UNIVERSITY OF CALIFORNIA
RIVERSIDE

Cannabinoid CB_1 Receptor Control of Gut-Derived Hormone Secretion

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

Doctor of Philosophy

in

Biomedical Sciences

by

Pedro Antonio Perez

September 2020

Dissertation Committee:
Dr. Nicholas V. DiPatrizio, Chairperson
Dr. Christian Y. Lytle
Dr. Declan F. McCole

The Dissertation of Pedro Antonio Perez is approved:

Committee Chairperson

University of California, Riverside

Acknowledgements

"…en esta vida, hay que echarle ganas…" -famous Mexican saying

Simply stated, one must put some life into every challenge you undertake. My dissertation, the culmination of years of *ganas*, shows that great things happen to those who put in a full faith effort and passionately work towards meeting their goals. Admittedly, this journey was not easy, and I am heavily indebted to my "pueblo"- the people who have lifted me along the way spiritually, emotionally, physically, and financially. Without their unwavering support, no amount of *ganas* would have made this successful journey possible.

First and foremost, I would like to thank my loving and supportive *Familia Pérez* for their ceaseless encouragement and inspiration. A simple conversation with anyone in the family was enough to refill my drive in finishing this degree. Additionally, to my friends who may not be blood-relatives but are included within the greater *Familia Pérez*, I am sincerely grateful for your numerous texts, emails, and phone calls during emotional highs and lows.

Next, I would like to highlight my Big Sib, Dr. Stephanie King, who has served as a dependable role model and is someone that I have continually turn to for guidance at every stage of my graduate career. Thank you for not allowing me to become complacent and for pushing me to try new things. Additionally, my Lil' Sibs, Mark Wiley and Bryant Avalos, have made my time in the lab enjoyable and worthwhile. Thank you for the laughs and the inside jokes.

I acknowledge the loyal members of the DiPatrizio Laboratory, both past and present. My undergraduate mentees Jasmin Sanchez and Vishal Gupta helped with preparing samples and maintaining the laboratory, as well as by making those long days behind the bench bearable with their witty remarks. Courtney Wood is a dependable lab mate with an infectious enthusiasm who has helped with the completion of some of my later experiments. Dr. Donovan Argueta, a truly gifted and ambitious scientist, taught me perseverance by mentoring me through rough patches. Dr. Argueta further challenged me to get out of my comfort zone and pursue various opportunities to network with world leaders in my field.

To the members of the McCole laboratory, thank you for the hands-on training you gave me. Dr. Ali Shawki patiently taught me the fundamentals of cell culture techniques as well as provided an interesting perspective on career challenges. Dr. Anica Sayoc helped me with experiments and openly listened to my doubts and concerns. Together, their steady voices helped ease my worries. To both of you, a sincere thank you for being admirable colleagues and friends.

Furthermore, I am indebted to my dissertation committee, Dr. Christian Y Lytle and Dr. Declan F. McCole. These two accomplished scientists have been guiding me in my graduate career since the beginning. I appreciate their questions and interesting perspectives that led to the completion of my dissertation project.

Last, but by no means least, my mentor: Dr. Nicholas V. DiPatrizio. Humbly, I thank you for your continual encouragement, unwavering support, and tailored guidance throughout my graduate career. Thank you for patience in teaching me

the intricacies of scientific design and for your passion for science. This work was only possible due to your commitment to improve the human condition.

The text of this dissertation, in part or in full, is a reprint of the material as it appears in “Impact of maternal western diet-induced obesity on offspring mortality and peripheral endocannabinoid system in mice” PLOS ONE, 2018 (Chapter 1), “Cannabinoid CB_1 Receptors Inhibit Gut-Brain Satiation Signaling in Diet-Induced Obesity” Frontiers in Physiology, 2019 (Chapter 2) and “Cannabinoid CB_1 Receptors in the Intestinal Epithelium are Required for Acute Western-Diet Preferences in Mice” *Under Review,* 2020 (Chapter 3). The co-author Nicholas V. DiPatrizio is listed in all publication and directed and supervised the research, which forms the basis for this dissertation.

Additional co-authors include a) Donovan A Argueta, b) Alexandros Makriyannis, c) Mark B Wiley, d) Bryant Avalos, e) Courtney P. Wood

a) Assisted with design and implementation for Chapter 2 and Chapter 3
b) Provided intellectual support and materials for Chapter 2
c) Assisted with implementation for Chapter 3
d) Assisted with implementation for Chapter 3
e) Assisted with implementation for Chapter 3

Funding

The work being presented has been funded by NIH awards DA034009, DK119498, and AI135500 to Nicholas V. DiPatrizio, Supplement to DA034009 to Donovan A. Argueta, Supplement to AI135500 to Pedro A. Perez. Additional funding was provided to DAA by the University of California, Riverside; Dean's Distinguished Fellowship and Dissertation Year Fellowship, and the Ford Foundation Dissertation Fellowship. Lastly, additional funding has been generously provided to PAP by the University of California, Riverside; Jumpstart/GradEdge Summer Award, Eugene Cota Robles Award, Burden Fellowship, Graduate Research Mentorship Program, and Dissertation Year Fellowship.

Dedication

To my sister for her for committed support.

ABSTRACT OF THE DISSERTATION

Cannabinoid CB_1 Receptor Control of Gut-Derived Hormone Secretion

by

Pedro Antonio Perez

Doctor of Philosophy, Graduate Program in Biomedical Sciences
University of California, Riverside, September 2020
Dr. Nicholas V. DiPatrizio, Chairperson

The endocannabinoid (eCB) system is an important regulator of food intake and energy conservation both within the central nervous system and in key metabolically relevant peripheral organs. Recent findings have shown the peripheral eCB system to be an important contributor in regulating energy homeostasis. With the increasing obesity epidemic, we sought to determine whether this system is altered during maternal obesity which might explain some predispositions for later weight gain. Notably, these studies were met with unexpected high neonate mortality, emphasizing how detrimental obesity can be and the further need for therapeutic targets to curtail this disease. Thus, we examined the eCB system in the context of obesity. Previous evidence shows an increase in eCB signaling in the proximal small intestine during obesity. We further examined these findings and determined that these changes occur in the same region as enteroendocrine I-cells, which secrete satiation factor cholecystokinin (CCK). Indeed, I-cells were shown to express cannabinoid receptor subtype 1 (CB_1Rs). Using pharmacological agents or increased endogenous signaling (as

seen during diet-induced obesity), we showed that CB_1R signaling controls CCK secretion. This suggests that CB_1R signaling in the proximal small intestine controls satiation through a CCK dependent mechanism. Furthermore, we examined the role of CB_1Rs on glucose regulatory peptides glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide 1 (GLP1), which are produced by enteroendocrine K- and L-cells, respectively, and both express CB_1Rs. CB_1R activation inhibited incretin secretion, an effect that was restored by co-administering a peripherally-restricted CB_1R antagonist. Lastly, through the use of transgenic mouse models, we demonstrate that CB_1R on enteroendocrine K-cells control GIP release, but CB_1R on enteroendocrine L-cells do not, suggesting an alternative CB_1R dependent, intestinal independent mechanism for GLP1. This body of work begins to elucidate eCB signaling in the small intestine as regulators of enteroendocrine incretin release, which regulate both food intake (CCK) and energy homeostasis (GIP, GLP1).

Table of Contents

List of Tables

Chapter 1

Chapter 2

List of Figures

Chapter 1

Chapter 2

Introduction

Endocannabinoid System

The endocannabinoid (eCB) system is an important regulator of food intake and energy conservation (1, 2). The eCB system includes the cannabinoid receptors (CB_1R/CB_2R), their bioactive lipid-derived endogenous ligands 2-arachidonoyl-*sn*-glycerol (2AG) and N-arachidonoyl ethanolamide (anandamide; AEA), and the machinery necessary for ligand biosynthesis and degradation (3). Notably, this system is also activated by the bioactive compounds found commonly in *Cannabis Indica* and *Cannabis sativa* (4). CB_1R is a $G_{i/o}$ protein-coupled receptor found on the presynaptic terminals of glutamatergic neurons. CB_1R signaling modulates neurotransmission by reducing the probability of vesicular neurotransmitter release through cAMP-dependent inhibition of voltage-gated calcium channels (5). Centrally, CB_1R is widely expressed in regions that govern different processes ranging from food intake, fear, memory, and even locomotion (6-9). Furthermore, there is high expression of these receptors in the hypothalamus, which is heavily implicated in controlling food intake and energy homeostasis (10). Peripherally, CB_1R is also expressed in low, yet significant, levels in key metabolically relevant tissues which include the liver, endocrine pancreas, adipose tissue, and throughout the intestinal epithelium (11-16). CB_1R signaling mechanisms controlling food intake and energy conservation in the periphery are less characterized. On the other hand, CB_2R is highly expressed in

different immune cell populations and governs immune responses (17). Both receptors are activated by 2AG and AEA.

2AG is synthesized from a diacylglycerol precursor, notably 1-stearoyl-2-arachidonoyl-*sn*-glycerol, by diacylglycerol lipase alpha and beta (DAGLα/β) and degraded by its major degradation enzyme monoacylglycerol lipase (MGL) into arachidonic acid and glycerol (18, 19). 2AG can also be hydrolyzed through its minor pathway by the enzyme α/β hydrolyzing domain 6 (ABHD6) (20). On the other hand, AEA, an acylethanolamide, is synthesized by N-acylphosphatydylethanolamine phospholipase-D (NAPE-PLD) and is degraded by fatty acid amide hydrolase (FAAH) (21, 22). Notably, these two natural ligands bind CB_1Rs and CB_2Rs yet have different biosynthesis and degradation pathways. These pathways have been studied as potential therapies in modulating food intake and energy storage (23, 24).

Activation of the eCB system leads to increased energy consumption while inhibiting the system blunts food intake in mammals (25). Furthermore, CB_1R signaling is a crucial pathway for establishing food seeking behavior from the moment of birth. By inhibiting early neonatal hypothalamic CB_1R signaling, *Fride et al.* reported that neonate rodents failed to initiate initial suckling behavior and eventually fatally succumbed to starvation a few days later (26). This showed that even acute disruption of early CB_1R signaling has lasting effects in developing appropriate food seeking behaviors. The eCB system may have evolved to

increase food consumption and energy storage in line with the “thrifty theory” outlined by geneticist James V. Neel (1, 27). The thrifty theory postulates that systems that enhance energy conservation are advantageous to enhance species survival through cycles of feast and famine. Indeed, the elevated eCB signaling during fasted states, which lead to increased food seeking behaviors and energy storage processes, may have been beneficial to mammals. Recently, *DiPatrizio et al* showed that eCBs are elevated in the proximal small intestine when tasting certain fatty acids (a macronutrient that is typically scarce in nature) (25). Thus, the eCB system increase food seeking behaviors in response to fat-tasting in an effort to store the calorie-dense nutrients to later use in times of famine. While this mechanism may have been beneficial for organisms at times when high energy foods were scarce, it may be maladaptive for modern humans who have access to diets which are high in sugars and oils.

Obesity

Obesity rates have been steadily increasing in the United States for the past several decades. Over 70% of adults are overweight (Body Mass Index; BMI >25.0) and about 40% are obese (BMI >30.0) (28). In 2013, the American Medical Association officially recognized obesity as a chronic disease that requires medical attention, which increased available treatment options for obese patients (29). Importantly, obesity increases the probability of developing other serious concomitant diseases such as certain cancers, hypertension, stroke, diabetes, and heart disease. Diet is a major contributing factor in developing obesity. Adults have

steadily increased their caloric intake (specifically in sugar and fat consumption) (30, 31). The ease of access and high palatability of these high-fat and high-sugar diets, termed a Western-style diet, has further impacted this increase in caloric intake.

Obesity prevalence is also increasing among women of childbearing age. Obese women are more likely to experience gestational issues during pregnancy which include developing gestational diabetes, undergoing spontaneous abortions, and delivering infants that fail to thrive, or are small (or large) for their gestational age (see Chapter 1) (32). Notably, both small and large for gestational age infants are more likely to develop obesity and metabolic syndrome (33, 34). This multifactorial condition may be contributing to the alarmingly increasing obesity prevalence among children and adolescents; this strongly emphasizes the need to identify safe and effective therapeutic targets to increase available obesity treatment options.

Hyperphagia and metabolic abnormalities involving glucose homeostasis are associated with diet-induced obesity. Indeed, leptin and other satiety hormones become dysregulated during obesity which may contribute to the hyperphagia phenotype (reviewed in 35). In 2001, *Kunos and colleagues* showed that central hypothalamic leptin signaling directly opposes eCB levels (8). Intracranial hypothalamic infusion of leptin reduced eCBs, a phenomenon that was lost in leptin receptor insensitive high fat diet-induced obese mice and in transgenic *db/db* mice. More recently, CB_1Rs have been linked to other food intake regulatory pathways

in POMC neurons and in control of Ghrelin mTOR signaling (10, 36); additionally, CB_1R expression has been localized to cells that produce hormones governing glucose homeostasis including insulin, glucagon, somatostatin, glucose-dependent insulinotropic peptide (GIP), and glucagon-like peptide-1 (GLP1)(37, 38). During obesity, studies have reported an elevated eCB content in different tissues and in circulation suggesting increased cannabinoid receptor signaling (39, 40). Interestingly, there is also a well described FAAH polymorphism that puts individuals at higher risk to develop obesity (41, 42). On the other hand, CB_1R polymorphisms appears to be protective against metabolic syndrome, while whole body CB_1R null mice are hypophagic and diet-induced obesity resistant (43, 44). Taken together, these studies suggest that an overactive eCB system may be contributing to diet-induced obesity and, alternatively, inhibition of eCB signaling may provide a promising target to develop therapeutic interventions.

In 2006, rimonabant (Acomplia™), a global CB_1R antagonist, was introduced into the European market to treat obesity. Obese patients on rimonabant lost weight while maintaining lean body mass, indicating a decrease in total adiposity (45). Furthermore, these treated patients also saw improvements in their HDL levels, TG levels, and had a decrease in HbA1c, reflecting a tighter regulation of circulating blood glucose (46-48). However, the patients also saw an increased in insomnia, depression, and suicidal ideation (45). The adverse psychiatric side effects outweighed the metabolic benefits of rimonabant, leading its discontinuation in Europe by early 2009.

Recent second and third generation eCB system inhibitors have been focused on targeting CB_1R in the periphery. These peripherally restricted CB_1R antagonists (ie. they do not cross the blood brain barrier) have shown promise in reducing obesity related hyperphagia while reducing adiposity and restoring insulin signaling in various preclinical models at levels at or approaching the levels of rimonabant (39, 49, 50).

Gut physiology and nutrient sensing

Food travels through the alimentary canal starting from the mouth and is eventually secreted through the anus. The mouth provides major mechanical digestion and limited enzymatic digestion before food is swallowed and travels down the esophagus into the stomach. Once in the stomach, food is further digested mechanically, enzymatically, and chemically into smaller components, referred to as chyme. The stomach's pyloric sphincter opens to slowly release chyme into the lumen of the proximal small intestine where it is met by pancreatic secretions that neutralize the high acidic content and enzymes to further break down nutrients, as well as by biliary secretions to emulsify lipids contents to aid in the breakdown of fats.

As nutrients travel through the jejunum, they are sensed by different enteroendocrine cells that control food intake and energy storage. Notably, proximal intestinal enteroendocrine I-cells sense luminal sugars and free fatty acids and secrete cholecystokinin, an important satiation hormone, following a meal. These open-type enteroendocrine cells with neuropod basolateral

extensions secrete cholecystokinin and act on cholecystokinin-A receptors on afferent vagal fibers (51). Recent data have indicated that CB_1R mRNA is present in these cells, which suggests a role for eCB regulation of cholecystokinin secretion (see Chapter 2).

Other enteroendocrine cells differentially secrete glucose regulatory peptides which enhance pancreatic insulin secretion following a meal to ensure full nutrient absorption. Open-type enteroendocrine K-cells (proximal small intestine) and L-cells (distal small intestine) secrete GIP and GLP1, respectively, into circulation upon sensation of luminal sugars and free fatty acids (52). These incretins act on their respective G-protein coupled receptors within the endocrine pancreas to enhance the secretion of insulin while limiting the secretion of glucagon (53, 54). In the presence of insulin, GIP further acts on its receptor on adipocytes to promote lipogenesis (55-57). Incretins, the enzymes responsible for their degradation, and their respective receptors have been successful drug targets to treat type 2 diabetes for their natural ability to enhance endogenous insulin secretion for better glycemic control, prolonging the need for diabetic patients to resort to exogenous insulin injections (58-60). Recent data have indicated that CB_1R mRNA are present in both of these cell types, but the exact role for intestinal CB_1R and regulation of incretin secretion is poorly studied (see Chapter 3) (37).

References

1. DiPatrizio NV, Piomelli D. The thrifty lipids: Endocannabinoids and the neural control of energy conservation. Trends in Neurosciences. 2012;35(7):403-11. PubMed PMID: 1017621058.

2. Bermudez-Silva FJ, Viveros MP, McPartland JM, de Fonseca FR. The endocannabinoid system, eating behavior and energy homeostasis: The end or a new beginning? Pharmacology, Biochemistry and Behavior. 2010;95(4):375-82. PubMed PMID: 622179579.

3. Piomelli D. The molecular logic of endocannabinoid signalling. Nat Rev Neurosci. 2003;4(11):873-84. PubMed PMID: 14595399.

4. Pertwee RG. Cannabinoid pharmacology: the first 66 years. British Journal of Pharmacology. 2006;147(S1):S163-S71. doi: 10.1038/sj.bjp.0706406.

5. Zou S, Kumar U. Cannabinoid Receptors and the Endocannabinoid System: Signaling and Function in the Central Nervous System. International Journal of Molecular Sciences. 2018;19(3):833. PubMed PMID: 2108449253.

6. Jenniches I, Ternes S, Albayram O, Otte DM, Bach K, Bindila L, Michel K, Lutz B, Bilkei-Gorzo A, Zimmer A. Anxiety, stress, and fear response in mice with reduced endocannabinoid levels. Biological Psychiatry. 2016;79(10):858-68. PubMed PMID: 1799002406.

7. Morena M, Aukema RJ, Leitl KD, Rashid AJ, Vecchiarelli HA, Josselyn SA, Hill MN. Upregulation of anandamide hydrolysis in the basolateral complex of amygdala reduces fear memory expression and indices of stress and anxiety. The Journal of Neuroscience. 2019;39(7):1275-92. PubMed PMID: 2211177922.

8. Di Marzo V, Goparaju SK, Wang L, Liu J, Batkai S, Jarai Z, Fezza F, Miura GI, Palmiter RD, Sugiura T, Kunos G. Leptin-regulated endocannabinoids are involved in maintaining food intake. Nature. 2001;410(6830):822-5. PubMed PMID: 11298451.

9. Cosenza M, Gifford AN, Gatley SJ, Pyatt B, Liu Q, Makriyannis A, Volkow ND. Locomotor activity and occupancy of brain cannabinoid CB1 receptors by the antagonist/inverse agonist AM281. Synapse. 2000;38(4):477-82. doi: 10.1002/1098-2396(20001215)38:4<477::aid-syn13>3.0.co;2-y.

10. Koch M, Varela L, Kim JG, Kim JD, Hernandez-Nuno F, Simonds SE, Castorena CM, Vianna CR, Elmquist JK, Morozov YM, Rakic P, Bechmann I, Cowley MA, Szigeti-Buck K, Dietrich MO, Gao XB, Diano S, Horvath TL. Hypothalamic POMC neurons promote cannabinoid-induced feeding. Nature. 2015;519(7541):45-50. doi: 10.1038/nature14260. PubMed PMID: 25707796; PMCID: PMC4496586.

11. Matias I, Belluomo I, Cota D. The Fat Side of the Endocannabinoid System: Role of Endocannabinoids in the Adipocyte. Cannabis and Cannabinoid Research. 2016;1(1):176-85. doi: 10.1089/can.2016.0014.

12. DiPatrizio NV, Igarashi M, Narayanaswami V, Murray C, Gancayco J, Russell A, Jung KM, Piomelli D. Fasting stimulates 2-AG biosynthesis in the small intestine: role of cholinergic pathways. Am J Physiol Regul Integr Comp Physiol. 2015;309(8):R805-13. Epub 2015/08/21. doi: 10.1152/ajpregu.00239.2015. PubMed PMID: 26290104; PMCID: PMC4666947.

13. Gonzalez-Mariscal I, Montoro RA, Doyle ME, Liu QR, Rouse M, O'Connell JF, Santa-Cruz Calvo S, Krzysik-Walker SM, Ghosh S, Carlson OD, Lehrmann E, Zhang Y, Becker KG, Chia CW, Ghosh P, Egan JM. Absence of cannabinoid 1 receptor in beta cells protects against high-fat/high-sugar diet-induced beta cell dysfunction and inflammation in murine islets. Diabetologia. 2018;61(6):1470-83. Epub 2018/03/03. doi: 10.1007/s00125-018-4576-4. PubMed PMID: 29497784; PMCID: PMC6201315.

14. Shin H, Han JH, Yoon J, Sim HJ, Park TJ, Yang S, Lee EK, Kulkarni RN, Egan JM, Kim W. Blockade of cannabinoid 1 receptor improves glucose responsiveness in pancreatic beta cells. J Cell Mol Med. 2018;22(4):2337-45. doi: 10.1111/jcmm.13523. PubMed PMID: 29431265; PMCID: PMC5867156.

15. Bermudez-Silva FJ, Baixeras E, Cobo N, Bautista D, Cuesta-Munoz AL, Fuentes E, Juan-Pico P, Castro MJ, Milman G, Mechoulam R, Nadal A, Rodriguez de Fonseca F. Presence of functional cannabinoid receptors in human endocrine pancreas. Diabetologia. 2008;51(3):476–87. Epub December 19, 2007.

16. Jourdan T, Nicoloro SM, Zhou Z, Shen Y, Liu J, Coffey NJ, Cinar R, Godlewski G, Gao B, Aouadi M, Czech MP, Kunos G. Decreasing CB1 receptor signaling in Kupffer cells improves insulin sensitivity in obese mice. Molecular Metabolism. 2017;6(11):1517-28. doi: https://doi.org/10.1016/j.molmet.2017.08.011.

17. Dotsey E, Ushach I, Pone E, Nakajima R, Jasinskas A, Argueta DA, Dillon A, DiPatrizio N, Davies H, Zlotnik A, Crompton PD, Felgner PL. Transient Cannabinoid Receptor 2 Blockade during Immunization Heightens Intensity and Breadth of Antigen-specific Antibody Responses in Young and Aged mice. Scientific Reports. 2017;7(1):42584. doi: 10.1038/srep42584.

18. Blankman JL, Simon GM, Cravatt BF. A comprehensive profile of brain enzymes that hydrolyze the endocannabinoid 2-arachidonoylglycerol. Chem Biol. 2007;14(12):1347-56. PubMed PMID: 18096503.

19. Gao Y, Vasilyev DV, Goncalves MB, Howell FV, Hobbs C, Reisenberg M, Shen R, Zhang M-Y, Strassle BW, Lu P, Mark L, Piesla MJ, Deng K, Kouranova EV, Ring RH, Whiteside GT, Bates B, Walsh FS, Williams G, Pangalos MN, Samad TA, Doherty P. Loss of retrograde endocannabinoid signaling and reduced adult neurogenesis in diacylglycerol lipase knock-out mice. The Journal of Neuroscience. 2010;30(6):2017-24. PubMed PMID: 622139936.

20. Thomas G, Betters JL, Lord CC, Brown AL, Marshall S, Ferguson D, Sawyer J, Davis MA, Melchior JT, Blume LC, Howlett AC, Ivanova PT, Milne SB, Myers DS, Mrak I, Leber V, Heier C, Taschler U, Blankman JL, Cravatt BF, Lee RG, Crooke RM, Graham MJ, Zimmermann R, Brown HA, Brown JM. The serine hydrolase ABHD6 Is a critical regulator of the metabolic syndrome. Cell Rep. 2013;5(2):508-20. Epub 2013/10/08. doi: 10.1016/j.celrep.2013.08.047. PubMed PMID: 24095738; PMCID: 3833083.

21. McKinney MK, Cravatt BF. Structure and function of fatty acid amide hydrolase. Annu Rev Biochem. 2005;74:411-32. PubMed PMID: 15952893.

22. Ahn K, McKinney MK, Cravatt BF. Enzymatic Pathways That Regulate Endocannabinoid Signaling in the Nervous System. Chemical Reviews. 2008;108(5):1687-707. doi: 10.1021/cr0782067.

23. Everard A, Plovier H, Rastelli M, Van Hul M, de Wouters d'Oplinter A, Geurts L, Druart C, Robine S, Delzenne NM, Muccioli GG, de Vos WM, Luquet S, Flamand N, Di Marzo V, Cani PD. Intestinal epithelial N-acylphosphatidylethanolamine phospholipase D links dietary fat to metabolic adaptations in obesity and steatosis. Nature Communications. 2019;10(1):457. doi: 10.1038/s41467-018-08051-7.

24. Vozella V, Ahmed F, Choobchian P, Merrill CB, Zibardi C, Tarzia G, Mor M, Duranti A, Tontini A, Rivara S, Piomelli D. Pharmacokinetics, pharmacodynamics and safety studies on URB937, a peripherally restricted fatty acid amide hydrolase inhibitor, in rats. Journal of Pharmacy and Pharmacology. 2019;71(12):1762-73. doi: 10.1111/jphp.13166.

25. DiPatrizio NV, Piomelli D. Intestinal lipid-derived signals that sense dietary fat. J Clin Invest. 2015;125(3):891-8. doi: 10.1172/JCI76302. PubMed PMID: 25642767; PMCID: PMC4362267.

26. Fride E, Ginzburg Y, Breuer A, Bisogno T, Di Marzo V, Mechoulam R. Critical role of the endogenous cannabinoid system in mouse pup suckling and growth. European Journal of Pharmacology. 2001;419(2–3):207-14. doi: http://dx.doi.org/10.1016/S0014-2999(01)00953-0.

27. Neel JV. Diabetes mellitus: a "thrifty" genotype rendered detrimental by "progress"? Am J Hum Genet. 1962;14:353-62. Epub 1962/12/01. PubMed PMID: 13937884; PMCID: 1932342.

28. Ogden CL, Carroll MD, Lawman HG, Fryar CD, Kruszon-Moran D, Kit BK, Flegal KM. Trends in Obesity Prevalence Among Children and Adolescents in the United States, 1988-1994 Through 2013-2014. JAMA. 2016;315(21):2292-9. doi: 10.1001/jama.2016.6361. PubMed PMID: 27272581.

29. Kyle TK, Dhurandhar EJ, Allison DB. Regarding Obesity as a Disease: Evolving Policies and Their Implications. Endocrinology and Metabolism Clinics of North America. 2016;45(3):511-20. doi: https://doi.org/10.1016/j.ecl.2016.04.004.

30. Medina-RemOn A, Kirwan R, Lamuela-Raventos RM, Estruch R. Dietary Patterns and the Risk of Obesity, Type 2 Diabetes Mellitus, Cardiovascular Diseases, Asthma, and Mental Health Problems. Crit Rev Food Sci Nutr. 2016:0. doi: 10.1080/10408398.2016.1158690. PubMed PMID: 27127938.

31. Poti JM, Duffey KJ, Popkin BM. The association of fast food consumption with poor dietary outcomes and obesity among children: is it the fast food or the remainder of the diet? Am J Clin Nutr. 2014;99(1):162-71. doi: 10.3945/ajcn.113.071928. PubMed PMID: 24153348; PMCID: PMC3862453.

32. Baugh N, Harris DE, AbouEl-Makarim A, Sarton C, Lichter E. The Impact of Maternal Obesity and Excessive Gestational Weight Gain on Maternal and Infant Outcomes in Maine: Analysis of Pregnancy Risk Assessment Monitoring System Results from 2000 to 2010. Journal of Pregnancy. 2016;2016. PubMed PMID: 1825204955.

33. WILLIAMS CB, MACKENZIE KC, GAHAGAN S. The Effect of Maternal Obesity on the Offspring. Clinical Obstetrics and Gynecology. 2014;57(3):508-15. doi: 10.1097/grf.0000000000000043. PubMed PMID: 00003081-201409000-00009.

34. Oken E, Gillman MW. Fetal Origins of Obesity. Obesity Research. 2003;11(4):496-506. doi: 10.1038/oby.2003.69.

35. Lean MEJ, Malkova D. Altered gut and adipose tissue hormones in overweight and obese individuals: cause or consequence? International Journal of Obesity. 2016;40(4):622-32. doi: 10.1038/ijo.2015.220.

36. Senin LL, Al-Massadi O, Folguiera C, Pardo M, Barja-Fernandez S, Roca-Rivada A, Amil M, Criujeiras AB, Garcia-Caballero T, Gabellieri E, Leis R, Dieguez C, Pagotto U, Casanueva FF, Seoane LM. The gasric CB1 receptor modulates ghrelin production through the mTOR pathway to regulate food intake. PLoS ONE. 2013;8(11):e80339.

37. Moss CE, Marsh WJ, Parker HE, Ogunnowo-Bada E, Riches CH, Habib AM, Evans ML, Gribble FM, Reimann F. Somatostatin receptor 5 and cannabinoid receptor 1 activation inhibit secretion of glucose-dependent insulinotropic polypeptide from intestinal K cells in rodents. Diabetologia. 2012;55(11):3094-103. Epub 2012/08/09. doi: 10.1007/s00125-012-2663-5. PubMed PMID: 22872212; PMCID: PMC3464380.

38. Matias I, Gonthier MP, Orlando P, Martiadis V, De Petrocellis L, Cervino C, Petrosino S, Hoareau L, Festy F, Pasquali R, Roche R, Maj M, Pagotto U, Monteleone P, Di Marzo V. Regulation, function, and dysregulation of endocannabinoids in models of adipose and beta-pancreatic cells and in obesity and hyperglycemia. J Clin Endocrinol Metab. 2006;91(8):3171-80. Epub 2006/05/11. doi: 10.1210/jc.2005-2679. PubMed PMID: 16684820.

39. Argueta DA, DiPatrizio NV. Peripheral endocannabinoid signaling controls hyperphagia in western diet-induced obesity. Physiol Behav. 2017;171:32-9. doi: 10.1016/j.physbeh.2016.12.044. PubMed PMID: 28065722.

40. Kuipers EN, Kantae V, Maarse BCE, van den Berg SM, van Eenige R, Nahon KJ, Reifel-Miller A, Coskun T, de Winther MPJ, Lutgens E, Kooijman S, Harms AC, Hankemeier T, van der Stelt M, Rensen PCN, Boon MR. High Fat Diet Increases Circulating Endocannabinoids Accompanied by Increased Synthesis Enzymes in Adipose Tissue. Frontiers in Physiology. 2019;9(1913). doi: 10.3389/fphys.2018.01913.

41. Sipe JC, Scott TM, Murray S, Harismendy O, Simon GM, Cravatt BF, Waalen J. Biomarkers of endocannabinoid system activation in severe obesity. PLoS ONE. 2010;5(1):e8792. Epub 2010/01/26. doi: 10.1371/journal.pone.0008792. PubMed PMID: 20098695; PMCID: 2808340.

42. Sipe JC, Waalen J, Gerber A, Beutler E. Overweight and obesity associated with a missense polymorphism in fatty acid amide hydrolase (FAAH). Int J Obes (Lond). 2005;29(7):755-9. PubMed PMID: 15809662.

43. Bordicchia M, Battistoni I, Mancinelli L, Giannini E, Refi G, Minardi D, Muzzonigro G, Mazzucchelli R, Montironi R, Piscitelli F, Petrosino S, Dessì-Fulgheri P, Rappelli A, Di Marzo V, Sarzani R. Cannabinoid CB1 receptor expression in relation to visceral adipose depots, endocannabinoid levels, microvascular damage, and the presence of the Cnr1 A3813G variant in humans. Metabolism. 2010;59(5):734-41. doi: https://doi.org/10.1016/j.metabol.2009.09.018.

44. Ravinet Trillou C, Delgorge C, Menet C, Arnone M, Soubrie P. CB1 cannabinoid receptor knockout in mice leads to leanness, resistance to diet-induced obesity and enhanced leptin sensitivity. Int J Obes Relat Metab Disord. 2004;28(4):640-8. PubMed PMID: 14770190.

45. Sam AH, Salem V, Ghatei MA. Rimonabant: From RIO to Ban. J Obes. 2011;2011:432607. doi: 10.1155/2011/432607. PubMed PMID: 21773005; PMCID: PMC3136184.

46. Sam AH, Troke RC, Tan TM, Bewick GA. The role of the gut/brain axis in modulating food intake. Neuropharmacology. 2012;63(1):46-56. doi: 10.1016/j.neuropharm.2011.10.008. PubMed PMID: 22037149.

47. Van Gaal LF, Rissanen AM, Scheen AJ, Ziegler O, Rossner S. Effects of the cannabinoid-1 receptor blocker rimonabant on weight reduction and cardiovascular risk factors in overweight patients: 1-year experience from the RIO-Europe study. Lancet. 2005;365(9468):1389-97. Epub 2005/04/20. doi: S0140-6736(05)66374-X [pii]10.1016/S0140-6736(05)66374-X. PubMed PMID: 15836887.

48. Scheen AJ, Finer N, Hollander P, Jensen MD, Van Gaal LF. Efficacy and tolerability of rimonabant in overweight or obese patients with type 2 diabetes: a randomised controlled study. The Lancet. 2006;368(9548):1660-72. doi: https://doi.org/10.1016/S0140-6736(06)69571-8.

49. Han JH, Shin H, Park J-Y, Rho JG, Son DH, Kim KW, Seong JK, Yoon S-H, Kim W. A novel peripheral cannabinoid 1 receptor antagonist, AJ5012, improves metabolic outcomes and suppresses adipose tissue inflammation in obese mice. The FASEB Journal. 2019;33(3):4314-26. doi: 10.1096/fj.201801152RR.

50. Tam J, Vemuri VK, Liu J, Batkai S, Mukhopadhyay B, Godlewski G, Osei-Hyiaman D, Ohnuma S, Ambudkar SV, Pickel J, Makriyannis A, Kunos G. Peripheral CB1 cannabinoid receptor blockade improves cardiometabolic risk in mouse models of obesity. J Clin Invest. 2010;120(8):2953-66. Epub 2010/07/29. doi: 42551 [pii]10.1172/JCI42551. PubMed PMID: 20664173; PMCID: 2912197.

51. Kaelberer MM, Buchanan KL, Klein ME, Barth BB, Montoya MM, Shen X, Bohórquez DV. A gut-brain neural circuit for nutrient sensory transduction. Science. 2018;361(6408):eaat5236. doi: 10.1126/science.aat5236.

52. Gribble FM, Reimann F. Enteroendocrine Cells: Chemosensors in the Intestinal Epithelium. Annual Review of Physiology. 2016;78(1):277-99. doi: 10.1146/annurev-physiol-021115-105439. PubMed PMID: 26442437.

53. Seino Y, Fukushima M, Yabe D. GIP and GLP-1, the two incretin hormones: Similarities and differences. J Diabetes Investig. 2010;1(1-2):8-23. doi: 10.1111/j.2040-1124.2010.00022.x. PubMed PMID: 24843404; PMCID: PMC4020673.

54. Holst JJ, Gromada J. Role of incretin hormones in the regulation of insulin secretion in diabetic and nondiabetic humans. Am J Physiol Endocrinol Metab. 2004;287(0193-1849 (Print)). doi: 10.1152/ajpendo.00545.2003; PMCID: 15271645.

55. Miyawaki K, Yamada Y, Ban N, Ihara Y, Tsukiyama K, Zhou H, Fujimoto S, Oku A, Tsuda K, Toyokuni S, Hiai H, Mizunoya W, Fushiki T, Holst JJ, Makino M, Tashita A, Kobara Y, Tsubamoto Y, Jinnouchi T, Jomori T, Seino Y. Inhibition of gastric inhibitory polypeptide signaling prevents obesity. Nat Med. 2002;8(7):738-42. Epub 2002/06/18. doi: 10.1038/nm727. PubMed PMID: 12068290.

56. Yip RG, Boylan MO, Kieffer TJ, Wolfe MM. Functional GIP receptors are present on adipocytes. Endocrinology. 1998;139(9):4004-7. Epub 1998/09/02. doi: 10.1210/endo.139.9.6288. PubMed PMID: 9724057.

57. Beck B, Max J-P. Gastric inhibitory polypeptide enhancement of the insulin effect on fatty acid incorporation into adipose tissue in the rat. Regulatory Peptides. 1983;7(1):3-8. doi: https://doi.org/10.1016/0167-0115(83)90276-8.

58. Decara J, Arrabal S, Beiroa D, Rivera P, Vargas A, Serrano A, Pavón FJ, Ballesteros J, Dieguez C, Nogueiras R, Rodríguez de Fonseca F, Suárez J. Antiobesity efficacy of GLP-1 receptor agonist liraglutide is associated with peripheral tissue-specific modulation of lipid metabolic regulators. BioFactors. 2016;42(6):600-11. doi: 10.1002/biof.1295.

59. Pathak R, Bridgeman MB. Dipeptidyl Peptidase-4 (DPP-4) Inhibitors In the Management of Diabetes. P & T : a peer-reviewed journal for formulary management. 2010;35(9):509-13. PubMed PMID: 20975810.

60. Marso SP, Daniels GH, Brown-Frandsen K, Kristensen P, Mann JFE, Nauck MA, Nissen SE, Pocock S, Poulter NR, Ravn LS, Steinberg WM, Stockner M, Zinman B, Bergenstal RM, Buse JB. Liraglutide and Cardiovascular Outcomes in Type 2 Diabetes. New England Journal of Medicine. 2016;375(4):311-22. doi: 10.1056/NEJMoa1603827. PubMed PMID: 27295427.

Chapter 1: Impact of Maternal Western Diet-induced Obesity on Offspring Mortality and Peripheral Endocannabinoid System in Mice

Authors: *Perez PA*[1] and DiPatrizio NV[1]

[1]University of California Riverside, School of Medicine, Division of Biomedical Sciences, Riverside, CA, USA

Abbreviations: 2-AG, 2-arachidonoylgylcerol; AEA, arachidonoylethanolamide; DIO, diet-induced obesity; CB_1R, cannabinoid receptor subtype 1; CB_2R, cannabinoid receptor subtype 2; DAGLα, diacylglycerol lipase α isoform; DAGLβ, diacylglycerol lipase β isoform; DHEA, docosahexaenoylethanolamide; DHG, docosahexaenoylglycerol; eCB, endocannabinoid; ESI, electrospray ionization; FAAH, fatty acid amide hydrolase; LC/MS, liquid chromatography mass spectrometry; MRM, multiple reaction monitoring; MGLL, monoacylglycerol lipase; NAPE-PLD, n-acyl phosphatidylethanolamine-specific phospholipase D; OEA, oleoylethanolamide; SD, Soy-free Standard rodent chow; WD, Western diet; UPLC, ultra-performance liquid chromatography.

Abstract

Over two-thirds of adults in the United States are obese or overweight, which is largely due to chronic overconsumption of diets high in fats and sugars (i.e., Western diet). Recent studies reveal that maternal obesity may predispose offspring to development of obesity and other metabolic diseases; however, the molecular underpinnings of these outcomes are largely unknown. The endocannabinoid system is an important signaling pathway that controls feeding behavior and energy homeostasis, and its activity becomes upregulated in the upper small intestinal epithelium of Western diet-induced obese mice, which drives overeating. In the current investigation, we examined the impact of chronic maternal consumption of Western diet on the expression and function of the endocannabinoid system in several peripheral organs important for food intake and energy homeostasis in offspring. Female C57BL/6Tac mice were fed a Western diet or low-fat/no-sucrose control chow for 10 weeks, then males were introduced for mating. Dams were maintained on their respective diets through weaning of pups, at which time pups were maintained on low-fat/no-sucrose chow for 10 weeks. Neonates born from dams fed Western diet, when compared to those born from mice fed control chow, unexpectedly displayed increases in mortality that occurred exclusively within six days following birth (greater than 50% mortality). Males comprised a larger fraction of surviving offspring from obese dams. Furthermore, surviving off- spring displayed transient increases in body mass for first two days post weaning, and no marked changes in feeding patterns and

endocannabinoid levels in upper small intestinal epithelium, pancreas, and plasma, or in expression of key endocannabinoid system genes in the upper small intestinal epithelium and pancreas at 10 weeks post-weaning. Collectively, these results suggest that maternal diet composition greatly influences survival of neonate C57BL/6Tac mice, and that surviving offspring from dams chronically fed a Western diet do not display marked changes in body mass, eating patterns, or expression and function of the endocannabinoid system in several peripheral organs important for feeding behavior and energy homeostasis.

Introduction

Over 70% of adults in the United States are overweight or obese, and childhood and adolescent obesity rates have more than tripled from the 1970s (1-4). Diet-induced obesity (DIO) is preventable and associated with overconsumption of foods high in fats and sugars [i.e. a Western diet (WD)], which greatly increases risk of developing type-2 diabetes and other metabolic diseases (5, 6). Furthermore, there is increasing incidence of obesity in women during childbearing age and increases in gestational diabetes during pregnancy (7, 8). Evidence also links gestational diabetes or obesity to increased risk of developing type-2 diabetes in, both, mothers and their offspring (9, 10). Studies in rodent models of maternal DIO reveal changes in offspring taste preference, stress responses, adiposity, and weight gain; however, the molecular mechanisms underlying these behavioral and metabolic outcomes are poorly understood but

may include dysregulation of the endocannabinoid (eCB) system (11-18). Indeed, substantial evidence suggests that the eCB system serves critical roles in food intake and energy balance (19-22).

The eCB system is comprised of the lipid-derived signaling molecules, the eCBs, which include the well-characterized 2-arachidonoyl-*sn*-glycerol (2-AG) and anandamide (AEA), and their biosynthetic and degradative enzymes, and receptors [i.e., cannabinoid receptor sub- type-1 and subtype-2 (CB_1R and CB_2R)] (23, 24). The eCB system plays important roles in nearly all physiological functions associated with energy balance, including pancreatic endocrine function (21). CB_1R activity on β-cells in the pancreas impact their function by directly inhibiting insulin receptor signaling, as well as in utero pancreatic endocrine islet development (25-27). Increasing evidence also suggests that CB_1R activation within the islets of Langerhans stimulates insulin secretion through a cAMP- and calcium-dependent mechanism (28-31); however, other groups report inhibitory actions on insulin secretion (32-34). Nonetheless, changes in pancreatic eCB signaling during development may predispose offspring to perturbations in glucose homeostasis.

The eCB system in, both, the brain and periphery plays an important role in controlling feeding behaviors (19-21, 35-38). CB_1R antagonists are widely reported to inhibit palatable food intake in lean and DIO rodents, and improve a multitude of metabolic parameters (39-43), which highlights the therapeutic potential for cannabinoid receptor inhibitors to combat the growing obesity epidemic. In

neonate mice, systemic administration of the CB_1R antagonist/inverse agonist, SR141716, led to the failure of suckling and ultimately death, which underscores the importance of the eCB system in developing early feeding signals (44). Furthermore, recent studies suggest a role for the eCB system in peripheral tissues–specifically the proximal small intestine–in the control of food intake (45, 46). Indeed, 2-AG levels in the proximal small intestinal epithelium were elevated in, both, fasted lean male mice and non- fasted male mice maintained on WD for 60 days (40, 47, 48). When compared to lean mice maintained on a low-fat/sugar control diet, mice fed WD rapidly gained body weight and were hyperphagic with increased daily caloric consumption and meal size (40). Importantly, inhibiting peripheral CB_1Rs with the peripherally restricted neutral CB_1R antagonist, AM6545, fully normalized eating patterns in mice fed WD for 60 days to those found in lean controls. These studies suggest that overeating associated with a WD is controlled by overactive eCB signaling at cannabinoid CB_1Rs in the upper small intestinal epithelium. Thus, it is plausible that maternal DIO may impact eCB signaling in the proximal small intestine in offspring, with functional outcomes that may include dysregulation of food intake and energy balance.

This study aimed to identify the impact of a maternal WD on offspring body weight gain, feeding patterns, and expression and function of the eCB system in select peripheral organs important for food intake and energy balance, which include pancreas, small-intestinal epithelium, and plasma in C57BL/6Tac mice. Neonate mortality was also evaluated.

Materials and Methods

Animals

Six-week old male and female C57BL/6Tac (Taconic, Oxnard, CA, USA) were grouped housed according to sex with free access to water and food and maintained on a 12-hour light/dark cycle (lights off at 1800 hours). Test diets composed of low-fat/no-sucrose standard lab rodent chow [(SD) Teklad 2020x Global Soy Protein-Free Extruded Rodent Diet; 16% kcal from fat, 60% kcals from carbohydrate, no sucrose], or Western diet [(WD) Research Diets D127098, New Brunswick, NJ, USA; 40% kcal from fat, 43% carbohydrates, mostly sucrose) (Table 1.1). All procedures met the U.S. National Institute of Health guidelines for care and use of laboratory animals, and were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, Riverside.

Breeding parameters

Female mice were fed either SD or WD during pre-gestation for 10 weeks, gestation, and lactation. Male mice were only given access to SD. Following 10 weeks, male mice were harem bred with female mice for mating at 1000 hr and separated at 1600 hr (see Figure 1.1 for experimental design). Food was removed during mating times and returned immediately after to avoid male consumption of WD diet. When a vaginal plug was observed during mating, females were single housed with their respective diets to give birth. Birth dates, litter sizes, neonate survival, and overall health of all animals were monitored daily. Body weights of offspring were recorded twice weekly. At postnatal day 21, pups were weaned, ear

tagged, grouped housed with mice of same condition (i.e., those born from the same maternal test diet), and given access to only SD for the duration of the 10-week study. Pregnancies all occurred approximately at the same date. Thus, the outcome from all litters occurred at approximately the same date.

Notably, increased mortality of neonates was not an expected outcome or anticipated endpoint of our experimental design, which was aimed at evaluating feeding patterns and endocannabinoid system expression and function in peripheral organs of offspring born from dams maintained on WD or SD. Thus, despite daily monitoring of health and adherence to well-defined IACUC-approved humane endpoints for determining when animals will be removed from studies, treated, or euthanized, their implementation was not possible for mice in this study given that no signs of suffering or distress were observed for non-surviving neonates and that mortality of neonates occurred unexpectedly and exclusively within the first six days following birth. The cause of increased mortality is unknown but is associated with chronic maternal consumption of WD. Out of a total of 70 pups, 44 failed to survive by six days post-birth, at which time the remaining pups survived throughout the entirety of the study. All dams born from WD fed mothers suffered from at least one dead pup, with several damns suffering from complete mortality. Thus, litter sizes could not be adjusted to the same number of pups in the groups due to the variability of litter sizes and concomitant survival rates in litters born from WD fed mothers.

Feeding behavior

Age-matched females (to those used in maternal studies) and surviving offspring were separated into single house feeding chambers (TSE Systems) and acclimated for 5 days prior to feeding behavior testing. Food and water intakes were obtained every 60 seconds. Baseline feeding behavior was monitored for 24 hours to assess daily intake patterns. In addition, a preference test between SD and WD was conducted after baseline reading. Animals had free access to either diet for 24 hours and their feeding behaviors were recorded. Feeding parameters included total caloric intake, average meal size, average rate of intake, average number of meals, average meal duration, average post-meal interval, and percentage of total meals between SD and WD (preference test).

Tissue processing

Tissue collection

Isoflurane was used to anesthetize animals at time of tissue harvest (0900 to 1100 hours). Blood was collected by cardiac puncture and stored in EDTA-lined tubes on ice. Tubes were centrifuged (1500g for 10 minutes at 4℃) to obtain plasma. Pancreas was rapidly collected, washed with ice-chilled phosphate-buffer solution (PBS), and flash frozen in liquid nitrogen. Jejunum was rapidly collected, washed with ice-chilled PBS, sliced open longitudinally on a stainless-steel plate kept on ice, scraped with a glass slide to separate mucosal layer, then mucosa was flash frozen in liquid nitrogen. All samples were stored at -80℃ until time of processing.

Lipid extraction

Tissues were weighed and homogenized in 1.0 mL of methanol solution containing internal standards: [$^{2}H_5$]-2-arachidonoyl-*sn*-glycerol (2-AG), [$^{2}H_4$]-arachidonoylethanolamide (AEA), and [$^{2}H_4$]-oleoylethanolamide (OEA) (Cayman Chemical, Ann Arbor, MI, USA). Lipids were extracted using chloroform (2.0 mL) and washed with ultra-pure (0.2 µm filtered) water. Lipids were extracted from plasma using sterile 0.9% saline solution in lieu of water (0.1 mL plasma at expense of saline). Organic phases were collected and separated using silica gel column chromatography as previously described (40). Eluate was gently dried under N_2 stream (99.998% pure) and resuspended in LCMS grade methanol:chloroform (9:1) solution [100 µL for plasma, 200 µL for tissue]. 1 µL was injected for analysis by ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) as described (40, 49).

Lipid analysis

Measurements of eCBs (2-AG and AEA) and related molecules [docosahexaenoylethanolamide (DHEA), docosahexaenoylglycerol (DHG)] were performed using methods previously described by our group (40, 49, 50). 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 C18 column (2.1 × 50 mm i.d., 1.7 µm, Waters) with inline Acquity guard column (UPLC BEH C18 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℃, and samples were maintained in the sample manager at 10℃. 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; AEA = 30v, 14v; OEA = 28v, 16v; DHEA = 30v, 16v; DHG = 34v, 14v; [$^2H_5$]-2-AG = 25v, 44v; [$^2H_4$]-AEA = 26v, 16v; [$^2H_4$]- OEA = 48v. 14v. Lipids were quantified using a stable isotope dilution method detecting protonated adducts of the molecular ions [M + H]+ in the multiple reaction monitoring (MRM) mode. Acyl migration from 2-AG to 1-AG is known to occur (51), 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* = 379.2>287.26), AEA (*m/z* = 348.3>62.04), OEA (*m/z* = 326.3 >62.08), DHEA (*m/z* = 372.3>91.02), DHG (*m/z* = 403.3>311.19), and [$^2H_5$] 2-AG (*m/z* = 384.2 >93.4), [$^2H_4$] AEA (*m/z* = 352.3>66.11) and [$^2H_4$] OEA (*m/z* = 330.3>66.05), which were used as internal standards. One “blank” sample was processed as a control and analyzed in the same manner as all samples, except no tissue or plasma were included. This control revealed no detectable endocannabinoids and related molecules included in our analysis [see (49) for description of contaminants in standard glassware].

Gene expression

Tissues were chosen at random to reflect a sample from each distinct litter in each of the two groups (SD vs. WD). Total RNA was extracted from jejunum mucosa and pancreas using QIAzol (Qiagen, Maryland, USA) and RNeasy (Qiagen, Germany) combined method, and generated first strand complementary DNA using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA). Surfaces for tissue collection and processing were sanitized using 70% EtOH solution followed by RNAse inhibitor (RNAse out, G-Biosciences, St. Louis, MO, USA) to maintain integrity of isolated RNA. Reverse transcription of RNA was performed with random hexamers (Invitrogen, Carlsbad, CA, USA) for 50 minutes at 37℃, RT-PCR was carried out using PrimePCR assays (BioRad, Irvine, CA, USA) with primers for cannabinoid receptor 1 and 2 (Cnr1, Cnr2), diacylglycerol α and β (DAGLα, DAGLβ), monoacylglycerol lipase (MGLL), N-acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD), and fatty acid amide hydrolase (FAAH) using preconfigured SYBR green assay (BioRad, Irvine, CA, USA). Reactions were run in triplicate. Hprt was selected as a housekeeping gene for jejunum mucosa studies; β-actin was chosen as a housekeeping gene for pancreas studies. No changes in expression were found in conditions tested between respective housekeeping genes [Cq values for conditions, n = 5; hprt: SD born offspring, 24.51±0.84, WD born offspring, 24.47±0.77, not significant; β-actin: SD born offspring, 25.59±0.56, WD born offspring, 25.12±0.32, not significant].

Statistical analyses

Data was analyzed using Graphpad Prism 7.0 software. Results are expressed as the mean ±S.E.M. Significant differences between groups were assessed using Student's two-tailed t-test or two-way analysis of variance (ANOVA) with repeated or non-repeated measures with Sidak or Newman-Keuls post hoc analysis, respectively. Survival curves were analyzed using Mentel- Cox and Gehan-Breslow-Wilcoxon tests. Differences were considered significant if $p<0.05$. Grubbs' tests for statistical outliers were performed on data from biochemical analyses. Statistical significance of any parameter was not affected by inclusion of outliers.

Results

Female mice fed Western diet display altered feeding behavior during pre-gestation phase

Female mice fed Western diet (WD) ad-libitum over a time course of 10 weeks (age-matched to mice used in maternal experiments below), when compared to mice maintained on standard rodent chow (SD), gained weight at a higher rate based on change in body weight [Figure 1.2A, diet effect on cumulative change in body weight, $F(1,5)= 18.89$, $p= 0.007$; interaction between diet and time, $F(18,90)= 4.182$, $p<0.0001$)]; however, effect of diet on cumulative gross body was only significant when accounting for interaction between diet and time [Figure 1.2B, diet effect on cumulative gross body weight, $F(1,5)= 5.3$, $p= 0.07$; interaction

between diet and time [$F(18,90)= 4.182$, $p<0.0001$)]. These effects were met with an increased average meal size (Figure 1.2C, $t= 2.085$, $p= 0.039$), increased rate of intake (Figure 1.2D, $t= 2.382$, $p= 0.019$), and lower meal duration (Figure 1.2E, $t= 2.247$, $p= 0.026$), over a 24-hour period. Other feeding parameters tested were not affected by diet, including meal frequency (Figure 1.2F, $t= 0.424$, $p= 0.68$), total caloric consumption (Figure 1.2G, $t= 1.146$, $p= 0.279$), dark cycle consumption (Fig 1.2H, $t= 0.282$, $p= 0.781$), light cycle consumption (Figure 1.2G, $t= 1.179$, $p= 0.104$), and post meal interval (Figure 1.2J, $t= 0.864$, $p= 0.389$). Total daily caloric consumption trended towards an increase but did not reach statistical significance (Figure 1.2G), which is in contrast to reports of significant increases in body weight and daily caloric intake in male mice fed a WD versus SD under similar conditions [see our (40)].

These results highlight important sex differences in feeding patterns in mice chronically maintained on WD and may represent differential expression of the eCB system and function among sexes in organs important for food intake and energy balance, including the upper small-intestinal epithelium (35, 52) and pancreas (21). Indeed, when compared to male mice fed WD for 60 days that display increases in levels of the eCBs anandamide and 2-AG in the upper small intestinal epithelium and plasma (40), female mice fed WD displayed decreases and increases in levels of plasma 2-AG (Table 1.2, $t= 2.654$, $p= 0.025$) and anandamide (Table 1.2, $t= 3.025$, $p= 0.0128$) respectively, and no changes in levels of these eCBs in upper small intestinal epithelium when compared to mice

fed SD (Table 1.2). Levels of the eCB-related molecule oleoylethanolamide (OEA) was increased in plasma of WD mice (Table 1.2, *t*= 3.223, p= 0.01), and docosahexaenoylethanolamide (DHEA) was decreased (Table 1.2, *t*= 3.605, p= 0.005) in upper small intestinal epithelium when compared to control mice fed SD.

Female mice fed WD display changes in endocannabinoid and related lipid profiles in plasma and jejunal epithelium during pre-gestation phase

Lipids were extracted from blood plasma, epithelium of the proximal small intestine (jejunum), and pancreas of female mice maintained on WD or SD for 10 weeks (age-matched to mice used in maternal experiments below). Female WD mice, when compared to those fed SD or 10 weeks, had significantly higher levels of plasma AEA (*t*= 3.025, p= 0.0128) and OEA (*t*= 3.223, p= 0.01), and reductions in 2-AG (*t*= 2.654, p= 0.024), but did not display differences in jejunal epithelium or pancreas (Table 1.2). Levels of the less characterized DHEA (*t*= 3.605, p= 0.005) and DHG (*t*= 2.963, p= 0.014) were also decreased and increased, respectively, in jejunal epithelium. These results are in contrast to male C57BL/6 mice similarly fed WD for 60 days, which were reported to have increases in plasma and jejunal epithelial 2-AG and AEA [see our (40)]. These data highlights important sex differences in the impact of DIO on the peripheral eCB system, which may underlie differential feeding patterns (i.e., total caloric intake) in females (Figure 1.2) versus males [see our (40)].

Offspring born from DIO mothers have lower rates of survival

Female mice were fed a SD or WD during pre-gestation (10 weeks) and lactation phases (see Figure 1.1). Neonates born from DIO dams had lower rates of survival when compared to mice born to lean dams (Figure 1.3A, HR 0.2309; 95% CI, 0.1032–0.5166, p= 0.0004). All mortality occurred within the first six days following birth. Average litter sizes of the total offspring born between DIO and lean mothers did not differ at time of birth (Figure 1.3B, *t*= 0.691, p= 0.528). However, the average litter sizes of surviving offspring born from DIO mothers decreased by post-natal day 21 (Figure 1.3C, t= 3.06, p= 0.012). The remaining offspring that survived the lactation period born from DIO mice had a higher male fraction than surviving pups born from lean mothers (Figure 1.3D, 60% vs. 45%, respectively).

Surviving offspring display similar feeding behaviors between groups

Male (Figure 1.4A) and female (Figure 1.5A) offspring born from dams fed WD had higher body mass at time of wean when compared to those born from dams maintained on SD; however, body weights rapidly normalized over the monitoring period of WD mice to levels found in offspring born from SD dams. Male offspring did not display a difference in meal size (Figure 1.4B, *t*= 1.16, p= 0.244), rate of intake (Figure 1.4C, *t*= 0.798, p= 0.426), meal duration (Figure 1.4D, *t*= 0.146, p= 0.146), meal frequency (Figure 1.4E, *t*= 1.023, p= 0.323), total caloric intake (Figure 1.4F, *t*= 0.468, p= 0.647), dark cycle caloric intake (Figure 1.4G, *t*= 1.918, p= 0.066), light cycle caloric intake (Figure 1.4H, *t*= 0.276, p= 0.787), post meal interval (Figure 1.4I, *t*= 0.449, p= 0.654), or preference between WD and SD

(Figure 1.4J, *t*= 0.618, 0.546). Female offspring did not display a difference in meal size (Figure 1.5B, *t*= 0.542, p= 0.589), meal duration (Figure 1.5D, *t*= 0.446, p= 0.656), meal frequency (Figure 1.5E, *t*= 1.609, p= 0.139), total caloric intake (Figure 1.5F, *t*= 0.883, p= 0.398), dark cycle caloric intake (Figure 1.5G, *t*= 0.938), light cycle caloric intake (Figure 1.5H, *t*= 0.543, p= 0.599), post meal interval (Figure 1.5I, *t*= 1.753, p= 0.082), or preference between WD and SD (Figure 1.5J, *t*= 1.048, p= 0.3191). Female offspring born from WD dams had a slightly decreased rate of intake (Figure 1.5C, *t*= 2.304, p= 0.023) when compared to female off- spring born from SD controls.

Surviving offspring display similar endocannabinoid and related lipid profiles irrespective of dam's diet

Lipids were extracted from blood plasma, epithelium of the proximal small intestine (jejunum), and pancreas from male and female offspring from dams maintained on WD or SD for 10 weeks prior to mating and through to weaning (Table 1.2). Irrespective of dams' diet, no changes were found in levels of 2-AG, AEA, or OEA in plasma, jejunal epithelium, or pancreas; however, plasma levels of DHG (*t*= 2.727, p= 0.017) in male offspring born from WD dams, and DHEA (*t*= 2.955, p= 0.018) in female offspring born from WD dams, were moderately decreased.

Male offspring display changes in expression of mRNA for select endocannabinoid system components in jejunal epithelium

We next evaluated expression of mRNA for components of the eCB system in jejunal epithelium and pancreas from male (Figure 1.6) and female (Figure 1.7) offspring from dams maintained on WD or SD for 10 weeks prior to mating and through to weaning. Expression of mRNA for the monoacylglyerol (e.g., 2-AG, DHG) degradative enzyme, monoacylglycerol lipase (MGL), was decreased in jejunal epithelium of male mice born from WD dams (Figure 1.6A), when compared to those born from SD dam, (t= 3.1, p= 0.017). In contrast, no changes in mRNA expression were found in the same tissue for CB_1Rs (Cnr1; t= 0.897, p= 0.396) and CB_2Rs (Cnr2; t= 0.069, p= 0.947), and the monoacylglycerol (e.g., 2-AG, DHG) biosynthetic enzymes, diacylglycerol lipase alpha (DagLa; t= 0.147, p= 0.888) and diacylglycerol lipase beta (DagLb; t= 0.491, p= 0.962), as well as no changes in expression of mRNA for the fatty acid ethanolamide (e.g., AEA, OEA, DHEA) biosynthetic enzyme, NAPE-PLD (t= 0.725, p= 0.492), or degradative enzyme, fatty acid amide hydrolase (FAAH; t= 0.475, p= 0.651). In male pancreas (Figure 1.6B), there were no changes in Cnr1 (t= 0.181, p= 0.861), Cnr2 (t= 0.042, p = 0.967), or DagLb (t= 1.516, p= 0.173) between groups born from mothers maintained on WD or SD. DagLa, MGL, NAPE-PLD, and FAAH were unable to be quantified due to low expression (LE). In female jejunal epithelium, (Figure 1.7A), no changes were found in Cnr1 (t= 0.331, p= 0.752), Cnr2 (t= 0.106, p= 0.919), DagLa (t= 0.513, p= 0.626), DagLb (t= 1.209, p= 0.272), MGL (t= 2.074, p= 0.083), NAPE-PLD (t= 0.449, p= 0.669), or FAAH (t= 0.192, p= 1.47). In female pancreas (Figure 1.7B), there were no changes in expression of Cnr1 (t= 0.921, p= 0.103),

Cnr2 (*t*= 1.302, p= 0.241), or DagLb (*t*= 892, p= 0.407). DagLa, MGL, NAPE-PLD, and FAAH were unable to be quantified due to low expression (LE).

Discussion

These studies suggest that maternal diet is an important predictor of survival in neonate C57BL/6Tac mice, and maternal WD is not associated with disruptions in feeding patterns or marked abnormalities in eCB system expression or function in jejunal epithelium, pancreas, or plasma in surviving offspring maintained on a SD for 10 weeks.

DIO is associated with a host of metabolic abnormalities that include type-2 diabetes (5, 6). Obesity rates have increased over the past several decades in human females of child-bearing age, and obesity during gestation is associated with abnormal metabolic profiles in offspring, which may include an epigenetic component in these outcomes (53). Thus, research aimed at exploring the impact of maternal diet on feeding behavior and glucose homeostasis is critical. In the present study, chronic exposure to WD greatly influenced offspring survival within the first 6 days following birth. Female mice were maintained on a WD for 10 weeks, at which time they displayed significantly increased body weight gain and altered feeding behaviors. Maternal obesity led to high rates of neonate mortality, with a higher male fraction in surviving pups. Our results are consistent with reports of increased mortality in rat pups born from mothers that were exposed to a highly palatable “cafeteria diet” (i.e., chocolate candy bars) for eight weeks prior to mating and through to weaning (54). Importantly, however, we found a much higher rate

of mortality in our studies (above 50% mortality by 6 days post-birth in pups born from WD dams) when compared to studies by Ramirez-Lopez and colleagues (4.3% pups died at birth and about 10% during lactation by 21 days) (54). It is plausible that dietary composition plays a large role in these effects and WD in our studies, which is high in milk fat (40% total diet kcals) and sucrose (29% total diet kcals), leads to considerably higher neonate deaths. A comprehensive analysis of the impact of specific dietary components (i.e., various types of fats and carbohydrates) on neonate survival in rodents remains.

Surviving offspring were then monitored for 10 weeks and feeding behaviors were assessed. Surviving offspring did not display notable differences in body weights or feeding parameters at 10 weeks of age (i.e., total caloric intake, meal size, meal duration, meal frequency, rate of intake, or preference tests). Other groups report increased body weights and food intake in mice or rats born from dams maintained on high-fat diets versus standard diets, but these effects were mostly apparent after 8–10 weeks post-weaning (13, 17, 55). Thus, changes in feeding behaviors and body weight change under our conditions may occur at later timepoints from those included in our analysis (i.e., 10 weeks post-weaning). Furthermore, in contrast to our findings in mice that offspring born from dams maintained on WD or SD displayed no differences in preference for WD during a 24-h test, rats born from dams maintained on a “cafeteria diet” were reported to display “an exacerbated preference for fatty, sugary and salty foods at the expense of protein-rich foods” (56). These results may reflect important species differences

and differential impact of specific nutrients on palatable food preference. Future studies should include tracking the impact of maternal DIO on feeding patterns of offspring born from WD or SD dams over extended periods of time (i.e., greater than 10 weeks).

We next analyzed levels of eCBs (i.e., the fatty acid ethanolamide, AEA, and the monoacylglycerol, 2-AG) and related fatty acid ethanolamides (i.e., OEA and DHEA) and monoacylglycerols (i.e., DHG) in plasma, upper small intestinal epithelium, and pancreas. We found no changes in the eCBs in organs tested between groups born from dams chronically fed WD or SD, and modest reductions in levels of DHG in male plasma and DHEA in female plasma of those born from WD dams. DHG and DHEA are synthesized from the omega-3 fatty acid, docosahexaenoic acid (DHA, 22:6n-3). Their physiological relevance, however, is not well-established but may include roles in inflammation and neural development (57, 58). It is plausible that lower levels of these molecules in plasma may play a role in systemic inflammation associated with DIO; however, a direct test of this hypothesis remains. Furthermore, we found no appreciable changes in expression of eCB biosynthetic and degradative enzymes in small intestinal epithelium and pancreas of males and females, with the exception of a small but significant reduction in expression of the 2-AG degradative enzyme, MGL, in males. The latter effect suggests a possible remodeling of the eCB metabolic machinery in the small intestinal epithelium in male mice born from mothers maintained on WD; however, no changes in levels of select monoacylglycerols (i.e., 2-AG and DHG) were found,

which raises the question of the physiological significance of this change in expression of MGL. A complete analysis of the expression in tissue of a wider variety of monoacylglycerols under our conditions is warranted. Notably, we analyzed eCB expression in organs of pups 10 weeks after weaning and maintenance on SD. Thus, we cannot rule out that changes in expression or function of the eCB system in pancreas and small intestinal epithelium occur in pups born from WD mothers at earlier time points and is transient, or at later time points from our analysis of eCB system expression and feeding behavior in pups (i.e., 10 weeks after weaning). Indeed, other groups reported sex-specific changes in eCB system expression in white and brown adipose depots in rat pups born from mothers maintained on a high-fat diet versus standard; however, in contrast to our experiments, their analysis occurred immediately after weaning (15). Similarly, Ramirez-Lopez and colleagues reported reductions at birth in levels of the eCBs, AEA and 2-AG, in the hypothalamus of male rats born from mothers maintained on a high-calorie test diet versus standard chow during the pregestational and gestational periods (54). Furthermore, rats born to mothers maintained on either a palatable chocolate diet in combination with standard chow (ad-libitum access to both) or control rats given access to only standard chow for 8 weeks displayed very small decreases in body weight gain over a period of nineteen weeks (16). The authors then analyzed expression of eCB system components in brain, liver, and adipose tissue in these rats at nineteen weeks postnatal and found a variety of sex-specific changes in expression of mRNA for several components of the eCB

system [see for details (16)]. Collectively, these studies reveal possible changes in eCB system function or expression in several organs immediately after birth (54) or weaning (15), and at much later timepoints [i.e., nine- teen weeks postnatal (16)] in offspring born from dams consuming high-energy diets. Further- more, under our conditions, similar expression and function of the eCB system between groups in the upper small intestinal epithelium–particularly given its critical role in feeding behavior (45–47, 49)–may underlie a lack of change in feeding patterns or palatable food preferences observed between mice born from WD or SD dams at the time point analyzed in our experiments.

Conclusion

Our studies reveal large increases in mortality in neonates born from dams maintained on a WD for 10 weeks before mating, during gestation, and through to weaning of pups. Mortality was restricted to the first six days after birth. Furthermore, changes in eCB system function and expression in these offspring, when compared to dams maintained on a control SD, were largely absent at 10 weeks post-weaning in small-intestinal epithelium, pancreas, and plasma. Future studies under our conditions should include a comprehensive temporal evaluation of eCB system expression and function in small intestinal epithelium, pancreas, and plasma of pups born from mothers maintained on WD and SD, from immediately after weaning through to time points after 10 weeks post-weaning. Furthermore, despite a lack of marked changes in eCB system profile or feeding

behavior in offspring born from dams maintained on WD, it is important to consider that behavioral and biochemical analysis of the animals tested under our conditions were performed on surviving pups, which may be considered “extraordinary”. It is possible that the neonates that died within six days following their birth had significant changes in eCB system and other regulatory factors affecting feeding and glucose homeostasis that led to their failure to thrive. A test of this hypothesis, however, remains, but is difficult given the inability to predict when mice will die, and which mice will survive.

Author Disclosure Statement:

The authors have declared that no competing interests exist.

References

1. Ogden CL, Carroll MD, Lawman HG, Fryar CD, Kruszon-Moran D, Kit BK, et al. Trends in Obesity Prevalence Among Children and Adolescents in the United States, 1988–1994 Through 2013–2014. JAMA. 2016; 315(21):2292–9. https://doi.org/10.1001/jama.2016.6361 PMID: 27272581.

2. Ogden CL, Carroll MD, Kit BK, Flegal KM. Prevalence of obesity in the United States, 2009–2010. NCHS data brief, no 82 Hyattsville, MD: National Center for Health Statistics 2012. 2012.

3. Ogden CL, Carroll MD, Kit BK, Flegal KM. Prevalence of childhood and adult obesity in the United States, 2011–2012. JAMA. 2014; 311(8):806–14. https://doi.org/10.1001/jama.2014.732 PMID: 24570244.

4. Fryar CD, Carroll MD, Ogden CL. Prevalence of overweight and obesity among children and adoles- cents: United States, 1963–1965 through 2011–2012. Atlanta, GA: National Center for Health Statistics. 2014.

5. Poti JM, Duffey KJ, Popkin BM. The association of fast food consumption with poor dietary outcomes and obesity among children: is it the fast food or the remainder of the diet? Am J Clin Nutr. 2014; 99 (1):162–71. https://doi.org/10.3945/ajcn.113.071928 PMID: 24153348; PubMed Central PMCID: PMCPMC3862453.

6. Medina-RemOn A, Kirwan R, Lamuela-Raventos RM, Estruch R. Dietary Patterns and the Risk of Obesity, Type 2 Diabetes Mellitus, Cardiovascular Diseases, Asthma, and Mental Health Problems. Crit Rev Food Sci Nutr. 2016:0. https://doi.org/10.1080/10408398.2016.1158690 PMID: 27127938.

7. Vahratian A. Prevalence of Overweight and Obesity among Women of Childbearing Age: Results from the 2002 National Survey of Family Growth. Maternal and child health journal. 2009; 13(2):268–73. https://doi.org/10.1007/s10995-008-0340-6 PMC2635913. PMID: 18415671

8. Ferrara A. Increasing Prevalence of Gestational Diabetes Mellitus. Diabetes Care. 2007; 30(Supplement 2):S141.

9. Buchanan TA, Xiang AH, Page KA. Gestational Diabetes Mellitus: Risks and Management during and after Pregnancy. Nature reviews Endocrinology. 2012; 8(11):639–49. https://doi.org/10.1038/nrendo. 2012.96 PMC4404707. PMID: 22751341

10. Pirkola J, Pouta A, Bloigu A, Hartikainen A-L, Laitinen J, Ja¨rvelin M-R, et al. Risks of Overweight and Abdominal Obesity at Age 16 Years Associated With Prenatal Exposures to Maternal Prepregnancy Overweight and Gestational Diabetes Mellitus. Diabetes Care. 2010; 33(5):1115–21. https://doi.org/10.2337/dc09-1871 PMC2858187. PMID: 20427685

11. Bayol SA, Farrington Sj Fau—Stickland NC, Stickland NC. A maternal 'junk food' diet in pregnancy and lactation promotes an exacerbated taste for 'junk food' and a greater propensity for obesity in rat off- spring. British Journal of Nutrition. 2007; 98:4(0007–1145 (Print)).

12. Ramirez-Lopez MT, Vazquez M, Bindila L, Lomazzo E, Hofmann C, Blanco RN, et al. Exposure to a highly caloric palatable diet during pregestational and gestational periods affects hypothalamic and hippocampal endocannabinoid levels at birth and induces adiposity and anxiety-like behaviors in male rat offspring. Frontiers in Behavioral Neuroscience. 2015; 9(339). PMID: 1805495129.

13. Masuyama H, Hiramatsu Y. Additive Effects of Maternal High Fat Diet during Lactation on Mouse Offspring. PLOS ONE. 2014; 9(3):e92805. https://doi.org/10.1371/journal.pone.0092805 PMID: 24664181

14. Nivoit P, Morens C Fau—Van Assche FA, Van Assche Fa Fau—Jansen E, Jansen E Fau—Poston L, Poston L Fau—Remacle C, Remacle C Fau—Reusens B, et al. Established diet-induced obesity in female rats leads to offspring hyperphagia, adiposity and insulin resistance. (1432–0428 (Electronic)).

15. Almeida MM, Dias-Rocha CP, Souza AS, Muros MF, Mendonca LS, Pazos-Moura CC, et al. Perinatal maternal high-fat diet induces early obesity and sex-specific alterations of the endocannabinoid system in white and brown adipose tissue of weanling rat offspring. Br J Nutr. 2017; 118(10):788–803. Epub 2017/11/08. https://doi.org/10.1017/S0007114517002884 PMID: 29110748.

16. Ramirez-Lopez MT, Arco R, Decara J, Vazquez M, Noemi Blanco R, Alen F, et al. Exposure to a Highly Caloric Palatable Diet during the Perinatal Period Affects the Expression of the Endogenous Cannabinoid System in the Brain, Liver and Adipose Tissue of Adult Rat Offspring. PLoS One. 2016; 11(11): e0165432. https://doi.org/10.1371/journal.pone.0165432 PMID: 27806128.

17. Elahi MM, Cagampang FR, Mukhtar D, Anthony FW, Ohri SK, Hanson MA. Long-term maternal high-fat feeding from weaning through pregnancy and lactation predisposes offspring to hypertension, raised plasma lipids and fatty liver in mice. Br J Nutr. 2009; 102(4):514–9. Epub 2009/02/11. https://doi.org/10.1017/S000711450820749X PMID: 19203419.

18. Hiramatsu L, Kay JC, Thompson Z, Singleton JM, Claghorn GC, Albuquerque RL, et al. Maternal expo- sure to Western diet affects adult body composition and voluntary wheel running in a genotype-specific manner in mice. Physiology & behavior. 2017; 179:235–45. Epub 2017/06/20. https://doi.org/10.1016/j. physbeh.2017.06.008 PMID: 28625550; PubMed Central PMCID: PMCPMC5581230.

19. DiPatrizio NV, Piomelli D. The thrifty lipids: endocannabinoids and the neural control of energy conservation. Trends Neurosci. 2012; 35(7):403–11. Epub 2012/05/25. https://doi.org/10.1016/j.tins.2012.04. 006 S0166-2236(12)00066-5 [pii]. PMID: 22622030.

20. DiPatrizio NV, Piomelli D. Intestinal lipid-derived signals that sense dietary fat. The Journal of clinical investigation. 2015; 125(3):891–8. https://doi.org/10.1172/JCI76302 PMID: 25642767; PubMed Central PMCID: PMCPMC4362267.

21. Simon V, Cota D. MECHANISMS IN ENDOCRINOLOGY: Endocannabinoids and metabolism: past, present and future. Eur J Endocrinol. 2017; 176(6):R309–R24. https://doi.org/10.1530/EJE-16-1044 PMID: 28246151.

22. Cristino L, Becker T, Di Marzo V. Endocannabinoids and energy homeostasis: An update. BioFactors. 2014. https://doi.org/10.1002/biof.1168 PMID: 24752980.

23. Piomelli D. The molecular logic of endocannabinoid signaling. Nature reviews. 2003; 4(11):873–84. https://doi.org/10.1038/nrn1247 PMID: 14595399.

24. Pertwee RG. Endocannabinoids and Their Pharmacological Actions. Handb Exp Pharmacol. 2015; 231:1–37. https://doi.org/10.1007/978-3-319-20825-1_1 PMID: 26408156.

25. Malenczyk K, Keimpema E, Piscitelli F, Calvigioni D, Bjorklund P, Mackie K, et al. Fetal endocannabinoids orchestrate the organization of pancreatic islet microarchitecture. Proc Natl Acad Sci USA. 2015; 112(45):E6185–94. https://doi.org/10.1073/pnas.1519040112 PMID: 26494286; PubMed Central PMCID: PMCPMC4653226.

26. Kim W, Doyle ME, Liu Z, Lao Q, Shin YK, Carlson OD, et al. Cannabinoids inhibit insulin receptor signaling in pancreatic beta-cells. Diabetes. 2011; 60(4):1198–209. Epub 2011/02/25. https://doi.org/10. 2337/db10-1550 PMID: 21346174; PubMed Central PMCID: PMCPMC3064093.

27. Kim W, Lao Q, Shin Y-K, Carlson OD, Lee EK, Gorospe M, et al. Cannabinoids Induce Pancreatic β- Cell Death by Directly Inhibiting Insulin Receptor Activation. Science Signaling. 2012; 5(216):ra23. PubMed Central PMCID: PMCPMC3524575. https://doi.org/10.1126/scisignal.2002519 PMID: 22434934

28. Malenczyk K, M J, Keimpema E, Silvestri C, Janikiewicz J, Mackie K, et al. CB1 cannabinoid receptors couple to focal adhesion kinase to control insulin release. Journal of Biological Chemistry. 2013; 288:32685–99. PubMed Central PMCID: PMCPMC3820903. https://doi.org/10.1074/jbc.M113.478354 PMID: 24089517

29. Bermudez-Silva FJ, Baixeras E, Cobo N, Bautista D, Cuesta-Munoz AL, Fuentes E, et al. Presence of functional cannabinoid receptors in human endocrine pancreas. Diabetologia. 2008; 51(3):476–87. Epub December 19, 2007. https://doi.org/10.1007/s00125-007-0890-y PMID: 18092149

30. Li C, Bowe JE, Jones PM, Persaud SJ. Expression and function of cannabinoid receptors in mouse islets. Islets. 2010; 2(5):293–302. PMID: 21099327.

31. De Petrocellis L, Marini P, Matias I, Moriello AS, Starowicz K, Cristino L, et al. Mechanisms for the coupling of cannabinoid receptors to intracellular calcium mobilization in rat insulinoma beta-cells. Exp Cell Res. 2007; 313(14):2993–3004. https://doi.org/10.1016/j.yexcr.2007.05.012 PMID: 17585904.

32. Nakata M, Yada T. Cannabinoids inhibit insulin secretion and cytosolic Ca2+ oscillation in islet beta- cells via CB1 receptors. Regul Pept. 2008; 145(1–3):49–53. https://doi.org/10.1016/j.regpep.2007.08. 009 PMID: 17884194.

31. Juan-Pico P, Fuentes E, Bermudez-Silva FJ, Diaz-Molina F, Ripoll C, Rodriguez de Fonseca F, et al. Cannabinoid receptors regulate Ca(2+) signals and insulin secretion in pancreatic beta-cell. Cell Calcium. 2006; 39(0143–4160 (Print)):155–62. PubMed Central PMCID: PMC16321437. https://doi.org/ 10.1016/j.ceca.2005.10.005 PMID: 16321437

34. Shin H, Han JH, Yoon J, Sim HJ, Park TJ, Yang S, et al. Blockade of cannabinoid 1 receptor improves glucose responsiveness in pancreatic beta cells. J Cell Mol Med. 2018; 22(4):2337–45. https://doi.org/ 10.1111/jcmm.13523 PMID: 29431265; PubMed Central PMCID: PMCPMC5867156.

35. DiPatrizio NV. Endocannabinoids in the Gut. Cannabis and Cannabinoid Research. 2016; 1(1):67–77. https://doi.org/10.1089/can.2016.0001 PMID: 27413788

36. Di Marzo V, Matias I. Endocannabinoid control of food intake and energy balance. Nature neuroscience. 2005; 8(5):585–9. https://doi.org/10.1038/nn1457 PMID: 15856067.

37. Maccarrone M, Bab I, Biro T, Cabral GA, Dey SK, Di Marzo V, et al. Endocannabinoid signaling at the periphery: 50 years after THC. Trends Pharmacol Sci. 2015; 36(5):277–96. https://doi.org/10.1016/j. tips.2015.02.008 PMID: 25796370; PubMed Central PMCID: PMCPMC4420685.

38. Di Marzo V, Goparaju SK, Wang L, Liu J, Batkai S, Jarai Z, et al. Leptin-regulated endocannabinoids are involved in maintaining food intake. Nature. 2001; 410(6830):822–5. https://doi.org/10.1038/ 35071088 PMID: 11298451.

39. Ravinet Trillou C, Arnone M, Delgorge C, Gonalons N, Keane P, Maffrand JP, et al. Anti-obesity effect of SR141716, a CB1 receptor antagonist, in diet-induced obese mice. Am J Physiol Regul Integr Comp Physiol. 2003; 284(2):R345–53. PMID: 12399252.

40. Argueta DA, DiPatrizio NV. Peripheral endocannabinoid signaling controls hyperphagia in western diet- induced obesity. Physiology & behavior. 2017; 171:32–9. https://doi.org/10.1016/j.physbeh.2016.12. 044 PMID: 28065722.

41. Jarbe TU, DiPatrizio NV. Delta9-THC induced hyperphagia and tolerance assessment: interactions between the CB1 receptor agonist delta9-THC and the CB1 receptor antagonist SR-141716 (rimonabant) in rats. Behavioural pharmacology. 2005; 16(5–6):373–80. PMID: 16148441.

42. Tam J, Vemuri VK, Liu J, Batkai S, Mukhopadhyay B, Godlewski G, et al. Peripheral CB1 cannabinoid receptor blockade improves cardiometabolic risk in mouse models of obesity. The Journal of clinical investigation. 2010; 120(8):2953–66. Epub 2010/07/29. 42551 [pii] https://doi.org/10.1172/JCI42551 PMID: 20664173; PubMed Central PMCID: PMC2912197.

43. Arnone M, Maruani J, Chaperon F, Thiebot MH, Poncelet M, Soubrie P, et al. Selective inhibition of sucrose and ethanol intake by SR 141716, an antagonist of central cannabinoid (CB1) receptors. Psychopharmacology. 1997; 132(1):104–6. PMID: 9272766.

44. Fride E, Ginzburg Y, Breuer A, Bisogno T, Di Marzo V, Mechoulam R. Critical role of the endogenous cannabinoid system in mouse pup suckling and growth. European Journal of Pharmacology. 2001; 419 (2–3):207–14. http://dx.doi.org/10.1016/S0014-2999(01)00953-0(01)00953-0. PMID: 11426843

45. DiPatrizio NV, Astarita G, Schwartz G, Li X, Piomelli D. Endocannabinoid signal in the gut controls dietary fat intake. Proceedings of the National Academy of Sciences of the United States of America. 2011; 108(31):12904–8. Epub 2011/07/07. 1104675108 [pii] https://doi.org/10.1073/pnas.1104675108 PMID: 21730161; PubMed Central PMCID: PMC3150876.

46. DiPatrizio NV, Joslin A, Jung KM, Piomelli D. Endocannabinoid signaling in the gut mediates preference for dietary unsaturated fats. Faseb J. 2013; 27(6):2513–20. Epub 2013/03/07. https://doi.org/10.1096/fj. 13-227587 fj.13-227587 [pii]. PMID: 23463697; PubMed Central PMCID: PMC3659363.

47. DiPatrizio NV, Igarashi M, Narayanaswami V, Murray C, Gancayco J, Russell A, et al. Fasting stimulates 2-AG biosynthesis in the small intestine: role of cholinergic pathways. Am J Physiol Regul Integr Comp Physiol. 2015; 309(8):R805–13. https://doi.org/10.1152/ajpregu.00239.2015 PMID: 26290104.

48. Izzo AA, Piscitelli F, Capasso R, Aviello G, Romano B, Borrelli F, et al. Peripheral endocannabinoid dysregulation in obesity: relation to intestinal motility and energy processing induced by food deprivation and re-feeding. British journal of pharmacology. 2009; 158(2):451–61. Epub 2009/04/18. BPH183 [pii] https://doi.org/10.1111/j.1476-5381.2009.00183.x PMID: 19371345; PubMed Central PMCID: PMC2757684.

49. Angelini R, Argueta DA, Piomelli D, DiPatrizio NV. Identification of a Widespread Palmitoylethanolamide Contamination in Standard Laboratory Glassware. Cannabis Cannabinoid Res. 2017; 2(1):123– 32. https://doi.org/10.1089/can.2017.0019 PMID: 28861512; PubMed Central PMCID: PMCPMC5510777.

50. Dotsey E, Ushach I, Pone E, Nakajima R, Jasinskas A, Argueta DA, et al. Transient Cannabinoid Receptor 2 Blockade during Immunization Heightens Intensity and Breadth of Antigen-specific Antibody Responses in Young and Aged mice. Sci Rep. 2017; 7:42584. https://doi.org/10.1038/srep42584 PMID: 28209996.

51. Stella N, Schweitzer P, Piomelli D. A second endogenous cannabinoid that modulates long-term potentiation. Nature. 1997; 388(6644):773–8. https://doi.org/10.1038/42015 PMID: 9285589.

52. Izzo AA, Sharkey KA. Cannabinoids and the gut: New developments and emerging concepts. Pharmacology & therapeutics. 2010; 126(1):21–38. Epub 2010/02/02. S0163-7258(10)00006-9 [pii] https://doi. org/10.1016/j.pharmthera.2009.12.005 PMID: 20117132.

53. Agarwal P, Morriseau TS, Kereliuk SM, Doucette CA, Wicklow BA, Dolinsky VW. Maternal obesity, diabetes during pregnancy and epigenetic mechanisms that influence the developmental origins of cardio- metabolic disease in the offspring. Crit Rev Clin Lab Sci. 2018; 55(2):71–101. Epub 2018/01/09. https:// doi.org/10.1080/10408363.2017.1422109 PMID: 29308692.

54. Ramirez-Lopez MT, Vazquez M, Bindila L, Lomazzo E, Hofmann C, Blanco RN, et al. Exposure to a Highly Caloric Palatable Diet During Pregestational and Gestational Periods Affects Hypothalamic and Hippocampal Endocannabinoid Levels at Birth and Induces Adiposity and Anxiety-Like Behaviors in Male Rat Offspring. Front Behav Neurosci. 2016; 9:339. Epub 2016/01/19. https://doi.org/10.3389/ fnbeh.2015.00339 PMID: 26778987; PubMed Central PMCID: PMCPMC4701936.

55. Nivoit P, Morens C, Van Assche FA, Jansen E, Poston L, Remacle C, et al. Established diet-induced obesity in female rats leads to offspring hyperphagia, adiposity and insulin resistance. Diabetologia. 2009; 52(6):1133–42. Epub 2009/03/17. https://doi.org/10.1007/s00125-009-1316-9 PMID: 19288075.

56. Bayol SA, Farrington SJ, Stickland NC. A maternal 'junk food' diet in pregnancy and lactation promotes an exacerbated taste for 'junk food' and a greater propensity for obesity in rat offspring. Br J Nutr. 2007; 98(4):843–51. Epub 2007/08/19. https://doi.org/10.1017/S0007114507812037 PMID: 17697422.

57. Kim HY, Spector AA. N-Docosahexaenoylethanolamine: A neurotrophic and neuroprotective metabolite of docosahexaenoic acid. Mol Aspects Med. 2018. Epub 2018/03/25. https://doi.org/10.1016/j.mam. 2018.03.004 PMID: 29572109.

58. Park T, Chen H, Kevala K, Lee JW, Kim HY. N-Docosahexaenoylethanolamine ameliorates LPS- induced neuroinflammation via cAMP/PKA-dependent signaling. J Neuroinflammation. 2016; 13 (1):284. Epub 2016/11/05. https://doi.org/10.1186/s12974-016-0751-z PMID: 27809877; PubMed Central PMCID: PMCPMC5096293.

Tables

Table 1.1. Relative energy content (% total kcal) of major nutrients in laboratory mouse diets.

Diet	Source	%Kcal Fat/ Source	%Kcal CHO/ Source	%Kcal Protein/ Source
Soy-Free Standard Rodent Chow (SD)	Teklad Diets	16%	60%	24%
	2020x	Soybean Oil	Mostly Starch	Wheat and Corn
Western Diet (WD)	Research Diets	40%	43%	17%
	D12079B	Milk Fat	Sucrose	Mostly Casein

Table 1.2. Lipid levels in blood plasma, jejunum mucosa, and pancreas. Values ± SEM for each analyte were compared across dietary conditions for all groups using Student's two-tailed t-tests. Significant differences were denoted: **= p<0.01, * = p<0.05, ns = p>0.05. Plasma = all analytes pmol per mL; jejunum mucosa = 2-AG and DHG nmol per mg tissue, and AEA, OEA, DHEA pmol per mg tissue; pancreas = 2-AG and DHG nmol per mg tissue, and AEA, OEA, DHEA pmol per mg tissue.

	Tissue	Diet	2AG	AEA	OEA	DHEA	DHAG
Mothers	Plasma	SD	26.3 ± 3.09	1.73 ± 0.19	13.82 ± 0.56	1.717 ± 0.12	14.78 ± 2.70
		WD	*16.6 ± 1.95	*2.596 ± 0.21	*19.49 ± 1.52	1.658 ± 0.14	7.516 ± 3.72
	Jej. Mucosa	SD	46.03 ± 2.80	2.672 ± 0.35	47.49 ± 2.64	2.477 ± 0.32	9.401 ± 0.86
		WD	44.13 ± 2.34	2.415 ± 0.14	53.07 ± 2.81	**1.152 ± 0.18	*12.69 ± 0.70
	Pancreas	SD	11.07 ± 3.45	3.248 ± 0.24	105.1 ± 22.09	4.28 ± 0.52	2.236 ± 0.84
		WD	11.92 ± 5.31	5.275 ± 1.608	116.7 ± 29.00	3.185 ± 0.62	2.36 ± 0.98
Offspring	Plasma	SD	42.1 ± 4.94	1.007 ± 0.072	17.15 ± 1.45	1.412 ± 0.09	24.79 ± 0.81
Males		WD	42.68 ± 4.35	1.013 ± 0.06	15.72 ± 1.41	1.17 ± 0.09	*20.04 ± 1.46
	Jej. Mucosa	SD	49.35 ± 7.71	1.475 ± 0.28	101.7 ± 10.38	2.662 ± 0.26	9.41 ± 1.54
		WD	50.42 ± 7.34	1.696 ± 0.34	110.6 ± 16.35	2.821 ± 0.21	9.596 ± 1.53
	Pancreas	SD	35.41 ± 4.31	1.411 ± 0.27	27.82 ± 4.50	2.335 ± 0.41	4.744 ± 0.57
		WD	31.32 ± 3.03	1.115 ± 0.21	41.53 ± 11.83	2.468 ± 0.39	4.083 ± 0.34
Offspring	Plasma	SD	42.02 ± 4.74	0.9473 ± 0.05	18.11 ± 1.83	1.454 ± 0.07	10.61 ± 1.51
Females		WD	44.83 ± 4.37	0.6069 ± 0.18	13.34 ± 0.18	*1.151 ± 0.06	11.9 ± 1.69
	Jej. Mucosa	SD	76.74 ± 11.88	1.518 ± 0.33	117.9 ± 14.53	3.274 ± 0.41	9.911 ± 1.601
		WD	84.35 ± 6.34	1.547 ± 0.22	125 ± 16.62	3.599 ± 0.41	14.03 ± 1.58
	Pancreas	SD	68.69 ± 6.63	1.084 ± 0.26	28.73 ± 4.52	1.82 ± 0.30	5.659 ± 0.80
		WD	50.3 ± 10.17	1.189 ± 0.13	17.41 ± 1.49	2.075 ± 0.25	4.445 ± 0.90

Values +/- SEM for each analyte were compared across dietary conditions for all groups using Student's two-tailed t-tests. Significant differences were denoted: ** = p<0.01, * = p<0.05, ns = p>0.05. Plasma = all analytes pmol per mL; jejunum mucosa = 2-AG and DHG nmol per mg tissue, and AEA, OEA, DHEA pmol per mg tissue; pancreas = 2-AG and DHG nmol per mg tissue, and AEA, OEA, DHEA pmol per mg tissue.

Figures

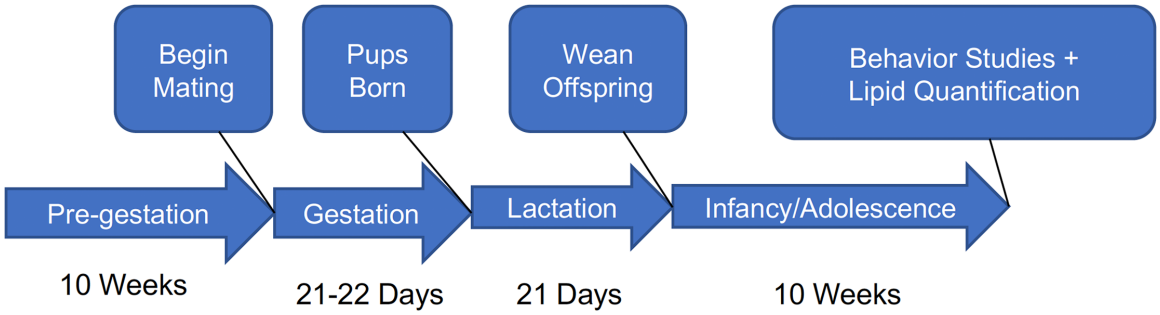

Figure 1.1. Experimental design. Female mice were fed a SD or WD for 10 weeks during the pregestation period, then males were introduced for mating. Females were then maintained on respective diets throughout the entirety of the experiment. Surviving offspring were weaned from damns at day 21 and placed on SD for 10 weeks of body mass monitoring. Behavioral analysis of feeding patterns were made after a 5 day acclimation period. At the conclusion of 10 weeks, tissues in offspring were collected and processed for analysis of endocannabinoid levels and expression of genes for components of the endocannabinoid system. Tissues from dams were collected during the pregestation phase and after 10 weeks on the corresponding diet. Behavioral analysis in damns also occurred at this timepoint.

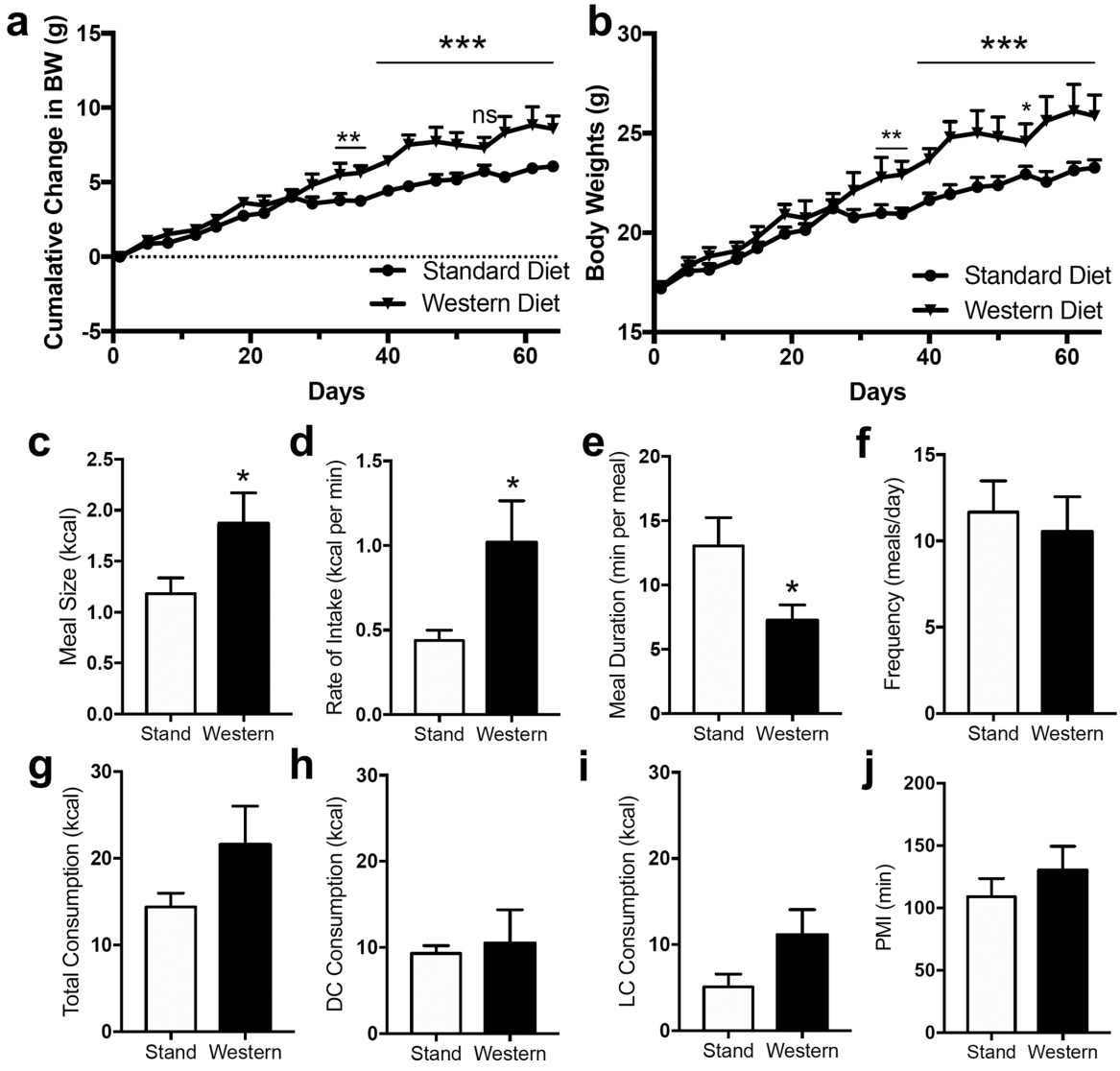
a
Cumalative Change in BW (g)
15
10
5
0
-5
***
**
ns
Standard Diet
Western Diet
0
20
40
60
Days
b
Body Weights (g)
30
25
20
15
***
**
*
Standard Diet
Western Diet
0
20
40
60
Days
c
Meal Size (kcal)
2.5
2.0
1.5
1.0
0.5
0.0
*
Stand
Western
d
Rate of Intake (kcal per min)
1.5
1.0
0.5
0.0
*
Stand
Western
e
Meal Duration (min per meal)
20
15
10
5
0
*
Stand
Western
f
Frequency (meals/day)
15
10
5
0
Stand
Western
g
Total Consumption (kcal)
30
20
10
0
Stand
Western
h
DC Consumption (kcal)
30
20
10
0
Stand
Western
i
LC Consumption (kcal)
30
20
10
0
Stand
Western
j
PMI (min)
200
150
100
50
0
Stand
Western

Figure 1.2. Chronic consumption of western diet results in altered feeding behavior in female mice. Female mice maintained for ten weeks (a, cumulative change in body weight; b, gross body weight) on a western diet (Western) become obese and display increases in 24 h meal size and rate of intake paired with a decrease in meal duration (c-e) when compared to mice maintained on standard chow diet (Stand). Meal frequency, total caloric intake, dark cycle intake, light cycle intake, and post meal interval do not significantly differ between diets (f-j). Repeated measures two-way ANOVA, with Sidak's multiple comparisons post hoc test, *** =$P<0.001$, ** = $p<0.01$, * =$p<0.05$ between Stand and Western. Results are expressed as means ± SEM; n = 6 per condition.

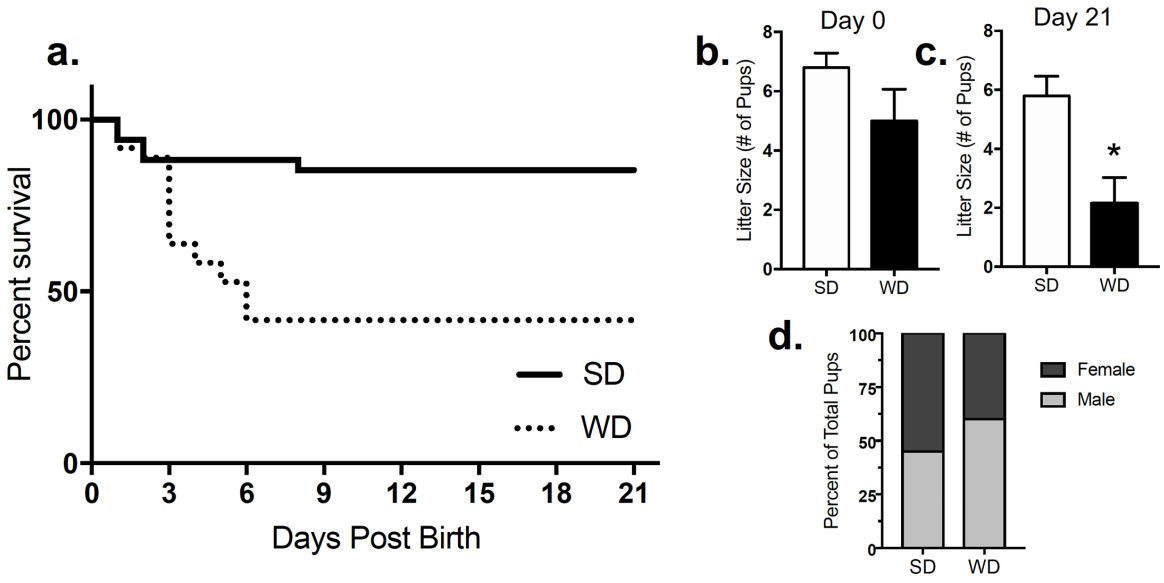

Figure 1.3. Chronic consumption of a maternal western diet increases mortality rate and male fraction in mice neonates. Mice born from mothers chronically fed a Western diet (WD) over 10 weeks have a lower survival rate when compared to mice born from dams fed a standard chow diet (SD) (a). The litter sizes between the two groups were similar in average number of pups at time of birth (b) but decreased in offspring born from DIO mothers at time of wean (c). An increase was found in the male fraction in groups born from WD fed mothers when compared to SD fed mothers (d, 60% vs. 45%). Survival data was analyzed using Mantel-Cox and Gehan-Breslow-Wilcoxon tests, HR 0.2309; 95% CI, 0.1032–0.5166, p = 0.0004. Litter size date was analyzed using unpaired Students t-tests (two-tailed): ns = p>0.05.

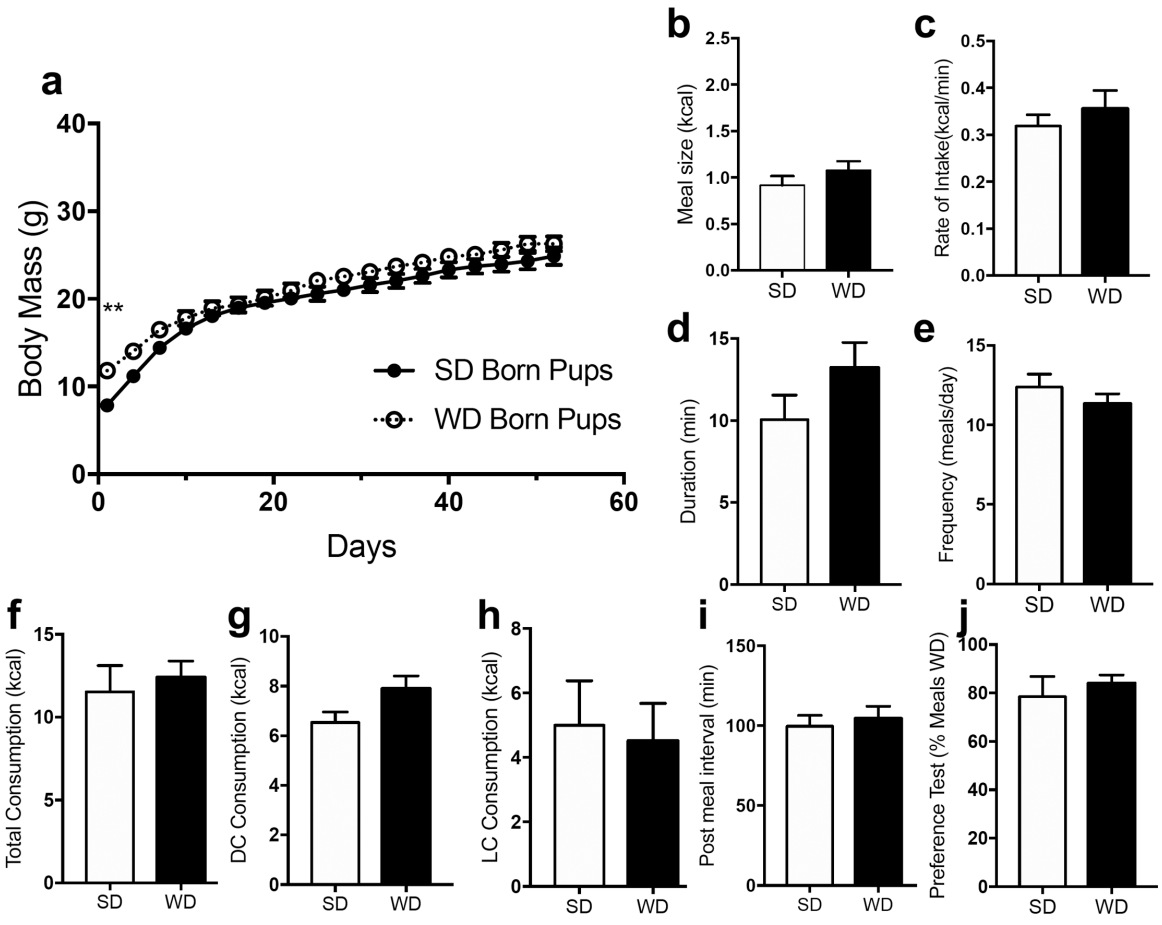
a
Body Mass (g)
Days
**
SD Born Pups
WD Born Pups
b
Meal size (kcal)
c
Rate of Intake(kcal/min)
d
Duration (min)
e
Frequency (meals/day)
f
Total Consumption (kcal)
g
DC Consumption (kcal)
h
LC Consumption (kcal)
i
Post meal interval (min)
j
Preference Test (% Meals WD)
SD
WD

Figure 1.4. Surviving male mice born from DIO or lean dams do not display differences in feeding behavior. Male offspring weaned from dams fed a standard chow diet (SD) or a Western diet (WD) were given ad libitum access to standard chow for 8 weeks. Body weights were monitored and feeding behavior was tested. WD offspring had higher body mass at time of wean but body weights were rapidly normalized over the monitoring period (a). There were no differences meal size, rate of intake, meal duration, meal frequency, total caloric intake, dark cycle intake, light cycle intake, or in post meal interval (b-i). The offspring also displayed no difference in preference when given the choice between consuming a standard chow and highly palatable western diet (j). Data analyzed using repeated measures two-way ANOVA, with Sidak's multiple comparison post hoc test, **= $p<0.01$, ns = $p>0.05$ (a); unpaired Student's t-test (two-tailed), ns = $p>0.05$ between SD and WD (b-j). Results are expressed as means ± SEM; n = 8/9 (SD/WD).

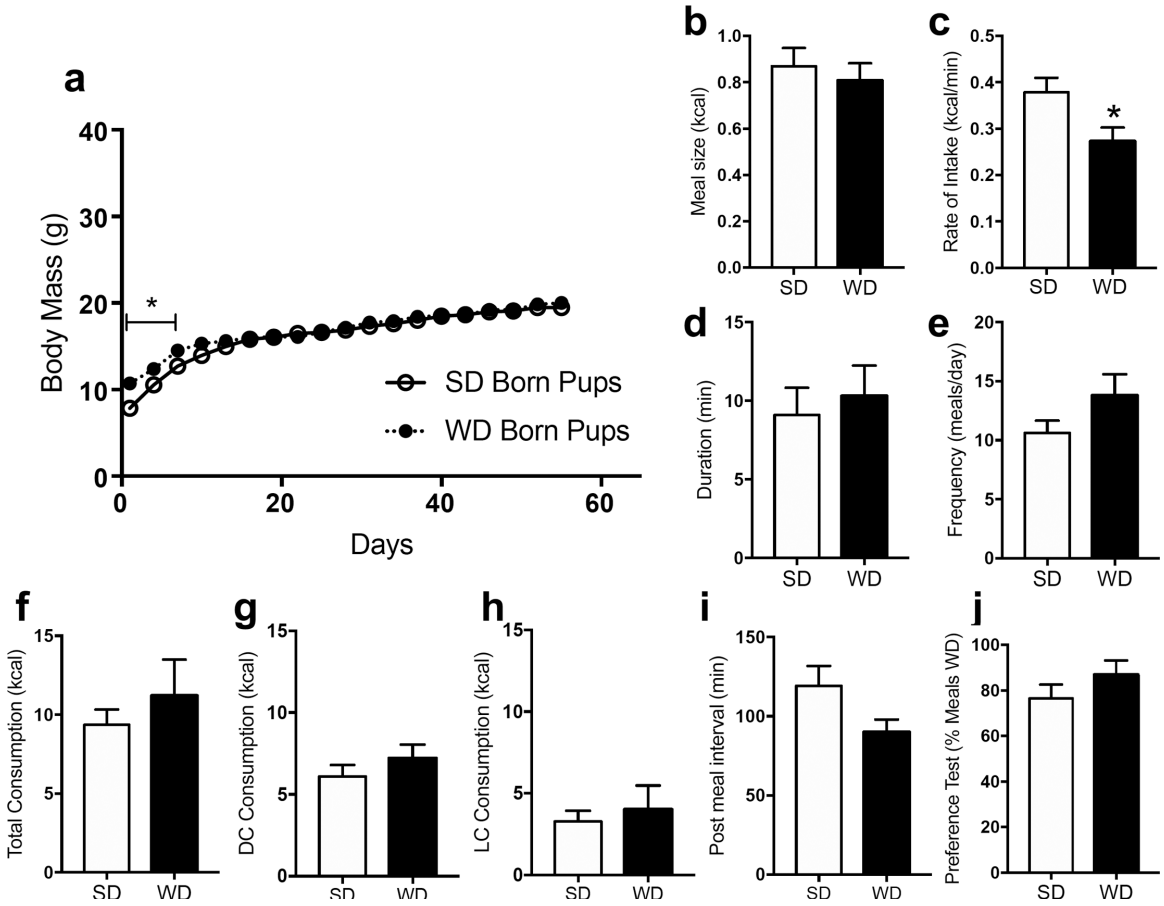
a
Body Mass (g)
Days
SD Born Pups
WD Born Pups
*
b
Meal size (kcal)
SD
WD
c
Rate of Intake (kcal/min)
*
d
Duration (min)
e
Frequency (meals/day)
f
Total Consumption (kcal)
g
DC Consumption (kcal)
h
LC Consumption (kcal)
i
Post meal interval (min)
j
Preference Test (% Meals WD)

Figure 1.5. Surviving female mice born from DIO or lean dams display a small decrease in rate of intake. Female offspring weaned from dams fed a standard chow (SD) or a Western diet (WD) were given ad libitum access to standard chow for 8 weeks. Body weights were monitored and feeding behavior was tested. WD offspring had higher body mass at time of wean but body weights were rapidly normalized over the monitoring period (a). There were no differences meal size (a) but slight decrease in rate of intake between groups (b). Further, there were no differences in meal duration, meal frequency, total caloric intake, dark cycle intake, light cycle intake, or in post meal interval (d-i). The offspring also displayed no difference in preference when given the choice between consuming a standard chow and highly palatable western diet (j). Data analyzed using repeated measures two-way ANOVA, with Sidak's multiple comparison post hoc test, **= $p<0.01$, ns = $p>0.05$ (a); unpaired Student's t-test (two-tailed), ns = $p>0.05$ between SD and WD (b-j). Results are expressed as means ± SEM; n = 8/4 (SD/WD).

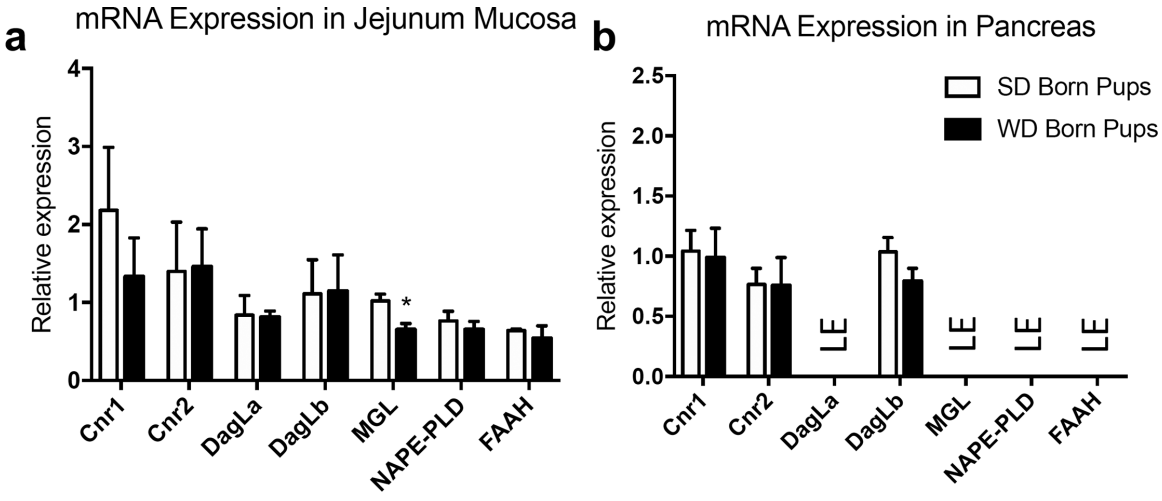

Figure 1.6. Male mice born from DIO dams display decreases in monoacylglycerol lipase (MGL) expression in jejunum mucosa. Expression of mRNA encoding MGL was significantly lower the in upper small intestinal epithelium of offspring born from WD dams versus SD dams. No changes were found between groups in expression of CB1R (Cnr1), CB2R (Cnr2), diacylglycerol lipase α/β (DagLa, DagLb), n-acyl phosphatidylethanolamine phospholipase-D (NAPE-PLD) and fatty acid amide hydrolase (FAAH) did not differ between tested groups in jejunum mucosa (a). In pancreas, Cnr1, Cnr2, and DagLb showed no differences between tested groups, while mRNA expression of DagLa, MGL, NAPE-PLD, and FAAH not detected (b). Data was analyzed using multiple student t tests. * = $p<0.05$, ns = not significant. Results are expressed as means ± SEM; n = 3–5 per condition in triplicate. LE = limited expression.

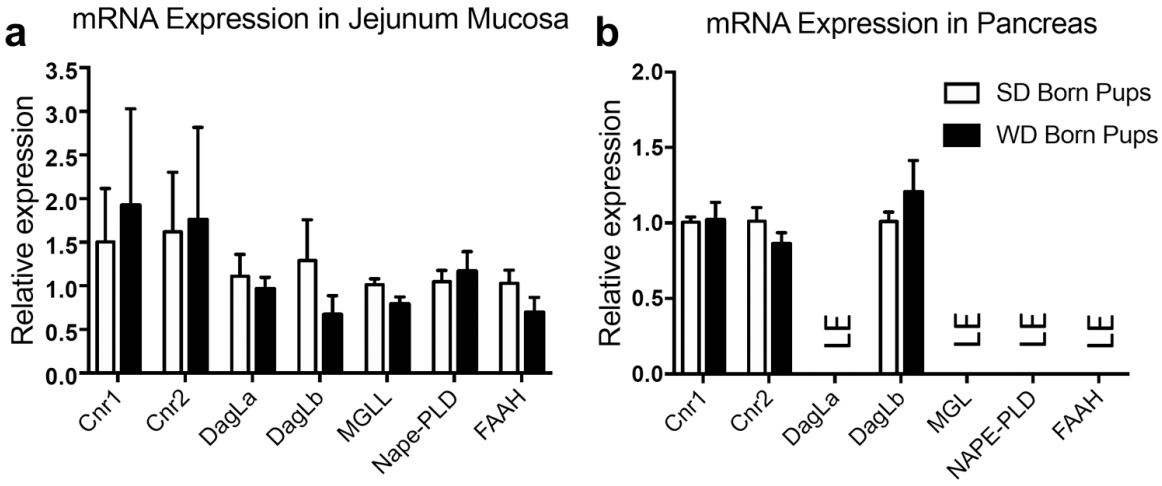

Figure 1.7. Female mice born from dams maintained on WD or SD display similar mRNA expression of key endocannabinoid system genes. Expression of mRNA encoding CB1R (Cnr1), CB2R (Cnr2), diacylglycerol lipase α/β (DagLa, DagLb), monoacylglycerol lipase (MGL), n-acyl phosphatidylethanolamine phospholipase-D (NAPE-PLD) and fatty acid amide hydrolase (FAAH) showed no changes between tested groups in jejunum mucosa (a). In pancreas, Cnr1, Cnr2, and DagLb showed no differences between tested groups while mRNA expression of DagLa, MGL, NAPE-PLD, and FAAH were unable to be quantified with the techniques described (b). Data was analyzed using multiple student t tests. ns = not significant. Results are expressed as means ± SEM; n = 4 per condition in triplicate. LE = limited expression.

Chapter 2: Cannabinoid CB_1 Receptors Inhibit Gut-Brain Satiation Signaling in Diet-Induced Obesity

Authors: Argueta DA[1], *Perez PA[1]*, Makriyannis A[2], DiPatrizio NV[1]

[1]University of California, Riverside, School of Medicine. 900 University Avenue, Riverside, CA, 92521 USA.

[2]Northeastern University Center for Drug Discovery. 360 Huntington Avenue, Boston, MA, 02115 USA.

Abbreviations: 2-AG, 2-arachidonoyl-*sn*-glycerol; 2-DG, 2-docosahexaenoylglycerol; 2-LG, 2-linoleoylglycerol; 2-PG, 2-palmitoylgylcerol; Abhd6, alpha-beta-hydrolyzing domain 6; AEA, Anandamide; AM, AM6545; CB_1R, Cannabinoid receptor subtype 1; CB_2R, 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; FFA, free fatty acid; MAG, Monoacylglycerol; MGL, Monoacylglycerol lipase; NAPE-PLD, N-acyl phosphatidylethanolamine-specific phospholipase D; SD, Standard diet; SIR, selected ion recording; UPLC-MS/MS, ultra-performance liquid chromatography tandem mass spectrometry; WD, Western diet; WIN, WIN 55,212-2

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 CB_1 receptor (CB_1Rs) in the gut of DIO mice inhibits nutrient-induced release of satiation peptides and promotes overeating. Immunoreactivity for CB_1Rs 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 CB_1Rs 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 CB_1R neutral antagonist, AM6545. Furthermore, monoacylglycerol metabolic enzyme function was dysregulated in the upper small-intestinal epithelium from mice fed a high-fat and high-sucrose diet for 60 days (DIO), 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 CB_1Rs in DIO mice inhibits nutrient-induced CCK release. Moreover, the hypophagic effect of AM6545 in DIO

mice was reversed by co-administration with the CCK_A receptor antagonist, devazepide. Collectively, these results provide evidence that hyperphagia associated with DIO is driven by a mechanism that includes CB_1R-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 [CB_1R and CB_2R, respectively (5, 6)]. CB_1Rs in the brain control food intake and energy homeostasis (3, 7); however, targeting central CB_1Rs 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, CB_1Rs 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); thus, the impact of disrupting

endocannabinoid signaling at peripheral CB_1Rs on these functions is largely unknown and warrants further investigation.

Studies from our lab and others suggest key roles for the peripheral eCB system in controlling feeding behavior and energy homeostasis (2, 7, 18). Indeed, eCB levels are increased in the small-intestinal epithelium of rodents *(i)* during a fast (11, 19-21), *(ii)* after oral exposure to dietary fats (9, 10), and *(iii)* in a mouse model of western diet-induced obesity (DIO) (21). Pharmacological inhibition of elevated eCB signaling at small-intestinal CB_1Rs with peripherally-restricted CB_1R 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, (21). 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 are largely unknown. Nonetheless, CB_1Rs 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 (22, 23). For example, expression of CB_1Rs in the rat nodose ganglion is upregulated after fasting, and refeeding or administration of the gut-derived satiation peptide, cholecystokinin (CCK), reversed this effect (22, 24). Moreover, both, fasting-induced increases in CB_1R expression in the nodose ganglion and the ability for CCK to decrease this response were blunted in rats fed a high-fat

diet (25). Vianna and colleagues, however, reported that select deletion of CB_1Rs 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 (26). These findings suggest that CB_1Rs 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, 27-29), which controls meal size and satiation by activating CCK_A receptors on the afferent vagus nerve (30-38). Furthermore, CCK-containing I-cells in the upper small-intestinal epithelium of mice express genes for CB_1Rs (39). Thus, CB_1Rs 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 CB_1Rs 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)2Mirn/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 5 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 CB_1R 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 CCK_A 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 (40, 41), 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_2. Samples were stored at -80^{o}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 (21) and resuspended in 0.1 mL methanol:chloroform (9:1) and analyzed via ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS).

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_{18} column (2.1 x 50 mm i.d., 1.7 µm, Waters Corporation) and inline Acquity guard column (UPLC BEH C_{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^{o}C, and samples were kept at 10^{o}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 in “DGL Activity Assay”) = 18v, 10v; and [$^2H_5$]-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 [$^2H_5$]-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 CB_1R 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 10min 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 CB_1R

antagonist, AM6545 (10 mg per kg). In addition, mice maintained for 60 days 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 Figures 1.1D,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, 320mM 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 [$^2H_5$]-2-AG as internal standard. Lipids were extracted and the product of the reaction, monononadecadienoin (19:2 monoacylglycerol), was analyzed via UPLC/MS/MS as described above for 19:2 monoacylglycerol (See “LCMS detection of 2-arachindoyl-sn-glycerol and other monoacylglycerols”).

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 (See "LCMS detection of 2-arachindoyl-sn-glycerol and other monoacylglycerols") 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^{o}C and samples were kept at 10^{o}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 (500 µL), then quantities of 19:2 FFA remaining the stomach were evaluated at the time of blood collection 30 min 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 (see "MGL Activity Assay").

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 (21).

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: CB_1R (Cnr1), CB_2R (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 (42)]. 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_1Rs 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 high-fat and sucrose diet for 60 days (DIO) when compared to lean controls maintained on a low-fat/sugar diet, and inhibiting peripheral CB_1Rs blocked overeating associated with DIO (i.e., increased meal size, rate of food intake, and total caloric intake) (21). To identify the molecular underpinnings of gut-brain eCB signaling important for feeding behavior and its dysregulation in DIO, we first evaluated whether CB_1Rs are expressed in cells that produce and secrete the satiation peptide, CCK. CCK controls meal size and induces satiation by activating CCK_A receptors on the afferent vagus nerve (30-36). CB_1R immunoreactivity was found in CCK-eGFP-positive cells from the upper small-intestinal epithelium (Figure 2.1) in a mouse line that expresses eGFP selectively in CCK-expressing cells [C57BL/6-Tg(Cck-EGFP)2Mirn/J] (43). 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 (Figure 2). 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 CB_1Rs (Cnr1) was enriched in CCK-eGFP-positive cells when compared to CCK-eGFP-negative cells (Figure 2.3A; 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 2.3B). 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 2.3C). Cells from wild-type mice (see Figure 2.3D) 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 Figure 2.4 for detailed FACS report). These results suggest that CCK-containing I-cells in the mouse upper small-intestinal epithelium are enriched in expression of CB_1Rs.

Peripheral CB_1Rs 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, 27, 29, 44, 45). We next tested the hypothesis that CB_1Rs 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 2.5A; 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 2.5A; 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 CB_1R-selective antagonist, AM6545 (Figure 2.5A; CO+WIN+AM6545 = 0.746 ± 0.141 ng per mL; $p< 0.05$ CO+WIN versus CO+WIN+AM, n=5). These results suggest that exogenous activation of CB_1Rs 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 CB_1Rs 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 DIO mice when compared to mice fed a standard diet (SD) that is low in fat and absent of sucrose (Figure 2.5B; 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 2.5B; 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 CB_1Rs 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 Figure 2.6), another gut-derived peptide that shares some common molecular features with CCK-8 (46-49). 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 K_i and EC_{50} values of sulfated CCK-8 in several binding and *in vitro* bioassays (e.g., amylase release from pancreatic acini in and ileum contractions in guinea pig) (50).

CB_1R activation is reported to decrease gastric emptying, an effect also found in mice fed a high-fat diet for 14 weeks (51, 52). 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 CB_1R 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 minutes after administration of drugs, we administered by oral gavage corn oil (500 µL) that contained 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 Figure 2.7A). Similarly, WD mice displayed no changes in gastric emptying of corn oil

when compared to SD mice (see Figure 2.7B). These data suggest that exogenous activation (WIN in SD mice) or endogenous activation (elevated small intestinal epithelial eCB levels in WD mice) of CB_1Rs 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.

CB_1Rs in pancreatic beta cells control insulin release and glucose homeostasis (53-57). 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 hormones 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 minutes later at time of kill (see Figure S5). Drug treatment had no significant impact on blood glucose levels at any point prior or after gavage of corn oil (see Figure 2.8). These data suggest that, under our conditions, activating CB_1Rs does not impact blood glucose levels following oral gavage of corn oil in mice.

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

We next aimed to identify the mechanism(s) of increased 2-AG and related monoacylglycerol levels (see Table 2.1) 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 Figure 2.9 for validation of enzyme assays). When compared to SD mice, WD mice displayed an increase in activity of DGL (Figure 2.10A; 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 2.10B; 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 2.1 and (21), these effects were met with increased levels of 2-AG in upper small-intestinal epithelium of separate mice (Figure 2.10C; SD = 45.71 ± 6.93, WD = 92.57 ± 16.41 nmol per g; *p*= 0.014, n = 9-10 per diet group). See Figure 2.10D 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, CB_1Rs, and CB_2Rs (Cnr2) was unchanged in whole upper small-intestinal epithelial scrapings from mice fed WD versus SD mice (Figure 2.11A: 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

2.11A; 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 2.11A; 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 2.11A: 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 2.11A: 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 2.11A 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 (21)], 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 (58-61)]. 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 (21); 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 2.11B; $p > 0.05$ not significant, data from 3 mice per diet group). Dagla, Mgll, 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 DIO mice that do not fully correspond to changes in activity of their proteins, including DGL and MGL (see Figure 2.10). 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, 20, 21, 62) – is generated in neighboring cells and therefore may act in a paracrine manner with I-cells that contain CB_1Rs. 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 CB_1Rs 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 CB_1Rs 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 (21), 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 Figure 2.12 and Table 2.2 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 section “Expression of Select eCB System Components in the Upper Small Intestinal Epithelium is Dysregulated in Mice Chronically fed WD and Partially

Conserved in CCK-Positive Cells", in contrast to our previous study (21), 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 2.1) and hyperphagia across relevant parameters in both studies [Table 2.2 and Figure 2.13 and (21)]. 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_A receptors blocks the anorexic effect of AM6545 in DIO.

We next tested the hypothesis that peripheral CB_1Rs 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 2.13A; vehicle = 1.47 $\pm$ 0.15 kcal, AM6545 = 1.13 $\pm$ 0.67 kcal; p<0.05, n=12), rate of intake (Figure 2.13B; vehicle = 0.76 $\pm$ 0.12 kcal per minute, AM6545 = 0.46 $\pm$ 0.05 kcal per minute; p< 0.01), and total caloric intake (Figure 2.13C; vehicle = 9.11 $\pm$ 0.67 kcal per minute, AM6545 = 6.62 $\pm$ 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 2.13A; vehicle = 0.74 $\pm$ 0.05 kcal, AM6545 = 0.71 $\pm$ 0.04 kcal; p> 0.05, n=12), rate of intake (Figure 2.13B; vehicle = 0.30 $\pm$ 0.03 kcal per minute, AM6545 = 0.32 $\pm$ 0.03 kcal per minute; p> 0.05), and total caloric intake (Figure 2.13C; 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 CCK_A receptor antagonist, devazepide (Dev; 0.1 mg per kg), in WD mice blocked the effects of AM6545 on reducing meal size (Figure 2.13A; vehicle = 1.47 ± 0.15 kcal, AM6545+devazepide = 1.49 ± 0.16 kcal; $p > 0.05$), rate of intake (Figure 2.13B; 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 2.13C; 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 2.13C; 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 2.13D), meal duration, (Figure 2.13E), post-meal interval (Figure 2.13F), or first-meal size (Figure 2.13G) 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_A receptors and ensuing 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_1Rs 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_1Rs 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_1Rs blocked this effect, which was reversed by inhibition of peripheral CB_1Rs with a peripherally-restricted CB_1R 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_1Rs in WD mice restored the ability for corn oil to increase CCK levels; *(v)* pharmacological inhibition of peripheral CB_1Rs 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_1R 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, 27, 29, 38) and controls meal size and induces satiation by activating CCK_A receptors on afferent vagal fibers (30-33, 37, 38) and possibly the brain (34, 35). Indeed, polymorphisms in CCK_A receptor genes in humans is associated with increased meal size and food intake, and obesity (63-65). 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 (66-68), and activating CCK_A receptors enhances the anti-obesity properties of GLP-1 agonists, amylin, and leptin (69-72).

Gene transcripts and immunoreactivity for CB_1Rs were found in CCK-containing I-cells in the upper small-intestinal epithelium of mice [see Figures 2.1 and 2.3, and (39)]. Furthermore, the hypophagic effects of AM6545 were blocked by co-administration of the CCK_A receptor antagonist, devazepide. These results suggest that when eCB activity is elevated at local CB_1Rs in the upper small-intestinal epithelium in DIO, increased CB_1R 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 CB_1Rs with AM6545 in DIO mice – at a dose that blocked overeating – restored the ability for corn oil to increase circulating levels of CCK-8.

The mechanisms of CB_1R 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 CB_1Rs block neurotransmitter release is by inhibiting calcium influx or mobilization (6, 73), and nutrient-induced CCK release is calcium-dependent (28, 74-77). Thus, CB_1R 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 (see Figure 2.14 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 (78) and a trend towards lower CCK release after intra-duodenal infusions of oleic acid in overweight or obese subjects (79). Fat-induced CCK secretion and satiation induced by CCK administration were also reduced in rats fed a high-fat diet (80). Other studies, however, reported no differences in CCK levels between obese or lean humans following a meal (81), and increases in CCK after a high-fat meal (82). Furthermore, several preclinical studies in rodents suggest that sensitivity of vagal afferent neurons to the satiating effects of CCK may be decreased in DIO (80, 83-85). 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 (86). Moreover, a variety of studies

conducted over the past several decades show that CCK-induced satiation is mediated by the vagus nerve (30-33, 36-38); however, selected studies show that gut-derived CCK may additionally interact with CCK-A receptors in the brain (34, 35). We used the brain-penetrant CCK-A receptor antagonist, devazepide, in our studies; therefore, we cannot rule out the possibility that CCK-A receptors in the brain participate in the appetite-suppressing effects of CCK release following the inhibition of peripheral CB_1Rs. 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 CB_1R control of nutrient-induced CCK release is one of several mechanisms by which peripheral CB_1Rs impact gut-brain signaling pathways (22, 23, 25). For example, administration of ghrelin – which is produced in the stomach and upper small intestinal epithelium and increases feeding [see for review (1, 87)] – blocked downregulation of CB_1Rs in the nodose ganglion after, both, re-feeding and CCK administration in fasted rats (23). Moreover, pharmacological inhibition of CB_1Rs blocked fasting-induced ghrelin production in rats (88-90), which suggests that CB_1Rs in the upper GI tract may control ghrelin signaling. Furthermore, Kunos and colleagues reported that a peripherally-restricted CB_1R 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). Bellocchio and colleagues reported that the hypophagic effects of CB_1R inhibition with the CB_1R inverse agonist, rimonabant, is blocked by pharmacological inhibition of peripheral beta-adrenergic neurotransmission (91), which suggests that CB_1Rs 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 CB_1Rs 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, 18, 21, 92-97), which may directly interact with CB_1Rs 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 (39)], CB_1Rs are also expressed in K-cells that produce and secrete glucose-dependent insulinotropic peptide [GIP (98, 99)]. Pharmacological activation of CB_1Rs inhibits GIP release in rodents, which suggests that local CB_1Rs 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 (38). 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 CB_1Rs may be at the interface of this

signaling. It is unknown, however, if CB_1Rs control glutamate signaling at these synapses in the small intestine as they do in the brain (100). Collectively, these studies – in combination with the present report – describe key roles for peripheral CB_1Rs in feeding behavior and energy homeostasis.

In summary, our results provide evidence of a previously unknown mechanism of CB_1R-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, 101). Despite the peripherally-restricted properties of these CB_1R 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

1. Steinert RE, Feinle-Bisset C, Asarian L, Horowitz M, Beglinger C, Geary N. Ghrelin, CCK, GLP-1, and PYY(3-36): Secretory Controls and Physiological Roles in Eating and Glycemia in Health, Obesity, and After RYGB. Physiol Rev. 2017;97(1):411-63. doi: 10.1152/physrev.00031.2014. PubMed PMID: 28003328.

2. DiPatrizio NV. Endocannabinoids in the Gut. Cannabis and Cannabinoid Research. 2016;1(1):67-77.

3. DiPatrizio NV, Piomelli D. The thrifty lipids: endocannabinoids and the neural control of energy conservation. Trends Neurosci. 2012;35(7):403-11. Epub 2012/05/25. doi: 10.1016/j.tins.2012.04.006S0166-2236(12)00066-5 [pii]. PubMed PMID: 22622030.

4. DiPatrizio NV, Piomelli D. Intestinal lipid-derived signals that sense dietary fat. The Journal of clinical investigation. 2015;125(3):891-8. doi: 10.1172/JCI76302. PubMed PMID: 25642767; PMCID: PMC4362267.

5. Piomelli D. The molecular logic of endocannabinoid signalling. Nature reviews. 2003;4(11):873-84. PubMed PMID: 14595399.

6. Pertwee RG. Endocannabinoids and Their Pharmacological Actions. Handb Exp Pharmacol. 2015;231:1-37. doi: 10.1007/978-3-319-20825-1_1. PubMed PMID: 26408156.

7. Simon V, Cota D. MECHANISMS IN ENDOCRINOLOGY: Endocannabinoids and metabolism: past, present and future. Eur J Endocrinol. 2017;176(6):R309-R24. doi: 10.1530/EJE-16-1044. PubMed PMID: 28246151.

8. Christensen R, Kristensen PK, Bartels EM, Bliddal H, Astrup A. Efficacy and safety of the weight-loss drug rimonabant: a meta-analysis of randomised trials. Lancet. 2007;370(9600):1706-13. doi: 10.1016/S0140-6736(07)61721-8. PubMed PMID: 18022033.

9. DiPatrizio NV, Astarita G, Schwartz G, Li X, Piomelli D. Endocannabinoid signal in the gut controls dietary fat intake. Proceedings of the National Academy of Sciences of the United States of America. 2011;108(31):12904-8. Epub 2011/07/07. doi: 1104675108 [pii]10.1073/pnas.1104675108. PubMed PMID: 21730161; PMCID: 3150876.

10. DiPatrizio NV, Joslin A, Jung KM, Piomelli D. Endocannabinoid signaling in the gut mediates preference for dietary unsaturated fats. Faseb J. 2013;27(6):2513-20. Epub 2013/03/07. doi: 10.1096/fj.13-227587fj.13-227587 [pii]. PubMed PMID: 23463697; PMCID: 3659363.

11. DiPatrizio NV, Igarashi M, Narayanaswami V, Murray C, Gancayco J, Russell A, Jung KM, Piomelli D. Fasting stimulates 2-AG biosynthesis in the small intestine: role of cholinergic pathways. Am J Physiol Regul Integr Comp Physiol. 2015;309(8):R805-13. doi: 10.1152/ajpregu.00239.2015. PubMed PMID: 26290104.

12. LoVerme J, Duranti A, Tontini A, Spadoni G, Mor M, Rivara S, Stella N, Xu C, Tarzia G, Piomelli D. Synthesis and characterization of a peripherally restricted CB1 cannabinoid antagonist, URB447, that reduces feeding and body-weight gain in mice. Bioorg Med Chem Lett. 2009;19(3):639-43. Epub 2009/01/09. doi: S0960-894X(08)01582-5 [pii]10.1016/j.bmcl.2008.12.059. PubMed PMID: 19128970.

13. Randall PA, Vemuri VK, Segovia KN, Torres EF, Hosmer S, Nunes EJ, Santerre JL, Makriyannis A, Salamone JD. The novel cannabinoid CB1 antagonist AM6545 suppresses food intake and food-reinforced behavior. Pharmacology, biochemistry, and behavior. 2010;97(1):179-84. Epub 2010/08/18. doi: S0091-3057(10)00233-9 [pii]10.1016/j.pbb.2010.07.021. PubMed PMID: 20713079.

14. Cluny NL, Vemuri VK, Chambers AP, Limebeer CL, Bedard H, Wood JT, Lutz B, Zimmer A, Parker LA, Makriyannis A, Sharkey KA. A novel peripherally restricted cannabinoid receptor antagonist, AM6545, reduces food intake and body weight, but does not cause malaise, in rodents. British journal of pharmacology. 2011;161(3):629-42. Epub 2010/10/01. doi: BPH908 [pii]10.1111/j.1476-5381.2010.00908.x. PubMed PMID: 20880401; PMCID: 2990160.

15. Maccarrone M, Bab I, Biro T, Cabral GA, Dey SK, Di Marzo V, Konje JC, Kunos G, Mechoulam R, Pacher P, Sharkey KA, Zimmer A. Endocannabinoid signaling at the periphery: 50 years after THC. Trends Pharmacol Sci. 2015;36(5):277-96. doi: 10.1016/j.tips.2015.02.008. PubMed PMID: 25796370; PMCID: PMC4420685.

16. Tam J, Cinar R, Liu J, Godlewski G, Wesley D, Jourdan T, Szanda G, Mukhopadhyay B, Chedester L, Liow JS, Innis RB, Cheng K, Rice KC, Deschamps JR, Chorvat RJ, McElroy JF, Kunos G. Peripheral cannabinoid-1 receptor inverse agonism reduces obesity by reversing leptin resistance. Cell Metab. 2012;16(2):167-79. Epub 2012/07/31. doi: 10.1016/j.cmet.2012.07.002S1550-4131(12)00277-X [pii]. PubMed PMID: 22841573.

17. Tam J, Szanda G, Drori A, Liu Z, Cinar R, Kashiwaya Y, Reitman ML, Kunos G. Peripheral cannabinoid-1 receptor blockade restores hypothalamic leptin signaling. Molecular metabolism. 2017;6(10):1113-25. Doi: https://doi.org/10.1016/j.molmet.2017.06.010.

18. Hillard CJ. Circulating Endocannabinoids: From Whence Do They Come and Where are They Going? Neuropsychopharmacology. 2017. doi: 10.1038/npp.2017.130. PubMed PMID: 28653665.

19. Gomez R, Navarro M, Ferrer B, Trigo JM, Bilbao A, Del Arco I, Cippitelli A, Nava F, Piomelli D, Rodriguez de Fonseca F. A peripheral mechanism for CB1 cannabinoid receptor-dependent modulation of feeding. J Neurosci. 2002;22(21):9612-7. PubMed PMID: 12417686.

20. Izzo AA, Piscitelli F, Capasso R, Aviello G, Romano B, Borrelli F, Petrosino S, Di Marzo V. Peripheral endocannabinoid dysregulation in obesity: relation to intestinal motility and energy processing induced by food deprivation and re-feeding. British journal of pharmacology. 2009;158(2):451-61. Epub 2009/04/18. doi: BPH183 [pii]10.1111/j.1476-5381.2009.00183.x. PubMed PMID: 19371345; PMCID: 2757684.

21. Argueta DA, DiPatrizio NV. Peripheral endocannabinoid signaling controls hyperphagia in western diet-induced obesity. Physiology & behavior. 2017;171:32-9. doi: 10.1016/j.physbeh.2016.12.044. PubMed PMID: 28065722.

22. Burdyga G, Lal S, Varro A, Dimaline R, Thompson DG, Dockray GJ. Expression of cannabinoid CB1 receptors by vagal afferent neurons is inhibited by cholecystokinin. J Neurosci. 2004;24(11):2708-15. PubMed PMID: 15028763.

23. Burdyga G, Varro A, Dimaline R, Thompson DG, Dockray GJ. Ghrelin receptors in rat and human nodose ganglia: putative role in regulating CB-1 and MCH receptor abundance. American journal of physiology. 2006;290(6):G1289-97. doi: 10.1152/ajpgi.00543.2005. PubMed PMID: 16423919.

24. Burdyga G, Varro A, Dimaline R, Thompson DG, Dockray GJ. Expression of cannabinoid CB1 receptors by vagal afferent neurons: kinetics and role in influencing neurochemical phenotype. American journal of physiology. 2010;299(1):G63-9. doi: 10.1152/ajpgi.00059.2010. PubMed PMID: 20430875; PMCID: 2904113.

25. Cluny NL, Baraboi ED, Mackie K, Burdyga G, Richard D, Dockray GJ, Sharkey KA. High fat diet and body weight have different effects on cannabinoid CB(1) receptor expression in rat nodose ganglia. Auton Neurosci. 2013;179(1-2):122-30. Epub 2013/10/23. doi: 10.1016/j.autneu.2013.09.015. PubMed PMID: 24145047; PMCID: 3866822.

26. Vianna CR, Donato J, Jr., Rossi J, Scott M, Economides K, Gautron L, Pierpont S, Elias CF, Elmquist JK. Cannabinoid receptor 1 in the vagus nerve is dispensable for body weight homeostasis but required for normal gastrointestinal motility. J Neurosci. 2012;32(30):10331-7. Epub 2012/07/28. doi: 10.1523/JNEUROSCI.4507-11.2012. PubMed PMID: 22836266.

27. McLaughlin J, Grazia Luca M, Jones MN, D'Amato M, Dockray GJ, Thompson DG. Fatty acid chain length determines cholecystokinin secretion and effect on human gastric motility. Gastroenterology. 1999;116(1):46-53. PubMed PMID: 9869601.

28. McLaughlin JT, Lomax RB, Hall L, Dockray GJ, Thompson DG, Warhurst G. Fatty acids stimulate cholecystokinin secretion via an acyl chain length-specific, Ca2+-dependent mechanism in the enteroendocrine cell line STC-1. The Journal of physiology. 1998;513 (Pt 1):11-8. PubMed PMID: 9782155; PMCID: PMC2231256.

29. Raybould HE, Glatzle J, Freeman SL, Whited K, Darcel N, Liou A, Bohan D. Detection of macronutrients in the intestinal wall. Auton Neurosci. 2006;125(1-2):28-33. Epub 2006/03/01. doi: S1566-0702(06)00014-2 [pii]10.1016/j.autneu.2006.01.016. PubMed PMID: 16504594.

30. Smith GP, Jerome C, Cushin BJ, Eterno R, Simansky KJ. Abdominal vagotomy blocks the satiety effect of cholecystokinin in the rat. Science. 1981;213(4511):1036-7. Epub 1981/08/28. PubMed PMID: 7268408.

31. Schwartz GJ, Moran TH. CCK elicits and modulates vagal afferent activity arising from gastric and duodenal sites. Annals of the New York Academy of Sciences. 1994;713:121-8. PubMed PMID: 8185153.

32. Raybould HE. Mechanisms of CCK signaling from gut to brain. Current opinion in pharmacology. 2007;7(6):570-4. doi: 10.1016/j.coph.2007.09.006. PubMed PMID: 17954038; PMCID: PMC2692370.

33. Smith GP, Jerome C, Norgren R. Afferent axons in abdominal vagus mediate satiety effect of cholecystokinin in rats. Am J Physiol. 1985;249(5 Pt 2):R638-41. PubMed PMID: 4061684.

34. Ripken D, van der Wielen N, van der Meulen J, Schuurman T, Witkamp RF, Hendriks HF, Koopmans SJ. Cholecystokinin regulates satiation independently of the abdominal vagal nerve in a pig model of total subdiaphragmatic vagotomy. Physiology & behavior. 2015;139:167-76. doi: 10.1016/j.physbeh.2014.11.031. PubMed PMID: 25449395.

35. Reidelberger RD, Hernandez J, Fritzsch B, Hulce M. Abdominal vagal mediation of the satiety effects of CCK in rats. Am J Physiol Regul Integr Comp Physiol. 2004;286(6):R1005-12. Epub 2004/01/01. doi: 10.1152/ajpregu.00646.200300646.2003 [pii]. PubMed PMID: 14701717.

36. Dockray GJ. Enteroendocrine cell signalling via the vagus nerve. Current opinion in pharmacology. 2013. doi: 10.1016/j.coph.2013.09.007. PubMed PMID: 24064396.

37. Schwartz GJ. Roles for gut vagal sensory signals in determining energy availability and energy expenditure. Brain research. 2018;1693(Pt B):151-3. Epub 2018/06/16. doi: 10.1016/j.brainres.2018.04.004. PubMed PMID: 29903617; PMCID: PMC6004821.

38. Kaelberer MM, Buchanan KL, Klein ME, Barth BB, Montoya MM, Shen X, Bohorquez DV. A gut-brain neural circuit for nutrient sensory transduction. Science. 2018;361(6408). Epub 2018/09/22. doi: 10.1126/science.aat5236. PubMed PMID: 30237325.

39. Sykaras AG, Demenis C, Case RM, McLaughlin JT, Smith CP. Duodenal Enteroendocrine I-Cells Contain mRNA Transcripts Encoding Key Endocannabinoid and Fatty Acid Receptors. PLoS ONE. 2012;7(8):e42373. doi: 10.1371/journal.pone.0042373.

40. Gregg LC, Jung KM, Spradley JM, Nyilas R, Suplita RL, 2nd, Zimmer A, Watanabe M, Mackie K, Katona I, Piomelli D, Hohmann AG. Activation of type 5 metabotropic glutamate receptors and diacylglycerol lipase-alpha initiates 2-arachidonoylglycerol formation and endocannabinoid-mediated analgesia. J Neurosci. 2012;32(28):9457-68. Epub 2012/07/13. doi: 10.1523/JNEUROSCI.0013-12.2012. PubMed PMID: 22787031; PMCID: 3652685.

41. Jung KM, Sepers M, Henstridge CM, Lassalle O, Neuhofer D, Martin H, Ginger M, Frick A, DiPatrizio NV, Mackie K, Katona I, Piomelli D, Manzoni OJ. Uncoupling of the endocannabinoid signalling complex in a mouse model of fragile X syndrome. Nat Commun. 2012;3:1080. Epub 2012/09/27. doi: 10.1038/ncomms2045ncomms2045 [pii]. PubMed PMID: 23011134.

42. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-8. Epub 2002/02/16. doi: 10.1006/meth.2001.1262. PubMed PMID: 11846609.

43. Schmidt MJ, Horvath S, Ebert P, Norris JL, Seeley EH, Brown J, Gellert L, Everheart M, Garbett KA, Grice TW, Caprioli RM, Mirnics K. Modulation of behavioral networks by selective interneuronal inactivation. Molecular psychiatry. 2014;19(5):580-7. Epub 2013/12/11. doi: 10.1038/mp.2013.167. PubMed PMID: 24322205; PMCID: PMC4179403.

44. Cvijanovic N, Isaacs NJ, Rayner CK, Feinle-Bisset C, Young RL, Little TJ. Duodenal fatty acid sensor and transporter expression following acute fat exposure in healthy lean humans. Clin Nutr. 2017;36(2):564-9. doi: 10.1016/j.clnu.2016.02.005. PubMed PMID: 26926575.

45. Raybould HE. Nutrient tasting and signaling mechanisms in the gut. I. Sensing of lipid by the intestinal mucosa. Am J Physiol. 1999;277(4 Pt 1):G751-5. PubMed PMID: 10516140.

46. Wolfe MM, Paquet RJ, Reel GM. Specificity of commercially available antibodies used for gastrin measurement. J Lab Clin Med. 1985;105(4):417-21. Epub 1985/04/01. PubMed PMID: 3981055.

47. Walsh JH, Lamers CB, Valenzuela JE. Cholecystokinin-octapeptidelike immunoreactivity in human plasma. Gastroenterology. 1982;82(3):438-44. Epub 1982/03/01. PubMed PMID: 6172314.

48. Eysselein VE, Reeve JR, Jr., Shively JE, Miller C, Walsh JH. Isolation of a large cholecystokinin precursor from canine brain. Proceedings of the National Academy of Sciences of the United States of America. 1984;81(21):6565-8. Epub 1984/11/01. PubMed PMID: 6093106; PMCID: PMC391970.

49. Shulkes A, Baldwin GS. Biology of gut cholecystokinin and gastrin receptors. Clin Exp Pharmacol Physiol. 1997;24(3-4):209-16. Epub 1997/03/01. PubMed PMID: 9131287.

50. Charpentier B, Pelaprat D, Durieux C, Dor A, Reibaud M, Blanchard JC, Roques BP. Cyclic cholecystokinin analogues with high selectivity for central receptors. Proceedings of the National Academy of Sciences of the United States of America. 1988;85(6):1968-72. Epub 1988/03/01. PubMed PMID: 3162318; PMCID: PMC279903.

51. Pertwee RG. Cannabinoids and the gastrointestinal tract. Gut. 2001;48(6):859-67. PubMed PMID: 11358910; PMCID: PMC1728337.

52. Di Marzo V, Capasso R, Matias I, Aviello G, Petrosino S, Borrelli F, Romano B, Orlando P, Capasso F, Izzo AA. The role of endocannabinoids in the regulation of gastric emptying: alterations in mice fed a high-fat diet. Br J Pharmacol. 2008;153(6):1272-80. Epub 2008/01/29. doi: 0707682 [pii]10.1038/sj.bjp.0707682. PubMed PMID: 18223666; PMCID: 2275439.

53. Juan-Pico P, Fuentes E, Bermudez-Silva FJ, Diaz-Molina F, Ripoll C, Rodriguez de Fonseca F, Nadal A. Cannabinoid receptors regulate Ca(2+) signals and insulin secretion in pancreatic beta-cell. Cell Calcium. 2006;39(0143-4160 (Print)):155-62; PMCID: 16321437.

54. De Petrocellis L, Marini P, Matias I, Moriello AS, Starowicz K, Cristino L, Nigam S, Di Marzo V. Mechanisms for the coupling of cannabinoid receptors to intracellular calcium mobilization in rat insulinoma beta-cells. Exp Cell Res. 2007;313(14):2993-3004. doi: 10.1016/j.yexcr.2007.05.012. PubMed PMID: 17585904.

55. Bermudez-Silva FJ, Baixeras E, Cobo N, Bautista D, Cuesta-Munoz AL, Fuentes E, Juan-Pico P, Castro MJ, Milman G, Mechoulam R, Nadal A, Rodriguez de Fonseca F. Presence of functional cannabinoid receptors in human endocrine pancreas. Diabetologia. 2008;51(3):476–87. Epub December 19, 2007.

56. Nakata M, Yada T. Cannabinoids inhibit insulin secretion and cytosolic Ca2+ oscillation in islet beta-cells via CB1 receptors. Regul Pept. 2008;145(1-3):49-53. doi: 10.1016/j.regpep.2007.08.009. PubMed PMID: 17884194.

57. Li C, Bowe JE, Jones PM, Persaud SJ. Expression and function of cannabinoid receptors in mouse islets. Islets. 2010;2(5):293-302. PubMed PMID: 21099327.

58. Thors L, Belghiti M, Fowler CJ. Inhibition of fatty acid amide hydrolase by kaempferol and related naturally occurring flavonoids. British journal of pharmacology. 2008;155(2):244-52. Epub 2008/06/17. doi: 10.1038/bjp.2008.237. PubMed PMID: 18552875; PMCID: PMC2538700.

59. Peroni RN, Abramoff T, Neuman I, Podesta EJ, Adler-Graschinsky E. Phytoestrogens enhance the vascular actions of the endocannabinoid anandamide in mesenteric beds of female rats. Int J Hypertens. 2012;2012:647856. Epub 2012/02/10. doi: 10.1155/2012/647856. PubMed PMID: 22319644; PMCID: PMC3272812.

60. McFarland MJ, Porter AC, Rakhshan FR, Rawat DS, Gibbs RA, Barker EL. A role for caveolae/lipid rafts in the uptake and recycling of the endogenous cannabinoid anandamide. J Biol Chem. 2004;279(40):41991-7. Epub 2004/08/05. doi: 10.1074/jbc.M407250200. PubMed PMID: 15292270.

61. Thors L, Eriksson J, Fowler CJ. Inhibition of the cellular uptake of anandamide by genistein and its analogue daidzein in cells with different levels of fatty acid amide hydrolase-driven uptake. British journal of pharmacology. 2007;152(5):744-50. Epub 2007/08/07. doi: 10.1038/sj.bjp.0707401. PubMed PMID: 17676056; PMCID: PMC2190009.

62. Perez PA, DiPatrizio NV. Impact of maternal western diet-induced obesity on offspring mortality and peripheral endocannabinoid system in mice. PLoS One. 2018;13(10):e0205021. doi: 10.1371/journal.pone.0205021. PubMed PMID: 30273406.

63. de Krom M, van der Schouw YT, Hendriks J, Ophoff RA, van Gils CH, Stolk RP, Grobbee DE, Adan R. Common genetic variations in CCK, leptin, and leptin receptor genes are associated with specific human eating patterns. Diabetes. 2007;56(1):276-80. Epub 2006/12/29. doi: 10.2337/db06-0473. PubMed PMID: 17192493.

64. Marchal-Victorion S, Vionnet N, Escrieut C, Dematos F, Dina C, Dufresne M, Vaysse N, Pradayrol L, Froguel P, Fourmy D. Genetic, pharmacological and functional analysis of cholecystokinin-1 and cholecystokinin-2 receptor polymorphism in type 2 diabetes and obese patients. Pharmacogenetics. 2002;12(1):23-30. Epub 2002/01/05. PubMed PMID: 11773861.

65. Miller LJ, Holicky EL, Ulrich CD, Wieben ED. Abnormal processing of the human cholecystokinin receptor gene in association with gallstones and obesity. Gastroenterology. 1995;109(4):1375-80. Epub 1995/10/01. PubMed PMID: 7557108.

66. Irwin N, Frizelle P, Montgomery IA, Moffett RC, O'Harte FPM, Flatt PR. Beneficial effects of the novel cholecystokinin agonist (pGlu-Gln)-CCK-8 in mouse models of obesity/diabetes. Diabetologia. 2012;55(10):2747-58. Epub 2012/07/21. doi: 10.1007/s00125-012-2654-6. PubMed PMID: 22814764.

67. Irwin N, Montgomery IA, O'Harte FP, Frizelle P, Flatt PR. Comparison of the independent and combined metabolic effects of subchronic modulation of CCK and GIP receptor action in obesity-related diabetes. International journal of obesity (2005). 2013;37(8):1058-63. Epub 2012/11/21. doi: 10.1038/ijo.2012.179. PubMed PMID: 23164696.

68. Pierson ME, Comstock JM, Simmons RD, Kaiser F, Julien R, Zongrone J, Rosamond JD. Synthesis and biological evaluation of potent, selective, hexapeptide CCK-A agonist anorectic agents. J Med Chem. 1997;40(26):4302-7. Epub 1998/01/22. doi: 10.1021/jm970477u. PubMed PMID: 9435899.

69. Irwin N, Pathak V, Flatt PR. A Novel CCK-8/GLP-1 Hybrid Peptide Exhibiting Prominent Insulinotropic, Glucose-Lowering, and Satiety Actions With Significant Therapeutic Potential in High-Fat-Fed Mice. Diabetes. 2015;64(8):2996-3009. Epub 2015/04/18. doi: 10.2337/db15-0220. PubMed PMID: 25883113.

70. Irwin N, Montgomery IA, Flatt PR. Comparison of the metabolic effects of sustained CCK1 receptor activation alone and in combination with upregulated leptin signalling in high-fat-fed mice. Diabetologia. 2013;56(6):1425-35. Epub 2013/03/07. doi: 10.1007/s00125-013-2878-0. PubMed PMID: 23462797.

71. Trevaskis JL, Sun C, Athanacio J, D'Souza L, Samant M, Tatarkiewicz K, Griffin PS, Wittmer C, Wang Y, Teng CH, Forood B, Parkes DG, Roth JD. Synergistic metabolic benefits of an exenatide analogue and cholecystokinin in diet-induced obese and leptin-deficient rodents. Diabetes, obesity & metabolism. 2015;17(1):61-73. Epub 2014/09/11. doi: 10.1111/dom.12390. PubMed PMID: 25204356.

72. Trevaskis JL, Turek VF, Griffin PS, Wittmer C, Parkes DG, Roth JD. Multi-hormonal weight loss combinations in diet-induced obese rats: therapeutic potential of cholecystokinin? Physiology & behavior. 2010;100(2):187-95. Epub 2010/03/09. doi: 10.1016/j.physbeh.2010.02.023. PubMed PMID: 20206194.

73. Howlett AC, Blume LC, Dalton GD. CB(1) cannabinoid receptors and their associated proteins. Curr Med Chem. 2010;17(14):1382-93. Epub 2010/02/20. PubMed PMID: 20166926; PMCID: PMC3179980.

74. Hira T, Elliott AC, Thompson DG, Case RM, McLaughlin JT. Multiple fatty acid sensing mechanisms operate in enteroendocrine cells: novel evidence for direct mobilization of stored calcium by cytosolic fatty acid. J Biol Chem. 2004;279(25):26082-9. Epub 2004/04/07. doi: 10.1074/jbc.M400098200. PubMed PMID: 15066999.

75. Liou AP, Sei Y, Zhao X, Feng J, Lu X, Thomas C, Pechhold S, Raybould HE, Wank SA. The extracellular calcium-sensing receptor is required for cholecystokinin secretion in response to l-phenylalanine in acutely isolated intestinal I cells. American Journal of Physiology - Gastrointestinal and Liver Physiology. 2011;300(4):G538-G46. doi: 10.1152/ajpgi.00342.2010. PubMed PMID: PMC3074990.

76. Nakajima S, Hira T, Hara H. Calcium-sensing receptor mediates dietary peptide-induced CCK secretion in enteroendocrine STC-1 cells. Mol Nutr Food Res. 2012;56(5):753-60. Epub 2012/06/01. doi: 10.1002/mnfr.201100666. PubMed PMID: 22648622.

77. Gevrey J-C, Cordier-Bussat M, Némoz-Gaillard E, Chayvialle J-A, Abello J. Co-requirement of Cyclic AMP- and Calcium-dependent Protein Kinases for Transcriptional Activation of Cholecystokinin Gene by Protein Hydrolysates. Journal of Biological Chemistry. 2002;277(25):22407-13. doi: 10.1074/jbc.M201624200.

78. Baranowska B, Radzikowska M, Wasilewska-Dziubinska E, Roguski K, Borowiec M. Disturbed release of gastrointestinal peptides in anorexia nervosa and in obesity. Diabetes, obesity & metabolism. 2000;2(2):99-103. Epub 2001/02/28. PubMed PMID: 11220530.

79. Stewart JE, Seimon RV, Otto B, Keast RS, Clifton PM, Feinle-Bisset C. Marked differences in gustatory and gastrointestinal sensitivity to oleic acid between lean and obese men. Am J Clin Nutr. 2011;93(4):703-11. Epub 2011/02/12. doi: 10.3945/ajcn.110.007583. PubMed PMID: 21310831.

80. Duca FA, Zhong L, Covasa M. Reduced CCK signaling in obese-prone rats fed a high fat diet. Hormones and behavior. 2013;64(5):812-7. doi: 10.1016/j.yhbeh.2013.09.004. PubMed PMID: 24100196.

81. Brennan IM, Luscombe-Marsh ND, Seimon RV, Otto B, Horowitz M, Wishart JM, Feinle-Bisset C. Effects of fat, protein, and carbohydrate and protein load on appetite, plasma cholecystokinin, peptide YY, and ghrelin, and energy intake in lean and obese men. American journal of physiology. 2012;303(1):G129-40. doi: 10.1152/ajpgi.00478.2011. PubMed PMID: 22556143.

82. French SJ, Murray B, Rumsey RD, Sepple CP, Read NW. Preliminary studies on the gastrointestinal responses to fatty meals in obese people. Int J Obes Relat Metab Disord. 1993;17(5):295-300. Epub 1993/05/01. PubMed PMID: 8389339.

83. Covasa M, Grahn J, Ritter RC. High fat maintenance diet attenuates hindbrain neuronal response to CCK. Regul Pept. 2000;86(1-3):83-8. Epub 2000/02/15. PubMed PMID: 10672906.

84. Daly DM, Park SJ, Valinsky WC, Beyak MJ. Impaired intestinal afferent nerve satiety signalling and vagal afferent excitability in diet induced obesity in the mouse. The Journal of physiology. 2011;589(Pt 11):2857-70. Epub 2011/04/14. doi: 10.1113/jphysiol.2010.204594. PubMed PMID: 21486762; PMCID: PMC3112560.

85. de Lartigue G. Role of the vagus nerve in the development and treatment of diet-induced obesity. The Journal of physiology. 2016. doi: 10.1113/JP271538. PubMed PMID: 26959077.

86. Lieverse RJ, Jansen JB, Masclee AA, Lamers CB. Satiety effects of a physiological dose of cholecystokinin in humans. Gut. 1995;36(2):176-9. Epub 1995/02/01. PubMed PMID: 7883212; PMCID: PMC1382399.

87. Kaelberer MM, Bohorquez DV. The now and then of gut-brain signaling. Brain research. 2018;1693(Pt B):192-6. Epub 2018/03/28. doi: 10.1016/j.brainres.2018.03.027. PubMed PMID: 29580839; PMCID: PMC6003878.

88. Al-Massadi O, Gabellieri E, Trujillo ML, Senaris R, Pagotto U, Pasquali R, Casanueva FF, Seoane LM. Peripheral endocannabinoid system-mediated actions of rimonabant on growth hormone secretion are ghrelin-dependent. J Neuroendocrinol. 2011;22(11):1127-36. Epub 2010/09/03. doi: JNE2065 [pii]10.1111/j.1365-2826.2010.02065.x. PubMed PMID: 20807320.

89. Senin LL, Al-Massadi O, Folguiera C, Pardo M, Barja-Fernandez S, Roca-Rivada A, Amil M, Criujeiras AB, Garcia-Caballero T, Gabellieri E, Leis R, Dieguez C, Pagotto U, Casanueva FF, Seoane LM. The gasric CB1 receptor modulates ghrelin production through the mTOR pathway to regulate food intake. PLoS ONE. 2013;8(11):e80339.

90. Cani PD, Montoya ML, Neyrinck AM, Delzenne NM, Lambert DM. Potential modulation of plasma ghrelin and glucagon-like peptide-1 by anorexigenic cannabinoid compounds, SR141716A (rimonabant) and oleoylethanolamide. Br J Nutr. 2004;92(5):757-61. Epub 2004/11/10. doi: S0007114504002363 [pii]. PubMed PMID: 15533263.

91. Bellocchio L, Soria-Gomez E, Quarta C, Metna-Laurent M, Cardinal P, Binder E, Cannich A, Delamarre A, Haring M, Martin-Fontecha M, Vega D, Leste-Lasserre T, Bartsch D, Monory K, Lutz B, Chaouloff F, Pagotto U, Guzman M, Cota D, Marsicano G. Activation of the sympathetic nervous system mediates hypophagic and anxiety-like effects of CB1 receptor blockade. Proceedings of the National Academy of Sciences of the United States of America. 2013;110(12):4786-91. Epub 2013/03/15. doi: 10.1073/pnas.1218573110 1218573110 [pii]. PubMed PMID: 23487769; PMCID: 3607008.

92. Little TJ, Cvijanovic N, DiPatrizio NV, Argueta DA, Rayner CK, Feinle-Bisset C, Young RL. Plasma endocannabinoid levels in lean, overweight and obese humans: relationships with intestinal permeability markers, inflammation and incretin secretion. Am J Physiol Endocrinol Metab. 2018. Epub 2018/02/14. doi: 10.1152/ajpendo.00355.2017. PubMed PMID: 29438631.

93. Bluher M, Engeli S, Kloting N, Berndt J, Fasshauer M, Batkai S, Pacher P, Schon MR, Jordan J, Stumvoll M. Dysregulation of the peripheral and adipose tissue endocannabinoid system in human abdominal obesity. Diabetes. 2006;55(11):3053-60. PubMed PMID: 17065342.

94. Cote M, Matias I, Lemieux I, Petrosino S, Almeras N, Despres JP, Di Marzo V. Circulating endocannabinoid levels, abdominal adiposity and related cardiometabolic risk factors in obese men. International journal of obesity (2005). 2007;31(4):692-9. Epub 2007/01/17. doi: 0803539 [pii]10.1038/sj.ijo.0803539. PubMed PMID: 17224929.

95. Di Marzo V, Cote M, Matias I, Lemieux I, Arsenault BJ, Cartier A, Piscitelli F, Petrosino S, Almeras N, Despres JP. Changes in plasma endocannabinoid levels in viscerally obese men following a 1 year lifestyle modification programme and waist circumference reduction: associations with changes in metabolic risk factors. Diabetologia. 2009;52(2):213-7. Epub 2008/10/31. doi: 10.1007/s00125-008-1178-6. PubMed PMID: 18972095.

96. Matias I, Gatta-Cherifi B, Tabarin A, Clark S, Leste-Lasserre T, Marsicano G, Piazza PV, Cota D. Endocannabinoids measurement in human saliva as potential biomarker of obesity. PLoS One. 2012;7(7):e42399. doi: 10.1371/journal.pone.0042399. PubMed PMID: 22860123; PMCID: PMC3409167.

97. Engeli S, Bohnke J, Feldpausch M, Gorzelniak K, Janke J, Batkai S, Pacher P, Harvey-White J, Luft FC, Sharma AM, Jordan J. Activation of the peripheral endocannabinoid system in human obesity. Diabetes. 2005;54(10):2838-43. PubMed PMID: 16186383.

98. Moss CE, Marsh WJ, Parker HE, Ogunnowo-Bada E, Riches CH, Habib AM, Evans ML, Gribble FM, Reimann F. Somatostatin receptor 5 and cannabinoid receptor 1 activation inhibit secretion of glucose-dependent insulinotropic polypeptide from intestinal K cells in rodents. Diabetologia. 2012;55(11):3094-103. Epub 2012/08/09. doi: 10.1007/s00125-012-2663-5. PubMed PMID: 22872212; PMCID: PMC3464380.

99. Reimann F, Gribble FM. Mechanisms underlying glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1 secretion. J Diabetes Investig. 2016;7 Suppl 1:13-9. doi: 10.1111/jdi.12478. PubMed PMID: 27186350; PMCID: PMC4854499.

100. Jung KM, Astarita G, Zhu C, Wallace M, Mackie K, Piomelli D. A key role for diacylglycerol lipase-alpha in metabotropic glutamate receptor-dependent endocannabinoid mobilization. Molecular pharmacology. 2007;72(3):612-21. Epub 2007/06/23. doi: mol.107.037796 [pii]10.1124/mol.107.037796. PubMed PMID: 17584991.

101. Khera R, Murad MH, Chandar AK, Dulai PS, Wang Z, Prokop LJ, Loomba R, Camilleri M, Singh S. Association of Pharmacological Treatments for Obesity With Weight Loss and Adverse Events: A Systematic Review and Meta-analysis. JAMA. 2016;315(22):2424-34. doi: 10.1001/jama.2016.7602. PubMed PMID: 27299618.

102. Tanaka T, Katsuma S, Adachi T, Koshimizu TA, Hirasawa A, Tsujimoto G. Free fatty acids induce cholecystokinin secretion through GPR120. Naunyn Schmiedebergs Arch Pharmacol. 2008;377(4-6):523-7. doi: 10.1007/s00210-007-0200-8. PubMed PMID: 17972064.

103. Liou AP, Lu X, Sei Y, Zhao X, Pechhold S, Carrero RJ, Raybould HE, Wank S. The G-protein-coupled receptor GPR40 directly mediates long-chain fatty acid-induced secretion of cholecystokinin. Gastroenterology. 2011;140(3):903-12. doi: 10.1053/j.gastro.2010.10.012. PubMed PMID: 20955703; PMCID: PMC4717904.

104. Wang Y, Chandra R, Samsa LA, Gooch B, Fee BE, Cook JM, Vigna SR, Grant AO, Liddle RA. Amino acids stimulate cholecystokinin release through the Ca2+-sensing receptor. American journal of physiology. 2011;300(4):G528-37. Epub 2010/12/25. doi: 10.1152/ajpgi.00387.2010. PubMed PMID: 21183662; PMCID: PMC3074989.

105. Rehfeld JF. Accurate measurement of cholecystokinin in plasma. Clin Chem. 1998;44(5):991-1001. Epub 1998/05/20. PubMed PMID: 9590372.

Tables

Table 2.1. Impact of diet on monoacylglycerols in mouse small-intestinal epithelium.

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.

MAG	20:4 (2-AG) (nmol g^{-1})	18:1 (nmol g^{-1})	18:2 (nmol g^{-1})	16:0 (nmol g^{-1})	22:6 (nmol g^{-1})	Total (nmol g^{-1})
SD	80.23 ± 8.542	49.57 ± 9.804	217.7 ± 52.09	33.99 ± 4.125	9.413 ± 1.996	390.9 ± 72.24
WD	132.5 ± 22.20	109.0 ± 22.03	415.3 ± 83.26	150.1 ± 21.53	22.63 ± 3.703	829.4 ± 144.2
P-value	**0.0353**	**0.0206**	0.0554	**<0.0001**	**0.0049**	**0.0122**

Table 2.2. Consumption of WD is associated with hyperphagia.

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.

	Δ Body Mass (g)	Meal Size (kcal)	Intake Rate (kcal min^{-1})	24h Intake (kcal)	First Meal (kcal)	Frequency (meals day^{-1})	Duration (Min)	PMI (Min)
SD	8.90 ±0.31	0.69 ±0.04	0.33 ±0.02	8.10 ±0.61	0.72 ±0.11	11.67 ±0.45	8.61 ±1.16	114.7 ±8.4
WD	18.14 ±0.46	1.29 ±0.10	0.71 ±0.08	13.28 ±0.81	1.99 ±0.66	9.83 ±1.42	6.85 ±0.98	129.3 ±14.3
P-value	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	0.07	0.23	0.26	0.31

Figures

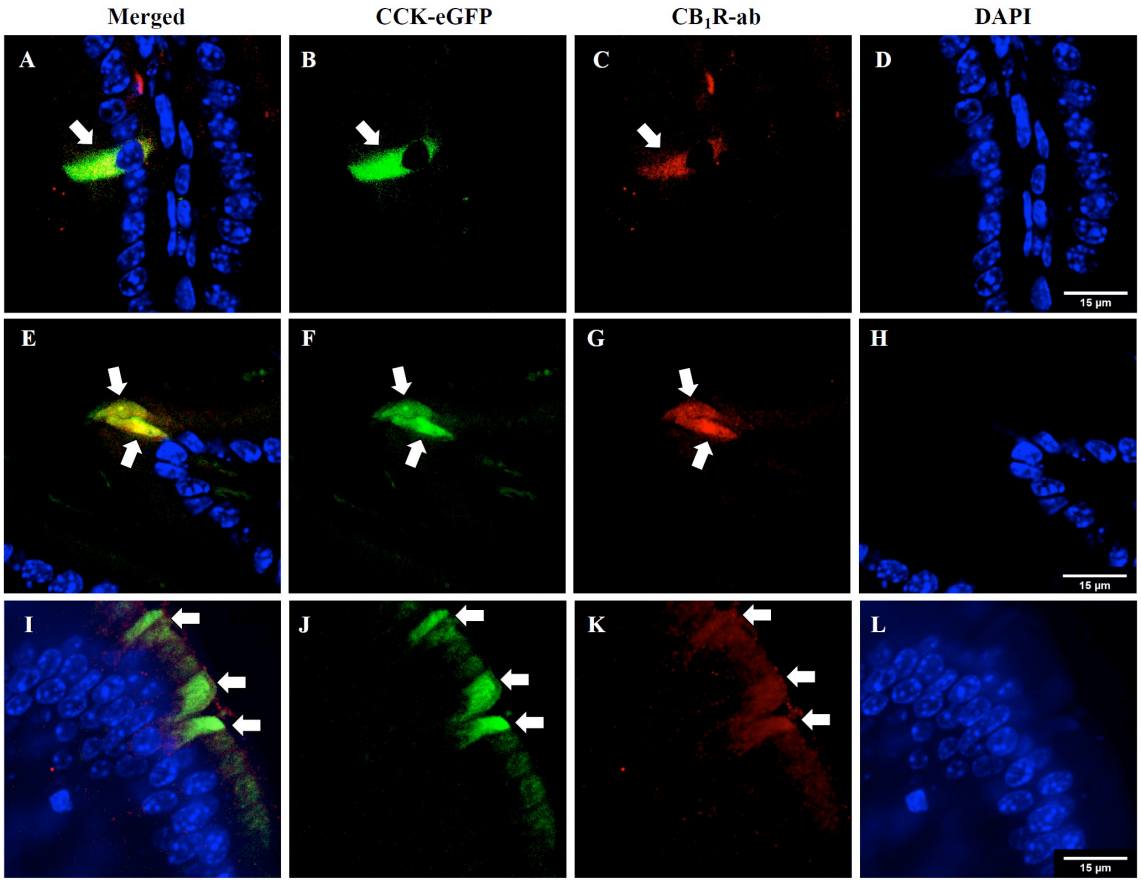

Figure 2.1. CB_1Rs co-localize with CCK-containing cells in the upper small-intestinal epithelium. Immunohistochemical detection of eGFP (CCK-eGFP: B, F, J) and CB_1Rs (CB_1R-ab: C, G, K) reveals co-localization (merge: A, E, I) in villi of intestinal epithelium. Arrows indicate separate enteroendocrine cells that contain immunoreactivity for CB_1Rs that co-localize with CCK-eGFP cells. Representative images from three CCK-eGFP mice. (DAPI stain D, H, L) (Scale bar 15 µm)

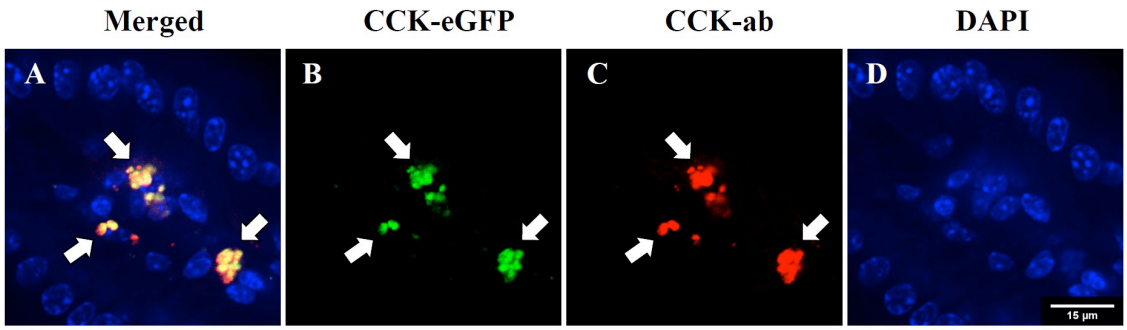

Figure 2.2. CCK receptors co-localize with eGFP in upper small-intestinal epithelium. Immunohistochemical detection of CCK (CCK-ab) in eGFP-containing cells (CCK-eGFP) confirms co-localization, which highlights the validity of this CCK-eGFP reporter mouse. Arrows indicate three separate enteroendocine 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)

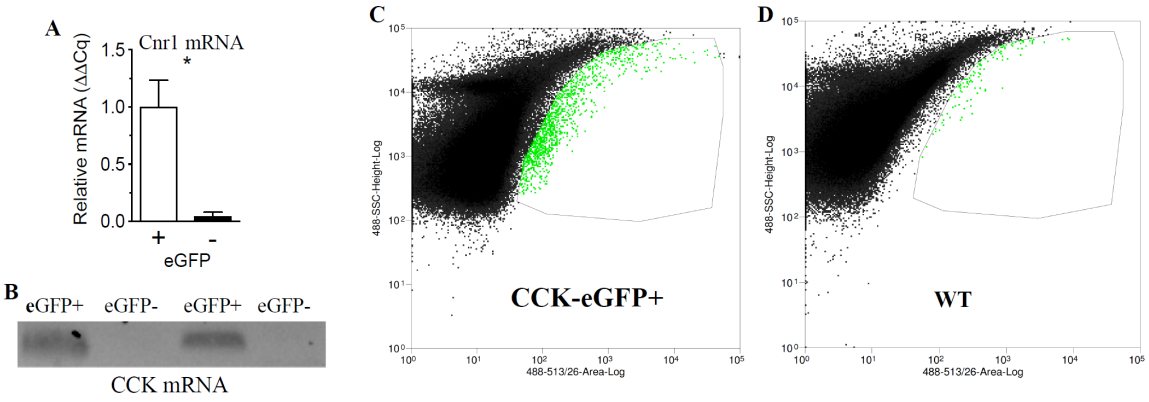

Figure 2.3. CB_1R mRNA expression is enriched in CCK-containing cells in the upper small-intestinal epithelium. Fluorescence-activated cell sorting (FACS) of eGFP-CCK-positive (+) and eGFP-CCK-negative (-) cells from the upper small-intestinal epithelium reveals enhanced Cnr1 expression in eGFP-CCK-positive 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.

A. CCK-eGFP-positive cells

Region	Count	% Hist	% All	Median	CV
Total	200212	100.00	100.00	12.53, 1253.25	1668.60, 194.75
R2	1255	0.63	* 0.63	137.17, 2955.21	404.63, 142.79

B. Wild-type cells

Region	Count	% Hist	% All	Median	CV
Total	199038	100.00	100.00	7.63, 2154.44	377.49, 150.24
R2	121	0.06	*0.06	387.47, 15706.47	158.69, 74.50

Figure 2.4. 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 2C) 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 2D), which represents less than 10% of CCK-eGFP-positive cells from CCK-eGFP reporter mice and likely reflects autofluorescence.

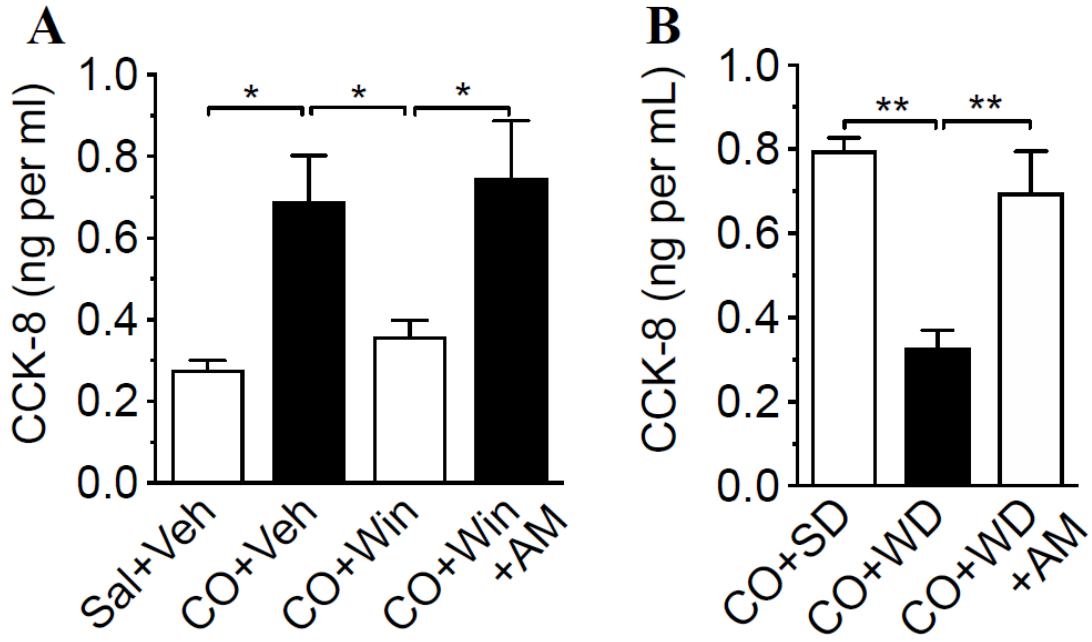
A
CCK-8 (ng per ml)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
Sal+Veh
CO+Veh
CO+Win
CO+Win +AM
B
CCK-8 (ng per mL)
1.0
0.8
0.6
0.4
0.2
0.0
**
**
CO+SD
CO+WD
CO+WD +AM

Figure 2.5. Exogenous or endogenous activation of peripheral CB_1Rs 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 CB_1R 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 CB_1R 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 CB_1Rs 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, * $p<0.05$, ** $p<0.01$.

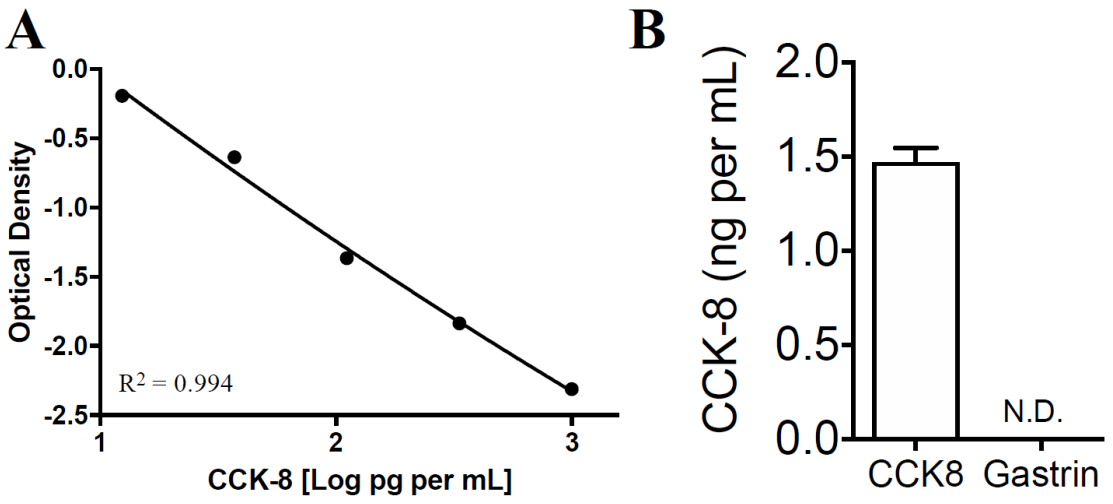

Figure 2.6. Standard curve for ELISA analysis of CCK-8 in plasma and analysis of gastrin cross-reactivity. All values of CCK-8 fall within the range of the standard curve (10 to 1000 pmol per mL) for CCK-8 quantitation by a sensitive and selective CCK-8 ELISA (A), which shows no cross-reactivity for 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.

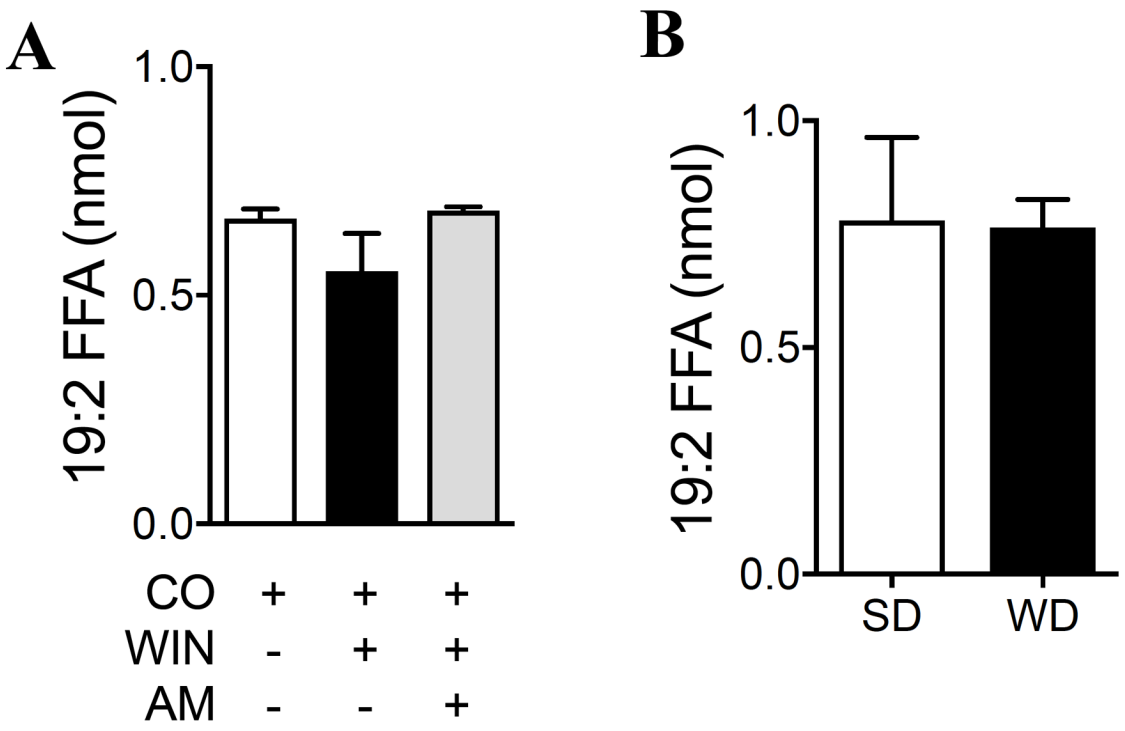

Figure 2.7. Analysis of gastric emptying. Gastric emptying was evaluated by quantitating via UPLC/MS/MS levels of 19:2 free fatty acid (19:2 FFA) recovered from stomach 30 min following oral gavage of corn oil (CO) in mice maintained on a standard low-fat no sucrose chow (SD). Thirty min prior to gavage, 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 ±SEM. Analyzed using one-way ANOVA (A) or student's t-test (B; two tailed). n=4 (A) or 3 (b) per condition, $p>0.05$.

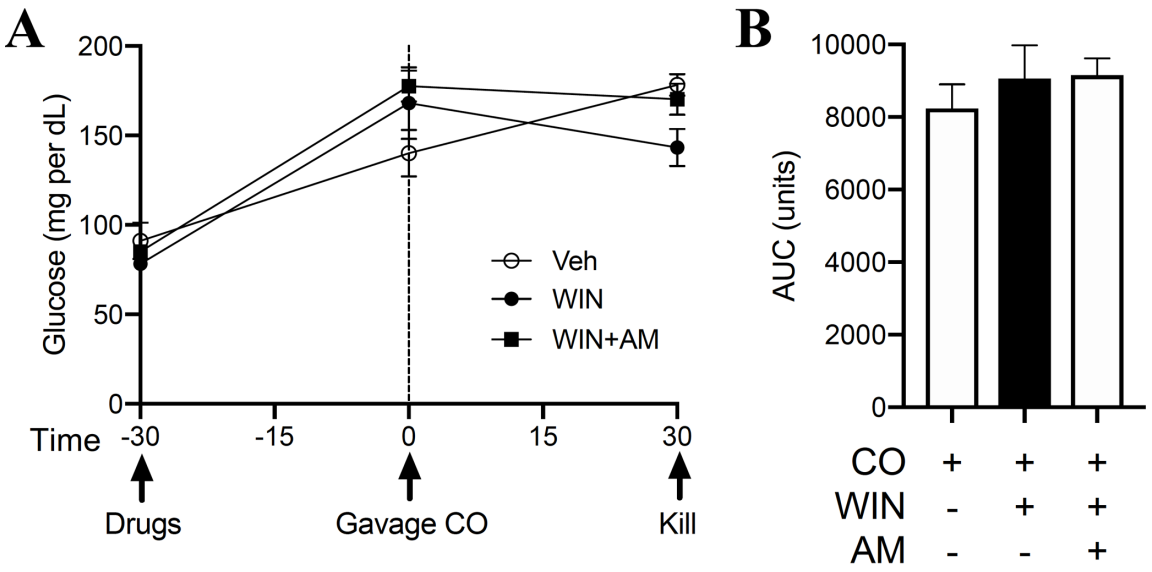

Figure 2.8. Effects of drug treatment on glucose levels in mice maintained on a standard low-fat no sucrose diet. Blood glucose levels were measured at time -30 just prior to administration of the drugs WIN 55,212-2 (WIN), AM6545 (AM), or vehicle (Veh). Blood glucose was again measured 30 min later at 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 is expressed as mean ±SEM. 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, $p>0.05$.

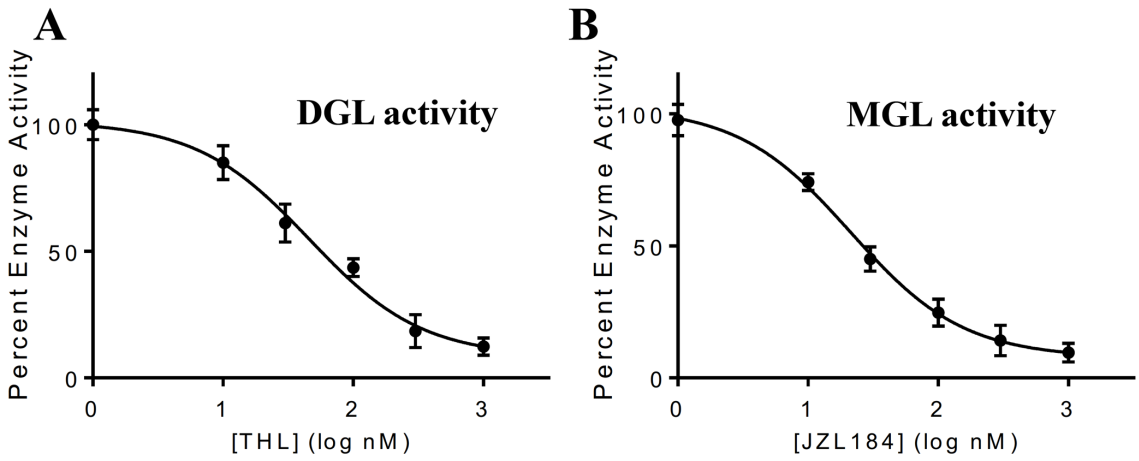

Figure 2.9. Validation of DGL and MGL functional enzyme assays. Inhibitors of DGL (A) and MGL (B) (THL and JZL184, respectively) dose-dependently inhibited hydrolytic activity of these enzymes in isolated protein from the upper small intestinal epithelium. Analyzed using nonlinear regression of log-inhibitor vs. response. n=3 per group and R^2=goodness of fit >0.8.

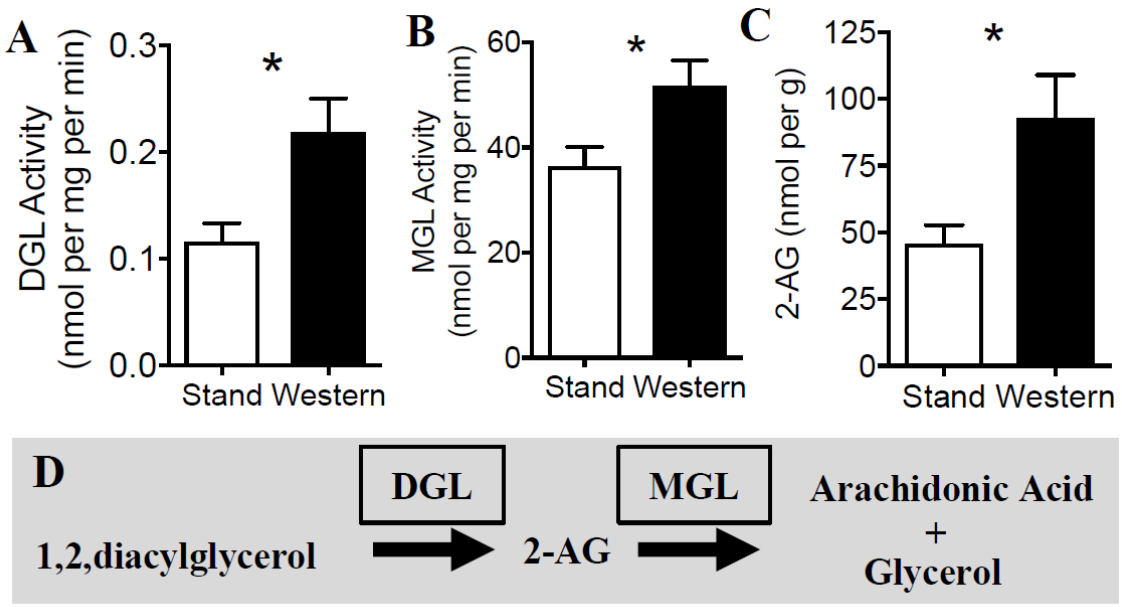

Figure 2.10. 2-AG biosynthesis and degradation are upregulated in small intestine during obesity. Hydrolytic activity of DGL (A) and MGL (B) are increased in mice maintained on western diet (WD) when compared to controls fed a standard diet (SD). Levels of the endocannabinoid, 2-AG, are increased in jejunum mucosa of WD mice, when compared to SD mice (C). 2-AG is formed by the hydrolysis of its 1,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.

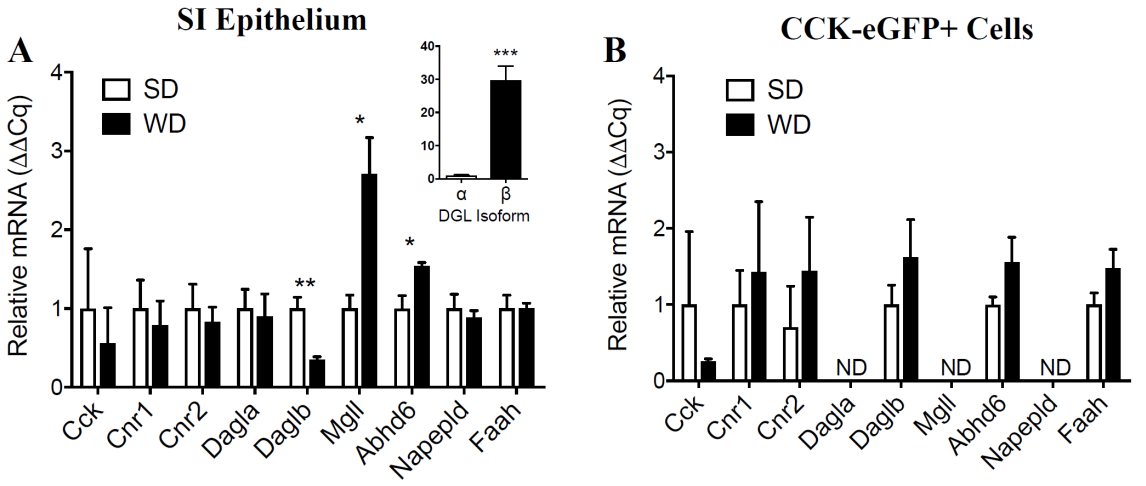

Figure 2.11. Expression of select components of the eCB system is dysregulated in the upper small intestine of DIO mice and partially conserved in CCK-eGFP+ cells. Expression of mRNA for cholecystokinin (Cck), cannabinoid receptor subtype 1 (Cnr1) and 2 (Cnr2), and other components of the eCB system 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).

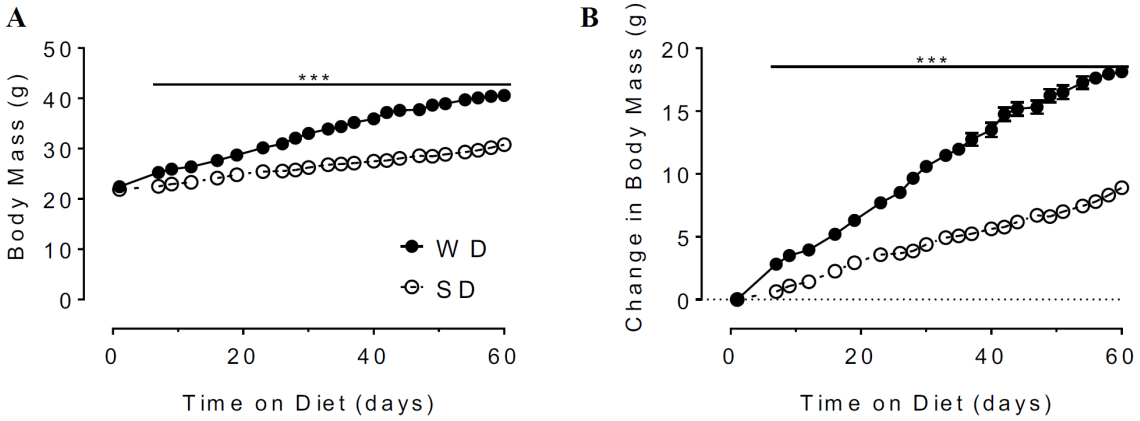

Figure 2.12. Mice fed WD displayed large increases in body weight. Mice maintained on a western diet for 60 days showed a significant increase in body weight (A) and change in body weight (B) when compared to littermates maintained on a chow diet. Data expressed as mean ± S.E.M. Analyzed using repeated measures (time) two-way ANOVA with Sidak's post hoc multiple comparison's test. n = 9-10, ***p<0.001.

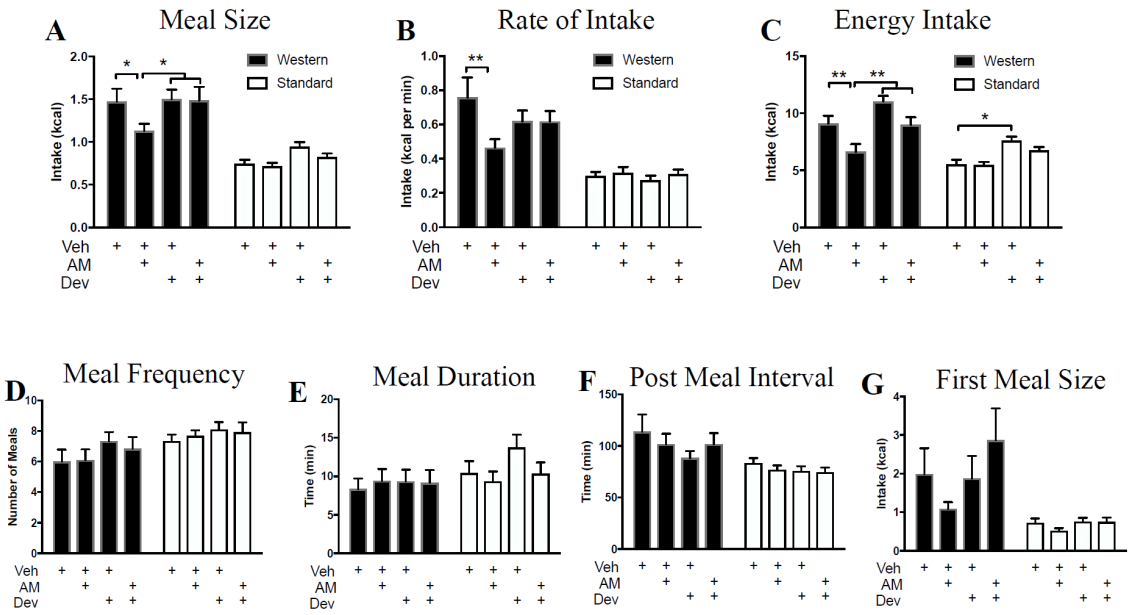

Figure 2.13. Peripheral eCB signaling drives hyperphagia in mice maintained on a WD via a CCK-dependent mechanism. Caloric intake (A), meal size (B), and rate of intake (C) of a western diet (closed bars) are significantly reduced during a 12 h test following inhibition of peripheral CB_1Rs with AM6545 (AM, 10mg per kg), an effect absent in low-fat chow fed mice (open bars) and that is blocked by co-administration with the CCK_A 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.

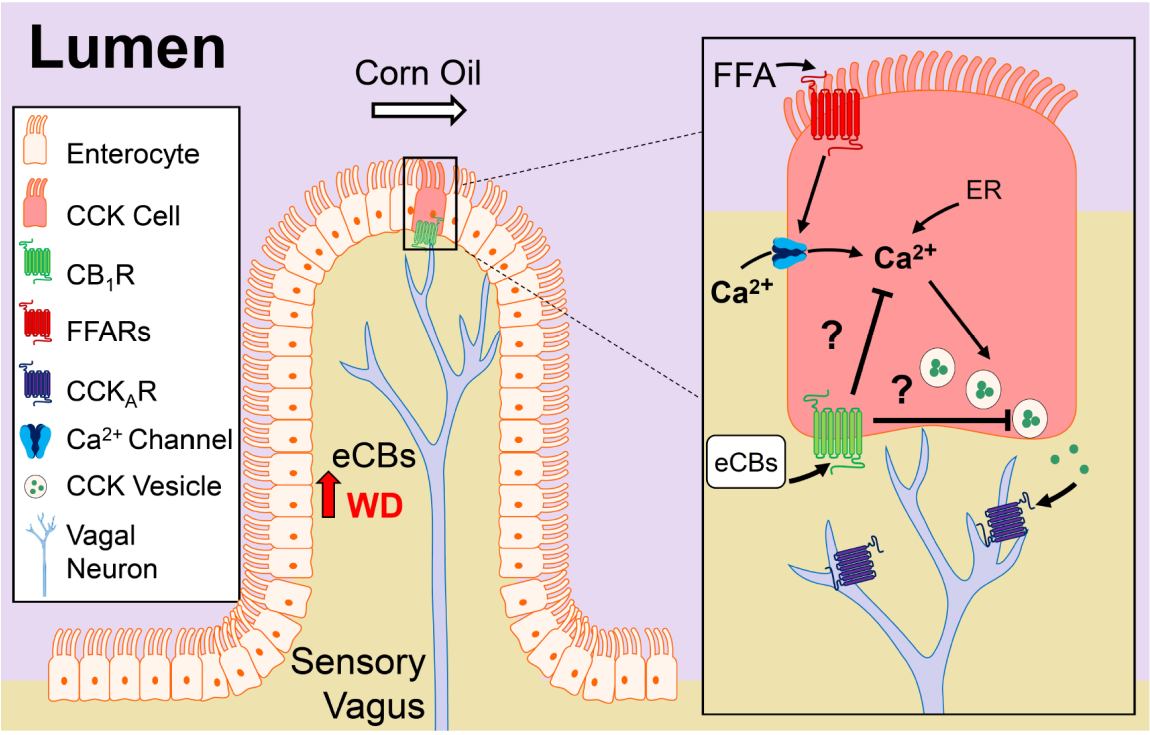
Lumen
Corn Oil
Enterocyte
CCK Cell
CB1R
FFARs
CCKAR
Ca2+ Channel
CCK Vesicle
Vagal Neuron
eCBs
WD
Sensory Vagus
FFA
ER
Ca2+
Ca2+
?
?
eCBs

Figure 2.14. Model of CB_1R control of nutrient-induced CCK release.

Our studies suggest that cannabinoid CB_1Rs 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 (81, 102-105). 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 (Ca^{2+}) influx and/or intracellular (i.e., endoplasmic reticulum, ER) mobilization (28, 74, 102). CCK activates CCK_A receptors located on adjacent afferent sensory vagal fibers, which in turn, communicate with the brain and control meal size and satiation (30, 31, 33, 38). Consumption of a Western diet (WD) is associated with increased levels of the endocannabinoids (eCBs) and their activity at CB_1Rs in the upper small-intestinal epithelium, which we propose inhibits CCK release and satiation signaling. The molecular mechanism(s) mediating CB_1R control of CCK release is unknown, but may include inhibition of Ca^{2+}-mediated CCK release. A future test of this hypothesis is warranted.

Chapter 3: Role for Intestinal CB_1 Receptors in controlling GIP and GLP1 secretion

Authors: *Perez PA*[1], Argueta DA[1], Wiley MB[1], Avalos B[1], Wood CP[1], and DiPatrizio NV[1]

[1]University of California, Riverside, School of Medicine. 900 University Avenue, Riverside, CA, 92521 USA.

Abbreviations: Abhd6, alpha-beta-hydrolyzing domain 6; AM, AM6545; CB_1R, Cannabinoid receptor subtype 1; CB_2R, Cannabinoid receptor subtype 2; CCK, Cholecystokinin CO, Corn oil; DGL, Diacylglycerol lipase; eCB, endocannabinoid; FAAH, Fatty acid amide hydrolase; FFA, free fatty acid; MGL, Monoacylglycerol lipase; NAPE-PLD, N-acyl phosphatidylethanolamine-specific phospholipase D; SIR, selected ion recording; UPLC-MS/MS, ultra-performance liquid chromatography tandem mass spectrometry; WIN, WIN 55,212-2

Abstract

Glucose homeostasis is regulated by a dynamic interplay between various hormones produced by endocrine cells along the entero-insular axis. Enteroendocrine K- and L- cells produce and secrete the incretin glucose-dependent insulinotropic peptide (GIP) and glucagon-like polypeptide-1 (GLP1), respectively. Following a meal, these cells detect intraluminal nutrients, including simple sugars and free fatty acids, and respond by secreting GIP and GLP1 into circulation. The circulating incretins act on their respective incretin receptors within the endocrine pancreas to enhance insulin secretion. Recent studies have shown the presence of cannabinoid CB_1 receptor (CB_1R) mRNA within these enteroendocrine populations. Here, we tested the hypothesis that CB_1Rs regulate nutrient induced incretin secretion from these cells. Oral gavage of corn oil increased levels of GIP and bioactive GLP1 (aGLP1) in mouse plasma. Pretreatment with the cannabinoid receptor agonist, WIN55,212-2, blocked this response, which was reversed by co-administration with the peripherally-restricted CB_1R neutral antagonist, AM6545. To further determine whether intestinal CB_1Rs controlled nutrient induced incretin release, we utilized an inducible transgenic mouse model in which mice lack intestinal CB_1Rs. In these mice, pretreatment with WIN55,212-2 failed to inhibit nutrient induced GIP release, but not aGLP1 release. Taken together, these results indicate that intestinal CB_1Rs control GIP secretion while peripheral CB_1Rs control aGLP1 release.

Introduction

Adult obesity has steadily increased in the United States largely due to the overconsumption of highly palatable foods rich in processed sugars and fats (i.e. the “Western Diet”) (1-4). Obesity increases the risk of developing other serious comorbidities, including heart disease and type 2 diabetes, by increasing adiposity and by dysregulating circulating glucose levels through changes in the efficacy of glucose regulatory hormones (5, 6). Indeed, insulin receptors become insensitive during type 2 diabetes which further limits glycemic regulation. Prediabetes and diabetes are multifactorial diseases that often have several therapeutic strategies (7). Some treatment options focus on directly reducing hyperglycemia by limiting hepatic glucose production (metformin) or by reducing renal glucose reuptake through sodium-glucose cotransporter-2 inhibitors (canagliflozin, dapagliflozin) (7-11). However, these treatments may result in hypoglycemia or kidney damage in some patients because they target glucose levels instead of the main metabolic pathways that govern the natural tightly regulated range of blood sugar levels. Other therapeutics focus on increasing insulin levels to compensate for insulin receptor insensitivity by directly promoting pancreatic insulin secretions (sulfonylureas) or by insulin or insulin-like hormone injections (7, 12). While sulfonylureas and insulin injections are still described in international guidelines, they have been used as auxiliary treatments due to their role to accelerate β-cell apoptosis and β-cell exhaustion (13). More recently, drugs have instead targeted the natural intestinal incretin system in efforts to enhance endogenous insulin

secretion while also limiting β-cell exhaustion, wide shifts in glycemic levels, and obesity related hyperphagia.

Jean La Barre first coined the term “incretin” to describe intestinal hormones that promoted “insulin secretion” (14). Research groups reported higher insulin secretion and faster clearance of blood glucose following glucose challenges administered orally compared to intravenously (15, 16). Decades later, two major incretins were identified as glucose-dependent insulinotropic polypeptide (GIP; renamed from gastric inhibitory peptide after discovery of its potent insulin enhancing effects) and glucagon-like peptide 1 (GLP1) (17-19). GIP and GLP1 are secreted by open-type enteroendocrine K- and L- cells, respectively, after sensing intraluminal sugars and free fatty acids (FFA) following a meal (Reviewed in (20)). Once in circulation, GIP and GLP1 act on their respective G-protein coupled receptors (GIPR and GLP1R, respectively) within the endocrine pancreas to enhance insulin secretion. Incretin signaling is responsible for between 50-70% of all insulin secretion and is a vital regulator of glucose homeostasis (21). Furthermore, double incretin receptor knockout mice ($GIPR^{-/-}$, $GLP1R^{-/-}$) demonstrated a decreased insulin secretion following a glucose challenge. Interestingly, mice with decreased GIPR signaling are resistant to diet induced obesity due to its role in fat accumulation (22-24). On the other hand, central GLP1Rs in the nucleus of the solitary tract regulate food intake and may synergize with peripheral GLP1Rs, through both vagus nerve dependent and independent pathways, to regulate satiety (25-28). Thus, therapeutics have targeted both

incretins for their abilities to enhance insulin secretion, but with particular emphasis on GLP1 to promote satiety while limiting the pro-adiposity effects of GIP (29, 30). Differentiating differences between the two incretin signaling pathways will inform treatment options to better manage insulin levels and hyperglycemia.

Recently, cannabinoid receptors have been identified in different enteroendocrine cell populations (see chapter 2). Studies from our group indicate that central and peripheral CB_1R signaling promotes preferential intake of highly palatable fatty foods to increase available nutrients (31-34). The endocannabinoid system includes the cannabinoid receptors (CB_1R and CB_2R), its endogenous lipid-derived bioactive ligands (the endocannabinoids; eCBs), and the enzymatic machinery for ligand biosynthesis and degradation (35, 36). The eCB system is an important regulator of energy homeostasis within metabolically-relevant tissues (35). Notably, CB_1R signaling has been reported within the endocrine pancreas, liver, adipose tissue, and small intestine to directly impact energy conservation pathways (31, 37-40). CB_1R mRNA expression has been localized to intestinal K- and L-cells (41). Limited studies have suggested a global role for CB_1R in incretin release, yet the exact role of peripheral intestinal CB_1Rs in incretin secretion requires further research (41, 42). Here, we investigate the role for peripheral and intestinal CB_1Rs in the control of nutrient induced GIP and GLP1 secretion.

Materials and Methods

Animals

Eight-week old C57BL/6 mice (Taconic, Oxnard, CA, USA) ,C57BL/6-Tg(1Lphi/J-Cnr1$^{tm1.1mll}$), (Control; IntCB$_1$+/+) or C57BL/6-Tg(Vil-CreERT/1Lphi/J-Cnr1$^{tm1.1mll}$) (IntCB$_1$-/-) mice were used for incretin secretion studies and were group-housed with *ad libitum* food and water access while maintained on a 12 h dark/light cycle. 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.

Generation of IntCB$_1$-/- mice

Conditional intestinal epithelium-specific CB$_1$R-deficient mice (Cnr1$^{tm1.1\ mrl}$/vil-cre ERT2) were generated by crossing Cnr1 floxed mice (Cnr1$^{tm1.1\ mrl}$; Taconic, Oxnard, CA, USA; Model # 7599) with Vil-CRE ERT2 mice donated by Dr. Randy Seeley (University of Michigan, Ann Arbor, MI) with permission from Dr. Sylvie Robin (Curie Institute, Paris, France). Cre expression in intestinal epithelium is driven by the villin promotor, which allows for conditional tamoxifen-dependent Cre recombinase action to remove the *Cnr1* gene from these cells, as described by el Marjou et al (43). Cnr1$^{tm1.1\ mrl}$/vil-cre ERT2 mice used in these experiments after tamoxifen treatment will be referred to as IntCB$_1$-/-, and Cnr1$^{tm1.1\ mrl}$ control mice will be referred to as IntCB$_1$+/+. Genotype was twice verified in tail snips by PCR using the following primers (5'-3'): GCAGGGATTATGTCCCTAGC (Cnr1, ALT), CTGTTACCAGGAGTCTTAGC (Cnr1, 1415-35), GGCTCAAGGAAT-

ACACTTATACC (Cnr1, 1415-37), GAACCTGATGGACATGTTCAGG (vilcre, AA), AGTGCGTTCGAACGCTAGAGCCTGT (vilcre, SS), TTACGTCCATCGTGG-ACAGC (vilcre, MYO F), TGGGCTGGGTGTTAGCCTTA (vilcre, MYO R). CB_1R deletion was induced at 6-8 weeks of age via intraperitoneal tamoxifen injections (100 µL IP, see below).

Chemicals and administration schedule

AM6545, a peripherally-restricted CB_1R neutral antagonist, was given by IP injection at 10 mg per kg (Northeastern University Center for Drug Discovery, Boston, MA, USA). WIN 55,212-2, a potent cannabinoid receptor agonist, was given by IP injection at 3 mg per kg (Cayman Chemicals, Ann Arbor, MI). 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. Tamoxifen (10 mg per mL) was dissolved in corn oil, sonicated and warmed in a water bath to ensure solubility, and stored away from light until use. Tamoxifen was given by IP injection at 40 mg per kg for 5 consecutive days.

Measurement of intestinal lipids

Tissue harvest

Animals were anesthetized with isoflurane at time of tissue harvest (0900-1100 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). Small intestine was quickly removed and washed in phosphate-buffered saline (PBS),

opened longitudinally on a stainless-steel tray on ice, and contents removed. Intestinal epithelium of different regions was isolated using glass slides to scrape the epithelial layer and was snap-frozen in liquid N_2. Samples were stored at -80^{o}C pending analysis.

ELISA analysis of GIP, and aGLP1

Mice were acclimated to cages fitted with elevated wire bottoms for 72 h to prevent coprophagia and fasted for 12 h in order to ensure an empty stomach. Mice were pretreated with CB_1R ligands, then administered corn oil (0.5 mL) by oral gavage 30 min later. Levels of GIP and aGLP1 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 10min at 4°C) by a sensitive and selective GIP ELISA (EMD Millipore, St. Louis, MI, USA) and aGLP1 ELISA (ALPCO, Salem, NH, USA). GIP ELISA reactions were measured using iMark microplate reader (BioRad, Hercules, CA, USA) and aGLP1 ELISA reactions were measured using Luminex MagPix instrument.

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 media (Fisher Healthcare, Chino, CA, USA) on dry ice. 16 μm sections were

collected in a cryostat (Leica) maintained at -20°C 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 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).

Gastric Emptying

To evaluate drug or endogenous endocannabinoid effects on gastric emptying, corn oil was spiked with 1.0 nmol or 2.5 nmol 19:2 FFA (as indicated) and administered by oral gavage (0.5 mL), then quantities of 19:2 FFA remaining in the stomach were evaluated at the time of blood collection 30 min 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 analyzed via

UPLC/MS/MS according to the following protocol. 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_{18} column (2.1 x 50 mm i.d., 1.7 µm, Waters Corporation) and inline Acquity guard column (UPLC BEH C_{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: 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^{o}C and samples were kept at 10^{o}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) and 17:1 FFA (*m/z* = 267.2) as internal standard.

Gene expression analysis

RNA isolation

Total RNA was extracted from tissues 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 (0.2-1.0 µg, tissue specific) was performed as previously described (31).

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: CB_1R (Cnr1), CB_2R (Cnr2), GPR55 (Gpr55), diacylglycerol lipase alpha (Dagla) and beta (Daglb), monoacylglycerol lipase (Mgll), alpha-beta-hydrolyzing domain 6 (Abhd6), n-acyl phosphatidyl ethanolamine-specific phospholipase D (Napepld), fatty-acid amide hydrolase (Faah), with Actb, (pancreas), B2m, (epididymal fat) and Hprt (all other tested tissues) as housekeeping genes. Values are expressed as relative mRNA expression using the delta-delta cq method (44). Reactions were run in duplicate for each animal.

Statistical analysis

Values are expressed as means ± SEM. Unpaired Student's two-tailed t-test was used to compare data for gene analysis in different tissues between

control and IntCB$_1$-/- mice. Additionally, two-way ANOVA were used to determine differences in multiple groups with post-hoc Holm-Sidak's multiple comparisons tests. Repeated measures two-way ANOVA was used for groups measured over time. Data were analyzed using GraphPad Prism8.4.2 software. Significance was determined as $p< 0.05$. Statistical outliers were determined using Grubb's test in all data sets.

Results

Peripheral CB$_1$Rs control corn oil induced incretion secretion.

We previously reported that CB$_1$Rs are colocalized on enteroendocrine I-cells and regulate the secretion of satiation hormone cholecystokinin (CCK); furthermore, increased eCB signaling resulted in decreased cholecystokinin secretion and was responsible for diet induced obesity related hyperphagia (see Chapter 2, (45)). Here, we investigated the role of CB$_1$Rs in regulating enteroendocrine incretin hormones involved in glucose homeostasis, namely GIP and GLP1. Oral gavage of corn oil (CO) potently increased plasma levels of GIP when compared to control mice that received oral gavage of saline (Figure 3.1A; CO= 2.74 ± 0.47 ng per mL vs saline= 0.12 ± 0.01 ng per mL, $p<0.001$, n = 5). Peripheral administration of the cannabinoid receptor agonist WIN 55,212-2 (WIN, 3 mg per kg), blocked CO-induced secretion of GIP (Figure 3.1A; CO + WIN = 0.68 ± 0.32 ng per mL, $p<0.05$, CO + WIN versus CO alone, n = 5). Additionally, the effect of WIN administration on CO-induced secretion of GIP was reversed by co-

treatment with the peripherally restricted CB_1R-selective antagonist, AM6545 (AM, 10 mg per kg) (Figure 3.1A; CO + WIN + AM = 2.59 ± 0.53 ng per mL, $p<0.05$, CO + WIN + AM versus CO + WIN, n = 5-6). CO also increased plasma levels of bioactive GLP1 (aGLP1) compared to control mice that received oral gavage of saline (Figure 3.1B; CO= 15.89 ± 2.29 pg per mL vs saline= 1.56 ± 0.38 pg per mL, $p<0.001$, n = 5-6). Peripheral administration of WIN blocked CO-induced secretion of aGLP1 (Figure 3.1B; CO + WIN = 4.10 ± 1.87 pg per mL, $p<0.01$, CO + WIN versus CO alone, n = 5). Lastly, the effect of WIN administration on CO-induced secretion of aGLP1 was reversed by co-treatment with AM (Figure 3.1B; CO + WIN + AM = 20.23 ± 3.09 pg per mL, $p<0.05$, CO + WIN + AM versus CO + WIN, n = 5-6). These results suggest that exogenous activation of CB_1Rs inhibits nutrient-induced incretin secretion from the small intestine.

CB_1R activation controls gastric emptying and intestinal motility (46, 47). With decreased gastric emptying, enteroendocrine cells may not respond to the free fatty acids in the CO gavage and appropriately secrete our incretins of interest. To identify the effects of WIN treatment on gastric emptying under our conditions, we administered 19:2 FFA (1 nmol, an odd chained FFA not endogenously produced in mammals) into the CO gavage (see chapter 2, (45)). WIN treatment alone or in combination with AM had no effect of gastric emptying of corn oil (see Chapter 2, Figure 2.7). Thus, this suggests that CB_1R activation under our conditions does not affect gastric emptying of CO and does not likely impact

incretin secretion through a mechanism that includes changes in gastric emptying in mice.

CB_1Rs in the endocrine pancreatic control insulin release and circulating blood glucose levels (37, 48-51). We tested if WIN treatment alone or in combination with AM impacted glucose levels in response to CO gavage, which in turn, could affect gastric emptying, motility, or hormones from enteroendocrine cells (see Chapter 2, (45)). Blood glucose levels were monitored *via* hand-held glucometer from mouse tail vein at (i) time of drug administration, (ii) 30 min later just prior to corn oil gavage, and (iii) 30 minutes later at time of tissue collection (see Chapter 2, Figure 2.8). WIN treatment alone or in combination with AM had no significant impact on glucose levels at any point during our conditions. These data suggest that activating CB_1Rs does not impact circulating blood glucose levels following oral gavage of CO in mice in the experimental conditions.

Acute intestinal CB_1R ablation does not alter expression of other eCB system related genes

To identify the molecular underpinnings of peripheral and intestinal eCB signaling, we selectively ablated intestinal CB_1Rs. Following 5 consecutive days of tamoxifen treatment (IP injections, 40 mg per kg in CO) and 10 days of recovery and acclimation, $IntCB_1$-/- mice did not show immunoreactivity for CB_1Rs compared to Control $IntCB_1$+/+ mice and untreated Wild-type mice (Figure 3.2). Messenger RNA (mRNA) for CB_1Rs (Cnr1) was significantly down regulated in the intestinal epithelium of the duodenum (Figure 3.3A; Control = 1.0 ± 0.19, $IntCB_1$-/-

= 0.23 ± 0.11; t=3.30, p<0.01) jejunum (Control = 1.0 ± 0.40, IntCB$_1$-/- = 0.20 ± 0.05; t=2.43, p<0.05), ileum (Control = 1.0 ± 0.30, IntCB$_1$-/- = 0.31 ± 0.10; t=2.31, p<0.05), and large intestine (Control = 1.0 ± 0.38, IntCB$_1$-/- = 0.27 ± 0.05; t=2.46, p<0.05). Cnr1 expression did not show significant changes in the small intestinal submucosa (Control = 1.0 ± 0.34, IntCB$_1$-/- = 0.79 ± 0.24; t=0.49, p>0.05), stomach (Control = 1.0 ± 0.34, IntCB$_1$-/- = 1.27 ± 0.35; t=0.54, p>0.05), liver (Control = 1.0 ± 0.48, IntCB$_1$-/- = 0.68 ± 0.32; t=0.55, p>0.05), pancreas (Control = 1.0 ± 0.23, IntCB$_1$-/- = 0.99 ± 0.17; t=0.13, p>0.05), or epididymal fat (Control = 1.0 ± 0.11, IntCB$_1$-/- = 0.70 ± 0.15; t=1.6, p>0.05).

Acute intestinal CB$_1$R ablation had limited off target effects on mRNA expression of eCB system genes. In the proximal intestinal epithelium, IntCB$_1$-/- mice did not have significant changes in mRNA expression when compared to control mice of receptors CB$_2$R (Cnr2; Figure 3.3B, Control = 1.0 ± 0.70, IntCB$_1$-/- = 1.11 ± 0.45; t=0.14, p>0.05) or GPR55 (Control = 1.0 ± 0.27, IntCB$_1$-/- = 0.89 ± 0.18; t=0.35, p>0.05). Additionally, IntCB$_1$-/- showed no changes in monoacylglycerol biosynthesis enzymes diacylglycerol lipase α (DagLa; Control = 1.0 ± 0.41, IntCB$_1$-/- = 1.14 ± 0.45; t=0.23, p>0.05) or β (DagLb; Control = 1.0 ± 0.24, IntCB$_1$-/- = 0.91 ± 0.24; t=0.27, p>0.05) as well as enzymes responsible monoacylglycerol degradation, monoacylglycerol lipase (mgl; Control = 1.0 ± 0.27, IntCB$_1$-/- = 1.06 ± 0.19; t=0.17, p>0.05) or alpha/beta hydrolyzing domain-6 (abhd6; Control = 1.0 ± 0.25, IntCB$_1$-/- = 0.89 ± 0.24; t=0.35, p>0.05). Lastly, fatty acid ethanolamide biosynthesis enzyme N-acyl phosphatidylethanolamine-

specific phospholipase D (nape-pld; Control = 1.0 ± 0.24, IntCB$_1$-/- = 0.69 ± 0.11; t=01.34, p>0.05) and degradation enzyme fatty acid amide hydrolase (faah; Control = 1.0 ± 0.28, IntCB$_1$-/- = 1.01 ± 0.35; t=0.02, p>0.05) remained unchanged between control and IntCB$_1$-/- mice. This data suggests that our acute intestinal CB$_1$R ablation was tissue specific and did not develop an eCB related compensatory mechanism 10 days following last tamoxifen injection.

Intestinal CB$_1$Rs differentially regulate incretin release

Next, we tested the hypothesis that intestinal CB$_1$Rs control CO-induced GIP and aGLP1 secretion. Oral gavage of CO increased plasma levels of GIP in control IntCB$_1$+/+ mice when compared to control IntCB$_1$+/+ mice that received oral gavage of saline (Figure 3.4A; Control CO = 2.13 ± 0.27 ng per mL vs Control saline = 0.26 ± 0.09 ng per mL, p<0.0001, n = 6); As expected, this effect was blunted by pretreatment with WIN (Control CO + WIN = 0.52 ± 0.08 ng per mL, Control CO + WIN vs Control CO alone, p<0.0001, n = 6). On the other hand, IntCB$_1$-/- mice had similar plasma levels of GIP when compared to control mice (IntCB$_1$-/- = 0.25 ± 0.07 ng per mL, Saline control vs saline IntCB$_1$-/-, p>0.05, n = 6-8), but gavage with CO did not increase plasma GIP in IntCB$_1$-/- as high as in control mice (IntCB$_1$-/- CO = 1.18 ± 0.09 ng per mL, IntCB$_1$-/- saline vs IntCB$_1$-/- CO, p<0.01; Control CO vs IntCB$_1$-/- CO, p<0.01, n = 6-8). Notably, IntCB$_1$-/- mice pretreated with WIN showed no effect on plasma GIP levels (IntCB$_1$-/- CO + WIN = 1.16 ± 0.21 ng per mL, IntCB$_1$-/- CO + WIN vs IntCB$_1$-/- CO alone, p>0.05, n = 7-8).

Oral gavage of CO increased plasma levels of aGLP1 in control IntCB$_1$+/+ mice when compared to control IntCB$_1$+/+ mice that received oral gavage of saline (Figure 3.4B; IntCB$_1$-/- CO = 17.60 ± 2.20 pg per mL vs IntCB$_1$-/- saline = 0.26 ± 0.09 pg per mL, $p<0.0001$, n = 6); Again, this effect was blunted by pretreatment with WIN (IntCB$_1$-/- CO + WIN = 3.73 ± 0.61 pg per mL, IntCB$_1$-/- CO + WIN vs IntCB$_1$-/- CO alone, $p<0.0001$, n = 6). Likewise, IntCB$_1$-/- mice had similar plasma levels of aGLP1 when compared to control mice (IntCB$_1$-/- = 1.78 ± 0.19 pg per mL, Saline control vs saline IntCB$_1$-/- vs $p>0.05$, n = 6-8), yet gavage with CO did not increase plasma aGLP1 in IntCB$_1$-/- as high as in control mice (IntCB$_1$-/- CO = 12.28 ± 1.59 pg per mL, IntCB$_1$-/- saline vs IntCB$_1$-/- CO, $p<0.0001$; Control CO vs IntCB$_1$-/- CO, $p<0.05$, n = 6-8). Interestingly, IntCB$_1$-/- mice pretreated with WIN continued to block aGLP1 secretion (IntCB$_1$-/- CO + WIN = 4.53 ± 0.46 pg per mL, IntCB$_1$-/- CO + WIN vs IntCB$_1$-/- CO alone, $p<0.001$, n = 7-8). Lastly, mice given a CO gavage showed no differences in gastric emptying (19:2 FFA, 2.5 nmol in 0.5 mL CO) or blood sugar levels regardless of treatment or genotype (Figure 3.5). Taken together, this data suggests a role for intestinal CB$_1$Rs in regulating GIP, but not aGLP1, release.

Discussion

The eCB system regulates food intake and energy homeostasis. Hypothalamic eCB signaling is crucial to develop food seeking behaviors and directly opposes the actions of leptin, a satiety hormone (52, 53). Additionally, eCB signaling in the forebrain controls energy use and dissipation within the adipose

tissue (54). During the development of diet induced obesity, there is increased eCB tone and increased eCB signaling in the hypothalamus, proximal small intestine, liver, pancreas, and adipose tissue (31, 38, 55, 56). Thus, inhibitors that disrupt eCB signaling showed promising potential to treat hyperphagia, glycemic dysregulation, and dyslipidemia often observed in obesity (31, 57-60). Rimonabant, a globally acting CB_1R antagonist, was prescribed to obese patients in Europe; treated patients lost significant weight while maintaining lean body mass (61, 62). Additionally, treated patients reported beneficial effects on HDL/LDL, blood triglycerides, and decreases in HbA1C% (61-63). However, while hypothalamic CB_1Rs are responsible for regulating energy homeostasis, recent findings have now shown central CB_1Rs to be involved in fear, anxiety, and memory (64). Treatment with Rimonabant resulted in increased incidence of depression and suicidal ideation, and thus was removed from the European market (65). Recent investigations have focused on peripherally-restricted CB_1R inhibitors to limit the centrally mediated side effects of central CB_1R inhibition.

Incretins are important peripheral hormones produced by enteroendocrine K- and L- cells that enhance insulin secretion and regulate blood sugar levels. Recently, CB_1R mRNA expression has been co-localized to K- and L- cells (41). Limited studies using globally acting agonists or transgenic full body CB_1R knockouts suggests a role for CB_1R in inhibiting GIP and GLP1 secretion following a meal, yet the functional role of these receptors on these cell types are poorly

characterized (41, 42, 66, 67). Here, we aimed to test the hypothesis that intestinal CB_1Rs control GIP and GLP1 release.

Enteroendocrine cells sense luminal contents through different FFA receptors. These G-protein coupled receptors are enriched in enteroendocrine populations and mobilize calcium through transient receptor potential channels and/or voltage gated calcium channels to depolarize the enteroendocrine cells, resulting in vesicular hormone release (68). Thus, we utilized corn oil, which contains a mix of different saturated and unsaturated FFAs, to stimulate incretin secretion in our investigation. A gavage with CO potently increased the blood plasma levels of GIP and aGLP1 in fasted mice (Figure 3.1). However, pretreatment with the general cannabinoid receptor agonist, WIN, blocked the CO induced incretin secretion. Our results are consistent with other groups showing decreased GIP or GLP1 release following cannabinoid receptor activation in primary murine cells, mouse models, and in humans (41, 42, 66). In contrast, we utilized a peripherally restricted CB_1R specific antagonist AM6545, not a globally CB_1R inhibitor, to delineate the location of this effect in our investigations. Cotreatment with WIN and AM restored the nutrient induced incretin secretion showing, for the first time, that this effect is mediated through peripheral CB_1R signaling. Additionally, we tested the possible effect of CB_1R activation on gastric emptying and circulating glucose levels under our conditions. Changes in gastric emptying could affect the accessibility of the FFAs from the CO gavage to the enteroendocrine cells and alter the release of the incretins of interest (46, 47, 69).

Also, CB_1R signaling has been implicated in regulating insulin secretion within the endocrine pancreas and this could, in turn, affect the release of GIP or GLP1 (37, 48). We show no differences in gastric emptying of CO in drug treated mice, nor do we report changes in blood glucose levels between conditions (see Chapter 2, Figure 2.7 and 2.8; (45)).

To further investigate the specific role of intestinal CB_1Rs in controlling incretin secretion, we utilized a novel inducible transgenic mouse model that lack intestinal CB_1Rs following tamoxifen injections. Inducible models allow for specificity and limited compensatory mechanisms that may develop due to lack of CB_1Rs in key tissues governing energy homeostasis. Indeed, CB_1R signaling is important in developing appropriate endocrine pancreas microarchitecture; full body CB_1R knockout mice have altered endocrine pancreas morphology, changes in adipose tissue, and are resistant to diet induced obesity (70-72). CB_1Rs have been shown to directly interfere with β-cell insulin receptor signaling and regulating overall β-cell mass (73, 74). On the other hand, adipose CB_1R signaling has also been reported to increase adiposity and inflammation during obesity (39). These pathways directly interfere with incretin insulinotropic effects, which makes it difficult to delineate between tissue specific outcomes of CB_1R signaling involved in governing glucose homeostasis. Our novel $IntCB_1$-/- mice showed no immunoreactivity to CB_1R antibodies in the proximal small intestine (Figure 3.2). Furthermore, $IntCB_1$-/- mice showed significant decreases in CB_1R mRNA in the small and large intestinal epithelium but not in the submucosal layer or in other

metabolically relevant tissues (Figure 3.3). The residual CB_1R mRNA may be attributed to resident immune cells, including macrophages, within the intestinal epithelium that derive from non-intestinal stem-cell lineages and were collected during the intestinal scraping process (75, 76). Lastly, we report no changes in mRNA expression of other genes in the eCB system at the time point tested in $IntCB_1$-/- mice. The intestinal CB_1R specific ablation with no reported changes in CB_1R expression in other tissues allows us to study the effect of intestinal CB_1R signaling.

Next, we investigated the effect of intestinal CB_1R ablation on incretin secretion. As expected, CO stimulated both GIP and aGLP1 secretion in control treated mice containing intestinal CB_1Rs which was blocked by WIN pretreatment (Figure 3.4). In $IntCB_1$-/- mice, CO stimulated GIP and aGLP1 secretion but at significantly lower levels when compared to control mice. This suggests that intestinal CB_1Rs may be involved in nutrient sensing, incretin production, or regulating enteroendocrine populations and are the subjects of future investigations. Interestingly, plasma GIP levels in $IntCB_1$-/- given a CO gavage were unaffected by WIN pretreatment (Figure 3.4A). On the other hand, WIN pretreatment in $IntCB_1$-/- mice blocked increases in plasma aGLP1 levels following a CO gavage (Figure 3.4B). Lastly, we evaluated gastric emptying and blood glucose levels between conditions and saw no difference between genotype or drug treatments (Figure 3.5). Thus, this indicates a role for intestinal CB_1Rs in controlling GIP release but not in controlling GLP1 secretion. The exact

peripherally mediated mechanism that controls GLP1 secretion requires further investigation.

Incretins also exhibit other non-insulinotropic outcomes that affect energy conservation. For example, GIP signaling within adipocytes has been reported to increase fat accumulation by improving insulin sensitivity and enhancing insulin induced glucose reuptake and fatty acid incorporation (23, 24, 77, 78). Also, GIP signaling increases lipoprotein lipase activity to increase free fatty acid availability from circulating triglycerides (78). Notably, circulating GIP levels are increased during obesity and blocking GIP signaling prevents diet induced obesity (23, 24). This study shows a role for intestinal CB_1Rs in controlling GIP release during a lean state. Without CB_1R regulation of GIP, it is possible that temporal increases in GIP overtime may contribute to obesity pathogenesis by increasing fat synthesis and energy storage. The long-term effects of removing intestinal CB_1R-mediated regulation of GIP release during chronic consumption of a high fat diet and the onset of obesity pathogenesis requires further research.

Finally, peripheral CB_1R control of GLP1 may also involve afferent vagal fibers and may overlap with CCK mediated gut-brain interactions. Our results show that peripheral CB_1R control of nutrient induced incretin release reflects a similar pattern when compared to peripheral CB_1R regulation of CCK secretion (see chapter 2, (45)), suggesting a redundant or overlapping mechanism between different enteroendocrine cell types. While enteroendocrine cells predominately produce a characteristic intestinal-derived hormone, specific enteroendocrine cell

types have the ability to produce multiple peptides (79-83). Open-type enteroendocrine cells (which include I- and L-cells) form functional synapses with afferent vagal fibers which in turn communicate with the brain via the gut-brain axis (20, 84-86). Importantly, about half of all neurons positive for CCK receptors also express GLP1 receptors, whereas nearly all neurons positive for GLP1 receptors contain CCK receptors (87). Once activated, GLP1 receptors on these neurons signal to the nucleus of the solitary tract to reduce food intake (88). Notably, GIP receptors were not detected in any vagal afferent neurons indicating a unique role for GLP1 over GIP in regulating caloric intake through the vagus nerve. Additionally, CB_1R expression was also colocalized to both types of CCK receptor positive neurons (those co-expressing and lacking GLP1 receptors), as well as on other neurons not expressing CCK receptors that innervate the intestinal mucosal layer (87). Thus, it may be possible that CB_1Rs on these neurons, and not intestinal CB_1Rs, may be controlling GLP1 secretion and may contribute to the hypophagic effects of CB_1R inhibition; however, a direct test to this hypothesis remains.

References

1. Ogden CL, Carroll MD, Lawman HG, Fryar CD, Kruszon-Moran D, Kit BK, Flegal KM. Trends in Obesity Prevalence Among Children and Adolescents in the United States, 1988-1994 Through 2013-2014. JAMA. 2016;315(21):2292-9. doi: 10.1001/jama.2016.6361. PubMed PMID: 27272581.

2. Ogden CL, Carroll MD, Kit BK, Flegal KM. Prevalence of obesity in the United States, 2009-2010. NCHS data brief, no 82 Hyattsville, MD: National Center for Health Statistics 2012. 2012.

3. Medina-RemOn A, Kirwan R, Lamuela-Raventos RM, Estruch R. Dietary Patterns and the Risk of Obesity, Type 2 Diabetes Mellitus, Cardiovascular Diseases, Asthma, and Mental Health Problems. Crit Rev Food Sci Nutr. 2016:0. doi: 10.1080/10408398.2016.1158690. PubMed PMID: 27127938.

4. Poti JM, Duffey KJ, Popkin BM. The association of fast food consumption with poor dietary outcomes and obesity among children: is it the fast food or the remainder of the diet? Am J Clin Nutr. 2014;99(1):162-71. doi: 10.3945/ajcn.113.071928. PubMed PMID: 24153348; PMCID: PMC3862453.

5. Reaven GM. Role of Insulin Resistance in Human Disease. Diabetes. 1988;37(12):1595-607. doi: 10.2337/diab.37.12.1595.

6. Stumvoll M, Goldstein BJ, van Haeften TW. Type 2 diabetes: principles of pathogenesis and therapy. The Lancet. 2005;365(9467):1333-46. doi: https://doi.org/10.1016/S0140-6736(05)61032-X.

7. Bennett WL, Maruthur NM, Singh S, Segal JB, Wilson LM, Chatterjee R, Marinopoulos SS, Puhan MA, Ranasinghe P, Block L, Nicholson WK, Hutfless S, Bass EB, Bolen S. Comparative Effectiveness and Safety of Medications for Type 2 Diabetes: An Update Including New Drugs and 2-Drug Combinations. Annals of Internal Medicine. 2011;154(9):602-13. doi: 10.7326/0003-4819-154-9-201105030-00336.

8. Hundal RS, Krssak M, Dufour S, Laurent D, Lebon V, Chandramouli V, Inzucchi SE, Schumann WC, Petersen KF, Landau BR, Shulman GI. Mechanism by which metformin reduces glucose production in type 2 diabetes. Diabetes. 2000;49(12):2063 9. doi: 10.2337/diabetes.49.12.2063.

9. Meng W, Ellsworth BA, Nirschl AA, McCann PJ, Patel M, Girotra RN, Wu G, Sher PM, Morrison EP, Biller SA, Zahler R, Deshpande PP, Pullockaran A, Hagan DL, Morgan N, Taylor JR, Obermeier MT, Humphreys WG, Khanna A, Discenza L, Robertson JG, Wang A, Han S, Wetterau JR, Janovitz EB, Flint OP, Whaley JM, Washburn WN. Discovery of Dapagliflozin: A Potent, Selective Renal Sodium-Dependent Glucose Cotransporter 2 (SGLT2) Inhibitor for the Treatment of Type 2 Diabetes. Journal of Medicinal Chemistry. 2008;51(5):1145-9. doi: 10.1021/jm701272q.

10. Komoroski B, Vachharajani N, Feng Y, Li L, Kornhauser D, Pfister M. Dapagliflozin, a Novel, Selective SGLT2 Inhibitor, Improved Glycemic Control Over 2 Weeks in Patients With Type 2 Diabetes Mellitus. Clinical Pharmacology & Therapeutics. 2009;85(5):513-9. doi: 10.1038/clpt.2008.250.

11. Devineni D, Morrow L, Hompesch M, Skee D, Vandebosch A, Murphy J, Ways K, Schwartz S. Canagliflozin improves glycaemic control over 28 days in subjects with type 2 diabetes not optimally controlled on insulin. Diabetes, Obesity and Metabolism. 2012;14(6):539-45. doi: 10.1111/j.1463-1326.2012.01558.x.

12. Del Prato S, Pulizzi N. The place of sulfonylureas in the therapy for type 2 diabetes mellitus. Metabolism. 2006;55:S20-S7. doi: https://doi.org/10.1016/j.metabol.2006.02.003.

13. Maedler K, Carr RD, Bosco D, Zuellig RA, Berney T, Donath MY. Sulfonylurea Induced β-Cell Apoptosis in Cultured Human Islets. The Journal of Clinical Endocrinology & Metabolism. 2005;90(1):501-6. doi: 10.1210/jc.2004-0699.

14. Rehfeld JF. The Origin and Understanding of the Incretin Concept. Frontiers in Endocrinology. 2018;9(387). doi: 10.3389/fendo.2018.00387.

15. McIntyre N Fau - Holdsworth CD, Holdsworth Cd Fau - Turner DS, Turner DS. New Interpretation of Oral Glucose Tolerance. Lancet. 1965(0140-6736 (Print)).

16. Vardarli I, Nauck MA, Köthe LD, Deacon CF, Holst JJ, Schweizer A, Foley JE. Inhibition of DPP-4 with Vildagliptin Improved Insulin Secretion in Response to Oral as well as “Isoglycemic” Intravenous Glucose without Numerically Changing the Incretin Effect in Patients with Type 2 Diabetes. The Journal of Clinical Endocrinology & Metabolism. 2011;96(4):945-54. doi: 10.1210/jc.2010-2178.

17. Seino Y, Fukushima M, Yabe D. GIP and GLP-1, the two incretin hormones: Similarities and differences. J Diabetes Investig. 2010;1(1-2):8-23. doi: 10.1111/j.2040-1124.2010.00022.x. PubMed PMID: 24843404; PMCID: PMC4020673.

18. Brown JC, Dryburgh JR. A gastric inhibitory polypeptide. II. The complete amino acid sequence. Can J Biochem. 1971;49(8):867-72. Epub 1971/08/01. doi: 10.1139/o71-122. PubMed PMID: 5120249.

19. Bell GI, Sanchez-Pescador R, Laybourn PJ, Najarian RC. Exon duplication and divergence in the human preproglucagon gene. Nature. 1983;304(5924):368-71. doi: 10.1038/304368a0.

20. Gribble FM, Reimann F. Enteroendocrine Cells: Chemosensors in the Intestinal Epithelium. Annual Review of Physiology. 2016;78(1):277-99. doi: 10.1146/annurev-physiol-021115-105439. PubMed PMID: 26442437.

21. NAUCK MA, HOMBERGER E, SIEGEL EG, ALLEN RC, EATON RP, EBERT R, CREUTZFELDT W. Incretin Effects of Increasing Glucose Loads in Man Calculated from Venous Insulin and C-Peptide Responses*. The Journal of Clinical Endocrinology & Metabolism. 1986;63(2):492-8. doi: 10.1210/jcem-63-2-492.

22. Ayala JE, Bracy DP, Hansotia T, Flock G, Seino Y, Wasserman DH, Drucker DJ. Insulin Action in the Double Incretin Receptor Knockout Mouse. Diabetes. 2008;57(2):288-97. doi: 10.2337/db07-0704.

23. Miyawaki K, Yamada Y, Ban N, Ihara Y, Tsukiyama K, Zhou H, Fujimoto S, Oku A, Tsuda K, Toyokuni S, Hiai H, Mizunoya W, Fushiki T, Holst JJ, Makino M, Tashita A, Kobara Y, Tsubamoto Y, Jinnouchi T, Jomori T, Seino Y. Inhibition of gastric inhibitory polypeptide signaling prevents obesity. Nat Med. 2002;8(7):738-42. Epub 2002/06/18. doi: 10.1038/nm727. PubMed PMID: 12068290.

24. Fulurija A, Lutz TA, Sladko K, Osto M, Wielinga PY, Bachmann MF, Saudan P. Vaccination against GIP for the Treatment of Obesity. PLOS ONE. 2008;3(9):e3163. doi: 10.1371/journal.pone.0003163.

25. Barrera JG, Jones KR, Herman JP, D'Alessio DA, Woods SC, Seeley RJ. Hyperphagia and Increased Fat Accumulation in Two Models of Chronic CNS Glucagon-Like Peptide-1 Loss of Function. The Journal of Neuroscience. 2011;31(10):3904-13. doi: 10.1523/jneurosci.2212-10.2011.

26. Chaudhri OB, Parkinson JRC, Kuo Y-T, Druce MR, Herlihy AH, Bell JD, Dhillo WS, Stanley SA, Ghatei MA, Bloom SR. Differential hypothalamic neuronal activation following peripheral injection of GLP-1 and oxyntomodulin in mice detected by manganese-enhanced magnetic resonance imaging. Biochemical and Biophysical Research Communications. 2006;350(2):298-306. doi: https://doi.org/10.1016/j.bbrc.2006.09.033.

27. Rüttimann EB, Arnold M, Hillebrand JJ, Geary N, Langhans W. Intrameal hepatic portal and intraperitoneal infusions of glucagon-like peptide-1 reduce spontaneous meal size in the rat via different mechanisms. Endocrinology. 2009;150(3):1174-81. Epub 2008/10/25. doi: 10.1210/en.2008-1221. PubMed PMID: 18948395; PMCID: PMC2654737.

28. Shah M, Vella A. Effects of GLP-1 on appetite and weight. Reviews in Endocrine and Metabolic Disorders. 2014;15(3):181-7. doi: 10.1007/s11154-014-9289-5.

29. Decara J, Arrabal S, Beiroa D, Rivera P, Vargas A, Serrano A, Pavón FJ, Ballesteros J, Dieguez C, Nogueiras R, Rodríguez de Fonseca F, Suárez J. Antiobesity efficacy of GLP-1 receptor agonist liraglutide is associated with peripheral tissue-specific modulation of lipid metabolic regulators. BioFactors. 2016;42(6):600-11. doi: 10.1002/biof.1295.

30. Drucker DJ. Enhancing Incretin Action for the Treatment of Type 2 Diabetes. Diabetes Care. 2003;26(10):2929-40. doi: 10.2337/diacare.26.10.2929.

31. Argueta DA, DiPatrizio NV. Peripheral endocannabinoid signaling controls hyperphagia in western diet-induced obesity. Physiol Behav. 2017;171:32-9. doi: 10.1016/j.physbeh.2016.12.044. PubMed PMID: 28065722.

32. DiPatrizio NV, Piomelli D. Intestinal lipid-derived signals that sense dietary fat. J Clin Invest. 2015;125(3):891-8. doi: 10.1172/JCI76302. PubMed PMID: 25642767; PMCID: PMC4362267.

33. DiPatrizio NV, Joslin A, Jung KM, Piomelli D. Endocannabinoid signaling in the gut mediates preference for dietary unsaturated fats. Faseb J. 2013;27(6):2513-20. Epub 2013/03/07. doi: 10.1096/fj.13-227587fj.13-227587 [pii]. PubMed PMID: 23463697; PMCID: 3659363.

34. DiPatrizio NV, Simansky KJ. Activating parabrachial cannabinoid CB1 receptors selectively stimulates feeding of palatable foods in rats. J Neurosci. 2008;28(39):9702-9. PubMed PMID: 18815256.

35. DiPatrizio NV, Piomelli D. The thrifty lipids: Endocannabinoids and the neural control of energy conservation. Trends in Neurosciences. 2012;35(7):403-11. PubMed PMID: 1017621058.

36. Piomelli D. The molecular logic of endocannabinoid signalling. Nat Rev Neurosci. 2003;4(11):873-84. PubMed PMID: 14595399.

37. Bermudez-Silva FJ, Baixeras E, Cobo N, Bautista D, Cuesta-Munoz AL, Fuentes E, Juan-Pico P, Castro MJ, Milman G, Mechoulam R, Nadal A, Rodriguez de Fonseca F. Presence of functional cannabinoid receptors in human endocrine pancreas. Diabetologia. 2008;51(3):476–87. Epub December 19, 2007.

38. Jourdan T, Nicoloro SM, Zhou Z, Shen Y, Liu J, Coffey NJ, Cinar R, Godlewski G, Gao B, Aouadi M, Czech MP, Kunos G. Decreasing CB1 receptor signaling in Kupffer cells improves insulin sensitivity in obese mice. Molecular Metabolism. 2017;6(11):1517-28. doi: https://doi.org/10.1016/j.molmet.2017.08.011.

39. Ruiz de Azua I, Mancini G, Srivastava RK, Rey AA, Cardinal P, Tedesco L, Zingaretti CM, Sassmann A, Quarta C, Schwitter C, Conrad A, Wettschureck N, Vemuri VK, Makriyannis A, Hartwig J, Mendez-Lago M, Bindila L, Monory K, Giordano A, Cinti S, Marsicano G, Offermanns S, Nisoli E, Pagotto U, Cota D, Lutz B. Adipocyte cannabinoid receptor CB1 regulates energy homeostasis and alternatively activated macrophages. The Journal of Clinical Investigation. 2017;127(11):4148-62. doi: 10.1172/JCI83626.

40. Everard A, Plovier H, Rastelli M, Van Hul M, de Wouters d'Oplinter A, Geurts L, Druart C, Robine S, Delzenne NM, Muccioli GG, de Vos WM, Luquet S, Flamand N, Di Marzo V, Cani PD. Intestinal epithelial N-acylphosphatidylethanolamine phospholipase D links dietary fat to metabolic adaptations in obesity and steatosis. Nature Communications. 2019;10(1):457. doi: 10.1038/s41467-018-08051-7.

41. Moss CE, Marsh WJ, Parker HE, Ogunnowo-Bada E, Riches CH, Habib AM, Evans ML, Gribble FM, Reimann F. Somatostatin receptor 5 and cannabinoid receptor 1 activation inhibit secretion of glucose-dependent insulinotropic polypeptide from intestinal K cells in rodents. Diabetologia. 2012;55(11):3094-103. Epub 2012/08/09. doi: 10.1007/s00125-012-2663-5. PubMed PMID: 22872212; PMCID: PMC3464380.

42. Weltens N, Depoortere I, Tack J, Van Oudenhove L. Effect of acute Δ9-tetrahydrocannabinol administration on subjective and metabolic hormone responses to food stimuli and food intake in healthy humans: a randomized, placebo-controlled study. The American Journal of Clinical Nutrition. 2019;109(4):1051-63. doi: 10.1093/ajcn/nqz007.

43. el Marjou F, Janssen KP, Chang BH, Li M, Hindie V, Chan L, Louvard D, Chambon P, Metzger D, Robine S. Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. Genesis. 2004;39(3):186-93. doi: 10.1002/gene.20042. PubMed PMID: 15282745.

44. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods. 2001;25(4):402-8. doi: https://doi.org/10.1006/meth.2001.1262.

45. Argueta DA, Perez PA, Makriyannis A, DiPatrizio NV. Cannabinoid CB1 Receptors Inhibit Gut-Brain Satiation Signaling in Diet-Induced Obesity. Frontiers in Physiology. 2019;10(704). doi: 10.3389/fphys.2019.00704.

46. Di Marzo V, Capasso R, Matias I, Aviello G, Petrosino S, Borrelli F, Romano B, Orlando P, Capasso F, Izzo AA. The role of endocannabinoids in the regulation of gastric emptying: alterations in mice fed a high-fat diet. Br J Pharmacol. 2008;153(6):1272-80. Epub 2008/01/29. doi: 0707682 [pii]10.1038/sj.bjp.0707682. PubMed PMID: 18223666; PMCID: 2275439.

47. Pertwee RG. Cannabinoids and the gastrointestinal tract. Gut. 2001;48(6):859-67. PubMed PMID: 11358910; PMCID: PMC1728337.

48. Juan-Pico P, Fuentes E, Bermudez-Silva FJ, Diaz-Molina F, Ripoll C, Rodriguez de Fonseca F, Nadal A. Cannabinoid receptors regulate Ca(2+) signals and insulin secretion in pancreatic beta-cell. Cell Calcium. 2006;39(0143-4160 (Print)):155-62; PMCID: 16321437.

49. De Petrocellis L, Marini P, Matias I, Moriello AS, Starowicz K, Cristino L, Nigam S, Di Marzo V. Mechanisms for the coupling of cannabinoid receptors to intracellular calcium mobilization in rat insulinoma beta-cells. Exp Cell Res. 2007;313(14):2993-3004. doi: 10.1016/j.yexcr.2007.05.012. PubMed PMID: 17585904.

50. Nakata M, Yada T. Cannabinoids inhibit insulin secretion and cytosolic Ca2+ oscillation in islet beta-cells via CB1 receptors. Regul Pept. 2008;145(1-3):49-53. doi: 10.1016/j.regpep.2007.08.009. PubMed PMID: 17884194.

51. Li C, Bowe JE, Jones PM, Persaud SJ. Expression and function of cannabinoid receptors in mouse islets. Islets. 2010;2(5):293-302. PubMed PMID: 21099327.

52. Di Marzo V, Goparaju SK, Wang L, Liu J, Batkai S, Jarai Z, Fezza F, Miura GI, Palmiter RD, Sugiura T, Kunos G. Leptin-regulated endocannabinoids are involved in maintaining food intake. Nature. 2001;410(6830):822-5. PubMed PMID: 11298451.

53. Fride E, Ginzburg Y, Breuer A, Bisogno T, Di Marzo V, Mechoulam R. Critical role of the endogenous cannabinoid system in mouse pup suckling and growth. European Journal of Pharmacology. 2001;419(2–3):207-14. doi: http://dx.doi.org/10.1016/S0014-2999(01)00953-0.

54. Quarta C, Bellocchio L, Mancini G, Mazza R, Cervino C, Braulke LJ, Fekete C, Latorre R, Nanni C, Bucci M, Clemens LE, Heldmaier G, Watanabe M, Leste-Lassere T, Maitre M, Tedesco L, Fanelli F, Reuss S, Klaus S, Srivastava RK, Monory K, Valerio A, Grandis A, De Giorgio R, Pasquali R, Nisoli E, Cota D, Lutz B, Marsicano G, Pagotto U. CB(1) signaling in forebrain and sympathetic neurons is a key determinant of endocannabinoid actions on energy balance. Cell Metab. 2010;11(4):273-85. Epub 2010/04/09. doi: S1550-4131(10)00070-7 [pii] 10.1016/j.cmet.2010.02.015. PubMed PMID: 20374960.

55. Miralpeix C, Fosch A, Casas J, Baena M, Herrero L, Serra D, Rodríguez-Rodríguez R, Casals N. Hypothalamic endocannabinoids inversely correlate with the development of diet-induced obesity in male and female mice. Journal of lipid research. 2019;60(7):1260-9. Epub 2019/05/28. doi: 10.1194/jlr.M092742. PubMed PMID: 31138606.

56. Matias I, Gonthier MP, Orlando P, Martiadis V, De Petrocellis L, Cervino C, Petrosino S, Hoareau L, Festy F, Pasquali R, Roche R, Maj M, Pagotto U, Monteleone P, Di Marzo V. Regulation, function, and dysregulation of endocannabinoids in models of adipose and beta-pancreatic cells and in obesity and hyperglycemia. J Clin Endocrinol Metab. 2006;91(8):3171-80. Epub 2006/05/11. doi: 10.1210/jc.2005-2679. PubMed PMID: 16684820.

57. Randall PA, Vemuri VK, Segovia KN, Torres EF, Hosmer S, Nunes EJ, Santerre JL, Makriyannis A, Salamone JD. The novel cannabinoid CB1 antagonist AM6545 suppresses food intake and food-reinforced behavior. Pharmacol Biochem Behav. 2010;97(1):179-84. Epub 2010/08/18. doi: S0091-3057(10)00233-9 [pii] 10.1016/j.pbb.2010.07.021. PubMed PMID: 20713079.

58. Jbilo O, Ravinet-Trillou C, Arnone M, Buisson I, Bribes E, Peleraux A, Penarier G, Soubrie P, Le Fur G, Galiegue S, Casellas P. The CB1 receptor antagonist rimonabant reverses the diet-induced obesity phenotype through the regulation of lipolysis and energy balance. Faseb J. 2005;19(11):1567-9. PubMed PMID: 16009704.

59. Poirier B, Bidouard JP, Cadrouvele C, Marniquet X, Staels B, O'Connor SE, Janiak P, Herbert JM. The anti-obesity effect of rimonabant is associated with an improved serum lipid profile. Diabetes Obes Metab. 2005;7(1):65-72. PubMed PMID: 15642077.

60. Verty AN, Allen AM, Oldfield BJ. The effects of rimonabant on brown adipose tissue in rat: implications for energy expenditure. Obesity (Silver Spring). 2009;17(2):254-61. Epub 2008/12/06. doi: oby2008509 [pii] 10.1038/oby.2008.509. PubMed PMID: 19057531.

61. Pi-Sunyer FX, Aronne LJ, Heshmati HM, Devin J, Rosenstock J. Effect of rimonabant, a cannabinoid-1 receptor blocker, on weight and cardiometabolic risk factors in overweight or obese patients: RIO-North America: a randomized controlled trial. JAMA. 2006;295(7):761-75. Epub 2006/02/16. doi: 295/7/761 [pii] 10.1001/jama.295.7.761. PubMed PMID: 16478899.

62. Van Gaal LF, Rissanen AM, Scheen AJ, Ziegler O, Rossner S. Effects of the cannabinoid-1 receptor blocker rimonabant on weight reduction and cardiovascular risk factors in overweight patients: 1-year experience from the RIO-Europe study. Lancet. 2005;365(9468):1389-97. Epub 2005/04/20. doi: S0140-6736(05)66374-X [pii]10.1016/S0140-6736(05)66374-X. PubMed PMID: 15836887.

63. Despres JP, Lemieux I, Almeras N. Contribution of CB1 blockade to the management of high-risk abdominal obesity. Int J Obes (Lond). 2006;30 Suppl 1:S44-52. PubMed PMID: 16570106.

64. Ruehle S, Rey AA, Remmers F, Lutz B. The endocannabinoid system in anxiety, fear memory and habituation. Journal of psychopharmacology (Oxford, England). 2012;26(1):23-39. Epub 2011/07/18. doi: 10.1177/0269881111408958. PubMed PMID: 21768162.

65. Moreira FA, Crippa JA. The psychiatric side-effects of rimonabant. Braz J Psychiatry. 2009;31(2):145-53. Epub 2009/07/07. doi: 10.1590/s1516-44462009000200012. PubMed PMID: 19578688.

66. Chia CW, Carlson OD, Liu DD, González-Mariscal I, Calvo SS-C, Egan JM. Incretin secretion in humans is under the influence of cannabinoid receptors. American Journal of Physiology-Endocrinology and Metabolism. 2017;313(3):E359-E66. doi: 10.1152/ajpendo.00080.2017. PubMed PMID: 28655715.

67. González-Mariscal I, Krzysik-Walker SM, Kim W, Rouse M, Egan JM. Blockade of cannabinoid 1 receptor improves GLP-1R mediated insulin secretion in mice. Molecular and Cellular Endocrinology. 2016;423:1-10. doi: https://doi.org/10.1016/j.mce.2015.12.015.

68. Lu VB, Gribble FM, Reimann F. Free Fatty Acid Receptors in Enteroendocrine Cells. Endocrinology. 2018;159(7):2826-35. doi: 10.1210/en.2018-00261.

69. Izzo AA, Coutts AA. Cannabinoids and the digestive tract. Handb Exp Pharmacol. 2005(168):573-98. PubMed PMID: 16596788.

70. Ravinet Trillou C, Delgorge C, Menet C, Arnone M, Soubrie P. CB1 cannabinoid receptor knockout in mice leads to leanness, resistance to diet-induced obesity and enhanced leptin sensitivity. Int J Obes Relat Metab Disord. 2004;28(4):640-8. PubMed PMID: 14770190.

71. Malenczyk K, Keimpema E, Piscitelli F, Calvigioni D, Bjorklund P, Mackie K, Di Marzo V, Hokfelt TG, Dobrzyn A, Harkany T. Fetal endocannabinoids orchestrate the organization of pancreatic islet microarchitecture. Proc Natl Acad Sci U S A. 2015;112(45):E6185-94. doi: 10.1073/pnas.1519040112. PubMed PMID: 26494286; PMCID: PMC4653226.

72. Gonzalez-Mariscal I, Montoro RA, Doyle ME, Liu QR, Rouse M, O'Connell JF, Santa-Cruz Calvo S, Krzysik-Walker SM, Ghosh S, Carlson OD, Lehrmann E, Zhang Y, Becker KG, Chia CW, Ghosh P, Egan JM. Absence of cannabinoid 1 receptor in beta cells protects against high-fat/high-sugar diet-induced beta cell dysfunction and inflammation in murine islets. Diabetologia. 2018;61(6):1470-83. Epub 2018/03/03. doi: 10.1007/s00125-018-4576-4. PubMed PMID: 29497784; PMCID: PMC6201315.

73. Kim W, Lao Q, Shin Y-K, Carlson OD, Lee EK, Gorospe M, Kulkarni RN, Egan JM. Cannabinoids Induce Pancreatic β-Cell Death by Directly Inhibiting Insulin Receptor Activation. Science Signaling. 2012;5(216):ra23; PMCID: PMC3524575.

74. Kim W, Doyle ME, Liu Z, Lao Q, Shin YK, Carlson OD, Kim HS, Thomas S, Napora JK, Lee EK, Moaddel R, Wang Y, Maudsley S, Martin B, Kulkarni RN, Egan JM. Cannabinoids inhibit insulin receptor signaling in pancreatic beta-cells. Diabetes. 2011;60(4):1198-209. Epub 2011/02/25. doi: 10.2337/db10-1550. PubMed PMID: 21346174; PMCID: PMC3064093.

75. Galiègue S, Mary S, Marchand J, Dussossoy D, Carrière D, Carayon P, Bouaboula M, Shire D, LE Fur G, Casellas P. Expression of Central and Peripheral Cannabinoid Receptors in Human Immune Tissues and Leukocyte Subpopulations. European Journal of Biochemistry. 1995;232(1):54-61. doi: 10.1111/j.1432-1033.1995.tb20780.x.

76. Wang S, Ye Q, Zeng X, Qiao S. Functions of Macrophages in the Maintenance of Intestinal Homeostasis. Journal of Immunology Research. 2019;2019:1512969. doi: 10.1155/2019/1512969.

77. Yip RG, Boylan MO, Kieffer TJ, Wolfe MM. Functional GIP receptors are present on adipocytes. Endocrinology. 1998;139(9):4004-7. Epub 1998/09/02. doi: 10.1210/endo.139.9.6288. PubMed PMID: 9724057.

78. Ceperuelo-Mallafré V, Duran X, Pachón G, Roche K, Garrido-Sánchez L, Vilarrasa N, Tinahones FJ, Vicente V, Pujol J, Vendrell J, Fernández-Veledo S. Disruption of GIP/GIPR Axis in Human Adipose Tissue Is Linked to Obesity and Insulin Resistance. The Journal of Clinical Endocrinology & Metabolism. 2014;99(5):E908-E19. doi: 10.1210/jc.2013-3350.

79. Grigoryan M, Kedees MH, Guz Y, Teitelman G. Phenotype of entero-endocrine L cells becomes restricted during development. Dev Dyn. 2012;241(12):1986-92. doi: 10.1002/dvdy.23875. PubMed PMID: 23027401.

80. Egerod KL, Engelstoft MS, Grunddal KV, Nøhr MK, Secher A, Sakata I, Pedersen J, Windeløv JA, Füchtbauer E-M, Olsen J, Sundler F, Christensen JP, Wierup N, Olsen JV, Holst JJ, Zigman JM, Poulsen SS, Schwartz TW. A Major Lineage of Enteroendocrine Cells Coexpress CCK, Secretin, GIP, GLP-1, PYY, and Neurotensin but Not Somatostatin. Endocrinology. 2012;153(12):5782-95. doi: 10.1210/en.2012-1595.

81. Habib AM, Richards P, Cairns LS, Rogers GJ, Bannon CAM, Parker HE, Morley TCE, Yeo GSH, Reimann F, Gribble FM. Overlap of Endocrine Hormone Expression in the Mouse Intestine Revealed by Transcriptional Profiling and Flow Cytometry. Endocrinology. 2012;153(7):3054-65. doi: 10.1210/en.2011-2170.

82. Svendsen B, Pedersen J, Albrechtsen NJW, Hartmann B, Toräng S, Rehfeld JF, Poulsen SS, Holst JJ. An Analysis of Cosecretion and Coexpression of Gut Hormones From Male Rat Proximal and Distal Small Intestine. Endocrinology. 2015;156(3):847-57. doi: 10.1210/en.2014-1710.

83. Theodorakis MJ, Carlson O, Michopoulos S, Doyle ME, Juhaszova M, Petraki K, Egan JM. Human duodenal enteroendocrine cells: source of both incretin peptides, GLP-1 and GIP. American Journal of Physiology-Endocrinology and Metabolism. 2006;290(3):E550-E9. doi: 10.1152/ajpendo.00326.2004. PubMed PMID: 16219666.

84. Kaelberer MM, Buchanan KL, Klein ME, Barth BB, Montoya MM, Shen X, Bohórquez DV. A gut-brain neural circuit for nutrient sensory transduction. Science. 2018;361(6408):eaat5236. doi: 10.1126/science.aat5236.

85. Bohorquez DV, Shahid RA, Erdmann A, Kreger AM, Wang Y, Calakos N, Wang F, Liddle RA. Neuroepithelial circuit formed by innervation of sensory enteroendocrine cells. J Clin Invest. 2015. doi: 10.1172/JCI78361. PubMed PMID: 25555217.

86. Bohorquez DV, Samsa LA, Roholt A, Medicetty S, Chandra R, Liddle RA. An enteroendocrine cell-enteric glia connection revealed by 3D electron microscopy. PLoS One. 2014;9(2):e89881. doi: 10.1371/journal.pone.0089881. PubMed PMID: 24587096; PMCID: 3935946.

87. Egerod KL, Petersen N, Timshel PN, Rekling JC, Wang Y, Liu Q, Schwartz TW, Gautron L. Profiling of G protein-coupled receptors in vagal afferents reveals novel gut-to-brain sensing mechanisms. Molecular Metabolism. 2018;12:62-75. doi: https://doi.org/10.1016/j.molmet.2018.03.016.

88. Iwasaki Y, Sendo M, Dezaki K, Hira T, Sato T, Nakata M, Goswami C, Aoki R, Arai T, Kumari P, Hayakawa M, Masuda C, Okada T, Hara H, Drucker DJ, Yamada Y, Tokuda M, Yada T. GLP-1 release and vagal afferent activation mediate the beneficial metabolic and chronotherapeutic effects of D-allulose. Nature Communications. 2018;9(1):113. doi: 10.1038/s41467-017-02488-y.

Figures

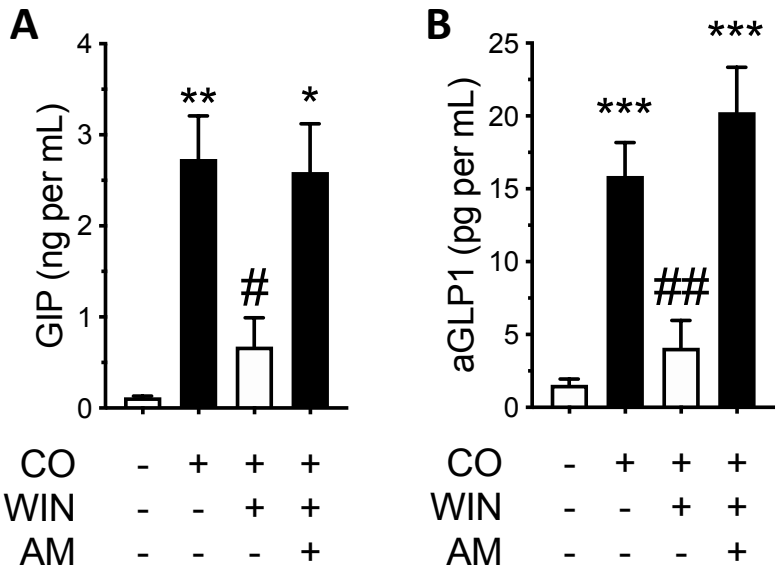

Figure 3.1. Peripheral CB_1Rs control GIP and aGLP1 secretion. Compared to control (0.5 mL saline by oral gavage and vehicle by IP injection), corn oil (CO; 0.5 mL by oral gavage) increased levels of GIP (A) and aGLP1 (B) in plasma of fasted mice an effect blocked by the CB_1R agonist, WIN 55,212-2 (WIN, IP 3 mg per kg 30 min before CO). The effects of WIN were inhibited by co-administration with the peripherally-restricted CB_1R antagonist, AM6545 (AM; IP 10 mg per kg 30 min before CO). Data expressed as means ± S.E.M. Analyzed by one-way ANOVA with post hoc Newman-Keuls multiple comparison test. n=5 per condition, * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

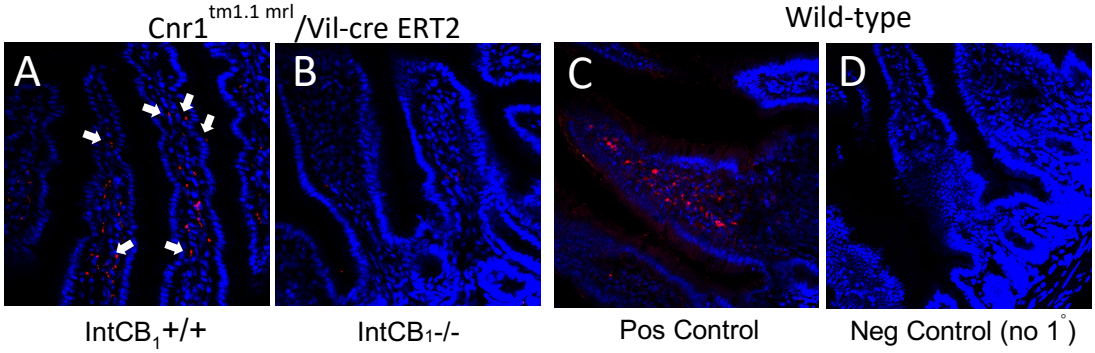

Figure 3.2. CB_1R immunoreactivity is absent in the proximal small intestinal epithelium in $IntCB_1$-/- mice. Compared to treated control mice (A, $IntCB_1$+/+) Immunohistochemical detection of CB_1R is absent in treated $IntCB_1$-/- mice (B). Wild-type C57Bl6/N mice display immunoreactivity for CB_1Rs, an effect that is lost when primary CB_1R antibody is omitted (D). Arrows indicate red immunoreactivity for CB_1Rs.

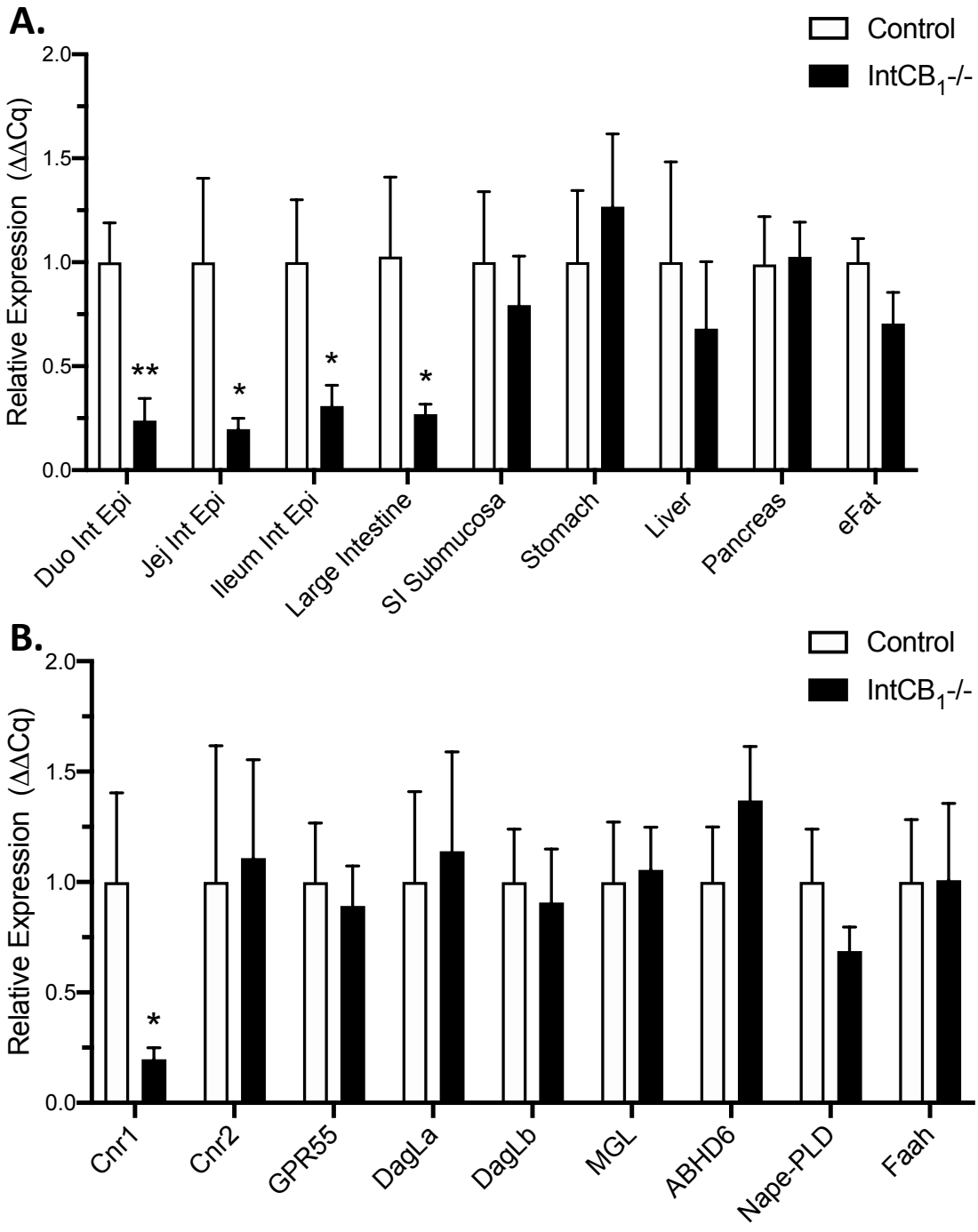
A.
Control
IntCB1-/-
Relative Expression (ΔΔCq)
2.0
1.5
1.0
0.5
0.0
**
*
*
*
Duo Int Epi
Jej Int Epi
Ileum Int Epi
Large Intestine
SI Submucosa
Stomach
Liver
Pancreas
eFat
B.
Control
IntCB1-/-
Relative Expression (ΔΔCq)
2.0
1.5
1.0
0.5
0.0
*
Cnr1
Cnr2
GPR55
DagLa
DagLb
MGL
ABHD6
Nape-PLD
Faah

Figure 3.3. CB_1R mRNA is downregulated in the intestinal epithelium in the small intestine and large intestine. Compared to tamoxifen treated control mice ($IntCB_1+/+$), $IntCB_1-/-$ showed a reduction in CB_1R (Cnr1) mRNA in the intestinal epithelium of the duodenum (duo int epi), jejunum (jej int epi), ileum (ileum int epi) and large intestine but not in the small intestinal submucosa (SI submucosa) or other metabolically relevant tissues (stomach, liver, pancreas, epididymal fat-eFat) (A). Additionally, in the proximal small intestine, there were no significant changes in mRNA of other genes involved in the extended endocannabinoid system (B). Data expressed as means ± S.E.M. Analyzed by regular student's unpaired t-test. n=6-8 per condition, * $p<0.05$, ** $p<0.01$.

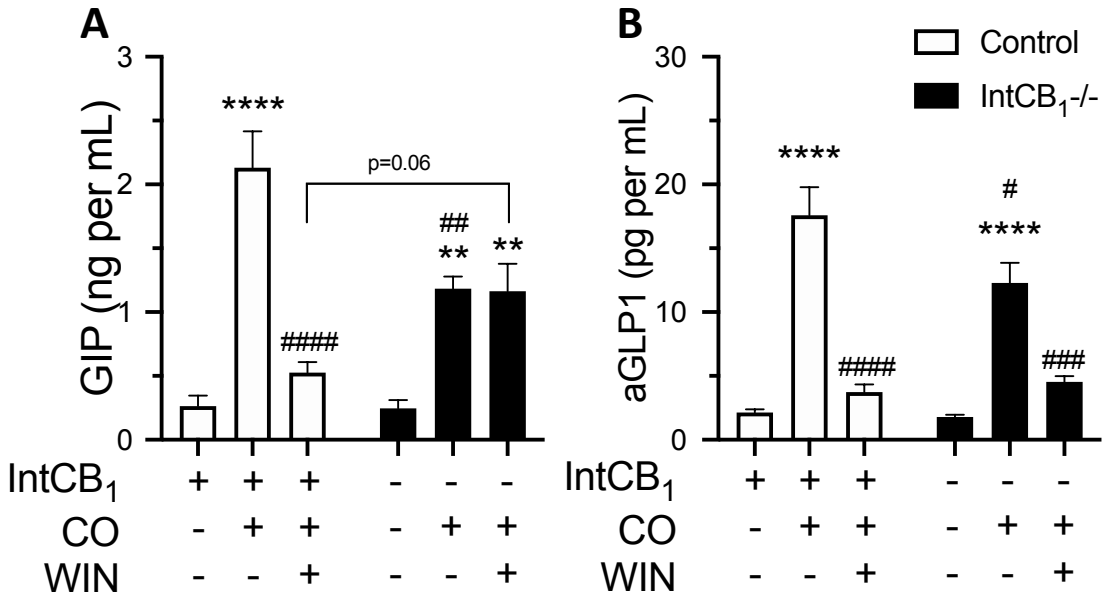

Figure 3.4. Intestinal CB_1Rs control GIP, but not aGLP1, secretion. Compared to saline-vehicle treated mice (0.5 mL saline by oral gavage and vehicle by IP injection), corn oil (CO; 0.5 mL by oral gavage) increased levels of GIP (A) and aGLP1 (B) in plasma of control ($IntCB_1$+) and $IntCB_1$-/- ($IntCB_1$-) mice. However, CO induced a less GIP and aGLP1 secretion in $IntCB_1$-/- mice when compared to control mice. The CB_1R agonist WIN 55,212-2 (WIN, IP 3 mg per kg 30 min before CO) blocked CO induced GIP and aGLP1 secretion in control mice. However, WIN did not block CO induced GIP secretion in $IntCB_1$-/- mice but did block CO induced aGLP1 secretion in $IntCB_1$-/- mice. Data expressed as means ± S.E.M. Analyzed by one-way ANOVA with post hoc Newman-Keuls multiple comparison test. n = 6-8 per condition, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ****$p<0.0001$.

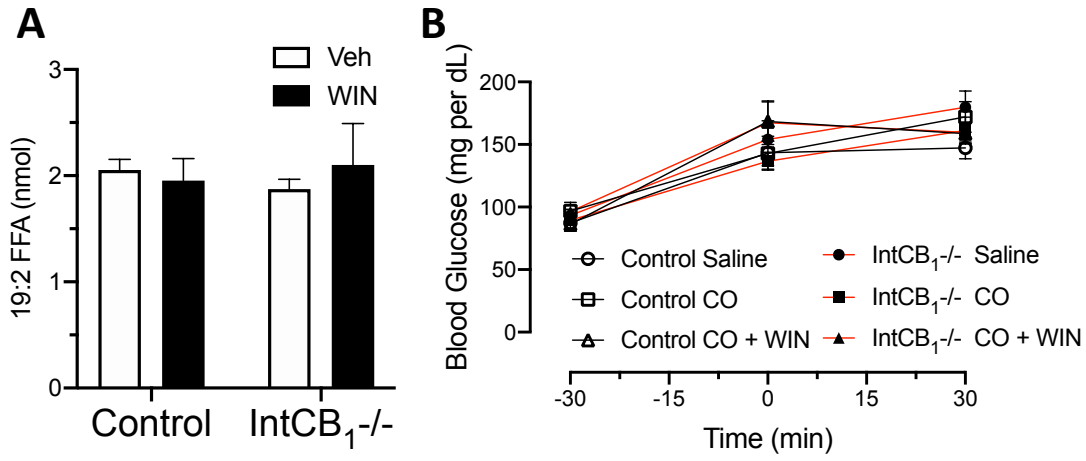

Figure 3.5. Analysis of effects of genotype and WIN treatment on gastric emptying and blood glucose levels. Gastric emptying was evaluated by quantitating via UPLC/MS/MS levels of 19:2 free fatty acid (19:2 FFA) recovered from stomach 30 min following oral gavage of corn oil (CO, 0.5 mL). Thirty min prior to gavage, mice were administered WIN 55,212-2 (WIN), or vehicle (Veh). WIN treatment had no significant effect on gastric emptying of CO. Additionally, intestinal CB_1R deletion did not affect gastric emptying (A). Blood glucose levels were measured at time -30 just prior to administration of WIN or vehicle. Blood glucose was again measured 30 min later at time 0 just prior to oral gavage of CO 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 (B). Data is expressed as mean ± SEM. Data expressed as mean ± SEM. Analyzed using two-way ANOVA with Holm-Sidak's post hoc multiple comparison's test (A) or repeated measures two-way ANOVA (B). $p>0.05$. n=4-8.

Conclusion

This body of work examines the endocannabinoid (eCB) system as a contributing factor to the onset of obesity. The eCB system governs food intake and energy conservation through different central (brain) and peripheral mechanisms; during diet induced obesity, there is increased eCB tone and increased signaling in different tissues. First, we examined the effect of chronic western diet consumption on maternal and neonate health in mice. Maternal diet and maternal obesity were associated with decreased neonate health and viability. Additionally, we examined the role of intestinal endocannabinoid signaling in controlling hyperphagia associated with western diet induced obesity. Indeed, increased eCB signaling in the proximal small intestine inhibited the secretion of satiation hormone cholecystokinin (CCK). Lastly, we revealed a differential role of intestinal cannabinoid receptors in controlling secretion of glucose regulatory peptides following a meal. Taken together, these data indicate a role for peripheral eCB signaling in controlling food intake and energy homeostasis, a phenomenon previously believed to be entirely centrally mediated.

Impact of Maternal Western Diet-Induced Obesity on Offspring

The American Medical Association declared obesity a disease in 2013 (1). Alarmingly, obesity rates have steadily increased over the past several decades and have led to a prevalence of roughly 40% among adults in the United States (2, 3). Dietary overconsumption of foods rich in sugars and fats (termed a western-

style diet, WD) is an important contributing factor to the onset of obesity (4). Diet-induced obesity (DIO) is associated with a host of metabolic abnormalities including hyperphagia, hyperglycemia, and increased adiposity (4, 5). Obesity in human females of childbearing age and obesity during gestation is also associated with abnormal metabolic profiles in offspring, which may include an epigenetic component in these outcomes (6). Thus, we aimed to examine the impact of maternal diet on feeding behavior and endocannabinoid signaling, a system involved in controlling food intake and energy conservation, on offspring of obese dams using a DIO mouse model.

Female mice were chronically fed a high fat/high sucrose WD for 10 weeks at which time they displayed significantly increased body weights and altered feeding behaviors compared to female mice fed a standard chow. The feeding behavior reflected similar changes in meal size and rate of intake as previously seen in DIO males (7). In males, peripheral endocannabinoid signaling controls hyperphagia associated with DIO (7). Furthermore, our recent findings suggest that hyperphagia during DIO may be due to increased eCB signaling in the proximal small intestine which inhibits secretion of satiation hormones, notably CCK (see chapter 2), following a meal (8). This data may suggest that a similar mechanism may exist in DIO females; however, a direct test of this hypothesis remains to be tested.

Maternal DIO led to high rates of neonate mortality including spontaneous stillbirths of individual offspring days before the rest of the litter's date of birth. Although the mortality rate in our study exceeded 50% in pups born from dams chronically fed a WD, the results are consistent with other rodent studies that report increased neonate mortality prior to weaning (9). Both of these studies indicate that maternal diet is an important indicator of neonate survival in rodents. Notably, human maternal obesity also increases the likelihood of spontaneous pre-term abortions, albeit at lower rates (10). A study that surveys the differences of specific dietary macronutrients and how these impact neonate survival in mammals is needed.

Surviving offspring were subsequently monitored and feeding behaviors were assessed after 10 weeks. Surviving offspring, both male and female, did not display significant differences in body weights or feeding parameters. Additionally, the surviving offspring showed little differences in levels of eCB and other related lipids in plasma, pancreas, and proximal small intestinal epithelium. We only report sex specific differences in plasma DHAG and DHEA, which are synthesized from omega-3 fatty acid docosahexaenoic acid and may regulate inflammation and neural development (11, 12). Furthermore, we found no appreciable changes in expression of the cannabinoid receptors or in the eCB biosynthetic and degradative enzymes within small intestinal epithelium and pancreas of males and females. Only male offspring born from obese dams saw a modest reduction of MGL mRNA expression in the proximal small intestine, which was paired with no

changes in monoacylglycerols 2AG or DHAG. This small decrease in MGL expression may have resulted in changes in the levels of other monoacylglycerols but a comprehensive analysis of these family of lipids is needed. Collectively, these subtle changes may predispose the offspring to develop obesity if challenged with a high fat diet, as is observed in human obesity (13, 14).

In contrast to other groups that examined changes in various other tissues immediately following weaning and at times later than our indicated 10-week timepoint, we report no appreciable changes in feeding behavior, in eCBs and related molecules, or in expression of key genes of the eCB system (9, 15). Additionally, our studies highlight a large increase in mortality in neonates born from dams chronically fed a WD which differs in overall macronutrient composition of diets tested in other studies. Notably, neonate mortality was restricted to the first six days after birth. Furthermore, it is important to consider that behavioral and biochemical analysis of the animals tested were performed on the surviving pup fraction, which may be considered “extraordinary” in their ability to survive. Thus, future studies under our conditions should include a comprehensive temporal evaluation of eCB signaling in metabolically relevant tissues. Lastly, it is possible that the neonates that died within six days following their birth had significant changes in eCB system and other regulatory factors affecting feeding and energy homeostasis that led to their failure to thrive. A test of this hypothesis remains but is difficult given the inability to predict when mice will die, and which mice will survive.

Cannabinoid CB_1 Receptors Inhibit Gut-Brain Satiation Signaling in Diet-Induced Obesity

The steady increase in obesity rates over the past several decades has escalated the effort to develop safe therapeutic obesity. Obesity is a multifactorial disease that affects food intake and energy balance which may increase morbidity and mortality (see chapter 1), and may increase the probability to develop other serious comorbidities such as heart disease, hypertension, or type 2 diabetes (16, 17). The eCB system regulates food intake and energy homeostasis. Hypothalamic eCB signaling is crucial to develop food seeking behaviors and directly opposes the actions of leptin, a satiety hormone (18, 19). Additionally, eCB signaling in the forebrain controls energy use and dissipation within the adipose tissue (20). Interestingly, circulating levels of the eCBs increase in human and rodent models of obesity (7, 21-27), which may directly interact with CB_1Rs in the brain and control feeding behavior and energy homeostasis. Thus, inhibitors that disrupt eCB signaling show promise to treat hyperphagia and other metabolic abnormalities often observed in obesity (7, 28-31).

Rimonabant, a globally acting CB_1R antagonist, was used to treat obese patients in Europe; patients treated with rimonabant demonstrated significant weight loss while maintaining lean body mass (32, 33). Additionally, treated patients had beneficial metabolic effects on HDL/LDL, blood triglycerides, and decreases in HbA1C% (32-34) However, while hypothalamic CB_1Rs are responsible for regulating energy homeostasis, recent findings have also shown

central CB_1Rs are involved in fear, anxiety, and memory (35). As a result of these centrally mediated effects, treatment with rimonabant resulted in increased incidence of depression and suicidal ideation, and was removed from the European market (36). Recent investigations have since focused on peripherally-restricted CB_1R antagonists to promote the pro-metabolic effects of eCB signaling inhibition while limiting the centrally mediated psychiatric side effects of central CB_1R disruption.

The small intestine is an important organ that produces a variety of neuropeptides that signal to the brain in order to govern food intake. The molecular underpinnings of gut-brain signaling and their dysregulation in DIO are poorly defined. Our previous research suggests a role for eCB signaling in the proximal small intestine in controlling food seeking behaviors (37-39). Furthermore, our follow-up studies suggest that eCB activity at CB_1Rs in the proximal small intestinal epithelium is upregulated in mice chronically fed a WD, a phenomenon that is associated with increased caloric consumption, meal size, and rate of intake (7). In our current study, we provide evidence to support the hypothesis that elevated intestinal eCB signaling promotes overeating by a mechanism that includes inhibiting nutrient-induced gut-brain CCK-dependent satiation signaling.

Sykaras and colleagues first reported the expression of CB_1R mRNA in enteroendocrine I-cells which are located in the proximal small intestine and secrete CCK, a satiation hormone involved in regulating meal size (40). Using transgenic CCK-eGFP mice, we confirmed CB_1R mRNA expression in sorted

CCK-eGFP producing cells and demonstrated CB_1R protein expression on CCK cells by immunohistochemical colocalization in the proximal small intestinal epithelium. In lean mice, oral gavage of corn oil (CO) increased circulating levels of bioactive CCK (CCK-8). Pharmacological activation of CB_1Rs blocked this effect, which was reversed by inhibition of peripheral CB_1Rs with a peripherally-restricted CB_1R-specific neutral antagonist. Mice fed a WD displayed increased levels of eCBs the proximal small intestinal epithelium and have elevated eCB signaling. In DIO mice, oral gavage of CO failed to affect circulating levels of CCK-8, and inhibition of peripheral CB_1Rs in WD mice restored the ability for CO to increase plasma CCK-8 levels. This indicated the presence of overactive eCB signaling that hindered normal secretion of CCK-8 during DIO.

We further examined the role of peripheral CB_1Rs in normalizing food intake in DIO-related hyperphagia through a CCK-dependent mechanism. We previously reported that mice chronically fed WD developed hyperphagia with increased meal size, rate of intake, and total caloric consumption (7). Pharmacological inhibition of peripheral CB_1Rs in DIO mice blocked these changes. However, the hypophagic effects of peripheral CB_1R antagonism in DIO mice were reversed by pretreatment with a low-dose CCK_A receptor antagonist. Thus, 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 data 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. Open-type CCK-producing enteroendocrine cells in the intestinal lining form functional synapses with afferent vagal fibers (41, 42). Termed “neuropods”, these cells sense luminal 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 along the gut-brain axis (43, 44). Our data suggest that CB_1Rs may be at the interface of this signaling. It is unknown, however, if CB_1Rs control glutamate signaling at these synapses in the small intestine as they do in the brain (45). Additionally, intestinal CB_1Rs may block neurotransmitter release by inhibiting calcium influx or mobilization as has been described in the brain considering nutrient-induced CCK release is calcium-dependent (46-51). However, a direct test of these hypotheses remains. Taken together, these studies describe key roles for peripheral CB_1Rs in feeding behavior and energy homeostasis.

Intestinal CB_1R control of nutrient-induced CCK release may be one of several mechanisms by which peripheral CB_1Rs impact gut-brain signaling pathways (52-54). Ghrelin, a hormone produced in the stomach that increases food intake, has been shown to be controlled by CB_1Rs (55-57). Additionally, other groups have developed and tested peripherally-restricted CB_1R inverse agonists and shown a reduction in food intake in DIO mice through a mechanism that may

include reversing hyperleptinemia and DIO associated leptin resistance and restoring anorexic melanocortin signaling in the arcuate nucleus of the hypothalamus (58, 59). Lastly, the hypophagic effects of CB_1R inhibition with the globally acting CB_1R inverse agonist, rimonabant, are blocked by pharmacological inhibition of peripheral beta-adrenergic neurotransmission, which suggests that CB_1Rs may additionally control feeding behavior via interactions with the peripheral sympathetic nervous system (60). Overall, peripheral CB_1R inhibition shows promise as a safer therapeutic option for treatment of hyperphagia during DIO by reducing food intake through various inhibitor-dependent pathways that may be devoid of centrally mediated psychiatric side effects (extensively reviewed in (61)).

Role for Intestinal CB_1 Receptors in Controlling GIP and GLP1 Secretion

The use of peripherally-restricted CB_1R inhibitors to treat metabolic abnormalities associated with obesity has also gained much attention. Importantly, many of the pro-metabolic effects resulting from peripheral CB_1R inhibition are comparable, if not equal, to the effects of global CB_1R inhibition by rimonabant. In various genetic and diet-induced obesity preclinical models, treatment with low brain-penetrant CB_1R inhibitors have decreased adiposity, increased insulin sensitivity, and improved obesity related non-alcoholic fatty liver disease and kidney injuries (59, 62-65). CB_1Rs are expressed in low, but significant, levels in key organs that govern energy conservation including the endocrine pancreas, liver, and adipose tissues (66-69). Recently, CB_1R mRNA expression was

localized to enteroendocrine cells that produce important glucose regulatory peptides that act within the entero-insular axis (70); the exact role of CB_1Rs requires further investigation.

Incretins are important peripheral hormones produced by enteroendocrine K- and L- cells that enhance insulin secretion, regulate blood sugar levels, and express mRNA for CB_1Rs. Limited studies using globally acting antagonists or transgenic full body CB_1R knockouts suggests a role for CB_1R in inhibiting GIP and GLP1 secretion following a meal, yet the functional role of these receptors on these cell types are poorly characterized (70-73). Here, we aimed to test the hypothesis that intestinal CB_1Rs control nutrient induced GIP and GLP1 release.

Enteroendocrine K- and L-cells sense luminal contents through different free fatty acid receptors. These G-protein coupled receptors are enriched in enteroendocrine populations and mobilize calcium through transient receptor potential channels and/or voltage gated calcium channels to depolarize the cells, resulting in vesicular hormone release (74). Thus, we utilized corn oil, which contains a mix of different saturated, mono-, and polyunsaturated FFAs, to stimulate incretin secretion in our investigation. Oral gavage of CO increased circulating levels of GIP and bioactive GLP1 (aGLP1), and pharmacological activation of CB_1Rs blocked this effect, which was reversed by inhibition of peripheral CB_1Rs with a peripherally-restricted CB_1R-specific neutral antagonist. Our results are consistent with other findings showing decreased GIP or GLP1 release following cannabinoid receptor activation in primary murine cells, mouse

models, and in humans (70-72). In contrast, we utilized a peripherally restricted CB_1R specific antagonist AM6545, not a globally-acting CB_1R inhibitor, to delineate the location of this effect in our investigations. Cotreatment with WIN and AM restored the nutrient induced incretin secretion showing, for the first time, that this effect is mediated through peripheral CB_1R signaling. These results also reflect a similar pattern when compared to CCK producing cells (see chapter 2, (8)), suggesting a redundant or overlapping mechanism between cell types. While enteroendocrine cells predominately produce a characteristic intestinal-derived hormone, specific enteroendocrine cell types have the ability to produce multiple peptides (75-79).

Next, we investigated role of intestinal CB_1Rs in controlling incretin secretion. we utilized a novel inducible transgenic mouse model that lack intestinal CB_1Rs. Our novel $iCB_1^{-/-}$ mice showed no immunoreactivity to CB_1R antibodies in the proximal small intestine. Additionally, we report significant decreases in CB_1R mRNA in the small and large intestinal epithelium but not in the submucosal layer or in other metabolically relevant tissues. As expected, CO stimulated both GIP and aGLP1 secretion in control treated mice containing intestinal CB_1Rs which was blocked by WIN pretreatment. In $IntCB_1$-/- mice, CO stimulated GIP and aGLP1 secretion but at significantly lower levels when compared to control mice. This suggests that intestinal CB_1Rs may be involved in nutrient sensing, incretin production, or regulating enteroendocrine populations and are the subjects of future investigations. Interestingly, plasma GIP levels in $IntCB_1$-/- given a CO

gavage were unaffected by WIN pretreatment. On the other hand, WIN pretreatment in IntCB_1-/- mice blocked increases in plasma aGLP1 levels following a CO gavage. This indicates a role for intestinal CB_1Rs in controlling GIP release but not in controlling GLP1 secretion.

Incretins also exhibit other non-insulinotropic outcomes that affect energy conservation. For example, GIP signaling within adipocytes has been reported to increase fat accumulation by improving insulin sensitivity and enhancing insulin induced glucose reuptake and fatty acid incorporation (80-83). GIP signaling also increases lipoprotein lipase activity to increase free fatty acid availability from circulating triglycerides (83). Notably, circulating GIP levels are increased during obesity and blocking GIP signaling prevents DIO (80, 81). This study shows a role for intestinal CB_1Rs in controlling GIP release during a lean state. Without CB_1R regulation of GIP, it is possible that temporal increases in GIP overtime may contribute to obesity pathogenesis by increasing fat synthesis and energy storage. The long-term effects of removing intestinal CB_1R-mediated regulation of GIP release during chronic consumption of a high fat diet and the onset of obesity pathogenesis require further research.

Finally, peripheral CB_1R control of GLP1 may also involve afferent vagal fibers and may overlap with CCK mediated gut-brain interactions. As previously described, intestinal CCK producing cells form functional synapses with afferent vagal fibers which in turn communicate with the brain via the gut-brain axis (41-44). Importantly, about half of all neurons positive for CCK receptors also express

GLP1 receptors, whereas nearly all neurons positive for GLP1 receptors contain CCK receptors (84). Once activated, GLP1 receptors on these cells signal to the nucleus of the solitary tract in the brainstem to reduce food intake (85). Additionally, CB_1R expression was also colocalized to both types of CCK receptor positive neurons (those co-expressing and lacking GLP1 receptors), as well as on other neurons not expressing CCK receptors that innervate the intestinal mucosal layer (84). Thus, it may be possible that CB_1Rs on these neurons, and not intestinal CB_1Rs, may be controlling GLP1 secretion and may contribute to the hypophagic effects of CB_1R inhibition; however, a direct test to this hypothesis remains.

References

1. Kyle TK, Dhurandhar EJ, Allison DB. Regarding Obesity as a Disease: Evolving Policies and Their Implications. Endocrinology and Metabolism Clinics of North America. 2016;45(3):511-20. doi: https://doi.org/10.1016/j.ecl.2016.04.004.

2. Ogden CL, Carroll MD, Lawman HG, Fryar CD, Kruszon-Moran D, Kit BK, Flegal KM. Trends in Obesity Prevalence Among Children and Adolescents in the United States, 1988-1994 Through 2013-2014. JAMA. 2016;315(21):2292-9. doi: 10.1001/jama.2016.6361. PubMed PMID: 27272581.

3. Ogden CL, Carroll MD, Kit BK, Flegal KM. Prevalence of obesity in the United States, 2009-2010. NCHS data brief, no 82 Hyattsville, MD: National Center for Health Statistics 2012. 2012.

4. Medina-RemOn A, Kirwan R, Lamuela-Raventos RM, Estruch R. Dietary Patterns and the Risk of Obesity, Type 2 Diabetes Mellitus, Cardiovascular Diseases, Asthma, and Mental Health Problems. Crit Rev Food Sci Nutr. 2016:0. doi: 10.1080/10408398.2016.1158690. PubMed PMID: 27127938.

5. Poti JM, Duffey KJ, Popkin BM. The association of fast food consumption with poor dietary outcomes and obesity among children: is it the fast food or the remainder of the diet? Am J Clin Nutr. 2014;99(1):162-71. doi: 10.3945/ajcn.113.071928. PubMed PMID: 24153348; PMCID: PMC3862453.

6. Agarwal P, Morriseau TS, Kereliuk SM, Doucette CA, Wicklow BA, Dolinsky VW. Maternal obesity, diabetes during pregnancy and epigenetic mechanisms that influence the developmental origins of cardiometabolic disease in the offspring. Critical Reviews in Clinical Laboratory Sciences. 2018;55(2):71-101. doi: 10.1080/10408363.2017.1422109.

7. Argueta DA, DiPatrizio NV. Peripheral endocannabinoid signaling controls hyperphagia in western diet-induced obesity. Physiol Behav. 2017;171:32-9. doi: 10.1016/j.physbeh.2016.12.044. PubMed PMID: 28065722.

8. Argueta DA, Perez PA, Makriyannis A, DiPatrizio NV. Cannabinoid CB1 Receptors Inhibit Gut-Brain Satiation Signaling in Diet-Induced Obesity. Frontiers in Physiology. 2019;10(704). doi: 10.3389/fphys.2019.00704.

9. Ramírez-López MT, Vázquez M, Bindila L, Lomazzo E, Hofmann C, Blanco RN, Alén F, Antón M, Decara J, Ouro D, Orio L, Suarez J, Lutz B, Rodríguez de Fonseca F, Gómez de Heras R. Exposure to a highly caloric palatable diet during pregestational and gestational periods affects hypothalamic and hippocampal endocannabinoid levels at birth and induces adiposity and anxiety-like behaviors in male rat offspring. Frontiers in Behavioral Neuroscience. 2015;9(339). PubMed PMID: 1805495129.

10. McDonald SD, Han Z, Mulla S, Beyene J. Overweight and obesity in mothers and risk of preterm birth and low birth weight infants: systematic review and meta-analyses. BMJ. 2010;341:c3428. doi: 10.1136/bmj.c3428.

11. Kim H-Y, Spector AA. N-Docosahexaenoylethanolamine: A neurotrophic and neuroprotective metabolite of docosahexaenoic acid. Molecular Aspects of Medicine. 2018;64:34-44. doi: https://doi.org/10.1016/j.mam.2018.03.004.

12. Park T, Chen H, Kevala K, Lee J-W, Kim H-Y. N-Docosahexaenoylethanolamine ameliorates LPS-induced neuroinflammation via cAMP/PKA-dependent signaling. Journal of Neuroinflammation. 2016;13(1):284. doi: 10.1186/s12974-016-0751-z.

13. Gaillard R, Felix JF, Duijts L, Jaddoe VW. Childhood consequences of maternal obesity and excessive weight gain during pregnancy. Acta Obstet Gynecol Scand. 2014;93(11):1085-9. Epub 2014/09/19. doi: 10.1111/aogs.12506. PubMed PMID: 25231923.

14. Gaillard R. Maternal obesity during pregnancy and cardiovascular development and disease in the offspring. European journal of epidemiology. 2015;30(11):1141-52. Epub 2015/09/16. doi: 10.1007/s10654-015-0085-7. PubMed PMID: 26377700.

15. Ramírez-López MT, Arco R, Decara J, Vázquez M, Noemí Blanco R, Alén F, Suárez J, Gómez de Heras R, Rodríguez de Fonseca F. Exposure to a Highly Caloric Palatable Diet during the Perinatal Period Affects the Expression of the Endogenous Cannabinoid System in the Brain, Liver and Adipose Tissue of Adult Rat Offspring. PLoS ONE. 2016;11(11):e0165432. doi:10.1371/journal.pone.0165432.

16. Reaven GM. Role of Insulin Resistance in Human Disease. Diabetes. 1988;37(12):1595-607. doi: 10.2337/diab.37.12.1595.

17. Stumvoll M, Goldstein BJ, van Haeften TW. Type 2 diabetes: principles of pathogenesis and therapy. The Lancet. 2005;365(9467):1333-46. doi: https://doi.org/10.1016/S0140-6736(05)61032-X.

18. Di Marzo V, Goparaju SK, Wang L, Liu J, Batkai S, Jarai Z, Fezza F, Miura GI, Palmiter RD, Sugiura T, Kunos G. Leptin-regulated endocannabinoids are involved in maintaining food intake. Nature. 2001;410(6830):822-5. PubMed PMID: 11298451.

19. Fride E, Ginzburg Y, Breuer A, Bisogno T, Di Marzo V, Mechoulam R. Critical role of the endogenous cannabinoid system in mouse pup suckling and growth. European Journal of Pharmacology. 2001;419(2–3):207-14. doi: http://dx.doi.org/10.1016/S0014-2999(01)00953-0.

20. Quarta C, Bellocchio L, Mancini G, Mazza R, Cervino C, Braulke LJ, Fekete C, Latorre R, Nanni C, Bucci M, Clemens LE, Heldmaier G, Watanabe M, Leste-Lassere T, Maitre M, Tedesco L, Fanelli F, Reuss S, Klaus S, Srivastava RK, Monory K, Valerio A, Grandis A, De Giorgio R, Pasquali R, Nisoli E, Cota D, Lutz B, Marsicano G, Pagotto U. CB(1) signaling in forebrain and sympathetic neurons is a key determinant of endocannabinoid actions on energy balance. Cell Metab. 2010;11(4):273-85. Epub 2010/04/09. doi: S1550-4131(10)00070-7 [pii] 10.1016/j.cmet.2010.02.015. PubMed PMID: 20374960.

21. Hillard CJ. Circulating Endocannabinoids: From Whence Do They Come and Where are They Going? Neuropsychopharmacology. 2018;43(1):155-72. doi: 10.1038/npp.2017.130.

22. Bluher M, Engeli S, Kloting N, Berndt J, Fasshauer M, Batkai S, Pacher P, Schon MR, Jordan J, Stumvoll M. Dysregulation of the peripheral and adipose tissue endocannabinoid system in human abdominal obesity. Diabetes. 2006;55(11):3053-60. PubMed PMID: 17065342.

23. Cote M, Matias I, Lemieux I, Petrosino S, Almeras N, Despres JP, Di Marzo V. Circulating endocannabinoid levels, abdominal adiposity and related cardiometabolic risk factors in obese men. Int J Obes (Lond). 2007;31(4):692-9. Epub 2007/01/17. doi: 0803539 [pii] 10.1038/sj.ijo.0803539. PubMed PMID: 17224929.

24. Di Marzo V, Cote M Fau - Matias I, Matias I Fau - Lemieux I, Lemieux I Fau - Arsenault BJ, Arsenault Bj Fau - Cartier A, Cartier A Fau - Piscitelli F, Piscitelli F Fau - Petrosino S, Petrosino S Fau - Almeras N, Almeras N Fau - Despres JP, Despres JP. Changes in plasma endocannabinoid levels in viscerally obese men following a 1 year lifestyle modification programme and waist circumference reduction: associations with changes in metabolic risk factors. Diabetologia. 2009;52:213(1432-0428 (Electronic)).

25. Matias I, Gatta-Cherifi B, Tabarin A, Clark S, Leste-Lasserre T, Marsicano G, Piazza PV, Cota D. Endocannabinoids measurement in human saliva as potential biomarker of obesity. PLoS One. 2012;7(7):e42399. doi: 10.1371/journal.pone.0042399. PubMed PMID: 22860123; PMCID: PMC3409167.

26. Engeli S, Bohnke J, Feldpausch M, Gorzelniak K, Janke J, Batkai S, Pacher P, Harvey-White J, Luft FC, Sharma AM, Jordan J. Activation of the peripheral endocannabinoid system in human obesity. Diabetes. 2005;54(10):2838-43. PubMed PMID: 16186383.

27. Little TJ, Cvijanovic N, DiPatrizio NV, Argueta DA, Rayner CK, Feinle-Bisset C, Young RL. Plasma endocannabinoid levels in lean, overweight, and obese humans: relationships to intestinal permeability markers, inflammation, and incretin secretion. American Journal of Physiology-Endocrinology and Metabolism. 2018;315(4):E489-E95. doi: 10.1152/ajpendo.00355.2017. PubMed PMID: 29438631.

28. Randall PA, Vemuri VK, Segovia KN, Torres EF, Hosmer S, Nunes EJ, Santerre JL, Makriyannis A, Salamone JD. The novel cannabinoid CB1 antagonist AM6545 suppresses food intake and food-reinforced behavior. Pharmacol Biochem Behav. 2010;97(1):179-84. Epub 2010/08/18. doi: S0091-3057(10)00233-9 [pii] 10.1016/j.pbb.2010.07.021. PubMed PMID: 20713079.

29. Jbilo O, Ravinet-Trillou C, Arnone M, Buisson I, Bribes E, Peleraux A, Penarier G, Soubrie P, Le Fur G, Galiegue S, Casellas P. The CB1 receptor antagonist rimonabant reverses the diet-induced obesity phenotype through the regulation of lipolysis and energy balance. Faseb J. 2005;19(11):1567-9. PubMed PMID: 16009704.

30. Poirier B, Bidouard JP, Cadrouvele C, Marniquet X, Staels B, O'Connor SE, Janiak P, Herbert JM. The anti-obesity effect of rimonabant is associated with an improved serum lipid profile. Diabetes Obes Metab. 2005;7(1):65-72. PubMed PMID: 15642077.

31. Verty AN, Allen AM, Oldfield BJ. The effects of rimonabant on brown adipose tissue in rat: implications for energy expenditure. Obesity (Silver Spring). 2009;17(2):254-61. Epub 2008/12/06. doi: oby2008509 [pii]10.1038/oby.2008.509. PubMed PMID: 19057531.

32. Pi-Sunyer FX, Aronne LJ, Heshmati HM, Devin J, Rosenstock J. Effect of rimonabant, a cannabinoid-1 receptor blocker, on weight and cardiometabolic risk factors in overweight or obese patients: RIO-North America: a randomized controlled trial. JAMA. 2006;295(7):761-75. Epub 2006/02/16. doi: 295/7/761 [pii] 10.1001/jama.295.7.761. PubMed PMID: 16478899.

33. Van Gaal LF, Rissanen AM, Scheen AJ, Ziegler O, Rossner S. Effects of the cannabinoid-1 receptor blocker rimonabant on weight reduction and cardiovascular risk factors in overweight patients: 1-year experience from the RIO-Europe study. Lancet. 2005;365(9468):1389-97. Epub 2005/04/20. doi: S0140-6736(05)66374-X [pii] 10.1016/S0140-6736(05)66374-X. PubMed PMID: 15836887.

34. Despres JP, Lemieux I, Almeras N. Contribution of CB1 blockade to the management of high-risk abdominal obesity. Int J Obes (Lond). 2006;30 Suppl 1:S44-52. PubMed PMID: 16570106.

35. Ruehle S, Rey AA, Remmers F, Lutz B. The endocannabinoid system in anxiety, fear memory and habituation. Journal of psychopharmacology (Oxford, England). 2012;26(1):23-39. Epub 2011/07/18. doi: 10.1177/0269881111408958. PubMed PMID: 21768162.

36. Moreira FA, Crippa JA. The psychiatric side-effects of rimonabant2009(1516-4446 (Print)).

37. DiPatrizio NV, Igarashi M, Narayanaswami V, Murray C, Gancayco J, Russell A, Jung KM, Piomelli D. Fasting stimulates 2-AG biosynthesis in the small intestine: role of cholinergic pathways. Am J Physiol Regul Integr Comp Physiol. 2015;309(8):R805-13. Epub 2015/08/21. doi: 10.1152/ajpregu.00239.2015. PubMed PMID: 26290104; PMCID: PMC4666947.

38. DiPatrizio NV, Joslin A, Jung KM, Piomelli D. Endocannabinoid signaling in the gut mediates preference for dietary unsaturated fats. Faseb J. 2013;27(6):2513-20. Epub 2013/03/07. doi: 10.1096/fj.13-227587 fj.13-227587 [pii]. PubMed PMID: 23463697; PMCID: 3659363.

39. DiPatrizio NV, Astarita G, Schwartz G, Li X, Piomelli D. Endocannabinoid signal in the gut controls dietary fat intake. Proc Natl Acad Sci U S A. 2011;108(31):12904-8. doi: 10.1073/pnas.1104675108. PubMed PMID: 21730161; PMCID: PMC3150876.

40. Sykaras AG, Demenis C, Case RM, McLaughlin JT, Smith CP. Duodenal Enteroendocrine I-Cells Contain mRNA Transcripts Encoding Key Endocannabinoid and Fatty Acid Receptors. PLoS ONE. 2012;7(8):e42373. doi: 10.1371/journal.pone.0042373.

41. Kaelberer MM, Buchanan KL, Klein ME, Barth BB, Montoya MM, Shen X, Bohórquez DV. A gut-brain neural circuit for nutrient sensory transduction. Science. 2018;361(6408):eaat5236. doi: 10.1126/science.aat5236.

42. Gribble FM, Reimann F. Enteroendocrine Cells: Chemosensors in the Intestinal Epithelium. Annual Review of Physiology. 2016;78(1):277-99. doi: 10.1146/annurev-physiol-021115-105439. PubMed PMID: 26442437.

43. Bohorquez DV, Shahid RA, Erdmann A, Kreger AM, Wang Y, Calakos N, Wang F, Liddle RA. Neuroepithelial circuit formed by innervation of sensory enteroendocrine cells. J Clin Invest. 2015. doi: 10.1172/JCI78361. PubMed PMID: 25555217.

44. Bohorquez DV, Samsa LA, Roholt A, Medicetty S, Chandra R, Liddle RA. An enteroendocrine cell-enteric glia connection revealed by 3D electron microscopy. PLoS One. 2014;9(2):e89881. doi: 10.1371/journal.pone.0089881. PubMed PMID: 24587096; PMCID: 3935946.

45. Jung KM, Astarita G, Zhu C, Wallace M, Mackie K, Piomelli D. A key role for diacylglycerol lipase-alpha in metabotropic glutamate receptor-dependent endocannabinoid mobilization. Mol Pharmacol. 2007;72(3):612-21. Epub 2007/06/23. doi: mol.107.037796 [pii] 10.1124/mol.107.037796. PubMed PMID: 17584991.

46. Pertwee RG. Endocannabinoids and Their Pharmacological Actions. Handb Exp Pharmacol. 2015;231:1-37. doi: 10.1007/978-3-319-20825-1_1. PubMed PMID: 26408156.

47. McLaughlin JT, Lomax RB, Hall L, Dockray GJ, Thompson DG, Warhurst G. Fatty acids stimulate cholecystokinin secretion via an acyl chain length-specific, Ca2+-dependent mechanism in the enteroendocrine cell line STC-1. The Journal of Physiology. 1998;513(1):11-8. doi: 10.1111/j.1469-7793.1998.011by.x.

48. Howlett AC, Reggio PH, Childers SR, Hampson RE, Ulloa NM, Deutsch DG. Endocannabinoid tone versus constitutive activity of cannabinoid receptors. Br J Pharmacol. 2011;163(7):1329-43. Epub 2011/05/07. doi: 10.1111/j.1476-5381.2011.01364.x. PubMed PMID: 21545414; PMCID: 3165945.

49. Hira T, Elliott AC, Thompson DG, Case RM, McLaughlin JT. Multiple Fatty Acid Sensing Mechanisms Operate in Enteroendocrine Cells: NOVEL EVIDENCE FOR DIRECT MOBILIZATION OF STORED CALCIUM BY CYTOSOLIC FATTY ACID. Journal of Biological Chemistry. 2004;279(25):26082-9. doi: 10.1074/jbc.M400098200.

50. Liou AP, Sei Y, Zhao X, Feng J, Lu X, Thomas C, Pechhold S, Raybould HE, Wank SA. The extracellular calcium-sensing receptor is required for cholecystokinin secretion in response to l-phenylalanine in acutely isolated intestinal I cells. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2011;300(4):G538-G46. doi: 10.1152/ajpgi.00342.2010. PubMed PMID: 21252045.

51. Nakajima S, Hira T, Hara H. Calcium-sensing receptor mediates dietary peptide-induced CCK secretion in enteroendocrine STC-1 cells. Molecular Nutrition & Food Research. 2012;56(5):753-60. doi: 10.1002/mnfr.201100666.

52. Cluny NL, Baraboi ED, Mackie K, Burdyga G, Richard D, Dockray GJ, Sharkey KA. High fat diet and body weight have different effects on cannabinoid CB(1) receptor expression in rat nodose ganglia. Auton Neurosci. 2013;179(1-2):122-30. Epub 2013/10/23. doi: 10.1016/j.autneu.2013.09.015. PubMed PMID: 24145047; PMCID: 3866822.

53. Burdyga G, Varro A, Dimaline R, Thompson DG, Dockray GJ. Ghrelin receptors in rat and human nodose ganglia: putative role in regulating CB-1 and MCH receptor abundance. Am J Physiol Gastrointest Liver Physiol. 2006;290(6):G1289-97. doi: 10.1152/ajpgi.00543.2005. PubMed PMID: 16423919.

54. Burdyga G, Lal S, Varro A, Dimaline R, Thompson DG, Dockray GJ. Expression of cannabinoid CB1 receptors by vagal afferent neurons is inhibited by cholecystokinin. J Neurosci. 2004;24(11):2708-15. PubMed PMID: 15028763.

55. Al-Massadi O, Gabellieri E, Trujillo ML, Senaris R, Pagotto U, Pasquali R, Casanueva FF, Seoane LM. Peripheral endocannabinoid system-mediated actions of rimonabant on growth hormone secretion are ghrelin-dependent. J Neuroendocrinol. 2011;22(11):1127-36. Epub 2010/09/03. doi: JNE2065 [pii] 10.1111/j.1365-2826.2010.02065.x. PubMed PMID: 20807320.

56. Senin LL, Al-Massadi O, Folguiera C, Pardo M, Barja-Fernandez S, Roca-Rivada A, Amil M, Criujeiras AB, Garcia-Caballero T, Gabellieri E, Leis R, Dieguez C, Pagotto U, Casanueva FF, Seoane LM. The gasric CB1 receptor modulates ghrelin production through the mTOR pathway to regulate food intake. PLoS ONE. 2013;8(11):e80339.

57. Cani PD, Montoya ML, Neyrinck AM, Delzenne NM, Lambert DM. Potential modulation of plasma ghrelin and glucagon-like peptide-1 by anorexigenic cannabinoid compounds, SR141716A (rimonabant) and oleoylethanolamide. Br J Nutr. 2004;92(5):757-61. Epub 2004/11/10. doi: S0007114504002363 [pii]. PubMed PMID: 15533263.

58. Tam J, Cinar R, Liu J, Godlewski G, Wesley D, Jourdan T, Szanda G, Mukhopadhyay B, Chedester L, Liow JS, Innis RB, Cheng K, Rice KC, Deschamps JR, Chorvat RJ, McElroy JF, Kunos G. Peripheral cannabinoid-1 receptor inverse agonism reduces obesity by reversing leptin resistance. Cell Metab. 2012;16(2):167-79. Epub 2012/07/31. doi: 10.1016/j.cmet.2012.07.002 S1550-4131(12)00277-X [pii]. PubMed PMID: 22841573.

59. Tam J, Szanda G, Drori A, Liu Z, Cinar R, Kashiwaya Y, Reitman ML, Kunos G. Peripheral cannabinoid-1 receptor blockade restores hypothalamic leptin signaling. Molecular Metabolism. 2017;6(10):1113-25. doi: https://doi.org/10.1016/j.molmet.2017.06.010.

60. Bellocchio L, Soria-Gomez E, Quarta C, Metna-Laurent M, Cardinal P, Binder E, Cannich A, Delamarre A, Haring M, Martin-Fontecha M, Vega D, Leste-Lasserre T, Bartsch D, Monory K, Lutz B, Chaouloff F, Pagotto U, Guzman M, Cota D, Marsicano G. Activation of the sympathetic nervous system mediates hypophagic and anxiety-like effects of CB1 receptor blockade. Proc Natl Acad Sci U S A. 2013;110(12):4786-91. Epub 2013/03/15. doi: 10.1073/pnas.1218573110 1218573110 [pii]. PubMed PMID: 23487769; PMCID: 3607008.

61. Quarta C, Cota D. Anti-obesity therapy with peripheral CB1 blockers: from promise to safe(?) practice. International Journal of Obesity. 2020. doi: 10.1038/s41366-020-0577-8.

62. Han JH, Shin H, Park J-Y, Rho JG, Son DH, Kim KW, Seong JK, Yoon S-H, Kim W. A novel peripheral cannabinoid 1 receptor antagonist, AJ5012, improves metabolic outcomes and suppresses adipose tissue inflammation in obese mice. The FASEB Journal. 2019;33(3):4314-26. doi: 10.1096/fj.201801152RR.

63. Jourdan T, Szanda G, Rosenberg AZ, Tam J, Earley BJ, Godlewski G, Cinar R, Liu Z, Liu J, Ju C, Pacher P, Kunos G. Overactive cannabinoid 1 receptor in podocytes drives type 2 diabetic nephropathy. Proceedings of the National Academy of Sciences. 2014;111(50):E5420-E8. doi: 10.1073/pnas.1419901111.

64. Cluny NL, Vemuri VK, Chambers AP, Limebeer CL, Bedard H, Wood JT, Lutz B, Zimmer A, Parker LA, Makriyannis A, Sharkey KA. A novel peripherally restricted cannabinoid receptor antagonist, AM6545, reduces food intake and body weight, but does not cause malaise, in rodents. Br J Pharmacol. 2011;161(3):629-42. Epub 2010/10/01. doi: BPH908 [pii] 10.1111/j.1476-5381.2010.00908.x. PubMed PMID: 20880401; PMCID: PMC2990160.

65. Boon MR, Kooijman S, van Dam AD, Pelgrom LR, Berbee JF, Visseren CA, van Aggele RC, van den Hoek AM, Sips HC, Lombes M, Havekes LM, Tamsma JT, Guigas B, Meijer OC, Jukema JW, Rensen PC. Peripheral cannabinoid 1 receptor blockade activates brown adipose tissue and diminishes dyslipidemia and obesity. FASEB J. 2014. doi: 10.1096/fj.13-247643. PubMed PMID: 25154875.

66. Bermudez-Silva FJ, Baixeras E, Cobo N, Bautista D, Cuesta-Munoz AL, Fuentes E, Juan-Pico P, Castro MJ, Milman G, Mechoulam R, Nadal A, Rodriguez de Fonseca F. Presence of functional cannabinoid receptors in human endocrine pancreas. Diabetologia. 2008;51(3):476–87. Epub December 19, 2007.

67. Jourdan T, Nicoloro SM, Zhou Z, Shen Y, Liu J, Coffey NJ, Cinar R, Godlewski G, Gao B, Aouadi M, Czech MP, Kunos G. Decreasing CB1 receptor signaling in Kupffer cells improves insulin sensitivity in obese mice. Molecular Metabolism. 2017;6(11):1517-28. doi: https://doi.org/10.1016/j.molmet.2017.08.011.

68. Starowicz KM, Cristino L, Matias I, Capasso R, Racioppi A, Izzo AA, Di Marzo V. Endocannabinoid dysregulation in the pancreas and adipose tissue of mice fed with a high-fat diet. Obesity (Silver Spring). 2008;16(3):553-65. Epub 2008/02/02. doi: 10.1038/oby.2007.106. PubMed PMID: 18239598.

69. Ruiz de Azua I, Mancini G, Srivastava RK, Rey AA, Cardinal P, Tedesco L, Zingaretti CM, Sassmann A, Quarta C, Schwitter C, Conrad A, Wettschureck N, Vemuri VK, Makriyannis A, Hartwig J, Mendez-Lago M, Bindila L, Monory K, Giordano A, Cinti S, Marsicano G, Offermanns S, Nisoli E, Pagotto U, Cota D, Lutz B. Adipocyte cannabinoid receptor CB1 regulates energy homeostasis and alternatively activated macrophages. The Journal of Clinical Investigation. 2017;127(11):4148-62. doi: 10.1172/JCI83626.

70. Moss CE, Marsh WJ, Parker HE, Ogunnowo-Bada E, Riches CH, Habib AM, Evans ML, Gribble FM, Reimann F. Somatostatin receptor 5 and cannabinoid receptor 1 activation inhibit secretion of glucose-dependent insulinotropic polypeptide from intestinal K cells in rodents. Diabetologia. 2012;55(11):3094-103. Epub 2012/08/09. doi: 10.1007/s00125-012-2663-5. PubMed PMID: 22872212; PMCID: PMC3464380.

71. Weltens N, Depoortere I, Tack J, Van Oudenhove L. Effect of acute Δ9-tetrahydrocannabinol administration on subjective and metabolic hormone responses to food stimuli and food intake in healthy humans: a randomized, placebo-controlled study. The American Journal of Clinical Nutrition. 2019;109(4):1051-63. doi: 10.1093/ajcn/nqz007.

72. Chia CW, Carlson OD, Liu DD, González-Mariscal I, Calvo SS-C, Egan JM. Incretin secretion in humans is under the influence of cannabinoid receptors. American Journal of Physiology-Endocrinology and Metabolism. 2017;313(3):E359-E66. doi: 10.1152/ajpendo.00080.2017. PubMed PMID: 28655715.

73. González-Mariscal I, Krzysik-Walker SM, Kim W, Rouse M, Egan JM. Blockade of cannabinoid 1 receptor improves GLP-1R mediated insulin secretion in mice. Molecular and Cellular Endocrinology. 2016;423:1-10. doi: https://doi.org/10.1016/j.mce.2015.12.015.

74. Lu VB, Gribble FM, Reimann F. Free Fatty Acid Receptors in Enteroendocrine Cells. Endocrinology. 2018;159(7):2826-35. doi: 10.1210/en.2018-00261.

75. Grigoryan M, Kedees MH, Guz Y, Teitelman G. Phenotype of entero-endocrine L cells becomes restricted during development. Dev Dyn. 2012;241(12):1986-92. doi: 10.1002/dvdy.23875. PubMed PMID: 23027401.

76. Egerod KL, Engelstoft MS, Grunddal KV, Nøhr MK, Secher A, Sakata I, Pedersen J, Windeløv JA, Füchtbauer E-M, Olsen J, Sundler F, Christensen JP, Wierup N, Olsen JV, Holst JJ, Zigman JM, Poulsen SS, Schwartz TW. A Major Lineage of Enteroendocrine Cells Coexpress CCK, Secretin, GIP, GLP-1, PYY, and Neurotensin but Not Somatostatin. Endocrinology. 2012;153(12):5782-95. doi: 10.1210/en.2012-1595.

77. Habib AM, Richards P, Cairns LS, Rogers GJ, Bannon CAM, Parker HE, Morley TCE, Yeo GSH, Reimann F, Gribble FM. Overlap of Endocrine Hormone Expression in the Mouse Intestine Revealed by Transcriptional Profiling and Flow Cytometry. Endocrinology. 2012;153(7):3054-65. doi: 10.1210/en.2011-2170.

78. Svendsen B, Pedersen J, Albrechtsen NJW, Hartmann B, Toräng S, Rehfeld JF, Poulsen SS, Holst JJ. An Analysis of Cosecretion and Coexpression of Gut Hormones From Male Rat Proximal and Distal Small Intestine. Endocrinology. 2015;156(3):847-57. doi: 10.1210/en.2014-1710.

79. Theodorakis MJ, Carlson O, Michopoulos S, Doyle ME, Juhaszova M, Petraki K, Egan JM. Human duodenal enteroendocrine cells: source of both incretin peptides, GLP-1 and GIP. American Journal of Physiology-Endocrinology and Metabolism. 2006;290(3):E550-E9. doi: 10.1152/ajpendo.00326.2004. PubMed PMID: 16219666.

80. Miyawaki K, Yamada Y, Ban N, Ihara Y, Tsukiyama K, Zhou H, Fujimoto S, Oku A, Tsuda K, Toyokuni S, Hiai H, Mizunoya W, Fushiki T, Holst JJ, Makino M, Tashita A, Kobara Y, Tsubamoto Y, Jinnouchi T, Jomori T, Seino Y. Inhibition of gastric inhibitory polypeptide signaling prevents obesity. Nat Med. 2002;8(7):738-42. Epub 2002/06/18. doi: 10.1038/nm727. PubMed PMID: 12068290.

81. Fulurija A, Lutz TA, Sladko K, Osto M, Wielinga PY, Bachmann MF, Saudan P. Vaccination against GIP for the Treatment of Obesity. PLOS ONE. 2008;3(9):e3163. doi: 10.1371/journal.pone.0003163.

82. Yip RG, Boylan MO, Kieffer TJ, Wolfe MM. Functional GIP receptors are present on adipocytes. Endocrinology. 1998;139(9):4004-7. Epub 1998/09/02. doi: 10.1210/endo.139.9.6288. PubMed PMID: 9724057.

83. Ceperuelo-Mallafré V, Duran X, Pachón G, Roche K, Garrido-Sánchez L, Vilarrasa N, Tinahones FJ, Vicente V, Pujol J, Vendrell J, Fernández-Veledo S. Disruption of GIP/GIPR Axis in Human Adipose Tissue Is Linked to Obesity and Insulin Resistance. The Journal of Clinical Endocrinology & Metabolism. 2014;99(5):E908-E19. doi: 10.1210/jc.2013-3350.

84. Egerod KL, Petersen N, Timshel PN, Rekling JC, Wang Y, Liu Q, Schwartz TW, Gautron L. Profiling of G protein-coupled receptors in vagal afferents reveals novel gut-to-brain sensing mechanisms. Molecular Metabolism. 2018;12:62-75. doi: https://doi.org/10.1016/j.molmet.2018.03.016.

85. Iwasaki Y, Sendo M, Dezaki K, Hira T, Sato T, Nakata M, Goswami C, Aoki R, Arai T, Kumari P, Hayakawa M, Masuda C, Okada T, Hara H, Drucker DJ, Yamada Y, Tokuda M, Yada T. GLP-1 release and vagal afferent activation mediate the beneficial metabolic and chronotherapeutic effects of D-allulose. Nature Communications. 2018;9(1):113. doi: 10.1038/s41467-017-02488-y.