MICROBIOTA AND THE MODULATION OF FOOD INTAKE AND HIPPOCAMPAL-DEPENDENT COGNITIVE FUNCTION

by

ALLISON WERNER RAUTMANN

(Under the Direction of Claire de La Serre)

ABSTRACT

The more we study gut microbiota, the more it becomes evident that alterations in bacterial relative abundances are associated with aspects of human health, including cognitive function and behavior. Human studies demonstrate links between weight status, high-fat (HF) diet, and cognition; and rodent studies implicate obese- and HF diet-type microbiota in disruption of hippocampal-dependent cognition and food intake. Evidence suggests that microbiota alterations are necessary for diet-driven cognitive disruption. We do not know whether microbiota changes alone are sufficient to induce these deficits. This thesis will first discuss mechanisms through which microbiota modulates food intake and cognition. Then, I will describe a study in which rats were given an antibiotic cocktail and recolonized with chow- or HF-type microbiota. We then assessed learning and memory function, neuroinflammation, and brain insulin signaling. We found that HF-type microbiota colonization is sufficient to induce neuroinflammation and alter insulin receptor gene expression and hippocampal-dependent cognition.

INDEX WORDS: Microbiota, Hippocampus, High Fat Diet, Memory, Learning, Food Intake

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ALLISON WERNER RAUTMANN

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ALLISON WERNER RAUTMANN

Major Professor:	Claire de La Serre
Committee:	Emily Noble
	Robert Pazdro

Electronic Version Approved:

Ron Walcott
Vice Provost for Graduate Education and Dean of the Graduate School
The University of Georgia
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DEDICATION

This work is dedicated to my grandfather, Steven C. Werner.

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I would like to thank Dr. Claire de La Serre for guiding me through this research and giving me the opportunity to grow as an academic.

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CHAPTER 1

INTRODUCTION

This thesis consists of three main chapters: two literature reviews, and an original study. Chapter 2, the first literature review, will discuss the current understanding of microbiota's role in food intake. The second review, Chapter 3, will discuss the mechanisms through which changes in microbiota composition might modulate cognitive function. Finally, Chapter 4 describes a study I conducted using selective recolonization of microbiota-depleted rats to determine whether microbiota composition resulting from high-fat feeding can 1) disrupt hippocampal-dependent cognition, and 2) cause hippocampal gliosis and insulin signaling gene expression disruptions independently of diet. Chapter 5 will discuss the conclusions from these works.

CHAPTER 2

MICROBIOTA'S ROLE IN DIET-DRIVEN ALTERATIONS IN FOOD INTAKE: SATIETY, ENERGY BALANCE, AND REWARD[1]

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[1] A.W. Rautmann and C.B. de La Serre. Submitted to *Nutrients,* 18 May 2021.

Abstract

The gut microbiota plays a key role in modulating host physiology and behavior, particularly feeding behavior and energy homeostasis. There is accumulating evidence demonstrating a role for gut microbiota in the etiology of obesity. In human and rodent studies, obesity and high-energy feeding are most consistently found to be associated with decreased bacterial diversity, changes in main phyla relative abundances and increased presence of pro-inflammatory products. Diet-associated alterations in microbiota composition are linked with weight gain, adiposity, and changes in ingestive behavior. There are multiple pathways through which microbiota influence food intake. This review discusses these pathways, including peripheral mechanisms such as regulation of gut satiety peptide release and alterations in leptin and cholecystokinin signaling along the vagus nerve; as well as central mechanisms, such as the modulation of hypothalamic neuroinflammation and alterations of reward signaling. Most research currently focuses on determining the role of the microbiota in the development of obesity and using microbiota manipulation to prevent diet-induced increase in food intake. More studies are necessary to determine whether microbiota manipulation after prolonged energy-dense diet exposure and obesity can reduce intake and promote meaningful weight loss.

Introduction

The gut microbiota is a collection of over 10^{13} microorganisms, including bacteria and fungi, that inhabits the gastrointestinal (GI) tract and plays a key role in regulating host physiology, particularly GI function and energy homeostasis [1, 2]. In response to the burgeoning obesity epidemic, research has focused on personal and environmental factors that might influence weight status. The discovery that in both humans and rodent models, obese individuals have a distinct microbiota profile compared to their lean counterparts, with an increased capacity to harvest energy from ingested food, has fueled over 15 years of research [2]. Microbiota is vital for proper GI function, as it is implicated in vitamin synthesis, digestion and metabolism of carbohydrate and other dietary components [3], and development and function of the GI immune system [4]. Gut microbes have also been shown to influence function of other peripheral organs, as well as the central nervous system (CNS), throughout development and the lifespan [5, 6]. The importance of gut microbiota in regulating host biology is evident from gnotobiotic studies: animals born germ-free (GF) present with altered intestinal, metabolic, and neural physiology [7, 8].

Recently, advances in sequencing technologies have allowed us to more comprehensively and thoroughly assess microbiota composition and its relation with disease states. Adverse changes in composition have been associated with an array of pathologies, including autoimmune diseases, neurological conditions, and metabolic disorders such as obesity and diabetes [9-12]. It is, however, important to note that any environmental modification is likely to impact microbiota composition, and differences in bacterial make-up associated with pathologies do not equate to a causal link between microbial changes and pathological development.

There is accumulating evidence supporting a role for microbiota in regulation of food intake through both peripheral and central mechanisms. Peripherally, bacteria and their metabolites interact with vagal afferent neurons (VANs) which transmit information about intestinal contents to the nucleus of the solitary tract (NTS) [13]. Microbiota influences gut-brain satiety signaling via modulation of gut peptide release [14] as well as sensitivity to satiety peptides (such as cholecystokinin, or CCK) and the energy storage hormone leptin [13]. Changes in microbiota composition have also been reported to affect the structural integrity of the gut-brain axis [15]. Centrally, unfavorable microbiota composition is associated with inflammation of key regions involved in regulation of feeding, particularly the NTS and the hypothalamus [16, 17]. Further, there is emerging evidence that certain taxa of bacteria play a role in modulating reward circuitry and motivation [18, 19]. The purpose of this review is to describe the microbiota's influence on food intake through the aforementioned mechanisms, including recent developments in the relationship between microbiota, reward, and eating behavior.

Energy-dense diets lead to altered food intake and microbiota composition

Energy-dense diets alter gut-brain communication and regulation of feeding

Chronic intake of energy-dense food has been linked to excessive weight gain [20]. Despite homeostatic signals that act protectively against food overconsumption, chronic intake of palatable, high-energy diets alters the physiological response to food and favors overeating. Ultimately, this results in increased body weight (BW) and fat deposition. Specifically, sensitivity to hedonic cues is altered, while homeostatic signals of meal termination are dampened [21].

The vagus nerve is a direct pathway that carries post-ingestive feedback from the gut to the brain [22]. Mechano- and chemosensitive VANs respond to nutrient composition of ingested food to regulate meal size [23]. VANs terminate in the NTS, where postprandial signals increase neuronal activity [24]. In addition, VANs project to limbic brain regions, and this gut-reward circuit is sufficient and necessary for meal termination [22]. Chronic consumption of high-fat (HF) diet reduces VAN sensitivity to tension [25, 26], satiation hormones (e.g. CCK) [25, 27-32], and intestinal nutrients [33-36]. As such, diet-induced disruption of vagal signaling coincides with the onset of hyperphagia [37]. In addition, diet-induced obese (DIO) rats also exhibit significantly decreased postprandial neuronal activation in the NTS compared to lean animals [28, 36].

Other neuronal networks involved in regulation of feeding are also altered by chronic HF consumption. Leptin is a key adiposity signal, with amounts produced proportional to the amount of fat stored in the body [38]. Hypothalamic leptin signaling is disrupted during chronic HF feeding [39] with increased expression of suppressor of cytokine signaling 3 (SOCS3) and decreased phosphorylated signal transducer and activator of transcription 3 (STAT3) in the arcuate nucleus [40]. Pro-opiomelanocortin (POMC) neurons in the hypothalamus in a normal physiological state are activated by leptin [41] to ultimately decrease food intake via production of α-melanocyte-stimulating hormone (α-MSH) [42, 43]. Thus, HF-induced disruption of leptin can directly alter hypothalamic inhibition of food intake. Another neuronal system altered by HF intake is the dopaminergic reward system. Food's hedonic value is an important factor in food consumption, and increased motivation for food intake is linked to obesity [44]. Palatable foods initially have a higher reward value [45], while as obesity progresses, reductions in reward signaling emerge and lead to compensatory overeating [46]. Among regions involved in the mesolimbic dopaminergic system, the nucleus accumbens (NAc) and striatum exhibit decreased dopamine release in rodents with long-term exposure to HF diet [47].

Microbiota alterations seen with energy-dense feeding

Dietary intake is a major and easily modifiable determinant of microbiota composition; other factors include age and genetics [48]. In humans, both short- and long-term intake of specific macronutrients, as well as fibers and other plant foods, are correlated with abundance distribution of specific bacterial taxa present in the GI tract [48]. Obesity is associated with changes in microbiota composition. While the vast majority of the composition is specific to the individual, small-scale studies have found that obesity has been associated with an increased ratio of Firmicutes to Bacteriodetes*,* the two main phyla present in the GI tract [11]. Conversely, weight loss through caloric restriction has led to an increase in Bacteriodetes abundance, whether that restriction was through a carbohydrate- or fat-restricted diet plan [11]. Similar results are observed in rodent models of DIO, with the addition of a bloom in the pro-inflammatory Proteobacteria sometimes reported in humans with obesity or type 2 diabetes [49]. In rats, 8 weeks of 45% HF-feeding is associated with decreased bacterial α-diversity, a measure of the variety of bacterial taxa colonizing the gut, and increased relative abundances in the Firmicutes orders *Clostridiales* [50], in particular the *Dorea* genus, and *Erysipelotrichiales* (*Erysipelotrichaceae* family) [51]. Three weeks of 60% HF-feeding has similar effect on microbiota composition in rats, with increases in relative abundances of several Firmicutes families, including *Streptococcaceae*, *Erysipelotrichaceae*, *Lachnospiraceae* (*Dorea* genus), *Peptococcaceae*, and *Staphylococcaceae*; as well as Proteobacteria families *Desulfovibrionaceae* and *Enterobacteriaceae* [15]. In rats, a mere 7 days of 60% HF-feeding is associated with decreased abundance of the Bacteroidetes orders *Bacteroidales* (*Prevotella* genus) and *Sphingobacteriales*; and increased Firmicutes order *Erysipelotrichales*, and several Proteobacteria orders, including *Rhodocyclales* and *Altermondales*, among others [52]. Diets high in sugars also affect gut microbiota composition, with alterations in the Firmicutes to Bacteroidetes ratio sometimes reported [16, 53], but not always [54]. Different results have also been observed with respect to α-diversity [16, 53-55]. In a study comparing high-glucose, high-fructose, and HF diets in mice, researchers found that all three similarly decreased diversity, decreased Bacteroidetes abundance (specifically *Muribaculum* spp.) and increased

Proteobacteria abundance (specifically *Desulfovibrio* spp.); however, diets high in sugars led to significant increase in *Akkermansia muciniphila* abundance compared to HF diet [53]. When compared to an unrefined chow diet, both refined low fat, high sugar (LFHS) and HF, high sugar (HFHS) diet consumption in rats result in decreased diversity and significant alterations in relative abundances within 1 week of feeding [16]. The LFHS diet increases Firmicutes*,* particularly *Ruminococcaceae* and *Lachnospiraceae,* as well as Proteobacteria genera *Sutterella* and *Bilophila,* and decreases Bacteroidetes abundances, though changes are more pronounced with HFHS-feeding. [16]. A mere daily 2-hour access to HFHS pellets alters gut microbiota composition in chow-fed rats with increased relative abundances of *Lachnospiraceae*, *Ruminoccoceae*, and *Erysipelotrichaceae* families [55].

In humans, specific dietary patterns and components have been reported to affect bacterial taxa relative abundances. Obese humans switched to a strict vegan diet low in fat and high in fiber display an increase in abundance of Bacteroidetes over a 4-week period [56]. Self-classified vegans tend to have lower *Bifidobacterium* spp., *Escherichia coli*, and *Enterobacteriaceae* spp. when compared to vegetarian and omnivorous humans [57]. Two recent studies published in 2021 associated specific dietary components with microbial taxa. In Japanese monozygotic twins, significant associations are found between *Lachnospiraceae* species: *Lachnospira* and *Lachnospiraceae* UCG-008 negatively correlate with protein intake and saturated fat intake, respectively; while *Lachnospiraceae* ND3007 group correlates positively with total fat intake [58]. A population study of 1,920 Chinese adults found that a calculated healthy diet score based on intakes of fruit, vegetables, seafood, nuts/legumes, refined grains, red meat, and processed meat, is associated with increased abundances within Firmicutes and Actinobacteria, particularly genera *Coprococcus* and *Bifidobacterium*. Dairy is positively associated with the family *Bifidocacteraceae* and genus *Bifidobacterium*; seafood with families *Alcaligenaceae* and *Desulfovibrionaceae*; and nuts and legumes with the phyla Proteobacteria. Inverse associations are found with processed meat and the family *Lachnospiraceae,* while it was positively associated with *Fusobacteriaceae* and *Acinetobacter* under Proteobacteria [59].

There is evidence that Western-type diet-driven changes in microbiota composition negatively affect host metabolism and energy homeostasis. Studies using microbiota-depleted and GF rodent models have established a relationship between diet-driven dysbiosis and excessive weight gain. GF mice have been shown to exhibit resistance to weight gain when fed a HFHS diet that leads to increased adiposity in a conventional mouse, showing that microbiota is necessary for DIO [60]. Conversely, GF rats and mice colonized with fecal and cecal contents from conventional HFHS-fed animals display a significant increase in BW compared to rodents colonized with chow-fed animal microbiota [15, 61]. Similar results have been observed when GF animals are re-colonized with microbiota from a genetically obese donor [2] or from an obese human donor [62]. We have successfully replicated these findings in an antibiotic depletion model [15]. These studies establish that an “obese microbiota,” from a host that is obese, or “HF-type microbiota,” from a host fed a HF diet, is sufficient to alter energy homeostasis and affect BW regulation, at least in the short-term. The GF studies cited here do not extend past 5 weeks post-colonization [2, 15, 62]. While there is evidence that microbiota composition may affect energy harvest [2], storage [17], and utilization [63], a major effect on BW may be driven by changes in regulation of energy intake.

Microbiota-depleted rats colonized with a HF-type microbiota have been shown to significantly increase weekly food intake compared to rats colonized with chow-type microbiota [15]. Conversely, modulation of the microbiota via supplementation of anti-, pro-, or prebiotics impacts weight and intake. In rats fed a 60% HF diet, administration of minocycline, a broad-spectrum antibiotic, lessens microbiota alterations and significantly reduces food intake [52]. In this experiment, 3 weeks of antibiotic administration normalizes HF-fed minocycline-treated rats' intake to that of the control rats. This occurs with restoration of the Firmicutes to Bacteroidetes ratio to a level comparable to that of the chow animals, prevention of the HF-induced decrease in *Bacteroidales* and *Sphingobacteriales*, and significant reduction in *Erysipelotrichales* [52]. Administration of oligofructose, a beneficial prebiotic fermented by intestinal microbes [64], restores populations of *Akkermancia muciniphila* in DIO mice and normalizes BW [65]. In addition to preventing weight gain, probiotics have been found to promote weight loss in mice fed HF diet for 12 weeks. In these animals, supplementation with a probiotic containing *Lactobacillus rhamnosus, Lactobacillus acidophilus,* and *Bifidobacterium bifidum* for 5 weeks decreases BW and food intake [66]. In young men, supplementation with a probiotic along with the initiation of a HF diet (55% kcal from fat, 25% of kcal from saturated fat) reduces the amount of weight gained over 4 weeks [67]. Based on these data, microbiota alterations appear 1) sufficient to alter food intake and 2) necessary for HF diet-induced increases in intake.

Microbiota composition influences peripheral intake mechanisms

The presence of food in the GI tract leads to release of satiety signals, such as CCK, by enteroendocrine cells (EECs) that can signal via the vagus nerve to regulate food intake, particularly meal size [22][23]. There is evidence that GI bacterial make-up modulate several aspects of this gut-brain communication.

GI satiety peptide expression/release

GI bacterial makeup may affect regulation of meal size via modulation of GI satiety peptide expression and release. GF mice, when compared to conventional mice of similar body weight, display decreased intestinal expression of CCK peptide [68]. Further, fructose malabsorption induces microbiota alterations, which in mice is associated with changes in CCK expression and secretion [69]. Ketohexokinase (KHK)-knockout mice are a model of fructose malabsorption. KHK catalyzes fructose phosphorylation and KHK deletion prevents GLUT5, a fructose transporter, upregulation [69]. KHK- KO mice do not absorb most fructose, and feeding these animals a diet of 20% fructose leads to increased fructose concentration in the colon and alterations in microbiota composition, including increased relative abundances of Actinobacteria (families *Coriobacteriaceae* and *Corynebacteriaceae*), Bacteroidetes and *Lactobacillaceae* (particularly *Lactobacillus johnsonii*); and decreased Proteobacteria (family *Desulfovibrionaceae*) [69]. These alterations are accompanied by a significant increase in CCK-positive EECs) which can be prevented via antibiotic administration, demonstrating that microbiota is necessary for fructose malabsorption-induced alterations in CCK release [69]. In addition to modulating CCK expression, microbiota may influence CCK release. In the murine EEC line STC-1, application of certain fatty acid metabolites produced by commensal lactic acid bacteria results in increased CCK release [70].

Satiety peptide	**Association with microbiota**
CCK	VANs exhibit decreased CCK sensitivity when the GI tract is colonized with HF-type microbiota [15].
GLP-1	SCFAs produced when microbiota ferments soluble fibers may promote GLP-1 secretion [14, 71].
PYY	Rats fed soluble fiber also exhibit increased PYY levels in the GI tract [71].

Table 2.1. Gastrointestinal (GI) satiety peptides and their association with microbiota.

Microbiota's influence on gut satiety peptides is not limited to CCK. Glucagon-like peptide (GLP) 1 is an incretin released from intestinal L-cells that decreases food intake via a vagally-mediated pathway [72]. There is evidence that short chain fatty acids (SCFAs) produced by a healthy microbiota [73] influence GLP-1 release [14]. Application of SCFAs – acetate, proprionate, and butyrate – to mouse colonic cell cultures leads to increased secretion of GLP-1 through activation of the free fatty acid receptor (FFAR) 2, a nutrient-sensing G-protein coupled receptor [14]. This suggests that bacterial metabolites may be able to directly interact with L-cells to regulate GLP-1 release [14]. SCFAs are produced through fermentation of soluble fibers such as inulin, and while most gut bacteria can produce acetate, there are specific taxa that produce proprionate and butyrate [74]. Proprionate producers include Bacteroides spp., *Salmonella* spp., *Megasphaera elsdenii, Coprococcus catus,* and *Ruminococcus obeum;* and butyrate producers include *Anaerostipes* spp., *Roseburia* spp., and *Coprococcus comes, eutactus,* and *catus* (this list is non-exhaustive) [74]. Supplementation of inulin and other prebiotic fibers has been shown to prevent hyperphagia associated with energy-dense diet consumption in rodents [75, 76] as well as increase cecal and portal GLP-1 concentrations in rats fed standard chow [71]. Similarly, rats pretreated with 35 days of oligofructose supplementation consumed less food, gained less weight, and had nearly twice the expression of portal and colonic GLP-1 when switched to a HF diet compared to control rats [75]. Oligofructose supplementation for 3 weeks in chow-fed rats leads to a decrease in intake accompanied by increased cecal and portal concentrations of GLP-1 and peptide YY (PYY), another anorexigenic gut peptide [71]. A study in humans found that acute supplementation of inulin-proprionate ester increased plasma GLP-1 and PYY and was associated with decreased food intake at a meal post-supplementation when compared to controls [77], showing that proprionate has acute effects on food intake. Interestingly, prebiotic supplementation results in increased colon length [71, 76] compared to non-supplemented controls, which leads the authors to conclude that GLP-1 increase may be due in part to an increased number of secretory cells [71]. However, GF mice with significantly decreased intestinal expression of satiety peptides (CCK, GLP-1, and PYY) exhibit increased cecal and decreased ileal counts of EECs compared to conventional mice [35]. A study found that GF mice have altered ileal expression of genes related to vesicle organization in L cells that was accompanied by increased GLP-1 in ileal L cells, while colonic transcriptome was not significantly altered [78]. The authors suggest that this is due to the colonic mucus barrier, which prevents bacteria from coming into direct contact with EECs, while microbes in the ileum may come into direct contact with the mucosa [78]. Microbiota may influence GLP-1 release through yet another mechanism. Administration of *Akkermansia muciniphila* as a probiotic in obese mice restores levels of acylglycerols in the gut [65]. Acylglycerols are products of fat digestion and components of the endocannabinoid system, and one acylglycerol, 2-oleoylglycerol, stimulates L-cells to secrete gut peptides, including GLP-1, through stimulation of a G-protein coupled receptor [80].

CCK and leptin signaling

In addition to affecting gut peptide expression and release [14, 69, 81], there is evidence that changes in microbiota composition modulate vagal afferent sensitivity to gut-originating satiety signals, particularly CCK [13]. CCK is released from the proximal GI tract (duodenum and early jejunum) in response to long chain fatty acids [82] and amino acids [83], and acts on VANs to promote meal termination [84]. Colonization of microbiota-depleted rats with a HF-type microbiota is sufficient to reduce CCK-induced satiety in the receiver animals [15]. Conversely, it has been shown that preventing HF diet-driven dysbiosis through prebiotic supplementation prevents HF diet-induced loss in CCK signaling [51], demonstrating that changes in microbiota composition are also necessary for HF diet-induced alterations in CCK signaling.

VAN sensitivity to CCK may be altered through bacterial metabolites and their effect on leptin signaling. Leptin, an anorexigenic adipokine, is released from adipocytes in proportion to fat mass [38] and enhances CCK signaling [85]. Peripheral leptin resistance has been linked to a reduction in CCK sensitivity and increased intake [13, 32]. Lipopolysaccharide (LPS), a pro-inflammatory by-product of Gram-negative bacteria, increases in circulation in DIO rodents [13, 86]. Cecal and serum concentrations of LPS are increased in animals fed both LFHS and HFHS diets [16]. In rats, chronic low-dose administration of LPS, resulting in serum levels comparable to those seen in HF-fed animals, leads to VAN leptin resistance and decreased sensitivity to CCK [13]. LPS leads to toll-like receptor (TLR) 4 activation at the level of the nodose ganglion (NG), where VAN cell bodies are located, which subsequently increases SOCS3 protein levels. SOCS3 inhibits activation of the leptin receptor, potentially abolishing the synergistic effect of leptin on CCK sensitivity and decreasing feeding suppression following CCK injection [13].

Inflammation

In rats, DIO is characterized by a leaky gut and low-grade inflammation, potentially driven by bacterial product such as LPS [50]. GI-originating inflammation may play a key role in mediating HF diet-associated alterations in post-ingestive gut-brain signaling. Interestingly, HF feeding rapidly activates microglia-like cells in the NG [16, 87], and this may be mediated by the microbiota. Microglia are the resident macrophages of the CNS [88], and chronic activation of microglia causes inflammation [89]. Colonization of microbiota-depleted rats with a HF-type microbiota leads to an increase in positive staining in the NG for the pan-microglia and monocyte marker ionized calcium binding adaptor molecule (Iba) 1 [15], while administration of antibiotics [16] or prebiotics [51] prevents $Iba1^+$ cell recruitment along the gut-brain axis. In the CNS, microglia alter synaptic function and axonal growth in response to bacterial products by releasing cytokines [90-92], and HF diet-driven microglial activation is associated with inflammation-mediated neuronal death [93]. It is therefore possible that microbiota-driven recruitment of $Iba1^+$ cells in the NG has a deleterious effect on VAN survival. Co-culture of VANs with Gram-negative bacteria isolated from HF-fed rats (specifically *Proteus mirablis* of the order *Enterobacteriales* mentioned previously) leads to a dramatic decrease in viable neurons, suggesting that bacterial products can directly influence VAN survival [52]. Further, increased serum LPS and decreased innervation of the cecum was found in rats fed HF diet [16]. These data would suggest that, in addition to altering vagal afferent signaling, microbiota composition could also affect the structural integrity of the gut-brain axis. A decrease in VAN number may explain the reduction in c-fibers observed in the NTS of GF rats conventionalized with a HF-type microbiota [15]. At the level of the NTS, c-fibers are of predominantly of vagal origin [94]. Conversely, administration of a broad-spectrum antibiotic [16] or prebiotic [51] concomitant with HF diet introduction can prevent both dysbiosis and c-fiber withdrawal from the NTS.

Microbiota influences central intake mechanisms

Neuroinflammation

Besides altering sensitivity to leptin and CCK, bacterial inflammatory products produced by obese-type microbiota are linked to inflammation and loss of function in key brain regions involved in food intake – the NTS, as previously discussed, and the hypothalamus [95-97]. The hypothalamus contain key anorexigenic and orexigenic neuronal populations involved in regulating appetite and energy expenditure. Signals related to energy stores within the body, especially leptin, can modulate neuropeptides expression and release within the hypothalamus to regulate energy homeostasis. Inflammation and cytokine signaling interfere with leptin sensitivity in neurons [98]. Conventionally raised mice exhibit increased hypothalamic SOCS3 expression as well as decreased suppression of orexigenic mRNA (*Npy* and *Agrp*) in response to intraperitoneal leptin injection compared to GF mice [99], hinting that presence of certain bacterial taxa may interfere with hypothalamic leptin sensitivity. Increased bacterial LPS may play a role. Female rats fitted with slow-release pellets set to deliver daily low (53 µg/d) or high (207 µg/d) dose of LPS were fed a chow or 60% HF diet for 8 weeks [100]. At the end of the study, both LPS groups had gained more weight and consumed more food than the control vehicle pellet group. LPS groups dose-dependently increased expression IL-1β in the hypothalamus while increasing the expression of orexin, a neuropeptide that increases food intake, in the low-dose group [100]. In DIO mice, increased TLR4 and IL-6 mRNA expression in the hypothalamus is associated with decreased leptin-induced STAT3 phosphorylation and a failure to decrease food intake in response to intraperitoneal leptin injection [66]. Supplementation with probiotic containing *Lactobacillus rhamnosus, Lactobacillus acidophilus,* and *Bifidobacterium bifidum* results in decreased BW and food intake in DIO animals, as well as normalization of TLR4 and IL-6 mRNA levels in the hypothalamus, and restoration of leptin-induced pSTAT3 expression [66]. Similar preservation of leptin signaling has been observed with *Lactobacillus rhamnosus* supplementation alone [101], demonstrating that the presence or absence of certain bacterial taxa can modulate hypothalamic leptin signaling.

Oxidative stress is another inflammatory measure that is increased in DIO rats and may be related to microbiota composition. In a study by Fouesnard et al., rats were placed on either chow or high-energy Western diet (WD; 45% fat) for 6 weeks [95]. WD-fed rats exhibited hyperphagia in the first week of feeding, increased weight gain, and adiposity. Metabolomic changes were observed in the hypothalamus within 2 hours of diet introduction, and these changes persisted after the first day of feeding [95]. Specific alterations included hypothalamic redox homeostasis (increased oxidized glutathione, among other measures, suggests increased oxidative stress) and cell membrane remodeling processes. Similarly, cecal microbiota composition was significantly altered within hours of diet introduction, with WD feeding leading to decreased α-diversity and increased Proteobacteria relative abundance, particularly *Desulfovibrionaceae* and *Tannerellaceae* families, as well as decreased *Lactobacillaceae* relative abundance. Cecal metabolites also correlated with hypothalamic metabolites – one notable association was seen between oxidative stress and indices of α-diversity. This demonstrates an immediate pro-inflammatory microbiota shift within 1 day of WD feeding that coincides with alterations in hypothalamic oxidative stress and hyperphagia. [95]. Interestingly, in conventionally raised, but not GF, rats, RNA expression of superoxide dismutase 2, glutaredoxin, and IL-6 are increased after 2 days of WD feeding, demonstrating that microbiota is necessary for the early pro-inflammatory effects of WD in the hypothalamus.

Reward pathways

External factors can override homeostatic regulation of intake, including food availability, social and contextual cues, and palatability [72]. The ventral tegmental area (VTA) contains receptors for peripheral energy signals, including ghrelin, insulin, and leptin [102]. It also receives input from the hypothalamus and the NTS [102]. Optogenetic activation of VANs that innervate the upper GI tract stimulates reward-associated behavior such as self-stimulation, place preference, and flavor conditioning; and is sufficient to increase dopamine (DA) levels in the dorsal striatum [22]. Further, ablation of the vagal-parabrachio-nigrostriatal pathway abolishes conditioned preference for gastric infusion of high-calorie nutritive lipids over low-calorie nutritive lipids, while vagal deafferentation alone decreases conditioned preference and avoidance learning in a number of other tests [22]. As microbiota has been found to modulate vagal signaling, changes in bacterial composition are expected to affect modulate central mechanisms regulating reward.

GF and antibiotic-depleted mice exhibit alterations in dopaminergic reward pathways [18]. GF mice have increased DA turnover in the striatum and lower expression of D1 receptor mRNA in the striatum and NAc [103], a region involved in food-seeking behavior [104], and display increased preference for even low concentrations of intralipid compared to conventional mice [68]. Antibiotic administration has resulted in increased L-3,4-dihydroxyphenylalanine (L-DOPA) in the amygdala of young mice [105] and decreased DA turnover in the amygdala and striatum in rats, suggesting that microbiota modulates DA neurochemistry in rodents. Colonization with fecal contents from mice with chronic ethanol exposure leads to depressive and anxiety-like behaviors similar to those evident in withdrawal [19], and administration of SCFAs to mice previously exposed to a small dose of cocaine abolishes conditioned place preference [106], which suggests that microbiota is directly involved in modulating addiction-like behaviors and may be relevant in food addiction. These data suggest that microbiota may affect food intake not only through homeostatic mechanisms, but also through regulation of the reward pathway and hedonic perception. There are studies emerging that support this hypothesis.

In adolescent rats with intermittent daily access to HFHS diet, overall energy intake increases and monoamine gene expression is altered in the hippocampus and prefrontal cortex, and these alterations correlate with bacterial relative abundances [55]. Specifically, prefrontal cortex expression of monoamine oxidase A is positively associated with an unspecified genus of the *Lachnospiraceae* family, while expression in the hippocampus is associated with a number of other families, including unspecified *Bifidobacteriales*, *Bifidobacteriaceae*, unspecified *Bacteroidales*, *Rikenellaceae*, *Lachnospiraceae*, *Ruminococcaceae*, and *Erysipelotrichaceae* [55]. Microbiota may play a role in food preferences as well. Increased preference for sucrose is evident in mice undergoing social stress, and this increased preference is abolished by SCFA supplementation, suggesting that microbiota modulate stress-induced sucrose preference via SCFA production [107]. In rats, chronic consumption of HFHS diets leads to decreased motivation to lever press to receive a sucrose pellet [108]. Fructooligosaccharide introduced along with the initiation of HFHS feeding restores motivation for the sucrose pellet, however supplementation beginning after 10 weeks of HFHS diet exposure is not able to rescue this measure of motivational behavior [108]. Further, supplementation leads to decreased preference for HFHS foods compared to rats without supplementation [108].

Low- and no-calorie artificial sweeteners are another point of contention in terms of their impact on reward and intake, as it has been found that some sweeteners, such as stevia, are metabolized by gut microbiota [109]. While short- and long-term studies in humans do not show that artificial sweetener intake leads to compensatory overeating [110], there is evidence in both humans and rodents that sweetener intake causes alterations in reward pathways. In humans, ingestion of sucralose has significantly different effects in VTA activation compared to glucose or sucrose [111]. Rats exposed to a chronic low-dose of rebaudioside A (RebA), a stevia glycoside, exhibit decreased tyrosine hydroxylase and dopamine transporter (DAT) mRNA expression in the NAc [112], which can be rescued by prebiotic oligofructose supplementation [112]. These data suggest that artificial sweeteners that are metabolized by microbiota may alter reward signaling. It should be noted that the evidence is limited as research in this area is still emerging.

Bacteria	**Intake alterations**
Lactobacillus rhamnosus, Lactobacillus acidophilus,* and *Bifidobacteria bifidum	5 weeks of supplementation decreased hypothalamic inflammation, food intake, and BW compared to DIO animals without the supplement [66]
Akkermansia muciniphila	May promote gut peptide release through increasing acylglycerols in the gut [65]
Gram-negative bacteria	• Produce LPS, which can o reduce VAN sensitivity to leptin and CCK [13] o hypothalamic inflammation and BW [100] • May decrease VAN survival (specifically, *Proteus mirablis*) [52]

Table 2.2. Bacteria associated with food intake alterations. It is important to note that there is currently insufficient evidence to consistently link specific bacterial species to altered intake. Further, these findings have only been demonstrated in rodent models and are not applicable in humans.

Summary

Microbiota exerts an undeniable influence in the regulation of food intake through central and peripheral mechanisms, including satiety peptide release and signaling, inflammation, and modulation of reward pathways. Microbiota-driven changes in gut-brain signaling may be linked to alterations in both homeostatic and hedonic regulation of feeding. Preventing adverse microbial alterations via supplementation of prebiotic fibers and probiotics can successfully prevent hyperphagia in animal models, especially when introduced concomitantly with a dietary challenge. However, changes to the gut-brain axis may have long-term consequences on feeding behavior. HF or HS feeding [16] and HF-type microbiota alone [15] lead to withdrawal of vagal c-fibers from the NTS. This remodeling coincides with onset of weight gain, and hyperphagia [37]. Nerve injury-induced [113] or diet-induced [52] vagal withdrawal can be followed by NTS reinnervation (sprouting). Crucially, reinnervation does not appear to restore function in HF-fed rats, as animals remain hyperphagic [37, 52], suggesting that gut-brain function may be permanently affected in obesity. It is still unclear if microbiota-based therapy could restore gut-brain signaling in obesity. While weight loss in both humans and rodents is associated with microbiota composition alterations [114, 115], there is limited evidence that restoring microbiota in obese individuals can lead to weight loss. Probiotic use may be circumstantially associated with weight loss [116], but no causal link has been established. Most animal studies in the realm of obesity research focus on preventing weight gain when introducing a HF diet and initiate treatments such as pre- and probiotics concomitantly [117-120]. While helpful when flushing out the etiology of obesity, these studies do not determine whether microbiota modulation can successfully and effectively decrease food intake and promote clinically meaningful weight loss. More studies should be executed in which pre- or probiotics are supplemented to DIO animals to determine whether microbiota composition can be restored to a pre-obese state, or if modulation is associated with weight loss. Such studies would help determining whether microbiota is an appropriate target to promote healthy eating behavior and weight loss.

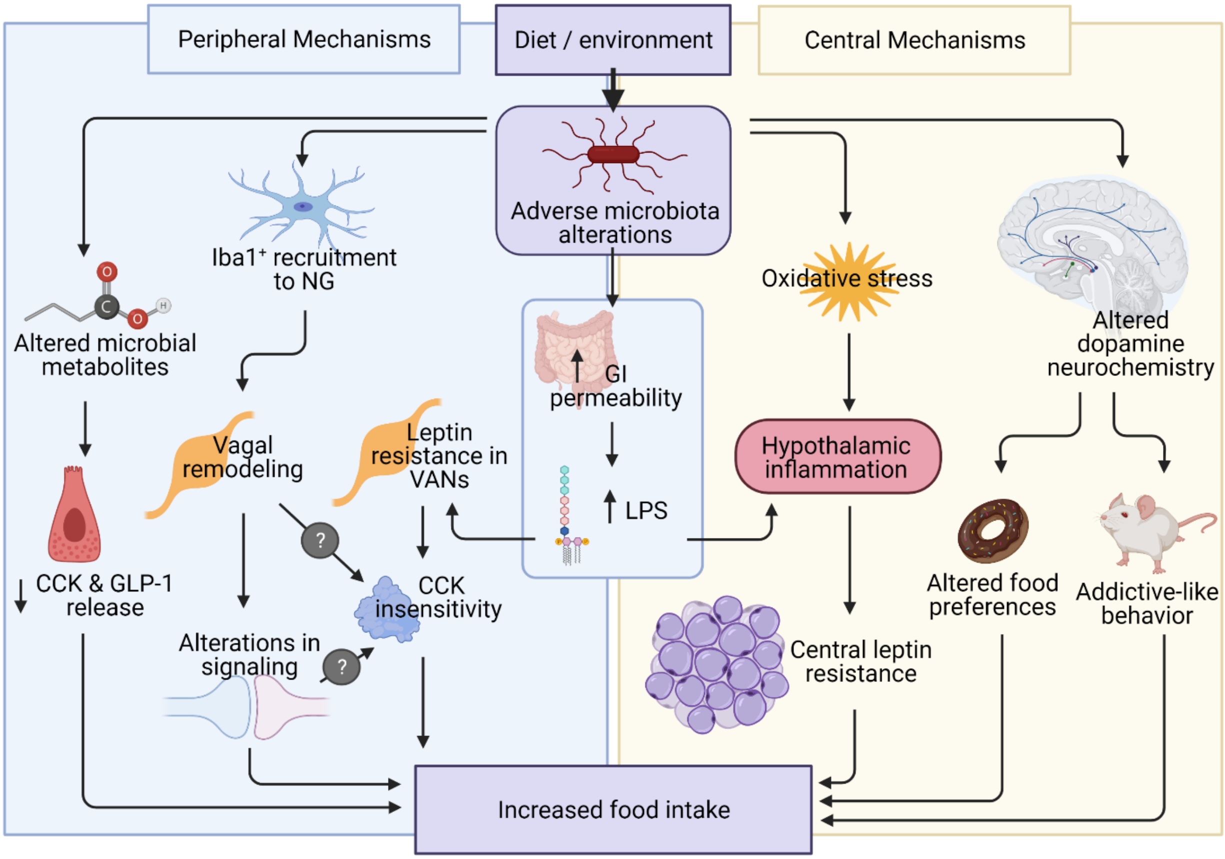

Figure 2.1. Summary depicting possible microbiota-mediated contributions to increasing food intake. Dietary and environmental changes lead to unfavorable microbiota alterations. Increased gastrointestinal (GI) permeability leads to increased systemic lipopolysaccharide (LPS), which increases leptin resistance in vagal afferent neurons (VANs), cholecystokinin (CCK) insensitivity, and increased food intake. Centrally, along with microbiota-induced oxidative stress, LPS increases hypothalamic inflammation and central leptin resistance. Microbial changes further result in altered production of microbial metabolites which can decrease CCK and glucagon-like peptide 1 (GLP-1) release. Microbiota resulting from high-fat feeding leads to ionized calcium binding adaptor molecule 1 (Iba1)-positive cell recruitment in the nodose ganglion (NG) and vagal remodeling, which contributes to alterations in signaling. Signaling alterations and vagal remodeling may also contribute to CCK insensitivity. Microbiota alterations can alter dopamine neurochemistry in reward centers, which alters food preferences and may promote addictive-like behaviors to increase food intake. Figure created with BioRender.

CHAPTER 3

MICROBIOTA MODULATES DIET-INDUCED COGNITIVE DYSFUNCTION: MECHANISMS

Introduction

Obesity is one of the most pressing public health epidemics of our time. In the United States, rates of both adult and, more disturbingly, childhood obesity are on the rise, with 39.6% of adults and 18.5% of children classified as obese in 2015-16 [115]. Adult obesity has large influence on a person's health and is associated with numerous comorbidities, including cardiovascular disease and diabetes, in addition to social consequences of weight stigma [116, 117]. Besides metabolic disorders, recent research has shown that obesity also affects brain functions. Obesity at mid-life increases the risk of developing vascular dementia and Alzheimer's disease in later life [118]. Further, obesity has a more immediate impact on cognition, with different domains of cognitive function negatively correlated with body mass index (BMI), including verbal learning, episodic memory, and working memory [119-121]. These domains are each linked to the hippocampus, an important brain structure that plays a key role in learning and memory processes and has been shown to be especially vulnerable to environmental and metabolic stress [122]. The interplay between diet, weight status, and the hippocampus is an important thread to follow in our understanding of the pathology of obesity.

This review will:

1) Explore the evidence that obesity is not only linked to decreased cognitive performance, but that changes in the brain – specifically, the hippocampus – that occur due to high-energy diets may further contribute to weight gain;
2) Examine alterations in the hippocampus, mainly increased neuroinflammation and alterations in insulin signaling, that occur in animals fed a high-energy diet; and
3) Discuss evidence that gut microbiota modulates the effect of high-energy diets on these hippocampal alterations and, consequently, poor performance on hippocampal-dependent tasks.

Obesity, High-Energy Diet, and the Hippocampus

Obesity itself has been linked to increased risk of developing Alzheimer's disease (AD) and other types of dementia [118]; atrophy of the hippocampus [123]; and decreased ability in various domains of cognitive function, including verbal learning [119], episodic memory [120], and working memory [121]. Cross-sectional studies have shown that young and middle-aged adults with obesity suffer poorer memory performance than their overweight or normal weight counterparts, even when adjusting for age, sex, education level, and metabolic and psychosocial covariables known to impact cognition [119, 124]. A prospective study of 2,223 healthy adults found that higher baseline BMI was associated with exacerbated cognitive decline at 5-year follow-up [119]. Gunstad et al. examined not only BMI but waist-to-hip ratio, a measure of central adiposity, and determined that both were associated with impaired memory performance [125]. In studies of young adults, BMI continues to be correlated with impaired performance on a variety of cognitive tests [121, 126] and is associated with deficits in executive function across the lifespan [127, 128], demonstrating that obesity can impact cognition across the lifespan.

However, in elderly adults (2,684 aged 65 to 94 years), Kuo et al. did find contradictory evidence, in which increased BMI had a nonlinear relationship with certain cognitive domains [129]. This may be because in elderly adults higher BMI indicates increased skeletal muscle mass and better cardiac output and stroke volume all of which are thought to improve cognition. Overall, there is significant evidence that midlife obesity can lead to current cognitive impairment and worsening cognitive decline with aging.

While the pathology of obesity is multifactorial, the World Obesity Federation has listed chronic consumption of high-energy-dense foods as one of the primary culprits of the obesity epidemic [130]. In fact, a diet high in saturated fat is so effective at increasing weight in rodents that a 45%-fat diet is a validated obesity model in rodent studies, proven to cause significant increases in weight and insulin resistance in as few as 4 weeks of feeding [131]. Fast food, which is associated with high energy intake, is currently consumed by 36.6% of Americans on a given day [132]. The 2020 Dietary Guidelines for Americans recommends consumption of fewer than 10% of daily calories each from sugar and saturated fat, though both of these limits are, more often than not, exceeded: the average American consumes 13% of calories from added sugar alone, and only 23% of Americans consume the recommended level of saturated fat [133].

Frequent consumption of energy-dense foods is also linked to impaired cognition in human studies. Relatively higher intake of simple sugars is associated with worse cognitive function in the elderly [134], young children [135], and young women [136]. Higher saturated fat intake is associated with worse cognitive outcomes with aging [137, 138]. In a study by Frances and Stevenson, increasing self-reported consumption of high-fat, high-sugar (HFHS) foods was negatively correlated with performance on memory tasks. Similarly, college students with a high-energy meal pattern were less successful on hippocampal-dependent memory tasks, accurately recalling food intake, and less sensitive to internal hunger cues in a cafeteria meal test compared to students consuming fewer HFHS foods [139]. Another study correlated increased HFHS intake with slower rate of learning on the hippocampal-dependent paired associate test, as well as decreased desire to consume salient foods when fasted compared to a fed state, an abnormality which the authors proposed is also hippocampal-related [140].

While high HFHS intake is related to poor cognition in humans, the reverse is also true: diets low in saturated fat and sugar are associated with decreased risk of cognitive impairment. A study by McEvoy examined dietary patterns during adulthood and cognitive performance in midlife. The 2,621 participants completed a food frequency questionnaire and had their diets scored as resembling the Mediterranean diet, Dietary Approaches to Stopping Hypertension (DASH), or the A Priori Diet Quality Score (APDQS). Diet score, verbal memory, processing speed, and executive function were assessed at baseline (participant average age of 25 years) and at 7 and 20 years follow-up. Participants with diets consistently scored as highly resembling the Mediterranean diet or APDQS were less likely to experience decline in cognitive function, with odds ratios of poor global cognitive function of 0.54 (CI 0.36-0.74) for the Mediterranean diet and 0.48 (CI 0.33-0.69) for APDQS [141]. Further, dietary pattern intervention, beyond merely supplementing or correcting for a single nutrient, has been shown to improve cognitive function or middle-aged adults [142, 143]. These studies, while telling, are only associational and cannot control for lifestyle and demographic factors that tend to go hand-in-hand with diet, such as physical activity, socioeconomic status, and education level.

Animal work has corroborated the previously stated human evidence. In rat studies in which male rats Sprague-Dawley rats were split into control, diet-induced obese (DIO), and DIO resistant groups, rats that became obese after feeding on a 40% high-fat (HF) diet exhibited decreased performance on hippocampal-dependent Y-maze [144] and feature-negative/simple discrimination tasks [145] compared to DIO-resistant rats. Rats maintained on a cafeteria diet suffered worse performance on place recognition task compared to rats fed standard chow after only 3 weeks of diet exposure [146]. Similarly to males, female Fisher rats fed HFS diet suffered progressively worse performance on the Morris water maze task after 1 and 2 months compared to their chow-fed counterparts [147]. Interestingly, non-obese rats display cognitive impairment in response to energy-dense feeding. Impairments in hippocampal-related tasks develop quickly after energy-dense diet exposure, with spatial memory disrupted by 3 days [148] and place memory by 5 days [149]; while another study found decreased performance on a maze task in rats after 9 days of HF feeding [150]. Long-term high-energy feeding while pair-feeding still results in comparatively poorer performance on the T maze in rats [151]. Identical results have been observed in mice and rat models where decreased hippocampal-dependent function was observed in response to high-energy diets [152-156]. These studies demonstrate that exposure to energy-dense diet, independently of hyperphagia or obesity, can affect hippocampal-dependent cognitive function.

The relationship between diet and hippocampal function is not one-way. While diet modulates hippocampal function, the hippocampus plays a key role in regulating food intake. Memory and learning are both important factors in appetite control, particularly when making the decision to initiate the next meal. This is evidenced by case studies of amnesic patients with damage to the hippocampus, who will continue to accept and consume meals despite having recently eaten [157, 158]. Simlarly, inactivation of the dorsal hippocampus in rats significantly decreased their inter-meal interval [159]. In humans, increasing salience of meals previously eaten has been shown to decrease intake of the current meal [160, 161]. These findings point to what has been termed the vicious cycle model, in which high-energy feeding causes hippocampal alterations and dysfunction, which in turn results in decreased inhibition of food intake and further consumption and weight gain (for review, see [162] and [163]). The hippocampus is particularly vulnerable to environmental insult, which includes high-energy diet [122]; is implicated in numerous cognitive functions, including learning and memory [122]; can be damaged by excessive intake of saturated fat and/or simple sugars [164]; and, when damaged, can promote excessive food intake [165]. In summary, the hippocampus is an important structure both impacted by obesity/high-energy feeding and a contributor toward further weight gain.

DIO animals exhibit hippocampal neuroinflammation and alterations in insulin signaling

In response to insult or injury, the brain's immune macrophage cells – microglia – become activated in order to remove pathogens or debris and keep the brain functional [81]. The activated, pro-inflammatory microglia phenotype is characterized by drastic changes in cell morphology and gene expression [166] which lead to release of inflammatory cytokines. Chronic activation can be harmful to neurons and is linked to neurodegenerative disease [167-169]. High-energy diet consumption has been shown to cause chronic microglial activation and is associated with neuronal inflammation and death [86]. Chronic neuroinflammation in the hippocampus is present in obese mice and is associated with altered synaptic function and cognitive impairment [170]. Microgliosis is evident in rat and mouse models of DIO and is linked with decreased performance on hippocampal-dependent tasks [171, 172]. In one study, high-energy feeding increased microglial activation and decreased performance on novel object recognition and Y-maze tests in mice, both microglia activation and test performances were rescued by returning the animals to chow diet for 2 months [170]. Microglia activation is necessary for diet-driven cognitive decline, as blocking microglia recruitment and activation in DIO mice prevented dendritic spine loss and obesity-associated impaired cognition [171]..

Astrocytes are supportive cells of the central nervous system (CNS) [173]. Like microglia, they become activated in response to injury to promote healing [173], and chronic activation is associated with nervous system pathology [174]. Chronic astrocyte activation is present in various models of obesity [175-177]. DIO and genetically obese mice exhibit increased glial fibrillary acidic protein (GFAP) staining, a marker of astrocyte activation [178], throughout the hypothalamus and, while the authors did not analyze other brain regions, they noted visually increased gliosis in extrahypothalamic areas, including the hippocampus [176]. Ovariectomized female rats that steadily gain weight exhibit increased astrocyte activation in the paraventricular nucleus of the hypothalamus over time compared to lean animals [175]. While one study reported decreased astrocyte activation in the hippocampus after 1 year of HF feeding compared to standard chow [179], DIO in rodents has been associated with increased astrocyte activation in the cortex [180], hypothalamus [181], and hippocampus [177]. Astrogliosis associated with high-energy feeding is further linked with hippocampal-dependent learning and memory dysfunction. Mice fed HFD have transient increases in GFAP staining in the dentate gyrus and impairments on novel object recognition and both the learning and memory phases of the Morris water maze [182]. Further, a study comparing two high-energy diets found that the diet higher in saturated fat resulted in increased astrocyte and microglial activation, increased markers of neuroinflammation, and impaired cognition on the Stone T maze [154]. This evidence suggests that neuroinflammation is a significant component in high-energy diet-related cognitive dysfunction.

Insulin is another important player in modulating cognitive function. While insulin receptor is found throughout the brain, it is particularly concentrated in the hippocampus [183]. Insulin signaling is involved in regulating neuroplasticity [184] and may be connected to the expression of brain-derived neurotrophic factor (BDNF), which promotes neurogenesis and plasticity [185]. Evidence suggests that insulin induces endocytosis of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors and thus contributes to long-term depression [186], while also playing a modulatory role in induction of long-term potentiation, both of which are crucial to the learning process [187]. Further, injection of insulin into the hippocampus improves performance on a spatial learning maze task which is believe to be linked to activation of the phosphoinositide 3-kinase (PI3K) cascade [184]. Clearly, maintaining optimal insulin signaling is imperative to maintain learning and memory function.

Indeed, brain insulin resistance is linked to cognitive decline [188]. Knockout of hippocampal insulin and IGF-1 receptors in mice results in abnormal spatial learning and memory on novel object, novel place, and Stone T maze tasks [189]. Similalry, lentiviral downregulation of the insulin receptor in rat hippocampus results in decreased performance on the water maze [190] and application of affibodies (antibody-like proteins) that target insulin in the hippocampus decreases performance on a maze task [184]. A model of type 2 diabetes in lean rats exhibit decreased performance on the Y-maze and increased signs of synaptic degeneration and astrogliosis [191], and higher i.p. doses of insulin impair performance on Y-maze in mice [192], demonstrating that peripheral hyperinsulinemia is detrimental to hippocampal function. Rats with diet-induced insulin resistance also suffer signs of altered synaptic plasticity and poor hippocampal-dependent water maze performance [152, 172] as well as microgliosis [172]. Further, DIO-prone Sprague-Dawley rats suffer worse performance on a spatial maze task compared to resistant animals and require higher doses of insulin injection to improve performance [184].

High-energy feeding induces activation of microglia and astrocytes, peripheral and central insulin resistance, and impaired hippocampal-dependent cognitive performance. While certain aspects of high-energy feeding may be directly linked to these effects, one important mediator has been shown to play a hand in both gliosis and insulin signaling – gut microbiota.

Microbiota modulates diet-induced gliosis and insulin signaling alterations in the CNS

Gastrointestinal microbiota composition is implicated in microglia maturation and function, as demonstrated by germ-free mice [193]. One study found that abundance of *Bacteroidetes* phylum is negatively correlated with recognition and spatial impairment [194]. Another study demonstrated that, in the 5XFAD mouse model of AD, the onset of significant increases in microglial recruitment and activation is associated with the change in microbiota composition – decreased *Bacteroidetes*, increased *Firmicutes,* and decreased overall diversity [195]. Rats with DIO show similar changes in microbiota diversity and composition [16]. Further, specific bacterial products have been shown to activate microglia and alter synaptic function and axonal growth, potentially altering neuronal function [196-198]. Specifically, lipopolysaccharide (LPS), a pro-inflammatory bacterial endotoxin, can directly activate microglia *in vivo* [199, 200], and in fact systemic LPS administration is used as a model for chronic neuroinflammation [201]. LPS is increased in plasma in both obese humans [10] and rodents fed on a HFD [52], and mice injected peripherally with LPS exhibit microglia recruitment [202] and impaired cognition [203], which suggests that a HFD-associated bacterial products can impact cognitive function. Additionally, gut-vagal signaling modulates hippocampal function and memory in rats [204] and dysbiosis is necessary for HF-diet-induced inflammation of the gut-brain axis, including microglial recruitment [51]. We have previously shown that colonization of antibiotic depleted and germ-free rats with a HF diet- type microbiota in is sufficient to increase microglial activation along the gut-brain axis [15]. Microbiota transplant from DIO mice to antibiotic-depleted recipients resulted in increased ionized calcium binding adapter protein 1 (Iba1), a marker for monocytes including microglia, in the cortex and impaired contextual fear conditioning [205]. Finally, in rats with high-fat diet-induced obesity and insulin resistance, microbiota dysbiosis, microglial activation, and performance on the Morris water maze were all rescued by supplementation of probiotics, prebiotics, or synbiotics [172]. Microbiota dysbiosis is both sufficient and necessary to cause diet-induced alterations in microglia activation and cognitive impairment.

Data from neurodegenerative and inflammatory disease models demonstrate that microbiota is connected to astrocyte activation as well. In a model of AD, both young and old AD rats displayed altered microbiota composition - including increased *Bifidobacteria* and *Firmicutes*, and decreased *Bacteroidetes* abundances - compromised gut barrier integrity, and increased activation of both microglia and astrocytes [206]. This relative increase in *Bifidobacteria* is curious, as it is generally believed to be beneficial to human health and is often found in probiotics [207]. One study of HF, high-fructose diet-induced nonalcoholic fatty liver disease in Iberian pigs correlated dysbiosis-associated metabolic alterations with astrogliosis and loss of neurons [208]. Oral supplementation of dietary compounds has been shown to modulate microbiota composition and astrogliosis. In a mouse model of AD, 7 months of supplementation of bioactive foods (in this case, a mixture of nopal, soy, chia oil, and turmeric) was associated with microbiota composition, reduced plasma LPS levels, and T-maze performance similar to that of wild-type mice, while partially or fully restoring GFAP staining across the stratum radiatum and stratum oriens layers of the hippocampus [209]. In amyloid precursor protein/PS1 transgenic mice, supplementation for 5 months with 5-heptadecylresorcinol, a compound found in whole grain rye, led to improvement of AD-related gut dysbiosis (specifically, increased abundances of *Akkermansia* and *Lactobacillus*), inhibited microglial and astrocyte activation, and improved learning and memory on the water maze task [210]. Acupuncture in a mouse model of Parkinson's disease (PD) resulted in alterations in 25 abundances in microbiota composition, improved motor activity, as well as decreased GFAP protein expression in the striatum and substantia nigra [211]. Further, fecal transplant from acupuncture-treated mice to PD mice resulted in improved motor activity, suggesting that microbiota modulates the beneficial effects of acupuncture in this model [211]. Modulation of the microbiota with probiotics and antibiotics impacts astrocyte activation. In a mouse model of autoimmune encephalomyelitis, supplementation of a mixture of Clostridia strains typically decreased in multiple sclerosis patients was associated with decreased astrocyte activation in spinal cord white matter and increased plasma butyrate, a short-chain fatty acid with immunoregulatory effects [212]. However, supplementation of butyrate alone did not result in a significant decrease in astrocyte activation, suggesting that Clostridia modulate astrocyte activation through multiple avenues [212]. In a mouse model of AD, antibiotic treatment resulted in decreased overall diversity and altered abundances of certain taxa, including blooms of genus *Akkermansia* and family *Lachnospiraceae* [213]. These changes were associated with decreases in AD pathology, including the number of plaque-localized GFAP-positive astrocytes [213]. While data connecting microbiota, astrocytes, and memory function are scarce, these studies demonstrate that microbiota dysbiosis in neurodegenerative disease is necessary for development of disease-associated astrogliosis.

Finally, microbiota alterations in insulin resistant and DIO models is associated with altered neural insulin signaling and cognitive impairment. Alternating 24-hour intermittent fasting (IF) in *db*/*db* mice improved peripheral insulin sensitivity, hippocampal insulin signaling markers, and learning and memory performance on the water maze task [214]. These improvements were seen along with changes in microbiota composition, namely increased diversity, *Lactobacillus*, and *Odoribacter* abundances; and decreased *Enterococcus*, *Streptococcus*, and unknown *Enterococcaceae* compared to controls [214]. Further, when microbiota was suppressed with antibiotic administration, the cognitive improvements associated with IF were not apparent [214]. As microbiota composition changes in response to HF feeding before metabolic, neural, and cognitive alterations develop [215], it is likely that microbiota modulates these effects. Supplementation of fiber to a HF, fiber-deficient model of obesity rescues microbiota richness, decreases abundance of *Proteobacteria*, and, among others, increased genus *Prevotella*, phylum *Actinobacteria*, and genus *Bifidobacterium* [216]. These changes reduced gut barrier leakiness and plasma endotoxemia, decreased intestinal inflammatory markers, increased serum short-chain fatty acid (SCFA) levels, reduced hippocampal gliosis, normalized insulin signaling markers (p-IRS-1, p-Akt, and p-GSK-3β), and performance on a temporal order memory test [216]. Performance on this test was significantly correlated with abundance of *Bacteroidetes*. Further, as in the study previously discussed, antibiotic administration eliminated the effects of fiber supplementation [216], suggesting that microbiota alterations are necessary for beneficial effects of diet manipulation on brain insulin signaling and cognitive function. Finally, mice colonized with microbiota from animals fed HF diet recapitulated the donor animals' insulin resistance of the nucleus accumbens and hypothalamus, and increased depressed and anxiety-like behavior [217]; demonstrating that HF diet-type microbiota is sufficient to induce insulin signaling abnormalities in mouse brain. Studies of the AD rodent model, in which β-amyloid is injected intracerebroventricularly, demonstrate how the microbiota modulates the effects of dietary components on improvements in hippocampal insulin resistance and function. Supplementation of β-glucans derived from yeast prevented microbiota dysbiosis, specifically normalizing abundances including *Oscillibacter*, *Alistipes*, *Rikenella*, *Lactobacillus*, *Bifidobacterium*, and *Desulfovibrio* to those of control, non-AD mice [218]. This accompanied decreased gliosis, normalization of proteins involved in insulin signaling (including p-IRS-1 and p-Akt), and improved learning and memory performance on the water maze [218]. Another study supplemented an ethanol extract of *Tetragonia tetragonioides* Kuntze, or New Zealand spinach, modulated gut dysbiosis (decreased *Clostridiales*, *Erysipelotrichales*, and *Desulfovibrionales*; and increased *Lactobacilales* and *Bacteroidales*), hippocampal insulin signaling, and improved cognitive function in AD rats [219]. In AD models of cognitive impairment, microbiota modulation improves hippocampal insulin signaling and spatial learning and memory function.

Conclusion

The hippocampus, a brain region implicated in learning, memory, and ingestive behavior, is particularly susceptible to environmental insult, including diet [122, 163]. Neuroinflammation, particularly gliosis, and insulin signaling alterations are associated with hippocampal dysfunction in obese, HF fed , and neurodegenerative disease models, and these changes are accompanied by alterations in microbiota composition. Microbiota depletion with antibiotics prevents these changes, and colonization of germ-free rodents with a HF-type dysbiotic microbiota results in behavior alterations similar to those seen in the donor animals. These studies point to microbiota as a major modulator of diet-related changes in hippocampal-dependent cognitive function.

CHAPTER 4

HIGH-FAT DIET-TYPE MICROBIOTA ALTERS COGNITION, NEUROINFLAMMATION, AND INSULIN RECEPTOR GENE EXPRESSION IN MALE WISTAR RATS[2]

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[2] A.W. Rautmann, C. Cawthon, R.A. Kirkland, J.S. Kim, S. Pandya, A. Brock, C.B. de La Serre. To be submitted to *Physiology & Behavior*.

Abstract

High-fat (HF) diet and obesity are associated with hippocampal-dependent cognitive impairment, namely spatial learning and memory, in humans and rodents. There are many causative factors involved, including neuroinflammation and alteration in central insulin signaling. It has been previously shown that microbiota transfer from HF-fed rats to microbiota-depleted animals lead to impairment in hippocampal-dependent cognitive tasks, alterations in insulin signaling in various brain regions, and increase microglial activation. However, data are inconsistent when it comes to hippocampal gliosis, insulin signaling gene expression, and cognitive performance. In this study, we utilized an antibiotic depletion model to colonize male Wistar rats with chow-type (Cconv) or HF diet-type (Hconv) microbiota. Recolonized animals underwent novel object recognition, novel place recognition, and Morris water maze behavior tests. Insulin tolerance and cholecystokinin (CCK) sensitivity tests were performed to determine whether HF diet-type microbiota is sufficient to alter peripheral insulin sensitivity and gut-brain satiety signaling, as it has been shown that the vagus influences hippocampal function and microbiota can alter gut-brain signaling. While insulin and CCK insensitivity were not recapitulated in recipients, HF and Hconv animals suffered increased microglial recruitment and decreased c-fiber staining in the nodose ganglion and NTS, demonstrating that HF microbiota was sufficient to alter gut-brain physiology. We then utilized immunohistochemistry to quantify hippocampal gliosis and RT-PCR to analyze hippocampal gene expression. Cconv and Hconv animals had similar weights and intakes throughout the length of the experiment. While recipient animal microbiota composition did not mimic their respective donors, differences between Cconv and Hconv were still associated with decreased memory on the novel place task,

increased microglial recruitment, and insulin receptor expression in Hconv animals. Some, but not all, aspects of HF diet-feeding were recapitulated in Hconv animals.

Introduction

The interactions between hippocampal function and diet have been evident ever since cases of amnesiacs, including the famous H.M., demonstrated that loss of hippocampal integrity resulted in an inability to regulate meal frequency [157, 158]. The hippocampus itself is particularly vulnerable to environmental insult and metabolic disruption [122] and, as a center implicated in such important cognitive functions as learning and memory [122], is a popular region of interest in obesity research. Hastened decline of cognitive function is well-documented in people who are obese at middle age [119, 124, 125, 127] or who become obese [119]. Further, altered cognition has been found in children and adolescents [128] as well as young adults [121, 126] with obesity. This is extremely concerning as rates of both adult and childhood obesity are increasing in the United States [115]. Energy-dense food consumption, which is directly linked to obesity, is also associated with altered cognition, with high intake of simple sugars associated with worse cognitive function in the elderly [134], young children [135], and young women [136]; and high-fat, high-sugar diets associated with slower rates of learning on hippocampal-dependent tasks [140] and memory performance [139]. Rodents with diet-induced obesity (DIO) similarly exhibit deficits on hippocampal-dependent tasks, ranging from various mazes [144, 147, 148, 151] to discrimination/recognition tasks [145, 146, 149]. In addition to these behavioral alterations, DIO rodents exhibit deleterious chronic activation of microglia [86, 170-172], and mixed evidence suggests that activation of astrocytes may also occur [154, 176-179, 182]. Neuroinflammation indicated by gliosis is associated not only with cognitive impairment, but with neurodegenerative disease [167-169]. A less-documented phenomenon, DIO rodents may also develop central insulin resistance [216, 217], which may interfere with insulin's contributions to plasticity [184, 185], long-term potentiation [187], and long-term depression [186] of hippocampal neurons, all of which are crucial to the learning process.

There is evidence that the gut microbiota, at least partially, mediates the effects of diet on the hippocampus. The gastrointestinal (GI) microbiota plays a role in the maturation and function of microglia [193]. Additionally, lipopolysaccharide (LPS) produced by high-fat (HF) diet-associated bacteria and found in increased levels in plasma of obese humans [10] and rodents fed a HF diet [52] has been shown to induce microglial recruitment [202] and impair cognition [203]. In Alzheimer's disease rodent models, modulation of the microbiota by supplementation of bioactive foods [209] and food-derived compounds [210] prevented astrocyte activation and improved performance on maze tasks. Further, supplementation of fiber in a DIO rodent model increases abundances of *Prevotella, Actinobacteria,* and *Bifidobacterium*, while decreasing abundance of *Proteobacteria*; and these changes are associated with normalization of hippocampal insulin signaling markers, including phosphorylated insulin receptor substrate (IRS)-1 and phosphorylated protein kinase B (Akt), and improvements on a temporal order memory test [216].

Previous microbiota transfer studies have demonstrated that, in rodents, HF diet-type microbiota is sufficient to induce microglial recruitment along the gut-brain axis [15] and in the cortex [205]; as well as insulin resistance in the nucleus accumbens and amygdala [217]. Further, saporin-induced ablation of vagal afferents results in hippocampal-dependent memory impairment [204], and we have previously shown that HF-associated microbiota transfer is sufficient to decrease c-fiber staining along the vagus [15]. Disruption may continue along the connections from the vagus to the hippocampus and result in memory impairment. Transfer has also been associated with behavior alterations, including impaired contextual fear conditioning [205] and anxiety-like behavior [217]. We aim to determine whether HF diet-type microbiota without exposure to HF diet itself can induce deficits in learning and memory function, gliosis, and alterations in insulin signaling gene expression in the hippocampus.

Materials and Methods

Animals

Two cohorts of 16 and one cohort of 12 male Wistar rats (240 ± 13 g, Envigo, Indianapolis, IN) were single-housed in a temperature-controlled vivarium on a 12-hour light-dark cycle. Body weights and food intake were assessed daily.

After 3 days of habituation, the donor cohort of 16 rats and the control cohort of 12 rats were given *ad libitum* access to either standard chow (Chow group; Picolab diet 5053; 13% fat; n = 8, n = 6) or high-fat diet (HF group; Research Diets D12451; 45% fat; n = 8, n = 6). The donor cohort continued on diets for 6 weeks before CCK sensitivity testing, insulin tolerance testing (ITT), novel object recognition, and sacrifice for collection of fecal and cecal contents. The control cohort was maintained on diets for 6 weeks before behavior testing and sacrifice.

The receiver cohort of rats had *ad libitum* access to chow diet. Receivers underwent antibiotic depletion for 17 days, including 3 days of antifungal (Amphotericin-B; 1 mg/kg BW, Gold Biotechnology, St. Louis, MO) and 14 days of antibiotic cocktail of ampicillin, gentamicin, neomycin (100 mg/kg BW; Gold Biotechnology), vancomycin (50 mg/kg BW, Gold Biotechnology), and metronidazole (100 mg/kg, MP-Biomedical LLC., Santa Ana, CA). Animals were gavaged daily with flexible tubing (plyethylene O.D. 2.42 mm) to decrease irritation. Following microbiota depletion, animals were placed in either chow receiver (Cconv; n = 8) or HF receiver (Hconv; n = 8) groups and were recolonized with respective donor fecal and cecal contents. Fecal/cecal inocula was prepared with 30 ug of intestinal matter in 500 uL of 20% glycerol in phosphate-buffered saline (PBS). Animals were gavaged with fecal slurry daily for 3 days and underwent booster gavages at weeks 8 and 10 post-depletion. Feces were collected from the receivers at 8 weeks post-depletion and at sacrifice to verify successful microbiota transplantation. CCK, ITT, and behavior testing occurred 6 weeks after transplantation.

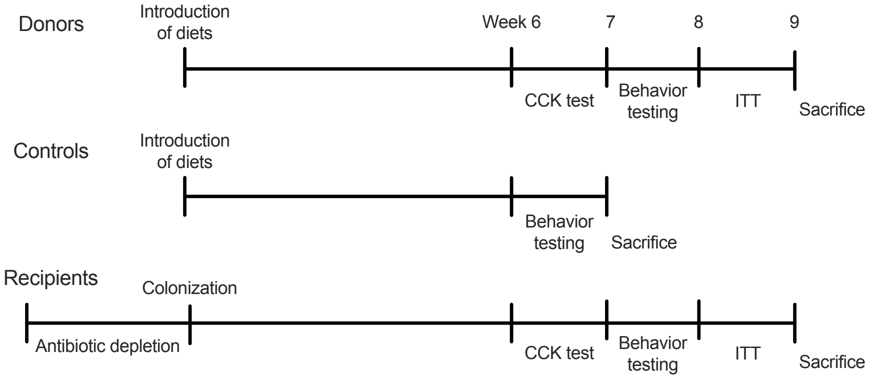

Figure 4.1. Timeline of diet introduction, microbiota depletion and colonization, and testing.

CCK sensitivity testing

CCK testing was performed after 6 weeks of feeding for the donor cohort and 6 weeks post-colonization in the recipient cohort. Animals were fasted for 12 h before intraperitoneal administration of CCK (0.22 nmL/kg, Bachem, Torrance, CA, USA) or saline (400 uL, vehicle). Testing was performed on two days, so that each rat received saline and CCK to serve as their own control. Food was returned to the animals and intake was measured 30, 60, and 120 minutes post-injection.

Insulin tolerance testing

Insulin tolerance testing was performed 10 weeks after initiation of diet challenge in the donor cohort and 10 weeks post-colonization in the recipient cohort. Insulin (0.75 units/kg) was injected intraperitoneally and blood glucose was measured before injection and at 15, 30, 45, 60, 90, and 120 minutes after injection via tail nick, using a glucose meter.

Novel object recognition (NOR)

Behavior testing commenced after 8 weeks post-diet introduction or post-colonization. Rats were placed in a clear plastic tub (50.5 x 34 x 32.7 cm;) with 2 identical objects for a 5-minute familiarization period before returning to their home cage for a 45-minute retention phase. For NOR, rats were then placed back in the tub with one of the objects replaced, and the time spent investigating each object was recorded for 3 minutes.

Morris Water Maze

A pool 1.8 meters in diameter was filled with water and black paint until opaque. The pool was divided into quadrants and a platform was placed in the center of one quadrant, designated the target quadrant, just beneath the surface of the water so that it was not visible to the rats. External visual cues (lamp, curtain, stuffed bear, and large cutouts of shapes) were placed around the room and kept constant throughout the duration of testing. A camera was fixed to the ceiling to record testing.

Testing occurred over five consecutive days and began at the onset of the dark cycle. The first two days of acquisition consisted of placing rats in the pool in the same spot and allowing them to swim until they found the platform. If they did not find the platform in 55 seconds, the rats were taken out of the water and placed on the platform for 15 seconds. During days 3 and 4 of the acquisition period, the rats were placed in the pool in different starting locations while the trial time period remained the same. During each day of the acquisition period, rats underwent 5 trials each at least 10 minutes apart. Day 5 of testing, the probe trial, consisted of placing each of the rats in the pool from the original start position and allowing them to swim for 60 seconds with the platform removed. ANY-maze® software (Stoelting Co., USA) was utilized to determine the latency to platform, time spent in each quadrant, time spent in thigmotaxis (the outer 15 cm of the pool), and time spent moving towards and away from the platform.

Tissue collection

Control animals were sacrificed after MWM testing, while donor and recipient animals were sacrificed after completion of insulin tolerance testing. Animals were euthanized via CO_2 inhalation and cardiac puncture. Brains were collected and cut in half. One half was immediately fresh-frozen on dry ice while the other half was post-fixed with 4% PFA for 2 h, then transferred to PBS with 20% sucrose for 1 week. Post-fixed brains were frozen in isopentane, then stored at -80°C.

Microbiota analysis

Fecal samples were collected from donor rats at sacrifice, and from recipient rats at week 8, after the first booster, and at sacrifice. Bacterial DNA was extracted with the ZR Fecal DNA MiniPrep (Zymo Research, Irvine, CA) and shipped to SeqMatic LLC (Fremont, CA) for library preparation and 16S sequencing of the V3-V4 region. Reads were processed by SeqMatic with Kraken2 to obtain OTUs and bacterial abundances. Abundances were normalized by log transformation for multivariate analysis on the METAGENAssist platform [220]. The Galaxy online platform was used to perform linear discriminant analysis effect size analysis (LefSe) to identify taxa that differed between groups [221].

Immunohistochemistry

Forebrains were sectioned at 20 microns between bregma -1.80 and -5.30 to obtain slices of the dorsal hippocampus. Sections were stained for glial fibrillary acidic protein (GFAP) (ab134436, 1:2000, Abcam, Cambridge, UK) to visualize reactive astrocytes or with ionized calcium binding adaptor (Iba)1 (100369-764, 1:1000 Wako Chemicals, Richmond, VA) and cluster of differentiation (CD) 86 (ab238468, 1:500, Abcam) to visualize pro-inflammatory microglia. Sections were incubated in primary GFAP antibody overnight at 4°C before incubation in Alexa Fluor ® 488-conjugated goat anti-chicken secondary antibody (A11039, Invitrogen, 1:500). Sections double-stained for Iba1 and CD86 were incubated with Iba1 primary overnight at 4°C and incubated in Alexa Fluor ® 555 secondary (A31572, Invitrogen, 1:500) at 37°C. Then, sections were incubated in CD86 primary overnight at 4°C and in Alexa Fluor secondary ® 448 donkey anti-mouse (A21202, 1:500, Abcam). All sections were mounted with Fluoro-Gel (Electron Microscopy Sciences, Hartfield, PA) and imaged at 10x using a BZ-X800 Fluorescence Microscope (Keyence, Itasca, IL). For each animal, the most preserved hippocampus was imaged by stitching together multiple 10x images to visualize the coverage of Iba1 or GFAP staining over the entire hippocampus using ImageJ software (US National Institutes of Health, Bethesda, MD). ImageJ was also used to quantify the area of each hippocampus section. To quantify the number of GFAP-positive cells, 2 images at 20x were sampled from the dentate gyrus, CA3, and CA1 regions for each section. Two blinded researchers manually counted GFAP-positive cell bodies in each image and an average of both counts was used in the analysis.

Gene expression

Fresh-frozen forebrains were sectioned until bregma -1.80 and punched medial to lateral with a blunt 15-guage needle to collect dorsal hippocampal tissue. Tissue was homogenized and RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA yield and purity was assessed using a Nanodrop spectrophotometer (Invitrogen). cDNA was prepared from the extracted RNA using the SSIV cDNA kit (Thermo Fisher, Waltham, MA) and stored at -80°C. RT-PCR was used to assess hippocampal expression of IR, IRS1, and Akt1 genes; and GAPDH reference gene using the $2^{-\Delta\Delta Ct}$ method. Primers were designed using PrimerQuest (Integrated DNA Technologies, Coralville, IA) and verified target specificity with PrimerBLAST (National Center for Biotechnology Information). RT-PCR was run with SYBR Green PCR master mix (Thermo Fisher) and run on a StepOnePlus real-time PCR system (Thermo Fisher).

Gene	FWD	REV
IR	5’ GCCCAACCATCTGTAAGT 3’	5’ GCCAATCCTGGAAGTGATAG 3’
IRS1	5’ GCCAATCTTCATCCAGTTGC 3’	5’ CAT CGTGAAGAAGGCATAGG 3’
Akt1	5’ TGTGCAAGGAGGGTATCA 3’	5’ TCTCATGGTCCTGGTTGT 3’
GAPDH	5’ GAGCATCTCCCTCACAATTC 3’	5’ GGGTGCAGCGAACTTTAT 3’

Table 4.1. Primer sequences used for PCR.

Statistical analysis

Except for microbiota analysis, Prism 8 (GraphPad Software Inc., La Jolla, CA) was used for statistical analysis. Two-way ANOVA with multiple comparisons or, for datasets with missing values, a mixed effects model, with Fisher’s LSD post hoc analysis was used to analyze NOR, water maze, gene expression, and immunohistochemistry results. ITT was analyzed with a 2-way ANOVA and area under the curve (AUC) was analyzed by T-test, and CCK was analyzed by paired T-test. Significance was considered as a $p<0.05$. Data are presented as mean ± standard error of the mean (SEM).

Results

Microbiota transfer

Taxonomic differences in bacterial abundances in the donor animals were similar to those found previously in the literature, with Chow animals exhibiting increased *Bacteroidetes* and HF increased *Firmicutes* [16]. Chow animals further had increased *Lactobacillus*, a probiotic [222]. Compared to Chow donors, HF animals had increased *Clostridia* families *Ruminococcaceae* and *Streptococcaceae*, as well as increased genus *Anaerotignum*, under the family *Ruminococcaceae*, seen in previous work [15, 16].

After antibiotic depletion, 3 days of colonization, and 2 booster gavages, unfortunately, microbiota transfer did not result in specific taxa recapitulated in the recipient animals. The resulting conventional biotas were unlike either donor biota, though Cconv and Hconv were different from each other.

Body weight

Each group steadily gained weight over the course of the experiment (Figure 4.3. Time factor: $F_{1.292,36.06}=818.9$, $p<0.0001$). HF animals gained significantly more weight compared to Chow and recipient animals.

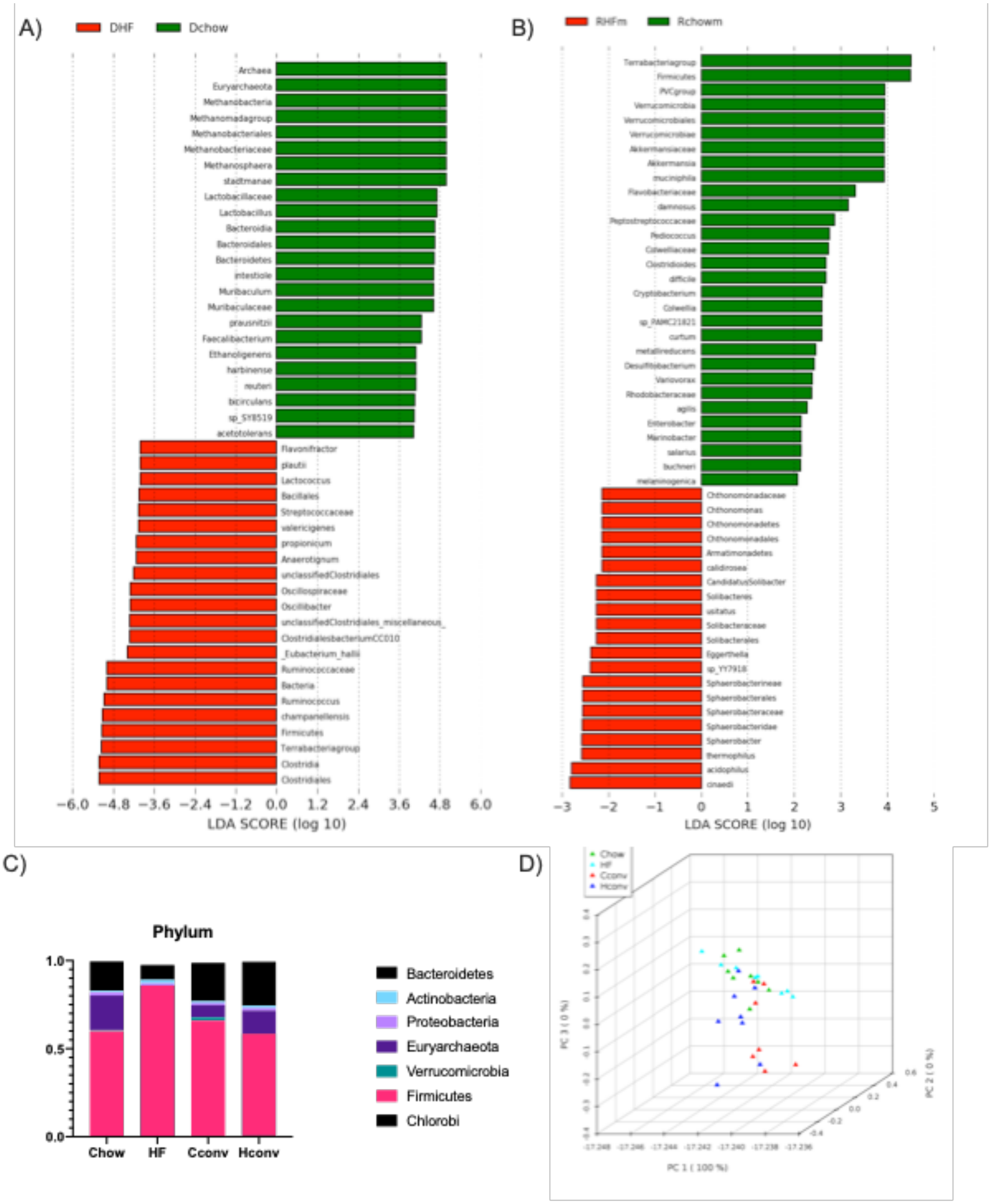

Figure 4.2. Microbiota analysis of the donor and recipient cohorts. Galaxy LeFSE results depicting the taxa significantly differing between (A) Chow and HF groups at sacrifice (LDA = 4.0) and (B) Cconv and Hconv groups before behavior testing (LDA = 2.0). (C) Relative abundances of the major phyla present in each group's microbiota. (D) 3D principal component analysis of all taxa in each sample. Chow, HF, and Hconv n = 8; Cconv n = 7.

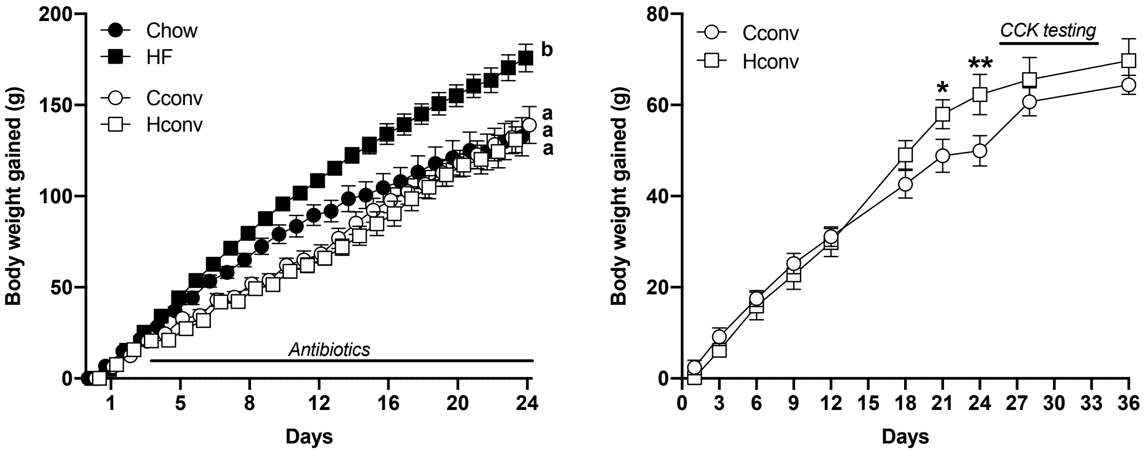

Figure 4.3. Body weight gain over time, in grams; n=8 per group. Data are presented as mean ± SEM. Groups that are statistically different have different letters. * $p<0.05$, ** $p<0.01$

CCK test

We studied vagally-mediated satiety by measuring food intake after i.p. injection of CCK. Cconv rats consumed significantly less food after 30 minutes when injected with CCK compared to control, demonstrating intact gut-brain signaling (paired T-test, $t_7=3.26$, $p<0.05$); while Hconv animals did not significantly reduce their intake following CCK injection ($t_7=0.93$, ns).

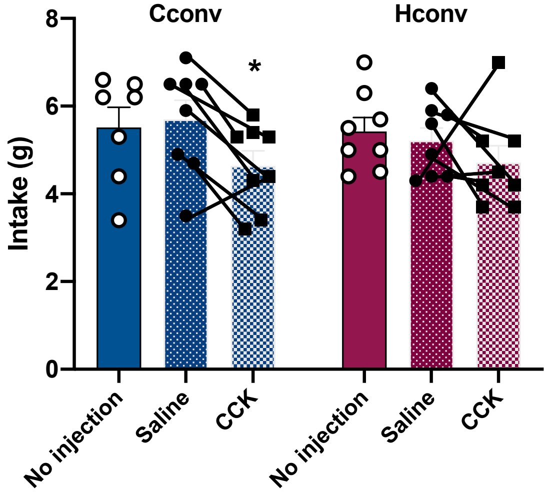

Figure 4.4. Overall food consumption, in grams, after CCK injection; n=8 per group. Data are presented as mean ± SEM. * $p<0.05$, ** $p<0.01$

ITT

Donor HF animals had significantly increased blood glucose at baseline and 15 minutes after insulin injection (Figure 5. $p<0.05$). However, calculated AUC was not different between groups (t=1.296, df=84, p=0.199). There were no differences between Cconv and Hconv animals at any timepoint or in calculated AUC (t=0.6718, df=97, p=0.503).

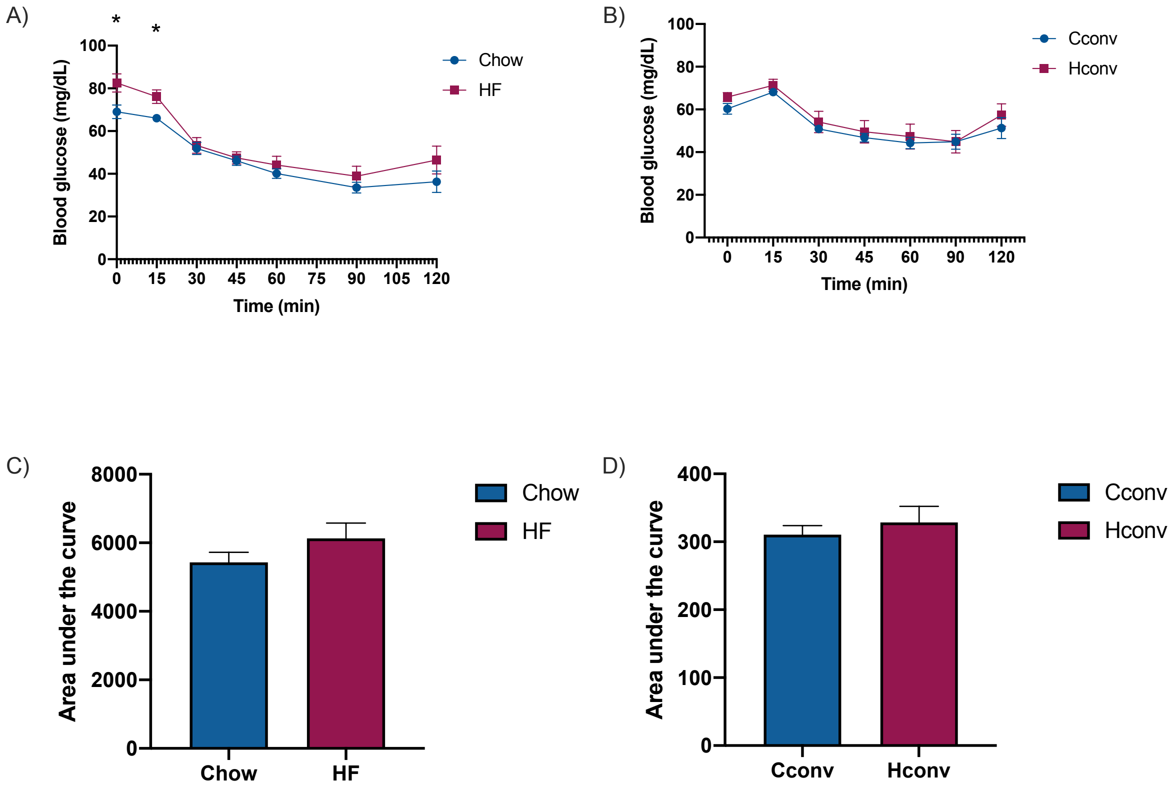

Figure 4.5. Insulin tolerance test (ITT) results. (A) Donor response to ITT test and (C) AUC; (B) Recipient ITT response and (D) AUC. Chow and HF n=7; Cconv and Hconv n=8. Data are presented as mean ± SEM. * $p<0.05$

Behavior testing

NOR

In the NOR test, microbiota was a significant factor (Figure 4.6. Two-way ANOVA; biota factor: $F_{1,24}=7.760$, p=0.010; cohort factor and interaction ns). Chow-fed donors' discrimination index was significantly higher than HF-fed ($p<0.05$), indicating that Chow rats spent more time investigating the new object. Cconv rats tended to have a higher discrimination index compared to Hconv (p=0.088), similar to the donor cohort.

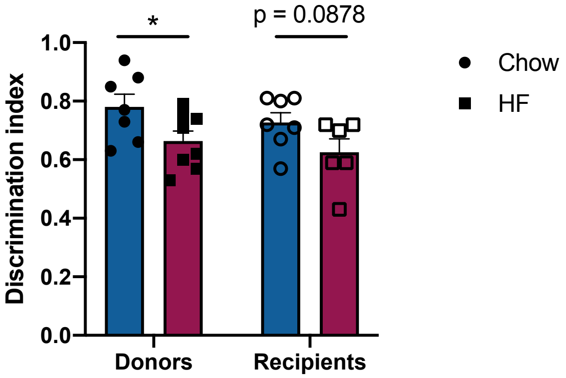

Figure 4.6. Novel object recognition test results. Chow are closed circles, HF are closed squares, Cconv are open circles, and Hconv are open squares. Discrimination index denotes how much time the animals spent investigating the familiar vs. the new place/object, where a value >0.5 means more time was spent on the new place/object. HF n=8, Chow and Cconv n=7, Hconv n=6. Data are presented as mean ± SEM. * p<0.05

Water maze

During the acquisition phase of the water maze, all animals learned the location of the platform, taking less time over the 4-day period to escape the pool (Figure 4.7. Control time factor: $F_{3,161}$=54.90, p<0.0001; recipient time factor: $F_{2.849,182.4}$=48.85, p<0.0001). Control chow and HF-fed rats performed significantly differently, with the chow animals escaping sooner (diet factor: $F_{1,58}$=4.468, p<0.05; interaction: $F_{3,161}$=2.416, p=0.0684), spending less time in the outer thigmotaxis zone (time factor: $F_{2.246,91.33}$=49.53, p<0.0001; diet factor: $F_{1,57}$=2.607, ns; interaction: $F_{3,122}$=3.493, p<0.05), and tending to spend more time in the target quadrant (time factor: $F_{2.4,119.2}$=34.47, p<0.0001; diet factor: $F_{1,58}$=3.949, p=0.0516; interaction ns). Cconv rats similarly performed better in the acquisition period, learning to find the platform faster (biota factor ns; interaction: $F_{3,192}$=5.98 p<0.001) and spending less time in thigmotaxis (time factor: $F_{2.53,121.4}$=37.44, p<0.0001; biota factor ns; interaction: $F_{3,144}$=5.743, p=0.001), though there was no difference between Cconv and Hconv in the time spent in the target quadrant (time factor: $F_{2.609,154}=4.935$, $p<0.01$; biota factor ns; interaction ns).

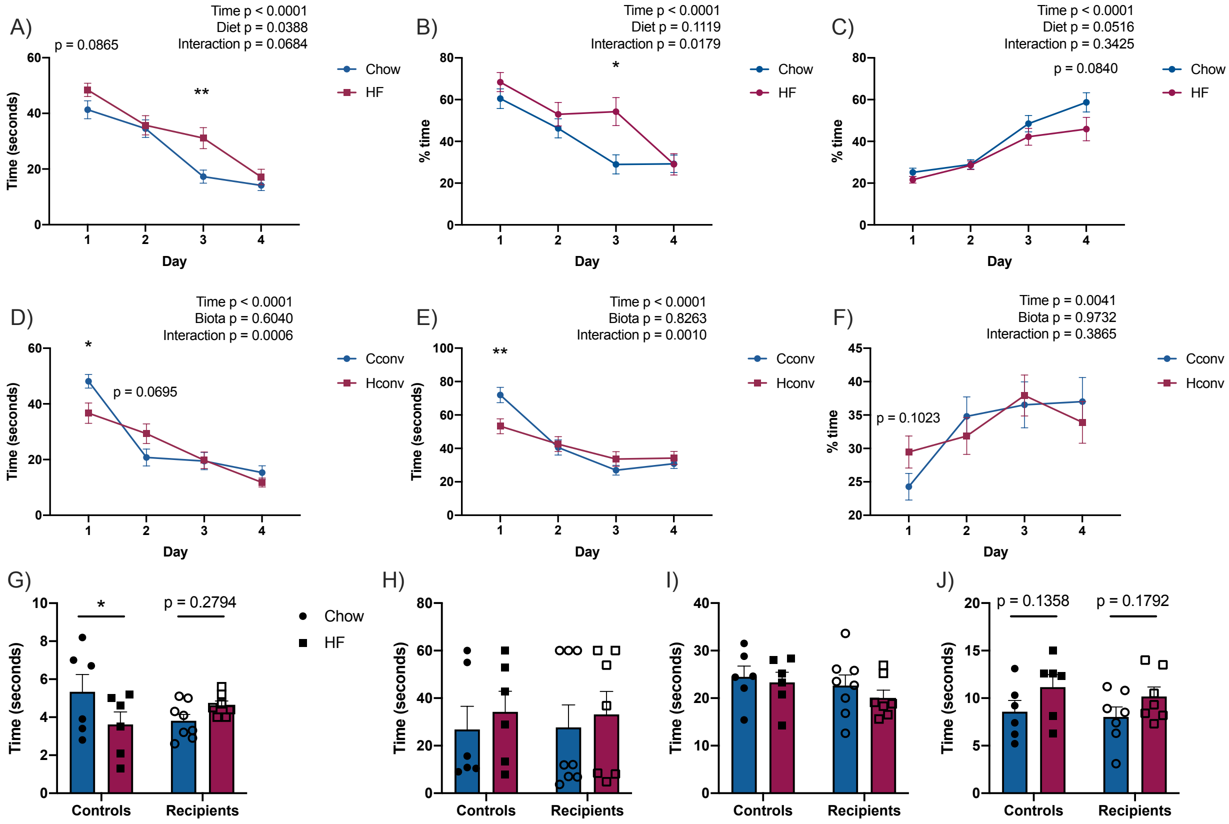

Figure 4.7. Water maze performance during the (A-F) acquisition and (G-J) probe periods. Escape latency for (A) controls and (D) recipients; percent of time spent in thigmotaxis for (B) controls and (E) recipients; and percent of time spent in the target quadrant for (C) controls and (F) recipients. Data were analyzed by 2-way ANOVA with multiple comparisons to measure differences between individual days and overall learning. For the probe trial, (G) represents the latency to the target quadrant, (H) the latency to the platform zone, (I) the time spent in target quadrant, and (J) time spent moving away from the platform. (A-C) Chow are blue circles and HF are red squares. (D-F) Cconv are blue circles and Hconv are red squares. (G-J) Chow are closed circles, HF are closed squares, Cconv are open circles, and Hconv are open squares. Chow and HF n=6; Cconv n=8, Hconv n=7. Data are presented as mean ± SEM. * $p<0.05$, ** $p<0.01$

During the probe trial, there was no consistent difference in memory performance among any of the groups. Unexpectedly, control HF rats took less time to enter the target quadrant during the trial (Figure 4.7. $p<0.05$), though there was no significant difference in the recipients.

There were no differences in latency to the platform zone (Chow vs. HF p=6.07; Cconv vs. Hconv p=0.674) or in the time spent in the target quadrant (Chow vs. HF p=0.716; Cconv vs. Hconv p=0.373). However, there was a small trended increase in time spent moving away from the platform in HF (p=0.136) and Hconv (p=0.179) animals, and diet/microbiota factor was significant ($F_{1,22}$=4.321, $p<0.05$).

Activation of microglia and astrocytes

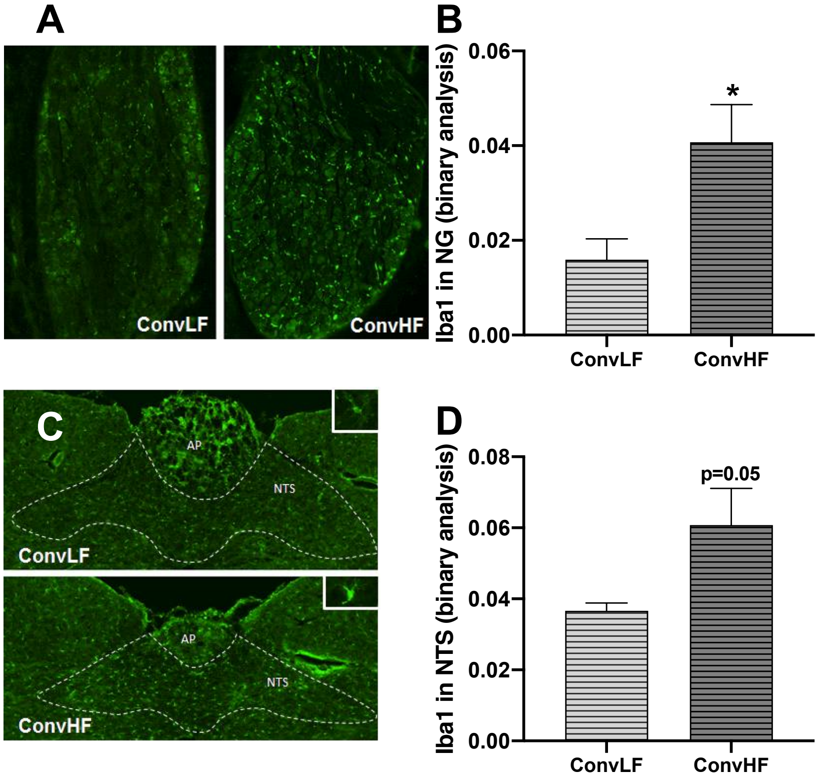

Figure 4.8. Iba1 stain in the NG and NTS. Figure taken from Kim et al., *Physiology & Behavior,* 2020. (A) Representative images of Iba1 staining in the nodose ganglion (NG) (A) and nucleus of the solitary tract (NTS) (C) at 20X magnification. (B) The area of the NG covered with Iba1 stain. (D) The area of the NTS stained. Insert images are 200X magnification. ConvLF rats received microbiota from rats fed a low-fat diet and ConvHF rats recived microbiota from rats fed a HF diet. Data are presented as mean ± SEM. * p<0.05

In a previous study, using the same antibiotic depletion paradigm, we found that colonization with HF-type microbiota results in increased microglial recruitment along the gut-brain axis, namely the nodose ganglion (NG) and nucleus of the solitary tract (NTS) depicted in

Figure 4.8 [15]. This measure of inflammation appears to further carry up into the hippocampus, as Hconv animals nearly recapitulated ($p=0.067$) the increased Iba1 staining in the hippocampus compared to Cconv rats seen in the donor animals (Figure 4.9. $p<0.05$). There was a significant relationship between hippocampus Iba1 coverage and cohort ($F_{1,23}=5.598$, $p<0.05$), as well as diet/microbiota ($F_{1,23}=8.554$, $p<0.01$). Hippocampal area was also significantly smaller in HF animals compared to Chows ($p<0.01$) and tended to be smaller than Cconv ($p=0.071$) and Hconv ($p=0.073$) hippocampi. There was no significant difference between Cconv and Hconv ($p=0.879$).

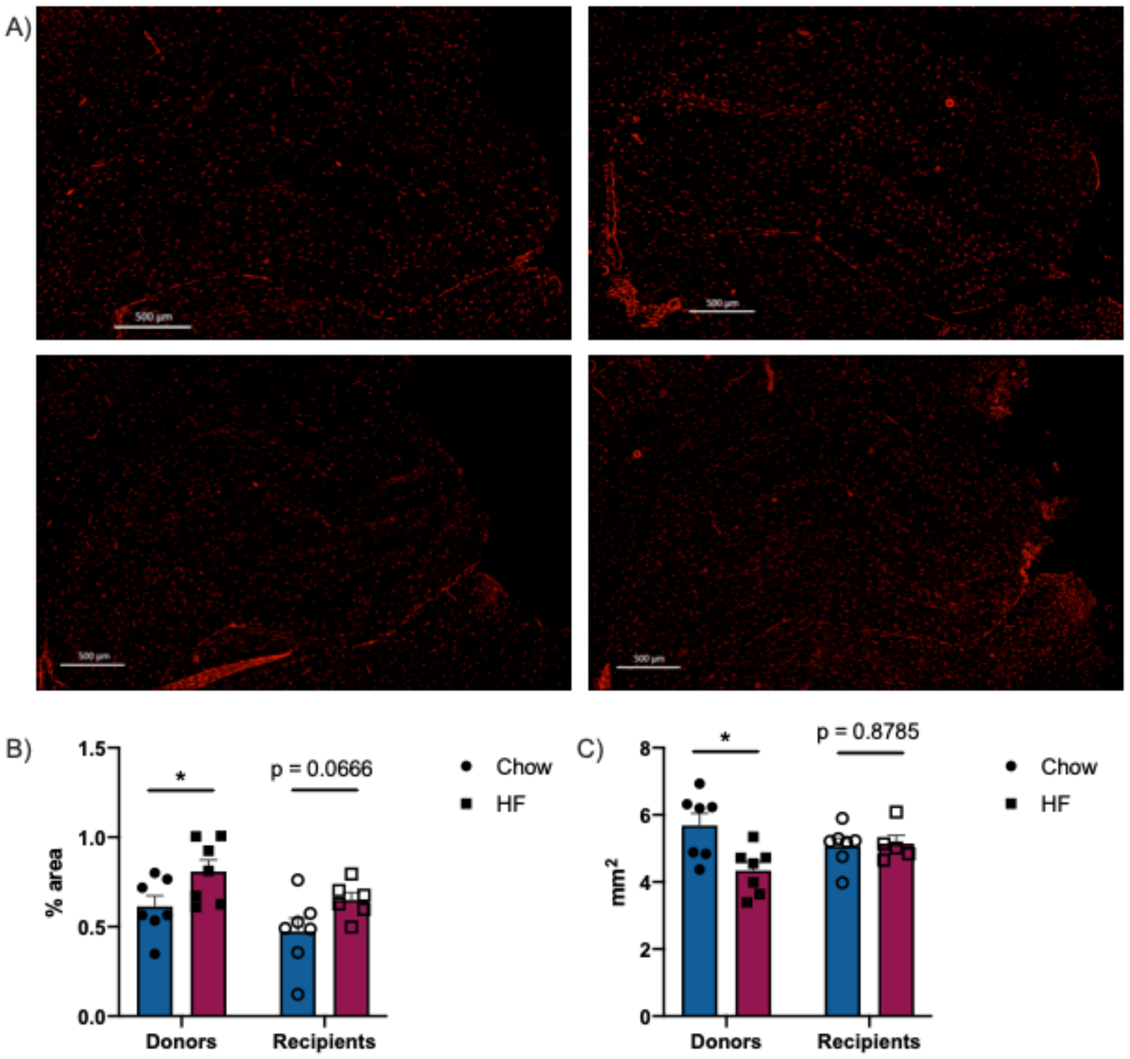

Figure 4.9. (A) Representative images of Iba1 staining for Chow (top-left), Cconv (top-right), HF (bottom-left), and Hconv (bottom-right) groups at 10X magnification. (B) The percent of the dorsal hippocampal section covered with Iba1 stain. (C) The average area of the dorsal hippocampus per group in mm^2. Chow are closed circles, HF are closed squares, Cconv are open circles, and Hconv are open squares. Data are presented as mean ± SEM. * $p<0.05$

There was no significant difference in coverage of GFAP staining among the groups, and no relationship between coverage and cohort of diet/microbiota was significant (Figure 4.10). When GFAP$^+$ cells were manually counted, there were no significant differences seen among groups when all regions were averaged or when considered by region (data not shown).

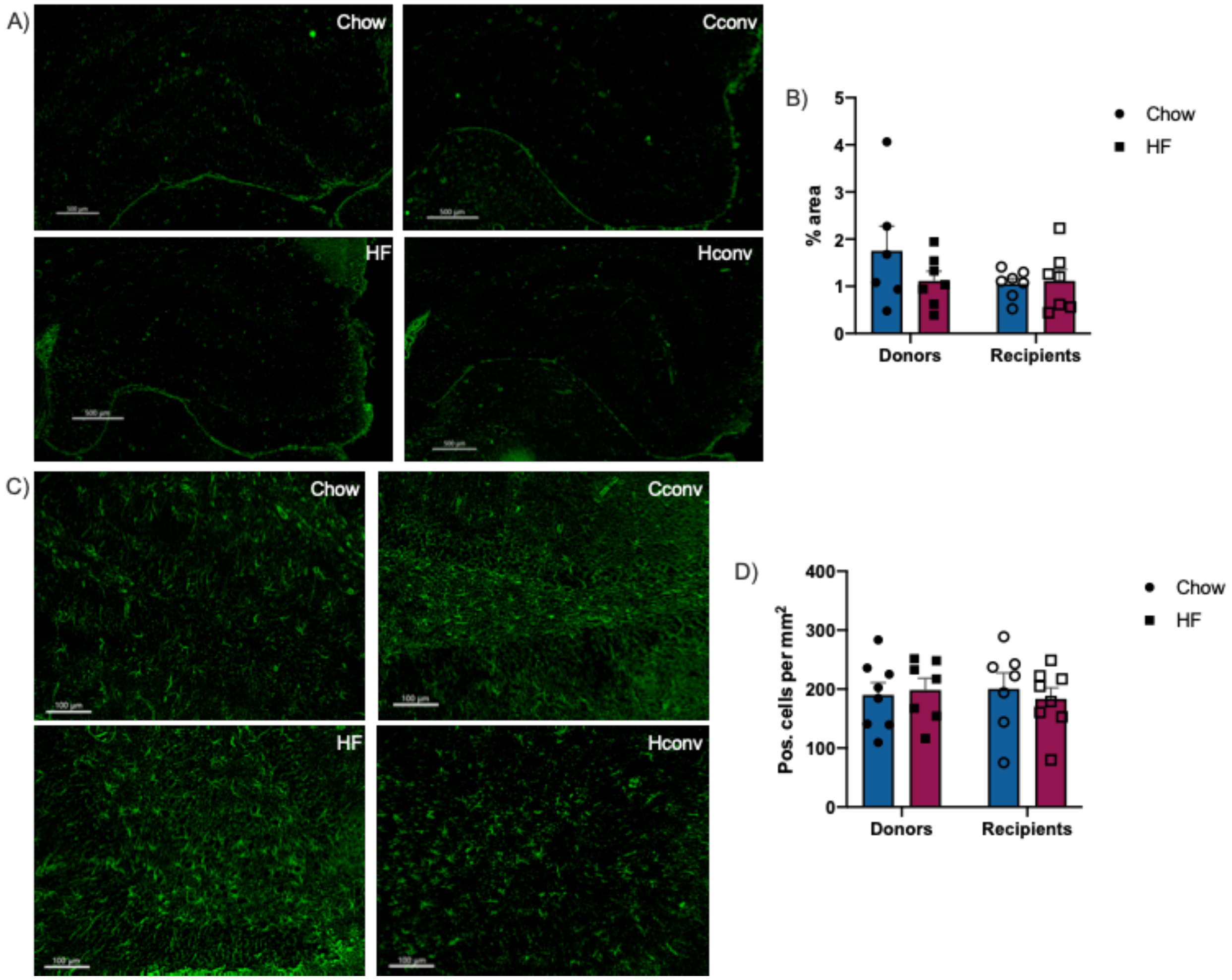

Figure 4.10. (A) Representative images of GFAP staining at (A) 10X and (C) 20X magnification. (B) The percent of the dorsal hippocampal section covered with GFAP stain. (D) The number of positive cells per mm^2 calculated by sampling and cell counting 2-20X images per region of the dorsal hippocampus (DG, CA3, and CA1). Chow are closed circles, HF are closed squares, Cconv are open circles, and Hconv are open squares. Data are presented as mean ± SEM.

Hippocampal insulin signaling gene expression

We analyzed hippocampal gene expression of IR, Atk1, and IRS1 to determine whether HF diet-associated microbiota caused alterations in insulin signaling gene expression. We found that Hconv had significantly greater IR expression than Cconv ($p<0.01$), and this result trended in the donor animals ($p=0.094$). There was a significant relationship between diet/microbiota and IR expression ($F_{1,23}=12.95$, $p<0.01$). There were no significant differences between groups in

IRS1 expression. Further, there were no significant differences between groups in Akt1 expression, though Cconv animals tended to have increased expression compared to Hconv animals (p=0.090).

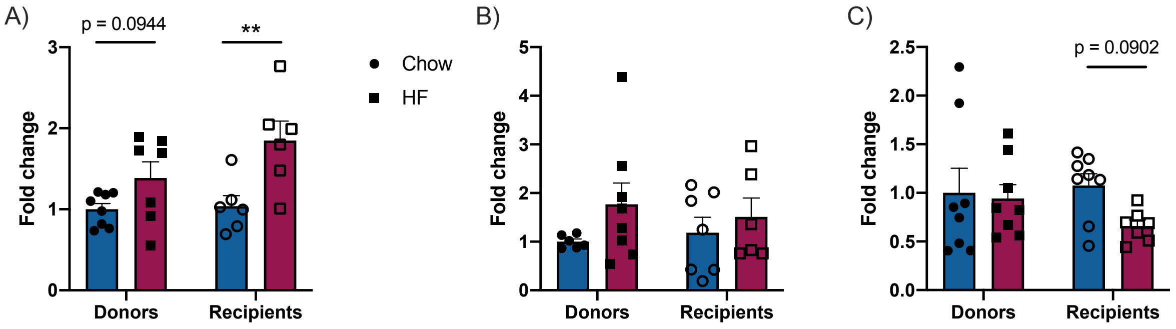

Figure 4.11. Fold change of (A) IR, (B) IRS1, and (C) Akt1 in the dorsal hippocampus. Chow are closed circles, HF are closed squares, Cconv are open circles, and Hconv are open squares. Data are presented as mean ± SEM. ** $p<0.01$

Discussion

While HF-feeding successfully drove significant differences in microbiota abundance in certain aspects similar to those seen previously [15, 16], these differences were not passed down to recipient animals. The resulting conventionalized microbiotas were different from each other, as the Cconv animals exhibited increased *Firmicutes* and *Akkermansia muciniphila*, and the Hconv animals increased *Chthonomonadacea*, *Candidatus Solibacter*, and *Sphaerobacter thermophilus*. There were significant differences in behavior testing and tissue analysis between recipients, suggesting that exposure to the different microbiotas was sufficient to alter behavior and physiology; though differences seen did not always mimic what was displayed by the respective donor animals. For example, while HF and Hconv animals similarly exhibited decreased performance in the acquisition period of the water maze compared to Chow and Cconv groups, significant increase in hippocampal IR expression was only evident in Hconv, but not HF animals. In many of the measures analyzed, increased sample size might have driven certain patterns that were trending to significance. It is curious, however, that certain measures, such as IR expression, are significant in the recipients, and not the donors. Again, sample size may play a role in this; as well as the fact that donor feces were sequenced but it was cecal contents that were used to colonize the recipients. Further, we did not sequence the slurry that ultimately colonized the recipients, which means that we do not know which taxa from the donors were viable and able to colonize. However, we have previously used this depletion and colonization protocol and found that there were fecal taxa successfully transplanted in recipients [15], though the rats in this study were younger Fishers. It is possible that the recipient rats in our current study were affected by drifting microbiota composition that occurs with age [223]. Another possibility is that exposure to air or increased production of reactive oxygen species by inflammatory Proteobacteria resulted in aerobes increasing in abundance. *Chthonomonas calidirosea* [224]*, Candidatus Solibacter usitatus* [225]*,* and *Sphaerobacter thermophilus* [226] are all aerobes that have increased abundance in Hconv.

As expected, HF feeding resulted in increased weight gain over the course of the experiment [15, 16], while overall there was no significant difference between Chow, Hconv, and Cconv groups [15]. Hconv animals briefly gained more weight than Cconv animals before behavior testing commenced, but rate of gain rejoined for the remainder of the experiment. This may be due to the fasting paradigm used for the CCK test. If HF-type microbiota results in feeding patterns similar to those of HF-fed animals, then food intake in Hconv animals would decrease moreso relative to Cconv, as HF rats tend to consume more food during the light period [227], when the rats were fasted.

HF feeding has been shown to result in decreased sensitivity compared to chow-fed animals [27] and we have successfully induced CCK insensitivity previously in rats by feeding them a HF diet [51]. Here we demonstrate that, along with vagal remodeling and microglia recruitment, HF-type microbiota can by itself induce disruption of gut-brain signaling. This occurred without exposure to HF diet or increased weight gain compared to Cconv animals. This might be due to HF microbiota's inflammatory properties; at least, relative to the Cconv rats. Hconv rat microbiota was characterized by increased abundances of Gram-negative bacteria, which produce the inflammatory product LPS [228]. LPS has been shown to disrupt gut-brain signaling by decreasing sensitivity to leptin, and diminishing its synergistic effect on satiety [13]. Further, Cconv was characterized by increased *Akkermansia muciniphila,* a commensal bacteria known for its contributions to maintaining the mucus layer of the gut epithelium [229]. It is possible that this prevented any LPS translocation and thus preserved gut-brain signaling in Cconv rats.

HF animals had increased blood glucose at baseline and 15 minutes after insulin injection, demonstrating a slight delay for insulin action. AUC was not significant. There was no significant difference in ITT performance between Cconv and Hconv. We have previously shown that HFD-induced signs of insulin resistance in the oral glucose tolerance test can be improved by modulation of the microbiota by blueberry supplementation [230] and both fasting insulin and insulin release in response to glucose tolerance test have been shown to be partially rescued by probiotic supplementation [65], so we hypothesized that HFD-associated microbiota transfer would be sufficient to induce peripheral insulin insensitivity; this was not the case. This may be due to the fact that Hconv animals were not significantly heavier than Cconv rats. A study in which microbiota transfer from lean to diabetic mice resulted in improved insulin resistance, though this change was also accompanied by a decrease in body weight relative to control diabetic mice [231].

HF animals had significantly lower discrimination indices on the NOR task compared to Chow, and Hconv trended having lower indices than Cconv ($p=0.0878$). Further, HF animals overall learned significantly slower than Chow when observing escape latency (time: $p<0.0001$, diet: $p<0.05$, interaction: $p=0.0684$) and percent of time spent in the outer thigmotaxis zone (interaction: $p<0.05$); and trended slower learning in terms of percent of time spent in the target quadrant (time: $p<0.0001$, diet: $p=0.0516$). Similarly, Hconv animals learned more slowly than Cconv animals in terms of escape latency (interaction: $p<0.001$) and percent time spent in thigmotaxis (interaction: $p=0.0010$). More time spent in thigmotaxis is an indication of the rat using non-spatial strategies, and in adult mice it has been shown that over the acquisition period animals tend to turn from non-spatial to spatial search strategies to solve the maze [232, 233], showing that peripheral cues have been successfully integrated [234]. There was no consistent pattern as to performance on the retention probe test. Chow donors took significantly longer to enter the target quadrant during the probe and would suggest poorer memory than HF, though this difference is only 1.7 seconds in magnitude. The remaining measures were nonsignificant, though both HF and Hconv animals tended to spend more time swimming away from the platform zone, the incorrect direction (HF: $p=0.1358$; Hconv: $p=0.1792$). Interestingly, one study found that acute neuroinflammation achieved by acute systemic LPS administration in rats resulted in impaired performance on NOR, but not context object discrimination or water maze probe trial [235]. They suggested that LPS impairs hippocampal-dependent pattern separation, which is not required on NOR and probe tasks. These data resemble our own results and suggest that, while microbiota composition in recipients was not permanently altered, LPS-producers may have transiently increased and resulted in increased systemic LPS, sufficient to induce cognitive deficiency. In fact, *Helicobacter cinaedi* [236], *Chthonomonadales calidirosea* [224] and *Candidatus Solibacter usitatus* [225], bacteria with increased abundance in Hconv, are Gram-negative.

Increased Iba1 stain coverage was found in the dorsal hippocampus of HF animals compared to Chow ($p<0.05$), and Hconv animals tended to have more staining than Cconv ($p=0.0666$). Again, increased sample size might have shifted this trend to significance. Microbiota transfer or colonization of germ-free rats has been shown to increase microglial recruitment along the gut-brain axis as shown in a previous study [15] and it has been found that transplant from DIO mice results in increased recruitment in the cortex [205]. We demonstrate that microbiota-induced neuroinflammation in the form of microglial recruitment also appears in the hippocampus. This alteration in physiology may also be due to increased LPS, as mice injected peripherally with LPS exhibit microglia recruitment [202] and impaired cognition [203]. GFAP staining, however, was nonsignificant in terms of both percent coverage and when counting GFAP^{+} cells. Astrogliosis was not induced by HFD in this study. Incidentally we found that the area of the dorsal hippocampus was significantly decreased in HF but not Hconv animals, logical as obesity is associated with decreased hippocampal size [123]. Hippocampal atrophy has also been associated with vascular disease and conditions that are associated with increased cardiovascular risk, such as diabetes; mental illness, such as depression; and physical trauma (for review, see [237]). Fittingly, Donor HF rats displayed increased fasting blood glucose relative to Chow, a measure that was associated with hippocampal atrophy in the female grey mouse lemur [238]. It appears that, in this study, hippocampal atrophy occurred due to obesity and/or the beginnings of blood glucose dysregulation as opposed to inflammation.

Duration of exposure to the HF microbiota may also not have been long enough to produce size changes in the hippocampus, though previous rat studies have demonstrated that 4 weeks of exposure to stress is sufficient to decrease hippocampus size [239, 240].

Finally, of the three insulin signaling genes analyzed, only IR trended in a pattern similar between the donor and receiver animals. Cconv animals had significantly less expression in the dorsal hippocampus compared to Hconv, and this trended in the donors (p=0.0944). Current data regarding diet-induced alterations in hippocampal insulin signaling markers are inconsistent. Out of four studies, all of male Wistars, one found no change in IR or IRS1 expression [241]; another found that HFD increased IRS1 expression [242]; a third found IR increased and no change in IRS1 and Akt1 [243]; and a fourth found IR and IRS1 increased and no change in Akt1 [244] due to HF diet. Protein expression of insulin signaling markers are also inconsistent, though most demonstrate decreases in some proteins analyzed in response to HF diet [177, 245-247]. Specifically, in rats, one study found that levels of phosphorylated IR remained unchanged, while phosphorylated IRS1 and Akt were decreased after HF feeding [177]. Another study found that when bathing hippocampal slices in insulin, the resulting IR, IRS1, and Akt phosphorylation were significantly decreased after 12 weeks of HF feeding [247]. Increased IR expression, as found here, might suggest a compensatory increase in expression due to onset of central insulin resistance, given that protein expression of IR has been shown to be unchanged or decreased in HF animals. Impaired activation of the insulin receptor may interfere with insulin's modulation of plasticity [184, 185], and thus have contributed to the learning impairment exhibited by HF and Hconv animals in aspects of the water maze task. It is interesting that Hconv rats did not display peripheral insulin resistance and yet have increased expression of IR. Neuroinflammation may be the culprit here as well – in rats with 6 days of LPS administration, insulin-induced phosphorylation of IRS1 and Akt in the hypothalamus was impaired compared to a single LPS dose [89]. The increase in Gram negative bacteria and decrease in gut barrier protecting *Akkermansia muciniphila* may have resulted in increased circulating LPS, which results in neuroinflammation [168] that moves up along the vagus and into the brain [15] and disrupts insulin signaling.

Conclusions

Microbiota transfer is sufficient to induce microglial recruitment [15, 205], central insulin resistance [217], and impairment on hippocampal-dependent tasks [205]. In this study, we demonstrate that impermanent transfer of HF-associated microbiota is sufficient to alter non-spatial memory-related behavior performance (NOR and the spatial learning portion of the water maze), cause microglial recruitment, and increase IR gene expression in the hippocampus. These changes may be due to increased LPS, as systemic LPS administration is linked to microglial activation [202] and impaired cognitive performance [203] and, while no specific taxa were similar between donors and recipients, Hconv did have increased abundances of Gram-negative, aerobic bacteria. Incomplete microbiota transfer was unable to induce peripheral insulin resistance, and CCK insensitivity was transferred from Chow to Cconv, but not HF to Hconv.

CHAPTER 5

CONCLUSIONS

The aim of this thesis was to explore the mechanisms through which gut microbiota influences food intake and hippocampal-dependent cognition. In Chapter 2, we discussed the pathways microbiota may act through to affect energy homeostasis. Changes in microbiota composition induced by high-fat (HF) feeding or associated with an obese state are linked to increased peripheral and central inflammation, which impacts sensitivity of vagal afferents to gut satiety peptides, specifically CCK, and therefore food intake. Microbial products such as short-chain fatty acids modulate release of GLP-1 and PYY, other satiety peptides. Further, HF-type microbiota is sufficient to alter the structure of the gut-brain axis, reducing vagal innervation to the brainstem, which may alter signaling and food intake. Centrally, unfavorable microbial alterations cause hypothalamic inflammation which contributes to altered food intake; and changes in dopamine neurochemistry, which might lead to altered food preferences and addictive-like behaviors in rodents. Microbiota-associated neuroinflammation in the form of gliosis and alterations in insulin signaling have further been identified as mechanisms by which gut microbiota may influence cognition, which were explored in Chapter 3.

In Chapter 4, we demonstrated that colonization with a HF-type microbiota, in the absence of HF feeding and significant differences in body weight, results in altered performance on novel object recognition and Morris water maze tasks. This was accompanied by significantly increased microgliosis along the vagus and at the level of the nucleus of the solitary tract, and a trending increase in the hippocampus. We also found an increase in insulin receptor expression in the hippocampus. We further showed that exposure to HF-type microbiota was sufficient to alter vagal function, as demonstrated by decreased CCK sensitivity in animals colonized with a HF-type microbiota (Hconv). We hypothesize that these changes are due to increased production of LPS, perhaps only transient, following colonization with a HF type microbiota.

These data contribute to the understanding of how microbiota shapes our behaviors, including food intake and hippocampal-dependent cognitive function. Targeting neuroinflammation and LPS production may prove useful as the scientific community searches for interventions to care for people with obesity and its associated complications.

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