Fall 2022

Distinct Roles for Free Fatty Acid Receptor 3 (FFAR3) in Vagal and Spinal Afferents Regulating Energy Balance and Glucose Homeostasis

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DISTINCT ROLES FOR FREE FATTY ACID RECEPTOR 3 (FFAR3) IN VAGAL AND SPINAL AFFERENTS REGULATING ENERGY BALANCE AND GLUCOSE HOMEOSTASIS

A DISSERTATION SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDACY FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

PROGRAM IN CELL & MOLECULAR PHYSIOLOGY

BY

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CHICAGO, ILLINOIS

DECEMBER 2022

ACKNOWLEDGEMENTS

Accomplishing this dream of earning my PhD has been a massive undertaking and privilege, which would not have been possible without the support and guidance of so many important people in my life. First and foremost, I would like to thank my family for supporting me in so many different ways. Thank you for all of the messages of encouragement, meals, beers, coffees, and fun times that carried me through this journey.

In a lot of ways, this journey began for me thanks to my amazing undergraduate professor Dr. Mike Grider. Dr. Grider's neurobiology class excited and inspired me to pursue this career path, and he encouraged me that I was ready to apply for PhD programs. I also want to thank my lacrosse coach, Jon Torpey, for teaching me what it truly means to work hard and devote yourself to a dream. The work ethic and perseverance that he instilled in me prepared me for the grueling stretches of study and research. After undergraduate study, as I was transitioning from a psychology degree into biomedical research, I had the invaluable opportunity to work in the research lab of Dr. Jocelyn Nolt. I came in with zero bench skills, but Dr. Nolt gave me the chance to grow as a scientist and she guided me through the application process for graduate school. I cannot thank Jocelyn enough for her support.

There are so many people to thank from my time at Loyola. Thank you to the faculty and members of the Cell & Molecular Physiology Program. Thank you to Dr. Mashkoor Choudhry for serving as co-sponsor on my F31. Also, I would like to thank Dr.

Lara Dugas for guiding me as I wrote my F31. Without the help of both Dr. Choudhry and Dr. Dugas, I would not have been able to receive my fellowship, and receiving that award was a massive step to advance my scientific career. I would like to give a special thanks to my committee members, Dr. Seth Robia, Dr. Mashkoor Choudhry, Dr. Ivana Kuo, and Dr. Mohamad Mokadem. Thank you for spending the time to guide me through my dissertation.

Finally, I want to thank my advisor Virginie, as well as the current and past lab members. Thank you, Chaitanya, Raiza, Nadia, Emily, Chelsea, and Jason. It has been such a fun and rewarding journey that would not have been the same without all of you. I would like to thank Dr. Greg Aubert for all of the time he dedicated to helping me with my research, troubleshooting in lab meetings, reading over manuscripts, and editing grants to provide a fresh perspective. I would also like to especially thank Chaitanya for mentoring me in the lab. I learned so much working alongside Chaitanya, and he really prepared me to succeed on my own. Lastly, I cannot spend enough time thanking my advisor, Dr. Virginie Mansuy-Aubert, for all of her mentorship throughout the different stages of my time in the lab. Virginie has always instilled confidence in me to try the risky experiment and use the exciting new technique. Nearly all of the successes that came along the way of my PhD study started with Virginie pushing me to take a chance and not worry about failing. I am so thankful for the time I have spent in the lab and for the guidance that Virginie has given me. I would not be the scientist I am today without her mentorship.

For my grandma, Sally

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LIST OF ABBREVIATIONS

CNS	Central Nervous System
T2D	Type 2 Diabetes
GI	Gastrointestinal
NG	Nodose Ganglia
NTS	Nucleus Tractus Solitarius (Nucleus of the Solitary Tract)
EE	Energy Expenditure
DRG	Dorsal Root Ganglia
CG	Celiac Ganglia
SMG	Superior Mesenteric Ganglia
IGLE	Intraganglionic Laminar Endings
IMA	Intramuscular Array
IPAN	Intrinsic Primary Afferent Neuron
GPCR	G Protein-Coupled Receptor
GLP-1	Glucagon-like Peptide 1
GLP-1R	Glucagon-like Peptide 1 Receptor
OxtR	Oxytocin Receptor
GPR65	G Protein-Coupled Receptor 65
CGRP	Calcitonin Gene-Related Peptide
PYY	Peptide YY
CCK	Cholecystokinin

NPY2R	Neuropeptide Y receptor type 2
IBS	Irritable Bowel Syndrome
FMT	Fecal Microbiome Transplantation
Abx	Antibiotics
PNS	Peripheral Nervous System
5-HT	Serotonin
GABA	Gamma-Aminobutyric Acid
SCFA	Short-Chain Fatty Acid
PROP	Propionate
EEC	Enteroendocrine Cell
ENS	Enteric Nervous System
AHR	Aryl Hydrocarbon Receptor
TLR	Toll-Like Receptor
TGR5	Takeda G-Protein Receptor 5
EC Cell	Enterochromaffin Cell
GIP	Gastric Inhibitory Polypeptide
FFAR2	Free Fatty Acid Receptor 2
FFAR3	Free Fatty Acid Receptor 3
OLFR78	Olfactory Receptor 78
HCAR2	Hydroxycarboxylic Acid Receptor 2
DS	Diet Switch
WT	Wild-Type
HFD	High Fat Diet

DIO	Diet-Induced Obesity
WD	Western Diet
NC	Normal Chow
GTT	Glucose Tolerance Test
AUC	Area Under the Curve
CGK	Glucokinase
PEPCK	Phosphoenolpyruvate Carboxykinase
G6PASE	Glucose-6-Phosphatase
KO	Knockout
CISH	Chromogenic in situ Hybridization
FISH	Fluorescent in situ Hybridization
DVC	Dorsal Vagal Complex
AGRP	Agouti-Related Neuropeptide
NPY	Neuropeptide Y
PANTHER	Protein Analysis through Evolutionary Relationships
I.P.	Intraperitoneal
TF	Transcription Factor
STAT3	Signal Transducer and Activator of Transcription 3
EGR1	Early Growth Response 1
ITPR1	Inositol 1,4,5-Trisphosphate Receptor Type 1
CXCL10	C-X-C Motif Chemokine Ligand 10
IRF7	Interferon Regulatory Factor 7
IFI44	Interferon Induced Protein 44

IFI3	Interferon Induced Protein 3
CART	Cocaine- and Amphetamine-Regulated Transcript
GNRH	Gonadotropin-Releasing Hormone
CDNA	Complementary DNA
GDNA	Genomic DNA
VGLUT2	Vesiculate Glutamate Transporter 2
CA2+	Calcium
CAMP	Cyclic AMP
CREB	cAMP Response Element-Binding Protein
IGN	Intestinal Gluconeogenesis

CHAPTER ONE

INTRODUCTION

Neuronal Control of Energy Balance and Glucose Homeostasis

Finding food and appropriately mobilizing the nutrients is essential for survival. The body must know when to seek and eat food or when to stop eating, to conserve energy or to store up excess, and how to utilize different energy stores for different body functions. The central nervous system (CNS) is integral to this process of sensing and controlling the energy balance of the body. The CNS achieves this balance by receiving signals from the gut directly after a meal, from adipose tissue, directly from the blood, and from other metabolic organs as well. Different neuronal networks in the brain integrate this nutrient information and responsively coordinate signals sent out from the brain through autonomic circuits and hormones. Importantly, the CNS primarily utilizes glucose for energy, so proper maintenance of blood glucose levels is essential for survival.

In the western world, food is plentiful, and the energy balance is often shifted positive, meaning more calories are consumed than burned. Ultimately this positive energy balance is the cause of obesity, but it is still unclear why some people are more prone to reach a positive energy balance than others. Despite attempts to change diet and exercise, individuals struggle to shift the balance back towards net burning calories.

Furthermore, obesity is often accompanied with other comorbidities such as heart disease and diabetes, which increase healthcare costs and personal distress. Furthering our understanding of the molecular mechanisms and neural circuitry of energy balance and glucose homeostasis is needed to help explain why certain individuals are predisposed to obesity and associated complications, and this basic knowledge might help tailor therapies.

Hypothalamic Control of Energy Metabolism

As early as the mid 1800's, physicians (notably von Mohr in 1840) began to notice that individuals with tumors or lesions in a particular area of the brain exhibited dysregulated body temperature, elevated blood glucose, and obesity[2]. Subsequent lesion studies[3], targeted mutations[4], optogenetic[5], chemogenetic[6], and pharmacological manipulations[7] have continued to reveal how signaling of this one brain area is crucial for proper regulation of glucose homeostasis and energy balance – the hypothalamus. The hypothalamus is comprised of several nuclei (clusters of neurons grouped by similar characteristics) that regulate many key homeostatic functions. For instance, the suprachiasmatic nucleus controls circadian rhythms, the preoptic area regulates several sex hormones, the paraventricular hypothalamus is important for regulating energy expenditure and food intake, and the arcuate nucleus also controls feeding behavior. As the basic understanding of hypothalamic signaling continues to grow, therapies have been developed to modulate different neuronal populations for the treatment of obesity and type 2 diabetes (T2D)[7].

The hypothalamus receives information from the body through different signal modalities including circulating nutrients, immune cell signals, endocrine hormones, and peripheral sensory neurons. For instance, neurons in several hypothalamic nuclei that control glucose homeostasis are directly modulated by local glucose levels[8]. Leptin is a hormone released from adipose tissue, which circulates to arcuate neurons that control feeding and energy expenditure[9]. Finally, neurons innervating the gastrointestinal (GI) tract, liver, and pancreas send sensory signals that are integrated in hypothalamic circuits controlling energy balance and glucose homeostasis[10,11]. My work focuses on the sensory (afferent) neuron route of communication between the gut and the brain for proper nutrient utilization.

Sensory Neurons in Gut-Brain Communication

Sensory neurons provide a direct line of communication from the viscera to provide the brain with important information regarding the energy status of the body. The two major lines of sensory neurons connect the gut and the brain: vagal and spinal afferent nerves. Vagal afferent neurons innervate the digestive tract, monitoring mechanical, chemical, thermal, and nociceptive signals related to the diet[10,12-16]. The cell bodies of vagal neurons are located in the nodose ganglia (NG), and they synapse into the solitary nucleus (NTS) in the brainstem (Figure 1). The NTS integrates vagal afferent signals and relays the information up to higher brain regions such as the hypothalamus, or reflexes back down to the dorsal motor nuclei (DMV) of the brainstem where vagal efferent neurons project out to effector organs[17]. The CNS integrates these vagal signals and responsively coordinates pancreatic hormone release, gut

motility, food intake, energy expenditure (EE), and gluconeogenesis[18-20]. Intriguingly, multiple studies report disrupted signaling of vagal afferent neurons which may contribute to dysregulated feeding behavior in obesity models[21,22]. Furthermore, electrical stimulation of the vagus nerve can prevent weigh gain and induce weight loss in both rodent[23] and human studies[19,24,25].

Spinal afferent neurons have cell bodies in dorsal root ganglia (DRG), and they project into the dorsal horn of the spinal cord. These signals are relayed up to the brain and integrated, or they induce reflex activation of motor neurons which may bypass the brain. The spinal nerves can be subdivided into five anatomical divisions: cervical, thoracic, lumbar, sacral, and coccygeal, based on their projections into and out of the vertebrae. More work is beginning to elucidate the contributions of spinal afferent neurons in controlling energy metabolism[26-28], particularly glucose homeostasis[29]. Additionally, dysfunctional spinal afferent signaling is major contributor to neuropathic pain, which presents as a common feature in obesity and T2D[30]. Neuropathic pain primarily manifests as somatic pain in the periphery, rather than in the viscera. However, visceral pain is a common feature of irritable bowel syndrome[31]. Recent reports are identifying sensory and autonomic nerve dysfunction in fibers projecting to the liver, pancreas, intestine, and other organs in obesity and associated comorbidities[32]. Degeneration of these fibers innervating key metabolic organs may even contribute to disrupted signaling in metabolic disease.

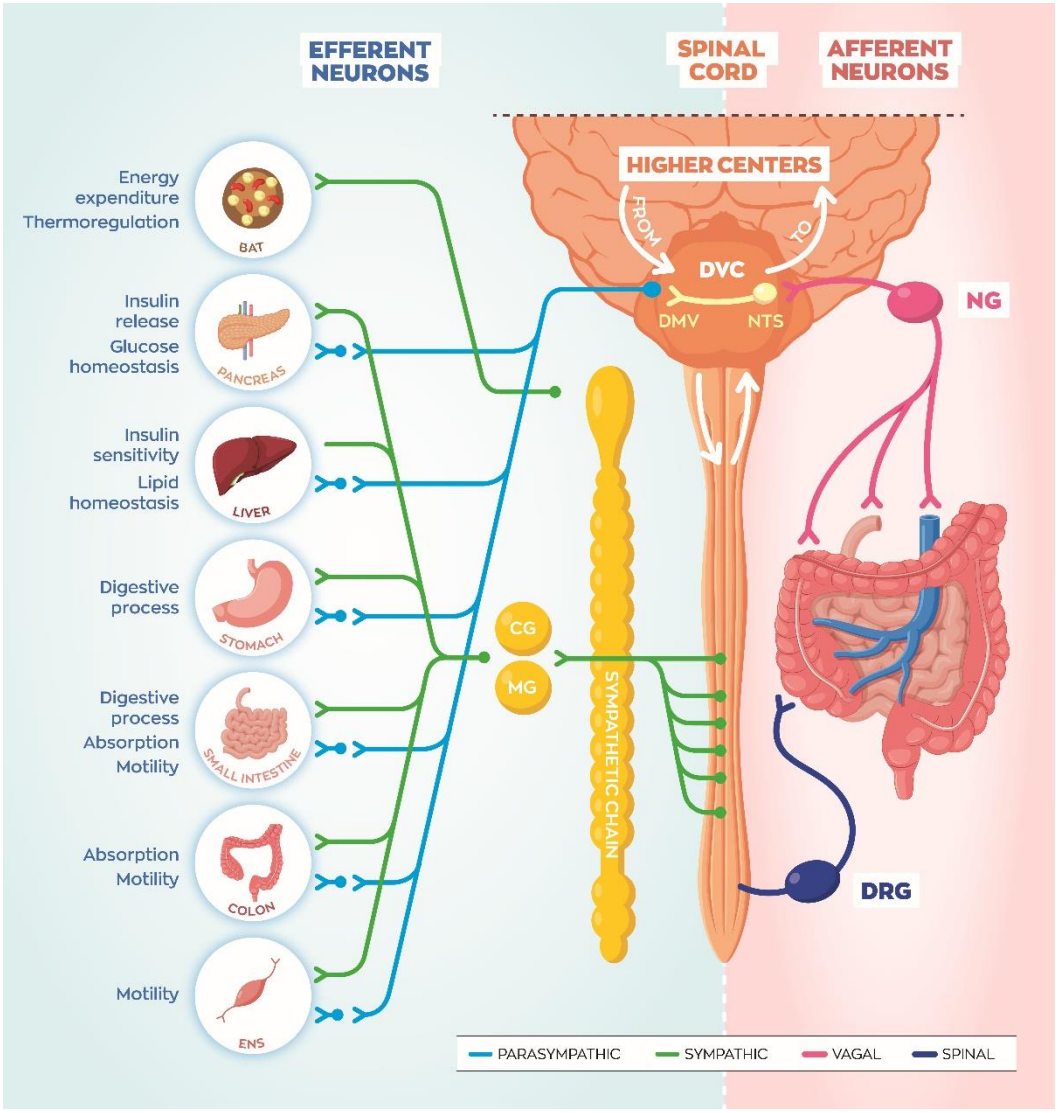

Figure 1: Overview of Peripheral Neurons Regulating Energy Balance and Glucose Homeostasis. Spinal and vagal sensory neurons innervate the gastrointestinal (GI) tract and portal vein. Vagal sensory neurons with cell bodies in the nodose ganglia (NG) project to the nucleus tractus solitarius (NTS). The NTS and dorsal motor vagus (DMV) comprise the dorsal vagal complex (DVC) in the hindbrain. Spinal sensory neurons with cell bodies in the dorsal root ganglia (DRG) project into the spinal cord to relay visceral signals to the brain. The vagal efferent neurons have long preganglionic projections out from the DMV and short postganglionic neurons that reach target organs. Short sympathetic efferent neurons leave the spinal cord and connect with postganglionic neurons in the sympathetic chain or discrete peripheral ganglia such as the celiac ganglia (CG) and mesenteric ganglia (MG). Figure from Cook *et al. (*2022)[1].

As illustrated Figure 2, vagal and spinal afferent neurons can be categorized by the morphology of innervation within the layers of the gastrointestinal tract. They are classified as intraganglionic laminar endings (IGLEs), intramuscular arrays (IMAs), or mucosal innervating neurons. IGLEs project into the myenteric plexus functioning as mechanoreceptors, and IMAs innervate the circular and longitudinal muscle layers. Mucosal primary afferents project all the way into the lamina propria and intestinal villi, likely functioning as nutrient- sensing chemo-receptors[33]. Recent work has identified mucosal afferents forming synapses with enteroendocrine cells[34,35], but most are thought to terminate as free nerve endings inside the villi[12,36]. Enteroendocrine cells synapsing

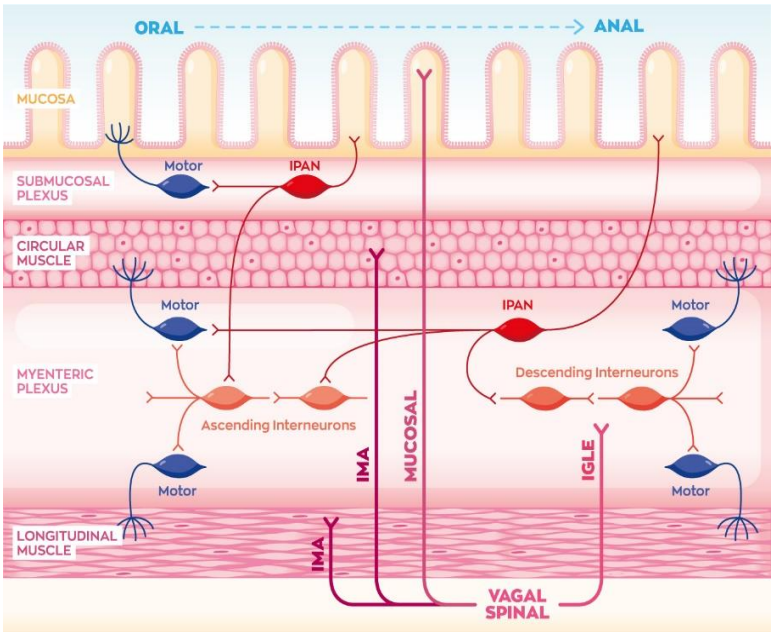

Figure 2. Morphology of Afferent Nerve Terminals. Vagal and spinal afferent are categorized based on their projections within the walls of the gastrointestinal (GI) tract. Intramuscular arrays (IMAs) terminate in the circular and longitudinal muscle, intraganglionic laminar ending (IGLEs) contact the myenteric plexus, and mucosal afferent neurons reach into the mucosa. In the enteric nervous system, intrinsic primary afferent neurons (IPANs) coordinate GI contraction and motility by sensing mechanical distension of the lumen and stimulating motor and interneurons. Motor neurons in the submucosal plexus mainly control blood flow and secretion, while myenteric interneurons and motor neurons control circular and longitudinal muscle contraction to propel food through the gut lumen[1].

onto vagal afferent neurons are known as “neuropod” cells, which likely regulate feeding behavior[35].

The different morphologies of vagal afferents appear to express distinct G protein-coupled receptors (GPCR) or neuropeptide markers. For instance, most IGLEs in the stomach express GLP-1 receptor (GLP-1R), while small intestine IGLEs express oxytocin receptor (OxtR). Mucosal afferent neurons generally are identified by GPR65 expression[33], stomach mucosal neurons express calcitonin-gene regulated peptide (CGRP), and small intestine mucosal afferent neurons express VIP. Interestingly, IGLEs appear to control food intake[37,38], while mucosal afferents regulate glucose production in the liver[38]. “Neuropod” cells are glutamatergic and express Peptide YY (PYY) and Cholecystokinin (CCK), so the vagal afferents synapsing with “neuropod” cells likely express the receptors for these neurotransmitters and hormones. More and more studies are beginning to elucidate the molecular mechanisms of how these different populations of neurons are also relaying signals from gut bacteria to regulate host physiology[39].

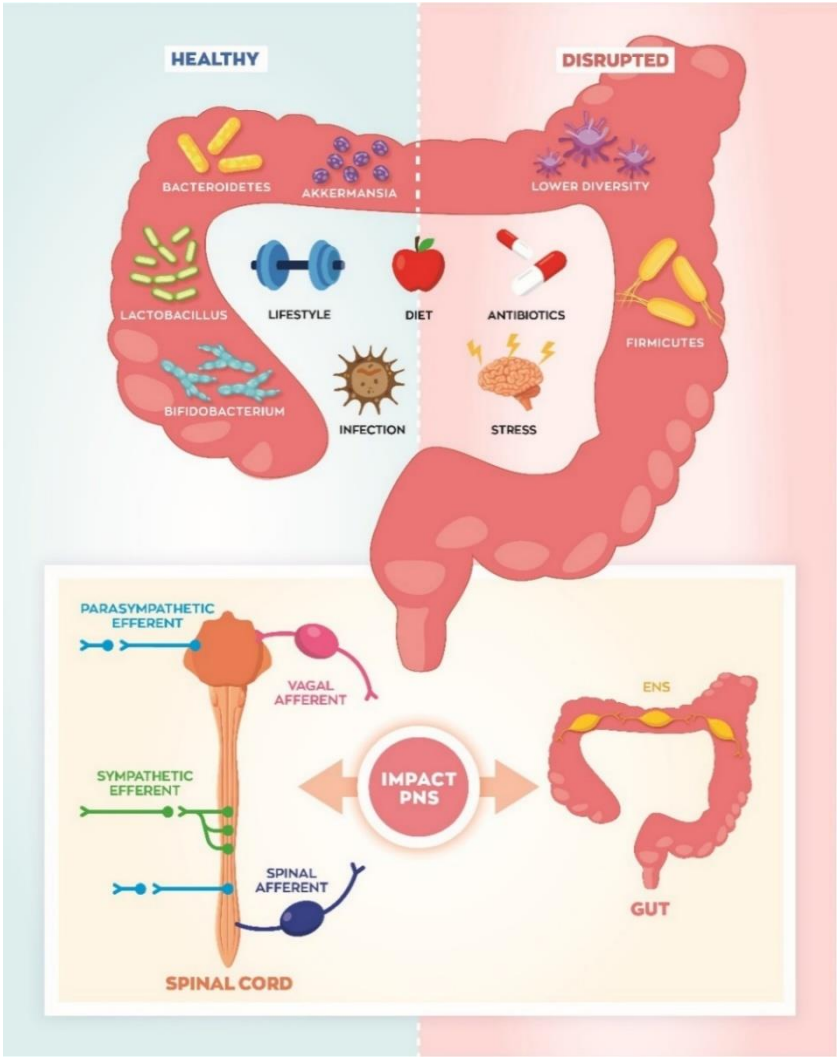

Figure 3. Environmental Influences on Gut Microbiota and Interaction with PNS. Gut microbiome composition is established at birth and is continually shaped by environmental factors such as lifestyle, diet, antibiotic use, infection, and stress. Generally, healthy individuals have a highly diverse microbiome, enriched in *Bacteroidetes*, *Lactobacillus*, *Bifidobacterium*, and *Akkermansia*. In chronic disease, microbiome diversity is often reduced and *Firmicutes* are expanded. The composition of gut microbes drastically impacts the peripheral nervous system (PNS) development and function. Vagal and spinal afferent (sensory) neurons relay microbial signals to the brain, and autonomic output is carried by sympathetic and parasympathetic efferent neurons. Enteric neurons form their own network in the gut, and they are ideally positioned to detect gut microbe signaling and reflexively alter gastrointestinal functions. The afferent, efferent, and enteric nervous systems are interconnected to respond to gut microbe signaling and cooperatively control a variety of homeostatic functions such as digestion, immunity, and visceral perception[1].

Gut Microbiota Influence on Host Energy Balance and Glucose Homeostasis

The gastrointestinal tract houses a dynamic community of trillions of bacteria, archaea, virus, and fungi, which is primarily established at birth from interaction with the mother[40,41]. Collectively referred to as the gut microbiota, there are more bacterial cells within the GI tract than cells in the entire human body. Environmental factors such as geographical location, diet, antibiotic exposure, and infection continue to gradually shape microbiota composition during first few years of life[42,43]. The bacterial community that ultimately colonizes the gut should ideally be evolved to symbiotically function with the host, aiding in various functions such as digestion and immune response. However, dramatic changes in diet and lifestyle within the last century have contributed to the explosion of non-communicable diseases such as obesity, type 1 & 2 diabetes, non-alcoholic fatty liver disease, and irritable bowel syndrome (IBS), and a loss of symbiosis between the gut microbiota and the host physiology likely contributes[44,45]. With increased processing of foods and usage of antibiotics, preservatives, and other additives, the western diet is apparently not suitable for the gut microbiota, and over time this diet may actively disrupt the balance and diversity of microbes within the gut[41].

In the past two decades thousands of studies have aimed to identify microbial signatures, genes, or key species which underly the increased prevalence of various diseases, or to identify potential bacteria with therapeutic potential. Dr. George Fox and Dr. Carl Woese were among the first to leverage the 16S ribosomal RNA gene as a unique marker to sequence the microbiome composition[46]. Subsequent advances in genomic sequencing technology sparked an explosion of studies proposing

associations between various bacteria and host processes or diseases. Elegant studies performed by Jeff Gordon's research team identified associations between the *Firmicutes* and *Bacteroidetes* phyla and adiposity, postulating the *Firmicutes* / *Bacteroidetes* ratio as a putative signature of the obese microbiome[47-49] (Figure 3). They showed that transplanting bacteria from genetically obese mice (ob/ob) caused wild-type littermates to gain more fat mass, as compared to mice transplanted with a lean wild-type microbiota[47]. Accompanying studies by Gordon's group found that the microbiota of obese mice and overweight humans is comprised of higher percentage of *Firmicutes*[49]. Conversely, lean individuals displayed higher abundance of *Bacteroidetes*, and as people lost weight via different diet regiments the population of *Bacteroidetes* expanded[48]. However, associations between the *Firmicutes*/*Bacteroidetes* ratio and body weight have not been consistently supported[50]. *Prevotella* species ferment fiber and often correlate with healthy metabolic outcomes and reduced inflammation[51-53], however there are conflicting findings on whether or not *Prevotella* are expanded in chronic diseases like IBS[54,55]. Reduced bacterial diversity, richness, and stability are often reported in obesity and metabolic syndrome[49,56], but overall, identifying reliable microbial disease signatures has been a major challenge[50].

Since numerous lines of evidence point to various members of the gut microbiota as contributors in chronic diseases, microbiota-focused therapies may provide benefits where other approaches have failed. Microbiota-targeted treatments for these diseases come in the form of prebiotics, probiotics, postbiotics, fecal microbiota transplantations (FMT), and antibiotics (Figure 4). Prebiotics consist of nutrients directly targeted to feed

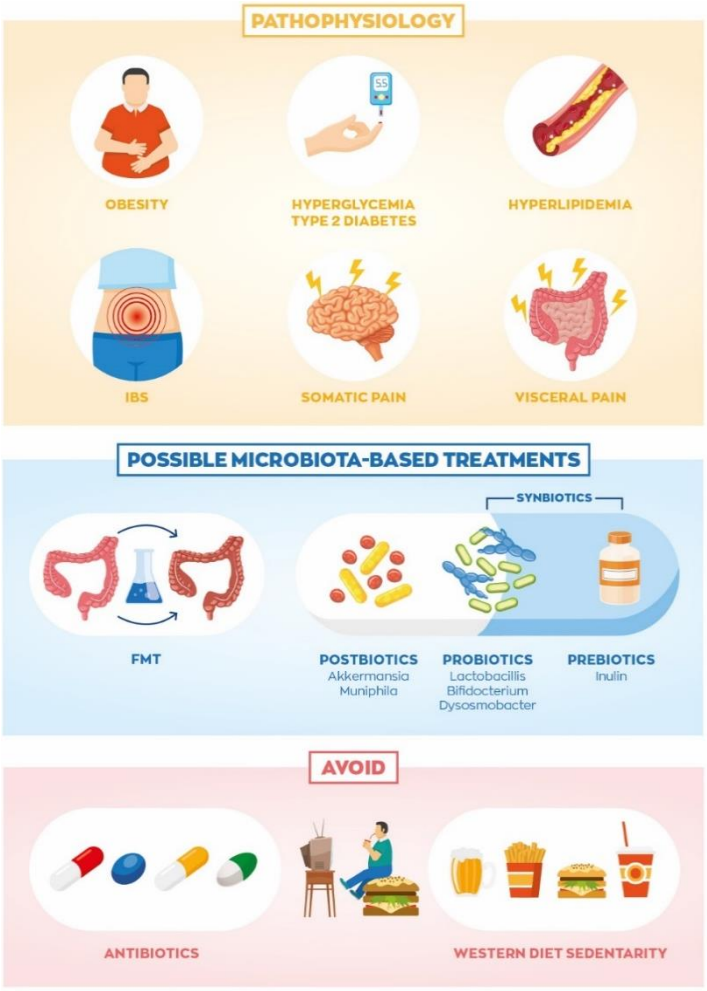

Figure 4. Microbiota-Targeted Therapies for Obesity-Associated Complications
Disrupted gut microbe to peripheral nervous system signaling can lead to obesity, irritable bowel syndrome, and associated comorbidities. Microbiota-targeted therapies such as fecal microbiome transplantation (FMT), postbiotics, probiotics, prebiotics, and combinations may help improve obesity- and IBS-associated complications. Additionally, avoiding antibiotics and western diet may prevent progression of these diseases[1].

or promote certain bacterial growth. Dietary fibers like inulin are prebiotics fermented by gut bacteria and can provide therapeutic benefits to people who are overweight[57], diabetic[58], or suffering from IBS[59]. Probiotics and postbiotics are viable or dead cultures of bacteria, respectively. Probiotics strains developed from bacterial genus such as *Akkermansia*[60-62], *Lactobacillus*[63,64], *Bifidobacterium*[65-67], and *Dysosmobacter welbionis*[68] have emerged as promising targets for obesity and associated comorbidities. Combinations of pre- and probiotics are referred to as synbiotics. FMT is a less specific approach where healthy donor bacteria are transferred to a diseased recipient in the hopes of restoring the perturbed microbiota back to healthy form. FMT is more commonly used

to combat aggressive infections like *Clostridium difficile*, but more research is linking dysfunctional gut microbiota signaling and chronic diseases, so FMT is being tested to treat obesity, diabetes, and cardiometabolic disorders[69,70]. Different studies and treatment strategies utilize various combinations of pre-, pro-, and/or or postbiotics with antibiotics (abx) and/or FMT. Thousands of products and supplements are currently being synthesized aimed at restoring a healthy gut microbiota, but there is still a large gap in basic knowledge of the signaling mechanisms by which the host detects gut microbe function.

Gut Microbiota-Brain Pathways

It is becoming increasingly clear that the western diet (high saturated fat, high sugar, high cholesterol, low fiber and ultra-processed foods) modifies the gut microbiome leading to improper communication between the gut, brain, and peripheral tissue. Mechanistically there are three general ways in which the gut microbiota and host can communicate to regulate energy balance and glucose metabolism: immunological, hormonal, and neuronal[71-74]. Interestingly, there is a high degree of intercommunication between these modalities within the gut. For instance, the peripheral nervous system (PNS) responds to and modulates immunological and hormonal responses to gut bacterial biochemical processes. As shown in Figures 1 and 5, gut microbe signals can be “sensed” via vagal[75,76] and spinal neurons[77], integrated in the brainstem and hypothalamus, and this ultimately influences the efferent signals to peripheral organs. Increasing efforts have been placed on understanding the molecular interactions between the gut microbiota and host PNS to identify causes and treatments

for diseases, such as obesity and diabetes. Several recent studies manipulating the gut microbiota composition illustrate the importance of the interaction between gut microbes and the PNS in regulating host physiology. Antibiotics treated mice exhibit reduced innervation and disrupted excitability of enteric neurons, which contributes to slowed intestinal transit time and reduced motility[78-81]. The maternal gut microbiota dramatically influences the development and maturation of the PNS, influencing host physiology[78-83]. Furthermore, different components of the peripheral nervous system express nuclear and GPCRs which allow these neurons to “sense” gut microbe signaling. Such signals emanating from or being altered by gut microbiota include, bacterial surface proteins, tryptophan metabolites, serotonin (5-HT), gamma-aminobutyric acid (GABA), and short-chain fatty acids (SCFA’s) (Figure 5). My dissertation work has focused on the signaling mechanisms by which vagal and spinal afferent neurons detect SCFAs and how this sensing alters host energy balance and glucose homeostasis.

Gut Microbiota Fermentation of Dietary Fiber Produces Short-Chain Fatty Acids

SCFAs (acetate, propionate, butyrate, and valerate) are monocarboxylic acids produced by fermentation of soluble fiber by various genera of bacteria including *Lactobacillus*, *Bifidobacterium*, *Prevotella*, and *Bacteroides*[84]. After being produced, primarily in the colon, luminal SCFAs are transported into epithelial cells via H+-dependent or sodium-dependent monocarboxylate transporters[85]. The majority of butyrate is consumed as an energy source by colonic epithelial cells, and larger concentrations of propionate and acetate are absorbed into portal circulation and transported to the liver[86].

Gut-microbiota fermentation of dietary fiber produces SCFAs which have been proposed to improve host metabolic health through various mechanisms[77,87], including reducing inflammation[88], lowering food intake[76,89], increasing energy expenditure[90] and altering various processes that modulate glucose homeostasis[52,77,87,91]. For example, *Lactobacillus* strains have been commercially developed as probiotics that can increase SCFA production [84] and both *Lactobacillus* and SCFAs may exert effects on the CNS via the vagus nerve[77,92,93]. Le Roy *et al* found that probiotic supplementation of the commensal bacteria *Dysosmobacter welbionis* induced weight loss in mice, likely via activation brown adipose thermogenesis. The authors speculated that production of the SCFA butyrate was the driver of increased energy expenditure[68]. Li *et al*. also demonstrated the thermogenic capability of butyrate to restore energy expenditure after antibiotics depletion[90]. Although the many studies show benefits of fiber fermentation, other studies show that SCFAs increase dietary energy harvest and weight gain[94], leading to disrupted glucose homeostasis[91,95].

Short-Chain Fatty Acids Signaling through the Gut-Brain Axis

Just like the other signals involved in gut-brain communication, SCFAs can signal via inflammatory, hormonal, and neuronal routes[86,96]. SCFAs regulate the release of enteroendocrine (EEC) hormones such as GLP-1 (Figure 5)[97] and Peptide YY (PYY) form L cells, as well as gastric inhibitory polypeptide (GIP)[98]. These hormones induce potent effects on the host to decrease food intake[99] and stimulate insulin release[100], resulting in a huge effort to produce GLP-1 and GIP mimetics for obesity and diabetes[101,102]. As shown in Figure 5, EEC hormones can accomplish these potent

functions by binding GPCRs expressed in vagal and spinal neurons innervating the GI tract to transmit this nutrient information to the brain. While the hormonal route of SCFA signaling has been widely studied, SCFAs have also been proposed to signal through a direct neuronal route, by altering the activity of peripheral sensory[103] and efferent neurons[104,105].

At the molecular level, SCFAs interact with host cells through multiple signaling mechanisms to influence physiology. SCFAs function as signaling molecules by altering histone acetylation of chromatin[106,107], and by binding several different GPCRs. SCFA-binding GPCRs include free fatty acid receptors 2 and 3 (FFAR2, FFAR3)[108], olfactory receptor 78 (Olfr78), and hydroxycarboxylic acid receptor 2 (HCAR2). HCAR2 (also referred to as GPR109a) weakly binds butyrate, and otherwise has low affinity of other SCFAs[109]. Olfr78 is expressed in a variety of tissues, and although it binds SCFAs a low affinity, the primary ligand for this receptor is lactate. In contrast to HCAR2 and Olfr78, FFAR2 and FFAR3 bind SCFAs at physiologically relevant levels, posing them as interesting mediators between the gut-microbiota and host physiology. FFAR2 is expressed in several different tissues and cell types including enteroendocrine cells[110], intestinal epithelial cells, immune cells[87,111], adipose tissue[112], pancreatic islets[113-116], and more.

FFAR3, is a particularly interesting modulator mediator between the gut microbiota and the brain because it is expressed in several sympathetic ganglia, enteric neurons, nodose ganglia, celiac/superior mesenteric (CG/SMG), and dorsal root ganglia[105,110,117,118]. Distinct roles have been suggested for FFAR3 in these different

neuronal populations to regulate intestinal gluconeogenesis[77,87], heart rate[105], energy expenditure[119], and food intake[39].

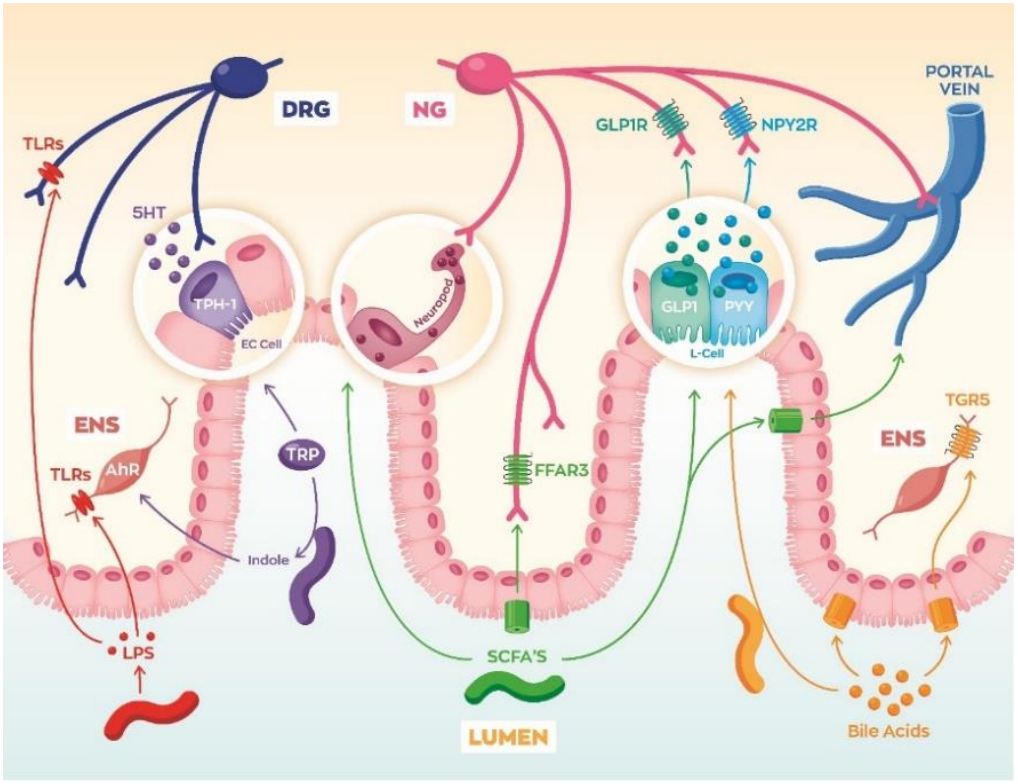

Figure 5. Molecular Mechanisms of Gut Microbiota-Brain Signaling via Sensory Neurons. Gut microbes signal to vagal, spinal, and enteric neurons via a variety of mechanisms. Lipopolysaccharide (LPS) from gram-negative bacteria can activate neuronal toll-like receptors (TLRs). Bacteria convert tryptophan (Trp) into indole metabolites which can alter gene programming of enteric neurons via aryl hydrocarbon receptor (Ahr) signaling. Trp can also be converted into serotonin (5-HT), which is release by enterochromaffin cells (EC). Bacterial fermentation of fiber produces short-chain fatty acids (SCFAs) which can bind free fatty acid receptor 3 (FFAR3). SCFAs can also trigger L-cells to release neuropeptides glucagon-like peptide 1 (GLP-1) and peptide YY (PYY). Gut microbe production of secondary bile acids binds Takeda G-protein receptor 5 (TGR5) on L-cells to trigger GLP-1 and PYY release. Secondary bile acids can also signal to TGR5 on enteric neurons to regulate motility[1].

At a broader level, FFAR3 signaling also influences fetal nervous system development. The gut microbiota is established after birth, but even before this SCFAs produced from the pregnant mothers microbiota can signal to the developing fetus via FFAR3 and promote sympathetic neuron innervation and neurite outgrowth[83]. Ultimately, lack of FFAR3 signaling in utero predisposed the mice to high-fat diet (HFD)-induced obesity via reduced energy EE[83].

Central Hypothesis and Aims

For my PhD dissertation I have tested the hypothesis that microbiome produced SCFA's alter glucose homeostasis and energy balance via direct action in vagal and spinal afferent neurons. The first aim of my dissertation was to comprehensively characterize the consequences of western diet-induced obesity on gut microbiome composition and short-chain fatty acid signaling, and then to assess how fecal microbiome transplantation from lean mice can reverse these outcomes. Given the abundance of literature suggesting that FFAR3 mediates gut-brain signaling of SCFAs, I then sought to delineate the role of this SCFA-binding receptor in vagal and spinal afferent neurons which innervate the GI tract. In my second aim I characterized the feeding behavior and metabolic consequences of deleting FFAR3 in vagal neurons, and this work was published in Molecular Metabolism[39]. For my final aim I have begun to explore how FFAR3 signaling in spinal afferent neurons may alter glucose homeostasis.

My work is of the first to utilize tissue specific models to genetically delete Ffar3, and comprehensively assess the physiological consequences. Furthermore, I utilized additional genetic tools to elucidate the signaling mechanisms of FFAR3 within different

sensory neuron populations, which had yet to be done. More studies utilizing cell-type specific and inducible knockout models are necessary to clearly elucidate adult vs. developmental signaling roles for peripheral neurons in sensing fiber fermentation by the gut microbiota, and my work has helped begin this process. Ultimately, my work highlights the potential for targeting FFAR3 signaling in the peripheral nervous system to combat obesity and the associated disturbances in glucose regulation.

CHAPTER TWO

LEAN FECAL MICROBIOME TRANSPLANTATION RESTORES WESTERN DIET-INDUCED DISRUPTION OF GUT MICROBIOME COMPOSITION AND LOWERS FASTING GLUCOSE

Introduction

The prevalence of obesity continues to rise, and along with it more people suffer from the associated comorbidities, such as type 2 diabetes (T2D). Although studies have identified genetic factors predisposing individuals to obesity[120,121], there is undoubtedly environmental contributors, as well. A growing body of research connects the gut microbiota with adiposity and dysregulation of glucose homeostasis[122]. The community of bacteria residing within the gastrointestinal tract begins colonizing at birth from interaction with the mother. Ideally, the microbes that ultimately colonize in the gut should form a symbiotic relationship with the host, aiding in various functions, particularly digestion. For instance, humans lack the necessary enzymes to digest dietary fibers, and thus microbes in the gut perform this process. However, fiber intake has decreased with the modernization of food[123], and this along with other major dramatic changes in the diet may contribute to disruption of gut microbiota composition and function[41]. A major product of fiber fermentation by gut bacteria are short-chain fatty acids (SCFAs), which have been proposed to alter host physiology in a myriad of ways.

SCFAs alter immune cell signaling, stimulate release of enteroendocrine hormones[86,97,98], are utilized as energy sources by colonocytes[124], form as substrates for gluconeogenesis[125], and even directly alter brain function. Furthering our understanding of how the western diet and lifestyle is altering the composition and function of our gut bacteria is necessary to prevent and treat obesity and the associated complications.

One particularly important challenge in microbiome research is finding reproducibility across different facilities and experimental conditions. Furthermore, many studies utilize a purely high fat diet, while our lab uses a western diet with added cholesterol and sucrose. Therefore, we sought to test the effects of western diet feeding on gut microbiota composition and SCFA production within our experimental conditions. Furthermore, we assessed how fecal microbiome transplantation (FMT) from lean to obese mice altered gut microbiota and obesity-associated glucose intolerance. Finally, we tested the therapeutic potential of exogenous propionate supplementation on obese mice. Finally, I began to explore the mechanisms by which SCFAs may regulate vagal gene programming of GPCRs controlling blood glucose.

Methods

Animal Studies

Animal studies were conducted in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and with the approval of the Loyola University Chicago Institutional Animal Care and Use Committee (IACUC). Wild-type C57BL/6J (#000664) were obtained from Jackson

laboratory (Maine, USA). Mice were group housed 12:12 hour light/dark cycle 22-25°C. Mice received either NC (Teklad LM-485) or WD (TD88137, Teklad Diets; 42%kcal from fat, 34% sucrose by weight, and 0.2% cholesterol total) (Envigo, Indiana, USA) starting at 7 weeks of age. Body weights (BW) were recorded weekly from weaning.

Fecal Microbiome Transplantation

For the FMT paradigm, obese mice received oral gavages of fecal slurry from lean mice daily, following three days of antibiotic treatment. In the diet switch (DS) groups, mice fed NC for the first five days of fecal slurry gavages. Controls either received saline gavage (saline) or gavages of slurry derived from the feces of obese WD-fed controls (Auto FMT).

Cecal DNA Isolation and 16S Sequencing

Cecal contents were collected, equal masses were homogenized, and DNA isolated using QIAamp Powerfecal DNA Kit (Qiagen). Quantitative PCR (qPCR) was performed with universal 16S primers. The Loyola Genomic Core performed PCR of 16S rRNA V4-5 regions sequenced by the Illumina HiSeq4500 platform, as done previously[126];16S sequences were aligned using the Silva Taxonomy Annotation, and files were uploaded to MicrobiomeAnalyst[127,128] for analysis (https://www.microbiomeanalyst.ca/).

Plasma Short-Chain Fatty Acid (SCFA) Quantification

After decapitation under anesthesia, blood was collected in K3EDTA tubes (Sarstedt), and centrifuged at 2,000 × g for 10 min. The LC/MS analysis was performed on AB Sciex Qtrap 5500 coupled to Agilent UPLC/HPLC system. All of the samples

were analyzed by the University of Illinois at Chicago Mass Spectrometry Core in triplicate and an internal control was used to evaluate inter-assay variability.

Propionate Supplementation

25mg/mL sodium propionate (Millipore Sigma) or equimolar saline was administered in drinking water and mice consumed *ad libitum*. Water was changed every other day. Daily water intake was multiplied by the concentration of propionate in the water, then divided by body mass to approximate the dose at 1.8g propionate/kg body mass. The lean group received propionate supplemented water at the same time diet was switched to WD (in metabolic cages), and the DIO group continued to feed WD with prop. supplemented or control water.

Liver Protein Extraction and Western Blot

Mice were fasted the morning before euthanasia under isoflurane and livers were removed, frozen on dry ice, and stored at -80°C. Livers were homogenized in RIPA buffer with Pierce Protease/Phosphatase inhibitor cocktail using bullet blender tubes. BCA was performed to measure total protein concentration and equal protein was loaded onto 4-20% precast polyacrylamide gels and transferred onto PVDF membranes.

Nodose Ganglia RNA Isolation

After undergoing the FMT paradigm, mice were fasted in the morning for four hours and euthanized under anesthesia via cervical dislocation and decapitation. Left and right nodose ganglia were exposed and the vagus nerve was cut to remove the

intact ganglia. NG were then stored at -80°C before RNA isolation. RNA was isolated using from left and right NG pairs using the Arcturus PicoPure RNA Isolation Kit.

Vagal Explants

Nodose ganglia were removed as previously described from either Wild-Type (WT) or Phox2b-Cre FFAR3KO mice and cultured on open air-interface membrane inserts (Millicell). After three days in culture, NG were serum starved (MEM, 2.5mM GlutaMax, 2.5% horse serum) for twelve hours before vehicle, SCFA (1mM butyrate or propionate), or Trichostatin A (TSA) treatments in low serum media for 12 hours. RNA was isolated as previously described using PicoPure Kit.

Statistics

Statistical analyses were performed using GraphPad Prism 9. Student's T-Test, Multiple T-tests, and Two-Way ANOVA were performed to assess differences between groups, and time or genotype served as co-variants. Fischer's LSD was used for all post-hoc analyses. The statistical test and sample size are indicated in Figure legends for all experiments. All data are expressed as means and error bars indicate SEM. For all experiments male and females were analyzed separately. Randomization and blinding were not used. Statistical significance is indicated by *$p<0.05$, **$p<0.01$, **$p<0.001$.

Results

Western Diet Disrupts Gut Microbiome Composition, Lowering Propionate

Given the disparity in rodent microbiome literature, I analyzed the microbiome composition and circulating SCFA levels of diet-induced obese (DIO) mice in our western diet (WD) model and under conditions at Loyola's animal facility. I fed 7-week-old male mice a WD for 12 weeks and performed 16S sequencing on DNA isolated from cecal contents. I found that DIO mice exhibited altered gut microbiome composition compared to age matched normal chow (NC)-fed littermates (Figure 6A, Table 1). DIO mice also exhibited reduced microbial diversity (Figure 6B) and richness (Figure 6C). The DIO gut microbiome was characterized by a reduction in Tenericutes and Unclassified Bacteria, and an expansion of Proteobacteria and Verrucomicrobia Phyla (Figure 6D, Table 1). Sparse correlations for compositional data (SparCC)[129] network analysis demonstrated that *Roseburia* and *Butyricicoccus* were among the key determinants of the NC-fed microbial landscape (Figure 6E), as their abundance was negatively correlated with several other genus that were expanded in DIO mice. We also found Bacteria Unclassified (Figure 6F), *Lachnospiraceae* (Figure 6G) and *Lactobacillus* (Figure 6H) were among the top genera decreased in DIO mice. Given the proposed role of *Lachnospiraceae*, *Lactobacillus*, *Roseburia*, and *Butyricicoccus* in producing SCFAs [130], we measured plasma levels between NC-fed and WD-fed DIO mice. DIO did not alter butyrate (Figure 6I) or acetate level (not shown), while significantly lowering circulating valerate (Figure 6J) and propionate (Figure 6K), which are known high affinity ligands for FFAR3[131].

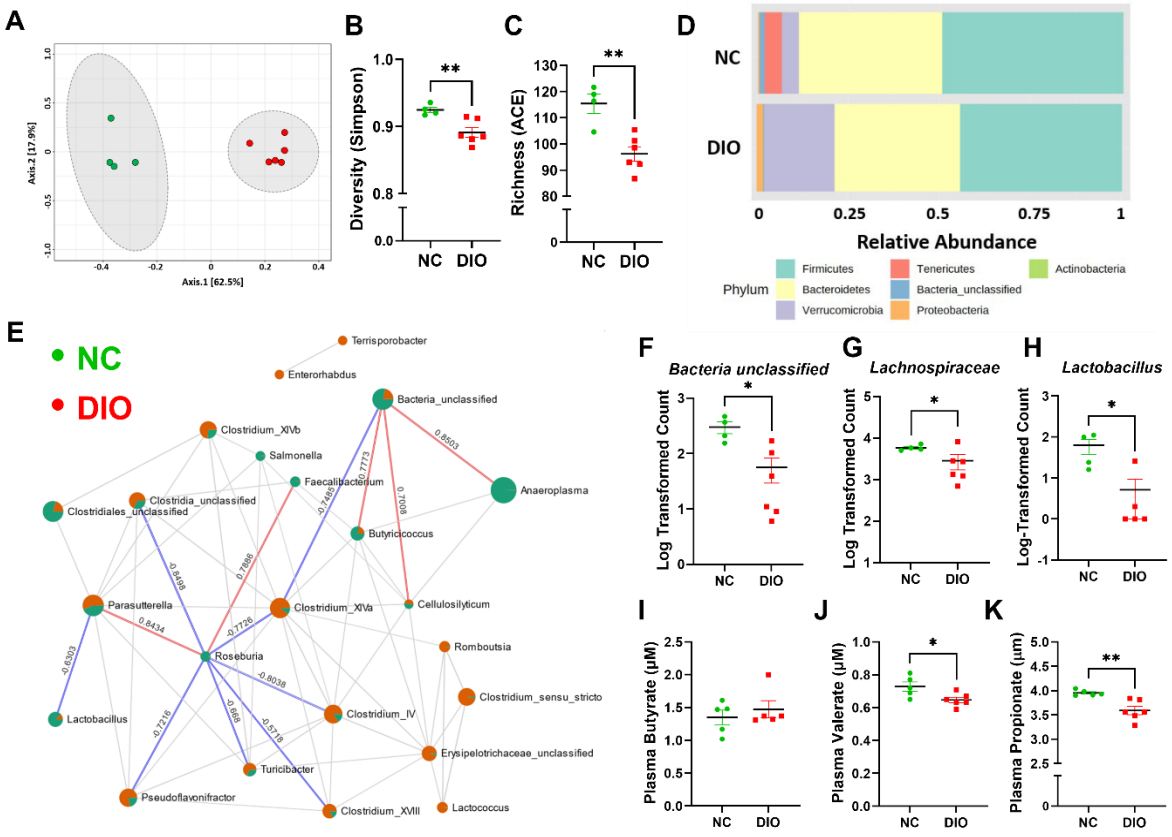

Figure 6. DIO disrupts microbiome composition lowering circulating propionate. (A-H) 16S sequencing of DNA isolated from cecal contents of DIO and NC littermates. NC vs. DIO Principal component analysis (PCA) (A), Simpson diversity (B) and ACE richness indices (C), relative phyla abundances (D), and SparCC correlation network analysis (E) (n=4-6 males/group, Zero-inflated Gaussian Fit FDR-adjusted *p < 0.05). Log-transformed actual counts of Unclassified (F), Lachnospiraceae (G), and Lactobacillus (H) genera (n=4-6 males / group, SparCC correlation analysis line indicates R > 0.5, p < 0.05). (I-K) Circulating SCFA concentrations (μM) of Butyrate (I), Valerate (J), and Propionate (K) (n=5-6 mice/group, Student's T-test, *p < 0.05, **p <0.01).

Lean FMT Restores Gut Microbiome Composition, Increasing Circulating Butyrate and Propionate Levels

I then performed fecal microbiome transplantations with or without a brief diet switch in an attempt to reverse some of the WD-induced disruptions in gut microbiota composition and signaling (see Figure 7 for experimental paradigm and groups). Male mice were fed WD for twelve weeks to develop DIO, given broad-spectrum antibiotics in the drinking water for three days, and then received ten gavages of fecal slurry from NC-fed mice (lean and age-matched). Control mice either received saline or autologous FMT from obese age-matched mice. Consistent with previous studies, FMT from lean donors to DIO mice restored characteristics of the obese mice microbiome to a "lean" phenotype. FMT, independent of diet switch, increased microbiome richness (Figure 8A) and diversity (Figure 8B). FMT with diet switch was most effective to increase *Lactobacillus* by total and relative counts (Figure 8C-D). Additionally, FMT with diet switch increased circulating levels of butyrate and propionate (Figure 8E-F).

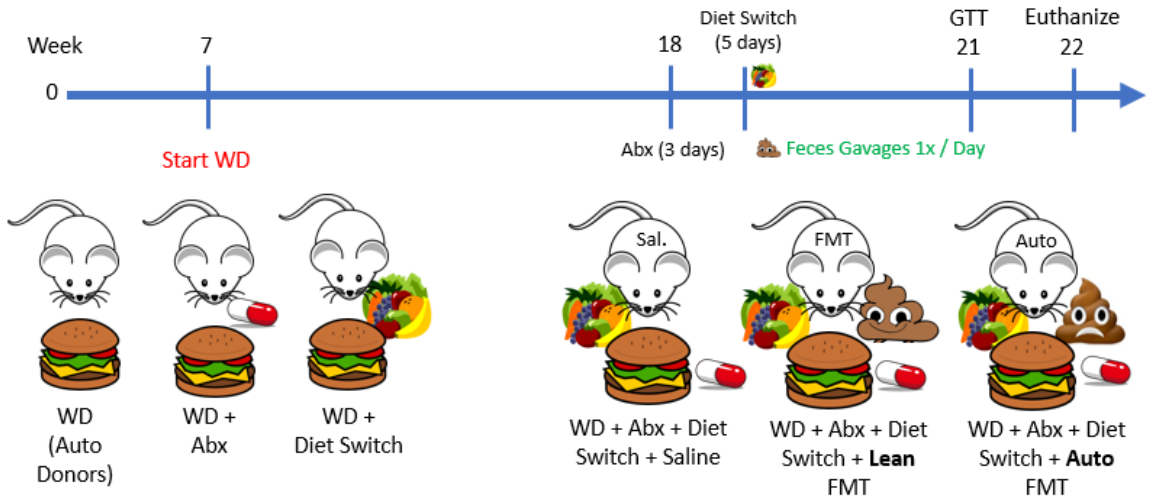

Figure 7. Fecal Microbiome Transplantation (FMT) Paradigm and Groups

Table 1. Significantly Altered Microbiome Features from 16S Sequencing			
Phylum (NC vs. WD)	log2FC	Pvalues	FDR
Tenericutes	-7.792	6.83E-07	**4.78E-06**
Bacteria_unclassified	-2.6247	2.13E-05	**7.45E-05**
Proteobacteria	3.2777	0.000924	**0.002157**
Verrucomicrobia	1.9966	0.006286	**0.011001**
Firmicutes	-0.49297	0.096993	0.13579
Actinobacteria	1.2146	0.16887	0.19702
Bacteroidetes	-0.34089	0.21202	0.21202
Genus (NC vs. WD)	log2FC	Pvalues	FDR
Anaeroplasma	-8.3595	2.18E-09	**8.06E-08**
Firmicutes_unclassified	-3.7947	1.70E-07	**3.14E-06**
Lactobacillus	-3.6995	1.58E-05	**0.000195**
Porphyromonadaceae_un	-1.4576	4.18E-05	**0.000387**
Parasutterella	2.8542	0.00051	**0.003778**
Lactococcus	2.5849	0.000807	**0.004975**
Clostridium_sensu_stricto	3.4969	0.001003	**0.005303**
Bacteria_unclassified	-2.7776	0.001191	**0.005509**
Erysipelotrichaceae_un	2.9098	0.00142	**0.005838**
Lachnospiraceae_un	-1.6061	0.001906	**0.006896**
Romboutsia	2.5348	0.00205	**0.006896**
Oscillibacter	-1.8843	0.004044	**0.012467**
Clostridium_XlVa	2.1389	0.013628	**0.038787**
Akkermansia	1.5648	0.038881	0.10276
Clostridiales_un	-1.7884	0.064662	0.1595
Butyricicoccus	-0.99282	0.12533	0.28982
Dorea	-1.3301	0.18034	0.3925

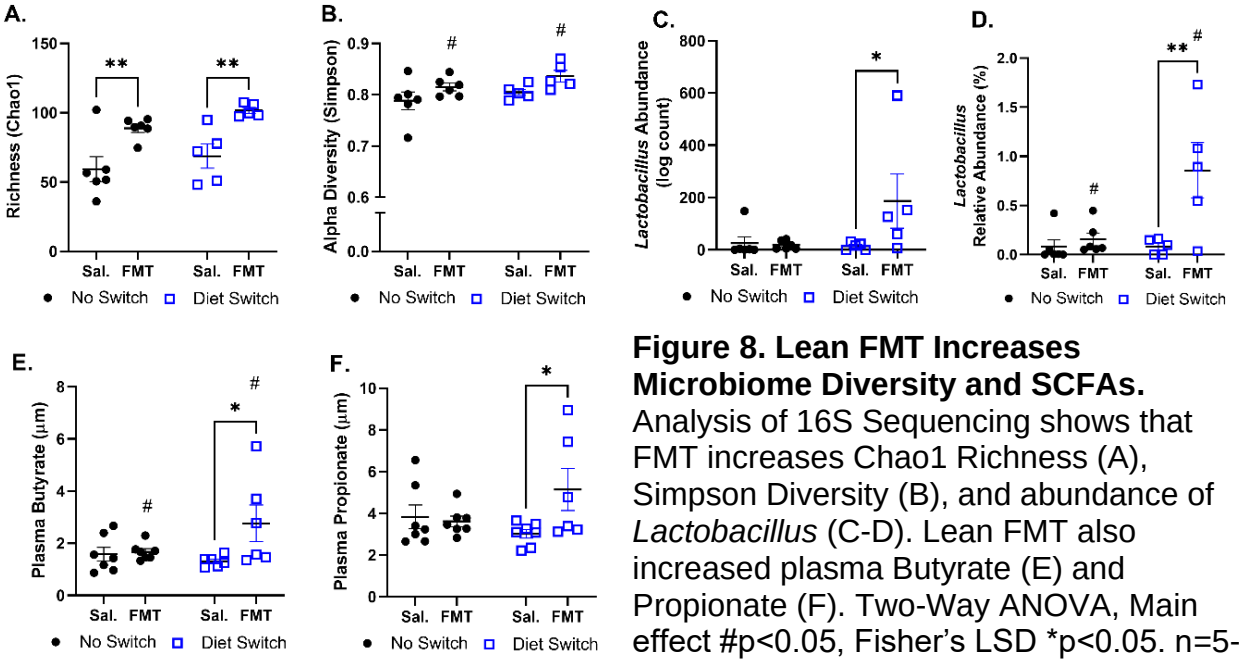

Figure 8. Lean FMT Increases Microbiome Diversity and SCFAs. Analysis of 16S Sequencing shows that FMT increases Chao1 Richness (A), Simpson Diversity (B), and abundance of *Lactobacillus* (C-D). Lean FMT also increased plasma Butyrate (E) and Propionate (F). Two-Way ANOVA, Main effect #p<0.05, Fisher's LSD *p<0.05. n=5-7 mice/group.

FMT from Lean Donors Lowers Fasting Glucose

Previous studies of HFD-fed mice have shown that lean FMT can improve glucose homeostasis. We tested several different variables within our WD FMT paradigm to determine the effects of diet and microbiota on glucose homeostasis. In the first cohort, switching the diet alone without FMT improved glucose tolerance (Figure 9A). However, among the FMT groups, there was no difference between saline and FMT with or without diet switch (Figure 9A-B). There was no difference in fasting glucose among the WD, WD+abx, and WD diet switch groups, yet FMT had a main effect of lowering fasting glucose compared to saline controls (Figure 9C). The FMT group that underwent a diet switch had the lowest overall fasting glucose. In a separate cohort, we tested an autologous FMT (Auto FMT) control which received FMT from obese donors. Compared to Auto FMT, lean FMT mice also had lower fasting glucose (Figure 9D). When comparing each animal's baseline (9 weeks western diet) and post-

FMT levels, lean FMT prevented an increase in fasting glucose even though these mice continued feeding WD (Figure 9E). Although there was no significant change in GTT area under the curve (AUC) between Auto and lean FMT groups (Figure 9F), lean FMT mice trended toward lower glucose levels 30 mins after glucose injection (Figure 9G), despite having similar glucose-stimulated insulin release (Figure 9H). Given the differences in fasting glucose, we tested fasting levels of the counter regulatory hormone glucagon, which trended lower in the lean FMT group (Figure 6I).

I hypothesized that insulin tolerance was improved between Auto and lean FMT. Consistent with this idea, lean FMT increased liver hepatic Akt phosphorylation (Figure 9J-K), which is a key marker of insulin signaling.. Hepatic uptake and production of glucose is regulated by several key enzymes, including glucokinase (Gck), phosphoenolpyruvate carboxykinase (Pepck), and glucose-6-phosphatase (G6Pase), which are transcriptionally regulated by insulin and glucagon levels. Glucokinase expression trended higher in lean FMT mice, whereas *Pepck* expression trended lower, and there was no difference in expression of *G6pase* (Figure 9L).

Altogether, our data suggested that hepatic insulin and glucagon sensitivity may have been improved after lean FMT, however more experiments would be needed to fully support this claim.

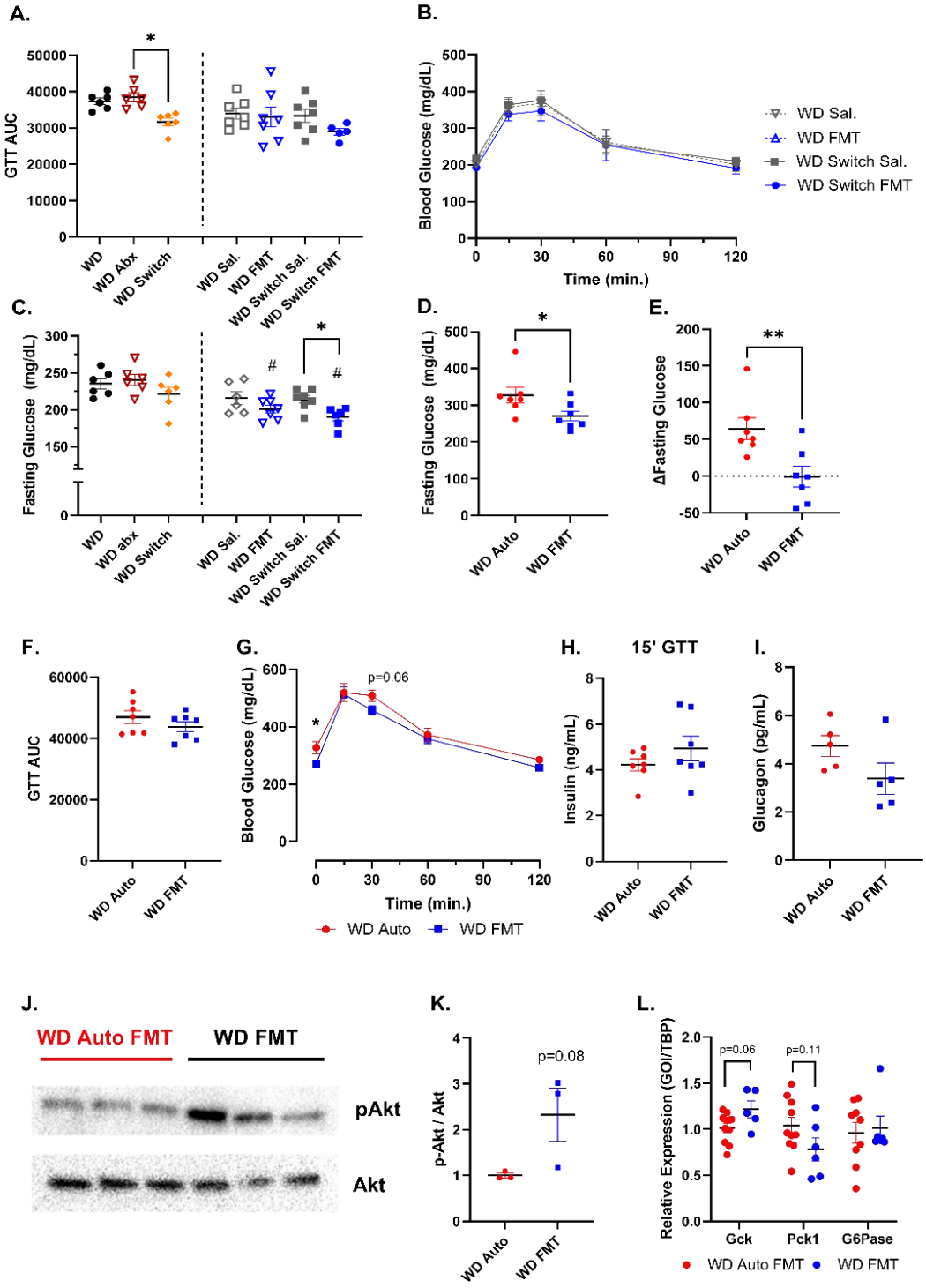
A.
GTT AUC
WD
WD Abx
WD Switch
WD Sal.
WD FMT
WD Switch Sal.
WD Switch FMT
B.
Blood Glucose (mg/dL)
Time (min.)
WD Sal.
WD FMT
WD Switch Sal.
WD Switch FMT
C.
Fasting Glucose (mg/dL)
WD abx
D.
WD Auto
WD FMT
E.
ΔFasting Glucose
F.
G.
p=0.06
H.
15' GTT
Insulin (ng/mL)
I.
Glucagon (pg/mL)
J.
WD Auto FMT
WD FMT
pAkt
Akt
K.
p-Akt / Akt
p=0.08
L.
Relative Expression (GOI/TBP)
p=0.06
p=0.11
Gck
Pck1
G6Pase

Figure 9. Lean FMT Lowers Fasting Glucose in Obese Mice. (A-C) Glucose tolerance test (GTT) data from first fecal microbiome transplantation cohort with antibiotics, diet switch, and saline controls. GTT area under the curve (AUC) (A), glucose traces (B), and fasting glucose (C). (D-I) GTT data from second cohort with lean and autologous FMT. Raw (D) and delta fasting glucose (E) from before and after the FMT paradigm. (F-G) GTT AUC (F) and glucose curves (G), glucose stimulated insulin secretion (H), and fasting glucagon (I). Western blot (J) and normalized intensity of phosphorylated Akt and total Akt from liver homogenates. RT-qPCR of livers (L) for glucokinase, pepck, and glucose-6-phosphatase.

Propionate Supplementation Reduces Food Intake

Given that propionate was reduced in DIO mice and restored upon lean FMT, I next wanted to directly test the effects of propionate supplementation on glucose homeostasis and energy balance. To begin testing this, I placed wild-type (WT) mice in PhenoMaster metabolic cages during the start of propionate supplementation. Previous studies have demonstrated positive benefits of propionate and other SCFAs in lowering food intake[76,132], increasing energy expenditure[76,105] and improving glucose homeostasis[52,58,77,87]. However, addition of SCFAs to the food in these studies may alter palatability, which could indirectly alter many of the variables we wanted to test.

To avoid the confounding variable of reduced food palatability, I supplemented 25mg/mL sodium propionate in the drinking water of 7-week-old lean WT mice at the same time that their food was switched from NC to WD. Propionate reduced water intake to approximately 2mL per day (Figure 10A), estimating the dose to 1.8g propionate per kg body mass. This dose was sufficient to lower food intake in lean male mice upon switching from NC to WD (Figure 10B), preventing WD-induced weight gain (Figure 10C) with nearly zero fat mass gain after one-week WD-feeding (Figure 10D). Given that propionate rapidly altered body weight and composition, it would be difficult

to distinguish propionate-induced alterations in glucose homeostasis separate from the lower body weight. Therefore, I chose to focus on the effects of this supplementation on energy balance.

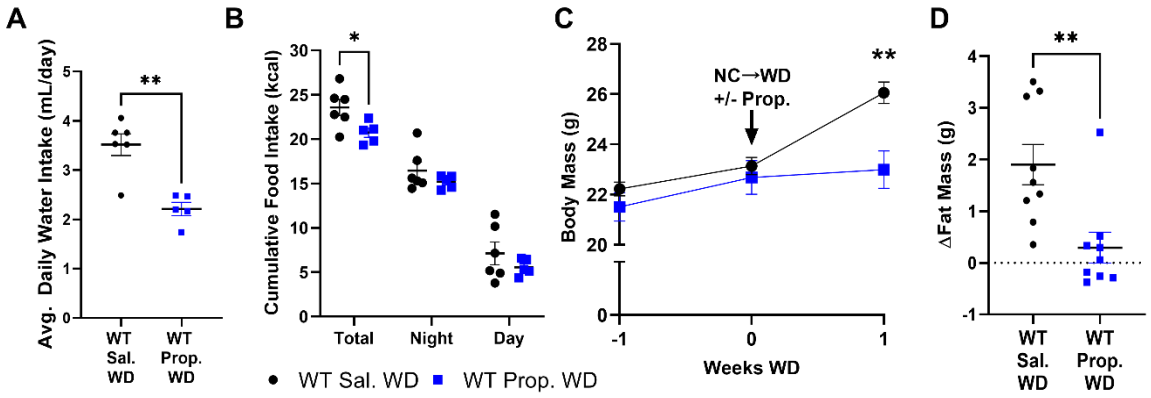

Figure 10. Propionate Supplementation Lowers Food Intake and Prevents WD-Induced Weight Gain. (A-D) Lean wild-type (WT) mice after switch to WD-feeding with or without 25mg/mL sodium propionate in the drinking water. Daily water intake (A) and cumulative food intake (B) during first 48 hours of switch to WD with or without propionate in water (n=5-6 male mice / group; Two-Way ANOVA with repeated measures, Fischer's LSD *p <0.05; Student's T-test **p <0.01). Longitudinal body mass over 2 weeks (C) and delta fat mass after the first week of WD-feeding (D) with or without propionate (n=8-9 mice / group, Student's T-test **p <0.01). Error bars indicate mean ±SEM

To further assess the effects of propionate on energy balance, I fed a separate cohort of WT mice on WD for 9 weeks to cause DIO, and then supplemented sodium propionate (25mg/mL) or equimolar saline in the drinking water. Again, propionate reduced food intake (Figure 11A-B) and water intake (Figure 11D), without altering energy expenditure (Figure 11E), inducing a loss in body mass (Figure 11C, F) and fat mass (Figure 11G). Thus, propionate supplementation in the drinking water at a high dose was effective to lower food intake, preventing weight gain and inducing weight loss in WD-fed mice.

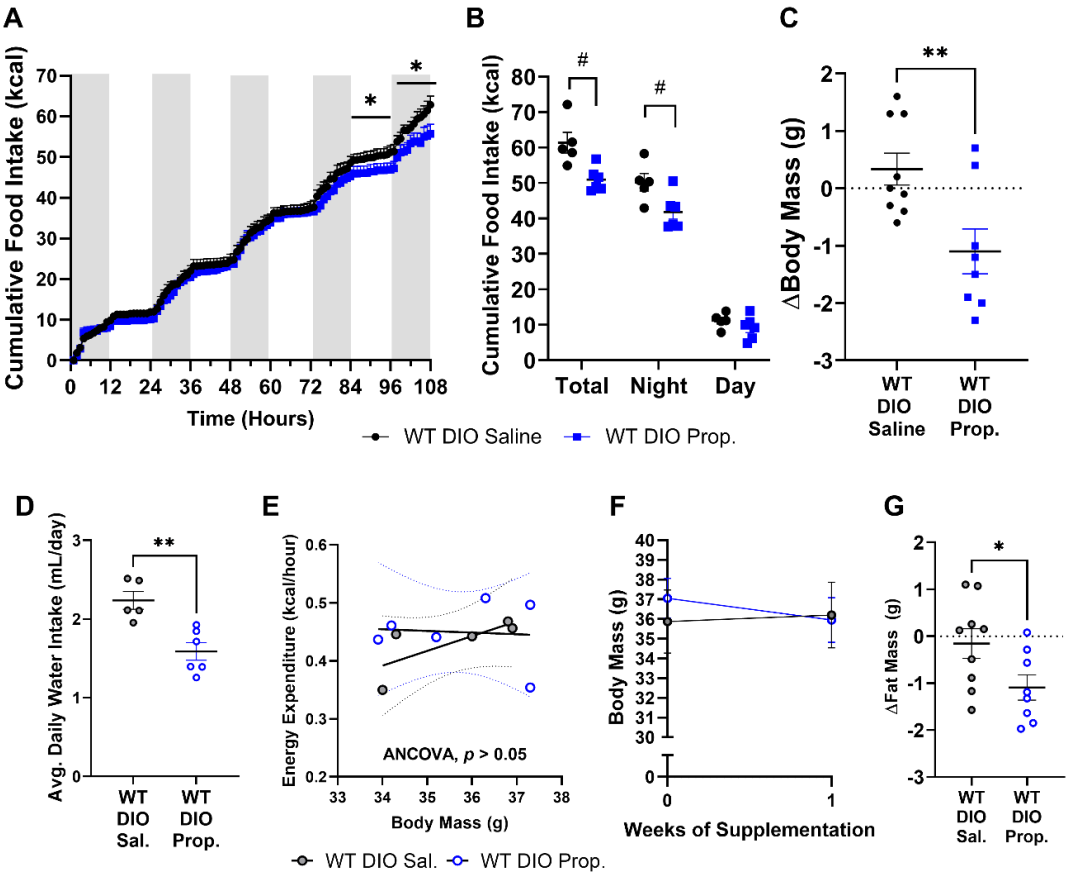

Figure 11. Propionate Lowers Food Intake and Induces Weight Loss in Obese Mice. Diet-induced obese (DIO) WT mice continued WD-feeding with 25mg/mL sodium propionate or equimolar saline administered *ad libitum* through drinking water. Hourly (A) and cumulative food intake (B) (Multiple T-tests, **p*<0.05; Two-way ANOVA with repeated measures, Fischer's LSD Multiple Comparison's, #*p*<0.05, n=5-6 males/group). (C) Subsequent change in body mass of WT DIO mice after 1 week of propionate supplementation in drinking water (Student's T-test, ***p*<0.01, n=8-9 males / group). Average daily water intake (E), and energy expenditure vs. body mass (F) (n=5-6 male mice / group, Two-Way ANOVA with repeated measures, Fisher's LSD **p* <0.05, Student's T-test ***p* <0.01). Body mass (G) and change in fat mass (H) after one week of propionate or saline drinking (n=8-9 mice / group, Student's T-test **p* <0.05 ***p*<0.01). Error bars indicate mean ±SEM.

FMT and SCFAs Alter Vagal Gene Expression

Gut microbiota signaling and SCFA supplementation are proposed to exert effects on host metabolism via the vagus nerve[16,74,92,103]. Therefore, I began to profile gene expression changes in the NG after FMT. Although I analyzed several different genes via RT-qPCR, I especially focused on GPCRs which bind EEC hormones known to control glucose homeostasis and feeding behavior. Interestingly, I found that compared to the Auto FMT control, lean FMT increased the expression of *Glp-1r* (Figure 12A) and *Npy2r* (Figure 12B) in nodose ganglia of obese mice. I did not see any difference in *Cckar* expression (Figure 12C).

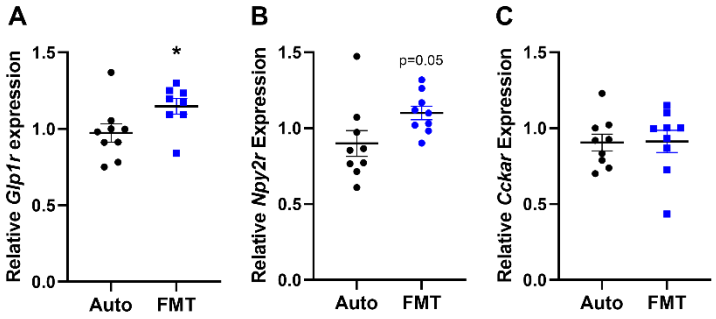

Figure 12. Lean FMT Increases Vagal *Glp-1r* and *Npy2r.* (A-C) RT-qPCR of nodose ganglia pairs isolated from FMT-treated mice. Gene expression relative to β-actin. Student's T-test *p<0.05, n= 8-9 NG pairs / treatment group.

I next wanted to test the possibility that altered SCFA production by gut microbiota after lean FMT contributed to these gene expression changes in vagal neurons. To do so, I

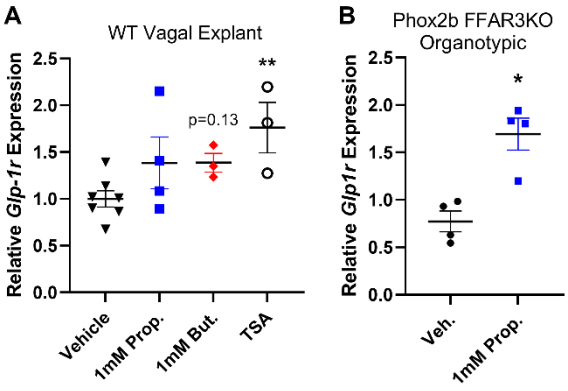

Figure 13. Histone Deacetylase Inhibition Increases Vagal *Glp-1r* Expression. RT-qPCR of vagal explants from wild-type mice (A) and Vagal-FFAR3KO mice (B) after different treatments. Gene expression relative to β-actin. (A) One-way ANOVA, **p <0.01, (B) Students T-Test **p<0.01. n=3-6 NG pairs/treatment.

cultured vagal explants from Wild-Type (WT) mice and stimulated with different SCFAs. While propionate did not significantly alter *Glp-1r* expression, butyrate trended toward increasing vagal *Glp-1r* (Figure 13A). As mentioned, SCFAs can signal through GPCRs or modify chromatin structure via histone deacetylase (HDAC) inhibition. I treated vagal explants with a general inhibitor of HDACs, Trichostatin A (TSA), which significantly increased *Glp-1r* expression (Figure 13A). To further rule out the contribution of FFAR3 signaling, I stimulated vagal explants lacking FFAR3 with propionate. Interestingly, propionate significantly increased *Glp-1r* in the absence of FFAR3 (Figure 13B). Extrapolating these *in vitro* findings to my FMT data might suggest that SCFAs increase *Glp-1r* expression *in vivo* via altering histone acetylation.

Discussion

Overall, I found that western diet feeding resulted in a disruption of gut microbiota diversity, which could be partially restored through FMT from lean donors. Lean FMT also increased circulating levels of propionate, without altering body composition. However, propionate supplementation rapidly lowered food intake and body weight in obese mice. Thus, endogenous microbiota production of propionate in NC-fed mice may not be sufficiently high enough to alter energy balance. Alternatively, propionate supplementation in drinking water may target host signaling mechanisms in the proximal GI tract that alter feeding behavior, while endogenous SCFA signaling in the colon and hepatoportal systems exerts different effects. This hypothesis would need to be directly tested, since I did not measure propionate concentrations after supplementation in the water. Two different studies by Dr. Gary Frost's group showed that increasing

propionate in the colon acutely alters food intake and long-term supplementation improved insulin sensitivity in humans[58,133]. In order to target propionate to the colon, participants received a modified compound in which propionate was bound to the fiber inulin, requiring bacterial fermentation to liberate the SCFA[133]. Development of additional compounds for targeted delivery of different SCFAs would likely clarify the complicated picture of SCFA signaling and increase therapeutic potential.

As mentioned, different studies often fail to reproducibly identify the same microbial pattern associated with diet-induced obesity. Differences in housing and experimental conditions likely contribute to these disparities in microbiome sequencing results across different studies. I wanted to characterize the microbiome composition and signaling within our own conditions and after prolonged WD feeding. Several studies have found the bacteria *Akkermansia muciniphila* to be depleted in diet-induced obesity models[62,134], restored by FMT[135], and associated with improved glucose homeostasis[134,136]. Thus, *A. muciniphila* is a promising microbiota-targeted therapy for metabolic syndrome[60,61]. However, contrary to these findings, I have consistently found *A. muciniphila* levels increased in WD-fed groups compared to NC-fed animals, even with different FMT manipulations. This is possibly a result of utilizing a “western diet” with added sucrose and cholesterol, while other studies often use a high fat diet[134]. Given the strong evidence that *Akkermansia Spp.* provide multiple metabolic benefits to the host, the expansion of this genus in our WD model may confer some protection against metabolic syndrome. Future studies directly comparing a HFD vs. WD may help clarify the contribution of commensal *Akkermansia* to host physiology.

Although outside of the scope of my dissertation research, a major aim of our FMT studies was to study pain behaviors. As published in Bonomo *et al.* (2019) we found that FMT from lean donors improved thermal and mechanical allodynia induced by WD-feeding[126]. We concluded that FMT and SCFAs likely alter neuroimmune communication via HDAC inhibition and TRPV1 desensitization to alleviate pain, however we did not rule out potential contributions of SCFA-binding GPCRs. Future work studying neuropathic pain after performing FMT in mice lacking SCFA-binding receptors in sensory and autonomic neurons would be interesting to directly test this possibility.

Previous studies have consistently demonstrated that FMT from lean rodents improves insulin sensitivity in obese mice[137,138]. Studies vary, however, in terms of metabolic outcomes when FMT is performed from HFD donors to lean recipients. For instance Zoll *et al*. showed that HFD-FMT disrupts insulin tolerance,[137] whereas Zhang *et al* did not observe disrupted gut microbiota structure or glucose homeostasis in rats receiving FMT from diabetic donors[138]. Although I hoped to verify these findings in our FMT paradigm, I did not perform an insulin tolerance test, because insulin injections may alter neuronal regeneration mechanisms[139] and complicate the interpretations of our pain behavior data. However, given that FMT lowered hepatic Akt phosphorylation, as well as fasting glucose, I suspect insulin tolerance was indeed improved by lean FMT in our model.

Many reports demonstrated that the glucagon like peptide receptor 1 (GLP-1R) is a key vagal regulator of glucose homeostasis[140,141]. However, very little is understood

about the mechanisms leading to dysregulated GLP1R signaling and expression in metabolic diseases. Different studies report mixed results regarding downregulation of CCK receptors[142-145] and GLP-1 receptors[146,147] in the vagus nerve in obese rodents. I found that FMT and SCFA treatments increased *Glp-1r* expression, which raises that possibility that differences in diet and environment across different laboratory settings may explain the lack of consistency in the literature. It is worth noting that the RT-qPCR results that I obtained showing increased *Glp-1r* were not confirmed when we performed RNA sequencing from the same groups. Therefore, more work is needed to confirm the interaction between SCFAs and regulation of vagal *Glp-1r* expression. Butyrate only trended toward increasing *Glp-1r in vitro.* In these vagal explant studies, I applied treatments in low serum media. Combinations of SCFAs with other nutrients (glucose, fatty acids) would be more physiologically relevant and possibly would more potently alter gene expression.

Overall, my work suggests that propionate production by lean gut microbiota improves host glucose homeostasis, while exogenous propionate supplementation can reduce food intake. In my next aims, I will investigate potential signaling mechanisms of vagal and spinal afferent neurons to sense SCFAs and contribute to these benefits in glucose homeostasis and energy balance.

CHAPTER THREE

VAGAL AFFERENT FREE FATTY ACID RECEPTOR 3 (FFAR3) REGULATES FEEDING BEHAVIOR

Introduction

The vagus nerve (cranial X) connects the brain and visceral organs, providing a bidirectional line of communication to control homeostatic functions, including feeding[148]. The majority of vagal neurons are sensory (afferent), with cell bodies located in the nodose ganglia (NG). Vagal sensing of meal size, caloric content, gastrointestinal (GI) hormones, and nutrient composition fine tunes an appropriate physiological and behavioral response to a meal[15,33,37]. Intriguingly, vagal afferent neurons also detect signals from gut microbes to regulate host physiology and behavior (Figure 3)[1,74]. Many studies have utilized surgical or chemical denervation of the vagus nerve to assess its role in mediating gut-brain communication[76,77,148]. These studies have advanced our understanding of how the vagus nerve contributes to metabolic homeostasis; however, they fail to address afferent vs. efferent contributions and can lead to massive whole body metabolic and immune dysfunction cofounding interpretations. The same limitation applies to vagal blockade or stimulation studies[148].

Gut-microbiota fermentation of dietary fiber produces short chain fatty acids (SCFAs) which have been proposed to improve host metabolic health through various

mechanisms[77,87]. Several studies have suggested vagus nerve-dependent effects of SCFAs, but molecular mechanisms have not been identified[75,76,89]. The SCFAs acetate, propionate, butyrate and valerate can signal through multiple G Protein-Coupled Receptors (GPCRs) including hydroxycarboxylic acid receptor 2 (HCAR2), Olfactory receptor 78 (Olfr78), and free fatty acid receptors 2 (FFAR2) and 3 (FFAR3)[131]. I investigated the expression patterns of these GPCRs in vagal neurons to begin investigating how SCFAs might regulate energy balance. I found Ffar3 expression in NG neurons innervating throughout the gut and characterized the genetic identity of these neurons. With a newly developed a mouse model for cell-type specific deletion of Ffar3, I sought to investigate if FFAR3 expressed in vagal neurons mediates gut microbiome-brain regulation of feeding behavior in lean and obese mice. I show for the first time that vagal neuron expression of Ffar3 is necessary for normal feeding behavior, and genetic deletion of this SCFA-binding receptor eliminates the positive benefits of propionate supplementation on energy balance.

Methods

Animal Studies

Animal studies were conducted in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and with the approval of the Loyola University Chicago IACUC. C57BL/6J (#000664), RiboTag (#011029), Phox2b-cre (#016223) and Vglut2-ires-cre (#028863) were obtained from Jackson laboratory (Maine, USA). Mice were group housed 12:12 hour light/dark cycle 22-25°C. Mice received either NC (Teklad LM-485) or WD (TD88137,

Teklad Diets; 42%kcal from fat, 34% sucrose by weight, and 0.2% cholesterol total) (Envigo, Indiana, USA) starting at 7 weeks of age. Body weights (BW) were recorded weekly from weaning.

Generation of FFAR3 Flox Mouse

FFAR3 flox mice were generated using the Targeted Knockout First, Reporter-tagged Insertion with Conditional Potential (conditional-ready) strategy by the International Knockout Mouse Consortium. The critical portion of exon 2 was flanked by loxP sites, with upstream elements including FRT – LacZ – loxP – neo – FRT. Flp-mediated recombination converted the mutated allele to conditional allele. In vitro fertilization (IVF) was performed at Jackson Laboratories to generate FFAR3 flox mice on C57BL/6J background, and subsequent breeding was carried out at Loyola University Chicago.

Indirect Calorimetry and Meal Pattern Analysis

Mice were single housed for indirect calorimetry and feeding behavior assessment using TSE Phenomaster home-cage system (TSE systems, Chesterfield, MO). Ambient temperature was maintained at 24.5°C for all studies using Phenomaster Climate Chamber. All experimental groups were BW matched upon start of metabolic assessment to avoid confound of BW on energy balance [149] as previously performed [150,151]. Furthermore, ANCOVA with BW as covariate was used to analyze energy expenditure as suggested by Tschöp *et al.* [149]. All food and water intake data shown was collected using Phenomoster cages. A meal was defined as a bout of feeding larger than 0.05g with a 15-minute intermeal interval when no further food removal

occurred [152]. Meal sizes were then broken down into categories: small (< 0.09), medium (> 0.09 - 0.13), large (> 0.13 - 0.18), very large (> 0.18) as described by Zhang *et al.* [99].

Fasting Refeeding and CCK injection

After overnight fast (18:00-8:00) in Phenomaster cages with free access to water, food was replenished, and mice were fed ad libitum. For CCK studies, after overnight fast, mice received intraperitoneal injection (i.p.) of 1µg/kg body mass CCK8s (Tocris) or saline 20 minutes before food was replaced in feeders.

Glucose Tolerance Test (GTT) and Fasting Insulin

Overnight (14hours) fasted mice were given i.p. dose of glucose (1g/kg BW) after measuring fasting glucose levels. Blood glucose levels were then monitored using AlphaTrak glucometer for rodents (Fisher Scientific, Pennsylvania, USA). Fasting blood was collected in and centrifuged at 2,000 × g for 10 min, and Insulin ELISA (Sigma-Aldrich EZRMI-13K) performed according to manufacturer's instructions.

Hypothalamic and Intestinal RNA Isolation and qPCR

Whole hypothalamus, duodenum, and colon tissue was homogenized using Trizol (Invitrogen) in Bullet Blender bead tubes (Next Advance). After BCP (Sigma) phase separation, RNA was purified in Zymo columns with DNAse I treatment according to manufacturer's instructions. RNA was converted to cDNA with High-Capacity Reverse Transcription Kit (Life Technologies) and qPCR was performed in triplicate with gene expression normalized to 18s.

RiboTag Immunoprecipitation

Nodose ganglia were isolated from Na$_V$1.8$^{Cre/WT}$/RiboTag$^{Flox/Flox}$ mice and translating mRNA was purified as previously described[153,154]. Briefly, NG were homogenized in bullet blender tubes and centrifuged at 10,000 g for 10 min at 4C. Supernatant was incubated at 4C with anti-HA antibody (Biolegend, #901513) at 1:150 dilution for four hours. Samples were then incubated overnight with magnetic beads (Pierce A/G magnetic beads, California, USA). Supernatant were collected and beads were washed with high salt buffer 3x10 mins at 4°C. Finally, RNA was purified using PicoPure RNA isolation Kit.

Single Cell Target Sequencing Data Utilization

RNA-sequencing data from Bai *et al*.[37] target-scSeq was downloaded from Gene Expression Omnibus ID GEO: GSE138651. Ln(cpm)>1 was considered “positive” for a given gene.

Chromogenic in situ Hybridization

Chromogenic in situ hybridization for Ffar3 (ACD Probe cat#447011) was done exactly as previously described[117] using a combination of chromogenic RNAScope (FastRed) and GFP immunohistochemistry.

Immunohistochemistry

NG sections (18μM) from RiboTag+/+:Nav1.8Cre+/-, RiboTag+/+:Vglut2-Cre+/-, and control mice were probed for HA-tag (Biolegend, #901513, California, USA; secondary- goat anti-mouse 647, ab150115, Abcam, Massachusetts, USA) as described before[153,154].

Vagal Explants

After weaning, mice were euthanized via cervical dislocation under isoflurane anesthesia and decapitated. Left and right nodose ganglia were exposed and the vagus nerve was cut to remove the intact ganglia. Ganglia were cultured on open air-interface membrane inserts (Millicell). After three days in culture, NG were serum starved (MEM, 2.5mM GlutaMax, 2.5% horse serum) for twelve hours before propionate treatment (1mM in low serum media). NG were then stored at -80°C before RNA isolation.

RNA Isolation, cDNA Library Construction, and Illumina Sequencing

Total RNA was extracted from the NG of mice using Arcturus PicoPure RNA Isolation Columns (Thermo Fisher). The Northwestern Genomic Core (NuSeq) performed experiments and biostatistical analyses. Briefly full-length cDNA synthesis and amplification were carried out with the Clontech SMART-Seq v4 Ultra Low Input RNA Kit. Subsequently, Illumina sequencing libraries were prepared from the amplified full-length cDNA with the Nextera XT DNA Library Preparation Kit. The sequencing of the libraries was conducted on an Illumina NextSEq 500 NGS System. Single 75-bp reads were generated with dual indexing. RNA sequencing analysis was done with STAR and DESeq2. The quality of reads, in FASTQ format, was evaluated using FastQC. Pathway analysis was performed using PANTHER classification system (www.pantherdb.org). Putative transcription factors were predicted using oPOSSUM single site analysis tool (http://opossum.cisreg.ca).

Vagal Primary Cultures

Left and right NG from FFAR3 Flox and Vagal-FFAR3KO mice (5 weeks or younger) were dissociated in trypsin collagenase using glass pipettes. NG neurons were plated on poly-L lysine coated dish in DMEM/F12 with AraC and pen/strep. After 3 days in culture, primary neurons were serum starved overnight in empty DMEM/F12, and media was collected and frozen at -80°C. CART levels were measured using CART (61-102) EIA Kit (Phoenix Pharmaceuticals).

Statistics

Statistical analyses were performed using GraphPad Prism 9. Student's T-Test, Multiple T-tests, and Two-Way ANOVA were performed to assess differences between groups, and time or genotype served as co-variants. Fischer's LSD was used for all post-hoc analyses. The statistical test and sample size are indicated in Figure legends for all experiments. All data are expressed as means and error bars indicate SEM. For all experiments male and females were analyzed separately. Randomization and blinding were not used. Energy expenditure was analyzed using ANCOVA with body mass as co-variate using Origin Pro. Statistical significance is indicated by *$p<0.05$, **$p<0.01$, **$p<0.001$.

Results

FFAR3 is Expressed in a Mixed Population of Vagal Sensory Neurons

Utilizing ribosome-tagged strategy, I determined which SCFA-binding GPCRs were translated in NG sensory neurons. Our data revealed that *Ffar3* was expressed and actively translated, along with Olfactory receptor 78 (Olfr78) to a lesser degree (Figure 14A). To better understand the innervation and gene expression patterns of FFAR3+ neurons we analyzed single-cell target RNA sequencing (Target-scSeq.) data sets published by Bai *et al.*[37] (see Figure15A).

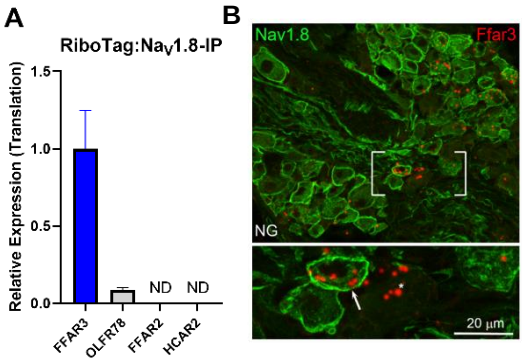

Figure 14. SCFA-Binding GPCRs Translated in Vagal Sensory Neurons. (A) IP and RT-qPCR of translating mRNA from the NG of Nav1.8-Cre: RiboTag mice. Gene expression normalized to β-actin. (B) Chromogenic *in situ* hybridization staining *Ffar3* mRNA (red) in Nav1.8-cre-ChR2 positive (green and arrow) and Nav1.8-negative (no stain and asterisk) vagal sensory neurons.

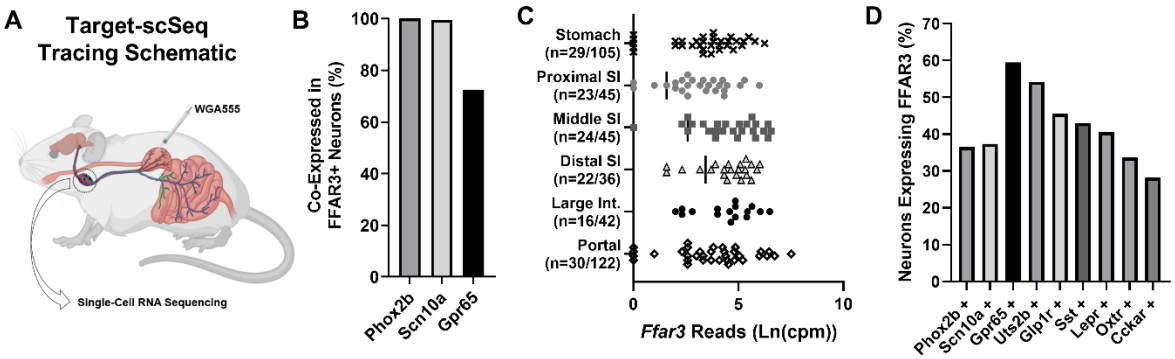

Figure 15. Genetic and Anatomical Characterization of FFAR3+ Vagal Neurons. (A-D) Characterization of *Ffar3* innervation pattern and co-expression from target single-cell (Target-scSeq.) RNA sequencing Tracing schematic showing retrograde membrane dye labeling vagal afferents from different intestinal regions (A). Percent co-expression of other nodose ganglia (NG) markers withing Ffar3+ neurons (B). *Ffar3* expression across vagal neurons innervating different parts of the intestine (C). Percent of neurons expressing Ffar3 among distinct clusters (D).

According to our secondary analyses, 37% of all Phox2b+ NG neurons (marker of all vagal neurons) expressed *Ffar3* (Figure 15B). All FFAR3+ neurons co-expressed Phox2b, 99% co-expressed Scn10a+ (marker of small diameter peripheral afferents), and 70% co-expressed Gpr65 (marker of mucosal innervation pattern) (Figure 15B). *Ffar3* mRNA was detected in NG neurons tracing back from the stomach, small and large intestine, and portal vein (Figure 15C). Furthermore, *Ffar3* expression was detected in NG neuronal populations known to control feeding, including 56% of oxytocin receptor (Oxtr), 45% of glucagon-like peptide receptor 1 (Glp1r), 40% of leptin receptor (LepR), and 28% of CCKaR+ neurons (Figure 15D) [33,37]. Consistent with Target-scSeq. data, we identified *Ffar3* in both Nav1.8 (Scn10a)-positive and negative neurons via chromogenic in situ hybridization (CISH) (Figure 14B). *Ffar3* mRNA, however, was not detected in the dorsal vagal complex (DVC) via CISH (not shown), in agreement with previous studies showing that *Ffar3* is not present in the CNS[118].

Genetic Deletion of Ffar3 from Vagal Neurons Disrupts Feeding Behavior

Many conflicting studies have suggested roles for SCFAs and FFAR3 in control of glucose homeostasis and feeding behavior[76,77,89,114]. Cell-specific roles are likely masked in whole body KO studies, and cell-specific studies are absent from literature, so we generated mice with loxP sites flanking exon 2 of Ffar3. We crossed phox2b-cre heterozygous mice ($phox2b^{Cre/WT}$) with Ffar3 flox homozygous ($Ffar3^{Fl/Fl}$) to generate vagal FFAR3 knockout mice (Vagal-FFAR3KO) and FFAR3 flox littermate controls (Figure 16A). As expected, we did not detect *Ffar3* transcripts in the NG of $phox2b^{Cre/WT}$/$Ffar3^{Fl/Fl}$ (Figure 16A, D), but *Ffar3* expression remained intact in the NG of

Ffar3$^{Fl/Fl}$ controls (Figure 16A,C). Importantly, our phox2b$^{Cre/WT}$/FFAR3$^{Fl/Fl}$ model also did not diminish intestinal *Ffar3* expression (Figure 16B).

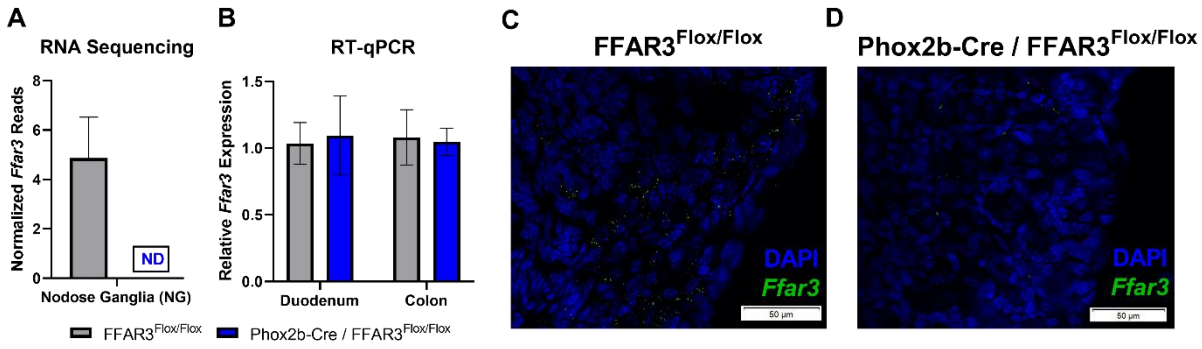

Figure 16. Validation of Vagus Nerve Specific Deletion of FFAR3. (A) RNA sequencing results for *Ffar3* reads in flox and Vagal-FFAR3KO nodose ganglia. (B) RT-qPCR of intestinal tissue for *Ffar3.* (C-D) Fluorescent in situ hybridization against *Ffar3* mRNA in FFAR3 flox and Vagal-FFAR3KO mice.

To determine if loss of FFAR3 in vagal neurons disrupted feeding behavior or energy balance, we performed indirect calorimetry on male and female mice at 8-9 weeks old (Figure 17A). When fed NC *ad libitum*, male and female vagal-FFAR3KO mice consumed larger meals compared to their FFAR3 flox littermates (Figure 17B), with a larger percentage of “very large meals” (over 0.18 grams) in males (Figure 17D). Many male vagal-FFAR3KO mice compensated for the increased meal size by reducing the number of meals (Figure 17C), resulting in equal cumulative food intake compared to FFAR3 flox controls (Figure 18A). On the other hand, female mice lacking vagal FFAR3 ate equal number of meals as control littermates, resulting in an increased cumulative food intake at baseline (Figure 18B).

Given the apparent deficit in meal-induced satiation caused by genetic ablation of vagal Ffar3, we challenged FFAR3 flox and vagal-FFAR3KO mice with an overnight

fast, and quantified refeeding over 48 hours. Female vagal-FFAR3KO mice consumed more calories within the first 24 hours (Figure 17E), while male vagal-FFAR3KO mice increased food intake during the second day of refeeding (Figure 17E). In both sexes, vagal-FFAR3KO's consumed more calories over 48 hours after an overnight fast (Figure 17F-G). Loss of vagal FFAR3 also caused a trend towards increased water consumption (Figure 18D).

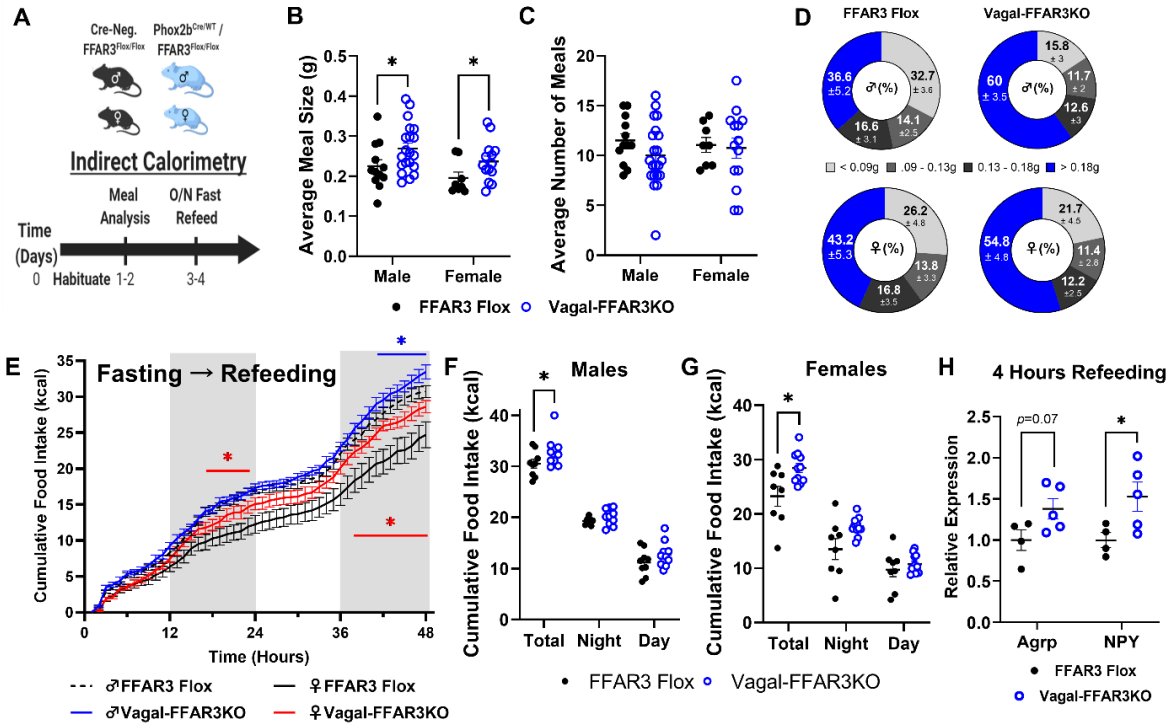

Figure 17. Vagal FFAR3KO Disrupts Feeding Behavior. (A) Experimental paradigm for panels B-G. (B-D) Average nightly meal size (B), number of meals (C), and relative percentage of different meal sizes (D) (n=12-20 males / group and 8-14 females / group, Two-Way ANOVA, Fischer's LSD *p <0.05). (E-G) Cumulative food intake after overnight fast in male (F) and female (G) FFAR3 flox and vagal-FFAR3KO mice (Multiple Paired T-tests, blue asterisk compares males FFAR3 flox vs Vagal-FFARKO, red asterisk compares females, n= 8-10 males / group and n=8-12 females / group). (H) Whole hypothalamic gene expression of the orexigenic neuropeptides *Agrp* and *Npy* (n=4-5 females / group, Student's T-test, *p < 0.05, Gene expression relative to 18s and confirmed with β-actin).

Consistent with the idea vagal FFAR3 relays meal termination signals to the hypothalamus[76], hypothalamic gene expression of the orexigenic neuropeptides agouti-related peptide (*Agrp)* and neuropeptide Y (*Npy)* were elevated four hours after reintroduction of food in vagal-FFAR3KO mice compared to FFAR3 flox controls (Figure17H). Hypothalamic *Npy* and *Agrp* mRNA are upregulated during fasting and subsequently downregulated during feeding[155]. Thus, our data suggests that SCFA sensing via vagal FFAR3 may contribute to post-absorptive downregulation of these orexigenic transcripts to terminate feeding.

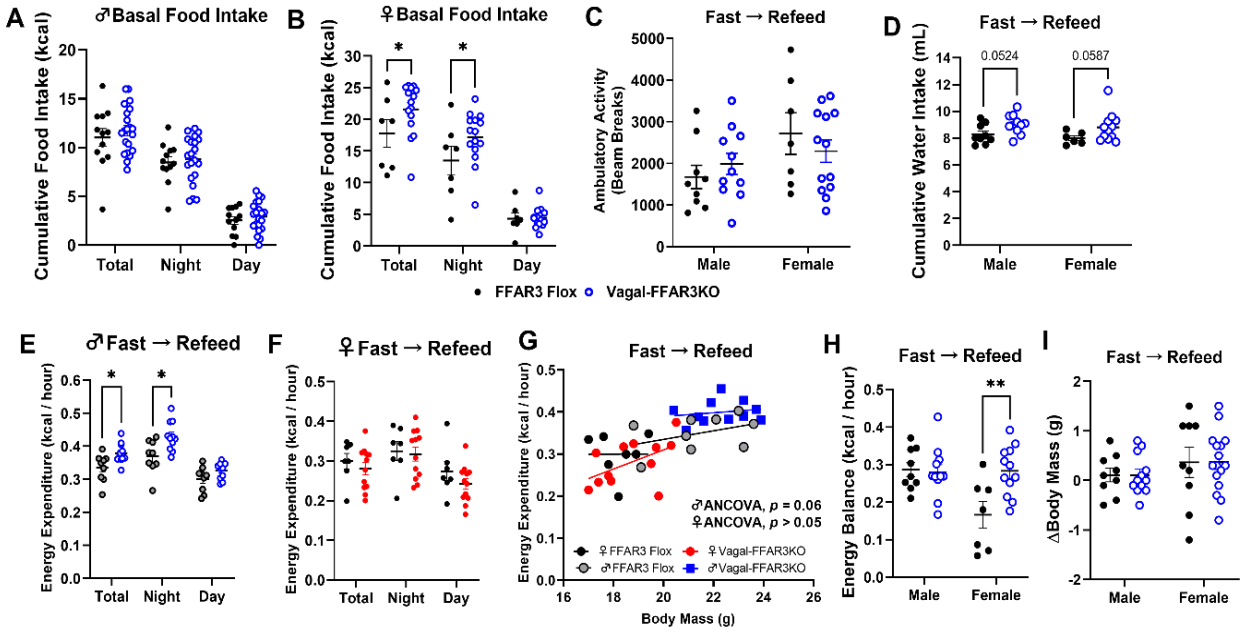

Figure 18. Sex Differences in Energy Balance of Vagal-FFAR3KO Mice. (A-B) Basal food intake of FFAR3 Flox and vagal-FFAR3KO littermates. (n=12-20 males/group, 7-14 females / group). (C-I) Ambulatory activity (C) water consumption (D), energy expenditure (E-G) and average energy balance (H) during refeeding. Subsequent change in body mass (I) after refeeding (n=8-12 females, n=9-11 males/group). Error bars indicate mean ±SEM, Two-Way ANOVA with repeated measures, Fischer's LSD *p <0.05.

Male mice lacking vagal FFAR3 did exhibit altered ambulatory activity (Figure 16C). Male Vagal-FFAR3KO mice compensated for the larger food intake by elevating energy expenditure (EE) (Figure 18E), resulting in a net energy balance equal to FFAR3 flox controls (Figure 18H) and no subsequent difference in body mass (Figure 18I). Conversely, female vagal-FFAR3KO's did not exhibit increased EE (Figure 18F-G), resulting in a higher energy balance during refeeding (Figure 18H), potentially indicating sex differences in diet-induced thermo-genesis.

Given the established role of vagal CCK signaling in proper regulation of feeding and because vagal neurons exhibit cooperative signaling of GI hormones[156-158], we wondered if FFAR3 deletion might alter CCK sensitivity. To test this, we injected (i.p.) male and female mice with saline or low dose (1µg/kg) CCK-8s after an overnight fast. We then measured subsequent refeeding behavior. CCK-8s significantly lowered food intake in FFAR3 flox controls, while vagal-FFAR3KO mice appeared less responsive (Figure 19A-B), suggesting that vagal FFAR3 signaling may weakly contribute to CCK sensitivity.

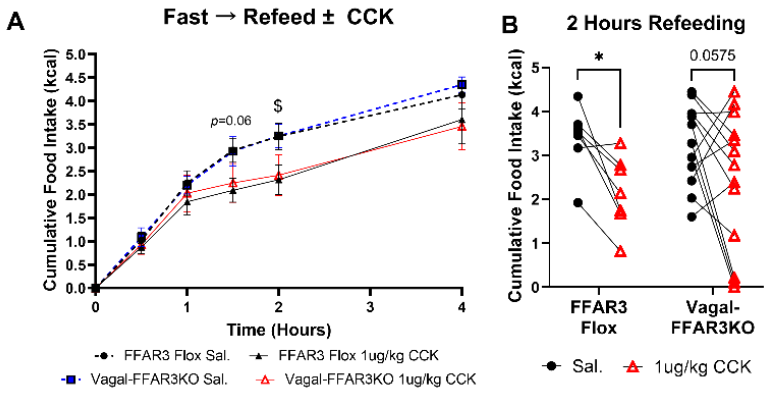

Figure 19. Vagal FFAR3 and CCK Sensitivity. (A-B) Cumulative food intake trace of lean NC-fed mice after i.p. saline or 1ug/kg CCK8s (A) (Multiple Paired T-tests $$p<0.05$ FFAR3 flox saline vs. FFAR3 flox CCK). Cumulative food intake two hours post-injection (N) (Two-Way ANOVA with repeated measures $*p<0.05$). (n=3 female, 4 male FFAR3 flox; 4 female, 9 male Vagal-FFAR3KO mice). Error bars indicate mean ±SEM.

We did not detect any differences in glucose tolerance (Figure 20A-B), fasting glucose (Figure 20C), or insulin levels (Figure 20D) in lean female vagal-FFAR3KO or DIO male vagal-FFAR3KO mice.

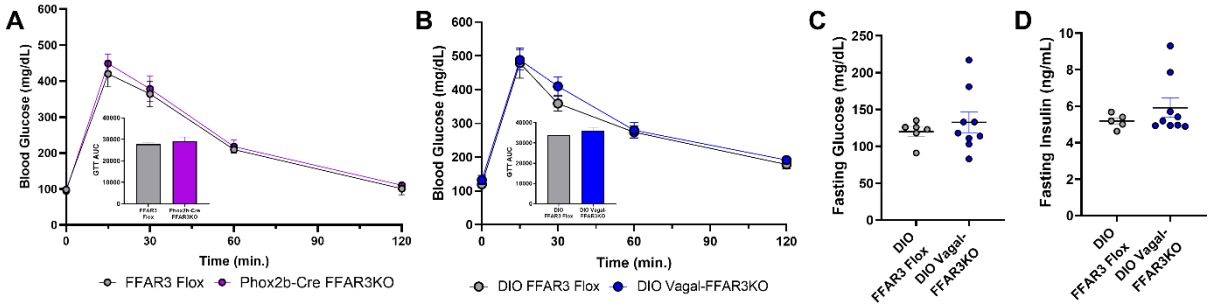

Figure 20. Deletion of FFAR3 from Vagal Neurons does not Alter Glucose Tolerance. (A) GTT trace and area under curve of lean female FFAR3 Flox and vagal-FFAR3KO mice (n=4-10 female mice / group, Student's T-test). (B-C) Glucose tolerance test trace and area under the curve (B), fasting glucose (C) and fasting insulin levels (D) of DIO FFAR3 Flox and vagal-FFAR3KO mice (n=6-9 male mice/group, Student's T-test). Error bars indicate mean ±SEM

Vagal-FFAR3KO Eliminates the Anorectic Effect of Propionate Supplementation

Since we found that genetic ablation of vagal FFAR3 disrupted meal satiation, we wondered how vagal-FFAR3KO mice might respond to the challenge of a more energy dense WD chow. To test this, we challenged lean male FFAR3 flox and vagal-FFAR3KO mice with WD, while performing indirect calorimetry. Upon switching to WD, vagal-FFAR3KO mice consumed more food (Figure 21A-B), without significantly increasing energy expenditure (Figure 21C), resulting in trends towards larger positive energy balance (Figure 21D) and a transient increase in weight gain (Figure 21E).

Having previously established the anorectic effect of propionate in lean and DIO WT mice, we next examined how vagal-FFAR3KO mice responded to the same paradigm of propionate supplementation. After 9 weeks of WD-feeding, male DIO mice lacking vagal FFAR3 were supplemented with either 25mg/mL sodium propionate or equimolar saline in the drinking water. Strikingly, prop. supplementation failed to lower food intake (Figure 22A-B) and water intake (Figure 22D) compared to saline controls. Without altering EE (Figure 22E), prop. supplementation caused a more positive energy balance (Figure 22F) and weight gain (Figure 22C, G).

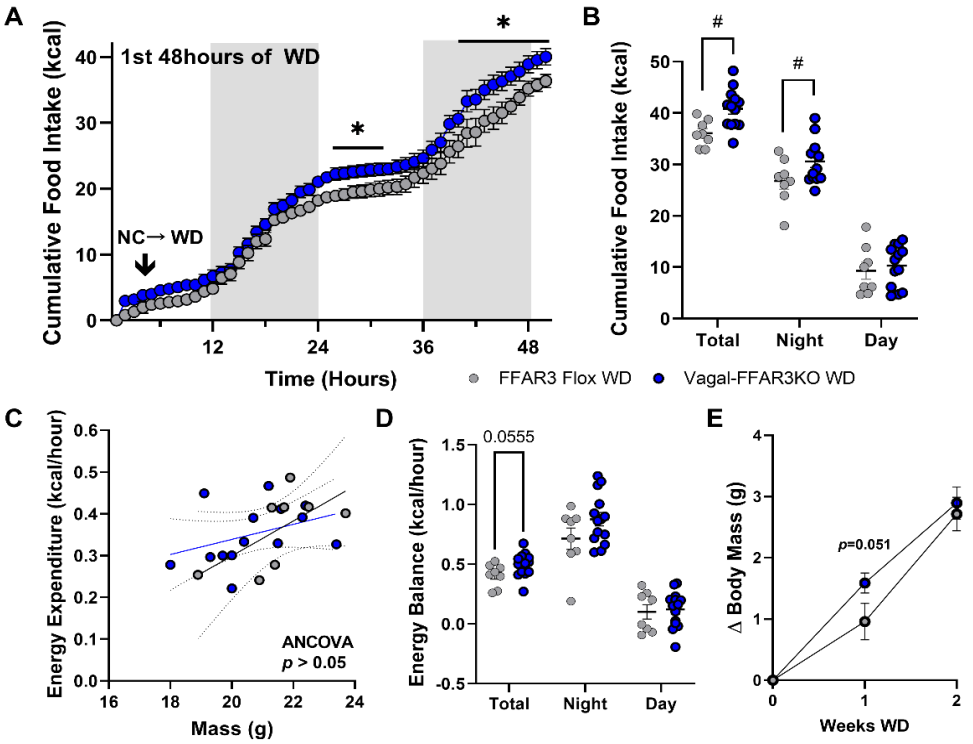

Figure 21. Vagal-FFAR3KO Increases WD-induced Food Intake and Weight Gain. (A-D) Indirect calorimetry of lean FFAR3 flox and vagal-FFAR3KO mice during first 48 hours of WD challenge. Hourly (A) and cumulative food intake (B), energy expenditure vs. body mass (C), and average energy balance (D) (Multiple T-tests, *p<0.05; Two-way ANOVA with repeated measures, Fischer's LSD Multiple Comparison's #p<0.05; n=8-13 males / group). (E) Change in body mass after two weeks of WD (Student's T-test, n= 8-17 male mice / group).

Altogether we found that genetic ablation of vagal Ffar3 disrupted ingestive behavior in a variety of contexts, and the anorectic effect of propionate was dependent upon expression of FFAR3 in vagal neurons. We next explored the signaling pathways through which propionate and FFAR3 activation might be acting in vagal neurons.

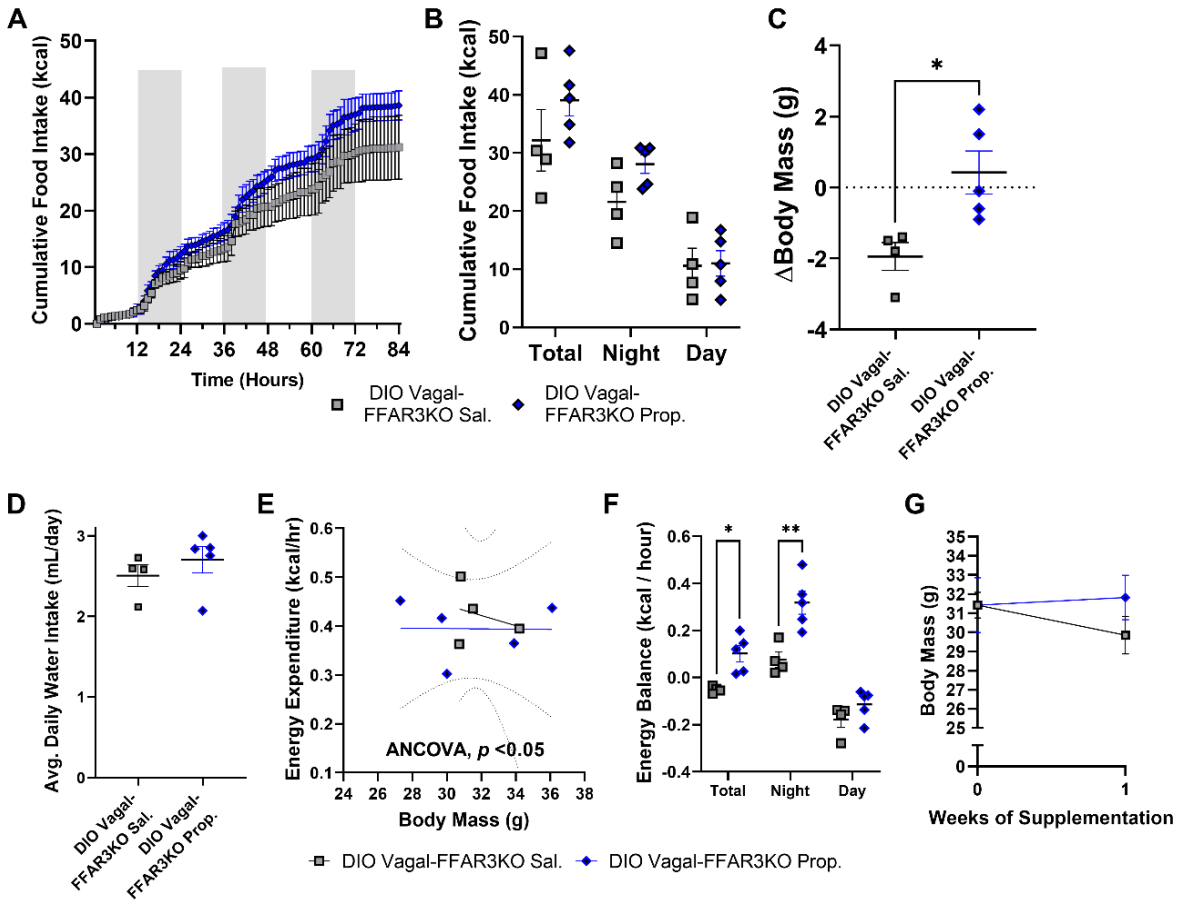

Figure 22. Vagal-FFAR3KO Eliminates Anorectic Effect of Propionate Supplementation. (A-B) Hourly (F) and cumulative food intake (A) of WT DIO mice (9 weeks fed WD) after supplementation of saline or 25mg/mL sodium propionate in drinking water (Multiple T-tests, **p*<0.05; Two-way ANOVA with repeated measures, Fischer's LSD Multiple Comparison's, #*p*<0.05, n=5-6 males/group). (H) Subsequent change in body mass of WT DIO mice after 1 week of propionate supplementation in drinking water (Student's T-test, ***p*<0.01, n=8-9 males / group). (I-K) Hourly (I) and cumulative food intake (J) of DIO Vagal-FFAR3KO mice (9 weeks fed WD) after supplementation of saline or 25mg/mL sodium propionate in drinking water and subsequent change in body mass (K) (n=4-5 males / group). Error bars indicate mean ±SEM.

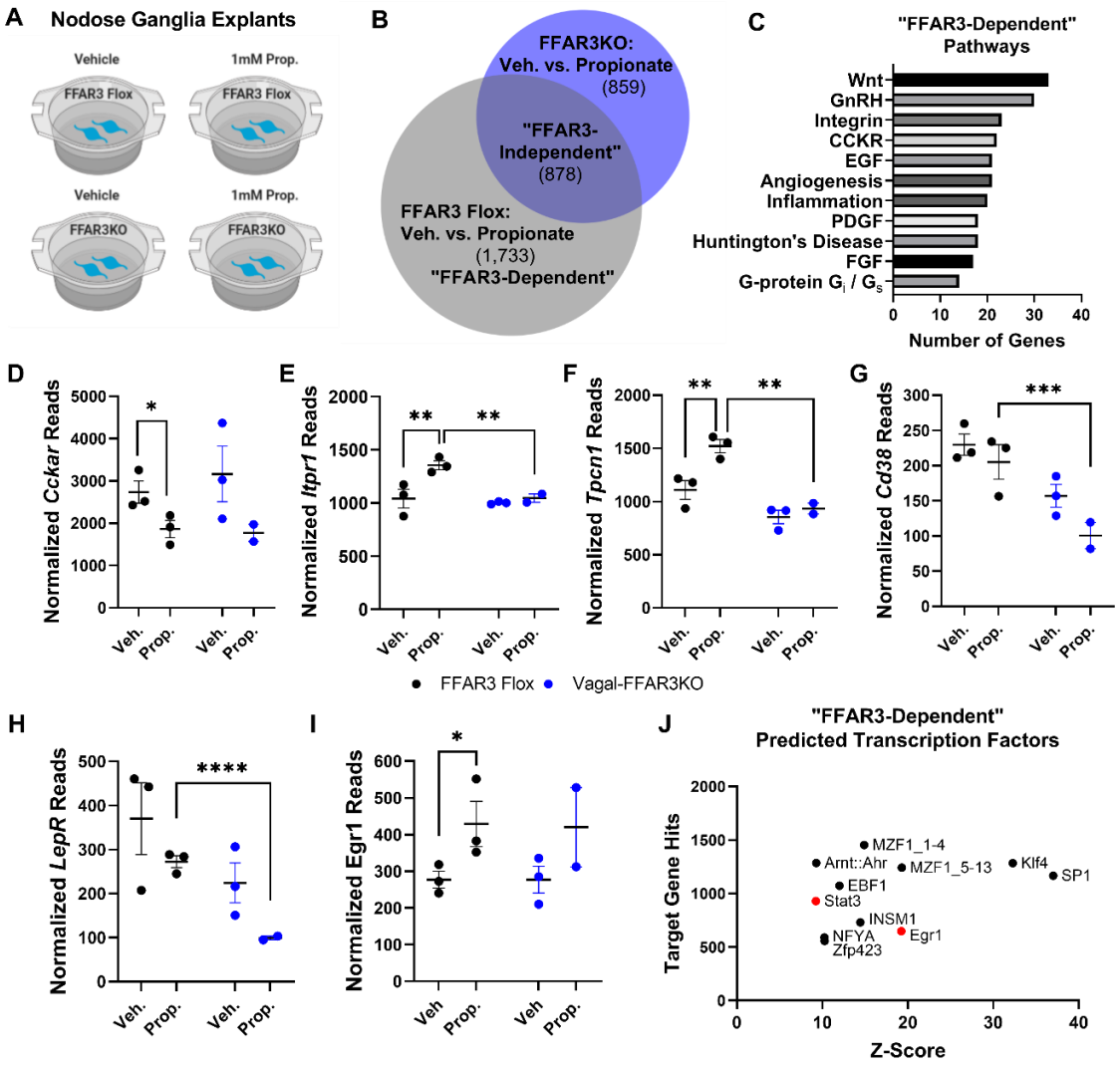

Figure 23. Propionate signals through FFAR3-Dependent and Independent Pathways in the Nodose Ganglion. (A) Graphical depiction of experimental paradigm. Nodose ganglion explants were cultured from FFAR3 flox or vagal-FFAR3KO mice and stimulated with vehicle or 1mM propionate for 12 hours, RNA was isolated and low-input sequencing performed. (B) Venn diagram demonstrating number of genes with expression significantly different between treatment groups. (C) PANTHER pathway classification of genes that were altered in FFAR3 Flox NG after propionate treatment, but not vagal-FFAR3KO NG treated with propionate. (D-I) RNA sequencing results of genes within “CCKR signaling pathway” (P06959) (D-G), leptin receptor (H), and early growth response 1 (I). (J) Prediction of altered transcription factors of “FFAR3-independent” group (see Figure3B) using oPOSSUM database. Error bars indicate mean ±SEM, n=2-3 NG pairs / treatment replicate, FDR-adjusted *p<0.05.

Propionate Signals through FFAR3-Dependent and Independent Pathways in Nodose Ganglia

To investigate the signaling pathways induced by propionate and downstream of FFAR3 within vagal neurons, we cultured NG explants from FFAR3 flox and Vagal-FFAR3KO mice and treated with either vehicle or sodium propionate (Figure 23A). Propionate directly altered a total of 2,611 transcripts in FFAR3-expressing (FFAR3 Flox) NG (Top 50 genes shown in Figure 24A), and 1,737 transcripts were differentially expressed in vagal-FFAR3KO NG after propionate treatment (Figure 23B). To gain insight into FFAR3-dependent pathways, we analyzed the list of transcripts altered by propionate in FFAR3-expressing ganglia, which were unchanged by propionate in vagal-FFAR3KO's (Figure 23C). Figure 24B shows top 50 genes significantly different between FFAR3 flox and Vagal-FFAR3KO ganglia treated with propionate. PANTHER (Protein Analysis through evolutionary relationships) Classification System[159] of these 1,733 "FFAR3-dependent" transcripts revealed Wnt, GnRH, and Integrin to be the top three signaling pathways downstream propionate-FFAR3 activation (Figure 23C), but these were also among the top "FFAR3-Independent" pathways altered by propionate (Figure 24C). Consistent with our data suggesting reduced CCK sensitivity in vagal-FFAR3KO mice (Figure 19), several transcripts within the "CCKR" signaling pathway were altered by propionate (Figure 23C-G), including *Cckar, Itpr1, Tpcn1,* and *Cd38*. Additionally, *Lepr* transcripts were reduced in propionate treated vagal-FFAR3KO NG (Figure 23H). We utilized the oPOSSUM database for prediction of transcription factors (TFs) from our RNA sequencing results[160], and many of the "FFAR3-dependent"

transcripts had signal transducer and activator of transcription 3 (STAT3) and early growth response 1 (Egr1) as a predicted TFs (Figure 23J). *Egr1* expression was increased in whole ganglia after 12-hour prop. treatment (Figure 23I), and after two-hour stimulation in sensory neuron transcripts isolated from NG of RiboTag mice (Figure 25A-B).

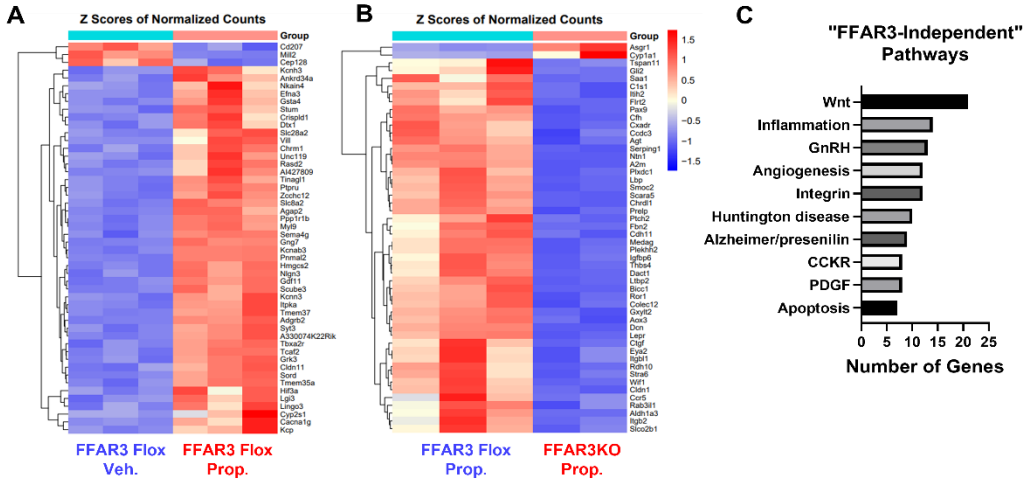

Figure 24. Propionate Alters Vagal Gene Expression. (A-B) Top 50 genes altered between FFAR3 Flox vehicle vs. Propionate (A), and FFAR3 Flox Propionate vs. vagal-FFAR3KO Propionate (B). (C) Pathway analysis of transcripts altered after propionate treatment, regardless of FFAR3 expression or deletion.

Previous work has demonstrated that leptin increases *Egr1* expression, and CCK induces translocation to the nucleus, ultimately potentiating the upregulating of the transcript for the satiety peptide cocaine-amphetamine regulated transcript (CART) [156]. Consistent with this possibility, we found that primary neurons isolated from vagal-FFAR3KO NG trended toward decreased secretion of CART, compared to FFAR3 flox primary neurons (Figure 25C). The potential crosstalk between FFAR3, leptin, and/or CCK receptors in vagal neurons is intriguing and will require further study.

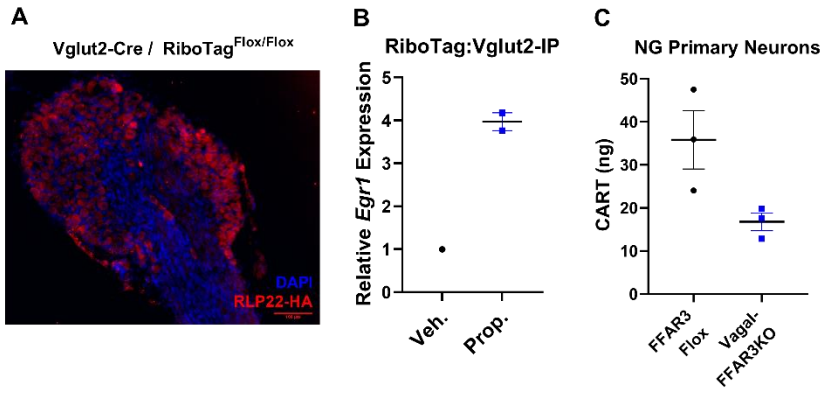

Figure 25. FFAR3 Crosstalk with Other GPCRs may Increase EGR1 and CART. (A) Immunofluorescence of HA-tagged ribosomes in NG from Vglut2-Ribotag mice. (B) RiboIP and RT-qPCR or translating *Egr1* after 1mM prop. Stimulation. (C) Levels of CART in media of primary neurons. Error bars indicate mean ±SEM

Finally, we performed RNA sequencing on NG isolated from NC vs. DIO mice (Figure 26A-B). Compared to NC-fed controls, DIO significantly altered 599 genes (Top 50 shown in Figure 26A), again many falling in Wnt, GnRH, Integrin and CCKR signaling pathways (Figure 26B). Many transcripts altered in DIO mice had Stat3 and Egr1 as predicted transcription factors (Figure 26C). Expression of Serca3 (*Atp2a3*) and the IP3-receptor (*Itpr1*) were decreased in DIO NG (Figure 26F, Table 2), potentially indicating altered upstream CCKaR or other $G_{\alpha q}$-mediated signaling. Given that DIO reduced circulating propionate (Figure 6K), we investigated whether any transcripts altered in DIO mice were also “FFAR3-dependently” altered by propionate. We identified 78 overlapping transcripts between “NC vs. DIO” and “FFAR3-dependent” groups (Figure 26D). Interestingly, several transcripts within the “inflammation mediated by chemokine and cytokine signaling pathway” (P00031) were upregulated in DIO mice and downregulated via propionate, including *Cxcl10, Irf7, Ifi44,* and *Ifi3* (Figure 24C,

26E, Table 2). However, propionate appeared to alter inflammation signaling pathway genes in an “FFAR3-Independent” (**Figure 24C**) fashion.

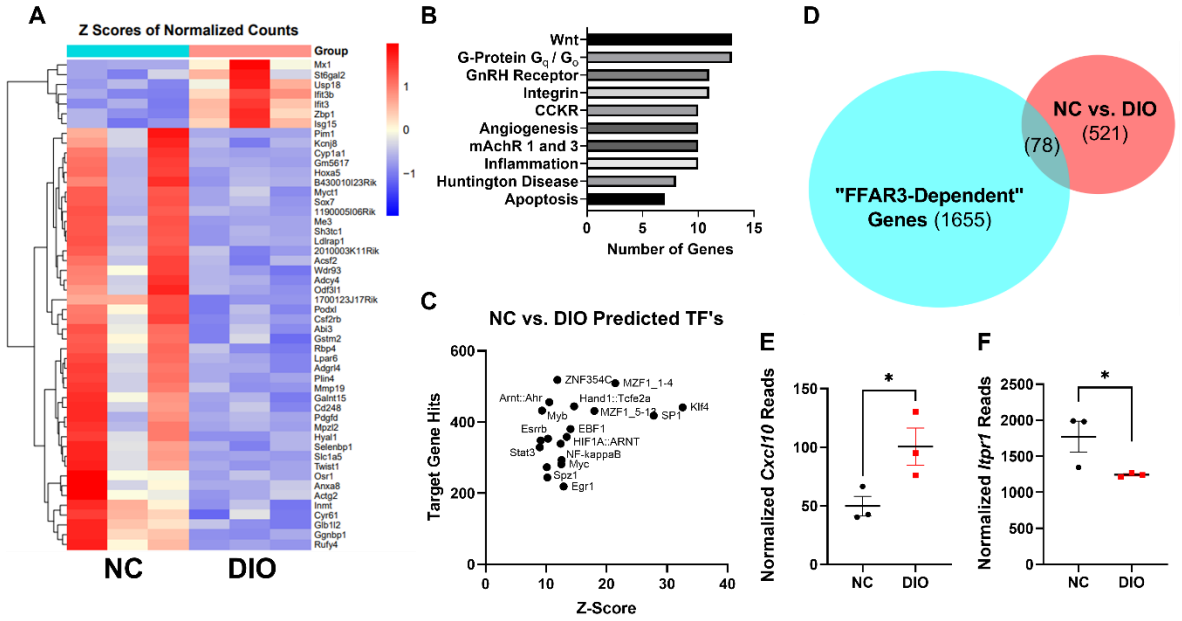

Figure 26. FFAR3-Dependent Pathways are Altered in Nodose Ganglia of Obese Mice. (A-C) Analysis of RNA sequencing of nodose ganglia from NC vs. DIO mice. Top 50 genes (A) altered PANTHER pathways (B), and oPossum predicted transcription factors (C). (D) Venn diagram showing overlapping “FFAR3-dependent” (see Figure 21A-B) and NC vs. DIO groups. (D-E) RNA sequencing results for C-X-C motif chemokine ligand 10 (E) and Inositol 1,4,5-Trisphosphate Receptor Type 1 (F). Error bars indicate mean ±SEM, n=2-3 NG pairs / treatment replicate, FDR-adjusted *p <0.05.

The heterogeneity of localization and co-expression patterns of FFAR3+ vagal neurons opens the possibility of cooperative signaling with several key GI hormones that control feeding such as GLP-1, CCK, and leptin. My RNA sequencing results further support this possibility given that propionate, dependent on FFAR3 presence, altered pathways associated with CCK and Leptin signaling in vagal neurons. Future studies should further test the ability of FFAR3 signaling to alter vagal sensitivity to satiety hormones.

Table 2. Transcripts Significantly Altered by Propionate and DIO in Nodose Ganglia		
	Log2 Fold Change	
	Veh. Vs Prop.	NC vs. DIO
Selenbp1	1.09	-1.05
Atp2a3	1.14	-0.63
Itpr1	0.36	-0.47
Per1	0.29	-0.42
Nedd9	0.43	-0.37
Piezo1	0.35	-0.36
Cdk5r1	-0.87	0.33
Kcnk10	-0.60	0.35
Gbp2	-0.88	0.41
Gbp3	-0.62	0.44
Cxcl10	-0.60	0.70
Irf7	-0.94	0.73
Ifi44	-0.63	0.74
Ifit3	-0.60	1.06

Discussion

I utilized several methods to characterize the expression patterns of SCFA-binding GPCRs in nodose ganglia including RNA sequencing on whole nodose ganglia, ribotag qPCR of vagal sensory neurons, and analysis of single cell data sets of vagal sensory neurons. In the RNA sequencing data of whole nodose ganglia, which also includes transcripts from non-neuronal cells, I detected expression of all four receptors FFAR3, Olfr78, FFAR2, and HCAR2. However, in my vagal neuron-specific ribotag data, only FFAR3 and Olfr78 were detected as actively translated in vagal sensory neurons. In support of this, the secondary analysis of single cell nodose neuron data

also showed that ~37% of vagal neurons express FFAR3, ~30% express Olfr78, only one neuron was detected expressing FFAR2, and one neuron expressed HCAR2. Two separate single cell analyses identified Olfr78 and a maker of a unique population of vagal sensory neurons[37,161]. Olfr78 also binds lactate and this signaling contributes to proper regulation of respiration [162]. Thus, future studies assessing the specific role of Olfr78+ vagal neurons innervating the lungs and heart would be of particular interest. Although I detected low and variable expression of FFAR2 and HCAR2, future studies may reveal important roles for these receptors in vagal neurons and/or non-neuronal cells residing in nodose ganglia, as suggested by Barki *et al.* [163].

Whole body knockout studies have generated conflicting data regarding the roles for FFAR3 in controlling energy balance. For instance, Samuel *et al.* found that global FFAR3KO decreased energy harvest (reduced caloric and SCFA absorption) and decreased adiposity[95], and Lednovich *et al.* recently identified intestinal cells as the locus for FFAR3 signaling to increase adiposity[94]. Conversely, multiple groups have shown global knockout to decrease energy expenditure and increase adiposity[105,119]. Thus, it is likely that FFAR3 signaling regulates multiple opposing components of energy balance through different cell-types, emphasizing the urgent need for the cell-type specific studies such as mine.

I discovered that FFAR3 in vagal sensory neurons regulates short-term feeding and drinking behavior in lean NC-fed mice. WD challenge led to an increase in food intake which caused a transient weight gain in vagal-FFARKO mice. Furthermore, propionate supplementation in the drinking water reduced food and water intake,

dependent upon vagal FFAR3. Although disrupted vagal signaling is not thought to primarily cause diet-induced obesity, several studies have shown diminished vagal sensitivity to key satiety hormones (ie. GLP-1 and CCK) in obesity[19,146]. There is also substantial evidence that the vagus nerve can be therapeutically targeted to combat obesity[14,148,164]. Thus, while dysfunctional vagal FFAR3 signaling is not likely a causal factor in obesity, the vagal-FFAR3-dependent anorectic effect of propionate offers therapeutic potential.

My RNA sequencing and food intake data suggested that FFAR3 signaling cross-talks with CCK and leptin receptors in vagal neurons to modify food intake. CCKaR signaling induces extracellular calcium influx in NG neurons[165] likely altering the gating of information to the CNS. Deletion of leptin receptors from vagal sensory neurons reduces satiation and CCK sensitivity, resulting in increased fat mass gain [22]. Furthermore, other studies have demonstrated synergistic actions of CCK and leptin in vagal calcium signaling, peptide secretion, and long-term feeding behavior[22,156-158]. My data suggests that propionate/FFAR3 may also be involved in this synergistic interaction.

Ffar3 expression was also found in a high percentage of Glp-1r+ and Oxtr+ vagal neurons, which are populations known to control feeding and drinking behavior[37,38]. Activation of these GPCRs triggers neuronal excitation and neurotransmitter and/or peptide release through intracellular and extracellular calcium pathways that we found altered in our sequencing data[166-168]. Vagal-FFAR3KO mice drank more water during fasting refeeding, and propionate reduced water intake in WT mice, yet it failed to do so

in vagal-FFARKO's. Given the overlap of Ffar3 expression in Oxtr+ neurons controlling water intake[37], it is likely that FFAR3 signaling contributes to vagal control of thirst satiation.

Vagal sensory neurons synapse onto the dorsal vagal complex (DVC) where they release glutamate and other neuropeptides to control feeding[15]. Thus, future investigation would be needed to fully decipher how FFAR3 impacts calcium dynamics and peptide secretion into the brainstem to regulate satiety. Future studies elucidating the precise signaling mechanisms of FFAR3 within vagal sensory neurons might help develop effective therapies for modulation of eating and drinking behavior. Ultimately, vagal satiation signals are integrated in the hypothalamus to control feeding behavior. Li *et al*. demonstrated that butyrate decreases food intake by attenuating activation of NPY+ neurons in the arcuate nucleus [76]. Our study supports the idea that vagal-FFAR3 signaling contributes to this process. Further work mapping the neurocircuitry of FFAR3+ vagal neurons may improve our understanding of how fiber fermentation via gut microbiota impacts post-absorptive satiety signals.

Recently, elegant work has established links between maternal gut microbiota and nervous system development and behavior, and the vagus nerve has been suspected to mediate this connection via unknown mechanisms[82,169]. It is possible that I uncovered a role for vagal FFAR3 in development that ultimately influences adult feeding behavior, aside from acute signaling of FFAR3 in the adult. Future work directly testing this hypothesis and utilizing conditional or chemogenetic models to temporally manipulate vagal FFAR3 may provide interesting insights in the role of FFAR3 in

nervous system development and function. My work stresses the need for further development of tissue specific genetic tools to elucidate the precise mechanisms by which FFARs, fibers and SCFAs control digestion, energy balance, glucose homeostasis, and behavior.

CHAPTER FOUR

FREE FATTY ACID RECEPTOR 3 (FFAR3) IN SPINAL AFFERENT NEURONS MAY REGULATE GLUCOSE HOMEOSTASIS

Introduction

Gut bacteria ferment indigestible dietary fiber to produce the SCFA's (acetate, propionate and butyrate). In humans, increasing fiber intake and SCFA production has been proven beneficial in lowering fasting glucose and hemoglobin A1c (Hba1c)[170]. Furthermore, increased plasma SCFA levels is associated with improved insulin sensitivity[171]. The mechanisms by which SCFA's improve glucose homeostasis and energy balance is not defined, but many studies have proposed the involvement of free fatty acid receptors 3 (FFAR3)[113,172-174].

Previous work utilizing global knockout models show conflicting data regarding the role of free fatty acid receptor 3 (FFAR3) in regulating blood glucose. For instance, Tolhurst *et al* found that FFAR3KO mice had impaired glucose tolerance despite normal insulin tolerance and release[97]. Conversely, other groups show either no effect[175] or improved glucose tolerance in mice lacking FFAR3[176]. However, none of the mentioned studies have utilized cell-specific models, despite FFAR3 being expressed in multiple tissues that may have opposing functions on regulation of glycemia. Just recently Lednovich *et al* identified an important function for intestinal FFAR3 in promoting fat

mass accumulation upon WD-feeding[94], supporting the findings of Samuel *et al*[95]. The mechanism by which FFAR3 alters glucose tolerance still remains unclear, but several studies have provided evidence that peripheral neurons expressing FFAR3 control blood glucose. First, Tirosh *et al.* showed that propionate stimulates glucagon secretion, not by direct action on the pancreas, but rather by activating the sympathetic nervous system[91]. In a study by De Vadder and colleagues, dietary propionate reduced hepatic gluconeogenesis and improved glucose tolerance and insulin sensitivity[77]. Interestingly, these benefits of propionate were lost when the rats underwent a procedure to destroy the sensory neurons innervating the portal vein. The portal vein carries high level of SCFA's from intestinal blood to the liver and is innervated by vagal and spinal afferent neurons[11]. The authors concluded that sensory neurons innervating the portal vein may mediate propionate sensing, potentially via FFAR3, which in turn regulated whole body glucose homeostasis[77]. It is known that spinal, and not vagal neurons, projecting to the portal vein mediate the counter regulatory response to slow drops in blood glucose[177]. Still, it remains to be directly tested whether propionate first signals through spinal afferent FFAR3 to ultimately increase sympathetic activation and glucagon release.

To begin addressing this important question, I utilized a vesicular glutamate transporter 2 (Vglut2)-Cre mouse line and performed a series of experiments assessing the long-term phenotype of Vglut2-FFAR3KO mice, as well as acute studies of propionate action. Vglut2 is generally expressed in peripheral sensory neurons and excitatory neurons of the CNS[178,179]. I did not detect any alterations in glucose tolerance in my vagal-FFAR3KO mice, and *Ffar3* expression has not been detected in the CNS.

Therefore, I began to test the hypothesis that spinal afferent neurons innervating the portal vein “sense” propionate via FFAR3, ultimately engaging an autonomic circuit driving glucagon release.

Methods

Animal Studies

Animal studies were conducted in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the NIH and with the approval of the Loyola University Chicago IACUC. C57BL/6J (#000664), Vglut2-IRES-cre (#028863), Gcamp6s (#028866), and TdTomato (#007914) were obtained from Jackson laboratory (Maine, USA). Mice were group housed 12:12 hour light/dark cycle 22-25°C. Mice received either NC (Teklad LM-485) or WD (TD88137, Teklad Diets; 42%kcal from fat, 34% sucrose by weight, and 0.2% cholesterol total) (Envigo, Indiana, USA) starting at 7 weeks of age. Body weights (BW) were recorded weekly from weaning.

Propionate Intraperitoneal Injection (I.P.)

Mice were fasted 4-5 hours in the morning and injected with 15mmol/kg sodium propionate in sterile filtered saline as done previously by Tirosh *et al.*[91] Mice were then either euthanized to collect blood and tissues, or blood glucose was measured at 15, 30, 60, and 120 minutes.

Von Frey

Mechanical allodynia was assessed with the experimenter blinded to genotype using calibrated Von Frey filaments (Stoelting) as previously described[126].

Primary Dorsal Root Ganglia Cultures

DRG neurons were dissociated in tryspin collagenase and cultured on 35mm dishes with cover slips in DMEM/F12 with added AraC and PenStrep. Neurons were cultured at least two days before experiments were performed.

Immunofluorescence

DRG and CG/SMG were fresh frozen and sectioned at 20μM using a cryostat. Sections were fixed in 4% PFA, permeabilized in 0.3% Triton X, blocked in 3%BSA in PBS with 0.2% (PBST) Triton X added. Antibody solutions were prepared in 2% BSA Similar fixation, permeabilization, and blocking protocols were performed on primary neuron cultures on glass cover slips.

Gcamp6s Calcium Imaging

Vglut2-Cre and Gcamp6s mice were crossed, and dorsal root ganglia were isolated from young mice. Neurons were serum starved on the morning of experiments (less than 4 hours), and imaged in physiological Tyrode solution (150mM NaCl, 4mM KCl, 2mM $CaCl_2$, 2mM $MgCl_2$, 10mM Glucose, 10mM HEPES, pH 7.4). Ca2+ free Tyrode solution was made as follows (130mM NaCl, 4mM KCl, 2mM $MgCl_2$, 10mM Glucose, 10mM HEPES, pH 7.4). Neurons were included in analysis if responsive to high KCl (45mM) stimulation.

Results

Ffar3 is Expressed in DRG Neurons and Alters Neuronal Activity

I performed quantitative PCR utilizing primers flanking the first intron of Ffar3 to distinguish genomic DNA (gDNA) from complementary DNA (cDNA), in order to appropriately identify Ffar3 mRNA expression and confirm a knockout. I confirmed previous findings that Ffar3 is indeed expressed in late thoracic and early lumbar DRG (Figure 27A). I then crossed Vglut2-IRES-Cre[178] with FFAR3 floxed mice to generate a tissue specific knockout. Vesicular glutamate transporter 2 (Vglut2) is expressed in the majority of small and large fiber DRG neurons[179], and it packages glutamate into neurotransmitter vesicles for synaptic transmission. Densitometry of the cDNA band for Ffar3 from Vglut2$^{Cre+/WT}$ / FFAR3$^{Flox/Flox}$ DRG showed a trend toward lower expression of Ffar3 (Figure 27B). FFAR3 is $G_{\alpha i}$-coupled, so classical signaling should lower cyclic AMP (cAMP) and lower activation of cAMP response element-binding protein (CREB). Indeed, immunofluorescence of primary DRG neurons revealed reduced intensity of pCREB staining in the nucleus after five-minute propionate stimulation (Figure 28A-C). Similarly, intraperitoneal injection of propionate decreased nuclear pCREB staining of DRG neurons *in vivo* (Figure 28D).

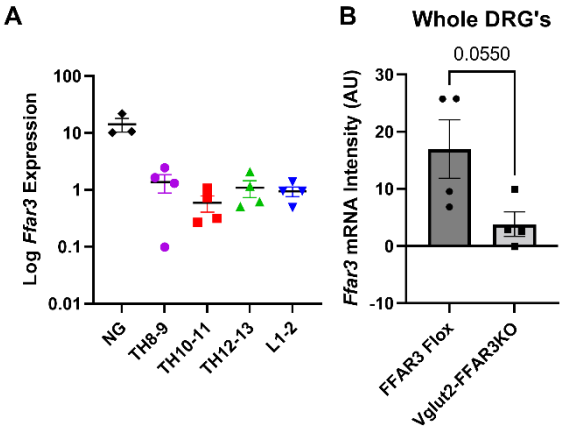

Figure 27. FFAR3 is expressed in DRG neurons and Reduced by Vglut2-Cre. (A) RT-qPCR of nodose ganglia and DRGs. (B) Densitometry of mRNA band of *Ffar3* qPCR products. Error bars indicate mean ±SEM

Previous reports have also shown that FFAR3 activation modulates calcium (Ca^{2+}) signaling[180,181]. To test this, I crossed Vglut2-Cre with mice transgenic expression of the fluorescent calcium indicator Gcamp6s. In a small percentage of primary DRG neurons, propionate stimulated low amplitude increases in cytosolic calcium (Figure 28E). I repeated this experiments in tyrode lacking extracellular Ca2+ and similar transients were observed (Figure 28E), suggesting that FFAR3 signaling couples to an intracellular calcium store.

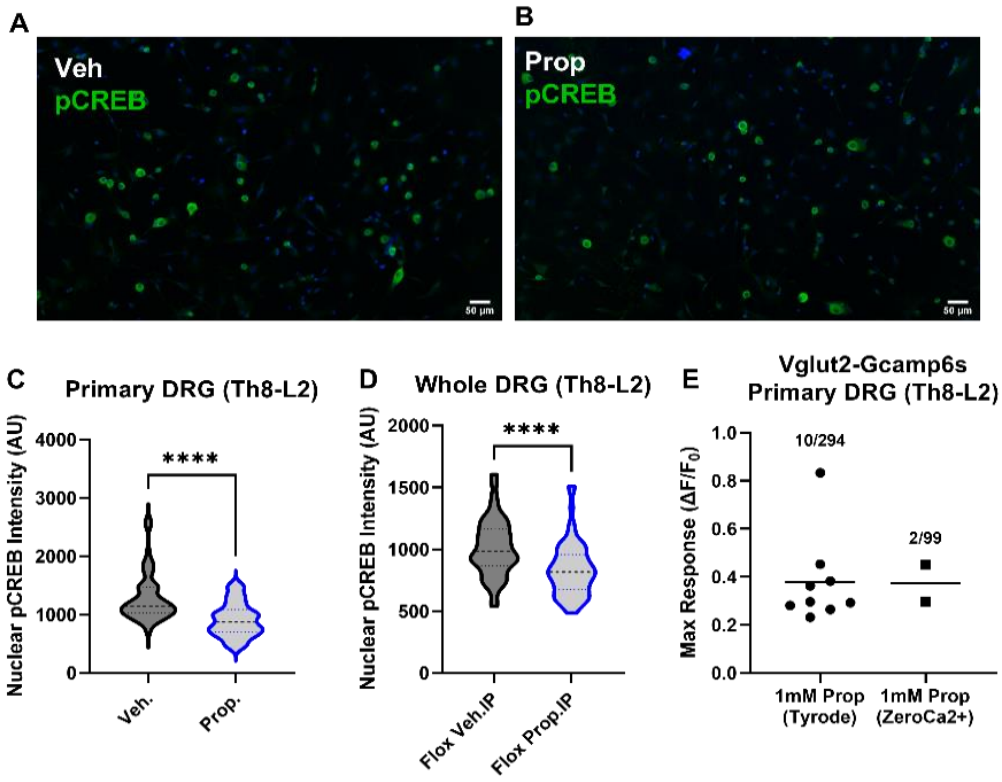

Figure 28. Propionate Alters DRG Neuron Activity. (A-D) Immunofluorescence staining of DRGs for phosphorylated CREB (pCREB). Example images of vehicle (A) or propionate (B) stimulated primary DRG neurons and calculated intensity of nuclear pCREB (C). Quantification of pCREB staining of whole DRG sections after propionate injection (D). Analysis of Calcium imaging of DRG primary neurons from Vglut2-Gcamp6s mice after propionate stimulation in tyrode and zero ca2+ Tyrode solution (E). Error bars indicate mean ±SEM

Genetic Deletion of Free Fatty Acid Receptor 3 (FFAR3) from Vglut2+ Cells Alters Glucose Homeostasis

Given that I found FFAR3 expressed in DRG neurons and FFAR3 agonists appeared to alter DRG neuron activity, I next wanted to characterize the physiology of mice lacking FFAR3 in DRG neurons. I previously did not detect any alterations in glucose homeostasis in vagal-FFAR3KO mice, but other studies have shown that peripheral sensory neurons sense SCFAs so regulate blood glucose. So, I began to assess glucose homeostasis in lean Vglut2-FFAR3KO mice. After insulin injection, lean blood glucose dropped lower in lean Vglut2-FFAR3KO mice compared to FFAR3 flox controls (Figure 29A-B). However, KO mice quickly responded to elevate their blood glucose during the ITT, resulting in no difference in area under the curve between groups (Figure 29A, C). Given that Tirosh *et al.* showed that propionate can engage a sympathetic circuit to increase blood glucose, we performed similar experiments in Vglut2-FFAR3KO mice to determine if sensory FFAR3 signaling might precede sympathetic activation. Similar to Tirosh *et al* propionate i.p. increased glucagon levels and stimulated rises in blood glucose in FFAR3 flox mice (Fig 29D-E). Interestingly, Vglut2-FFAR3KO mice exhibited even higher rises in blood glucose after propionate injection compared to FFAR3 flox controls (Figure 29D). Thus, propionate likely stimulates glucagon through direct activation of sympathetic neurons, rather than through a spinal afferent-sympathetic circuit as I had previously hypothesized. Additionally, I performed indirect calorimetry on lean, NC-fed, mice. Vglut2-FFAR3KO

mice had lower energy expenditure compared to their littermate FFAR3 flox controls (Figure 29G-H).

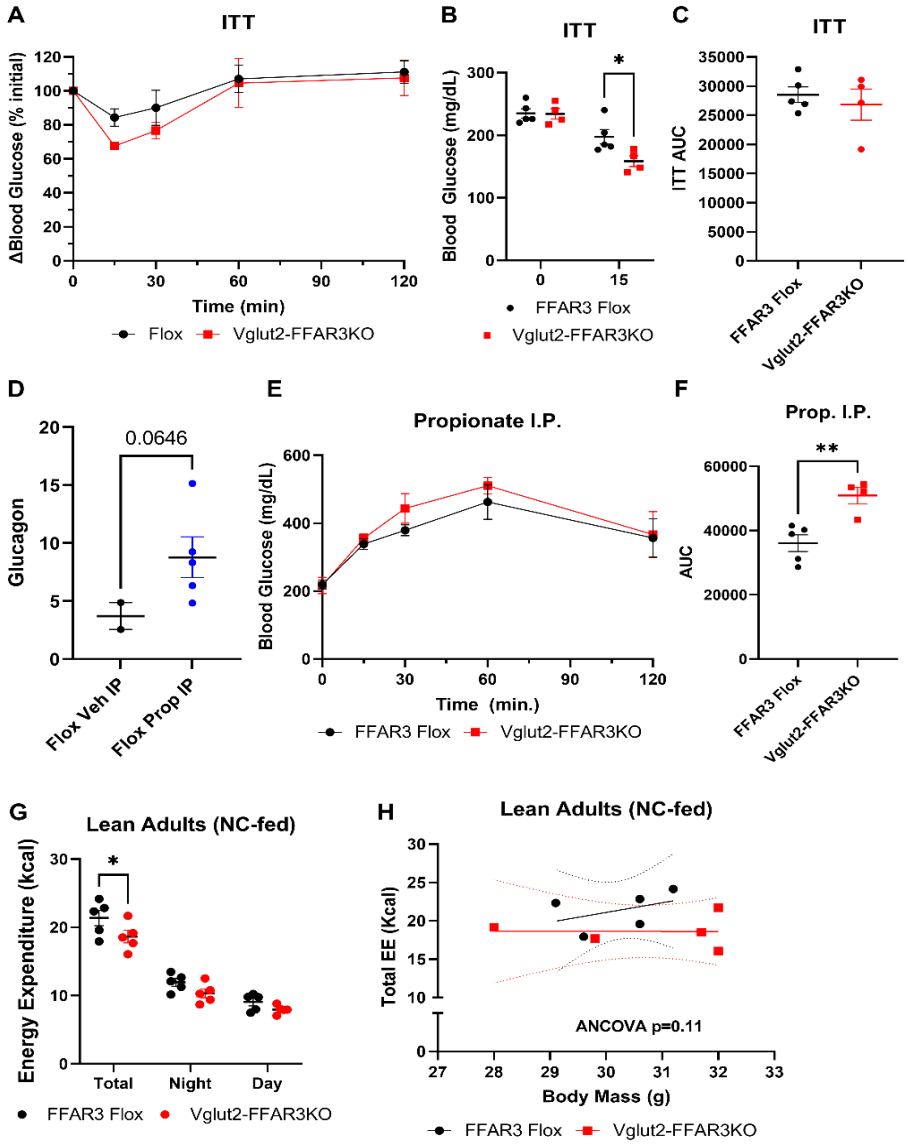

Figure 29. Genetic Deletion of FFAR3 from Vglut2+ Cells Improves Insulin Sensitivity. (A-C) Insulin tolerance test of lean Flox and Vglut2-FFAR3KO male mice. Percent change in blood glucose after insulin i.p.(A) raw fasting and 15-minute glucose levels (B) and ITT area under the curve (C). (D-F) Propionate tolerance test results. Glucagon levels 15 minutes after prop i.p. (D), glucose curves (E), and AUC (F). (G-H) Indirect calorimetry of lean adult male mice. Cumulative energy expenditure (G) and EE vs. body mass plot (H) during 48 hours of NC-feeding. Student's T-test *p<0.05, **p<0.01. Analysis of co-variance (ANCOVA). Error bars indicate mean ±SEM, dotted lines show 95% confidence intervals.

Vglut2-FFAR3KO Mice are Protected from WD-Induced Glucose Intolerance

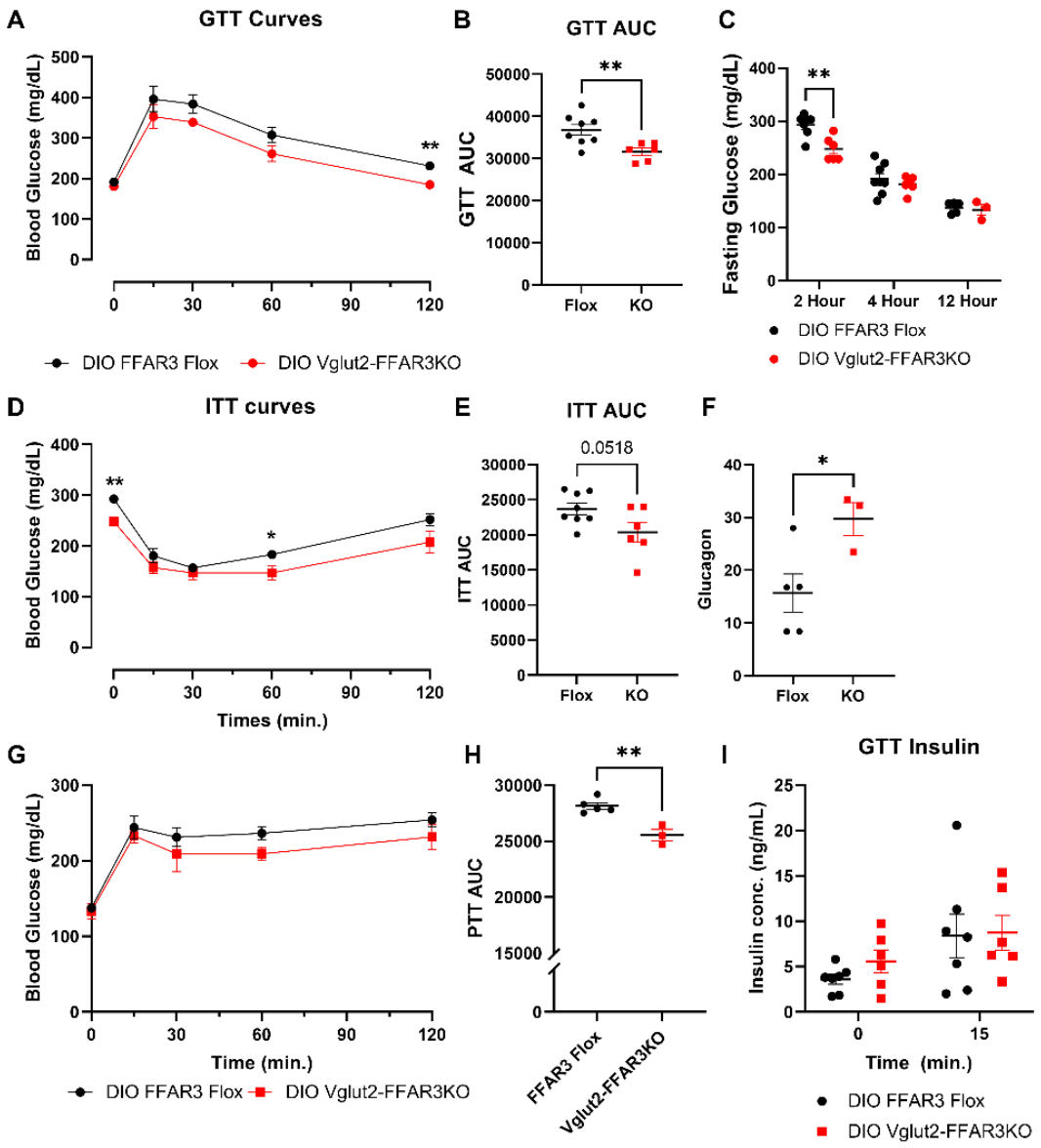

Figure 30. Vglut2-FFAR3KO Protects Mice from Obesity-Induced Disturbances in Glucose Homeostasis. (A) Glucose tolerance test curves (B) area under the curve (C) and fasting glucose levels of obese (WD-fed) Flox and Vglut2-FFAR3KO male mice. Insulin tolerance test curves (D) and AUC (E). Fasting glucagon levels (F). Blood glucose levels after pyruvate i.p. (G) and calculated AUC (H). Insulin levels after fasting and glucose i.p. during ITT (I). Error bars indicate mean ±SEM. Student's T-Test *p<0.05, **p<0.01.

Next, I challenged male FFAR3 flox and Vglut2-FFAR3KO mice with a WD to develop diet-induced obesity and to test associated disturbances in glucose control. Vglut2-FFAR3KO mice exhibited improved glucose tolerance (Figure 30A-B), lower 2-hour fasting glucose (Figure 30C), and improved insulin tolerance (Figure 30D). Looking more closely at the ITT curves, the Vglut2-FFAR3KO mice showed similar glucose disposal during the first 30 minutes (Figure 30D), but blood glucose rose back up more slowly after the insulin was no longer active. Consistent with the idea that these mice differ in counter-regulatory response to dropping glucose, Vglut2-FFAR3KO had lower area under the curve during a pyruvate tolerance test (PTT) (Figure 30C-D). Additionally, I did not detect any differences in fasting or glucose stimulated insulin levels (Figure 30E). Paradoxically, despite showing signs of blunted counter-regulation, Vglut2-FFAR3KO mice had higher fasting glucagon levels (Figure 30F). Additionally, I assessed mechanical and thermal pain thresholds of these mice, since the Vglut2-Cre model would target the dorsal root ganglia, and we previously found that SCFA's can alleviate neuropathic pain[126]. Interestingly, even with lower fasting glucose levels, DIO Vglut2-FFAR3KO mice trended towards lower pain threshold as measured by Von Frey filaments (Figure 31).

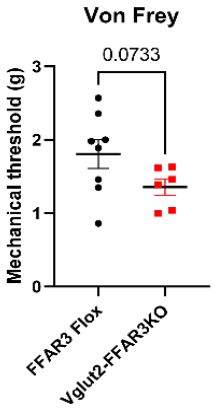

Figure 31. Mechanical Allodynia in Obese Vglut2-FFAR3KO Mice

As previously mentioned, lean Vglut2-FFAR3KO mice had lower energy expenditure. However, after 24 weeks feeding WD to develop DIO, obese Vglut2-FFAR3KO mice had equivalent body weight (Figure 32A), fat mass (Figure 32B), and

energy expenditure (Figure 32C). Similar to the Phox2b-Cre model, Vglut2-FFAR3KO mice consumed more water than FFAR3 flox controls (Figure 32D).

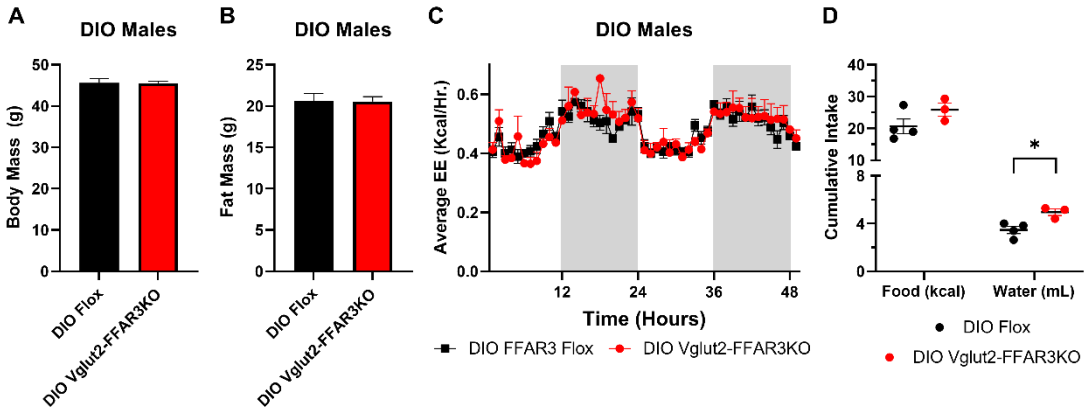

Figure 32. Vglut2-Driven Deletion of FFAR3 does not alter Long-term Energy Balance in DIO Male Mice. Body mass (A) and fat mass (B) of flox and vglut2-FFAR3KO mice after 22 weeks feeding WD. (C-D) Phenomaster indirect calorimetry data of male DIO mice after 24 weeks feeding WD. Energy expenditure (C) and total food and water intake (D) over a 48-hour period. Student's T-Test *p<0.05, n=3-4 mice / genotype. Error bars indicate mean ±SEM

DRG Neurons Project to the Celiac/Superior Mesenteric Ganglia (CG/SMG) to form a Spinal Reflex that may Control Glucose Counter-Regulation

Finally, to begin to understand the neural circuitry by which spinal afferent FFAR3 signaling may regulate glucose homeostasis, I performed immunofluorescence staining of celiac / superior mesenteric ganglia (CG/SMG) isolated from Na$_V$1.8/TdTomato mice. Sympathetic and parasympathetic neurons in the CG/SMG neurons project to the liver, GI tract, and pancreas to control blood glucose and motility. As shown in Figure 33, TdTomato+ were detected projecting throughout the ganglia, and the neurons within the ganglia stained positive for tyrosine hydroxylase (TH). While the TdTomato+ fibers I detected could also be vagal afferent neurons, previous studies suggest that they are more likely spinal[182].

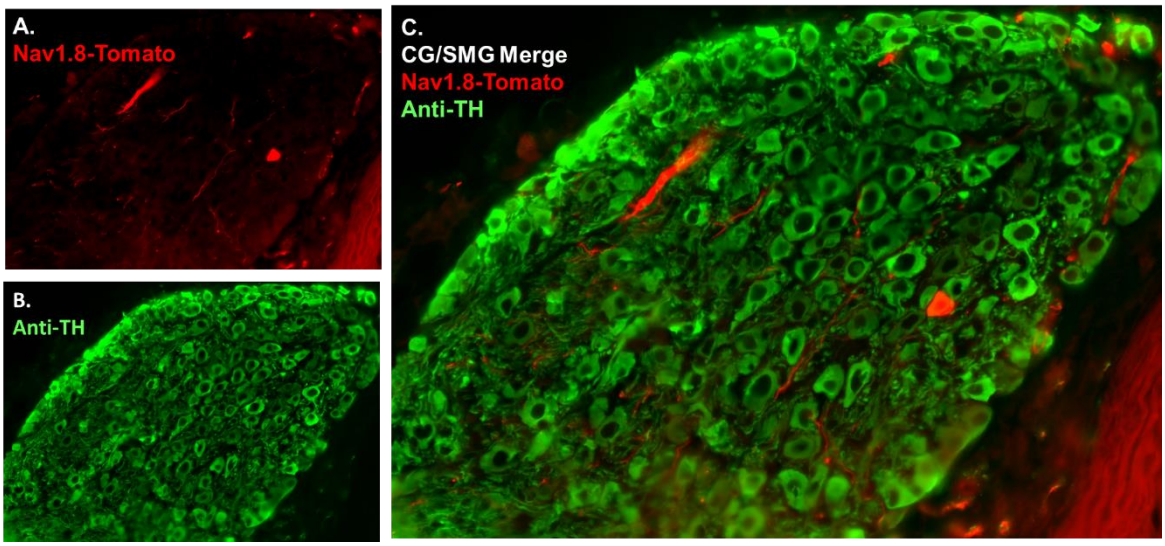

Figure 33. Na$_V$1.8+ Fibers Innervate the Celiac / Superior Mesenteric Ganglia (CG/SMG). (A-C) Celiac / Superior Mesenteric Ganglia (CG/SMG) sections from Na$_V$1.8-Cre / TdTomato mice. TdTomato signal (A), anti-TH immunofluorescence (B), and merge (C).

Additionally, I performed immunofluorescence staining of portal veins from Na$_V$1.8/TdTomato mice. TdTomato staining of vagal and or spinal afferent neurons which express Na$_V$1.8 was also detected along the portal vein, as expected. I also detected TH+ staining along the portal vein (Figure 34), which could be detecting either vagal, spinal, or sympathetic fibers. Thus, it is theoretically possible that spinal afferent neurons innervating the portal vein detect SCFAs via FFAR3 and form a reflex onto CG/SMG to alter autonomic control of glucose homeostasis. However, much more work is needed to substantiate this hypothesis. Furthermore, spinal afferent neurons expressing FFAR3 may not participate in a short reflex, but rather these projections may go to the CNS and alter autonomic outflow via a multi-neuron circuit.

Overall, our data shows that Vglut2-Cre-driven deletion of Ffar3 alters insulin sensitivity and energy expenditure in lean mice and possibly blunts counter-regulatory

responses in DIO mice. I likely ruled out the possibility of spinal afferent FFAR3 mediating propionate-induced release of glucagon and mobilization of glucose. Additionally, I found that propionate alters the activity of DRG neurons, possibly via $G_{\alpha i}$ signaling of FFAR3, ultimately leading to reduced nuclear CREB signaling. However, the results I have obtained are preliminary and require further study to directly link spinal FFAR3 signaling and glucose homeostasis.

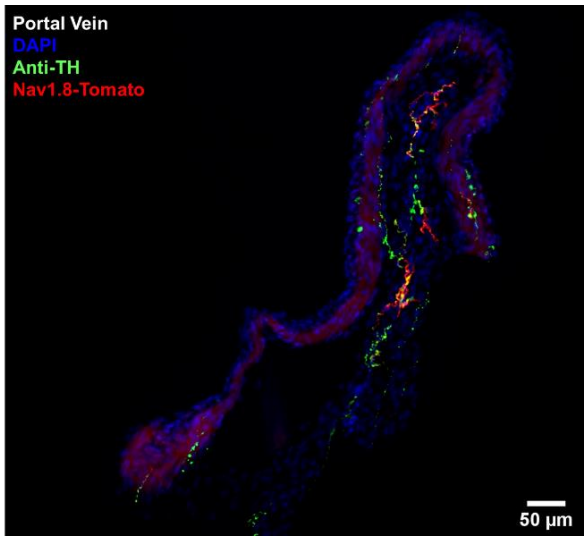

Figure 34. $Na_V1.8$+ Fibers Innervate the Portal Vein. Immunofluorescent staining against tyrosine hydroxylase (TH) (green) in the portal vein of a $Na_V1.8$-TdTomato (red) mouse.

Discussion

Limitations of Vglut2-Cre Driven Deletion of FFAR3

The Vglut2-Cre model serves as a broad model to target most peripheral sensory neurons, particularly the DRGs which I have identified to express FFAR3. Besides peripheral sensory neurons, this model may also target other tissues which express FFAR3, including enteroendocrine cells (EEC)[183] and the pancreas[184]. Given the importance of these tissues for controlling glucose homeostasis, it is important to consider the contribution of FFAR3 in these cells to the phenotype we have begun to uncover. We did not detect any difference glucose stimulated insulin release, so we would not expect altered incretin release from enteroendocrine cells or a global

disruption in beta cell secretion. However, Vglut2-FFAR3KO mice had lower two-hour fasting glucose levels, which could indicate altered glucose control after a night of feeding. In future studies, we will likely perform meal tolerance and oral glucose tolerance tests to assess this possibility. Additionally, it will be necessary to measure hormones such as GLP-1, GIP, and PYY, which could be altered if FFAR3 is deleted in EECs in our model.

Table 3. Summary of Relevant Cre Lines and the Targeted Cell Populations

Marker / Cre Line	**Phox2b-Cre**	**Vglut2-Cre**	**Nav1.8-Cre**	**TH-Cre**
Vagal Efferent (brain to periphery)	100%	0%	0%	Unknown
Vagal Afferents (periphery to brain)	100%	100%	~80%	+
Spinal afferents	0%	~60-70%	~75%	+
Sympathetic Neurons	0%	0%	0%	+
Other	Brainstem	CNS, EEC, Pancreas	PNS	CNS

Tirosh *et al* did not observe a direct effect of propionate stimulating glucagon release, so we also would not expect this to be the case in our model. Therefore, to our knowledge, the most likely loci of FFAR3 expression that is targeted in our model are spinal afferent neurons. To complement this model, the Na_v1.8-Cre would also be of interest. I chose not to utilize this model originally because the knock-in disrupts the Na_v1.8 gene itself, essentially creating a knockout. Furthermore, not all vagal or spinal afferent neurons express Nav1.8, and I actually identified a population of non-Na_v1.8 vagal neurons which express FFAR3. Therefore, I focused on the Phox2b-Cre and

Vglut2-Cre models which more comprehensively targeted vagal and spinal afferent neurons. However, a $Na_V1.8$-FFAR3KO would be important to test in future studies to avoid the broader targeting of the Vglut2-Cre model (Table 3 shows the summary of Cre lines and targeted tissues).

FFAR3-Mediated Intracellular Calcium Release

Barki *et al* performed similar calcium imaging experiments on primary DRG neurons, and they found a large percentage of neurons responding to propionate mobilizing calcium through a $G_{\alpha i}$-dependent pathway[163]. Conversely, I identified a low percentage of neurons responding to propionate with low-amplitude calcium signals, potentially from intracellular calcium release. Although we did not directly test it, I do not expect that FFAR3 is expressed in a high percentage of DRG neurons. Given the low expression compared to nodose ganglia, I suspect that only a small population of spinal afferent neurons express FFAR3. Therefore, Barki *et al* likely identified a FFAR3-independent effect of propionate on calcium dynamics in DRG neurons, and differences in imaging solution or neuronal culturing methods could have resulted in stronger calcium responses in their experiments. Additionally, I found that propionate stimulation in both primary cultures and *in vivo* injections lowered CREB phosphorylation in DRG neurons. Large calcium transients would likely, but not necessarily, induce CREB phosphorylation at serine 133 where the antibody I used targets[185]. Therefore, my immunofluorescence and Gcamp6s data are consistent that propionate does not produce large calcium transients in DRG neurons. Interestingly, Mizuta *et* found similar results of FFAR3 signaling in smooth muscle[181]. They found that FFAR3 activation

reduced adenylyl cyclase activity and produced minor calcium influxes. However, they also found that FFAR3 ligands potentiated calcium transients evoked by acetylcholine. Therefore, it would be interesting to test if FFAR3 agonism potentiates DRG calcium influxes in response to known excitatory stimuli such as capsaicin. Overall, I suspect that FFAR3-$G_{\alpha i}$ signaling reduced CREB phosphorylation, and FFAR3 may couple to intracellular calcium stores via another pathway.

Spinal Afferent Neurons Connecting Gut Microbiota and Glucose Homeostasis

Many studies have shown that gut microbiota production of SCFAs alters host glucose regulation. De Vadder *et al* showed that propionate engages spinal neurons innervating the portal vein that ultimately alter intestinal gluconeogenesis (IGN)[77]. We did not directly measure IGN, but this would be interesting to assess in future studies. We found that deletion of FFAR3 from glutamatergic cells was protective, increasing insulin sensitivity in lean mice. Vglut2-FFAR3KO mice were also protected against obesity-induced glucose intolerance, however we did not clearly see an improvement in insulin sensitivity in the DIO KO mice. Tirosh *et al* showed that propionate stimulates glucagon secretion by increasing sympathetic drive to the pancreas. I began to test whether FFAR3 activation in DRG neurons was the first step in a circuit, rather than direct activation of sympathetic ganglia as suggested by others. Contrary to my hypothesis, propionate still appeared to mobilize glucose, regardless of FFAR3 expression in Vglut2+ cells. In fact, propionate stimulated an even larger increase in blood glucose, in Vglut2-FFAR3KO mice suggesting that spinal FFAR3 may even inhibit glucagon release. This hypothesis would require further testing, but it would help

explain the confusing literature surrounding the effects of SCFAs and FFAR3 signaling in regulation glucose homeostasis. As this work is currently ongoing, we still plan to test propionate-stimulated glucagon secretion in FFAR3 flox and Vglut2-FFAR3KO mice, and we expect that propionate will indeed still increase glucagon secretion in the KO mice. Propionate most likely activates FFAR3 in sympathetic neurons directly to increase glucagon and mobilize glucose, so testing a sympathetic-specific FFAR3KO is of high interest. Regardless of the Cre line utilized, the complexity of inter-organ signaling to control insulin sensitivity likely requires glucose clamps studies to truly understand the contribution of FFAR3 to glucose homeostasis in this model.

Razavi et al. showed that transient receptor potential cation channel subfamily V member 1 (TRPV1+) neurons innervating the pancreas contribute to pancreatic inflammation in NOD mice, and ablating these neurons actually normalized insulin sensitivity[186]. Sensory neurons can indeed alter immune cell signaling in a phenomenon known as neurogenic inflammation[187]. Thus, it would be interesting in future studies to assess if spinal FFAR3 signaling alters neuro-immune communication, particularly in the context of metabolic disease when DRG neurons are hyperactive. Indeed, we saw a trend toward heightened mechanical allodynia in DIO Vglut2-FFAR3KO mice suggesting that FFAR3 signaling is somehow protective towards hypersensitivity of DRG neurons caused by diet-induced obesity. Furthermore, the FFAR3 agonist, propionate reduced nuclear CREB phosphorylation, suggesting reduced activity of these neurons. We have yet to repeat these experiments of DRGs from Vglut2-FFAR3KO mice, but I would expect that propionate would fail to alter pCREB when

FFAR3 is absent in these neurons. Further elucidating this signaling could be very interesting for study of pain associated with metabolic disease. In future studies, we will likely test the effects of SCFAs on nerve activity in mice expressing and lacking FFAR3 different sensory neuron populations.

Propionate-FFAR3 Signaling Regulating Sensory Neuron Development

Kimura *et al* showed that FFAR3 signaling influences the development of the sympathetic nervous system by promoting neurite outgrowth[83]. Given this interesting finding, we cannot rule out potential developmental effects of FFAR3 deletion in glutamatergic neurons regulating glucose homeostasis. It would be worthwhile to repeat similar experiments done by Kimura *et al* measuring neurite outgrowth in spinal afferent neurons after propionate in FFAR3-expressing and KO neurons. Furthermore, manipulating the maternal diet to alter SCFA levels in the developing embryo would be very interesting to test the effects on peripheral nervous system development.

Future Directions

As mentioned, the majority of work for this aim is unfinished and preliminary. Going forward, there are several different experiments that our lab will likely perform to deepen our understanding of how FFAR3 signaling in DRG neurons regulates glucose homeostasis. First, we will further characterize the dynamics of glucagon secretion in lean Vglut2-FFAR3KO mice. To do so we will take blood samples longitudinally throughout a period of fasting and after insulin injection to assess the response to hypoglycemia. Additionally, we will perform I.P. and oral glucose tolerance tests. The oral glucose tolerance test may especially inform us of potential contributions of Vglut2-

driven deletion of FFAR3 in enteroendocrine cells. On this note, we will also measure GI hormones that are possibly regulated by FFAR3 signaling in enteroendocrine cells, including GLP-1, GIP, and PYY. Additionally, measuring expression of proglucagon (Gcg) may also be helpful. Ultimately, all of these experiments will guide our decision-making process in choosing the appropriate type of clamp study[188]. Replicating these findings in a $Na_V1.8$-Cre model would be ideal to complement the findings of the Vglut2-Cre model.

We will also perform more experiments to characterize the neural circuitry of FFAR3-expressing spinal neurons controlling glucose homeostasis. First, we will need to replicate the calcium imaging and immunofluorescence experiments utilizing FFAR3KO mice. Additionally, staining brainstem and CG/SMG for cFos after propionate injection would be interesting. RNA sequencing of primary neurons and whole DRG may also provide a more unbiased approach to elucidate pathways downstream FFAR3 signaling in spinal afferents.

CHAPTER 5

CONCLUSIONS AND DISCUSSION

Summary of Results

I found that in a western diet model of diet-induced obesity, the composition and function of gut microbiota was disrupted. Bacterial diversity and specific genus known to ferment fiber to produce SCFAs were lowered, and circulating SCFAs were lowered, as well. Fecal microbiome transplantation from lean mice to obese mice, and a brief diet switch, improved bacterial diversity, and increased abundance of beneficial commensal *Lactobacillus.* Furthermore, butyrate and propionate levels were increased by FMT, and fasting glucose was lowered. FMT increased vagal *Glp-1r* potentially via SCFAs altering chromatin acetylation. Additionaly *ex vivo* and *in vitro* studies confirmed that propionate can alter gene expression of GPCRs controlling feeding behavior and glucose homeostasis via FFAR3-dependent and –independent mechanisms.

Given our FMT studies, and previous studies demonstrating positive effects of SCFAs, I began to test the contribution of the SCFA-binding receptor free fatty acid receptor 3 (FFAR3) in sensory neurons mediating gut-brain control of energy balance and glucose homeostasis. I found that FFAR3 signaling in vagal neurons contributes to meal satiation, and propionate supplementation lowers food intake through vagal FFAR3+ neurons likely innervating the upper gastrointestinal tract. Propionate also

stimulated glucagon secretion, however independent of FFAR3 in vagal or spinal sensory neurons. Propionate likely activates FFAR3 in sympathetic neurons to stimulate glucose counter-regulatory mechanisms, but this requires further study. FFAR3 signaling likely decreases activity of DRG neurons and modulates insulin sensitivity. Mechanistically, propionate activation of FFAR3 may couple to intracellular calcium stores in both vagal and spinal neurons.

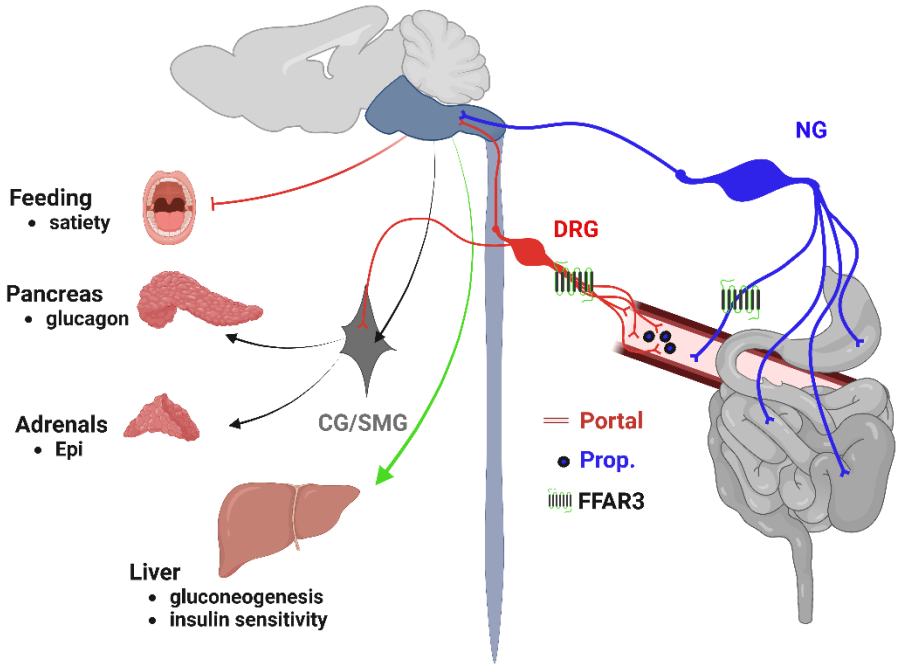

Figure 35. Proposed Outline of SCFA Sensing by Vagal and Spinal Neurons Regulating Energy Balance and Glucose Homeostasis. Vagal neurons (Blue) innervating the upper gastrointestinal tract likely sense exogenous SCFAs to decrease food intake. Vagal afferent neurons projecting to the colon and portal vein sense endogenous SCFAs produced by gut microbiota to enhance satiety and terminate a bout of feeding. SCFAs absorbed into portal circulation are also detected by spinal afferent neurons (Red) via FFAR3. Spinal afferent FFAR3 signaling controls insulin sensitivity and glucose counter-regulation, likely in part via glucagon and epinephrine release. Sympathetic neurons in the celiac ganglia and superior mesenteric ganglia (CG/SMG) likely detect SCFA directly by FFAR3 to stimulate glucagon and epinephrine release, mobilizing glucose from storage into the blood.

Overall, my work has identified substantial roles for FFAR3 signaling in sensory neurons innervating the rodent gastrointestinal tract. More work is needed to expound upon my studies to determine the intracellular signaling mechanisms within different sensory neuron populations, as well as the translational impact of these studies to humans.

Discussion and Future Directions

Obstacles in Microbiome Research

There is still large gap in the knowledge of molecular mechanisms driving the interaction between the gut microbiota and host physiology. A shortfall of microbiome sequencing approaches is that there likely exists a high level of redundancy in the genes and metabolic pathways across different genus of bacteria. Conversely, different species within the same genus may express distinct genes that affect the microbiota landscape or directly signal to the host in a unique way, yet many sequencing techniques cannot resolve beyond genus level. Furthermore, it is difficult to identify a clear microbial signature driving disease or physiological process when the microbiome differs in individuals across different regions. The functional output of an individual's microbiota may provide more physiological information than purely microbiome composition. Thus, future studies utilizing proteomic approaches to determine alterations in metabolites or peptides produced by the gut microbiota may prove very insightful.

Western Diet Disrupts SCFA Signaling

Despite the many obstacles in microbiome research, we were able to confirm other studies showing that an obesogenic diet disrupts gut microbiome composition, specifically lowering diversity, reducing *Lactobacillus* abundance, and diminishing SCFA production. Several human studies have found fecal SCFAs to be positively associated with body mass index (BMI) and obesity. However, another study found that circulating but not fecal SCFAs were associated with improved insulin sensitivity[171], and it is well accepted that a high fiber diet is effective for lowering body weight[86,189,190]. Therefore, it is surprising that multiple studies have found higher SCFAs in the feces of obese individuals. One possible explanation for this apparent contradiction could be disrupted SCFA absorption in obese individuals. Many reports have found that gastric emptying, GI motility, and energy harvest are altered in obese individuals and rodents[191,192]. In fact, Samuel *et al* found that fecal SCFAs were increased in global FFAR3 knockout mice[95]. Thus, increased fecal SCFAs in obesity may actually indicate disrupted intestinal FFAR3 signaling and inadequate nutrient absorption in the gut. If this is indeed the case, it would be interesting to test how altered SCFA absorption influences the dynamics of SCFA sensing by sensory neurons. Additionally, it could be interesting to see if colonocytes of obese animals or individuals shift fuel preferences away from SCFAs.

Endogenous SCFA Sensing versus Exogenous Supplementation

Many human and rodent studies administer SCFAs exogenously in order to better understand what may be happening physiologically. However, caution must be

taken when generalizing effects of SCFAs as supplements given that the route of administration and dose are often largely different. Some evidence points to SCFA production by gut microbiota to even promote adiposity, yet studies including mine consistently show that SCFA supplementation at high levels reduces fat mass. It then becomes difficult to assess the effects of exogenous SCFA supplementation on glucose homeostasis since propionate induced weight loss which would by itself improve fasting glucose and insulin tolerance. Indeed, there is conflicting evidence on whether SCFAs are beneficial or not for improving glucose homeostasis. For instance, Tirosh *et al* showed that low amounts of propionate added to food as a preservative impede insulin signaling in both rodents and humans[91]. Conversely, a series of studies by Dr. Gary Frost's team and collaborators show that a modified propionate supplementation synthesized to target the distal gut can induce fat loss, increase insulin sensitivity, and improve beta cell function and insulin release[58,133,193]. These are just two examples of contradicting findings in human studies, while the literature varies even more in rodents[116,194].

As mentioned, another key difference between endogenous SCFAs and exogenous supplementation is that gut microbiota SCFA production is highest in the colon, while dietary or water supplementation delivers high concentrations to the stomach and small intestine. This distinction is particularly interesting regarding vagal FFAR3 signaling because FFAR3 is expressed in vagal neurons throughout the GI tract. Thus, FFAR3+ projections to the stomach would likely mediate propionate-induced anorexia, while endogenous propionate production might primarily engage vagal

afferent neurons which control meal size. Indeed, Li *et al.* found that butyrate could lower food intake and increase thermogenesis when administered orally, but not systemically[76]. Along these lines, characterization of the innervation pattern of FFAR3-expressing spinal afferent neurons would help clarify how these sensory neurons respond to different sources of SCFAs.

Other studies have shown different effects of SCFAs when administered intraperitoneally[76,91]. While circulating acetate levels reach hundreds of micromolar in rodents, the majority of butyrate is cleared in the GI tract, and very little propionate passes the liver to reach circulation. Therefore, intraperitoneal injection of propionate or butyrate may produce profound effects on energy balance and glucose metabolism, but these findings should be considered in the context of SCFAs as supplements and would not likely reflect physiological SCFA sensing. However, it is interesting that multiple GPCRs and transporters that can bind SCFAs are expressed throughout the body, where physiological concentrations are rarely high enough to activate. Additionally, it may be interesting to further test how SCFA-binding GPCRs respond to repeated high doses of ligand. For instance, the assumption of many studies is that the propionate or butyrate are acting as agonists for FFAR3, however little is known about this receptor's desensitization. Similarly, multiple studies disagree as to whether the ketone body, β-hydroxybutyrate is an agonist or antagonist for FFAR3[77,104,105,174].

Vagal FFAR3 Signaling Modulates Feeding Behavior in Mice

Deletion of FFAR3 from vagal neurons increased average meal size and increased consumption of WD and NC after a fast. Interestingly, vagal-FFAR3KO mice

did not consume more calories immediately after refeeding, but rather during the second night after food was reintroduced. It is well established that vagal afferent neurons promote satiety, by relaying mechanical and hormonal signals to terminate a meal, thus inhibiting food intake. Therefore, I expected to see a change in food intake within 4 hours after refeeding. However, several recent reports have found that vagal afferents also can promote food intake through rewarding and appetitive pathways. Groundbreaking work by Han *et al.* showed that sensory neurons in the right vagus nerve mediate reward pathways to promote preference towards particular nutrients in the gut[195]. Given these recent findings, I suspect that some components of the altered feeding behavior of vagal-FFAR3KO mice may be due to altered interoceptive reward signaling. Specifically, I hypothesize that SCFA-FFAR3 signaling inhibits a rewarding component of the meal, and deletion of FFAR3 may have caused an even stronger reward and subsequent preference. Consistent with this idea, FFAR3 is expressed in GPR65+ neurons which are known to have mucosal nerve endings (**Figure 2**). Stimulation of GPR65+ neurons does not elicit strong satiation signals[37], however it remains to be tested if this subpopulation of vagal neurons mediates the flavor preference identified by Han *et al* (2018). If this is indeed the case, future studies assessing nutrient preference conditioning by SCFAs or FFAR3 stimulation would be very interesting. Furthermore, determining the expression pattern and signaling of FFAR3 in left vs. right vagal afferent neurons could provide more insight into the role of the receptor in nutrient sensing.

SCFAs and FFAR3 Signaling May Regulate Peripheral Neuron Development and Prevent Neuropathy

SCFA and FFAR3 signaling may alter the growth, survival, and/or plasticity of peripheral sensory neurons, as it does in sympathetic neurons[83,105]. In our FMT studies, we found that transplantation from lean mice alleviated mechanical allodynia and increased nerve fiber density. We hypothesized that butyrate either prevented distal nerve degeneration caused by WD or promoted nerve regeneration. These studies focused on sensory fibers in the skin, but it would also be interesting to assess fiber density of autonomic nerves innervating various metabolic organs such as the liver, pancreas, or intestines. Liu *et al.* (2021) observed profound morphological changes in sympathetic innervation of the liver after mice fed a HFD for 20 weeks, and deterioration of these nerves worsened after 24 weeks[32]. Testing the efficacy of microbial-derived or exogenously supplemented SCFAs in the prevention of autonomic neuropathy in metabolic disease would be particularly interesting. Alternatively, it would be interesting to see if pretreatment of SCFAs could protect autonomic nerves from subsequent damage induced by diet-induced obesity. Kimura *et al.* (2020) showed that maternally produced SCFAs influence the embryonic development of sympathetic neurons, ultimately protecting the pups from diet-induced obesity and associated metabolic syndrome[83]. The authors showed that SCFAs signaled through FFAR3 to promote neurogenesis in sympathetic neurons. It remains to be tested whether this phenomenon occurs in vagal and spinal afferent neurons as well. It is possible that acute signaling of vagal afferent FFAR3 in the adult is less as important as is the role of FFAR3 in the

developing fetus and pup. Utilizing an inducible-Cre model to temporally delete FFAR3 would directly test whether FFAR3 plays a more prominent role in development or acute "sensing". Given that propionate rapidly altered feeding behavior and energy balance in wild-type mice and failed to alter feeding in vagal-FFAR3KO mice, I suspect the host does acutely sense SCFAs via FFAR3. However, additional development effects of FFAR3 signaling in vagal sensory neurons may exist, as well.

Afferent FFAR3 Signaling and Neuro-Immune Communication

Previous work has suggested links between dysfunctional sensory neuron signaling and inflammation. For example, Razavi et al argue that neurogenic inflammation influences islet autoimmunity in T1D, and inhibiting this spinal afferent signaling helps prevent the disease[186]. Since many studies have indeed found anti-inflammatory effects of SCFA's, it would be intriguing for future studies to assess the contribution of spinal afferent FFAR3 signaling in the attenuation of neurogenic inflammation. Spinal afferent neurons release neuropeptides such as Substance P and CGRP, which control blood flow and immune cell function[196], while possibly possessing antimicrobial properties[197]. As I have extensively discussed, endogenous SCFA production from fiber fermentation may be disrupted in obesity and T2D, and exogenous SCFA supplementation is an intriguing therapy for improving energy balance and glucose homeostasis. Interestingly, more recent work has potentially identified a functional link between gut microbiome signaling and type 1 diabetes progression (T1D), as well. The Environmental Determinants of Diabetes in the Young (TEDDY) study showed that microbial fermentation is disrupted in T1D individuals, and the

authors argued that SCFA production is key for preventing the disease[198]. I began to test whether signaling through FFAR3 in sensory neurons was also involved in proper glucose regulation. Although my work is preliminary, I suggest that SCFAs may signal through FFAR3 in spinal afferent neurons to regulate glucose counter-regulation. Both peptides are implicated in visceral and peripheral pain[199], and levels of Substance P have been seen in the colon after antibiotic treatment[63] and in colitis models[200].

Additionally, RNA sequencing of nodose ganglia explants revealed that propionate altered several genes within the “inflammation mediated by chemokine and cytokine signaling pathway” (P00031). This was a particularly intriguing result suggesting either the presence of immune cells within the vagal explant cultures, or that vagal neurons express genes known to regulate inflammation. Comparing sequencing results of explants and primary neurons, and then repeating these experiments in DRG neurons would be very interesting to clarify the interaction between FFAR3 signaling in different sensory neuron populations and local immune cell function. Additionally, utilizing cell sorting and immunofluorescence techniques to further address this question would be particularly interesting.

FFAR3 Signaling Regulates Energy Expenditure

Previous work has shown that global deletion of FFAR3 reduces energy expenditure[105,119]. It is generally thought that SCFAs elevate energy expenditure through direct activation of sympathetic neurons, however this has never been directly tested. My studies have been of the first to assess the physiological consequences of tissue specific deletion of FFAR3. Interestingly, Phox2b-cre targeted deletion resulted in

a sex difference in energy expenditure after fasting refeeding. While male Vagal-FFAR3KO mice exhibited a larger increase in EE after refeeding, female mice did not. Additionally, Vglut2-Cre mediated deletion of FFAR3, which targets both vagal and spinal afferents neurons, caused a decrease in basal EE in lean males. However, neither male Vglut2-FFAR3KO nor Phox2b-FFAR3KO mice exhibited increased weight gain or adiposity upon long-term WD-feeding. Therefore, FFAR3 signaling in vagal and spinal neurons may slightly increase energy expenditure, but likely sympathetic FFAR3 drives changes in long-term energy balance. Future studies deleting FFAR3 in sympathetic neurons will help clarify this question.

Why Would Peripheral Neurons "Sense" SCFAs?

Given that several SCFA binding GPCRs are expressed in different peripheral neuron populations, there must be utility for the host to rapidly sense bacterial fermentation. One might expect that microbial metabolites could elicit an aversive response to reduce food intake, however studies have shown that dietary fiber does not result in taste aversion[189]. Rather, it has been consistently shown that fiber and SCFAs reduce food intake by promoting satiety, and likely involving the vagus nerve[76,175,201,202]. So, the question stands, why do vagal would vagal SCFA sensing promote satiety? SCFAs are utilized as major energy sources by gut epithelial cells, so it is reasonable that sensory mechanisms exist to rapidly transmit this nutrient information to the brain. Additionally, soluble fibers bind water and can result in fluid loss through the feces. Ideally, this fluid-fiber mix slows digestion to allow for appropriate nutrient absorption. SCFAs could therefore serve as signals to inform the host that fiber has been properly

digested in the presence of enough water, and therefore may promote thirst satiation. Recent work by Dr. Scott Sternson's lab showed that rodents struggle to distinguish hunger and thirst until after they consume food or water. It is not until rodents consume either food or water that interoceptive signals are integrated into the brain that the animal can determine whether the appropriate need was met or not[203]. Both the phox2b-cre and vglut2-cre driven knockouts of FFAR3 increased water intake, and propionate dramatically reduced water intake in WT, but not vagal-FFAR3KO mice. Vagal-FFAR3KO mice drank particularly more water after the fasting refeeding challenge as well. Therefore, it would be very interesting if some of the food intake data we observed was a byproduct of disrupted thirst satiation.

With respect to glucose homeostasis, propionate can be utilized as a substrate for gluconeogenesis, and both propionate and butyrate stimulate de novo glucose production in the intestine via separate mechanisms[77]. Furthermore, propionate stimulates glucagon release and increases blood glucose[91]. Thus, propionate can stimulate gluconeogenesis via promoting glucagon release, and then propionate itself can be used as a substrate for this process. Presumably, gluconeogenesis would cease to be promoted once the SCFAs are used up. Therefore, sensory SCFA sensing may allow for rapid detection of energy substrates to allow the brain to fine tune which fuels are to be used.

Concluding Remarks

Through my dissertation study I have utilized several different techniques to study peripheral neurons and elucidate potential gut-brain signaling mechanisms

regulating physiology. My work has uncovered important roles for the GPCR free fatty acid receptor 3 in different populations of sensory and efferent neurons controlling energy balance and glucose homeostasis. Furthermore, I have identified ways in which SCFA supplementation may activate FFAR3 in peripheral neurons to reduce food intake and regulate blood glucose.

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VITA

Tyler Michael Cook was born in Warrenville, Illinois to Ken and Lori Cook. Tyler has four siblings: Garrett, Allie, and Emma. Throughout his PhD study Tyler became the proud uncle of three nephews. His brother Garrett and wife Jonelle had two boys: Judah and Elias. Tyler's sister Allie and her husband Kyle had a boy named Kory James (KJ).

Tyler received his Bachelor of Science in Psychology from High Point University, where he also played Division 1 Lacrosse. Tyler spent a gap year after undergraduate study in a research lab at Northwestern Medicine Central DuPage Hospital led by Dr. Jocelyn Nolt. In the fall of 2017, Tyler began his PhD study in the Integrated Program in Biomedical Sciences at Loyola University Chicago. Tyler joined the lab of Dr. Virginie Mansuy-Aubert and began his research investigating short-chain fatty acid (SCFA) sensing by peripheral neurons. Tyler was awarded the prestigious Ruth L. Kirschstein Predoctoral Individual National Research Service Award (NRSA) from the NIH (NIDDK) to fund his research.

Upon graduation, Tyler will move to Denver, Colorado to work as a post-doctoral research fellow in the lab of Dr. Darleen Sandoval, studying molecular mechanisms of gut-brain signaling regulating glucose homeostasis and energy balance after bariatric surgery.