is mediated by the vagus nerve (31-37); however, select studies show that gut-derived CCK may additionally interact with CCK₆ receptors in the brain (38, 39). We used the brain-penetrant CCK₆ receptor antagonist, devazepide, in our studies; therefore, we cannot rule out the possibility that CCK₆ receptors in the brain participate in the appetite-suppressing effects of CCK release following
inhibition of peripheral CB₁Rs. Thus, given discrepancies in the literature regarding the underlying mechanisms of gut-brain signaling and its dysregulation in DIO, it is critical to examine the impact of diet and obesity on gut-brain satiation signaling using reliable and reproducible model systems.

It is plausible that CB₁R control of nutrient-induced CCK release is one of several mechanisms by which peripheral CB₁Rs impact gut-brain signaling pathways (23-26). For example, administration of ghrelin – which is produced in the stomach and upper small intestinal epithelium and increases feeding [see for review (1, 89)] – blocked downregulation of CB₁Rs in the nodose ganglion after, both, re-feeding and CCK administration in fasted rats (24). Moreover, pharmacological inhibition of CB₁Rs blocked fasting-induced ghrelin production in rats (90-92), which suggests that CB₁Rs in the upper GI tract may control ghrelin signaling. Furthermore, Kunos and colleagues reported that a peripherally-restricted CB₁R inverse agonist improved a host of metabolic parameters as well as reducing food intake in DIO mice by a mechanism that may include reversing hyperleptinemia and leptin resistance associated with DIO (16) and restoring anorexic melanocortin signaling in the arcuate nucleus of the hypothalamus (17). Marsicano and colleagues reported that the hypophagic effects of CB₁R inhibition with the CB₁R inverse agonist, rimonabant, is blocked by pharmacological inhibition of peripheral beta-adrenergic neurotransmission (93), which suggests that CB₁Rs may additionally control feeding behavior via interactions with the peripheral sympathetic nervous system. This study also showed that intact
afferent vagal signaling was required for the hypophagic effects of rimonabant, and CB₁Rs in the brain were not required for its pharmacological actions. Nonetheless, circulating levels of the eCBs increase in human and rodent models of obesity (7, 19, 22, 94-99), which may directly interact with CB₁Rs in the brain and control feeding behavior and energy homeostasis. A comprehensive analysis of this possibility remains to be performed. In addition to I-cells in the small-intestinal epithelium [see Figures 1 and 2, and (40)], CB₁Rs are also expressed in K-cells that produce and secrete glucose-dependent insulinotropic peptide [GIP (100, 101)]. Pharmacological activation of CB₁Rs inhibits GIP release in rodents, which suggests that local CB₁Rs may impact glucose homeostasis via a mechanism that includes controlling nutrient-induced incretin release. Lastly, enteroendocrine cells in the intestinal lining form functional synapses with afferent vagal fibers (37). Termed “neuropods” by Bohorquez and colleagues, these cells sense nutrients and release glutamate and CCK in a coordinated manner that interact with corresponding receptors on local afferent vagal fibers, which in turn, communicate with the brain. Our data suggest that CB₁Rs may be at the interface of this signaling. It is unknown, however, if CB₁Rs control glutamate signaling at these synapses in the small intestine as they do in the brain (102). Collectively, these studies – in combination with the present report – describe key roles for peripheral CB₁Rs in feeding behavior and energy homeostasis.
In summary, our results provide evidence of a previously unknown mechanism of CB₁R-mediated inhibition of gut-brain satiation signaling in DIO that promotes overeating. Pharmacological manipulation of these pathways in the periphery may provide a therapeutic advantage for the treatment of obesity and related metabolic disorders when compared to anti-obesity drugs that interact with the brain and display psychiatric side-effects (8, 103). Despite the peripherally-restricted properties of these CB₁R antagonists, however, their impact on cognition and brain function by altering gut microbe activity is unknown and remains to be reported.

**Author Disclosure Statement:** The authors declare no conflict of interest.
References


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Table 1. Impact of diet on monoacylglycerols in mouse small-intestinal epithelium.

<table>
<thead>
<tr>
<th></th>
<th>MAG 20:4 (2-AG)</th>
<th>18:1 (nmol g⁻¹)</th>
<th>18:2 (nmol g⁻¹)</th>
<th>16:0 (nmol g⁻¹)</th>
<th>22:6 (nmol g⁻¹)</th>
<th>Total (nmol g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD</td>
<td>80.23 ± 8.542</td>
<td>49.57 ± 9.804</td>
<td>217.7 ± 52.09</td>
<td>33.99 ± 4.125</td>
<td>9.413 ± 1.996</td>
<td>390.9 ± 72.24</td>
</tr>
<tr>
<td>WD</td>
<td>132.5 ± 22.20</td>
<td>109.0 ± 22.03</td>
<td>415.3 ± 83.26</td>
<td>150.1 ± 21.53</td>
<td>22.63 ± 3.703</td>
<td>829.4 ± 144.2</td>
</tr>
<tr>
<td>P-value</td>
<td><strong>0.0353</strong></td>
<td><strong>0.0206</strong></td>
<td>0.0554</td>
<td>&lt;0.0001</td>
<td><strong>0.0049</strong></td>
<td><strong>0.0122</strong></td>
</tr>
</tbody>
</table>

MAG, Monoacylglycerol represented as fatty acid chain. SD, Standard Diet n=10. WD, Western Diet n=9. Mean values are shown as ± S.E.M. Bold values are significantly different determined by Two-tailed Unpaired T-Test.
Table 2. Consumption of WD is associated with hyperphagia.

<table>
<thead>
<tr>
<th></th>
<th>Δ Body Mass (g)</th>
<th>Meal Size (kcal)</th>
<th>Intake Rate (kcal min⁻¹)</th>
<th>24h Intake (kcal)</th>
<th>First Meal (kcal)</th>
<th>Frequency (meals day⁻¹)</th>
<th>Duration (Min)</th>
<th>PMI (Min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD</td>
<td>8.90 ±0.31</td>
<td>0.69 ±0.04</td>
<td>0.33 ±0.02</td>
<td>8.10 ±0.61</td>
<td>0.72 ±0.11</td>
<td>11.67 ±0.45</td>
<td>8.61 ±1.16</td>
<td>114.7 ±8.4</td>
</tr>
<tr>
<td>WD</td>
<td>18.14 ±0.46</td>
<td>1.29 ±0.10</td>
<td>0.71 ±0.08</td>
<td>13.28 ±0.81</td>
<td>1.99 ±0.66</td>
<td>9.83 ±1.42</td>
<td>6.85 ±0.98</td>
<td>129.3 ±14.3</td>
</tr>
</tbody>
</table>

P-value: <0.0001 <0.0001 <0.0001 <0.0001 0.07 0.23 0.26 0.31

PMI, Post Meal Interval. Mean values are shown as ± S.E.M. n=10. Bold values are significantly different determined by Two-tailed Unpaired T-Test.
Figure 1. CB₁Rs co-localize with CCK-containing cells in the upper small-intestinal epithelium. Immunohistochemical detection of eGFP (CCK-eGFP: B, F, J) and CB₁Rs (CB₁R-ab: C, G, K) reveals co-localization (merge: A, E, I) in villi of intestinal epithelium. Arrows indicate separate enteroendocrine cells that contain immunoreactivity for CB₁Rs that co-localize with CCK-eGFP cells. Representative images from three CCK-eGFP mice. (Scale bar 15 µm)
Supplementary Figure 1. CCK receptors co-localize with eGFP in upper small-intestinal epithelium. Immunohistochemical detection of CCK (CCK-ab) in eGFP-containing cells (CCK-eGFP) confirms co-localization, which highlights the validity of this CCK-eGFP reporter mouse. Arrows indicate three separate enteroendocrine cells that contain immunoreactivity for CCK that co-localizes with CCK-eGFP-positive cells. Representative images from three CCK-eGFP mice. (Scale bar 15 µm)
Figure 2. CB₁R mRNA expression is enriched in CCK-containing cells in the upper small-intestinal epithelium. Fluorescence-activated cell sorting (FACS) of eGFP-CCK-positive (+) and eGFP-CCK-negative (-) cells from the upper small-intestinal epithelium reveals enhanced Cnr1 expression in eGFP-CCK-positive cells (A). Expression of mRNA for CCK is found in eGFP-CCK-positive cells but not in eGFP-CCK-negative cells, (B). Gating strategy shown for sorting of eGFP-positive and eGFP-negative events, with eGFP-positive cells highlighted in green and demarked by thin line (C) and compared to upper small-intestinal epithelial cells from a wild-type (WT) mouse (D). Data expressed as mean ± S.E.M. Analyzed using Student’s T-test, two-tailed (c); n=3 per group; *P<0.05.
Supplementary Figure 2. Details of gating strategy for fluorescence-assisted cell sorting (FACS) of CCK-eGFP-positive cells from upper small-intestinal epithelium of CCK-eGFP reporter mice, and wild-type control. CCK-eGFP-positive cells (A; associated graphic Fig 2D) represent 0.63% of total cells (value denoted by *). Wild-type cells show minimal fluorescence at 0.06% total cells (B, value denoted by *; associated graphic Fig 2E), which represents less than 10% of CCK-eGFP-positive cells from CCK-eGFP reporter mice and likely reflects autofluorescence.
Figure 3. Exogenous or endogenous activation of peripheral CB1Rs inhibits fat-induced CCK release. Compared to control [0.5 mL saline (Sal) by oral gavage and vehicle (Veh) by IP injection], corn oil (CO; 0.5 mL by oral gavage) increased levels of CCK-8 in plasma of lean mice fed a low-fat no-sugar standard diet (SD), an effect blocked by the CB1R agonist, WIN 55,212-2 (WIN, IP 3 mg per kg 30 min before CO)(A). The effects of WIN were inhibited by co-administration with the peripherally-restricted CB1R antagonist, AM6545 (AM; 10 mg per kg 30 min before CO). When compared to control SD mice (CO+SD), CO failed to elicit changes in levels of CCK-8 in plasma in mice fed western diet (WD) for 60 days, and inhibition of peripheral CB1Rs with AM6545 normalized levels of CCK-8 to those found in SD CO controls (B). Data expressed as means ± S.E.M. Analyzed by one-way ANOVA with post hoc Newman-Keuls multiple comparison test. n=3-5 per condition, * p<0.05, ** p<0.01.
Supplementary Figure 3. Standard curve for ELISA analysis of CCK-8 in plasma and analysis of gastrin cross-reactivity. All values of CCK-8 fall within the range of the standard curve (10 to 1000 pmol per mL) for CCK-8 quantitation by a sensitive and selective CCK-8 ELISA (A), which shows no cross-reactivity for gastrin (B). Gastrin and CCK-8 (1 ng per mL each) were analyzed side-by-side. Data from plasma from two mice ± S.E.M. Analyzed with linear regression. ND = not detected.
Supplementary Figure 4. Analysis of gastric emptying. Gastric emptying was evaluated by quantitating via UPLC/MS/MS levels of 19:2 free fatty acid (19:2 FFA) recovered from stomach 30 min following oral gavage of corn oil (CO) in mice maintained on a standard low-fat no-sucrose chow (SD). Thirty min prior to gavage, mice were administered WIN 55,212-2 (WIN), AM6545 (AM), or vehicle (CO). Drug treatment had no significant effect on gastric emptying of CO (A). Similarly, mice fed a western diet (WD) for 60 days displayed no changes in gastric emptying of CO, when compared to SD mice (B). Data expressed as mean ± S.E.M. Analyzed using one-way ANOVA (A) or student's t-test (B; two-tailed). n = 4 (A) or 3 (B) per condition, p>0.05
Supplementary Figure 5. Effects of drug treatment on glucose levels in mice maintained on a standard low-fat no-sucrose diet. Blood glucose levels were measured at time -30 just prior to administration of the drugs WIN 55,212-2 (WIN), AM6545 (AM), or vehicle (Veh). Blood glucose was again measured 30 min later at time 0 just prior to oral gavage of corn oil (CO, 500 µL), then again at time of kill at time 30. Drug treatment had no significant effect on blood glucose levels at any time point, and CO did not impact blood glucose levels 30 minutes later at time 30 (A). Drug treatment also had no impact on area under the curve (AUC, all timepoints included) for blood glucose levels (B). Data expressed as mean ± S.E.M. Analyzed using repeated measures (time) two-way ANOVA with Sidak’s post hoc multiple comparison’s test (A) and one-way ANOVA (B). n = 4 per condition, p>0.05.
Figure 4. 2-AG biosynthesis and degradation are upregulated in small intestine during obesity. Hydrolytic activity of DGL (A) and MGL (B) are increased in mice maintained on western diet (WD) when compared to controls fed a standard diet (SD). Levels of the endocannabinoid, 2-AG, are increased in jejunum mucosa of WD mice, when compared to SD mice (C). 2-AG is formed by the hydrolysis of its 1,2-stearoyl-2-arachidonoyl-sn-glycerol precursor by DGL and is subsequently degraded by MGL into arachidonic acid and glycerol (D). Data expressed as mean ± S.E.M. Analyzed using Student's two-tailed T-test. n=6 per condition, *P <0.05.
Supplementary Figure 6. Validation of DGL and MGL functional enzyme assays. Inhibitors of DGL (A) and MGL (B) (THL and JZL184, respectively) dose-dependently inhibited hydrolytic activity of these enzymes in isolated protein form the upper small-intestinal epithelium. Analyzed using nonlinear regression of log-inhibitor vs. response. n=3 per group and $R^2$ = goodness of fit $> 0.8$. 
Figure 5. Expression of select components of the eCB system is dysregulated in the upper small intestine of DIO mice and partially conserved in CCK-eGFP+ cells. Expression of mRNA for cholecystokinin (Cck), cannabinoid receptor subtype 1 (Cnr1) and 2 (Cnr2), and other components of the eCB system in upper small-intestinal mucosal scrapings are not impacted by western diet (WD) exposure when compared to controls fed a standard diet (SD)(A). Expression of diacylglycerol lipase beta (Daglb) is decreased, and expression of the degradative enzymes monoacylglycerol lipase (Mgll) and alpha-beta hydrolyzing domain 6 (Abhd6) are increased in WD mice. Expression of mRNA for CCK or components of the eCB system were not significantly affected by diet in eGFP (+) sorted cells (B). Expression of mRNA for diacylglycerol lipase alpha (Dagla), Mgll, and N-acyl phosphatidylethanolamine specific phospholipase D (Napepld) was not detected (ND)(B). Data expressed as mean ± S.E.M. Analyzed using Student’s two-tailed T-test. n = 3 per group in triplicate and *P <0.05, **P <0.01 (A); n = 3 per group in triplicate, P >0.05 (B).
Figure 6. Peripheral eCB signaling drives hyperphagia in mice maintained on a WD via a CCK-dependent mechanism. Caloric intake (A), meal size (B), and rate of intake (C) of a western diet (closed bars) are significantly reduced during a 12 h test following inhibition of peripheral CB₁Rs with AM6545 (AM, 10mg per kg), an effect absent in low-fat chow fed mice (open bars) and that is blocked by co-administration with the CCKA receptor antagonist, devazepide (Dev; 0.1mg per kg). Diet and drug had no effect on meal frequency (D), meal duration (E), post meal interval (F), or first meal size (G). All data represented as means ± SEM. Analyzed using regular 2-Way ANOVA with post hoc Newman-Keuls multiple comparison’s test. n=11-12 per condition, *p<0.05, **p<0.01.
Supplementary Figure 7. Mice fed WD displayed large increases in body weight. Mice maintained on a western diet for 60 days showed a significant increase in body weight (A) and change in body weight (B) when compared to littermates maintained on a chow diet. Data expressed as mean ± S.E.M. Analyzed using repeated measures (time) two-way ANOVA with Sidak’s post hoc multiple comparison’s test. n = 9-10, ***P<0.001.
Our studies suggest that cannabinoid CB₁Rs in the upper small-intestinal epithelium control nutrient-induced satiation signaling, and their signaling is increased in diet-induced obesity, which drives overeating by delaying satiation. The upper small-intestinal epithelium contains enteroendocrine I-cells, which are a subpopulation of enterocytes that secrete cholecystokinin (CCK) when nutrients – including dietary fats – enter the lumen (83, 104-107). Dietary fats (e.g., corn oil), in the form of triacylglycerols, are hydrolyzed in the lumen into mostly monoacylglycerols and free-fatty acids (FFAs) that are sensed by free-fatty acid receptors (FFARs) located on enteroendocrine cells in the small-intestinal epithelium. Activation of FFARs stimulates secretion of CCK by a mechanism that requires calcium (Ca²⁺) influx and/or intracellular (i.e., endoplasmic reticulum, ER) mobilization (29, 76, 104). CCK activates CCKₐ receptors located on adjacent afferent sensory vagal fibers, which in turn, communicate with the brain and control meal size and satiation (31-33, 37).

Consumption of a Western diet (WD) is associated with increased levels of the endocannabinoids (eCBs) and their activity at CB₁Rs in the upper small-intestinal epithelium, which we propose inhibits CCK release and satiation signaling. The molecular mechanism(s) mediating CB₁R control of CCK release is unknown but may include inhibition of Ca²⁺-mediated CCK release. A future test of this hypothesis is warranted.
Chapter 4: Host and Helminth-Derived Endocannabinoids Are Generated During Infection with Functional Effects on Host Immunity


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Abbreviations: 2-AG, 2-arachidonoyl-sn-glycerol; AEA, anandamide; CB1R, cannabinoid subtype 1 receptor; DGL, diacylglycerol lipase; MGL, monoacylglycerol lipase; OEA, oleoylethanolamine; Th2, T helper type 2.

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Abstract

Helminths have coevolved with their hosts, resulting in the development of specialized host immune mechanisms and parasite-specific regulatory products. Identification of new pathways that regulate helminth infection could provide a better understanding of host-helminth interaction and may identify new therapeutic targets for helminth infection. Here we identify the endocannabinoid system as a new mechanism that influences host immunity to helminths. Endocannabinoids are lipid-derived signaling molecules that control important physiologic processes, such as feeding behavior and metabolism. Following murine infection with *Nippostrongylus brasiliensis*, an intestinal nematode with a life cycle similar to that of hookworms, we observed increased levels of endocannabinoids (2-arachidonoylglycerol [2-AG] or anandamide [AEA]) and the endocannabinoid-like molecule oleoylethanolamine (OEA) in infected lung and intestine. To investigate endocannabinoid function in helminth infection, we employed pharmacological inhibitors of cannabinoid subtype receptors 1 and 2 (CB1R and CB2R). Compared to findings for vehicle-treated mice, inhibition of CB1R but not CB2R resulted in increased *N. brasiliensis* worm burden and egg output, associated with significantly decreased expression of the T helper type 2 cytokine interleukin 5 (IL-5) in intestinal tissue and splenocyte cultures. Strikingly, bioinformatic analysis of genomic and transcriptome sequencing (RNA-seq) data sets identified putative genes encoding endocannabinoid biosynthetic and degradative enzymes in many parasitic nematodes. To test the novel hypothesis...
that helminth parasites produce their own endocannabinoids, we measured endocannabinoid levels in *N. brasiliensis* by mass spectrometry and quantitative PCR and found that *N. brasiliensis* parasites produced endocannabinoids, especially at the infectious larval stage. To our knowledge, this is the first report of helminth- and host-derived endocannabinoids that promote host immune responses and reduce parasite burden.

**Introduction**

Parasitic helminths infect an estimated two billion individuals worldwide (1). Although helminth infection is not typically fatal, it is associated with a multitude of pathologic conditions, including malnutrition and growth retardation. A majority of soil-transmitted helminths reside in the gastrointestinal tract, where they can negatively impact the host’s nutritional status by stealing nutrients or preventing nutrient absorption by damaging or causing inflammation of the intestinal tissue (2). Additionally, recent studies have identified new mechanisms by which helminths impact host feeding and metabolism (3). Gastrointestinal helminth infection was reported to decrease food intake (4), and was beneficial in mice fed a high-fat diet, in which it improved glucose metabolism and reduced adiposity (5, 6). This effect was partly mediated through T helper type 2 (Th2) cytokine-activated M2 macrophages in the adipose tissue, which have a known beneficial effect in metabolic homeostasis (7). In the intestine, helminth infection induced a Th2 cytokine-dependent expansion of tuft cells, which express taste
receptors, and cholecystokinin (CCK)-positive enteroendocrine cells, which secrete hormones that regulate feeding behavior (8). Overall, these findings support a multifactorial relationship between helminth and host immune response that affects host feeding behavior and metabolism. Identification of new helminth or host-derived factors that regulate this process, and how they affect host health and helminth killing, could provide a better understanding of the pathologic or beneficial effects of helminth infection that could be exploited therapeutically.

Among the many host-derived molecules that affect feeding and metabolism, endocannabinoids are an important class of lipid molecules that regulate these physiologic processes (9, 10). Endocannabinoids are the body’s natural cannabis-like molecules that signal through cannabinoid receptors, which are highly expressed on neurons (11). Unsurprisingly, a highly recognized function of endocannabinoids is promoting neural-mediated behaviors such as food intake and reward (9). Endocannabinoids, however, are generated throughout the body, and cannabinoid receptors are present on extraneuronal cells, including intestinal epithelial cells and immune cells (12-14). Signaling by the endocannabinoids, 2-arachidonoylglycerol (2-AG) or anandamide (AEA), at cannabinoid receptors on intestinal cells impacts feeding behavior (15, 16), while signaling on immune cells can promote anti-inflammatory pathways (17). Despite functional effects on intestinal physiology and immune responses, no studies reported to date have investigated the role of endocannabinoids in intestinal parasite infection.
In this study, we investigated the expression and function of endocannabinoids in murine infection with *Nippostrongylus brasiliensis*, a rodent nematode parasite that has a life cycle similar to that of human hookworms (18). We show that *N. brasiliensis* infection significantly induces the biosynthesis of endocannabinoids and endocannabinoid-like molecules in the infected lung and intestine. We also performed functional assays to measure endocannabinoid biosynthetic and degradative enzyme activity in infected jejunal tissue, and we observed significantly increased endocannabinoid synthetic but not degradative enzyme activity. Endocannabinoid levels were negatively correlated with early infection-induced weight loss, associated with reduced food intake, and *N. brasiliensis* egg output, suggesting that endocannabinoids are associated with improved host immunity. To test this hypothesis, we employed validated peripheral pharmacological inhibitors of the cannabinoid subtype 1 receptor (CB₁R) and CB₂R, AM6545 and AM630, respectively, which act peripherally and do not cross the blood-brain barrier (16, 19). Pharmacological inhibition of CB₁R, but not CB₂R, significantly increased *N. brasiliensis* worm burdens and fecal egg output. Increased parasite burden was associated with reductions in Th2 cytokines (interleukin 5 [IL-5] and IL-4) but not in the Th1 cytokine gamma interferon (IFN-γ), suggesting that *N. brasiliensis*-induced endocannabinoid signaling through CB₁R was important for optimal host Th2 immune responses. Strikingly, bioinformatic analyses of the genomes and transcriptome sequencing (RNA-seq) data sets from *N. brasiliensis* and other parasitic nematodes,
including the hookworms *Ancylostoma ceylanicum* and *Necator americanus*, revealed putative genes encoding endocannabinoid synthetic and degradative enzymes. We validated the bioinformatic predictions for *N. brasiliensis* by quantitative real-time PCR and mass spectrometry (MS) and showed that *N. brasiliensis* produces endocannabinoids at all life cycle stages. Taken together, these studies show for the first time the production of endocannabinoids by parasitic helminths and suggest that helminth infection-induced endocannabinoids functionally influence the host immune response and parasite burden. These findings support a new area of investigation into the function of the endocannabinoid system in infectious diseases.

**Materials and methods**

**Parasite**

The *Nippostrongylus brasiliensis* life cycle was maintained in Sprague-Dawley rats, as previously described (20, 21). Hatched infectious L3 larvae were recovered from 1- to 2-week-old fecal egg cultures by a Baermann apparatus. L4 larvae were recovered from lung tissue of day 2-infected rats by manual picking from coarsely minced lung tissue in media after 2h incubation at 37°C. Adult *N. brasiliensis* parasites were recovered from day 6 to 8 *N. brasiliensis* infected mice by dissection and slitting of the whole small intestine, followed by 2 hour incubation in warm PBS and manual picking of the worms from the intestinal tissue or supernatant. Eggs in the feces of infected mice and rats were counted.
using a McMaster counting chamber. For endocannabinoid quantification, parasites were washed 3x in excess PBS, counted, and weighed.

**Mice and tissue recovery**

C57BL/6 mice were purchased from the Jackson Laboratory or bred in-house. All mice in the experiment were age-matched (6- to 8-week-old) males and females housed in a specific-pathogen-free facility. Mice were anesthetized with isoflurane and injected subcutaneously with 500 *N. brasiliensis* L3 larvae. Behavior was assessed using single-housing units (TSE Systems, Chesterfield, MO). Mice were placed into units 3 days prior to recording for acclimation, and daily feeding was monitored using Phenomaster software (TSE Systems). Where indicated, mice were treated intraperitoneally with a vehicle control (7.5% DMSO, 7.5% Tween 80, and 85% saline), CB₁R antagonist AM6545 (10 mg/kg of body weight), or CB₂R antagonist AM630 (10 mg/kg). Blood recovery was done by cardiac puncture into tubes containing 7.2 mg of EDTA. Following excision of the small intestine, mucosa was stripped and recovered for endocannabinoid quantification. One-centimeter jejunal tissues were weighed and homogenized in 0.5 mL of PBS with Mini-Beadbeater-96 (BioSpec Products) at 4°C, and supernatant was collected after centrifugation (4000 x g for 15 min at 4°C) for cytokine quantification. All protocols for animal use and euthanasia were approved by the University of California, Riverside Institutional Animal Care and Use Committee (https://or.ucr.edu/ori/committees/iacuc.aspx; protocols A-20150028E and A-20170036) and were in accordance with the National Institutes
of Health Guidelines. Animal studies are in accordance with the provisions established by the Animal Welfare Act and the Public Health Services (PHS) policy on the humane care and use of laboratory animals.

**Lipid extraction and FAEs and MAGs analysis**

Lipid extraction and analysis were performed as previously described (16, 22). Frozen tissue or worms were homogenized in 1.0 mL of methanol solution containing the internal standards, $[\text{H}_5]^2$-AG and $[\text{H}_4]$-AEA (Cayman Chemical, Ann Arbor, MI, USA). Lipids were extracted with chloroform (2 mL) and washed with water (1 mL). Lipids were similarly extracted from serum samples, with the exception of a 0.9 % saline wash replacing water (0.1 mL serum at the expense of saline). Organic phases were collected and separated by open-bed silica gel column chromatography as previously described (15). Eluate was gently dried under $N_2$ stream (99.998% pure) and resuspended in 0.1 mL of methanol:chloroform (9:1), with 1μL injection for ultra-performance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS) analysis.

Data was acquired using an Acquity I Class UPLC with in-line connection to a Xevo TQ-S Micro Triple Quadrupole Mass Spectrometer (Waters Corporation, Milford, MA, USA) with electrospray ionization (ESI) sample delivery. Lipids were separated using an Acquity UPLC BEH C$_{18}$ column (2.1 x 50 mm i.d., 1.7 μm, Waters) and inline Acquity guard column (UPLC BEH C$_{18}$ VanGuard PreColumn; 2.1 x 5 mm i.d.; 1.7 μm, Waters), and eluted by a gradient of water and methanol (containing 0.25% acetic acid, 5 mM ammonium acetate)
at a flow rate of 0.4 mL/min and gradient: 80% methanol 0.5 min, 80% to 100% methanol 0.5 to 2.5 mins, 100% methanol 2.5 to 3.0 mins, 100% to 80% methanol 3.0 to 3.1 mins, and 80% methanol 3.1 to 4.5 mins. Column was maintained at 40°C and samples were kept at 10°C in sample manager. MS detection was in positive ion mode with capillary voltage maintained at 1.10 kV and Argon (99.998%) was used as collision gas. Cone voltages and collision energies for respective analytes: 2-AG = 30v, 12v; 2-DG = 34v, 14v; 19:2 MAG = 18v, 10v; AEA = 30v, 14v; OEA = 28v, 16v; DHEA = 30v, 16v; [^2H_5]-2-AG = 25v, 44v; [^2H_4]-AEA = 26v, 16v; [^2H_4]-OEA = 48v, 14v. Lipids were quantified using a stable isotope dilution method detecting proton or sodium adducts of the molecular ions [M + H/Na]^+ in multiple reaction monitoring (MRM) mode. For many MAGs acyl migration from sn-2 to sn-1 glycerol positions is known to occur; for these analytes, the sum of these isoforms is presented. Tissue processing and LCMS analyses for experiments occurred independently of other experiments. Extracted ion chromatograms for MRM transitions were used to quantify analytes: 2-AG (m/z = 379.3 > 287.3), 2-DG (m/z = 403.3 > 311.2), AEA (m/z = 348.3 > 62.0), OEA (m/z = 326.4 > 62.1), DHEA, (m/z = 372.3 > 62.0), 19:2 MAG (m/z = 386.4 > 277.2), with [^2H_5]-2-AG (m/z = 384.3 > 93.4), [^2H_4]-AEA (m/z = 352.4 > 66.1), and [^2H_4]-OEA (m/z = 330.4 > 66.0) as internal standards. Controls included one “blank” sample that was processed and analyzed in the same manner as all samples, except no tissue was included. This control
revealed no detectable endocannabinoids and related lipids included in our analysis.

**Functional enzyme assays of DGL and MGL activity**

*Free-fatty acid measurements*

Data for free fatty acids (FFA) (19:2 FFA, product of MGL assay) were measured using the UPLC/MS/MS instrument as described above. Mobile phase compositions for 19:2 FFA were the same as described above, but the flow gradient began at flow rate 0.4 mL/min: 90% Methanol 0.1 min, 90% to 100% methanol 0.1 to 2.0 mins, 100% methanol 2.0 to 2.1 mins, 100% to 90% methanol 2.1 to 2.2 mins, and 90% methanol 2.2 to 2.5 mins. 19:2 MAG, product of DGL assay, was analyzed exactly as described above as for other MAGs. MS detection of fatty acids was in negative ion mode with capillary voltage maintained at 1.10 kV. Cone voltages for respective analytes: 19:2 FFA = 48v; 17:1 FFA = 64v. Lipids were quantified using a dilution series detecting deprotonated molecular ions (23)⁻ in selected ion recording (SIR) mode. Extracted ion chromatograms for SIR masses were used to quantify the analytes: 19:2 FFA (m/z = 293.3) and the internal standard 17:1 FFA (m/z = 267.2).

*Tissue Preparation*

Intestinal epithelium was collected as described above and approximately 100 mg of frozen tissue was homogenized in 2 mL of ice-cold 50mM Tris-HCl, 320mM sucrose (pH 7.5) buffer. Homogenates were centrifuged at 800g for 10 minutes while kept at 4°C and supernatant collected. Protein supernatants were
sonicated twice for 10 s and then freeze-thawed in liquid nitrogen twice. Samples were spun again, as described above, and supernatant was quantified with BCA assay and diluted with Tris-HCl/sucrose buffer.

**DGL activity assay**

25 µg tissue homogenates, in 0.1 mL Tris-HCl/sucrose (pH7.5), were incubated with 0.3 µM JZL184 [MGL inhibitor; Cayman Chemical, Ann Arbor, Michigan (24)] and accompanying treatments for 10 min at room temperature. Homogenates were incubated with 0.1 mL solution of Tris-HCL with 0.2% Triton X-100 (pH 7.0) containing 20 nmol dinonadecadienoin (Nu-Chek Prep, Waterville, MN; final volume 0.2 mL/reaction) at 37°C for 30 min. Reaction was stopped by adding 1 mL of MeOH containing 25 pmol [2H5]-2-AG. Lipids were extracted as described above and analyzed via UPLC/MS/MS.

**MGL activity assay**

10 µg tissue homogenates, in 0.1 mL Tris-HCl/sucrose (pH 7.5) were incubated with 0.4 mL solution of Tris-HCL with 0.1% BSA (pH 8.0) containing 50 nmol nonadecadienoin (Nu-Chek Prep; final volume 0.5 mL/reaction) at 37°C for 10 min. Reaction was stopped by adding 1 mL of MeOH containing 10 nmol heptadecanoic acid (Nu-Chek Prep). Lipids were extracted as described above and analyzed via UPLC/MS/MS.
Cytokine quantification

ELISA

IL-5 quantification of intestinal homogenate and spleen supernatants was performed by standard sandwich enzyme-linked immunosorbent assay (ELISA) according to previously described protocols (20).

Cytokine bead array

IL-4, IFN-γ, and IL-10 quantification was performed on intestinal homogenate and supernatants from 72-h-stimulated splenocytes (5 x 10^6/well; 0.5 µg/ml of anti-CD3 and anti-CD28) using the Th1/Th2 CBA assay (BD Biosciences) according to the manufacturer’s instructions.

RNA quantification

Mouse tissue recovered for RNA extraction was first incubated overnight in RNAlater (Qiagen) at 4˚C then extracted by TRIzol (Life Technologies). iScript Reverse Transcriptase (Biorad) was used for cDNA synthesis. Relative quantification of cDNA was measured by real-time PCR using the Biorad CFX Connect. 18S primers were purchased from Qiagen, and Cnr1 and Cnr2 primers were purchased from Biorad.

L3 infective juveniles (3 replicates of 500 to ~1,000) were collected from cultured rat fecal plates. L4 worms (3 replicates of 100 to 500) were collected from dissected lungs of infected rats 2 days post infection. Adult worms (3 replicates of 100 to 200) were collected from dissected intestines of infected rats. Worms were washed in double-distilled water (ddH₂O), flash-frozen, and then
homogenized in RiboZol (VWR) using pellet pestles and a pestle motor (Fisher Scientific and VWR). RNA was extracted according to RiboZol manufacturer instructions. Reverse transcription was done using Proto-Script II reverse transcriptase (New England BioLabs [NEB]), followed by real-time PCR as described above. Primer sequences were as follows: N. brasiliensis actin gene, ACGACGTGGCAGCTCTCGTTGTGG (forward) and GGTGCTTCGGTCAGCAGCGGA (reverse); N. brasiliensis faah gene, TCGGAGCAGGTGTTGAAGA (forward) and AGCCGGTACCACGGATCTGA (reverse); and N. brasiliensis nape gene, GGCATACGTCCACGATGGTT (forward) and GGTGCTTCGGCTCGAGGTAG (reverse).

**Gene orthology analysis and protein alignment**

To identify enzymes and regulatory proteins involved in lipolysis and endocannabinoid signaling we leveraged previously identified orthologs in *C. elegans* (25, 26). WormBase ParaSite (version WBPS9) was consulted to identify putative orthologs of these genes in several parasitic nematodes. To validate the putative parasitic nematode orthologs, we performed an orthology analysis using available predicted protein datasets from WormBase release WSPS9 — *Ancylostoma ceylanicum, Ancylostoma duodenale, Ascaris lumbricoides, Ascaris suum, C. elegans, Necator americanus, N. brasiliensis, Steinernema carpocapsae, Strongyloides ratti, Strongyloides stercoralis*, and *Toxocara canis*. Version 1.4 of the OrthoMCL pipeline was used to cluster proteins into families of
orthologous genes, with default settings and the BLAST parameters recommended in the OrthoMCL documentation (27).

The NAPE-PLD gene was further explored by aligning the sequence of mouse NAPE-PLD and the putative homolog from *N. brasiliensis*. Protein sequences were aligned using MUSCLE (28) and visualized using Mesquite (version 3.2). The accession numbers of the proteins used are NP_848843 for the mouse NAPEPLD from GenBank and NBR_0001270801-mRNA-1 for the *N. brasiliensis*NAPEPLD from WormBase ParaSite. The B_2 lactamase domain in the alignment was identified using Smart protein database (29).

**Gene expression analysis**

To determine if genes for the endocannabinoid system are expressed in other parasitic nematodes, we downloaded the RNA-seq data for *T. canis* (30) (SRR1707010, SRR1707031-6), *S. ratti* (31) (ERR299168-79, ERR225783-4), *S. stercoralis* (32) (ERR146945-6, ERR146948-9), *A. suum* (33) (SRR851186-95), *N. americanus* (34) (SRR609895, SRR609951), *N. brasiliensis* (35); PRJEB16076, and *A. ceylanicum* (36) (SRR1124912-4, SRR1124985-6) from NCBI. The published RNA-seq data for *N. brasiliensis* was downloaded from European Nucleotide Archive (http://www.ebi.ac.uk/) (58). The reads were mapped to each species' indexed transcriptome (downloaded from WormBase ParaSite, WSPS9) with bowtie 1.0.0 in paired-end mode with the following settings: bowtie –X 800 -m 200 -S --seedlen 25 --trim3 [50 or 100] -n 2 --offrate 1 (37). Gene expression was quantified with RSEM version 1.2.31 (38).
Statistical Analysis

GraphPad Prism software was used for statistical analyses. Where appropriate, Student’s t test (for normal distribution data), one-way analysis of variance (ANOVA) (for analysis of more than two groups), two-way ANOVA (for analysis of more than one experiment), linear regression, and nonparametric Spearman correlation (for correlation analysis) were performed. Statistical significance is indicated in Table 1 and in figures as follows: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.0001$. To evaluate CB1R inhibitor effect on *N. brasiliensis* burdens across experimental repeats, we employed linear mixed models. The inhibitor was tested as the main effect, which was adjusted by baseline weight and the weight on day 3; the experimental cooperation was the random effect. On the basis of the distribution types for egg and worm, the corresponding distribution families were Gamma and Poisson, respectively.

Results

*Nippostrongylus brasiliensis* infection induces lung and intestinal endocannabinoids production

Endocannabinoids are lipid signaling mediators that affect a variety of behaviors (e.g. feeding and memory) and metabolic processes (e.g. glucose homeostasis) (see Fig. S1 in the supplemental material) (9). Additionally, endocannabinoids can regulate the immune response and dampen inflammation (39). Despite reported immunoregulatory function, however, whether
endocannabinoids are generated in parasite infection and the functional consequence for the host or pathogen are unknown. *N. brasiliensis* infects the small intestine of mice and has been shown to affect food intake and metabolism(3-5). Given that endocannabinoids and endocannabinoid receptors are expressed in the intestine, we hypothesized that *N. brasiliensis* infection may affect endocannabinoid signaling. Similar to the hookworm life cycle, *N. brasiliensis* infects both the lung and the intestine as part of its life cycle and feeds on host blood(40). We therefore measured tissue and circulating endocannabinoid levels in naive and *N. brasiliensis*-infected mice at days 2 and 7 postinfection, when the parasites had infected the lung and jejunum, respectively. We observed a modest but significant increase in 2-AG and AEA in *N. brasiliensis*-infected lung tissue and a trend toward increased levels of endocannabinoid-like molecule oleoylethanolamine (OEA) (Fig. 1A). Strikingly, 2-AG levels in jejunal tissue were almost 10-fold higher than in the lung, and we observed a >2-fold increase following *N. brasiliensis* infection (Fig. 1B). AEA and OEA, both of which regulate feeding and are anti-inflammatory(15, 41, 42), were also significantly elevated in the jejunum in response to *N. brasiliensis* infection (Fig. 1B). In contrast, circulating endocannabinoid levels in the plasma were unchanged following *N. brasiliensis* infection (Fig. 1C), suggesting that *N. brasiliensis*-induced endocannabinoids were restricted to the tissue infection site. Given that the 2-AG levels were highest in the infected jejunum, we next evaluated the activity of the biosynthetic and degradative enzymes responsible
for 2-AG metabolism in jejunal epithelium of infected versus naive mice. Specifically, we measured the enzymatic activities of diacylglycerol lipase (DGL, biosynthetic) and monoacylglycerol lipase (MGL, degradative) by functional enzyme assays (15). We found significant increases in the activity of DGL in jejunal tissue of infected mice compared to noninfected mice (Fig. 1D, left), which suggests that levels of 2-AG in jejunum are elevated in infected mice by a mechanism that includes increases in jejunal DGL-mediated 2-AG biosynthesis. In contrast, we did not observe any changes in enzymatic activity of MGL (Fig. 1D, right). Endocannabinoids signal through the G protein-coupled cannabinoid receptors subtype 1 and 2; therefore, we measured the cannabinoid receptor coding genes Cnr1 and Cnr2 mRNA by quantitative PCR of the jejunal tissue. *N. brasiliensis* infection induced increases in both Cnr1 and Cnr2 (Fig. 1E).

Collectively, these data demonstrate that *N. brasiliensis* infection increases endocannabinoid levels locally in the infected lung and intestinal tissue and promotes intestinal endocannabinoid receptor expression, suggesting that the endocannabinoid system is induced in helminth infection.

**Intestinal endocannabinoids are negatively correlated with *Nippostrongylus brasiliensis* infection-induced weight loss and parasite egg burdens**

We examined if *N. brasiliensis*-induced endocannabinoids were associated with health outcomes for the host or parasite by correlative analyses between endocannabinoid levels and infection-induced weight loss or parasite egg burdens. *N. brasiliensis* infectious larval stage 3 (L3) helminths migrate to
the lung, where they develop into L4, followed by infection of the small intestine, where they develop into adults and produce eggs (Fig. 2A and B). At the infectious dose of 500 L3 helminths, *N. brasiliensis* infection of the lung, which occurs between day 1 and 3 postinfection, causes lung hemorrhaging and inflammation likely due to the physical damage of the worm burrowing through the lung tissue. During this acute infection of the lung, we observe significant weight loss that was remarkably resolved once the *N. brasiliensis* parasites were established in the intestine, at day 6 (Fig. 2A). Consistent with this weight loss, analysis of feeding patterns in naive and infected mice revealed reduced food intake (*P* < 0.05) and motor activity (*P* < 0.05) at day 1 postinfection, when the *N. brasiliensis* parasites had reached the lung, that was also resolved by day 6 postinfection (Fig. 2C). We also observed a significant positive correlation between *N. brasiliensis*-induced acute weight loss at day 3 and *N. brasiliensis* egg burdens at day 7 post infection (Fig. 2D, left). This correlation suggests that acute infection-induced weight loss at day 3 may be a good predictor of subsequent parasite establishment in the intestine. The effect of *N. brasiliensis* infection on mouse weight changes may be due to lung tissue inflammation or changes in mouse feeding behavior.

Given that endocannabinoids can regulate both these processes, we investigated correlations between endocannabinoids and *N. brasiliensis* parasite burdens. We observed that 2-AG intestinal levels from day 7 infected mice were negatively correlated with early infection-induced weight loss (day 3) and day 7
parasite egg burden (Fig. 2D, right). To comprehensively define the relationship between endocannabinoids versus host and parasite fitness, we performed Spearman correlation analyses across all experiments (Table 1). We observed a negative correlation between day 7 infected jejunum 2-AG and OEA and (i) early (day 3) infection-induced weight changes and (ii) day 7 parasite egg burden. In contrast, we observed a positive correlation between plasma 2-AG and OEA, weight loss, and parasite egg burden. These data indicate that high endocannabinoid levels locally in the intestine are associated with reduced early infection-induced weight loss and decreased parasite egg burden.

**Disruption of CB1 receptor but not CB2 receptor signaling increases** *Nippostrongylus brasiliensis* egg burden and impairs intestinal IL-5 responses

The endocannabinoids, 2-AG and AEA, both of which were upregulated following *N. brasiliensis* infection (Fig. 1), signal through CB1R and CB2R (12). We investigated the function of endocannabinoid signaling in *N. brasiliensis* infection by treatment with the peripherally restricted neutral CB1R antagonist AM6545 (43, 44), CB2R antagonist AM630 (19), and dimethyl sulfoxide (DMSO) as a vehicle control. To rule out potential confounding effects on parasite establishment in the intestine, mice were treated daily with antagonists or vehicle starting at day 4 postinfection, when all *N. brasiliensis* parasites had reached the intestine. At this time point, CB1R or CB2R inhibition had no effect on mouse weight (Fig. 3A). Interestingly, CB1R but not CB2R inhibition led to increased fecal *N. brasiliensis* egg output and intestinal *N. brasiliensis* worm counts (Fig.
3B), suggesting that CB₁R signaling is necessary for optimal *N. brasiliensis* expulsion. We validated these CB₁R inhibitor-mediated differences in *N. brasiliensis* burdens across three experimental repeats, using generalized linear models, and found that CB₁R inhibitor treatment led to a 2.21-fold-higher egg burden (*P* < 0.01) and 1.46-fold-higher worm burden (*P* < 0.01) (Table 2). CB₁R inhibition resulted in significantly decreased intestinal levels of the Th2 cytokine IL-5 but not IL-4, IFN-γ or IL-10 (Fig. 3C). Further, in vitro CD3/CD28-activated splenocytes from CB₁R inhibitor-treated mice secreted significantly less IL-5, IL-4, and IL-10 than vehicle-treated mice but exhibited no defect in IFN-γ secretion (Fig. 3D). This cytokine effect was specific to CB₁R signaling, as inhibition of CB₂R signaling had no significant effect. Given that host immunity to *N. brasiliensis* is dependent on Th2 cytokines, the CB₁R inhibitor-induced decrease in IL-5 and IL-4 may explain the increase in parasite egg burden. Additionally, the significant reduction in IL-10 secretion is consistent with an anti-inflammatory function for CBR signaling (41). These data support the functional link between the endocannabinoid system and immunity to *N. brasiliensis* and suggest that CB₁R signaling has a beneficial impact for the host following *N. brasiliensis* infection by promoting Th2 cytokine expression and reducing parasite burdens.

Endocannabinoid system is present in *Nippostrongylus brasiliensis* and other parasitic helminths

The endocannabinoid system is conserved in vertebrates and some invertebrates (45), and endocannabinoid biosynthetic enzymes are expressed in
Caenorhabditis elegans (46). However, whether the endocannabinoid system exists in parasitic helminths is unknown. We conducted bioinformatic analyses of available parasitic nematode genomes for genes involved in the endocannabinoid synthetic and signaling pathway (Table 4). Within the N. brasiiliensis genome, genes encoding synthetic enzymes for the monoacylglycerols, 2-AG and DHAG (dagl), and the fatty acid ethanolamides, AEA, OEA, and docosahexaenoylethanolamide (DHEA) (nape) were identified. We also found orthologs of the fatty acid ethanolamide degradative enzyme, fatty acid amide hydrolase (faah-1), the proposed monoacylglycerol degradative enzymes, alpha beta hydroxylases (abhd-12 and abhd-5). Although nematodes did not have obvious orthologs of monoacylglycerol lipase (magl), the major degradative enzyme for 2-AG found in mammals, we identified a putative nematode gene encoding the minor 2-AG degradative enzyme, abhd-6 (47). Moreover, the synthetic and degradative endocannabinoid genes were conserved in other nematodes, including human hookworms Ancylostoma duodenale and Necator americanus, and human roundworm Ascaris lumbricoides.

Analysis of available RNA-seq data set revealed the expression of putative genes for the endocannabinoid pathway in parasitic nematodes of humans (A. ceylanicum, Ascaris suum, N. americanus, Toxocara canis, and Strongyloides stercoralis), rodents (N. brasiiliensis and Strongyloides ratti), and even insects (Steinernema carpocapsae) (Fig. 4; see also Fig. S2 in the supplemental
material). Further, these genes were expressed in a tissue- or stage-specific manner in some of these parasites. For example, the hookworm *A. ceylanicum*, which infects humans and other mammals, showed expression of abhd-5 in all stages sampled but showed highest expression of abhd-5 in the infective L3 stage. In the human hookworm *N. americanus*, both nape-1 and abhd-12 were more highly expressed in L3 larvae than in adult nematodes. Although it was previously thought that no obvious orthologs of CB₁ and CB₂ receptors were present in nematodes, a recent study identified the neuropeptide receptor NPR-19 as a cannabinoid-like receptor in *C. elegans* (26, 48-51). We were able to find putative orthologs of the cannabinoid like receptor NPR-19 in other nematodes, including *A. ceylanicum*, *N. americanus*, and *Wuchereria bancrofti* (Table 3). However, we were not able to find an obvious ortholog of NPR-19 in *N. brasiliensis*, though this may be due to the incomplete state of the available *N. brasiliensis* genome (N50 = 33.5 kb; i.e., nearly 30% of the predicted protein-coding genes are on contigs smaller than 10 kb), rather than the absence of an NPR-19 ortholog(52, 53).

*Nippostrongylus brasiliensis* produces endocannabinoids

Given the presence of genes encoding endocannabinoid synthetic enzymes in the *N. brasiliensis* genome, we investigated if *N. brasiliensis* could produce endocannabinoids at any stage of its life cycle. We isolated infectious *N. brasiliensis* L3 from hatched fecal cultures, *N. brasiliensis* L4 from day 2 infected lungs, and *N. brasiliensis* adults from day 7 infected jejenum, performed thorough
washes in phosphate-buffered saline (PBS), and measured endocannabinoid levels. Although there were no endocannabinoids in the control washes, we measured detectable levels of endocannabinoids and endocannabinoid-like molecules in worm extracts from all life cycle stages (Fig. 5A). These exhibited identical chromatogram patterns to reference endocannabinoids, AEA (Fig. 5B) and 2-AG (Fig. 5C), and endocannabinoid-like molecules (see Fig. S3A to C in the supplemental material). On a per-weight basis, adult *N. brasiliensis* produced the most 2-AG and DHAG, whereas L4s produced OEA. Strikingly, infectious L3 produced over 100-fold more AEA than the other life cycle stages or the host (Fig. 1B). L4 and adult *N. brasiliensis* worms feed on host tissue and blood; therefore, it may be possible that some endocannabinoids detected may be host-derived. However, the infectious L3 were isolated from culture and concentrations of AEA reached 1000 pmol per gram of worm, which is 100-200 times levels found in upper intestinal mucosal scrapings (5 to 10 pmol per g of tissue) and 300-1000 times found in circulation (1 to 3 pmol per mL) (Fig. 1, and (16)). Thus, the endocannabinoids measured in L3 worms are likely generated by the parasite. Consistent with the presence of a functional endocannabinoid system in *N. brasiliensis*, sequence alignment of the predicted *N. brasiliensis* gene encoding NAPE-PLD, the dominant enzyme catalyzing the biosynthesis of fatty acids ethanolamides, including AEA, OEA, and DHEA, revealed 43% identity, and sequence conservation in the functional lactamase domain (Fig. 5D). To validate that the predicted *N. brasiliensis* genes encoding
endocannabinoid biosynthetic and degradative enzymes were expressed, we performed quantitative PCR for predicted *nape* and *faah* and the actin gene as a housekeeping gene control (Fig. 5E). *nape* and *faah* mRNAs were present in all *N. brasiliensis* life cycle stages, with the highest expression in the infectious L3 stage. Overall, these data show for the first time that endocannabinoids are produced by *N. brasiliensis* and suggest that the endocannabinoid system is also present in parasitic nematodes that infect humans.

**Discussion**

In this study, we investigated the endocannabinoid signaling system following infection with *N. brasiliensis*. While recognized for its critical function in the central and enteric nervous system, the endocannabinoid system is also activated by and can influence inflammatory immune responses (22, 39, 54). For example, 2-AG and AEA are anti-inflammatory, which has provided the basis for the potential therapeutic use of synthetic cannabinoids, or cannabis, in autoimmune or inflammatory diseases (55). Oral treatment of mice with AEA promoted a tolerogenic immune response and regulatory macrophages in the intestine that were protective in a non-obese diabetic model (56). Despite an immune function in the intestine, the functional significance of endocannabinoids in intestinal parasite infection has not been examined. Our finding that helminth infection triggers significant endocannabinoid expression that is correlated with
both host health outcomes and parasite fecundity suggests that endocannabinoids may be important players in host-helminth dynamics.

Inhibition of CB₁R led to decreased expression of the Th2 cytokine IL-5 in the intestine and IL-4, IL-5, and IL-10 in the spleen but no difference in the Th1 cytokine IFN-γ. This was associated with increased parasite egg and worm burdens, suggesting that CB₁R signaling may be important for the optimal host immune response to keep helminth burdens in check. Endocannabinoids also signal through CB₂R (39); however, CB₂R antagonist treatment did not significantly change cytokine responses or helminth burdens. CB₁R and CB₂R inhibition was conducted during a short time frame, days 4 to 6 post-infection, and we cannot confirm complete abrogation of CB₁R or CB₂R signaling in the intestine with this treatment regime. Future studies with earlier treatment regimes or CB₁R/CB₂R-deficient mice may delineate functional differences between these signaling pathways in helminth infection. Cannabinoid CB₁Rs are expressed in cholecystokinin (CCK)-positive enteroendocrine cells in the duodenum of mice (57) and ghrelin-expressing cells of the stomach of rats (58), and N. brasiliensis infection in rats is associated with elevations in circulating levels of CCK (4, 59). Thus, it is possible that helminth infection may induce changes in feeding behavior, as seen in Fig. 2, by a mechanism that includes endocannabinoid-mediated changes in the production and/or release of peptides important for feeding behavior, including CCK. We observed significant correlations between endocannabinoid levels, infection-induced weight loss, and parasite burdens.
However, further functional studies are necessary to determine causal relationships between these multiple parameters and the contribution, if any, of infection-induced endocannabinoids to feeding behavior.

In addition to host endocannabinoid expression, we show for the first time that *N. brasiliensis* produces endocannabinoids and that genes encoding endocannabinoid biosynthetic and degradative enzymes are present in the genomes of multiple parasitic nematodes, including some of the most common helminth parasites of humans. One proposed strategy by which parasites modulate host immunity is by releasing molecules that are already native within the host, or at least native-like molecules (60). For example, *A. suum* and *T. canis* have been shown to synthesize opioid or opioid-like substrates, and morphine is a known immunomodulator (61, 62). It is well recognized that the endocannabinoid system is conserved in a diverse variety of vertebrates, including pythons (63) and goldfish (64); however, whether it evolved earlier and is functional in more primitive eukaryotic organisms is less well understood (48). We observed that *N. brasiliensis* has a predicted gene encoding the NAPE-PLD and FAAH enzymes that catalyze endocannabinoid synthesis and breakdown. Quantitative PCR analysis confirmed *N. brasiliensis* expression of both of these predicted genes. Moreover, mass spectrometry analysis revealed that infectious L3 *N. brasiliensis* produced extremely high levels of AEA, reaching 100 to 1000 times that found in tissue or blood of mice. A recent study showed that truffles, the fruiting body of fungi produce AEA potentially as an attractant and feeding
stimulant for animals to ensure its dissemination (65). Given that AEA is anti-inflammatory, high level synthesis at the infectious stage may also function to dampen the host immune response. It is possible that in addition to endocannabinoids, *N. brasiliensis* may produce and release other signaling molecules endogenous to the host, such as opioids, and that there is overlap in the biological effects of these molecules on host immunity or behavior. Given the difference in mass between *N. brasiliensis* and the host, the endocannabinoids detected in the infected mice are likely host derived. However, it is possible that *N. brasiliensis* derived endocannabinoids may functionally impact the host at the cellular level. Future studies are necessary to test these hypotheses.

Inhibition of CB₁R signaling significantly increased *N. brasiliensis* egg burden; however, whether the functional effect was through influencing the host or alteration of the endocannabinoid system in *N. brasiliensis* is unclear. In the host, the increased *N. brasiliensis* burdens when CB₁R signaling is inhibited could be due to the altered immune response or to reduced intestinal motility. Indeed, immune cells express both CB₁ and CB₂ receptors (66) (i.e., macrophages, dendritic cells, and T cells) and intestinal epithelial cells express CB₁R (14). Whether the CB₁R inhibitor-mediated increase in *N. brasiliensis* burden was through direct effects on immune cells, effects on the intestinal epithelial cells, or a combination of such effects is unclear and would require further studies. In addition to regulating immune responses, the endocannabinoid system throughout the gastrointestinal tract plays a variety of physiological roles,
including the control of motility, immune function, mucosal barrier function, and feeding behavior (67-69). Although outside the scope of the present study, future studies should include an evaluation of the impact that endocannabinoid-induced changes in intestinal motility have on egg burden. Furthermore, it is possible that endocannabinoid metabolism and/or release may be secondarily affected by perturbations in physiological responses governed by endocannabinoids themselves. It is also possible that CB1R inhibition could directly affect *N. brasiliensis*. Although the *N. brasiliensis* genome does not have canonical cannabinoid receptor genes, genes encoding endocannabinoid degradative enzymes are present in the *N. brasiliensis* genome, suggesting that *N. brasiliensis* may also respond to the endocannabinoids it produces through an as-yet-unidentified receptor. Consistent with this, *C. elegans* produces and responds to cannabinoids through NPR-19 (26, 50, 51). Inhibiting CB1R signaling was ultimately beneficial to *N. brasiliensis*, leading to improved parasite fertility. Why *N. brasiliensis* would produce endocannabinoids that adversely affect its fertility is unclear at present. Our data, however, support a functional impact of the host and parasite endocannabinoid system and suggest that further studies delineating the beneficial or detrimental function of endocannabinoids in the host versus the helminth are warranted. For example, the timing of cannabinoid receptor signaling inhibition may be critical. In our studies, we inhibited CB1R signaling after adult parasite establishment in the intestine; however, *N. brasiliensis* produces the most AEA during the initial infection, possibly as an
anti-inflammatory mechanism to downregulate the host immune response. \( N. \) 
\textit{brasiliensis}-derived AEA may also prevent excessive host inflammation that 
could lead to host mortality, which would be an equally adverse outcome for the 
helminth. Notwithstanding this, our discovery of parasite-derived 
endocannabinoids implicates the endocannabinoid system as a primitive 
pathway that contributes to host-pathogen interaction and suggests that 
investigation of the existence of the endocannabinoid system in other pathogens 
is warranted.

The complexity of host-helminth interaction and the numerous factors that 
influence the health outcomes for the parasite and the host are increasingly 
recognized. In addition to an optimized Th2 response for parasite expulsion, 
parasitic helminths trigger a multitude of non-immune pathways that affect 
physiologic processes, such as feeding and metabolism, but can also influence 
the immune response (70-72). Our findings suggest that the endocannabinoid 
system is a previously unrecognized contributor to this dynamic process and may 
therefore have a significant impact on the host’s health outcome beyond parasite 
expulsion.
References


Tables

**Table 1:** Correlation analysis between endocannabinoids, infection-induced weight loss and parasite egg burden (n=26-28, ns=not significant, n/a=not applicable).

<table>
<thead>
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<th>Jejunum eCB</th>
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- 2AG: 2-arachidonoylglycerol
- OEA: Oleoylethanolamide
- AEA: Anandamide
- DHAG: Docosahexaenoylglycerol
- DHEA: Docosahexaenoic acid
Table 2: Association test of *Nippostrongylus brasiliensis* egg burden with endocannabinoids and prohibitive effect of CB₁R inhibition (n=6/group) S.E. standard error.

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Table 3: Association test by the principal component (PC) analysis best selection between endocannabinoid and *N. brasiliensis* egg burden and effect of CB1R inhibition (n=6/group), CI, confidence interval.

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Table 4: Putative genes in endocannabinoid signaling and degradation identified in the genome of *Nb*, human hookworms and other parasitic nematodes.

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Figures

**Figure 1.** *N. brasiliensis* infection induces endocannabinoid production and cannabinoid receptor expression. C57BL/6 mice were left naïve or infected for 2 or 7 days with 500 *N. brasiliensis* L3 worms. 2-AG, AEA, and OEA levels in the lung at day 2 (A), jejunum at day 7 (B), and plasma at day 2 and day 7 (C). (D) 2-AG biosynthetic (DGL, left) and degradative (MGL, right) enzyme activities were measured in jejunal tissue from naive or day 7 infected mice. (E) *N. brasiliensis* infection-induced *Cnr1* and *Cnr2* mRNA in the jejunal tissue at day 7 was quantified as fold induction over naive after normalization with the 18S rRNA housekeeping gene. Data are presented as means ± SEM (*n* = 4 to 6/group) and are representative of results from four experiments.
Figure 2. Intestinal endocannabinoid levels are negatively correlated with infection-induced weight loss and fecal egg burdens. (A) Time course of *N. brasiliensis* infection-induced weight loss. (B) Compiled data of *N. brasiliensis* parasite burdens in lung and small intestine. (C) Food, water intake, and motor activities of naive (N) and *N. brasiliensis*-infected (INF) mice were evaluated in a feeding chamber. (D) Correlation analysis between parasite egg burden and weight loss (left) and jejunal 2-AG (right) was performed. Data are presented as means ± SEM and are representative of results from two to four experiments (n = 4 to 6/group).
Figure 3. Pharmacologic inhibition of cannabinoid receptor 1 but not cannabinoid receptor 2 increases helminth burdens associated with decreased IL-5 expression. C57BL/6 mice were left naive (black) or infected with *N. brasiliensis*. At days 4 to 7 postinfection, mice were treated intraperitoneally with AM6545 (CB₁R inhibitor [Inh]; red), AM630 (CB₂R inhibitor; gray), or DMSO (VEH; blue). (A) Infection induced weight loss was monitored and compared to that in naive mice. (B) Fecal egg burdens (left) and intestinal worm counts (right) were quantified in *N. brasiliensis*-infected mice. (C and D) IL-5, IL-4, IFN-γ, and IL-10 were quantified in day 8 infected (C) intestinal tissue homogenate and (D) supernatant from 72-h anti-CD3/anti-CD28-stimulated splenocytes. Data are presented as means ± SEM (n = 4 to 6/group) and representative of results from three separate experiments.
Figure 4. Expression of genes encoding endocannabinoid biosynthetic and degradative enzymes in parasitic nematodes. Nematode expression data for
endocannabinoid biosynthetic enzyme gene nape-1 (A) and degradative enzyme gene faah-1 (B), the monoacylglycerol biosynthetic enzyme gene dagl-2 (C), and genes for the potential degradative enzymes alpha beta hydrolases (abhd-5 [D]) and (abhd-12 [E]), and the minor 2-AG degradative enzyme (abhd-6 [F]) are shown in transcripts per million (TPM). The life stages for S. ratti expression are: free-living female (FLF), parasitic female (PF), free-living male (FLM), and infective L3 (L). The tissues for expression data for T. canis expression are female gut (1), female reproductive tract (2), female anterior body (3), male gut (4), male reproductive tract (5), male anterior body (6), and L3 (7). The life stages for N. americanus expression are adult (A) and infectious L3 (L). The life stages for S. carpocapsae expression are nonactivated infective juveniles (IJs) (NA IJ), 12-h in vitro activated IJs (VIT 12 h), 9-h in vivo activated IJs (VIV 9h), 12-h in vivo activated IJs (VIV 12h), and 15 h in vivo activated IJs (VIV 15h). The life stages for S. stercoralis expression are nonactivated IJ (NA IJ) and activated IJ (A IJ). The life stages for A. ceylanicum expression are 17 days postinfection (1), 12 days postinfection (2), 5 days postinfection (3), 24-h incubation in hookworm culture media (4), and infective L3 (5). The life stages for N. brasiliensis expression are adult (1), L4 from mouse lung (2), infectious L3 (3), and eggs (4).
Figure 5. *N. brasiliensis* produces endocannabinoids and cannabinoid-like molecules. *N. brasiliensis* parasites from life cycle stages were isolated, washed, and assessed for levels of endocannabinoids and endocannabinoid-like molecules by UPLC/MS/MS. (A) Levels of analytes at various life stages: anandamide (AEA), 2-arachidonoyl-sn-glycerol (2-AG), oleoylethanolamide (OEA), docosahexaenoylethanolamide (DHEA), and docosahexaenoylglycerol (DHAG). (B and C) Representative chromatograms of AEA (B) and 2-AG (C). (D) A 68-amino-acid (aa) region of an alignment of mouse and *N. brasiliensis* NAPE-PLD (43% shared identity). The alignment region shown is the first 68 aa in a 202-aa lactamase (B_2) domain identified in mouse NAPE-PLD. (E) Quantitative reverse transcription-PCR of *Nippostrongylus* life cycle stages for predicted *nape* and *faah*.
Supplementary Figure 1. Endocannabinoid metabolism and signaling pathways. Endocannabinoids are lipid-derived signaling molecules that belong to two lipid classes: fatty acid ethanolamides (FAEs) and monoacylglycerols (MAGs). FAEs include the endocannabinoid, anandamide (AEA), and the endocannabinoid-related molecules, oleoylethanolamide (OEA) and docosahexaenoyl ethanolamide (DHEA), as well as others. FAEs are generated by the activity of N-acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) and are degraded by fatty acid amide hydrolase (FAAH). The MAG endocannabinoid, 2-AG, on the other hand, is synthesized primarily by the activities of diacylglycerol lipase α or β (DGLα/β) and is degraded primarily by monoacylglycerol lipase (MGL) or to a lesser extent by several alpha beta hydrolase domain enzymes (ABHD). Both, AEA and 2-AG, bind and activate cannabinoid type-1 (CB₁) and type-2 receptors (CB₂), and activation of CB₁ receptor is known to generally increase feeding and is associated with decreased inflammation. OEA is known to act through peroxisome proliferator-activated receptor α (PPARα) to decrease feeding and possibly inflammation. The physiological relevance and cognate receptors of DHEA and the MAG, docosahexaenoyl glycerol (DHAG), are unclear.
Supplementary Figure 2. RNA-seq expression of *Ascaris suum*. The life stages for *A. suum* expression were not clearly identified in the RNA-seq data available in the public database and are therefore not defined in the figure.
Supplementary Figure 3. Chromatograms for endocannabinoid-like molecules in *Nb*. Representative chromatograms of (A) OEA, (B) DHEA, and (C) DHAG.
Conclusion

This body of work provides novel mass spectrometric analyses for components of the endocannabinoid (eCB) system, while describing the pitfalls of the current technique. Using the described methods, a role for eCBs signaling via peripheral cannabinoid type 1 receptor (CB₁R) in the control of hyperphagia is discussed in the context of obesity. This work was enhanced by further scrutiny of the intestinal eCB system, which revealed that corn-oil induced secretion of intestine-derived cholecystokinin is blunted by CB₁R activation; activation occurred via pharmacological agonists or enhanced eCB levels following diet induced obesity. Additionally, the role for eCB control of feeding was assessed in a separate disease model; helminth infection was used to evaluate intestinal and lung eCB levels in a model of hookworm infection. Infection revealed an enhancement of eCB levels that were met with reduced feeding, but this is suggestive of feeding independent mechanisms of eCB control of immune response. Together, this body of work provides novel insight to the varied roles that intestinal and peripheral eCBs have during obesity and hookworm infection in the modulation of feeding behaviors and immune response.
Identification of a Widespread Palmitoylethanolamide Contamination in Standard Laboratory Glassware

Current GC/MS (1) and LC/MS (2) analytical techniques for detection and subsequent quantification of eCBs and eCB-like fatty acid ethanolamines (FAEs) and monoacylglycerols (MAGs) of biological origin, including human serum (3), exist, but may sometimes lack proper controls to determine false positive results. During development of novel, sensitive quantitative LC/MS method to quantify blood lipid content, literature review revealed discrepancies in the observed eCBs and eCB-like concentrations in human blood plasma and serum (Table 1.1). The EC$_{50}$ for the eCBs N-arachidonoyl ethanolamine (AEA) and 2-arachidonoyl-sn-glycerol (2-AG) vary between assays, tissue types and source; however, reported levels (see Table 1.1) for both eCBs in plasma/serum are below the reported concentrations required for functional activation of the CB receptors [comprehensively reviewed in specific assays performed by a multiple investigators (4)]. Relative levels of the FAEs palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) have been reported on the same order of magnitude by some groups (5-9), however others have observed PEA at two-fold higher concentrations than OEA (3, 10-14). Following absolute quantitation of PEA and OEA, two separate groups reported plasma levels that were far greater than the amount required to activate their native receptor (15, 16). The homeostatic amounts of eCBs in healthy plasma/serum may reflect an equilibrium of secreted eCBs from peripheral tissues and their subsequent enzymatic breakdown in
circulation. In rodent tissues (e.g., brain, upper small intestine), PEA and OEA levels are present in the same order of magnitude (17, 18); therefore, the expectation was that levels would be similar across studies evaluating samples of human origin. In our method validation, OEA levels were in agreement with reported value. By contrast, PEA was one order of magnitude higher than expected. This finding prompted us to carefully evaluate all possible sources of contamination including solvents, reagents, and glassware used for lipid extraction and quantitative analysis.

In the present study, we identify glass Pasteur pipets (5¾”) that are used during sample preparation for mass spectrometric analysis as the source of PEA contamination. PEA was identified as the contaminant by its exact mass and liquid chromatography retention time using three similarly functioning, but different chromatographic systems. Additionally, the contaminant’s fragmentation pattern during tandem mass spectrometric detection were identical to those of standard PEA, purchased commercially. Further, we provide evidence that the polyurethane foam used by manufacturers to wrap and transport the pipets contained PEA; this suggests a source by which PEA “leaks” onto the glass pipets. Moreover, accurate exact mass measurements with ppm deviation lower than 5 unambiguously confirmed the identity of the contaminant as PEA. Quantification of pipet contamination revealed a substantial amount of PEA in each individual piece of glassware. Unfortunately, all 5¾” glass Pasteur pipets tested – purchased from various manufacturers – were contaminated (see
Figure 1. S6). PEA was undetectable in 9" pipets from only one vendor (see Figure 1.3A), allowing the use of these consumables in the overall procedure.

Lipidomics research is rapidly expanding; however, reproducible standard procedures across multiple laboratories have not been established. Therefore, discrepancies in lipidomics data are common between independent laboratories (19). These discrepancies are generally thought to be caused by variation in instrumentation, preparation procedures, and technical expertise. The present study provides results confirmed by two independent laboratories using different liquid chromatography (LC) systems and triple quadrupole (QQQ) mass spectrometers (Agilent 1200 LC system coupled to an Agilent G6410A QQQ Mass Spectrometer in the Piomelli laboratory, and Waters Acquity I Class UPLC system coupled to a Xevo TQ-S QQQ Mass Spectrometer in the DiPatrizio laboratory). Furthermore, accurate mass data were acquired on a third Shimadzu IT-TOF High Resolution Mass Spectrometer for definitive confirmation that the contaminant was indeed PEA. Putatively, various contaminations derive experimental artifacts. Lipids are often present in laboratory equipment including glassware and solvents due to being common contaminants in detergents, mineral oils, greases, and plasticizers involved in manufacturing. FAEs are not sheltered from this pitfall of lipidomics. We provide evidence that glass Pasteur pipets, commonly used in lipid extraction protocols to transfer organic solvents, may and often contain PEA as a contaminant. This contamination gives rise to artifacts in the measurement of PEA in biological samples, especially when the
procedure for sample preparation includes fractionation of the lipid extract, which concentrates the contaminant. The present study is an alert to lipidomics researchers about possible PEA analytical artifacts. Therefore, it is essential that proper controls are in place to avoid misinterpretation of false positive results; future application of these findings include proper inclusion of “blank” samples to monitor analytes of non-biological origin. Additionally, use of the analytic techniques presented here will provide for accurate and reproducible measurement of eCB and eCB-like molecules and other bioactive lipid compounds.

Future directions for mass spectrometric analysis of the endocannabinoid system will involve development of novel assays for targeted detection of the biosynthetic and degradative enzymes. Utilizing non-endogenous lipid substrates for the enzymes responsible for endocannabinoid production and degradation will allow us a glimpse into the cellular function and demand for endocannabinoids. Additionally, this work will provide a platform for drug development and identification of compounds to selectively reduce or enhance endocannabinoid levels in situ.

Peripheral Endocannabinoid Signaling Controls Hyperphagia in Western Diet-Induced Obesity

The analytical tools described above were used in several contexts, including the dysregulation of eCB lipid production that occurs during diet
induced obesity (DIO)(20). In 2013, the American Medical Association declared obesity a disease; presently it affects nearly one-third of adults in the United States and carries a significant burden while reducing life expectancy (21). A growing body of evidence suggests that overconsumption of high-energy foods – rich in fats and sugars – is a primary contributing factor of climbing obesity rates (22, 23). The eCB system has been identified as a controller of feeding behaviors, especially those related to hedonic eating via central mechanisms (24, 25). However, the role for eCBs in the control of feeding during obesity have not been delineated.

Central attenuation of eCB signaling has positive impacts on metabolism (26-28), but interventions targeting CBRs in the brain have been stopped due to deleterious side effects (29, 30). Recent evidence, however, indicates that obesity causes a dysregulation of feeding and peripheral endocannabinoid tone (31-33). Similarly, taste of dietary fats, termed oleogustus, and 24 h fast mobilized intestinal 2-AG in rats through a cholinergic mechanism of vagal efferent neurons (34-36). These observations are suggestive of a yet-unknown mechanism of peripheral endocannabinoid control of feeding.

We evaluated the impact of a western style diet (i.e. high in fat and sucrose; WD) on body weight, feeding behavior, and endocannabinoid levels – measured using ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) – in small intestinal epithelium (jejunum mucosa) and blood plasma. We observed that mice maintained on WD for 60 days
become hyperphagic due to increases in both meal size and rate of consumption. We also showed pharmacologically that feeding responses are driven – in part – by increases in small intestinal and circulating eCB tone following WD exposure. Specifically, treatment with the peripherally-restricted CB₁R neutral antagonist, AM6545, normalized hyperphagia in diet induced obese mice only. Investigations of the specific downstream pathways by which peripheral eCBs signal are essential to further our understanding of peripheral communications to the brain that control feeding; these studies may include possible neural (e.g., vagal) and endocrine (i.e., circulation) mechanisms. Additionally, various other dietary lipids (e.g., high in mono- or di-unsaturated fats versus saturated and polyunsaturated fats) and carbohydrates (e.g., fructose, sucrose) will need to be evaluated to determine their effect on eCB signaling in the gut and brain; further parsing of dietary components can give insight to their impact on hyperphagic behaviors that often lead to obesity.

Our findings suggest peripheral CB₁Rs may be an effective target of therapeutic value for attenuation of eating disorders that may or may not be associated with western diet-induced obesity. The proposed, novel approach has advantage over previously used CB₁Rs antagonists/inverse agonists (e.g., rimonabant), which may permeate the blood-brain barrier and influence central mechanisms. Rimonabant, for example, displayed positive clinical results for the treatment of obesity and metabolic syndrome. However, central mechanisms associated with rimonabant use resulted in reports of severe psychiatric side
effects, including depression and suicidal ideation (54); thereby eliminating rimonabant from clinical trials and sale in the United States and Europe. Alternatively, our work and the work of others (12, 24-34) suggests that peripheral CB₁Rs may be targeted with antagonists (e.g., AM6545) incapable of reaching the brain. These new compounds may provide effective treatment for metabolic syndrome and eating disorders, without the deleterious psychiatric side-effects that are consequent of brain-penetrant CB₁R inhibitors.

Future directions for this study include a discreet analysis of the specific dietary components (i.e. specific sugars and fatty acids) that contribute to the obese phenotype and impaired endocannabinoid levels. The role for specific dietary fatty acids in endocannabinoid production is largely unknown, so this work could provide a foundation for translational or behavioral interventions to combat obesity. Additionally, upstream mechanisms that contribute to intestinal endocannabinoid production during a fast – which include cholinergic vagal efferents – will be investigated for their potential role in obesity.

Cannabinoid CB₁ Receptors Inhibit Gut-Brain Satiation Signaling in Diet-Induced Obesity

Feeding behavior and energy balance are controlled by a dynamic crosstalk of gut-brain signaling pathways that remain poorly defined but are suggested to become dysregulated during obesity (37). Recent studies human and rodent studies suggest the endocannabinoid (eCB) system plays a key role
in regulating these processes (38-40). One of the primary mechanisms of gut-brain control of feeding is by intestinal release of gut neuropeptides from enteroendocrine cells in response to sensing dietary nutrients in the small intestinal lumen (37, 41-44). These gut-derived neuropeptides include cholecystokinin (CCK), which is released from enteroendocrine I-cells in the proximal small intestine and regulates meal size and induces satiation by activating CCK\textsubscript{A} receptors on vagal afferent fibers (45-50) and possibly the brain (51, 52). Recent evidence indicates a functional interaction between CB\textsubscript{1}Rs present on CCK-producing cells in central regions responsible for fear and learning (53, 54); other findings show that I-cells express mRNA encoding CB\textsubscript{1}R (55), although a functional link in intestinal CB\textsubscript{1}R and CCK signaling has not been identified.

Our present study suggests that eCB signaling in small-intestinal epithelium controls feeding behavior by a mechanism that involves inhibition of nutrient-induced CCK release, which subsequently increases meal size and caloric intake. We confirm mRNA transcripts encoding CB\textsubscript{1}R in isolated CCK-producing cells, sorted using enhanced green fluorescent protein (eGFP), and provide the first immunohistochemical evidence of co-localization of the receptor to CCK cells (see Figures 3.1 and 3.2). Furthermore, the anorexigenic effects of AM6545 were blocked by co-administration of the CCK\textsubscript{A} receptor antagonist, devazepide. These results suggest that when eCB activity is elevated at local CB\textsubscript{1}Rs in the upper small-intestinal epithelium in DIO – measured as described
above – increased CB₁R activation may inhibit nutrient-induced release of bioactive CCK-8 from small-intestinal enteroendocrine I-cells and lead to increased meal size and caloric intake. In further support of this hypothesis, oral gavage of corn oil – which potently stimulates bioactive CCK-8 release into circulation in lean mice that have low levels of small-intestinal eCB levels – failed to increase circulating amounts of CCK-8 in DIO mice that have elevated eCB levels in the small-intestinal epithelium. Moreover, inhibiting elevated eCB signaling at peripheral CB₁Rs with AM6545 in DIO mice – at a dose that blocked hyperphagia – restored the ability for corn oil to increase circulating levels of CCK-8.

In summary, our data provide evidence of a previously unknown mechanism of CB₁R-mediated inhibition of gut-brain satiation signaling in DIO that controls hyperphagia. Pharmacological manipulation of these pathways in the periphery may provide a therapeutic advantage for the treatment of obesity and related metabolic disorders when compared to anti-obesity drugs that interact with the brain and display psychiatric side-effects (56, 57).

Future work involves deeper investigation of the molecular mechanisms that control CCK release in response to manipulation of the eCB system. It has been well-described that CB₁Rs reduce probability of neurotransmitter release by inhibiting calcium influx or mobilization in neurons (4, 58), and nutrient-induced CCK secretion is calcium-dependent (43, 59-62). Thus, it may be likely that CB₁R
activity inhibits release of gut peptides by a mechanism that involves inhibition of calcium influx or mobilization; however, a direct test of this hypothesis remains.

Host and Helminth-Derived Endocannabinoids Are Generated During Infection with Functional Effects on Host Immunity

In addition to obesity, many other disease-states have a major impact on body weight, feeding, and metabolism. Of particular interest, intestinal hookworm infections present with a major reduction in body weight and decreased nutrient absorption (63). Helminth parasites infect approximately two billion individuals worldwide (64). Although not typically fatal, helminth infection is associated with many pathologic conditions, including malnutrition and growth retardation. Recently described mechanisms may indicate how helminths impact feeding and metabolism in their host (65). Gastrointestinal helminth infection decreases food intake (66), and has been beneficial in mice maintained on a high-fat diet, improving glucose metabolism and reducing adiposity (67, 68). Immune responses in the intestine following helminth infection caused an expansion of cholecystokinin (CCK)-positive enteroendocrine I-cells, which regulate feeding behavior (69). Despite mounting evidence for intestinal endocannabinoid control of feeding and immune responses(70), no studies reported to date have investigated the role of eCBs in intestinal helminth infection.

In the present study, we investigated components of the eCB system following infection with Nippostrongylus brasiliensis – a murine model of human
hookworm infection – as well the impact that modulating eCB signaling has on disease phenotype and inflammatory outcomes. While recognized for its critical functions in the central and enteric nervous systems, the endocannabinoid system is also affect by and can modulate inflammatory immune responses (70-72). For example, 2-AG and AEA mobilization are anti-inflammatory, which has provided the premise for the potential therapeutic value of synthetic cannabinoids, or cannabis, in autoimmune or inflammatory diseases (73). Oral AEA administration in mice promoted a tolerogenic immune response and enhanced regulatory macrophages in the intestine, which proved to be protective in a non-obese diabetic model (74). Despite playing a role in intestinal immunity, the functional significance of endocannabinoids during intestinal parasite infection has not been evaluated. Our finding that helminth infection triggers significant endocannabinoid expression that is correlated with both host health outcomes and parasite fecundity suggests that endocannabinoids may be important players in host-helminth dynamics.

Pharmacological CB₁R inhibition led to decreased IL-5 expression in the intestine and IL-4, IL-5, and IL-10 in the spleen, but no difference was observed for IFN-γ. CB₁R inhibition was associated with increased parasite egg and worm burdens, suggesting that functional CB₁R signaling is important for optimal host immune response during helminth infection. eCBs also signal through CB₂R (71); however, we observed that treatment with a CB₂R-selective antagonist did not significantly affect cytokine or helminth responses. Pharmacological CB₁R and
CB₂R inhibition occurred only during days 4 to 6 post-infection (during intestinal infection), and thus we cannot confirm complete abrogation of intestinal CB₁R or CB₂R signaling with this treatment schedule. Future studies may incorporate treatment schedules at earlier time points during infection or utilize genetic CB₁R/CB₂R-knockout mice to delineate functional differences between these signaling pathways during helminth infection. Additionally, CCK-containing cells in the proximal small intestine express mRNA encoding CB₁Rs in mice (55), and rat *N. brasiliensis* infection is associated with increased circulating CCK (66, 75). Thus, it is possible that helminth infection modulates feeding behavior by a mechanism that involves eCB-mediated changes in the production and/or secretion of gut-derived peptides that control feeding behavior, including CCK. We observed significant correlations between eCB levels, infection-induced weight loss, and parasite burdens. However, further functional studies are necessary to determine causal relationships between these multiple parameters and the contribution of infection-induced eCBs to feeding behavior.

In addition to changes in host eCB expression, we provide the first evidence that *N. brasiliensis* produces eCBs and possesses genes encoding eCB biosynthetic and degradative enzymes. One proposed strategy by which parasites evade host immunity is release of anti-inflammatory molecules that are endogenous to the host, or similar to endogenous molecules (76). For example, *A. suum* and *T. canis* possess the ability to synthesize morphine or morphine-like substrates, and morphine is a known immunomodulator (77, 78). Putatively, the
eCB system is conserved in vertebrates across multiple families, including pythons (79) and goldfish (80); however, whether the system evolved earlier and is present/functional in more primitive eukaryotic organisms is less well understood (81). We observed that *N. brasiliensis* has a gene predicted to encode *N*-acyl phosphatidyl ethanolamine-specific phospholipase D and fatty acid amide hydrolase, enzymes that catalyze FAE synthesis and breakdown. Quantitative PCR analysis confirmed *N. brasiliensis* expression of both of these predicted genes. Moreover, mass spectrometry analysis revealed that infectious L3-life cycle *N. brasiliensis* produced extremely high levels of AEA, reaching 100 to 1000 times the amounts found in mouse tissue or blood. Recently it has been observed that truffles, the fruiting body of fungi, may produce AEA as a potential attractant and feeding stimulant for animals as a method to ensure its dissemination (82). Given that AEA is anti-inflammatory, high level synthesis at the infectious stage may also function to dampen the host immune response. It is possible that in addition to eCBs, *N. brasiliensis* may produce and release other signaling molecules endogenous to the host, such as opioids, and that there is overlap in the biological effects of these molecules on host immunity or behavior. Given the difference in mass and distribution between *N. brasiliensis* and the host, the eCBs detected in the infected mice are likely host derived. However, it is possible that *N. brasiliensis* derived eCBs may functionally impact the host at the cellular level. Future studies are necessary to test these hypotheses. These findings suggest that the eCB system is a newly observed contributor to the
dynamic process of helminth infection, and eCB signaling may have a significant impact on a host’s health outcome beyond parasite clearance.

Future work involves a detailed assessment of the mechanism by which *N. brasiliensis* is creating endocannabinoids. Utilization of the current analytical tools for the assessment of endocannabinoid synthesis and degradation (i.e. functional MGL and DGL assays) will be paired with pharmacology to target candidate enzymes. Further scrutiny of the endocannabinoids being produced in helminths will help to delineate their potential role in evading or enhancing host immunity.
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