Title
Vagal interoception of microbial metabolites from the small intestinal lumen

Author
Jameson, Kelly

Publication Date
2023

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Vagal interoception of microbial metabolites from the small intestinal lumen

A dissertation submitted in partial satisfaction of the requirements

for the degree Doctor of Philosophy in Neuroscience

by

Kelly Jameson

2023

ABSTRACT OF THE DISSERTATION

Vagal interoception of microbial metabolites from the small intestinal lumen

by

Kelly Jameson

Doctor of Philosophy in Neuroscience

University of California, Los Angeles, 2023

Professor Elaine Yih-Nien Hsiao, Chair

The vagus nerve is proposed to enable communication between the gut microbiome and brain, but activity-based evidence is lacking. Herein, we assess the extent of microbial influences on vagal activity and metabolite signaling mechanisms involved. We find that mice reared without microbiota (germ-free, GF) exhibit decreased vagal afferent tone relative to conventionally colonized mice (specific pathogen-free, SPF), which is reversed by colonization with SPF microbiota. Perfusing non-absorbable antibiotics (ABX) into the duodenum of SPF mice, but not GF mice, acutely decreases vagal activity, which is restored upon re-perfusion with bulk lumenal contents or sterile filtrates from the small intestine and cecum of SPF, but not GF, mice. Of several candidates identified by metabolomic profiling, microbiome- and diet-dependent short-chain fatty acids, bile acids, and 3-indoxyl sulfate stimulate vagal activity with varied response kinetics, which is blocked by co-perfusion of pharmacological antagonists of FFAR2, TGR5, and TRPA1, respectively, into the small intestine. At the single-unit level, duodenal serial perfusion of each metabolite class elicits more singly responsive neurons than dually responsive neurons, suggesting distinct neuronal detection of different microbiome- and macronutrient- dependent metabolites. Finally, microbial metabolite-induced increases in vagal activity correspond with

activation of neurons in the nucleus of the solitary tract in the corresponding receptor-dependent manner. Results from this study reveal that the gut microbiome regulates select lumenal metabolites that differentially activate chemosensory vagal afferent neurons, thereby enabling microbial signaling across the gut-brain axis.

The dissertation of Kelly Jameson is approved.

Elissa A. Hallem

Emeran Mayer

Felix Erich Schweizer

Elaine Yih-Nien Hsiao, Committee Chair

University of California, Los Angeles

2023

For my family and friends whose unwavering support made this work possible.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

I would first like to offer my immense gratitude and appreciation for the mentorship I have received from Dr. Elaine Hsiao. As one of the first handful of graduate students to enter the lab, I have had the distinct pleasure of watching it grow, and have grown myself personally in ways that I could not have thought possible due to your guidance, support, and faith in my abilities. You gave me the inspiration to tackle novel experiments in the lab, and supported me throughout the ups and downs of seemingly endless optimization. You also allotted me copious amounts of opportunity to grow outside of the lab through peer review, writing, and leadership opportunities- so for all of that, I thank you for being an amazing mentor throughout this journey. I would also like to thank my previous mentors who inspired me to get this degree in the first place, and showed me the love I have for research; Dr. Andrew Bean and Dr. Adron Harris. I would also like to thank the members of my thesis committee; Dr. Elissa Hallem, Dr. Emeran Mayer for all of their invaluable input and feedback regarding sensory neuroscience and clinical perspectives surrounding this work, respectively. I would also like to thank my third thesis committee member, Dr. Felix Schweizer, for adopting me into his lab during the first few years and helping immensely to get all of the electrophysiological experiments off the ground. Your guidance and feedback throughout this process has made this work possible, quite literally.

I would like to acknowledge my lab mates past and present for their support, camaraderie, and collaboration during my doctoral studies- I could not have done this without y'all! Dr. Geoff Pronovost, Dr. Helen Vuong, Dr. Thomas Fung, Dr. Christine Olson, Alonso Iniguez, Dr. Ping Fang, Dr. Jon Lynch, Jorge Paramo, Kristie Yu, Sabeen Kazmi, Elena Coley, Dr. Gregory Lum, Dr. Gulistan Agirman, Dr. Ezgi Ozcan, Lewis Yu, Celine Son, Michael Quicho, Hannah Espey, Julissa Alvarado, Arlene Lopez-Romero. I also cannot express my gratitude enough for my two amazing undergraduate mentees who I have missed very much this past year while running the

last of my IHC: Donya Mazdeyasnan and Emma Leshan. I would also like to acknowledge my collaborators from the Masmanidis Lab, Dr. Long Yang.

I would like to acknowledge the animals who gave their lives for the advancement of this work.

Lastly, I would like to thank my friends and family for standing by my side through this journey, despite having to hear every single iteration of my elevator pitch in the process. To my parents, Gretchen Jameson and Peter Jameson, thank you for always inspiring me to forge my own path, and support me in the pursuit of my passion every step of the way. I am so thankful for all of the love and guidance you have provided me with throughout not only my life but also particularly during this time as a graduate student. To MFLOD, Penguins, Charcuteries, my Anderson Crew, Natalie Falkner, and Matthew Didero- Thank you for your endless support and ability to make life as a graduate student relatively balanced, haha. I love each and every one of you and cannot thank you enough for your friendships that made completing this degree possible.

VITA

EDUCATION

The University of California Los Angeles, Los Angeles, CA — July 2016 – Present
Neuroscience Interdisciplinary Ph.D.

- GPA: 3.8

The University of Texas at Austin, Austin, TX
May 2015
Bachelor of Science in Neuroscience (Concentration in Psychology)

- GPA: 3.67
- University Honors: 2011, 2012, 2013, 2014

HONORS AND AWARDS

Keystone Symposia Future of Science Fund Scholarship — November 2021

NIH Ruth L. Kirschstein Predoctoral Individual National Research Service Award (F31) — June 2020

Committee Chair, UCLA Neurodegenerative Diseases Symposium — April 2021

Chan-Zuckerberg Initiative Community Project Award — April 2019

UCLA Cellular and Molecular Biology Training Grant (T32) — May 2018

UCLA Hyde Fellowship — May 2018

College of Natural Sciences Excellence in Neuroscience Research Award — April 2015

University of Texas Health Science Center Graduate School for Biomedical Sciences Summer Research Program Awardee — June 2013

RESEARCH AND WORK EXPERIENCES

E.Y. Hsiao Lab, University of California, Los Angeles — September 2016-September 2023
Graduate Student Researcher

R.A. Harris Lab, University of Texas at Austin — August 2014 – December 2015
Undergraduate Research Assistant

A. Bean Lab, University of Texas Heath Science Center at Houston — May 2014 – August 2014
Summer Research Program Participant

R. Poldrack Lab, University of Texas at Austin — July 2013 – May 2014
Undergraduate Research Assistant

PUBLICATIONS

Jameson KG, Kazmi SA, Son C, Mazdeyasnan D, Leshan E, Vuong HE, Paramo J, Romero-Lopez A, Yang L, Schweizer FE, Hsiao EY. Vagal interoception of microbial metabolites from the small intestinal lumen. Submitted, *Neuron.*

Olson CA, Iñiguez AJ, Yang GE, Fang P, Pronovost GN, Jameson KG, Rendon TK, Paramo J, Barlow JT, Ismagilov RF, Hsiao EY. Alterations in the gut microbiota contribute to cognitive impairment induced by the ketogenic diet and hypoxia. *Cell Host Microbe*. (2021). 29(9), 1378-1392, PMID: 34358434; PMCID: PMC8429275.

Fang P, Kazmi SA, Jameson KG, Hsiao EY. The Microbiome as a Modifier of Neurodegenerative Disease Risk. *Cell Host Microbe*, (2020). 28(2), 201-222. PMID: 32791113; PMCID: PMC7430034.

Jameson, K.G., Olson, C.A., Kazmi, S.A., Hsiao, E.Y. (2020). Toward Understanding Microbiome-Neuronal Signaling. *Mol Cell*, (2020). 78(4), 577-583. PMID: 32275853

Jameson, K. G., & Hsiao, E. Y. (2019). A novel pathway for microbial metabolism of levodopa. *Nat Med, 25*(8), 1195-1197. PMID: 31388180 ; PMCID: PMC7004239.

Jameson, K. G., & Hsiao, E. Y. (2018). Linking the Gut Microbiota to a Brain Neurotransmitter. *Trends Neurosci, 41*(7), 413-414. PMID:29933773 ; PMCID: PMC7004240

Truitt JM, Blednov YA, Benavidez JM, Black M, Ponomareva O, Law J, Merriman M, Horani S, Jameson K, Lasek AW, Harris RA, Mayfield RD. (2016). Inhibition of IKKbeta Reduces Ethanol Consumption in C57BL/6J Mice. *eNeuro, 3*(5). PMID: 27822501; PMCID: PMC4901333.

Warden A, Truitt J, Merriman M, Ponomareva O, Jameson K, Ferguson LB, Mayfield RD, Harris RA. (2016). Localization of PPAR isotypes in the adult mouse and human brain. *Sci Rep, 6*, 27618. PMID: 27283430; PMCID: PMC5086799

PRESENTATIONS

Jameson, KG, Kazmi SA , Mazdeyasnan D , Leshan E , Vuong HE , Paramo J , Yang L , Masmanidis SC, Schweizer FE, Hsiao EY. Dissecting influences of the gut microbiome on vagal neuronal activity. Keystone Symposium: Gut-Brain Axis. March 2022, Banff, AB Canada.

Jameson K, Truitt JM, Merriman M, Horani S, Warden A, Ponomareva O, Harris RA, Mayfield RD. Brain region and cell type-specific expression profile of IKKb in C57BL/6J mice. 38th Annual Research Society on Alcoholism Meeting. June 2015, San Antonio, TX.

Jameson K, Truitt JM, Merriman M, Horani S, Warden S, Ponomareva O, Harris RA, Mayfield RD. IKK isoform expression in brain regions associated with alcohol dependence. College of Natural Sciences Undergraduate Research Forum. April 2015, Austin, TX.

Jameson K, Truitt JM, Merriman M, Horani S, Warden A, Ponomareva O, Harris RA, Mayfield RD. Brain region and cell type-specific expression profile of IKKb in C57BL/6J mice. 3rd Annual Waggoner Center for Alcohol and Addiction Research Advance. March 2015, Austin, TX.

Chapter 1: Overview

My dissertation research has been primarily focused on elucidating the effects of the microbiota and associated metabolites on vagal afferent neuronal activity along the microbiota-gut-brain axis. In particular, my dissertation work seeks to improve our understanding of the following questions: (i) How does the microbiota modify vagal afferent nerve physiology? (ii) Does the microbiome acutely regulate vagal afferent fiber activity? (iii) What is the landscape of *lumenal* microbially-modulated metabolites in the small intestinal and cecal lumens? (iv) Which classes of lumenal microbial metabolites activate vagal afferent neurons, and through which receptor-dependent pathways? (v) Do distinct classes of microbiota- and macronutrient- derived metabolites work through shared or distinct subpopulations of vagal neurons? (vi) Does receptor-dependent signaling from microbial metabolites in the small intestinal lumen alter central nervous system (CNS) activity?

The impetus for my graduate work stems from a need to more deeply probe the precise mechanisms underlying microbiota-dependent effects on CNS physiology and associated behaviors that have been reported to be vagally-mediated. The gut microbiota is an important regulator of brain function and behavior, with the capacity to affect neurogenesis, blood brain barrier integrity, microglial activation, and symptoms of neurological disease. Despite this evidence supporting a “microbiome-gut-brain axis”, the molecular and cellular pathways underlying communication between intestinal microbes, associated metabolites, and the brain remain unclear. It is hypothesized that the gut microbiota signals directly to the brain via the vagus nerve, which contains afferent and efferent fibers that innervate the intestinal epithelium and extend into the brainstem. However, direct support for this signaling pathway until recently has stemmed largely from studies wherein vagotomy abrogates behavioral alterations in response to microbiota perturbation in mice, thus only indicating *whether* vagal signaling is important, not *how*. Based on these fundamental gaps in knowledge, my dissertation work has

therefore aimed to elucidate the precise cell-type specific mechanisms underlying vagally-mediated interoception along the microbiota-gut-brain axis.

In **Chapter 2**, I present a version of the commentary titled, "Linking the Gut Microbiota to a Brain Neurotransmitter," from *Trends in Neuroscience.* In this work we discuss examples of foundational work in the field that contributed to our collective understanding of microbial influences on brain physiology, and associated sexual dimorphisms. This work has been published as:

Jameson, K. G., & Hsiao, E. Y. (2018). Linking the Gut Microbiota to a Brain Neurotransmitter. *Trends Neurosci, 41*(7), 413-414. PMID:29933773 ; PMCID: PMC7004240

In **Chapter 3**, I present a version of the perspective piece titled, "Toward Understanding Microbiome-Neuronal Signaling," from *Molecular Cell*. In this work we sought to review the existing work contributing to our understanding of microbiota-neuron crosstalk in health and disease. In particular, we focus on microbial regulation of short-chain fatty acids, neurotransmitters, uncharacterized biochemicals, and derivatives of neuromodularory drugs. Moreover, we discuss the cumulative state of the field and highlight important limitations in technology and gaps in understanding that should be focused on moving forward in order to foster scientific advancement. This work is published as:

Jameson, K.G., Olson, C.A., Kazmi, S.A., Hsiao, E.Y. (2020). Toward Understanding Microbiome-Neuronal Signaling. *Mol Cell*, (2020). 78(4), 577-583. PMID: 32275853

In **Chapter 4**, I present a version of the literature review titled, "The Microbiome as a Modifier of Neurodegenerative Disease Risk," published in *Cell Host & Microbe*. In this work we discuss roles for the microbiome in aging and neurodegenerative diseases (NDs), as well as summarize findings from human studies on microbiome alterations in Parkinson's Disease, Alzheimer's disease, amyotrophic lateral sclerosis, and Huntington's disease. Moreover, we discuss environmental and genetic animal studies as models for NDs, as well as evaluate the

role of immunological, neuronal, and metabolic mechanisms for how the gut microbiota may modulate risk for NDs. This work is published as:

Fang P, Kazmi SA, **Jameson KG,** Hsiao EY. The Microbiome as a Modifier of Neurodegenerative Disease Risk. *Cell Host Microbe*, (2020). 28(2), 201-222. PMID: 32791113; PMCID: PMC7430034.

In **Chapter 5**, I describe recent work where we aim to assess the extent of microbial influences on vagal activity and metabolite signaling mechanisms involved. In these studies we first sought to examine the role for the microbiota in modulating vagal afferent nerve physiology under homeostatic conditions. In order to address this question we performed baseline whole-nerve vagal electrophysiological recordings *in vivo* in specific-pathogen free (SPF) mice harboring a complex microbiota, germ-free (GF) mice reared without a microbiota, antibiotic-treated (ABX) mice wherein the microbiota was depleted in adulthood via oral gavage of antibiotics, and conventionalized (CONV) mice reared GF and then re-colonized with donor SPF fecal contents in adulthood. Interestingly, we found that GF mice exhibit decreased vagal afferent nerve tone relative to SPF mice, which is reversed by colonization. We then sought to determine whether or not these effects could be recapitulated acutely. In order to address this question we performed *in vivo* whole-nerve vagal electrophysiology in SPF mice with concurrent lumenal perfusion of various stimuli into the small intestine (SI). We found that vagal afferent nerve activity decreases with lumenal perfusion of non-absorbable antibiotics, and is restored upon re-perfusion with bulk lumenal contents or sterile filtrates from the small intestine and cecum of SPF, but not GF, mice. We then sought to elucidate the particular microbially-modulated small molecules present in such samples that may mediate our observed effects. Therefore, we performed global untargeted metabolomics on SI and cecal contents from SPF, GF, ABX and CONV mice. We identified hundreds of microbially-modulated metabolites in the SI and cecal lumen. We then cross-referenced these metabolites with existing vagal single-cell RNA sequencing data to identify candidates to screen with known receptors on vagal neurons.

Of several candidates identified, microbiome- and diet-dependent short-chain fatty acids, bile acids, and 3-Indoxyl sulfate were found to activate vagal afferents with varied response kinetics. Moreover, we found that these effects are ablated by co-perfusion of pharmacological antagonists of FFAR2, TGR5, and TRPA1, respectively, into the small intestine. We then investigated whether or not there were differences at the single-unit level between vagal neuronal responsivity to each metabolite class. In order to do so we performed intravital calcium imaging on vagal nodose neuronal cell bodies expressing the calcium indicator GCaMP6s, alongside lumenal perfusion of microbial metabolites. We found that serial duodenal-ileal perfusion of each metabolite class activates more singly responsive neurons than dually responsive neurons, suggesting distinct neuronal detection of different microbiome- and macronutrient-dependent metabolites. Finally, we sought to determine whether or not our identified signaling pathways result in alterations in CNS neuronal activity. We thus performed immunofluorescence experiments in order to detect changes in the immediate-early gene cFos in neurons within the nucleus of the solitary tract (NTS), where vagal afferents terminate. We reveal that indeed, microbial metabolite-induced increases in vagal activity correspond with activation of neurons in the nucleus of the solitary tract in the corresponding receptor-dependent manner.

Chapter 2: Linking the Gut Microbiota to a Brain Neurotransmitter

Abstract

The past decade has yielded substantial evidence that the gut microbiome modulates brain function, including for instance behaviors relevant to anxiety and depression, pointing to a need to identify the biological pathways involved. In 2013 Clarke and colleagues reported that the early-life microbiome regulates the hippocampal serotonergic system in a sex-dependent manner, findings that opened up numerous lines of inquiry on the effects of the microbiome on neurodevelopment and behavior.

1. Linking the Gut Microbiota to a Brain Neurotransmitter

In the early 2000s evidence was mounting for a link between the gut microbiome and stress-related behaviors. Based on longstanding associations of psychological stress with gastrointestinal disorders, Bailey and Coe [1] revealed that early-life stress alters the composition of the gut microbiota in rhesus monkeys and that these changes in the microbiota correlate with anxiety-related behaviors and serum levels of stress hormones. Sudo et al. [2] further demonstrated that mice raised devoid of microbial colonization (germ-free mice) exhibit elevated stress hormone responses and decreased hippocampal levels of brain-derived neurotrophic factor (BDNF), a protein important for neurogenesis, compared to conventionally colonized controls. These phenotypes were reversed by mono-colonizing germ-free mice with select gut bacteria, revealing a causal role for the gut microbiota in regulating stress responses. In subsequent years several investigators, including Neufeld et al. [3], Diaz Heijtz et al. [4], Nishino et al. [5], and Davis et al. [6], extended these studies by examining various models including germ-free or antibiotictreated mice, rats, and zebrafish to demonstrate that changes in the gut microbiome alter stress-related behaviors across different organisms. To date, anxiety-

associated changes in exploratory drive and risk avoidance have been the most frequently studied host behaviors in microbiota–gut–brain research.

While early work on microbial modulation of anxiety-related behaviors had revealed changes in stress hormones and brain BDNF levels, it seemed likely that additional neurotransmitter systems or modulators may underlie microbial alterations in host behavior, and insights into such pathways were at the time lacking. In 2009, a metabolomics study by Wikoff et al. [7] revealed deficiencies in serum serotonin and elevated serum tryptophan in germ-free mice compared to conventional controls. This was later corroborated by additional research indicating that the gut microbiota regulates peripheral serotonin levels (Sjögren et al. [8], Yano et al. [9]). Serotonin is a hormone and 4 excitatory neurotransmitter that is produced in large quantities in the gastrointestinal tract, but is most prominently known for its central contributions to anxiety and depression. Whether microbial effects on the peripheral serotonergic system were similarly seen in the central serotonergic system had not been explored.

The study by Clarke et al. pioneered the efforts to examine effects of the microbiome on the brain serotonergic system [10]. It found that germ-free mice display increased levels of hippocampal serotonin, hippocampal 5-hydroxyindoleacetic acid (5-HIAA, the main catabolic product of serotonin), plasma tryptophan, and decreased tryptophan metabolism to kynurenine, as measured by HPLC. This was correlated with decreased expression of hippocampal BDNF. These latter findings corroborated previous studies [4,7] by replicating germ-free increases in peripheral tryptophan and decreases in hippocampal BDNF expression, respectively.

In addition to revealing a link between the gut microbiota and brain serotonin, the Clarke et al. study also highlighted the importance of considering sex in microbiota–gut–brain studies. At the time many studies involving the microbiota examined consequences on the immune response

particularly in female animals, leaving sexually dimorphic effects largely unconsidered. Prior reports of microbial effects on stress response and behavior similarly tended to be performed in animal cohorts of only one sex. Interestingly, in the Clarke et al. study the microbiota-mediated changes in hippocampal serotonin, 5-HIAA, BDNF, and plasma tryptophan were observed only in male mice [10]. This consideration aligned with existing evidence that regulation of the brain serotonergic system is sexually dimorphic (Llorente et al. [11]) and contributed to growing efforts to examine sex as a biological variable. Today, studies continue to probe for sex-specific effects of the microbiome on the host. Notably, Thion et al. [12] recently demonstrated that the absence of the microbiome 5 has a sexually dimorphic impact both prenatally and postnatally on microglia function and maturation in mice.

The Clarke et al. study [10] strengthened the notion that microbial effects on the brain may be dependent on critical periods of host development. To determine whether the neurochemical changes seen in germ-free mice could be corrected postnatally, the researchers colonized adult mice with a conventional microbiome (also referred to as conventionalization). This conventionalization corrected abnormalities in plasma levels of tryptophan and tryptophan metabolism through the kynurenine pathway, but had no effect on any of the observed hippocampal neurochemical phenotypes. This was consistent with prior work by Sudo et al. [2] which revealed that postnatal conventionalization of germ-free mice in adulthood fails to correct abnormalities in stress responses. Surprisingly, the altered anxiety-like behavior observed in germ-free mice was ameliorated by microbiota colonization despite no effects on brain serotonergic phenotypes. These results suggested that alterations in the brain serotonin system could be decoupled from altered stressinduced behavior as well as from peripheral serotonin metabolism. The work of Clarke et al. [10] contributed to numerous lines of inquiry surrounding the role of the microbiome during neurodevelopment. Studies have since elucidated roles for the

early-life microbiome in hippocampal neurogenesis, microglial maturation, and neuronal myelination [13].

The several early behavioral studies conducted in microbiome-depleted animals have positioned the field to begin to consider not only whether the microbiome has effects on neurodevelopment and behavior but also, importantly, how. Advances in integrating functional genomic and metabolomic data with mechanistic studies in animal models have allowed interrogation of how select bacterial species and microbe-derived products affect 6 distinct immune, endocrine, and neuronal pathways [13]. In addition, these studies have motivated the examination of microbiota–gut–brain interactions in systems more relevant to the human condition. In particular, Clarke et al. wrote: 'The current study is limited by the common difficulty all germ-free studies have in directly translating the results to the clinical situation where no equivalent obliteration of the microbiota can be said to exist' [10]. Approaches involving the transplantation of human-derived microbiomes into germfree mice have recently allowed researchers to directly investigate the effects of human disease-associated microbes on host neurophysiology and behavior [14,15]. Together, these developments better position the field to face the current challenge of dissecting the biological circuits that enable bidirectional communication between the gut microbiome and the brain. Although a formidable endeavor, detailed investigation along this line of inquiry will continue to push the field toward a better understanding of how our microbial symbionts influence human biology.

References:

Bailey, M.T. and Coe, C.L. (1999) Maternal separation disrupts the integrity of the intestinal microflora in infant rhesus monkeys. Dev Psychobiol 35, 146–155

Sudo, N. et al. (2004) Postnatal microbial colonization programs the hypothalamicpituitary-adrenal system for stress response in mice. J Physiol 558, 263–275

Neufeld, K.M. et al. (2011) Reduced anxiety-like behavior and central neurochemical change in germ-free mice. Neurogastroenterol Motil 23, 255–264

4 Diaz Heijtz, R. et al. (2011) Normal gut microbiota modulates brain development and behavior. Proc Natl Acad Sci U S A 108, 3047–3052

5 Nishino, R. et al. (2013) Commensal microbiota modulate murine behaviors in a strictly contamination-free environment confirmed by culture-based methods. Neurogastroenterol Motil 25, 521–528

Davis, D.J. et al. (2016) Microbial modulation of behavior and stress responses in zebrafish larvae. Behav Brain Res 311, 219–227

Wikoff, W.R. et al. (2009) Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. Proc Natl Acad Sci U S A 106, 3698– 3703

8 Sjögren, K. et al. (2012) The gut microbiota regulates bone mass in mice. J Bone Miner Res 27, 1357–13567

Yano, J.M. et al. (2015) Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. Cell 161, 264–276

Clarke, G. et al. (2013) The microbiome–gut–brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. Mol Psychiatry 18, 666–73

Llorente, R. et al. (2010) Sex-dependent maternal deprivation effects on brain monoamine content in adolescent rats. Neurosci Lett 479, 112–117 8

Thion, M.S. et al. (2018) Microbiome influences prenatal and adult microglia in a sex-specific manner. Cell 172, 500–516

Vuong, H.E. et al. (2017) The microbiome and host behavior. Annu Rev Neurosci 40, 21–49

Sampson, T.R. et al. (2016) Gut microbiota regulate motor deficits and neuroinflammation in a model of Parkinson's disease. Cell 167, 1469–1480

Zheng, P. et al. (2016) Gut microbiome remodeling induces depressive-like behaviors through a pathway mediated by the host's metabolism. Mol Psychiatry 21, 786–796

Chapter 3: Toward Understanding Microbiome-Neuronal Signaling

Abstract

Host-associated microbiomes are emerging as important modifiers of brain activity and behavior. Metabolic, immune, and neuronal pathways are proposed to mediate communication across the so-called microbiota-gut-brain axis. However, strong mechanistic evidence, especially for direct signaling between microbes and sensory neurons, is lacking. Here, we discuss microbial regulation of short-chain fatty acids, neurotransmitters, as-yet-uncharacterized biochemicals, and derivatives of neuromodulatory drugs as important areas for assessing microbial interactions with the nervous system.

1. Introduction

We have co-evolved with trillions of indigenous microorganisms that comprise the human microbiota. Over the past decade, the notion that the microbiome is a key regulator of host physiology and behavior has skyrocketed with the advancement of multi-omics technologies, gnotobiotic tools, intersectional genetics, and live imaging. Early studies linking alterations in the gut microbiome with neurobehavioral phenotypes launched the concept of a microbiota-gut-brain axis whereby intestinal microbes influence brain and behavior through immune, neuronal, and metabolic pathways. In particular, emerging evidence suggests that select members of the microbiota have the ability to synthesize and/or regulate various neurochemicals known to modulate neurotransmission as well as a vast milieu of other metabolites that may directly or indirectly impact neuronal activity. As such, the role of mutualistic microbes in regulating sensory neuronal communication along the gut-brain axis is of active scientific interest. Microbial modulation of dietary molecules, neurotransmitters, as-yet-uncharacterized metabolites, and neurological drugs represent major areas for research toward uncovering mechanisms for microbial modulation of neuronal activity (Figure 1).

2. Microbial Regulation of Short-Chain Fatty Acids

Early studies on feeding behavior, led by such pioneers as Claude Bernard and Ivan Pavlov, laid the foundation for the concept of a gut-brain axis through dietary modulation (Leulier et al., 2017). With the advent of germ-free rodent models in the 1920s (Gustafsson, 1946-1947), gut microbes were identified as important mediators of dietary metabolism and host nutrition. Germ-free animals exhibited substantially deficient levels of the short-chain fatty acids (SCFAs) butyrate, propionate, acetate, and valerate in the intestine and blood, indicating a crucial role for the microbiota in regulating local and systemic SCFA bioavailability in the host (Høverstad and Midtvedt, 1986). Continued research in this area has uncovered molecular mechanisms underlying microbial production of SCFAs through their fermentation of complex polysaccharides, propelled by the discovery and characterization of polysaccharide utilization loci present in *Bacteroidetes* (Bjursell et al., 2006).

A wealth of evidence has further demonstrated that alterations in the microbiota and SCFAs are associated with conditions in which food intake behaviors are dysregulated (Byrne et al., 2015). In particular, alterations in the gut microbiota are seen in obese mice and humans, which correlate with alterations in the levels of acetate and butyrate (Ridaura et al., 2013, Turnbaugh et al., 2006). Propionate administration to patients with obesity enhanced gut hormone secretion while reducing adiposity and overall weight gain (Chambers et al., 2015). While some of the animal studies highlight microbial regulation of appetite as a basis for the observed differences in weight gain, exactly how microbial regulation of SCFAs impacts host feeding behaviors remains unclear. The SCFA free fatty acid receptors 2 and 3 (FFAR2 and FFAR3, respectively) are expressed in the enteric nervous system and the portal nerve, as well as various sensory ganglia (De Vadder et al., 2014, Egerod et al., 2018), suggesting a role for activation of the nervous system in mediating these effects. Consistent with this, propionate feeding

induces *fos* expression in the dorsal vagal complex of the brainstem, the hypothalamus, and the spinal cord (De Vadder et al., 2014), raising the question of whether SCFA-induced stimulation of peripheral sensory neuronal activity could mediate the effects of SCFAs on host feeding behavior.

As the list of host behaviors that are modified by the gut microbiome continues to grow (Vuong et al., 2017), a key open question is the extent to which the microbial regulation of molecules relating broadly to nutrition underlies the reported effects of the microbiome on complex host behaviors, spanning homeostatic feeding and social, stress-related, and cognitive domains. SCFAs are fundamental molecules involved in regulating energy homeostasis, and SCFA receptors are expressed by a wide variety of non-neuronal cell subtypes as well. In immune cells, for example, SCFAs can regulate T regulatory cell differentiation (Arpaia et al., 2013, Furusawa et al., 2013, Smith et al., 2013) and microglial maturation (Erny et al., 2017), whereas in enteroendocrine cells, SCFAs can stimulate the release of gut hormones (Larraufie et al., 2018). In addition to promoting SCFAs, the gut microbiota is integral to secondary metabolism of bile acids, another class of diet-related metabolites for which cognate receptors are expressed by various cell types, including subsets of sensory neurons, to regulate diverse host phenotypes (Mertens et al., 2017). In light of their pleiotropic effects, studies that dissect the precise signaling pathways by which SCFAs and bile acids alter host behaviors are warranted. Efforts to determine the functional roles of specific neuronal pathways in SCFA and bile acid signaling would be particularly illuminating toward uncovering roles for the microbiota in regulating neuronal activity via dietary metabolism.

3. **Microbial Regulation of Neurochemicals**

While the gut microbiota may affect host behavior through the regulation of dietary metabolites, like SCFAs and bile acids, emerging research indicates that select gut microbes also regulate levels of host neurotransmitters. The finding that microbes can synthesize neurotransmitters is rooted in the first discovery of chemical transmitters by Sir Henry Dale in the early 1900s (Valenstein, 2002). In studying ergot on wheat rye, he discovered the transmitter acetylcholine over a decade before it was extracted from mammalian tissue. Together with George Barger, Dale found that acetylcholine mimicked the effects of parasympathetic nerve stimulation, suggesting chemically mediated neurotransmission. It was realized later that the acetylcholine itself was likely derived from *Bacillus* contaminants in the ergot rather than from the ergot itself. Since this landmark discovery, additional neurotransmitters, including norepinephrine (NE), serotonin (5-HT), γ-aminobutyric acid (GABA), and dopamine (DA) have been found to be produced by bacteria in culture and to be regulated by the microbiota in animals (Strandwitz, 2018). Despite these tantalizing associations, all kingdoms of life produce the amino-acid derivatives that form common "neurotransmitters," raising the questions: What are the functional roles of neurotransmitters in microbes, and can host-associated microbes impact the nervous system through neurotransmitter modulation?

As yet, only a few studies have examined the effects of canonical neurotransmitters on bacterial physiology. One relatively early series of studies revealed that the catecholamines NE and epinephrine exhibit a structural similarity to the quorum-sensing molecule autoinducer-3 and that, therefore, each stimulates enterohemorrhagic *E. coli* motility and virulence (Clarke et al., 2006). Researchers hypothesize that this direct effect of NE and epinephrine on bacterial pathogenesis may contribute to the ability of stress to increase susceptibility to infection. More recently, a study utilizing *in vitro* co-culture screens and metagenomic datasets revealed GABA-producing versus GABA-consuming bacteria from the human gut microbiota (Strandwitz et al.,

2019). In particular, GABA synthesized by *Bacteroides fragilis* supported the growth of KLE1738, suggesting that select neurotransmitters may serve as growth substrates for bacteria. A separate study found that 5-HT promotes intestinal colonization of the bacterium *Turicibacter sanguinis*, similarly suggesting a role for a neurotransmitter in promoting microbial fitness (Fung et al., 2019). Beyond these initial findings, little is known regarding the extent of neurotransmitter modulation across various members of the gut microbiota, the specific microbial genes and gene products used for their synthesis and catabolism, and the molecular pathways underlying microbial sensing and response to neurotransmitters. Integrated microbiological, biochemical, and bioinformatic approaches are needed to support *in silico* predictions informed by multi-omic datasets, *in vitro* determination of microbial gene and protein function, and *in vivo* investigation of microbial community responses. Identifying the molecular underpinnings for microbial synthesis, transformation, and physiological response to neurotransmitters would further enable mechanistic interrogation of the potential consequences of microbiota-dependent neurotransmitter modulation on host physiology.

Despite evidence that select host-associated bacteria regulate neurotransmitter levels locally in the intestine and, in some cases, systemically in the blood or distantly in the brain itself, whether microbial modulation of neurotransmitters actually influences neuronal activity and behavior remains poorly understood. In mice, the gut microbiota is responsible for promoting the biosynthesis of up to 60% of colonic and blood 5-HT levels by enterochromaffin cells (ECs) in the intestinal epithelium (Yano et al., 2015). In the intestine, microbially modulated 5-HT activates intrinsic afferent primary neurons of the myenteric plexus to promote gastrointestinal motility, but whether extrinsic intestinally innervating nerves are also affected remains unknown. Separate studies suggest that subsets of ECs may synapse with 5-HT-receptive afferent fibers of chemosensory vagal or dorsal root neurons (Bellono et al., 2017, Bohórquez et al., 2015), suggesting a direct path for microbial regulation of local 5-HT to impact the central nervous

system. While evidence for microbiome-gut-sensory neuronal signaling is currently lacking, a growing number of studies reporting effects of the microbiome on host behavior have applied subdiaphragmatic vagotomy to demonstrate that severely impaired vagal signaling abrogates microbial effects on behavior (Bravo et al., 2011, Sgritta et al., 2019). Additional studies that circumvent the confounds of vagotomy and that carefully examine functional neuronal responses to microbially modulated neurochemicals are needed to evaluate the potential for microbes to directly affect neural activity. These efforts would be aided greatly by the development of synthetic biological tools to identify, regulate, and manipulate microbial genes for neurochemical modulation, coupled with host gnotobiotic and intersectional genetic tools for selective microbial colonization and targeted neurophysiological assessments. In addition to evaluating sensory neuronal pathways, efforts to examine the humoral transport of microbially modulated neurochemicals or their precursors are warranted. Consistent with this possibility, heavy-isotope-labeled acetate in the colon enters the bloodstream, crosses the blood-brain barrier, elevates hypothalamic acetate, and feeds into GABA neuroglial cycling to increase central GABA production (Frost et al., 2014). Novel tools to selectively label target neuromodulators that are produced or regulated by the microbiota, along with technologies for spatiotemporal tracking in animals, would help enable efforts to evaluate the ability of the microbiota to impact distant sites in the central nervous system.

4. Uncharacterized Microbial Products and the Nervous System

Aside from SCFAs, bile acids, and neurotransmitters, there are likely many additional microbiota-dependent biochemicals that have the potential to interact with neurons. The human microbiota regulates a vast repertoire of metabolites not only in the intestinal lumen but also in the circulating blood and various organ systems of the host. However, the identity, cognate receptors, signaling pathways, and physiological functions of many microbially modulated

metabolites remain poorly understood (Milshteyn et al., 2018). Recent functional metagenomics studies have begun to reveal the scope of bacterial genes for metabolite synthesis and signaling to the host. By screening cosmid metagenomic libraries, researchers identified host-associated bacterial effector genes, which, upon bioassaying the gene products, resulted in the discovery of commendamide, an N-acyl amide capable of activating the host G-protein-coupled receptor (GPCR; also referred to as GPR) G2A (Cohen et al., 2015). Continuing their work on N-acyl amides, the researchers also demonstrated that bacterially produced N-acyl serinol activated the endocannabinoid receptor GPR119A (Cohen et al., 2017). These studies illustrate that functional metagenomics can be a powerful tool to not only discover novel bacterial metabolites but also reveal how bacterial metabolites can affect host physiology through mimicking endogenous GPCR ligands.

Recent studies have begun to identify GPCRs and orphan receptors that are activated by bacterial metabolites *in vitro*. In a screen of supernatants from individually cultured bacteria from the human gut microbiota, receptors for DA, histamine, and 5-HT were highly responsive to soluble bacterial products. Among many additional candidates, bacterially derived phenethylamine and tyramine activated DA receptors, while bacterial production of histamine itself activated histamine receptors (Chen et al., 2019). In addition to these, as-yet-unidentified bacterial products activated a wide range of other neuropeptide and hormone receptors, classically known to be expressed in the nervous system. In another study, fractionated supernatants from a simplified human microbiome consortium were similarly found to robustly activate neurotransmitter GPCRs. In addition to histamine itself, bacterially produced cadaverine, putrescine, and agmatine also activated histamine receptors (Colosimo et al., 2019). Bacterial supernatants containing 9,10-methylenehexadecanoic acid activated brain angiogenesis factor 1, while 12-methyltetradecanoic acid activated neuromedin receptor 1.

Overall, these studies provide proof of concept that select microbial products could activate GPCRs known to be expressed by neurons.

Further research is required to identify specific microbial metabolites that are capable of signaling to neurons and to determine whether they are bioavailable to the host when produced by microbes within complex host-associated communities. While existing studies demonstrate the potential for bacteria to activate select GPCRs, the authentic identities of the bacterial molecules that affect individual receptors remain largely unknown. Additionally, our knowledge as yet relies primarily on bacteria grown in culture, alone or in limited communities, raising the question of whether there are additional molecules left unassayed from microbes that were not cultured and whether the data capture physiologically relevant outputs of complex microbial community interactions. Culture-independent approaches to screen metabolites directly from host biospecimens would greatly aid in this regard. Beyond bacteria from the microbiome, the roles for the mycobiome and virome in altering neuronal activity remain understudied. While sensory nerve fibers in the skin directly sense infectious *Candida albicans* (Kashem et al., 2015), whether non-pathogenic members of the mycobiome influence neuronal activity is poorly understood. Moreover, bacteriophages alter levels of the neurotransmitters tryptamine and tyramine in the gut (Hsu et al., 2019), but whether these alterations ultimately impact neuronal activity is unclear. These studies highlight a need for novel tools to selectively modulate non-bacterial members of the microbiome in order to fully understand the complex role of the entire microbiome in modulating the host nervous system.

While initial evidence suggests that microbes are capable of synthesizing molecules that could directly bind to neuron-relevant GPCRs *in vitro*, additional research is needed to determine whether they bind neuronal GPCRs in host tissues and to further evaluate the physiological consequences of their signaling. Accordingly, greater attention to spatial variations in metabolite

production and receptor activation *in vivo* is warranted. Microbial communities exhibit distinct spatial structures, or “microbiogeographies,” along and across the gastrointestinal tract (Donaldson et al., 2016). In addition, recent single-cell RNA-sequencing studies suggest that there is cellular, and potentially spatial, heterogeneity in the receptor profiles of intestinally innervating dorsal root and vagal neurons (Hockley et al., 2019, Kupari et al., 2019). Advances in technologies for high-throughput *in situ* microbial imaging, metabolite profiling, and GPCR mapping would help to establish the physiological landscape of the intestine to inform functional microbiome-nervous system interactions.

5. Microbial Interactions with Drugs for Neurological Disease

The finding that select microbes can synthesize, modulate, sense, and/or respond to neurochemicals raises the question of whether they would additionally interact with medical drugs that modulate neurotransmission. The gut microbiota encodes a diverse array of enzymes capable of metabolizing pharmacological agents, thus potentially influencing their bioavailability to the host and contributing to the wide range of intra-patient variability in drug efficacy. Early work describing how the process of glucuronidation promotes drug clearance, coupled with the identification of bacterial beta-glucuronidases from gut microbes, have set the foundation for pioneering studies on microbiomes as modulators of xenobiotic metabolism (Wallace et al., 2010). Since then, xenobiotic metabolism by the microbiome has been extended to numerous drugs targeting neurological indications. In sequencing studies of the human microbiota and culture-based screens of bacterial interactions with common medications, many antipsychotics, antidepressants, opioids, and anticholinergic drugs greatly affected bacterial physiology and correlated with alterations in the composition of the gut microbiota (Jackson et al., 2018, Maier et al., 2018, Zimmermann et al., 2019a). While the distinct contribution of the microbiota to the metabolism of drugs can be difficult to quantify

alongside host-derived enzymes carrying out the same metabolic functions, a recent study utilized gnotobiotic, pharmacological, and bacterial genetic approaches to disentangle microbial versus host xenobiotic transformations. By comparing the metabolism of the antiviral drug brivudine in multiple tissues of germ-free mice that vary in a single microbiome-encoded enzyme, researchers were able to generate a pharmacokinetic model to predict the contribution of the microbiota to features of drug metabolism, including oral bioavailability, host drug-metabolizing activity, metabolite absorption, and intestinal transit (Zimmermann et al., 2019b). This modeling approach was further applied to dissect microbiota contributions to the metabolism of the antiviral drug sorivudine and the benzodiazepine clonazepam (Zimmermann et al., 2019b).

Separate studies have utilized biochemical and metagenomic approaches to identify particular bacterial species and novel bacterial enzymes that modulate the metabolism of drugs, including those for neurodegenerative diseases. The mainstay treatment for Parkinson's disease, levodopa (L-dopa), is a natural precursor of DA that, when administered peripherally, is able to cross the blood-brain barrier for local conversion to DA in the brain. However, the gastrointestinal tract is a site of extensive metabolism of the drug, leading to reduced bioavailability and unwanted side effects caused by elevations in peripheral DA. Informed by mechanisms for host metabolism of DA, a recent study identified a novel interspecies pathway for microbial metabolism of L-dopa, whereby *Enterococcus faecalis* decarboxylates L-dopa to DA, which is subsequently dehydroxylated by *Eggerthella lenta* to m-tyramine (Maini Rekdal et al., 2019). Remarkably, the presence of a single-nucleotide polymorphism in the bacterial gene encoding dopamine dehydroxylase was predictive of the capacity for certain patients to metabolize the drug. As such, the field has begun to appreciate the microbiota as a potential therapeutic target not only to aid in drug efficacy for the treatment of neurological disorders but also as a means for developing additional personalized medical treatments.

Despite these exciting advancements toward our understanding of the molecular mechanisms behind the microbial metabolism of neuromodulatory drugs, a gap remains in our understanding of the relevance of these findings to the clinic. Few, if any, studies to date have rigorously assessed the symptomatic outcomes resulting from altering the microbial metabolism of drugs for neurological disorders. As a result, it remains unclear whether these mechanisms are ultimately impactful for clinically relevant outcomes in the host. Experiments utilizing genetically tractable bacterial species alongside gnotobiotic tools in animal models of disease are needed to assess the role of microbe-specific functions on drug bioavailability and neurobehavioral outcomes. Advancements such as these are paramount for our ability to better understand roles for the microbiome in regulating inter-patient variability in responsiveness to drugs for neurological conditions and to assess the potential to inform tractable strategies for clinical intervention.

6. **Conclusions**

A growing body of evidence indicates that disruptions in host-associated microbiomes can modify animal behavior and further supports the notion of signaling across a microbiota-gut-brain axis. To date, several studies highlight sensory neuronal signaling, humoral metabolic communication, and immunomodulation as likely direct and indirect pathways that mediate microbiota-nervous system interactions, but studies that clearly evaluate and dissect these signaling mechanisms are lacking. Recent advances in sequencing, viral targeting, and intersectional genetic and imaging tools, combined with gnotobiotic and bacterial genetic systems, can better our understanding of the molecular and cellular mechanisms underlying microbiota-gut-brain communication and the nuances that arise from the coordinated signaling of heterogeneous cell types in response to pleiotropic microbial cues. In particular, studies

profiling sensory neurons and intestinal epithelial cells have uncovered the possibility for both direct and indirect activation of sensory neurons by microbiota-dependent dietary products, neurotransmitters, and as-yet-uncharacterized metabolites either through binding of receptors on afferent fibers themselves or via signaling to enteroendocrine cells in the gut epithelium. However, experiments that use conditional receptor knockouts in specific neuronal or epithelial subpopulations and gain- or loss-of-function constructs in bacteria may aid in identifying pathway-specific effects of microbial signals in regulating host brain function and behavior. Additionally, few studies to date have used *in vivo* electrophysiological- and geneticallyencoded calcium-indicator-based tools to directly assess the functional role of microbial-metabolite effects on neuronal activity. Understanding the distinct circuitry and functional signatures involved in mediating neuronal communication along the gut-brain axis is imperative for our understanding of how the gut microbiota modifies host physiology. While such studies can be performed in animal models, an added challenge is in assessing the relevance of findings to human health outcomes. Interrogating whether microbes from the human microbiota interact with neuromodulatory drugs, and whether such interactions have measurable consequences on drug efficacy and clinical outcomes, may serve a tractable context. Overall, the future offers the exciting prospect of uncovering fundamental principles for how microbes and microbial products are detected and interpreted by host sensory systems, toward understanding the co-evolution of animals with their associated microbiomes.

Figures and Tables:

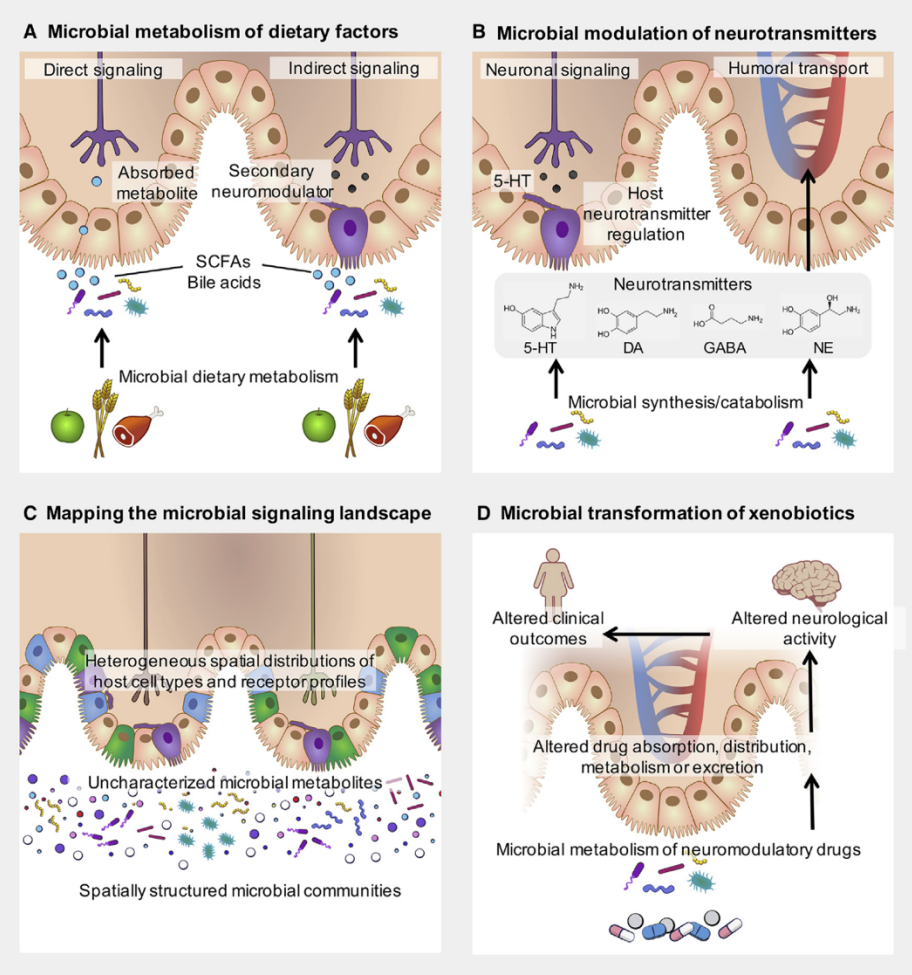


Figure 3.1 (A) Select bacteria from the gut microbiota produce short-chain fatty acids (SCFAs) and modify bile acids through dietary metabolism. Metabolites from the microbiota can signal directly to mucosal afferent fibers of sensory neurons (left) or can signal to neurons via intermediate interactions with enteroendocrine or epithelial cells (right). (B) Select bacteria from the gut microbiota can directly synthesize, consume, or sense neurotransmitters such as serotonin (5-HT), dopamine (DA), gamma-aminobutyric acid (GABA), and norepinephrine (NE) (center) or regulate host biosynthesis of neurotransmitters, like serotonin (5-HT) (center left). Microbially

modulated neurotransmitters have the potential to interact with sensory neurons (left) or be circulated humorally (right) to reach the blood-brain barrier. (C) The physiological landscape for microbial interactions with the nervous system is complex. Emerging evidence suggests that microbial communities are spatially structured (bottom), which yields ‘‘microbiogeographies’’ that vary in physiological function. In addition, the microbiome regulates various metabolites in the host, many of which remain uncharacterized (center). Further complexity is introduced when considering the heterogeneity of host cell types within the intestine, spanning various types of epithelial, endocrine, immune, and neuronal cells that are also spatially distributed and can vary temporally in their localization via turnover and remodeling. Spatial maps of signaling receptors, especially those available for mediating neural communication across intestinal cell types, will help inform functional pathways for microbe-host interactions. (D) The microbiome is increasingly appreciated as an important modulator of xenobiotic metabolism, particularly for neuromodulatory drugs, including anti-psychotics, anticholinergics, antidepressants, and opioids. Microbial transformation of drugs for neurological conditions could alter their absorption, distribution,metabolism, and/or excretion in the host, with potential downstream consequences on host neural activity and symptoms of neurological disease.

References:

Arpaia, N., Campbell, C., Fan, X., Dikiy, S., van der Veeken, J., deRoos, P., Liu, H., Cross, J.R., Pfeffer, K., Coffer, P.J.*, et al.* (2013). Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. Nature *504*, 451-455.

Bellono, N.W., Bayrer, J.R., Leitch, D.B., Castro, J., Zhang, C., O'Donnell, T.A., Brierley, S.M., Ingraham, H.A., and Julius, D. (2017). Enterochromaffin Cells Are Gut Chemosensors that Couple to Sensory Neural Pathways. Cell *170*, 185-198.e116.

Bjursell, M.K., Martens, E.C., and Gordon, J.I. (2006). Functional genomic and metabolic studies of the adaptations of a prominent adult human gut symbiont, Bacteroides thetaiotaomicron, to the suckling period. J Biol Chem *281*, 36269-36279.

Bohórquez, D.V., Shahid, R.A., Erdmann, A., Kreger, A.M., Wang, Y., Calakos, N., Wang, F., and Liddle, R.A. (2015). Neuroepithelial circuit formed by innervation of sensory enteroendocrine cells. The Journal of clinical investigation *125*, 782-786.

Bravo, J.A., Forsythe, P., Chew, M.V., Escaravage, E., Savignac, H.M., Dinan, T.G., Bienenstock, J., and Cryan, J.F. (2011). Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proceedings of the National Academy of Sciences of the United States of America *108*, 16050-16055.

Byrne, C.S., Chambers, E.S., Morrison, D.J., and Frost, G. (2015). The role of short chain fatty acids in appetite regulation and energy homeostasis. Int J Obes (Lond) *39*, 1331-1338.

Chambers, E.S., Viardot, A., Psichas, A., Morrison, D.J., Murphy, K.G., Zac-Varghese, S.E., MacDougall, K., Preston, T., Tedford, C., Finlayson, G.S*., et al.* (2015). Effects of targeted delivery of propionate to the human colon on appetite regulation, body weight maintenance and adiposity in overweight adults. Gut *64*, 1744-1754.

Chen, H., Nwe, P.K., Yang, Y., Rosen, C.E., Bielecka, A.A., Kuchroo, M., Cline, G.W., Kruse, A.C., Ring, A.M., Crawford, J.M*., et al.* (2019). A Forward Chemical Genetic Screen Reveals Gut Microbiota Metabolites That Modulate Host Physiology. Cell *177*, 1217-1231 e1218.

Clarke, M.B., Hughes, D.T., Zhu, C., Boedeker, E.C., and Sperandio, V. (2006). The QseC sensor kinase: a bacterial adrenergic receptor. Proc Natl Acad Sci U S A *103*, 10420-10425.

Cohen, L.J., Esterhazy, D., Kim, S.H., Lemetre, C., Aguilar, R.R., Gordon, E.A., Pickard, A.J., Cross, J.R., Emiliano, A.B., Han, S.M*., et al.* (2017). Commensal bacteria make GPCR ligands that mimic human signalling molecules. Nature *549*, 48-53.

Cohen, L.J., Kang, H.S., Chu, J., Huang, Y.H., Gordon, E.A., Reddy, B.V., Ternei, M.A., Craig, J.W., and Brady, S.F. (2015). Functional metagenomic discovery of bacterial effectors in the human microbiome and isolation of commendamide, a GPCR G2A/132 agonist. Proc Natl Acad Sci U S A *112*, E4825-4834.

Colosimo, D.A., Kohn, J.A., Luo, P.M., Piscotta, F.J., Han, S.M., Pickard, A.J., Rao, A., Cross, J.R., Cohen, L.J., and Brady, S.F. (2019). Mapping Interactions of Microbial Metabolites with Human G-Protein-Coupled Receptors. Cell Host Microbe *26*, 273-282 e277.

De Vadder, F., Kovatcheva-Datchary, P., Goncalves, D., Vinera, J., Zitoun, C., Duchampt, A., Backhed, F., and Mithieux, G. (2014). Microbiota-generated metabolites promote metabolic benefits via gut-brain neural circuits. Cell *156*, 84-96.

Donaldson, G.P., Lee, S.M., and Mazmanian, S.K. (2016). Gut biogeography of the bacterial microbiota. Nat Rev Microbiol *14*, 20-32.

Egerod, K.L., Petersen, N., Timshel, P.N., Rekling, J.C., Wang, Y., Liu, Q., Schwartz, T.W., and Gautron, L. (2018). Profiling of G protein-coupled receptors in vagal afferents reveals novel gut-to-brain sensing mechanisms. Mol Metab *12*, 62-75.

Erny, D., Hrabe de Angelis, A.L., and Prinz, M. (2017). Communicating systems in the body: how microbiota and microglia cooperate. Immunology *150*, 7-15.

Frost, G., Sleeth, M.L., Sahuri-Arisoylu, M., Lizarbe, B., Cerdan, S., Brody, L., Anastasovska, J., Ghourab, S., Hankir, M., Zhang, S.*, et al.* (2014). The short-chain fatty acid acetate reduces appetite via a central homeostatic mechanism. Nature communications *5*, 3611.

Fung, T.C., Vuong, H.E., Luna, C.D.G., Pronovost, G.N., Aleksandrova, A.A., Riley, N.G., Vavilina, A., McGinn, J., Rendon, T., Forrest, L.R.*, et al.* (2019). Intestinal serotonin and fluoxetine exposure modulate bacterial colonization in the gut. Nat Microbiol *4*, 2064-2073.

Furusawa, Y., Obata, Y., Fukuda, S., Endo, T.A., Nakato, G., Takahashi, D., Nakanishi, Y., Uetake, C., Kato, K., Kato, T.*, et al.* (2013). Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature *504*, 446-450.

Gustafsson, B. (1946). Germ-free rearing of rats. Acta Anat (Basel) *2*, 376-391.

Hallen-Adams, H.E., and Suhr, M.J. (2017). Fungi in the healthy human gastrointestinal tract. Virulence *8*, 352-358.

Hockley, J.R.F., Taylor, T.S., Callejo, G., Wilbrey, A.L., Gutteridge, A., Bach, K., Winchester, W.J., Bulmer, D.C., McMurray, G., and Smith, E.S.J. (2019). Single-cell RNAseq reveals seven classes of colonic sensory neuron. Gut *68*, 633-644.

Hoverstad, T., and Midtvedt, T. (1986). Short-chain fatty acids in germfree mice and rats. J Nutr *116*, 1772-1776.

Hsu, B.B., Gibson, T.E., Yeliseyev, V., Liu, Q., Lyon, L., Bry, L., Silver, P.A., and Gerber, G.K. (2019). Dynamic Modulation of the Gut Microbiota and Metabolome by Bacteriophages in a Mouse Model. Cell Host Microbe *25*, 803-814 e805.

Jackson, M.A., Verdi, S., Maxan, M.E., Shin, C.M., Zierer, J., Bowyer, R.C.E., Martin, T., Williams, F.M.K., Menni, C., Bell, J.T.*, et al.* (2018). Gut microbiota associations with common diseases and prescription medications in a population-based cohort. Nat Commun *9*, 2655.

Kashem, S.W., Riedl, M.S., Yao, C., Honda, C.N., Vulchanova, L., and Kaplan, D.H. (2015). Nociceptive Sensory Fibers Drive Interleukin-23 Production from CD301b+ Dermal Dendritic Cells and Drive Protective Cutaneous Immunity. Immunity *43*, 515-526.

Kupari, J., Haring, M., Agirre, E., Castelo-Branco, G., and Ernfors, P. (2019). An Atlas of Vagal Sensory Neurons and Their Molecular Specialization. Cell Rep *27*, 2508-2523 e2504.

Larraufie, P., Martin-Gallausiaux, C., Lapaque, N., Dore, J., Gribble, F.M., Reimann, F., and Blottiere, H.M. (2018). SCFAs strongly stimulate PYY production in human enteroendocrine cells. Sci Rep *8*, 74.

Leulier, F., MacNeil, L.T., Lee, W.J., Rawls, J.F., Cani, P.D., Schwarzer, M., Zhao, L., and Simpson, S.J. (2017). Integrative Physiology: At the Crossroads of Nutrition, Microbiota, Animal Physiology, and Human Health. Cell Metab *25*, 522-534.

Maier, L., Pruteanu, M., Kuhn, M., Zeller, G., Telzerow, A., Anderson, E.E., Brochado, A.R., Fernandez, K.C., Dose, H., Mori, H.*, et al.* (2018). Extensive impact of non-antibiotic drugs on human gut bacteria. Nature *555*, 623-628.

Maini Rekdal, V., Bess, E.N., Bisanz, J.E., Turnbaugh, P.J., and Balskus, E.P. (2019). Discovery and inhibition of an interspecies gut bacterial pathway for Levodopa metabolism. Science *364*.

Mertens, K.L., Kalsbeek, A., Soeters, M.R., and Eggink, H.M. (2017). Bile Acid Signaling Pathways from the Enterohepatic Circulation to the Central Nervous System. Front Neurosci *11*, 617.

Milshteyn, A., Colosimo, D.A., and Brady, S.F. (2018). Accessing Bioactive Natural Products from the Human Microbiome. Cell Host Microbe *23*, 725-736.

Mukhopadhya, I., Segal, J.P., Carding, S.R., Hart, A.L., and Hold, G.L. (2019). The gut virome: the 'missing link' between gut bacteria and host immunity? Therap Adv Gastroenterol *12*, 1756284819836620.

Ridaura, V.K., Faith, J.J., Rey, F.E., Cheng, J., Duncan, A.E., Kau, A.L., Griffin, N.W., Lombard, V., Henrissat, B., Bain, J.R.*, et al.* (2013). Gut microbiota from twins discordant for obesity modulate metabolism in mice. Science *341*, 1241214.

Sgritta, M., Dooling, S.W., Buffington, S.A., Momin, E.N., Francis, M.B., Britton, R.A., and Costa-Mattioli, M. (2019). Mechanisms Underlying Microbial-Mediated Changes in Social Behavior in Mouse Models of Autism Spectrum Disorder. Neuron *101*, 246-259 e246.

Smith, P.M., Howitt, M.R., Panikov, N., Michaud, M., Gallini, C.A., Bohlooly, Y.M., Glickman, J.N., and Garrett, W.S. (2013). The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science *341*, 569-573.

Strandwitz, P. (2018). Neurotransmitter modulation by the gut microbiota. Brain research *1693*, 128-133.

Strandwitz, P., Kim, K.H., Terekhova, D., Liu, J.K., Sharma, A., Levering, J., McDonald, D.,

Dietrich, D., Ramadhar, T.R., Lekbua, A.*, et al.* (2019). GABA-modulating bacteria of the human gut microbiota. Nat Microbiol *4*, 396-403.

Takeuchi, O., and Akira, S. (2009). Innate immunity to virus infection. Immunol Rev *227*, 75-86.

Turnbaugh, P.J., Ley, R.E., Mahowald, M.A., Magrini, V., Mardis, E.R., and Gordon, J.I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. Nature *444*, 1027-1031.

Valenstein, E.S. (2002). The discovery of chemical neurotransmitters. Brain Cogn *49*, 73-95.

Vuong, H.E., Yano, J.M., Fung, T.C., and Hsiao, E.Y. (2017). The Microbiome and Host Behavior. Annu Rev Neurosci *40*, 21-49.

Wallace, B.D., Wang, H., Lane, K.T., Scott, J.E., Orans, J., Koo, J.S., Venkatesh, M., Jobin, C., Yeh, L.A., Mani, S.*, et al.* (2010). Alleviating cancer drug toxicity by inhibiting a bacterial enzyme. Science *330*, 831-835.

Yano, Jessica M., Yu, K., Donaldson, Gregory P., Shastri, Gauri G., Ann, P., Ma, L., Nagler, Cathryn R., Ismagilov, Rustem F., Mazmanian, Sarkis K., and Hsiao, Elaine Y. (2015). Indigenous Bacteria from the Gut Microbiota Regulate Host Serotonin Biosynthesis. Cell *161*, 264-276.

Zheng, Y.M., Lin, F.L., Gao, H., Zou, G., Zhang, J.W., Wang, G.Q., Chen, G.D., Zhou, Z.H., Yao, X.S., and Hu, D. (2017). Development of a versatile and conventional technique for gene disruption in filamentous fungi based on CRISPR-Cas9 technology. Sci Rep *7*, 9250.

Zimmermann, M., Zimmermann-Kogadeeva, M., Wegmann, R., and Goodman, A.L. (2019a). Mapping human microbiome drug metabolism by gut bacteria and their genes. Nature *570*, 462-467.

Zimmermann, M., Zimmermann-Kogadeeva, M., Wegmann, R., and Goodman, A.L. (2019b). Separating host and microbiome contributions to drug pharmacokinetics and toxicity. Science *363*.

Chapter 4: The Microbiome as a Modifier of Neurodegenerative Disease Risk

Abstract

The gut microbiome is increasingly implicated in modifying susceptibility to and progression of neurodegenerative diseases (NDs). In this review, we discuss roles for the microbiome in aging and in NDs. In particular, we summarize findings from human studies on microbiome alterations in Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis and Huntington's disease. We assess animal studies of genetic and environmental models for NDs that investigate how manipulations of the microbiome causally impact the development of behavioral and neuropathological endophenotypes of disease. We additionally evaluate the likely immunological, neuronal and metabolic mechanisms for how the gut microbiota may modulate risk for NDs. Finally, we speculate on cross-cutting features for microbial influences across multiple NDs and consider the potential for microbiome-targeted interventions for NDs.

1. Introduction

Neurodegenerative diseases (NDs) are characterized by progressive functional loss of neurons in the central nervous system (CNS) or peripheral nervous system (PNS), which leads to long-term motor and/or cognitive impairments. While genetic susceptibilities are major risk factors for developing NDs, environmental factors experienced throughout life also impact the onset, progression and severity of NDs. For example, Parkinson's disease (PD) has been positively associated with pesticide exposure (Menegon et al., 1998) and gastrointestinal infection (Fasano et al., 2015), and Alzheimer's disease (AD) has been increasingly associated with herpes simplex virus (HSV) (Itzhaki, 2017). Exactly how genetic and environmental risk factors are conveyed to together predispose to NDs remains poorly understood. However, emerging evidence suggests that genetically and environmentally-induced alterations in the gut microbiome

may contribute in part to ND risk.

The gastrointestinal tract harbors trillions of microorganisms, collectively called the gut microbiota, that is shaped by both host genetics and environmental exposures (Lozupone et al., 2012), and that profoundly modulates the development and function of the CNS and PNS across model organisms (Fung et al., 2017). Relevant to aging-related disorders, like some NDs, the composition and activity of the gut microbiota changes over the lifespan, and manipulating the gut microbiota in animal models alters aging-associated immune function, metabolic activity and ultimately longevity (Kundu et al., 2017). Additional environmental risk factors for NDs, such as diet, chemical exposures and infection, also shape the gut microbiota in humans and animal models. Consistent with this, an increasing number of studies report changes in the microbiota in individuals with PD, AD, amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD), as well as in a subset of animal models for these disorders (Table 1). Across animal models for NDs, disruptions in the gut microbiome can positively or negatively impact the manifestation of neuropathological and behavioral endophenotypes of disease. While precise mechanisms underlying these phenomena remain unclear, microbial modulation of neuroimmune function, sensory neuronal signaling and metabolic activity are implicated. Altogether, these findings raise timely interest in how the microbiome is shaped by risk factors for NDs, how functional activities of the microbiome impact the nervous system and how microbiome-gut-nervous system interactions may predispose to NDs.

2. The Gut Microbiome on Aging and Longevity

Preceding interest in the microbiome and aging-related NDs were several pioneering studies on the gut microbiome and healthy aging. Aging is commonly accompanied by chronic inflammation, increased intestinal permeability, impaired digestion and disrupted nutrient absorption, each of which exhibits bidirectional interactions with the gut microbiome. Consistent with these interactions, a preponderance of sequencing studies reports shifts in the composition of the gut microbiota from adulthood to old age (Langille et al., 2014; O'Toole and Jeffery, 2015). However, there is little consensus on the particular aging-associated taxa found across these studies. For example, increases in *Bacteroidetes* were correlated with aging in some studies (Claesson et al., 2011; Odamaki et al., 2016), whereas decreases in *Bacteroidaceae*, *Lachnospiraceae* and *Ruminococcaceae*, and increases in *Akkermansia*, *Bifidobacterium* and *Christensenellaceae* were reported in another (Biagi et al., 2016). Aside from differences in study design and technical approaches, several confounding factors are likely to play a role. In a study of hospitalized elderly and healthy controls, for example, medication-intake correlated negatively with *Massilia* and *Lachnospiraceae* and positively with *Bradyrhizobium*, *Coprobacter*, *Helicobacter* and *Prevotella* (Ticinesi et al., 2017). In a sixty-year longitudinal study, married individuals exhibited greater microbial diversity and richness compared to individuals living alone (Dill-McFarland et al., 2019), which may be related to reported influences of social interaction on the gut microbiota.

Given the technical challenges of conducting mechanistic aging-related research in humans, animal models have been particularly useful for demonstrating proof-of-principle for microbial effects on aging. Manipulation of the gut microbiota can modify lifespan across rodents, fish, flies and worms (Gordon et al., 1966; Lee and Hase, 2014;

Obata et al., 2018; Smith et al., 2017; Thevaranjan et al., 2017). In *Drosophila melanogaster*, age-related alterations in the gut microbiota preceded and predicted age-associated intestinal barrier dysfunction and subsequent decline (Clark et al., 2015). Specific depletion of *Acetobacter* from the gut in response to early life oxidant exposure extended longevity (Obata et al., 2018), whereas overgrowth of *Lactobacillus plantarum* shortened lifespan via lactic acid-mediated overproduction of reactive oxygen species (Iatsenko et al., 2018). In the African turquoise killifish, transplanting microbiota from young donors into middle-aged recipients extended lifespan, which correlated with engraftment of *Exiguobacterium*, *Planococcus*, *Propionigenium* and *Psychrobacter* (Smith et al., 2017). Consistent with reported age-associated reductions in *Akkermansia muciniphila* in mice and humans (Fransen et al., 2017; Langille et al., 2014; Sovran et al., 2019), supplementation of *A. muciniphila* to the $Lmna^{G609G/G609G}$ mouse model of progeria significantly extended lifespan (Barcena et al., 2019). While little is known regarding the exact molecular and cellular mechanisms underlying these distinct phenomena, several studies suggest that microbial modulation of immune homeostasis may be involved. Germ-free mice colonized with microbiota from old, but not young, conventional mice displayed elevated inflammatory cytokines in the serum and higher intestinal permeability, suggesting that aging-associated changes in the gut microbiota enhance systemic inflammation (Thevaranjan et al., 2017). Consistent with this, impaired proliferation of B cells in the Peyer's patches of aged mice was corrected by fecal microbiota transplantation from young mice (Stebegg et al., 2019), indicating protective effects of the microbiota from young mice against aging-associated immune dysregulation. These foundational studies on roles for the microbiome during normal aging highlight temporal

changes in the microbiome that impact healthspan and lifespan, likely through immune and stress responses. They further pave the way for active interest in microbial influences on aging-related disorders, including subsets of NDs.

3. The Gut Microbiome and Parkinson's Disease

Of all NDs, PD has the richest history of interest in peripheral contributions to central neural dysfunction. PD is a multifactorial neurodegenerative disease, believed to be caused by both environmental and genetic risk factors. It is characterized by the presence of neurotoxic alpha-synuclein inclusions, known as Lewy bodies, that result in striatal dopaminergic cell death and motor deficits. A role for the GI tract in the pathogenesis of PD has been hypothesized since the 1980's when Lewy bodies were first observed in enteric tissues from PD patients (Wakabayashi et al., 1988). In the years to follow, Braak and colleagues went on to characterize the topographical spread of alpha-synuclein inclusions, noting that regions receiving input from peripheral nerve fibers such as the dorsal vagal brainstem motor nuclei and olfactory bulb are affected early on (Braak and Braak, 2000; Braak et al., 2002; Braak et al., 2000). These observations lead to the development of the "dual-hit" staging hypothesis, suggesting that PD pathology originates in the periphery where it is seeded before translocating to the brain (Hawkes et al., 2007). In line with this hypothesis, many PD patients experience hyposmia and gastrointestinal issues prior to the onset of motor symptoms, and patients with inflammatory bowel disease (IBD) are at a greater risk of developing PD (Cersosimo and Benarroch, 2012; Peter et al., 2018; Pfeiffer, 2011).

In the past decade, this interest in gastrointestinal associations with PD has

extended to include alterations in the gut microbiota as a potential disease biomarker and/or disease modifier. Clinical studies profiling the microbiota of PD patients reported many alterations relative to healthy controls, including increases in the relative abundance of *Bifidobacterium* (Hasegawa et al., 2015; Peng et al., 2018), *Lactobacillus* (Hasegawa et al., 2015; Petrov et al., 2017), and *Verrucomicrobiaceae* (Hasegawa et al., 2015; Petrov et al., 2017; Unger et al., 2016). Consistent with the latter, increases in the abundance of *Akkermansia* have been widely reported in individuals with PD (Hill-Burns et al., 2017; Keshavarzian et al., 2015; Scheperjans et al., 2015; Unger et al., 2016). Decreases in *Blautia* and *Coprococcus* (Bedarf et al., 2017; Hasegawa et al., 2015; Unger et al., 2016), as well as *Prevotellaceae* (Hasegawa et al., 2015; Heintz-Buschart et al., 2018; Hill-Burns et al., 2017) have also appeared across studies. One metagenomic study reported that increased abundance of *A. municiphila*, *Prevotella copri*, and *Eubacterium* in L-DOPA-naïve patients correlated with alterations in microbial metabolism of tryptophan and beta-glucoronide (Bedarf et al., 2017). Interestingly, a longitudinal study assessing the temporal dynamics of microbiota composition during the development of PD indicates that microbial changes remain relatively stable over years following the onset of PD symptoms (Aho et al., 2019). Despite these advances, there remain many contradictory findings likely due to inter-study variability in patient cohort design, DNA preparation and sequencing methodology, and data analysis relative to confounding variables. Rigorous, standardized methodology and study design are thus required in order to draw stronger conclusions regarding whether there exist common microbiome signatures for PD.

Changes in the gut microbiota are also seen in some genetic and pharmacological

animal models with face and construct validity for PD. In one study, mice overexpressing human alpha-synuclein (ASO) exhibited reduced *Verrucomicrobiaceae*, which contrasts the increased levels of this taxon reported in some aforementioned human studies (Gorecki et al., 2019). When raised as germ-free or treated with antibiotics in order to deplete the microbiota during adulthood, ASO mice displayed improved motor function compared to transgenic littermates harboring a complex microbiota (Sampson et al., 2016), which suggested that ASO-associated alterations in the microbiota contribute to motor symptoms of PD. Furthermore, transplantation of ASO mice with human microbiota from PD patients induced more severe motor dysfunction and reactive microglia relative to mice transplanted with human microbiota from healthy controls, suggesting that select human-derived microbes from PD patients can exacerbate motor deficits and neuroinflammation in mice (Sampson et al., 2016). In a rat model for PD, chronic treatment with the pesticide, rotenone, altered the small intestinal and colonic microbiota, with significant increases in *Actinobacteria* and *Proteobacteria*, but decreases in *Bacteroidetes* and *Cyanobacteria*. Alongside these observed shifts, their model was able to reproduce symptoms of gastroparesis before the development of nigrostriatal pathology (Johnson et al., 2018). Mice receiving similar exposures to rotenone show decreases in the relative abundance of *Bifidobacterium* and alterations in associated metabolic pathways such as glycosaminoglycan degradation in the cecum (Perez-Pardo et al., 2018). Another neurotoxin model for PD involving low-dose, chronic administration of methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) also alters the microbiota, increasing *Prevotellaceae and Erysipelotrichaceae* while decreasing *Lachnospiraceae* (Lai et al., 2018). Another group reported similar changes, where MPTP-treated mice

exhibited decreased *Firmicutes* and *Clostridiales* and increased *Proteobacteria, Turicibacterales, and Enterobacteriales* (Sun et al., 2018). Notably, transplantation of the MPTP-associated microbiota into conventional mice impaired motor function and reduced striatal dopamine levels relative to controls. Additionally, administration of a single bacterium, *Proteus mirabilis*, to mice was able to exacerbate MPTP-induced motor deficits, dopaminergic neuronal damage, and inflammation in brain structures relevant to PD such as substantia nigra (Choi et al., 2018). Altogether, these studies indicate that alterations in the gut microbiome can contribute to the presentation of PD-associated endophenotypes in animals.

3.2 The Microbiome and Intestinal Inflammation in PD

Exactly how the microbiome may impact PD-related symptoms remains unclear. However, several lines of inquiry are now converging on the notion that microbial regulation of intestinal inflammation may be involved. Some PD patients experience gastrointestinal disturbances, such as chronic gastritis and constipation, several years prior to the onset of motor symptoms, leading scientists to consider gastrointestinal issues as early prodromal features of PD in subsets of patients (Cersosimo and Benarroch, 2012; Pfeiffer, 2011). In a large Danish cohort where individuals with inflammatory bowel disease (IBD) were tracked over 30 years, IBD patients exhibited a 22% increase in PD incidence compared to the unaffected controls (Villumsen et al., 2019). In this clinical investigation, patients with ulcerative colitis exhibited a significantly higher risk of PD compared to patients with Crohn's disease (Villumsen et al., 2019). In a nationwide Swedish cohort, the incidence of PD was 30% higher in the individuals with IBD compared

to those without IBD (Weimers et al., 2019). Another study of an American cohort also observed elevated risk of PD in patients with IBD compared to non-IBD individuals (Peter et al., 2018). Accordingly, alpha-synuclein accumulation was observed in the submucosal layer of the intestine in PD patients with colitis (Grathwohl et al., 2019), providing a potential link between IBD and PD pathology. Notably, the IBD patients who received anti-TNF therapy had a 78% reduction in the incidence of PD, suggesting that suppressing peripheral inflammation may protect against PD (Peter et al., 2018). While the biological links between intestinal autoimmune disease and PD remain elusive, genetic studies of IBD and PD have converged on some shared genetic risk factors. For example, mutations in the kinase domain of leucine-rich repeat kinase 2 (LRRK2) has been identified in both the IBD patients and PD patients, with the N2081D mutation found in IBD and the G2019S mutation representing the most common monogenic cause of PD (Hui et al., 2018). Notably, both N2081D and G2019S mutations result in increased kinase activity of LRRK2, which is associated with impaired function of autophagy and lysosomal formation (Hui et al., 2018). Overall, these studies reveal associations between PD and IBD, a condition for which the gut microbiome is strongly implicated (as reviewed in (Johnson et al., 2019)).

An increasing number of animal studies provide causal evidence that intestinal disruptions can trigger PD-related pathology. For example, intestinal injury by dextran sodium sulfate triggered the aggregation of alpha-synuclein in enteric neurons and dopaminergic neurons of the substantia nigra, ultimately leading to neuronal degeneration in aged alpha-synuclein transgenic mice (Grathwohl et al., 2019). In ASO mice, lipopolysaccharide injection reduced intestinal barrier integrity and expedited the onset of

motor symptoms (Gorecki et al., 2019). Intestinal infection of *Pink1*$^{-/-}$ mice with *Citrobacter rodentium* induced severe motor deficits and loss of striatal dopaminergic axons as compared to uninfected *Pink1*$^{-/-}$ mice and infected wildtype littermates (Matheoud et al., 2019), suggesting interactions between intestinal inflammation and genetic risk for PD. Moreover, in rotenone-treated mice, depletion of Toll-like receptor (TLR) 4 ameliorates rotenone-induced intestinal infiltration of $CD3^+$ T cells and intestinal dysfunction, as well as rotenone-induced motor deficits and loss of dopaminergic neurons (Perez-Pardo et al., 2019). These studies provide proof-of-principle that intestinal inflammation can induce or exacerbate behavioral and pathological symptoms in mouse models of PD. Additional research is needed to examine potential relationships between the microbiome, intestinal disease and PD, and to further determine whether microbial regulation of peripheral immunity may underlie any of the existing links between the microbiome and PD in humans and animal models.

3.3 The Microbiome and Amyloidogenesis in PD

Beyond potential immune-mediated pathways linking the microbiome to PD-related neurophysiological and behavioral symptoms, several studies suggest that select microbes and microbial products could stimulate alpha-synuclein aggregation, leading to the presence of neuronal inclusions comprised of misfolded alpha-synuclein protein. Early clinical characterization of Lewy body pathology in postmortem brains of sporadic PD patients by Braak and colleagues uncovered particular regions susceptible to aggregates at different stages in the development of PD. It was reported that CNS Lewy body pathology first arises in the olfactory bulb and dorsal vagal brainstem nuclei, followed by

midbrain structures such as substantia nigra, ultimately spreading to cortical structures during later stages of disease (Braak and Braak, 2000; Braak et al., 2002). However, later histological examination of post-mortem intestinal tissue from non-symptomatic elderly patients uncovered the early presence of Lewy bodies in the intestine, in the absence of Parkinsonian motor symptoms (Bloch et al., 2006; Braak et al., 2006; Shannon et al., 2012). These findings led to Braak's "dual-hit" staging hypothesis, suggesting that sporadic PD arises from ingestion of an unknown pathogen through the nasal cavity that is subsequently swallowed and responsible for seeding Lewy body pathology in the periphery before ultimately traveling to the CNS (Braak et al., 2003; Hawkes et al., 2007).

Subsequent studies in animal models of PD have supported this hypothesis, demonstrating the presence of intestinal alpha-synuclein during disease (Drolet et al., 2009; Kelly et al., 2014; Wang et al., 2012) that coincides with alterations in gastric motility and permeability (Kelly et al., 2014). Further, intestinal injection of alpha-synuclein pre-formed fibrils (PFFs) (Pan-Montojo et al., 2010) or alpha-synuclein from human brain isolates (Holmqvist et al., 2014) results in the appearance of CNS inclusions. Duodenal inoculation of alpha-synuclein PFFs to aged ASO mice leads to loss of striatal dopamine in the mid brain (Challis et al., 2020). Together, these data implicate the ability for peripheral alpha-synuclein aggregation to propagate to the CNS and induce PD-related pathology. As alpha-synuclein overexpression is associated with alterations in vagal neuronal gene expression (Noorian et al., 2012), the dorsal motor vagal nucleus is one of the first brain regions in which Lewy bodies appear, and there is a decreased risk of developing PD in vagotomized patients (Svensson et al., 2015). The vagus nerve

possesses dense varicose projections to the intestinal villi of the small intestine, situating its terminals immediately adjacent to the intestinal lumen whereby the members of the host microbiota reside. One proposed mode of transport is via trans-neuronal propagation of alpha-synuclein along the vagus nerve. Indeed, alpha-synuclein released from enteric neurons or alpha-synuclein PFFs injected into the gastrointestinal tract are unable to reach the brain following vagotomy (Kim et al., 2019; Pan-Montojo et al., 2012; Uemura et al., 2018), suggesting a role for the vagus nerve in propagating alpha-synuclein spread from the periphery to the brain in a prion-like manner.

CNS alpha-synuclein burden is decreased in germ-free animals lacking a microbiome compared to specific-pathogen free mice harboring a complex microbiome (Sampson et al., 2016). One possible mechanism by which the microbiota may contribute to the development of PD alpha-synuclein pathology is through pathogenic cross-seeding, whereby one amyloidogenic protein causes another to adopt a beta-sheet structure, between alpha-synuclein and bacterial amyloids, such as curli in *Escherichia coli* in the gut. Curli are highly-conserved surface organelles that mediate binding of bacteria to soluble matrix proteins (Olsen et al., 1989). Additionally, the key element, CsgA, contains amyloidogenic peptide repeat motifs shared by human prions (Miraglia et al., 2018). Two genes in the curli operon, *CsgE* and *CsgC*, have been shown to modulate alpha-synuclein formation (Chorell et al., 2015) and oral administration of curli-producing bacteria, but not curli-deficient mutant strains, to wild-type rats results in intestinal accumulation of alpha-synuclein (Chorell et al., 2015). A recent study reproduced these findings in mice overexpressing alpha-synuclein, and additionally demonstrated that introduction of curli-producing bacteria to a healthy, complex human microbiota

exacerbated alpha-synuclein pathology and behavioral abnormalities in a CsgA-dependent manner (Sampson et al., 2020). These findings correlated with morphological alterations in midbrain microglia, suggesting an inflammatory state. However, curli are also considered microbial-associated molecular patterns and are recognized by TLRs to induce activation of the innate immune system (Chorell et al., 2015). Due to the role of inflammation in facilitating alpha-synuclein aggregation, more research is needed to determine whether bacterial amyloids from the gut microbiome may act to either directly or indirectly to increase peripheral alpha-synuclein aggregation. This amyloid hypothesis has also recently been extended to non-proteinaceous metabolites that are able to form fibrillar amyloid-like assemblies that seed alpha-synuclein aggregation *in vitro* (Tavassoly et al., 2018) . Due to the vast array of metabolic biotransformations carried out by the microbiota, additional efforts are warranted to evaluate effects of microbially-modulated metabolites on amyloidogenesis.

4. The Gut Microbiome and Alzheimer's Disease

In addition to PD, the gut microbiome is increasingly implicated in the manifestation of AD-related symptoms. AD is characterized by the accumulation of extracellular Aβ plaques and intracellular hyperphosphorylated tau tangles in the brain. The overwhelming burden of these amyloid deposits leads to neuronal loss and brain atrophy resulting in cognitive and motor decline. A few lines of evidence motivate interest in the microbiome and AD. From fundamental behavioral observations in laboratory animals, several studies reveal that manipulations of the microbiome, like germ-free rearing, antibiotic-treatment and severe dietary alterations, alter learning and memory-related behavior. In addition,

the microbiome is emerging as an important regulator of the development and function of the neuroimmune system (Fung et al., 2017), which is implicated in AD among several other neurological disorders (Giau et al., 2018; Kowalski and Mulak, 2019). As yet, however, there is only some evidence of microbiome alterations in human AD. In one study, decreases in the *Negativicutes* and *Lachnospirceae* and an increase in *Ruminococcaceae* were seen in AD participants compared to healthy controls (Zhuang et al., 2018). In another study, analysis of fecal samples obtained from AD participants reported alterations in the gut microbiota with decreased relative abundance of *Ruminococcaceae, Turicibacteraceae, Peptostreptococcaceae, Clostridiaceae,* and *Mogibacteriaceae* and increased relative abundance of *Bacteroidaceae and Rikenellaceae* compared to the healthy control group (Vogt et al., 2017). Notably, increases in bacterial abundance were correlated with alterations in cerebrospinal fluid (CSF) biomarkers suggestive of increased amyloid burden in the brain. In particular, elevated abundance of *Bacteroides* and *Blautia* corresponded to a decrease in CSF $A\beta_{42}/A\beta_{40}$ and increase in CSF p-tau and p-tau/$A\beta_{42}$ (Vogt et al., 2017). Microbiome profiling of mouse models for AD has also suggested alterations in the gut microbiota. Fecal samples from the 5xFAD and APP/PS1 mouse models for AD exhibited increased *Firmicutes* and decreased *Bacteroidetes* relative to wildtype controls (Brandscheid et al., 2017; Zhang et al., 2017a). In the 5xFAD mouse model, *Firmicutes* increased from 6 weeks of age to 18 weeks while *Bacteroidetes* decreased (Brandscheid et al., 2017). In the APP/PS1 mouse model, *Proteobacteria* doubled in relative abundance by 6 months of age, and *Verrucomicrobia* increased by 6-fold by 12 months of age (Zhang et al., 2017). However, a longitudinal study of the microbiota in the P301L tau mouse model of AD

reported the opposite relationship, wherein the abundance in *Bacteriodetes* increased starting at 3 months of age while *Firmicutes* decreased (Sun et al., 2019a). By 10 months of age, *Candidatus_saccharimonas, Alistipes, Rikenella, Odoribacter, Blautia, Ruminococcaceae, Eubacterium_xylanophilum, Paraprevotella, Butyricicoccus,* and *Parvibacter* were among the bacteria that were negatively associated with tau pathology in the brain. In contrast, *Bacteroides, Parabacteroides, Escherichia-Shigella, and Clostridium_innocuum* were positively correlated with tau pathology. Across these studies examining the microbiota in human AD and AD mouse models, there is as yet no clear microbial signature for AD.

4.2 The Microbiome and Neuroinflammation in AD

Despite limited evidence for a defined shift in the composition of the microbiota in human AD and AD mouse models, a growing number of studies report that manipulating the gut microbiota impacts the severity of Aβ pathology and cognitive impairment in genetic models for AD. Several of these studies suggest that microbial regulation of neuroimmunity may contribute. For example, rearing APP/PS1 mice as germ-free resulted not only in reduction of Aβ aggregates in the brain, but also a 40% decrease in Iba-1 positive microglia leading to an overall decrease in neuroinflammation compared to conventionalized mice (Harach et al., 2017). The pro-inflammatory cytokine IL-1β was also significantly decreased by 36% in germ-free APP/PS1 mice compared to conventionally colonized APP/PS1 controls. Another study using antibiotics to acutely deplete the microbiota of APP/PS1 mice saw similar results. One week of antibiotic treatment led to a decrease in Aβ load and down regulation of pro-inflammatory cytokine

IL-6 compared to the vehicle-treated group (Minter et al., 2017). These alterations corresponded with reductions in numbers of plaque-associated microglia and astrocytes, suggesting that depletion of the microbiome reduced neuroinflammation and gliosis in the brain. Tg2576 mice, which express a variant of human amyloid precursor protein, exhibited an aging-related increase in intestinal *Bacteroides fragilis* relative to wildtype controls. Notably, administering *B. fragilis* to APP/PS1 mice promoted Aβ plaque deposition in the cortex, which was rescued by calorie restriction (Cox et al., 2019). While exact mechanisms are unknown, the findings appear to counter existing literature reporting that *B. fragilis* elicits immunosuppressive effects that may ameliorate symptoms of other neurological diseases. Overall, these studies provide some evidence that large alterations in the gut microbiota can influence the progression and manifestation of AD-related features in genetically susceptible animals.

4.3 Infection and AD

While many studies on the microbiota and AD focus on symbiotic microorganisms indigenous to the gut, a growing body of literature links AD to bacterial and viral pathogens. RNA sequencing of brain tissue from deceased patients with a variety of neurodegenerative diseases revealed the presence of infectious agents in the CNS (Bennett et al., 2019). For AD in particular, reads aligning to *Toxoplasmosis gondii, Trichinella sp. T6, Babesia microti, Borrella burgdorferi, Porphyromonas gingivalis,* and *Treponema denticola* were observed in samples of the frontal cortex from post-mortem AD brains. Similarly, another transcriptomics study mapped HSV6 and 7 to multiple brain regions in AD patients (Readhead et al., 2018), which aligns with multiple case and

epidemiological studies on HSV and AD. This could be due to direct interactions between neurotropic viruses and amyloids, as HSV1 was capable of catalyzing Aβ plaque formation by binding Aβ-peptides using the protein corona (Ezzat et al., 2019). In addition, data from the National Health and Nutrition Examination Surveys database revealed a positive association between AD mortality and *H. pylori* (Beydoun et al., 2018). One study showed that *H. pylori* is able to induce tau hyperphosphorylation by activating the tau kinase glycogen synthase kinase-3β (Wang et al., 2015). Additional studies reported that Aβ precursor was upregulated in tissues from AD patients with periodontitis relative to unaffected AD controls (Nezu et al., 2017). Consistent with this, across several mouse models for AD, infection with *Porphyromonas gingivalis* exacerbated AD pathology resulting in an increase in neuroinflammation, neurodegeneration and amyloidosis (Dominy et al., 2019; Ilievski et al., 2018; Ishida et al., 2017). This evidence linking bacterial and viral infection to AD may be related to some studies proposing that native function of Aβ is an antimicrobial peptide involved innate immune defense (Moir et al., 2018). Whether the gut microbiome may play a role in regulating host responses to AD-associated infections remains poorly understood.

5. **The Gut Microbiome in Amyotrophic Lateral Sclerosis and Huntington's Disease**

ALS is a fatal neurological disease accompanied with the progressive degeneration of motor neurons in the brain and spinal cord. A few studies have compared the gut microbial composition in patients with ALS and healthy controls and highlighted certain species. In an investigation of 37 patients with ALS and 29 healthy familial controls, metagenomic sequencing of gut microbiome revealed a significantly distinct

microbial composition compared with healthy controls, among which the abundance of *Anaerostipes hadrus*, *Bacteroidales bacterium*, *Bifidobacterium pseudocatenulatum*, are marginally increased whereas *Clostridium leptum* and *Escherichia coli* are marginally decreased in ALS-patients compared to non-affected controls (Blacher et al., 2019). In addition, reduced levels of nicotinamide (NAM), correlated with decreased abundance of *Bifidobacterium pseudocatenulatum*, was observed in the serum and CSF of patients with ALS by metabolomic analysis (Blacher et al., 2019). Another study of 6 ALS-patients highlights the reduced ratio of *Firmicutes*/*Bacteroidetes* in fecal microbiota of ALS-patients, accompanied with increased abundance of *Dorea* and decreased abundance of *Oscillibacter*, *Anaerostipes* and *Lachnospiraceae* (Fang et al., 2016). By contrast, a study involving 25 ALS-patients did not find significant differences in the gut microbial composition between ALS patient and healthy controls, even though an increase of the overall number of microbial species was observed in ALS patients compared with healthy control (Brenner et al., 2018). The contradictory results from different studies may be due at least in part to the limited power of the studies, which highlights the need for systematic investigation of the microbiota in large cohorts of ALS patients and controls.

In addition to independent studies reporting correlations between microbiota composition and ALS, one study reported a role for the microbiota in protecting against ALS symptoms in the Sod1 transgenic mouse model. Depletion of gut microbiota by broad-spectrum antibiotics exacerbated the motor deficits in Sod1 mice, as evaluated in the rotarod test and wire-hanging test (Blacher et al., 2019). In line with this, Sod1 mice reared as germ-free exhibited higher mortality rates than those raised as conventionally colonized. This contrasts findings from mouse models of PD and AD, where absence of

the microbiota abrogates pathology and behavioral abnormalities. 16S rRNA sequencing and shotgun metagenomic sequencing revealed that Sod1 mice exhibited reduced abundance of *A. muciniphila* compared to wildtype littermates. Colonizing antibiotic-treated Sod1 mice with *A. muciniphila* not only extended lifespan but also ameliorated the brain atrophy and motor deficits of the mice. Consistent with the association of reduced NAM with ALS incidence in a companion clinical study, increased levels of NAM were observed in the sera of Sod1 mice administered *A. muciniphila,* and administering NAM to Sod1 mice recapitulated the protective effect of *A. muciniphila* (Blacher et al., 2019). Overall, this study suggests that gut microbes can regulate motor and neurophysiological endophenotypes of ALS by altering the systemic bioavailability of select metabolites. The findings contribute to growing interest in uncovering functions for previously uncharacterized metabolites that are regulated by the gut microbiome.

Similar links between the microbiota and HD are just beginning to be explored. HD is a progressive neurological disorder caused by a trinucleotide expansion in the huntingtin gene, which causes death of neurons in various brain regions, leading to chorea and abnormal alterations in behavior, emotion and cognition. While no studies to date have sequenced the human gut microbiome from HD patients, one study reported the presence of various fungal and bacterial species in the striatum of HD patients, including *Candida*, *Pseudomonas*, *Acinetobacter*, and *Burkholderia* (Alonso et al., 2019). While the study suggests that microbial infection may be associated with HD, the technical contamination of brain tissue needs to be excluded. Another study compared the gut microbiota in the R6/1 mouse model of HD with wildtype littermates and reported sexual dimorphism in the bacterial signature of R6/1 mice. An increase in *Bacteroidales*,

Lactobacillales and a decrease in *Clostridiales* were observed in male R6/1 mice, while an increase in *Coriobacteriales*, *Erysipelotrichales*, *Bacteroidales*, *Burkholderiale* and a decrease in *Clostridiales* were observed in females. In addition, male R6/1 mice show higher microbial diversity compared to both female R6/1 mice and wildtype littermates (Kong et al., 2018). Whether these microbiota alterations contribute to symptoms of the R6/1 model remain unclear. However, in the BACHD model, mice reared as germ-free exhibited reduced levels of myelin-related proteins and decreased numbers of mature oligodendrocytes in the prefrontal cortex compared to mice reared conventionally-colonized (Radulescu et al., 2019). This generally aligns with findings from the Sod1 model, where absence of the microbiota exacerbates endophenotypes of disease. Additional studies are warranted to replicate and extend interrogations into the microbiota in ALS and HD, among other neurodegenerative diseases.

6. Future Outlooks

Despite our limited mechanistic understanding of how the microbiota may predispose to symptoms of NDs, efforts to manipulate the microbiota through transplantation and probiotic treatment highlight the potential for microbial amelioration of ND-related pathology and behavior in laboratory animals. Transplantation of conventional microbiota into MPTP mice ameliorated motor deficits, increased dopaminergic neurons in the substantial nigra and reduced numbers of activated microglia and astrocytes (Sun et al., 2018). Similarly, AD mouse models exhibited improved learning and memory behavior, reductions in brain Aβ and tau aggregation, decreased neuroinflammation, and attenuated synaptic dysfunction after microbiota transplantation (Kim et al., 2020; Sun et

al., 2019b). In the ADLP mouse model, daily fecal microbiota transplant (FMT) from wildtype mice over 4 months resulted in improved spatial short-term memory and long-term memory (Kim et al., 2020). In addition, measuring the total plaque area of the frontal cortex and hippocampus revealed a decrease in Aβ plaque burden in treated mice compared to control ADLP mice. Furthermore, FMT decreased tau aggregates, reduced the number of activated microglia and attenuated gliosis. In the APP/PS1 mouse model, daily FMTs over 4 weeks also resulted in improved cognition as indicated by better performance in the Morris water maze and object recognition test (Sun et al., 2019b). Aβ deposition and hyperphosphorylated tau in the hippocampus were reduced in the treated mice compared to controls. Targeting select bacteria for treatment has also revealed beneficial effects in ND animal models. Administration of probiotic bacterium *Bacillus subtilis* to transgenic *C. elegans* expressing human alpha-synuclein increased clearance of alpha-synuclein, ultimately leading to a reversal of aggregation (Goya et al., 2020). Chronic treatment of 3xTg mice with a cocktail of *Lactobacillus* and *Bifidobacteria* (SLAB51) promoted working memory, increased cortical thickness and decreased amyloid burden in the brain (Bonfili et al., 2017). Furthermore, bacterial treatment activated sirtuin-1 dependent pathways to promote antioxidative responses (Bonfili et al., 2018), improved glucose reuptake in the brain, and reduced levels of hyperphosphorylated tau (Bonfili et al., 2019). Similarly, APP/PS1 mice treated with *Clostridium butyricum* had reduced microglial activation and decreased signs of neurodegeneration (Sun et al., 2020). These studies provide proof-of-concept for microbiome interventions in animal models. However, there is as yet no evidence that such approaches would be safe or effective for clinical NDs.

Other microbiome-based interventions that aim to enhance the efficacy of existing treatments for NDs may be more tractable for clinical translation. With the finding that select bacteria metabolize L-dopa, a common treatment for PD (Maini Rekdal et al., 2019; van de Steeg et al., 2018; van Kessel et al., 2019), there is increased interest in whether inhibiting or reducing microbial metabolism of L-dopa could promote drug efficacy in PD. Similar approaches of modulating microbial metabolism of existing medications may be relevant more broadly to neurology, as select microbes from the gut microbiome have been reported to interact with a variety of common medications for neurological diseases (Cryan et al., 2020; Maini Rekdal et al., 2019). In addition, a wealth of evidence suggests that adherence to the Mediterranean diet reduces susceptibility to AD (Berti et al., 2018; Rainey-Smith et al., 2018), but whether the microbiome may mediate the effects of the diet on host physiology remains unclear. Indeed, the microbiome was altered in patients with mild cognitive impairment after treatment with the modified Mediterranean-ketogenic diet (Nagpal et al., 2019), and the microbiome was reported to mediate the neuromodulatory effects of the ketogenic diet in mouse models of refractory epilepsy (Olson et al., 2018).

Overall, studies in animal models provide intriguing evidence that perturbing the microbiota can modify risk for developing pathological and behavioral endophenotypes of PD, AD, ALS and HD (Table 2). In various genetic models of PD and AD, complete lack of or depletion of the microbiota yields animals with reduced amyloid pathology and improved motor or cognitive behavior. While only a few such studies have been conducted in mouse models of ALS and HD, they commonly report that the absence or depletion of the microbiota exacerbates neuropathological and behavioral symptoms of

disease. Microbiota transplant and bacterial treatments are capable of reducing symptoms, suggesting that modifying the gut microbiota in animal models of NDs can ameliorate neuroinflammatory, neuropathological and/or behavioral abnormalities triggered by genetic risk. These findings raise the question of whether there are cross-cutting features and shared influences of the gut microbiota across subsets of ND models (Figure 1). Microbial regulation of peripheral immune and neuroimmune function may be a point of convergence across the disorders that warrants future study. Direct effects of microbial products or microbially-regulated biochemicals on protein misfolding is another area for continued investigation. The future holds tremendous opportunity to uncover how the gut microbiota modifies risk for NDs, with the potential to reveal fundamental interactions across the microbiota-gut-brain axis and inform new approaches for identifying and treating NDs.

Figures and Tables:

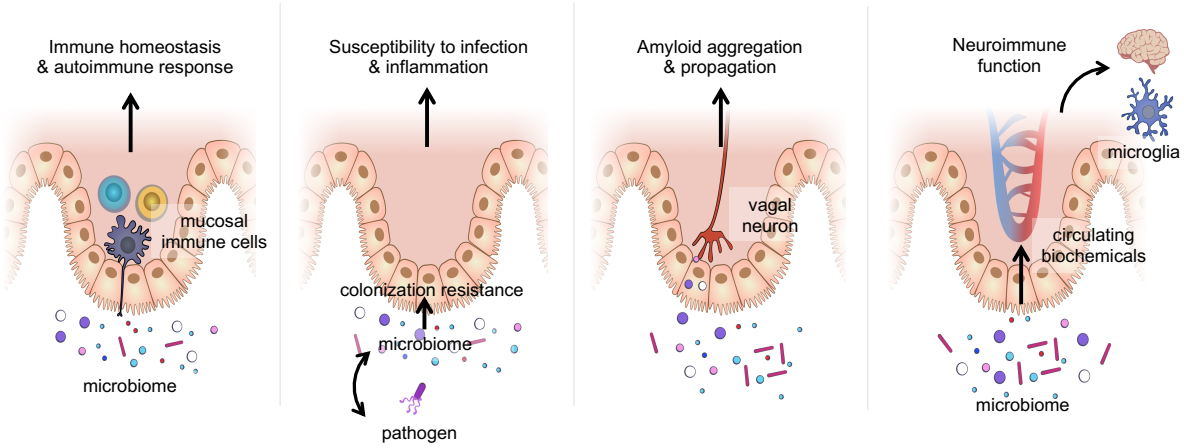


Figure 4.1. Proposed pathways for microbial modulation of neurodegenerative diseases. The gut microbiome is increasingly implicated in modifying risk for NDs. While precise mechanisms are unknown, a few pathways have been proposed. The gut microbiome is critical for conditioning peripheral immune homeostasis and autoimmune responses, which could influence the manifestation of NDs. In addition, the gut microbiome is important for promoting intestinal colonization resistance and protecting against pathogens, which could influence the likelihood of host exposure to infection and inflammation, as risk factors for subsets of NDs. The gut microbiome regulates a vast repertoire of biochemicals, some of which may interact directly with amyloids to promote aggregation in neurons and propagation from the periphery to the brain. The gut microbiome modulates biochemicals that modulate neuroimmune development and function, including the activity of brain microglia, which could impact the manifestation of subsets of NDs.

Table 4.1. Microbiome alterations in clinical cohorts and animal models for NDs

Parkinson's disease: Microbiota alterations in humans and animal models				
Subject	**Microbiota Alterations**	**Sample**	**Method**	**Reference**
Human, PD Patients	Increased: *Bifidobacterium*. Decreased: *Prevotellaceae, Roseburia*	n = 64, feces	16S rRNA gene sequencing	(Aho et al., 2019)
Human, L-Dopa Naïve PD Patients	Family/Genus Increased: *Verrumicrobiaceae* (genus *Akkermansia*), unclassified Bacteria, Firmicutes Family/Genus Decreased: *Prevotellaceae (Prevotella), Erysipelotrichaceae (Eubacterium)* Species Increased: *Akkermansia muciniphila*, *Alistipes shahii* Species Decreased: *Prevotella copri, Eubacterium bioforme, Clostridium saccharolyticum*	n = 31, feces	Shotgun metagenomic sequencing	(Bedarf et al., 2017)
Human, PD Patients	Increased: *Lactobacillus* Decreased: *Clostridium coccoides, Bacteroides fragilis*	n = 52, feces	16S rRNA gene qPCR	(Hasegawa et al., 2015)
Human, PD Patients	Increased *Akkermansia*	n = 76, feces	16S/18S rRNA gene sequencing	(Heintz-Buschart et al., 2018)
Human, PD Patients	Family Increased: *Bifidobacteriaceae, Lactobacillaceae, Tissierellaceae, Christensenellaceae, Verrucomicrobiaceae* Family Decreased: *Lachnospiraceae, Pasteurellaceae* Genus Increased: *Akkermansia, Christensenellaceae, Lactobacillus, Bifidobacterium* Genus Decreased: *Blautia, Roseburia, Lachnospiraceae, Faecalibacterium*	n = 197, feces	16S rRNA gene sequencing	(Hill-Burns et al., 2017)
Human, PD Patients	Increased: *Lactobacillaceae, Barnesiellaceae, Enterococcaceae*	n = 29, feces	16S rRNA gene sequencing	(Hopfner et al., 2017)
Human, PD Patients	Mucosal Genus Increased: *Faecalibacterium, Ralstonia* Fecal Genus Increased: *Blautia, Coprococcus, Roseburia*.	n = 38, sigmoid mucosal biopsies, feces	16S rRNA gene sequencing	(Keshavarzian et al., 2015)
Human, PD Patients	Increased: *Escherichia-Shigella, Streptococcus, Proteus, Enterococcus* Decreased: *Blautia, Faecalibacterium, Ruminococcus*	n = 24, feces	16S rRNA gene sequencing	(Li et al., 2017)
Human, PD Patients	Increased: *Ruminococcaceae, Verrucomicrobiaceae, Porphyromondaceae, Hydrogenoanaerobacterium, Lachnospiraceae* Decreased: *Bacteroides, Prevotellaceae*	n = 10, feces	16S rRNA gene sequencing	(Li et al., 2019)

Human, PD Patients	Increased: *Eubacteriaceae, Bifidobacteriaceae, Aerococcaceae, Desulfovibrionaceae* Decreased: *Streptococcaceae, Methylobacteriaceae, Comamonadaceae, Halmonadaceae, Brucellaceae, Xanthomonadaceae, Lachnospiraceae, Actinomycetaceae, Aphingomonadaceae, Pasturellaceae, Micrococcaceae, Intrasporangiacea, Methanobacteriacea, Idomarinaceae, Brevibacteriaceae, Gemellaceae*	n = 75, feces	16S rRNA gene sequencing	(Lin et al., 2018)
Human, PD Patients	Genus Increased: *Christensenella, Catabacter, Lactobacillus, Oscillospira, Bifidobacterium* Genus Decreased: *Dorea, Bacteroides, Prevotella, Faecalibacterium* Species Increased*: Christensenella minuta, Catabacter hongkongensis, Lactobacillus mucosae, Ruminococcus bromii Palpillibacter cinnamivorans* Species Decreased: *Bacteroides massiliensis, Stoquefichus massiliensis, Bacteroides coprocola, Blautia glucerasea, Dorea longicatena, Bacteroides dorei, Bacteroides plebeus, Prevotella copri, Coprococcus eutactus, Ruminococcus callidus*	n = 89, feces	16S rRNA gene sequencing	(Petrov et al., 2017)
Human, PD Patients	Increased: *Clostridium IV, Aquabacterium, Holdemania, Sphingomonas, Clostridium XVII, Butyricicoccus, Anaetroncus*	n = 45, feces	16S rRNA gene sequencing	(Qian et al., 2018)
Human, PD Patients	Family Increased: *Enterobacteriaceae* Phylum Decreased: *Bacteroidetes* Family Decreased*: Prevotellaceae*	n = 43, feces	16s RT-qPCR	(Unger et al., 2016)
Human, PD Patients	Increased: *Akkermansia, Bifidobacterium* (trending), Decreased: *Prevotella* (trending)	n = 9, feces	16S rRNA gene sequencing	(Vidal-Martinez et al., 2020)
Human, PD Patients with normal cognition (NC) or mild cognitive impairment (MCI)	PD-NC vs. PD-MCI and HC: Increased: *Blautia, Ruminococcus* PD- MCI vs PD-NC and HC: Increased: *Alistipes, Barnesiella, Butyricmonas, Odoribacter, Anaerotruncus*	n = 13 (MCI), n = 14 (NC), feces	16s rRNA gene sequencing	(Ren et al., 2020)
Mice, ASO	Decreased: *Verrumicrobiae*	n=26, feces	16S rRNA gene sequencing	(Gorecki et al., 2019; Ren et al., 2020)
Mice; Rotenone	No observed differences with FDR-*P*. With less stringent analysis, Decreased: *Actinobacteria*	n = 14, feces	16S rRNA gene sequencing	(Dodiya et al., 2020)
Rats, Rotenone	(RT-qPCR) Increased: *Bifidobacterium* (Sequencing, SI) Phylum Increased: *Actinobacteria, Proteobacteria* (Sequencing, SI) Phylum Decreased:	n=12, distal small intestine	qPCR, shotgun metagenomic	(Johnson et al., 2018)

	Bacteroidetes, Cyanobacteria, Firmicutes (Sequencing, colon) Phylum Decreased: *Cyanobacteria, Firmicutes* (Sequencing, SI/colon) Family Increased: *Bifidobacteriaceae, Alcaligenaceae, Clostridiaceae* (Sequencing, SI/colon) Family Decreased*: S24-7, Prevotellaceae, Paraprevotellaceae, Lachnospiraceae* (Sequencing, SI/Colon) Genus Increased: *Bifidobacterium, Lactobacillus, Turicibacter, Sutterella* (Sequencing, SI/Colon) Genus Decreaased: *Prevotella, S24-7, Oscillospira*	and colon contents	sequencing	
Mice, MPTP	Phylum Decreased: Proteobacteria (2 days and 3 weeks following treatment) Order Increased: *Erysipelotrichales* (3 weeks following treatment) Order Decreased: *Clostridiales* (3 weeks following treatment) Family Increased: *Prevotellaceae* (2 days following treatment), *Erysipelotrichaceae* (3 weeks following treatment) Family Decreased: *Lachnospiraceae* (2 days and 3 weeks following treatment)	n = 10, feces	16S rRNA gene sequencing	(Lai et al., 2018)
Mice, MPTP	Mucosa Phylum Increased: Bacteroidetes, Firmicutes Mucosa Phylum Decreased: Actinobacteria Lumenal Content Phylum Increased: Proteobacteria Lumenal Content Phylum Decreased: Actinobacteria Mucosa and Lumenal Content Family Increased: *Rikenellaceae, S24-7, Clostridiales* (unclassified), *Ruminococcaceae* Mucosa and Lumenal Content Family Decreased: *Bifidobacterium* Mucosa Genus Increased: *Allobaculum*	n = 9-10, cecum mucosal-associated and lumenal contents	16S rRNA gene sequencing	(Perez-Pardo et al., 2018)
Mice, MPTP	Phylum Increased: *Proteobacteria* Phylum Decreased: *Firmicutes* Order Increased: *Turicibacterales, Enterobacteriales* Order Decreased: *Clostridiales*	n = 7, feces	16S rRNA gene sequencing	(Sun et al., 2018)
Mice, Rotenone, within-comparison over 4 weeks	Phylum Increased: *Firmicutes* (3w, 4w of treatment) Phylum Decreased: *Bacteroidetes*(3w, 4w of treatment) Genus Increased: *Lactobacillus* (3 and 4w of treatment) Genus Decreased: *Clostridium* (1-4w of treatment), *Sutterella* (1-4w of treatment), *Lactococcus*(1-3w of treatment), *Desulfovibrio* (2-4w of treatment), *Aldercreutzia* (2w of treatment), *Paraprevotella* (1 and 3w of treatment)	n = 5-8, feces	16S rRNA gene sequencing	(Yang et al., 2017)

Alzheimer's disease: Microbiota alterations in humans and animal models				
Subject	**Microbiota Alterations**	**Sample**	**Method**	**Reference**
Human, AD Patients	Phylum level: mild decrease in *Bacteriodetes* decrease in *Verrucomicrobia* Class level: increase in *Actinobacteria* and *Bacilli* decrease in *Negativicutes* and *Bacteroidia* Order level: increase in *Lactobacillales* decrease in *Bacteroidales and Selenomonadales* Family level: increase in *Ruminococcaceae, Enterococcaceae,* and *Lactobacillaceae* decrease in *Lanchnospiracea, Bacteroidaceae* and *Veillonellaceae*	n=43, feces	16S rRNA gene sequencing	(Zhuang et al., 2018)
Human, AD Patients	Phylum level: increase in *Bacteriodetes* decrease in *Firmicutes and Actinobacteria* Family level: increase in *Gemellaceae, Bacteroidaceae and Rikenellaceae* decrease in *Bifidobacteriaceae, Ruminococcaceae, Turicibacteraceae, Peptostreptococcaceae, Clostridiaceae,* and *Mogibacteriaceae* Genus level: increase in *Bacteroides, Alistipes, Bilophila, Blautia, Phascolarctobacterium* and *Gemella* decrease in *SMB53, Dialister, Clostridium, Turicibacter, cc115, Bifidobacterium* and *Adlercreutzia*	n=25, feces	16S rRNA gene sequencing	(Vogt et al., 2017)
Mice, Tau P301L	Phylum level: increase in *Bacteroidetes* decrease in *Firmicutes, Actinobacteria* and *Tenericutes* Genus level: increase in *Ruminococcaceae_NK4A214_group, Bacteroides, Anaeroplasma, norank_f_Clostridiales_vadinBB60_group, norank_f_Bacteroidales_24_7_group, Phascolarctobacterium, Parabacteroides,* and *unclassified_o_Bacteroidales, Anaerovorax, Peptococcus, Caproiciproducens, Ruminoclostridium,* and *Oscillibacter* decrease in *Lactobacillus, Enterorhabdus, Staphylococcus, Bifidobacterium, Gemella, Roseburia, norank_f_Mycoplasmataceae, Lachnoclostridium, Klebsiella,* and *Streptococcus*	n=32, feces	16S rRNA gene sequencing	(Sun et al., 2019a)
Mice, APP/PS1	Phylum level: increase in *Proteobacteria* and *Verrucomicrobia* Genus level: decrease in *Ruminococcus* and *Butricicocus*	n=24, feces	16S rRNA gene sequencing	(Zhang et al., 2017a)

Mice, 5xFAD	Phylum level: increase in *Firmicutes* decrease in *Bacteriodetes*	n=18, feces	16S rRNA gene qPCR	(Brandscheid et al., 2017)
Amyotrophic lateral sclerosis: Microbiome alterations in humans and animal models				
Subject	**Microbiome Alterations**	**Sample**	**Method**	**Reference**
Human, ALS Patients	Increased *Methanobrevibacter* Decreased *Faecalibacterium and Bacteroides*	n=8, feces	16S rRNA gene sequencing	(Zhai et al., 2019)
Human, ALS Patients	No significant alterations	n=25-32, feces	16S rRNA gene sequencing	(Brenner et al., 2018)
Human, ALS Patients	Increased *Dorea* Decreased *Oscillibacter, Anaerostipes, Lachnospiraceae*	n=5-6, feces	16S rRNA gene sequencing	(Fang et al., 2016)
Human, ALS Patients	Marginally increased *Anaerostipes hadrus and Bacteroidales bacterium* Marginally decreased *Bifidobacterium pseudocatenulatum*, *Clostridium leptum* and *Escherichia coli*	n=29-37, feces	Shotgun metagenomic sequencing	(Blacher et al., 2019)
Mice, $SOD1^{G93A}$	Decreased *Butyrivibrio Fibrisolvens*	n=3, feces	16S rRNA gene sequencing	(Zhang et al., 2017b)
Mice, $SOD1^{G93A}$	Decreased *Akkermansia muciniphila*, *Parabaceroides distasonis*, *Rikenellaceae*, *Prevotella*, *Lactobacillus murinus*, *Alistipes unclassified*, *Eggertella unclassified*, Increased *Sutterella*, *Allobaculum*, *Desulfovibrionaceae*, *Coprococcus*, *Oscillospira*, *Bifidobacterium*, *Helicobacter hepaticus*, *Lactobacillus johnsonii and Lactobacillus reuteri*	n=6, feces	16S rRNA gene sequencing, Shotgun metagenomic sequencing	(Blacher et al., 2019)
Huntington's disease: Microbiota alterations in humans and animal models				
Subject	**Microbiota Alterations**	**Sample**	**Method**	**Reference**
Mice, R6/1	Sex difference in the bacterial signature of R6/1 mice. Male: an increase in *Bacteroidales*, *Lactobacillales* and a decrease in *Clostridiales* Female: an increase in *Coriobacteriales*, *Erysipelotrichales*, *Bacteroidales*, *Burkholderiale* and a decrease in *Clostridiales*	n=7-8, feces	16S rRNA gene sequencing	(Kong et al., 2018)

Table 4.2: Effects of microbiome manipulations in animal models for NDs

Parkinson's disease: Microbial effects on pathology and behavior in animal models					
Subject	**Perturbation**	**Sample**	**Test**	**Result**	**Reference**
Pink1$^{-/-}$ mice	Oral administration of *Citrobacter rodentium*	n=5-72 males and females	Grip strength test Pole test Open field test Flow cytometry for brain infiltrated immune subsets Histology for dopaminergic neurons in the striatum	Impaired motor ability shown in *Citrobacter rodentium* treated Pink1$^{-/-}$ mice Decreased density of dopaminergic axonal varicosities and enhanced CD8^{+} T cells in the brain of *Citrobacter rodentium* treated Pink1$^{-/-}$ mice	(Matheoud et al., 2019)
Thy1-αSyn mice	Administration of microbiota from PD patients	n=3-6 males	Beam traversal test Pole descent test Nasal adhesive removal test Hindlimb clasping reflexes	PD-derived gut microbiota promotes motor impairments	(Sampson et al., 2016)
Thy1-αSyn mice	GF vs SPF	n=4-6 males	Beam traversal test Pole descent test Nasal adhesive removal test Hindlimb clasping reflex Western blot and immunofluorescence staining for a-Syn aggregation Representative 3D reconstructions of Iba1-stained microglia in the caudoputamen	Reduced locomotor deficits, a-syn accumulation and decreased activation of microglia in GF Thy1-αSyn mice	(Sampson et al., 2016)
Thy1-αSyn mice	Drinking water containing 10 µg/ml LPS 12 consecutive days treatment	n is unknown males and females	Hindlimb clasping reflex	Mildly exacerbated motor impairments in LPS treated Thy1-αSyn mice	(Gorecki et al., 2019)
MPTP mouse model Rotenone mouse model	Probiotic cocktail containing *Lactobacillus rhamnosus GG*, *Bifidobacterium animalis lactis* and *Lactobacillus acidophilus* 4 weeks treatment	n=3-4 males	Cylinder test Beam traversal test Challenge beam test Stride length test Histology for dopaminergic neurons and glial activation in the striatum and substantial nigra	Mitigated behavioral impairments, ameliorated dopaminergic neuronal loss and gliosis in probiotics treated MPTP and rotenone mouse model	(Srivastav et al., 2019)

	before animal model establishment				
6-OHDA rat model	Antibiotic (neomycin, 2 mg/ mL; vancomycin 0.2 mg/mL; bacitracin, 0.5 mg/mL; pimaricin 1.2 µg/ mL) treatment for 14 days before animal model establishment	n=4-12 males	Cylinder test Forepaw adjusted steps test Amphetamine-induced rotation Histology for TH neuron loss in striatum and substantial nigra Q-PCR for expression of proinflammatory cytokines in striatum	Attenuated motor deficits, decreased dopaminergic neuron loss and lower expression of proinflammatory cytokines in antibiotic treated 6-OHDA rat model	(Koutzoumis et al., 2020)
MPTP mouse model	Fecal microbiota of transplantation after animal model establishment	n=15 males	Pole test Traction test Histology for dopaminergic neuron loss and glial activation in the substantial nigra Western blot for striatal TH expression	Attenuated motor deficits, decreased dopaminergic neuron loss and glial activation in the MPTP mouse model transplanted fecal microbiota from wildtype littermates	(Sun et al., 2018)
Caenorhabditis elegans model of synucleinopathy	*Bacillus subtilis* probiotic strain PXN21 feeding	n=25 sex unknown	Histology for αSyn aggregation	reduced αSyn aggregation in the C. elegans feed with Bacillus subtilis	(Goya et al., 2020)
MPTP mouse model	Oral administration of *Proteus mirabilis*	n=9-12 males	Rotarod test Open field test Histology for dopaminergic neuron loss and glial activation in the substantial nigra Histology for TH positive axons loss in the striatum	Exacerbated motor deficits, loss of dopaminergic neurons and glial activation in MPTP mouse model received *Proteus mirabilis*	(Choi et al., 2018)
MPTP mouse model	antibiotic (ampicillin 1 g/L, neomycin sulfate 1 g/L, metronidazole 1 g/L) treatment before animal model establishment	n=10 males	Histology for dopaminergic neuron loss in the substantial nigra	Attenuated dopaminergic neuron loss in the antibiotic treated MPTP mouse model	(Pu et al., 2019)

Alzheimer's disease: Microbial effects on pathology and behavior in mice					
Subject	**Perturbation**	**Sample**	**Test**	**Result**	**Reference**
APP/PS1 mice	GF vs CONV	n=5-8 males and females	Histopathology for Aβ in brain Immunostaining for microglia Western blot for Aβ ELISA for Aβ38, Aβ40, Aβ42, and cytokines	For GF-APP/PS1 mice: lower Aβ deposition in cortex and hippocampus, Aβ levels in western blot and Aβ42 levels in ELISA reduction in Iba-1 positive microglial leading to reduction in cortical neuroinflammation For CONV-APP/PS1 mice: increase in IL-1β, INF-χ, IL-2, IL-5	(Harach et al., 2017)
APP/PS1 mice	Antibiotics (gentamycin 1mg/mL, vancomycin 0.5mg/mL, metronidazole 2mg/mL, neomycin 0.5mg/mL, ampicillin 1mg/mL, kanamycin 3mg/mL, colistin 6000U/mL, cefaperazone 1mg/mL) for 1 week	n=5-14 males	Flow cytometry for immune cells Immunohistochemistry for Aβ, Iba-1, GFAP ELISA for Aβ Cytokine/chemokine array	For ABX treated mice: reduced Aβ deposition in cortex and hippocampus reduction in Aβ plaque localized microglial and astrocytes leading to reduction in neuroinflammation elevation in $FOXP3^+$ T_{regs} upregulation of CCL11, IL-1β, IL-2, IL-3, SCF and downregulation of IL-6	(Minter et al., 2017)
ADLP mice	Fecal microbiota transplant daily for 4 months	n=14-16 sex unknown	Behavioral tests: Y-maze, contextual fear conditioning, open field ELISA for Aβ Immunohistochemistry for Aβ, tau, GFAP	For FMT treated mice: better performance on behavioral tests reduction in Aβ deposition in frontal cortex and hippocampus reduction in cortex Aβ40 levels reduction in tau aggregates in hippocampus reduction in activated microglia and astrocytes in frontal cortex	(Kim et al., 2020)
APP/PS1 mice	Fecal microbiota transplant daily for 4 weeks	n=4-6 males	Behavioral tests: Morris Water Maze, object recognition test ELISA for Aβ40 and Aβ42 Western blot for tau Immunostaining for	For FMT treated mice: better performance on behavioral tests compared to controls reduction in Aβ deposition in cortex and hippocampus and in Aβ40/Aβ42 levels reduction in tau phosphorylation	(Sun et al., 2019b)

			PSD-95, CD11b and COX-2 NMR for SCFAs	increase in PSD-95 staining reduction in COX-2 and CD11b levels increase in butyrate levels	
APP/PS1 mice	Oral administration of *Clostridium butyricum* for 4 weeks	n=20 sex unknown	Behavioral tests: Morris Water Maze, object recognition test Histology for Aβ and CD11b ELISA for Aβ42 Butyrate assay	Treated mice performed better on behavioral tests compared to controls Decrease of Aβ levels in the brain for treated mice Reduction of activated microglial in treated mice Reduction of IL-1β and TNF-α in treated mice Higher levels of butyrate in treated mice	(Sun et al., 2020)
3xTg mice	SLAB51 probiotic treatment	n=48-64 males	Behavioral test: open field, novel object recognition and elevated maze GC for SCFAs ELISA for cytokines Histology and immunostaining for Aβ Western blot for tau	For SLAB51 treated mice: better performance on behavioral tests Reduction in Aβ deposition in hippocampus Reduction in tau phosphorylation Reduction in oxidative stress	(Bonfili et al., 2017; Bonfili et al., 2018; Bonfili et al., 2019)

Amyotrophic lateral sclerosis: Microbial effects on pathology and behavior in mice

Subject	Perturbation	Sample	Test	Result	Reference
$SOD1^{G93A}$ mice	2% sodium butyrate in water	n=5-10 sex unknown	ALS progression (body weight loss, lifespan) Intestinal tight junction (western blot and immunofluorescence staining for ZO-1)	Delayed ALS progression Increased the intestinal tight junction	(Zhang et al., 2017b)
$SOD1^{G93A}$ mice	Repeated oral administration of *Akkermansia muciniphila* into antibiotic pre-treated Sod1 mice at 6-day intervals for a total of 15 treatments.	n= 5-61 males and females	lifespan analysis rotarod locomotor test Hanging-wire grip test Histological staining for spinal cord T2 relaxation time	Extended lifespan Ameliorated locomotor deficits Increased number of motor neurons in spinal cord Reduced brain atrophy	(Blacher et al., 2019)

Huntington's disease: Microbial effects on pathology and behavior in mice

Subject	Perturbation	Sample	Test	Result	Reference
BACHD mice	GF vs SPF	n=4-12 males and	Transmission electron microscopy for myelination	Reduced levels of myelin-related proteins and decreased numbers of	(Radulescu et al., 2019)

		females	Q-PCR and western blot for myelin based protein	mature oligodendrocytes in the prefrontal cortex of BACHD mice reared in GF condition	

References:

Aho, V.T.E., Pereira, P.A.B., Voutilainen, S., Paulin, L., Pekkonen, E., Auvinen, P., and Scheperjans, F. (2019). Gut microbiota in Parkinson's disease: Temporal stability and relations to disease progression. EBioMedicine *44*, 691-707.

Alonso, R., Pisa, D., and Carrasco, L. (2019). Brain Microbiota in Huntington's Disease Patients. Front Microbiol *10*, 2622.

Barcena, C., Valdes-Mas, R., Mayoral, P., Garabaya, C., Durand, S., Rodriguez, F., Fernandez-Garcia, M.T., Salazar, N., Nogacka, A.M., Garatachea, N.*, et al.* (2019). Healthspan and lifespan extension by fecal microbiota transplantation into progeroid mice. Nat Med *25*, 1234-1242.

Bedarf, J.R., Hildebrand, F., Coelho, L.P., Sunagawa, S., Bahram, M., Goeser, F., Bork, P., and Wullner, U. (2017). Functional implications of microbial and viral gut metagenome changes in early stage L-DOPA-naive Parkinson's disease patients. Genome Med *9*, 39.

Bennett, J.P., Jr., Keeney, P.M., and Brohawn, D.G. (2019). RNA Sequencing Reveals Small and Variable Contributions of Infectious Agents to Transcriptomes of Postmortem Nervous Tissues From Amyotrophic Lateral Sclerosis, Alzheimer's Disease and Parkinson's Disease Subjects, and Increased Expression of Genes From Disease-Activated Microglia. Front Neurosci *13*, 235.

Berti, V., Walters, M., Sterling, J., Quinn, C.G., Logue, M., Andrews, R., Matthews, D.C., Osorio, R.S., Pupi, A., Vallabhajosula, S.*, et al.* (2018). Mediterranean diet and 3-year Alzheimer brain biomarker changes in middle-aged adults. Neurology *90*, e1789-e1798.

Beydoun, M.A., Beydoun, H.A., Elbejjani, M., Dore, G.A., and Zonderman, A.B. (2018). Helicobacter pylori seropositivity and its association with incident all-cause and Alzheimer's disease dementia in large national surveys. Alzheimers Dement *14*, 1148-1158.

Biagi, E., Franceschi, C., Rampelli, S., Severgnini, M., Ostan, R., Turroni, S., Consolandi, C., Quercia, S., Scurti, M., Monti, D.*, et al.* (2016). Gut Microbiota and Extreme Longevity. Curr Biol *26*, 1480-1485.

Blacher, E., Bashiardes, S., Shapiro, H., Rothschild, D., Mor, U., Dori-Bachash, M., Kleimeyer, C., Moresi, C., Harnik, Y., Zur, M.*, et al.* (2019). Potential roles of gut microbiome and metabolites in modulating ALS in mice. Nature *572*, 474-480.

Bloch, A., Probst, A., Bissig, H., Adams, H., and Tolnay, M. (2006). Alpha-synuclein pathology of the spinal and peripheral autonomic nervous system in neurologically unimpaired elderly subjects. Neuropathol Appl Neurobiol *32*, 284-295.

Bonfili, L., Cecarini, V., Berardi, S., Scarpona, S., Suchodolski, J.S., Nasuti, C., Fiorini, D., Boarelli, M.C., Rossi, G., and Eleuteri, A.M. (2017). Microbiota modulation counteracts Alzheimer's disease progression influencing neuronal proteolysis and gut hormones plasma levels. Sci Rep *7*, 2426.

Bonfili, L., Cecarini, V., Cuccioloni, M., Angeletti, M., Berardi, S., Scarpona, S., Rossi, G., and Eleuteri, A.M. (2018). SLAB51 Probiotic Formulation Activates SIRT1 Pathway Promoting Antioxidant and Neuroprotective Effects in an AD Mouse Model. Mol Neurobiol *55*, 7987-8000.

Bonfili, L., Cecarini, V., Gogoi, O., Berardi, S., Scarpona, S., Angeletti, M., Rossi, G., and Eleuteri, A.M. (2019). Gut microbiota manipulation through probiotics oral administration restores glucose homeostasis in a mouse model of Alzheimer's disease. Neurobiol Aging.

Braak, H., and Braak, E. (2000). Pathoanatomy of Parkinson's disease. J Neurol *247 Suppl 2*, II3-10.

Braak, H., de Vos, R.A., Bohl, J., and Del Tredici, K. (2006). Gastric alpha-synuclein immunoreactive inclusions in Meissner's and Auerbach's plexuses in cases staged for Parkinson's disease-related brain pathology. Neurosci Lett *396*, 67-72.

Braak, H., Del Tredici, K., Bratzke, H., Hamm-Clement, J., Sandmann-Keil, D., and Rub, U. (2002). Staging of the intracerebral inclusion body pathology associated with idiopathic Parkinson's disease (preclinical and clinical stages). J Neurol *249 Suppl 3*, III/1-5.

Braak, H., Del Tredici, K., Rub, U., de Vos, R.A., Jansen Steur, E.N., and Braak, E. (2003). Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol Aging *24*, 197-211.

Braak, H., Rub, U., Sandmann-Keil, D., Gai, W.P., de Vos, R.A., Jansen Steur, E.N., Arai, K., and Braak, E. (2000). Parkinson's disease: affection of brain stem nuclei controlling premotor and motor neurons of the somatomotor system. Acta Neuropathol *99*, 489-495.

Brandscheid, C., Schuck, F., Reinhardt, S., Schafer, K.H., Pietrzik, C.U., Grimm, M., Hartmann, T., Schwiertz, A., and Endres, K. (2017). Altered Gut Microbiome Composition and Tryptic Activity of the 5xFAD Alzheimer's Mouse Model. J Alzheimers Dis *56*, 775-788.

Brenner, D., Hiergeist, A., Adis, C., Mayer, B., Gessner, A., Ludolph, A.C., and Weishaupt, J.H. (2018). The fecal microbiome of ALS patients. Neurobiol Aging *61*, 132-137.

Cersosimo, M.G., and Benarroch, E.E. (2012). Pathological correlates of gastrointestinal dysfunction in Parkinson's disease. Neurobiol Dis *46*, 559-564.

Challis, C., Hori, A., Sampson, T.R., Yoo, B.B., Challis, R.C., Hamilton, A.M., Mazmanian, S.K., Volpicelli-Daley, L.A., and Gradinaru, V. (2020). Gut-seeded alpha-synuclein fibrils promote gut dysfunction and brain pathology specifically in aged mice. Nat Neurosci *23*, 327-336.

Choi, J.G., Kim, N., Ju, I.G., Eo, H., Lim, S.M., Jang, S.E., Kim, D.H., and Oh, M.S. (2018). Oral administration of Proteus mirabilis damages dopaminergic neurons and motor functions in mice. Sci Rep *8*, 1275.

Chorell, E., Andersson, E., Evans, M.L., Jain, N., Gotheson, A., Aden, J., Chapman, M.R., Almqvist, F., and Wittung-Stafshede, P. (2015). Bacterial Chaperones CsgE and CsgC Differentially Modulate Human alpha-Synuclein Amyloid Formation via Transient Contacts. PLoS One *10*, e0140194.

Claesson, M.J., Cusack, S., O'Sullivan, O., Greene-Diniz, R., de Weerd, H., Flannery, E., Marchesi, J.R., Falush, D., Dinan, T., Fitzgerald, G.*, et al.* (2011). Composition, variability, and temporal stability of the intestinal microbiota of the elderly. Proc Natl Acad Sci U S A *108 Suppl 1*, 4586-4591.

Clark, R.I., Salazar, A., Yamada, R., Fitz-Gibbon, S., Morselli, M., Alcaraz, J., Rana, A., Rera, M., Pellegrini, M., Ja, W.W.*, et al.* (2015). Distinct Shifts in Microbiota Composition during Drosophila Aging Impair Intestinal Function and Drive Mortality. Cell Rep *12*, 1656-1667.

Cox, L.M., Schafer, M.J., Sohn, J., Vincentini, J., Weiner, H.L., Ginsberg, S.D., and Blaser, M.J. (2019). Calorie restriction slows age-related microbiota changes in an Alzheimer's disease model in female mice. Sci Rep *9*, 17904.

Cryan, J.F., O'Riordan, K.J., Sandhu, K., Peterson, V., and Dinan, T.G. (2020). The gut microbiome in neurological disorders. Lancet Neurol *19*, 179-194.

Dill-McFarland, K.A., Tang, Z.Z., Kemis, J.H., Kerby, R.L., Chen, G., Palloni, A., Sorenson, T., Rey, F.E., and Herd, P. (2019). Close social relationships correlate with human gut microbiota composition. Sci Rep *9*, 703.

Dodiya, H.B., Forsyth, C.B., Voigt, R.M., Engen, P.A., Patel, J., Shaikh, M., Green, S.J., Naqib, A., Roy, A., Kordower, J.H.*, et al.* (2020). Chronic stress-induced gut dysfunction exacerbates Parkinson's disease phenotype and pathology in a rotenone-induced mouse model of Parkinson's disease. Neurobiol Dis *135*, 104352.

Dominy, S.S., Lynch, C., Ermini, F., Benedyk, M., Marczyk, A., Konradi, A., Nguyen, M., Haditsch, U., Raha, D., Griffin, C.*, et al.* (2019). Porphyromonas gingivalis in Alzheimer's disease brains: Evidence for disease causation and treatment with small-molecule inhibitors. Sci Adv *5*, eaau3333.

Drolet, R.E., Cannon, J.R., Montero, L., and Greenamyre, J.T. (2009). Chronic rotenone exposure reproduces Parkinson's disease gastrointestinal neuropathology. Neurobiol Dis *36*, 96-102.

Ezzat, K., Pernemalm, M., Palsson, S., Roberts, T.C., Jarver, P., Dondalska, A., Bestas, B., Sobkowiak, M.J., Levanen, B., Skold, M.*, et al.* (2019). The viral protein corona directs viral pathogenesis and amyloid aggregation. Nat Commun *10*, 2331.

Fang, X., Wang, X., Yang, S., Meng, F., Wang, X., Wei, H., and Chen, T. (2016). Evaluation of the Microbial Diversity in Amyotrophic Lateral Sclerosis Using High-Throughput Sequencing. Front Microbiol *7*, 1479.

Fasano, A., Visanji, N.P., Liu, L.W., Lang, A.E., and Pfeiffer, R.F. (2015). Gastrointestinal dysfunction in Parkinson's disease. Lancet Neurol *14*, 625-639.

Fransen, F., van Beek, A.A., Borghuis, T., Aidy, S.E., Hugenholtz, F., van der Gaast-de Jongh, C., Savelkoul, H.F.J., De Jonge, M.I., Boekschoten, M.V., Smidt, H.*, et al.* (2017). Aged Gut Microbiota Contributes to Systemical Inflammaging after Transfer to Germ-Free Mice. Front Immunol *8*, 1385.

Fung, T.C., Olson, C.A., and Hsiao, E.Y. (2017). Interactions between the microbiota, immune and nervous systems in health and disease. Nat Neurosci *20*, 145-155.

Giau, V.V., Wu, S.Y., Jamerlan, A., An, S.S.A., Kim, S.Y., and Hulme, J. (2018). Gut Microbiota and Their Neuroinflammatory Implications in Alzheimer's Disease. Nutrients *10*.

Gordon, H.A., Bruckner-Kardoss, E., and Wostmann, B.S. (1966). Aging in germ-free mice: life tables and lesions observed at natural death. J Gerontol *21*, 380-387.

Gorecki, A.M., Preskey, L., Bakeberg, M.C., Kenna, J.E., Gildenhuys, C., MacDougall, G., Dunlop, S.A., Mastaglia, F.L., Akkari, P.A., Koengten, F.*, et al.* (2019). Altered Gut Microbiome in Parkinson's Disease and the Influence of Lipopolysaccharide in a Human alpha-Synuclein Over-Expressing Mouse Model. Front Neurosci *13*, 839.

Goya, M.E., Xue, F., Sampedro-Torres-Quevedo, C., Arnaouteli, S., Riquelme-Dominguez, L., Romanowski, A., Brydon, J., Ball, K.L., Stanley-Wall, N.R., and Doitsidou, M. (2020). Probiotic Bacillus subtilis Protects against alpha-Synuclein Aggregation in C. elegans. Cell Rep *30*, 367-380 e367.

Grathwohl, S., Quansah, E., Maroof, N., Steiner, J.A., Spycher, L., Benmansour, F., Duran-Pacheco, G., Siebourg-Polster, J., Oroszlan-Szovik, K., Remy, H.*, et al.* (2019). Experimental colitis drives enteric alpha-synuclein accumulation and Parkinson-like brain pathology. 505164.

Harach, T., Marungruang, N., Duthilleul, N., Cheatham, V., Mc Coy, K.D., Frisoni, G., Neher, J.J., Fak, F., Jucker, M., Lasser, T.*, et al.* (2017). Reduction of Abeta amyloid pathology in APPPS1 transgenic mice in the absence of gut microbiota. Sci Rep *7*, 41802.

Hasegawa, S., Goto, S., Tsuji, H., Okuno, T., Asahara, T., Nomoto, K., Shibata, A., Fujisawa, Y., Minato, T., Okamoto, A.*, et al.* (2015). Intestinal Dysbiosis and Lowered Serum Lipopolysaccharide-Binding Protein in Parkinson's Disease. PLoS One *10*, e0142164.

Hawkes, C.H., Del Tredici, K., and Braak, H. (2007). Parkinson's disease: a dual-hit hypothesis. Neuropathol Appl Neurobiol *33*, 599-614.

Heintz-Buschart, A., Pandey, U., Wicke, T., Sixel-Doring, F., Janzen, A., Sittig-Wiegand, E., Trenkwalder, C., Oertel, W.H., Mollenhauer, B., and Wilmes, P. (2018). The nasal and gut microbiome in Parkinson's disease and idiopathic rapid eye movement sleep behavior disorder. Mov Disord *33*, 88-98.

Hill-Burns, E.M., Debelius, J.W., Morton, J.T., Wissemann, W.T., Lewis, M.R., Wallen, Z.D., Peddada, S.D., Factor, S.A., Molho, E., Zabetian, C.P.*, et al.* (2017). Parkinson's disease and Parkinson's disease medications have distinct signatures of the gut microbiome. Mov Disord *32*, 739-749.

Holmqvist, S., Chutna, O., Bousset, L., Aldrin-Kirk, P., Li, W., Bjorklund, T., Wang, Z.Y., Roybon, L., Melki, R., and Li, J.Y. (2014). Direct evidence of Parkinson pathology spread from the gastrointestinal tract to the brain in rats. Acta Neuropathol *128*, 805-820.

Hopfner, F., Kunstner, A., Muller, S.H., Kunzel, S., Zeuner, K.E., Margraf, N.G., Deuschl, G., Baines, J.F., and Kuhlenbaumer, G. (2017). Gut microbiota in Parkinson disease in a northern German cohort. Brain Res *1667*, 41-45.

Hui, K.Y., Fernandez-Hernandez, H., Hu, J., Schaffner, A., Pankratz, N., Hsu, N.Y., Chuang, L.S., Carmi, S., Villaverde, N., Li, X.*, et al.* (2018). Functional variants in the LRRK2 gene confer shared effects on risk for Crohn's disease and Parkinson's disease. Sci Transl Med *10*.

Iatsenko, I., Boquete, J.P., and Lemaitre, B. (2018). Microbiota-Derived Lactate Activates Production of Reactive Oxygen Species by the Intestinal NADPH Oxidase Nox and Shortens Drosophila Lifespan. Immunity *49*, 929-942 e925.

Ilievski, V., Zuchowska, P.K., Green, S.J., Toth, P.T., Ragozzino, M.E., Le, K., Aljewari, H.W., O'Brien-Simpson, N.M., Reynolds, E.C., and Watanabe, K. (2018). Chronic oral application of a periodontal pathogen results in brain inflammation, neurodegeneration and amyloid beta production in wild type mice. PLoS One *13*, e0204941.

Ishida, N., Ishihara, Y., Ishida, K., Tada, H., Funaki-Kato, Y., Hagiwara, M., Ferdous, T., Abdullah, M., Mitani, A., Michikawa, M.*, et al.* (2017). Periodontitis induced by bacterial infection exacerbates features of Alzheimer's disease in transgenic mice. NPJ Aging Mech Dis *3*, 15.

Itzhaki, R.F. (2017). Herpes simplex virus type 1 and Alzheimer's disease: possible mechanisms and signposts. FASEB J *31*, 3216-3226.

Johnson, M.E., Stecher, B., Labrie, V., Brundin, L., and Brundin, P. (2019). Triggers, Facilitators, and Aggravators: Redefining Parkinson's Disease Pathogenesis. Trends Neurosci *42*, 4-13.

Johnson, M.E., Stringer, A., and Bobrovskaya, L. (2018). Rotenone induces gastrointestinal pathology and microbiota alterations in a rat model of Parkinson's disease. Neurotoxicology *65*, 174-185.

Kelly, L.P., Carvey, P.M., Keshavarzian, A., Shannon, K.M., Shaikh, M., Bakay, R.A., and

Kordower, J.H. (2014). Progression of intestinal permeability changes and alpha-synuclein expression in a mouse model of Parkinson's disease. Mov Disord *29*, 999-1009.

Keshavarzian, A., Green, S.J., Engen, P.A., Voigt, R.M., Naqib, A., Forsyth, C.B., Mutlu, E., and Shannon, K.M. (2015). Colonic bacterial composition in Parkinson's disease. Mov Disord *30*, 1351-1360.

Kim, M.S., Kim, Y., Choi, H., Kim, W., Park, S., Lee, D., Kim, D.K., Kim, H.J., Choi, H., Hyun, D.W.*, et al.* (2020). Transfer of a healthy microbiota reduces amyloid and tau pathology in an Alzheimer's disease animal model. Gut *69*, 283-294.

Kim, S., Kwon, S.H., Kam, T.I., Panicker, N., Karuppagounder, S.S., Lee, S., Lee, J.H., Kim, W.R., Kook, M., Foss, C.A.*, et al.* (2019). Transneuronal Propagation of Pathologic alpha-Synuclein from the Gut to the Brain Models Parkinson's Disease. Neuron *103*, 627-641 e627.

Kong, G., Cao, K.L., Judd, L.M., Li, S., Renoir, T., and Hannan, A.J. (2018). Microbiome profiling reveals gut dysbiosis in a transgenic mouse model of Huntington's disease. Neurobiol Dis, 104268.

Koutzoumis, D.N., Vergara, M., Pino, J., Buddendorff, J., Khoshbouei, H., Mandel, R.J., and Torres, G.E. (2020). Alterations of the gut microbiota with antibiotics protects dopamine neuron loss and improve motor deficits in a pharmacological rodent model of Parkinson's disease. Exp Neurol *325*, 113159.

Kowalski, K., and Mulak, A. (2019). Brain-Gut-Microbiota Axis in Alzheimer's Disease. J Neurogastroenterol Motil *25*, 48-60.

Kundu, P., Blacher, E., Elinav, E., and Pettersson, S. (2017). Our Gut Microbiome: The Evolving Inner Self. Cell *171*, 1481-1493.

Lai, F., Jiang, R., Xie, W., Liu, X., Tang, Y., Xiao, H., Gao, J., Jia, Y., and Bai, Q. (2018). Intestinal Pathology and Gut Microbiota Alterations in a Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) Mouse Model of Parkinson's Disease. Neurochem Res *43*, 1986-1999.

Langille, M.G., Meehan, C.J., Koenig, J.E., Dhanani, A.S., Rose, R.A., Howlett, S.E., and Beiko, R.G. (2014). Microbial shifts in the aging mouse gut. Microbiome *2*, 50.

Lee, W.J., and Hase, K. (2014). Gut microbiota-generated metabolites in animal health and disease. Nat Chem Biol *10*, 416-424.

Li, F., Wang, P., Chen, Z., Sui, X., Xie, X., and Zhang, J. (2019). Alteration of the fecal microbiota in North-Eastern Han Chinese population with sporadic Parkinson's disease. Neurosci Lett *707*, 134297.

Li, W., Wu, X., Hu, X., Wang, T., Liang, S., Duan, Y., Jin, F., and Qin, B. (2017). Structural changes of gut microbiota in Parkinson's disease and its correlation with clinical features. Sci China Life Sci *60*, 1223-1233.

Lin, A., Zheng, W., He, Y., Tang, W., Wei, X., He, R., Huang, W., Su, Y., Huang, Y., Zhou, H.*, et al.* (2018). Gut microbiota in patients with Parkinson's disease in southern China. Parkinsonism Relat Disord *53*, 82-88.

Lozupone, C.A., Stombaugh, J.I., Gordon, J.I., Jansson, J.K., and Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. Nature *489*, 220-230.

Maini Rekdal, V., Bess, E.N., Bisanz, J.E., Turnbaugh, P.J., and Balskus, E.P. (2019). Discovery and inhibition of an interspecies gut bacterial pathway for Levodopa metabolism. Science *364*.

Matheoud, D., Cannon, T., Voisin, A., Penttinen, A.M., Ramet, L., Fahmy, A.M., Ducrot, C., Laplante, A., Bourque, M.J., Zhu, L.*, et al.* (2019). Intestinal infection triggers Parkinson's disease-like symptoms in Pink1(-/-) mice. Nature *571*, 565-569.

Menegon, A., Board, P.G., Blackburn, A.C., Mellick, G.D., and Le Couteur, D.G. (1998). Parkinson's disease, pesticides, and glutathione transferase polymorphisms. Lancet *352*, 1344-1346.

Minter, M.R., Hinterleitner, R., Meisel, M., Zhang, C., Leone, V., Zhang, X., Oyler-Castrillo, P., Zhang, X., Musch, M.W., Shen, X.*, et al.* (2017). Antibiotic-induced perturbations in microbial diversity during post-natal development alters amyloid pathology in an aged APPSWE/PS1DeltaE9 murine model of Alzheimer's disease. Sci Rep *7*, 10411.

Miraglia, F., Ricci, A., Rota, L., and Colla, E. (2018). Subcellular localization of alpha-synuclein aggregates and their interaction with membranes. Neural Regen Res *13*, 1136-1144.

Moir, R.D., Lathe, R., and Tanzi, R.E. (2018). The antimicrobial protection hypothesis of Alzheimer's disease. Alzheimers Dement *14*, 1602-1614.

Nagpal, R., Neth, B.J., Wang, S., Craft, S., and Yadav, H. (2019). Modified Mediterranean-ketogenic diet modulates gut microbiome and short-chain fatty acids in association with Alzheimer's disease markers in subjects with mild cognitive impairment. EBioMedicine *47*, 529-542.

Nezu, A., Kubota, T., Maruyama, S., Nagata, M., Nohno, K., Morozumi, T., and Yoshie, H. (2017). Expression of neprilysin in periodontitis-affected gingival tissues. Arch Oral Biol *79*, 35-41.

Noorian, A.R., Rha, J., Annerino, D.M., Bernhard, D., Taylor, G.M., and Greene, J.G. (2012). Alpha-synuclein transgenic mice display age-related slowing of gastrointestinal motility associated with transgene expression in the vagal system. Neurobiol Dis *48*, 9-19.

O'Toole, P.W., and Jeffery, I.B. (2015). Gut microbiota and aging. Science *350*, 1214-1215.

Obata, F., Fons, C.O., and Gould, A.P. (2018). Early-life exposure to low-dose oxidants can increase longevity via microbiome remodelling in Drosophila. Nat Commun *9*, 975.

Odamaki, T., Kato, K., Sugahara, H., Hashikura, N., Takahashi, S., Xiao, J.Z., Abe, F., and Osawa, R. (2016). Age-related changes in gut microbiota composition from newborn to centenarian: a cross-sectional study. BMC Microbiol *16*, 90.

Olsen, A., Jonsson, A., and Normark, S. (1989). Fibronectin binding mediated by a novel class of surface organelles on Escherichia coli. Nature *338*, 652-655.

Olson, C.A., Vuong, H.E., Yano, J.M., Liang, Q.Y., Nusbaum, D.J., and Hsiao, E.Y. (2018). The Gut Microbiota Mediates the Anti-Seizure Effects of the Ketogenic Diet. Cell *173*, 1728-1741 e1713.

Pan-Montojo, F., Anichtchik, O., Dening, Y., Knels, L., Pursche, S., Jung, R., Jackson, S., Gille, G., Spillantini, M.G., Reichmann, H.*, et al.* (2010). Progression of Parkinson's disease pathology is reproduced by intragastric administration of rotenone in mice. PLoS One *5*, e8762.

Pan-Montojo, F., Schwarz, M., Winkler, C., Arnhold, M., O'Sullivan, G.A., Pal, A., Said, J., Marsico, G., Verbavatz, J.M., Rodrigo-Angulo, M.*, et al.* (2012). Environmental toxins trigger PD-like progression via increased alpha-synuclein release from enteric neurons in mice. Sci Rep *2*, 898.

Peng, C., Gathagan, R.J., Covell, D.J., Medellin, C., Stieber, A., Robinson, J.L., Zhang, B., Pitkin, R.M., Olufemi, M.F., Luk, K.C.*, et al.* (2018). Cellular milieu imparts distinct pathological alpha-synuclein strains in alpha-synucleinopathies. Nature *557*, 558-563.

Perez-Pardo, P., Dodiya, H.B., Engen, P.A., Forsyth, C.B., Huschens, A.M., Shaikh, M., Voigt, R.M., Naqib, A., Green, S.J., Kordower, J.H.*, et al.* (2019). Role of TLR4 in the gut-brain axis in Parkinson's disease: a translational study from men to mice. Gut *68*, 829-843.

Perez-Pardo, P., Dodiya, H.B., Engen, P.A., Naqib, A., Forsyth, C.B., Green, S.J., Garssen, J., Keshavarzian, A., and Kraneveld, A.D. (2018). Gut bacterial composition in a mouse model of Parkinson's disease. Benef Microbes *9*, 799-814.

Peter, I., Dubinsky, M., Bressman, S., Park, A., Lu, C., Chen, N., and Wang, A. (2018). Anti-Tumor Necrosis Factor Therapy and Incidence of Parkinson Disease Among Patients With Inflammatory Bowel Disease. JAMA Neurol *75*, 939-946.

Petrov, V.A., Saltykova, I.V., Zhukova, I.A., Alifirova, V.M., Zhukova, N.G., Dorofeeva, Y.B., Tyakht, A.V., Kovarsky, B.A., Alekseev, D.G., Kostryukova, E.S.*, et al.* (2017). Analysis of Gut Microbiota in Patients with Parkinson's Disease. Bull Exp Biol Med *162*, 734-737.

Pfeiffer, R.F. (2011). Gastrointestinal dysfunction in Parkinson's disease. Parkinsonism Relat Disord *17*, 10-15.

Pu, Y., Chang, L., Qu, Y., Wang, S., Zhang, K., and Hashimoto, K. (2019). Antibiotic-induced microbiome depletion protects against MPTP-induced dopaminergic neurotoxicity in the brain. Aging (Albany NY) *11*, 6915-6929.

Qian, Y., Yang, X., Xu, S., Wu, C., Song, Y., Qin, N., Chen, S.D., and Xiao, Q. (2018). Alteration of the fecal microbiota in Chinese patients with Parkinson's disease. Brain Behav Immun *70*, 194-202.

Radulescu, C.I., Garcia-Miralles, M., Sidik, H., Bardile, C.F., Yusof, N., Lee, H.U., Ho, E.X.P., Chu, C.W., Layton, E., Low, D.*, et al.* (2019). Manipulation of microbiota reveals altered callosal myelination and white matter plasticity in a model of Huntington disease. Neurobiol Dis *127*, 65-75.

Rainey-Smith, S.R., Gu, Y., Gardener, S.L., Doecke, J.D., Villemagne, V.L., Brown, B.M., Taddei, K., Laws, S.M., Sohrabi, H.R., Weinborn, M.*, et al.* (2018). Mediterranean diet

adherence and rate of cerebral Abeta-amyloid accumulation: Data from the Australian Imaging, Biomarkers and Lifestyle Study of Ageing. Transl Psychiatry *8*, 238.

Readhead, B., Haure-Mirande, J.V., Funk, C.C., Richards, M.A., Shannon, P., Haroutunian, V., Sano, M., Liang, W.S., Beckmann, N.D., Price, N.D*., et al.* (2018). Multiscale Analysis of Independent Alzheimer's Cohorts Finds Disruption of Molecular, Genetic, and Clinical Networks by Human Herpesvirus. Neuron *99*, 64-82 e67.

Ren, T., Gao, Y., Qiu, Y., Jiang, S., Zhang, Q., Zhang, J., Wang, L., Zhang, Y., Wang, L., and Nie, K. (2020). Gut Microbiota Altered in Mild Cognitive Impairment Compared With Normal Cognition in Sporadic Parkinson's Disease. Front Neurol *11*, 137.

Sampson, T.R., Challis, C., Jain, N., Moiseyenko, A., Ladinsky, M.S., Shastri, G.G., Thron, T., Needham, B.D., Horvath, I., Debelius, J.W*., et al.* (2020). A gut bacterial amyloid promotes alpha-synuclein aggregation and motor impairment in mice. Elife *9*.

Sampson, T.R., Debelius, J.W., Thron, T., Janssen, S., Shastri, G.G., Ilhan, Z.E., Challis, C., Schretter, C.E., Rocha, S., Gradinaru, V*., et al.* (2016). Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease. Cell *167*, 1469-1480 e1412.

Scheperjans, F., Aho, V., Pereira, P.A., Koskinen, K., Paulin, L., Pekkonen, E., Haapaniemi, E., Kaakkola, S., Eerola-Rautio, J., Pohja, M*., et al.* (2015). Gut microbiota are related to Parkinson's disease and clinical phenotype. Mov Disord *30*, 350-358.

Shannon, K.M., Keshavarzian, A., Mutlu, E., Dodiya, H.B., Daian, D., Jaglin, J.A., and Kordower, J.H. (2012). Alpha-synuclein in colonic submucosa in early untreated Parkinson's disease. Mov Disord *27*, 709-715.

Smith, P., Willemsen, D., Popkes, M., Metge, F., Gandiwa, E., Reichard, M., and Valenzano, D.R. (2017). Regulation of life span by the gut microbiota in the short-lived African turquoise killifish. Elife *6*.

Sovran, B., Hugenholtz, F., Elderman, M., Van Beek, A.A., Graversen, K., Huijskes, M., Boekschoten, M.V., Savelkoul, H.F.J., De Vos, P., Dekker, J.*, et al.* (2019). Age-associated Impairment of the Mucus Barrier Function is Associated with Profound Changes in Microbiota and Immunity. Sci Rep *9*, 1437.

Srivastav, S., Neupane, S., Bhurtel, S., Katila, N., Maharjan, S., Choi, H., Hong, J.T., and Choi, D.Y. (2019). Probiotics mixture increases butyrate, and subsequently rescues the nigral dopaminergic neurons from MPTP and rotenone-induced neurotoxicity. J Nutr Biochem *69*, 73-86.

Stebegg, M., Silva-Cayetano, A., Innocentin, S., Jenkins, T.P., Cantacessi, C., Gilbert, C., and Linterman, M.A. (2019). Heterochronic faecal transplantation boosts gut germinal centres in aged mice. Nat Commun *10*, 2443.

Sun, B.L., Li, W.W., Wang, J., Xu, Y.L., Sun, H.L., Tian, D.Y., Wang, Y.J., and Yao, X.Q. (2019a). Gut Microbiota Alteration and Its Time Course in a Tauopathy Mouse Model. J Alzheimers Dis *70*, 399-412.

Sun, J., Xu, J., Ling, Y., Wang, F., Gong, T., Yang, C., Ye, S., Ye, K., Wei, D., Song, Z.*, et al.* (2019b). Fecal microbiota transplantation alleviated Alzheimer's disease-like pathogenesis in APP/PS1 transgenic mice. Transl Psychiatry *9*, 189.

Sun, J., Xu, J., Yang, B., Chen, K., Kong, Y., Fang, N., Gong, T., Wang, F., Ling, Z., and Liu, J. (2020). Effect of Clostridium butyricum against Microglia-Mediated Neuroinflammation in Alzheimer's Disease via Regulating Gut Microbiota and Metabolites Butyrate. Mol Nutr Food Res *64*, e1900636.

Sun, M.F., Zhu, Y.L., Zhou, Z.L., Jia, X.B., Xu, Y.D., Yang, Q., Cui, C., and Shen, Y.Q. (2018). Neuroprotective effects of fecal microbiota transplantation on MPTP-induced Parkinson's disease mice: Gut microbiota, glial reaction and TLR4/TNF-alpha signaling pathway. Brain Behav Immun *70*, 48-60.

Svensson, E., Horvath-Puho, E., Thomsen, R.W., Djurhuus, J.C., Pedersen, L., Borghammer, P., and Sorensen, H.T. (2015). Vagotomy and subsequent risk of Parkinson's disease. Ann Neurol *78*, 522-529.

Tavassoly, O., Sade, D., Bera, S., Shaham-Niv, S., Vocadlo, D.J., and Gazit, E. (2018). Quinolinic Acid Amyloid-like Fibrillar Assemblies Seed alpha-Synuclein Aggregation. J Mol Biol *430*, 3847-3862.

Thevaranjan, N., Puchta, A., Schulz, C., Naidoo, A., Szamosi, J.C., Verschoor, C.P., Loukov, D., Schenck, L.P., Jury, J., Foley, K.P.*, et al.* (2017). Age-Associated Microbial Dysbiosis Promotes Intestinal Permeability, Systemic Inflammation, and Macrophage Dysfunction. Cell Host Microbe *21*, 455-466 e454.

Ticinesi, A., Milani, C., Lauretani, F., Nouvenne, A., Mancabelli, L., Lugli, G.A., Turroni, F., Duranti, S., Mangifesta, M., Viappiani, A.*, et al.* (2017). Gut microbiota composition is associated with polypharmacy in elderly hospitalized patients. Sci Rep *7*, 11102.

Uemura, N., Yagi, H., Uemura, M.T., Hatanaka, Y., Yamakado, H., and Takahashi, R. (2018). Inoculation of alpha-synuclein preformed fibrils into the mouse gastrointestinal tract induces Lewy body-like aggregates in the brainstem via the vagus nerve. Mol Neurodegener *13*, 21.

Unger, M.M., Spiegel, J., Dillmann, K.U., Grundmann, D., Philippeit, H., Burmann, J., Fassbender, K., Schwiertz, A., and Schafer, K.H. (2016). Short chain fatty acids and gut microbiota differ between patients with Parkinson's disease and age-matched controls. Parkinsonism Relat Disord *32*, 66-72.

van de Steeg, E., Schuren, F.H.J., Obach, R.S., van Woudenbergh, C., Walker, G.S., Heerikhuisen, M., Nooijen, I.H.G., and Vaes, W.H.J. (2018). An Ex Vivo Fermentation Screening Platform to Study Drug Metabolism by Human Gut Microbiota. Drug Metab Dispos *46*, 1596-1607.

van Kessel, S.P., Frye, A.K., El-Gendy, A.O., Castejon, M., Keshavarzian, A., van Dijk, G., and El Aidy, S. (2019). Gut bacterial tyrosine decarboxylases restrict levels of levodopa in the treatment of Parkinson's disease. Nat Commun *10*, 310.

Vidal-Martinez, G., Chin, B., Camarillo, C., Herrera, G.V., Yang, B., Sarosiek, I., and Perez, R.G. (2020). A Pilot Microbiota Study in Parkinson's Disease Patients versus Control Subjects, and Effects of FTY720 and FTY720-Mitoxy Therapies in Parkinsonian and Multiple System Atrophy Mouse Models. J Parkinsons Dis *10*, 185-192.

Villumsen, M., Aznar, S., Pakkenberg, B., Jess, T., and Brudek, T. (2019). Inflammatory bowel disease increases the risk of Parkinson's disease: a Danish nationwide cohort study 1977-2014. Gut *68*, 18-24.

Vogt, N.M., Kerby, R.L., Dill-McFarland, K.A., Harding, S.J., Merluzzi, A.P., Johnson, S.C., Carlsson, C.M., Asthana, S., Zetterberg, H., Blennow, K.*, et al.* (2017). Gut microbiome alterations in Alzheimer's disease. Sci Rep *7*, 13537.

Wakabayashi, K., Takahashi, H., Takeda, S., Ohama, E., and Ikuta, F. (1988). Parkinson's disease: the presence of Lewy bodies in Auerbach's and Meissner's plexuses. Acta Neuropathol *76*, 217-221.

Wang, L., Magen, I., Yuan, P.Q., Subramaniam, S.R., Richter, F., Chesselet, M.F., and Tache, Y. (2012). Mice overexpressing wild-type human alpha-synuclein display alterations in colonic myenteric ganglia and defecation. Neurogastroenterol Motil *24*, e425-436.

Wang, X.L., Zeng, J., Yang, Y., Xiong, Y., Zhang, Z.H., Qiu, M., Yan, X., Sun, X.Y., Tuo, Q.Z., Liu, R.*, et al.* (2015). Helicobacter pylori filtrate induces Alzheimer-like tau hyperphosphorylation by activating glycogen synthase kinase-3beta. J Alzheimers Dis *43*, 153-165.

Weimers, P., Halfvarson, J., Sachs, M.C., Saunders-Pullman, R., Ludvigsson, J.F., Peter, I., Burisch, J., and Olen, O. (2019). Inflammatory Bowel Disease and Parkinson's Disease: A Nationwide Swedish Cohort Study. Inflamm Bowel Dis *25*, 111-123.

Yang, X., Qian, Y., Xu, S., Song, Y., and Xiao, Q. (2017). Longitudinal Analysis of Fecal Microbiome and Pathologic Processes in a Rotenone Induced Mice Model of Parkinson's Disease. Front Aging Neurosci *9*, 441.

Zhai, C.D., Zheng, J.J., An, B.C., Huang, H.F., and Tan, Z.C. (2019). Intestinal microbiota composition in patients with amyotrophic lateral sclerosis: establishment of bacterial and archaeal communities analyses. Chin Med J (Engl) *132*, 1815-1822.

Zhang, L., Wang, Y., Xiayu, X., Shi, C., Chen, W., Song, N., Fu, X., Zhou, R., Xu, Y.F., Huang, L.*, et al.* (2017a). Altered Gut Microbiota in a Mouse Model of Alzheimer's Disease. J Alzheimers Dis *60*, 1241-1257.

Zhang, Y.G., Wu, S., Yi, J., Xia, Y., Jin, D., Zhou, J., and Sun, J. (2017b). Target Intestinal Microbiota to Alleviate Disease Progression in Amyotrophic Lateral Sclerosis. Clin Ther *39*, 322-336.

Zhuang, Z.Q., Shen, L.L., Li, W.W., Fu, X., Zeng, F., Gui, L., Lu, Y., Cai, M., Zhu, C., Tan, Y.L.*, et al.* (2018). Gut Microbiota is Altered in Patients with Alzheimer's Disease. J Alzheimers Dis *63*, 1337-1346.

Chapter 5: Vagal Interoception of Microbial Metabolites From the Small Intestinal Lumen

SUMMARY

The vagus nerve is proposed to enable communication between the gut microbiome and brain, but activity-based evidence is lacking. Herein, we assess the extent of gut microbial influences on afferent vagal activity and metabolite signaling mechanisms involved. We find that mice reared without microbiota (germ-free, GF) exhibit decreased vagal afferent tone relative to conventionally colonized mice (specific pathogen-free, SPF), which is reversed by colonization with SPF microbiota. Perfusing non-absorbable antibiotics (ABX) into the small intestine of SPF mice, but not GF mice, acutely decreases vagal activity, which is restored upon re-perfusion with bulk lumenal contents or sterile filtrates from the small intestine and cecum of SPF, but not GF, mice. Of several candidates identified by metabolomic profiling, microbiome-dependent short-chain fatty acids, bile acids, and 3-indoxyl sulfate stimulate vagal activity with varied response kinetics, which is blocked by co-perfusion of pharmacological antagonists of FFAR2, TGR5, and TRPA1, respectively, into the small intestine. At the single-unit level, serial perfusion of each metabolite class elicits more singly responsive neurons than dually responsive neurons, suggesting distinct neuronal detection of different microbiome- and macronutrient- dependent metabolites. Finally, microbial metabolite-induced increases in vagal activity correspond with activation of neurons in the nucleus of the solitary tract, which is also blocked by co-administration of their respective receptor antagonists. Results from this study reveal that the gut microbiome regulates select metabolites in the intestinal lumen that differentially activate chemosensory vagal afferent neurons, thereby enabling microbial modulation of interoceptive signals for gut-brain communication.

INTRODUCTION

The gut microbiota is emerging as a key modulator of brain function and behavior, as several recent studies reveal effects of gut microbes on neurophysiology, complex animal behaviors, and endophenotypes of neurodevelopmental, neurological, and neurodegenerative diseases.[1-3] Despite these findings supporting a "microbiome-gut-brain axis", the mechanisms that underlie interactions between gut microbes and the brain remain poorly understood. While many studies highlight neuroimmune pathways for microbial influences on the brain,[4,5] it is also believed that the gut microbiota may directly signal to the brain via gut-innervating vagal neurons.[6] However, existing evidence for the vagal route is largely derives from studies wherein subdiaphragmatic vagotomy abrogates behavioral alterations in response to microbiota perturbation in mice.[2,7-10] This ablates signaling in both afferent and efferent directions, not only to the intestine, but also with other peripheral organs. While the approach provides an important initial indication that the vagus nerve contributes to behavioral phenotypes that are modified by the microbiome, *in vivo* evidence of microbial signaling through vagal neurons is needed, and fundamental questions remain regarding the nature of microbial effects on vagal activity, the particular molecular constituents involved, and the diversity of neuronal responses elicited.

The gut microbiome is central to dietary metabolism and modulates hundreds of biochemicals in the intestine, as well as the blood and various distal tissues.[11,12] Biochemical screens of supernatants from cultured human-derived gut microbes find that soluble microbial products (as yet uncharacterized) have the capacity to directly bind to numerous G-protein-coupled receptors (GPCRs) that mediate neurotransmitter and neuropeptide signaling,[13,14] some of which are reportedly expressed by vagal neurons.[15-19] As such, microbial metabolites generated in the intestinal lumen have the potential to directly or indirectly activate vagal neurons via receptors on mucosal sensory afferents or receptors on intestinal epithelial cells that synapse onto the mucosal

sensory afferents, as has been described for select lumenal nutrient stimuli[20-22] and microbial antigens.[23,24] In this study, we assess afferent vagal nerve activity in response to the presence, absence, depletion, and restoration of the gut microbiota. We further profile microbiome-dependent metabolites within the proximal small intestine and cecum, which are poised to signal to gut mucosal vagal afferents. We identify select microbial metabolites that induce vagal afferent neuronal activity with varied kinetics when administered to the lumen of the small intestine. Finally, we apply pharmacological approaches to probe the potential for direct and/or indirect receptor-mediated signaling of microbial metabolites to vagal neurons. This study provides fundamental functional and mechanistic evaluation of the gut microbiome as a regulator of lumenal metabolites that modulate vagal chemosensory signaling across the gut-brain axis.

RESULTS

The gut microbiome promotes afferent vagal nerve activity

To determine the extent to which the microbiome contributes to gut-brain signaling via the vagus nerve, we began by applying whole nerve electrophysiology to measure bulk activity of vagal afferents in wildtype C57BL/6J mice reared in the presence or absence of microbial colonization (**Figure 1A, left**). Germ-free (GF) mice exhibited significantly decreased afferent vagal nerve activity (**Figure 1B-1C**) as compared to conventionally colonized (specific pathogen-free, SPF) controls. These reductions were reversed by colonizing GF mice with the SPF microbiota during adulthood (conventionalized, CONV), suggesting active interactions between the microbiome and vagus nerve that occur independently of developmental colonization. Treating adult SPF mice with oral broad-spectrum antibiotics (ABX; ampicillin, neomycin, vancomycin, and metronidazole) for 7 days to reduce bacterial load yielded modest, but statistically insignificant, reductions in afferent vagal nerve activity. We hypothesized that the inconsistent phenotype between the ABX

and GF conditions may be due to incomplete depletion of bacteria by ABX treatment, enrichment of native ABX-resistant bacteria over the one-week treatment period, [25] off-target effects of ABX absorbed into the systemic circulation, [26] the activity of non-bacterial members of the microbiota, [27] and/or confounding effects of developmental GF rearing.[28] To gain further insight, we recorded afferent vagal nerve activity acutely while introducing a constant flow of the subset of the ABX cocktail that is non-absorbable (i.e., vancomycin and neomycin) directly into the lumen of the duodenum[29] and through the first ~10 cm of the small intestine, a site of dense vagal innervation[16] (**Figure 1A, right**). Perfusing nonabsorbable ABX into the intestinal lumen of SPF mice decreased afferent vagal nerve activity, as compared to vehicle (VEH)-perfused controls (**Figure 1D-1F**). ABX-induced decreases in afferent vagal nerve activity were not seen in GF mice perfused with ABX (**Figure 1D-1F**). These results suggest that intestinal perfusion with non-absorbable ABX decreases afferent vagal nerve activity via the bactericidal actions of ABX on microbes in the small intestine.

The gut microbiota influences many aspects host biology, in large part by bacterial metabolism of dietary substrates, synthesis of secondary metabolites, and modification of host-derived molecules in the intestine.[4,30-33] To evaluate the effects of lumenal microbial molecules on afferent vagal nerve activity, we administered a solution of small intestinal (SI) and cecal contents collected from donor SPF or GF mice into the SI lumen of ABX-perfused SPF mice. SI perfusion with non-absorbable ABX reduced afferent vagal nerve activity, as reported above, whereas re-perfusion of SI/cecal contents from SPF mice acutely increased activity toward levels seen at pre-ABX baselines ("+ SPF" in **Figure 1G-1I**). No such effect was seen with re-perfusion of SI/cecal contents from GF mice ("+ GF" in **Figure 1G-1I**), suggesting that the observed vagal response is due the presence of SI and cecal microbes and/or microbial molecules. To investigate the contribution of microbial small molecules, we sterile-filtered the equivalent solution of SPF SI/cecal contents and administered it to the SI lumen of ABX-perfused SPF mice. Sterile-filtered

SPF SI/cecal contents (SPF-SF) increased afferent vagal nerve activity to levels similar to SPF on average, albeit with shorter latency and more variability (**Figure 1G-1I**). These differences could be due in part to a lack of macromolecules present in SPF-SF samples which have been shown to consistently activate larger proportions of vagal neurons *in vivo*[20], or possibly variability in the fidelity of small molecules upon filtration or the distribution of small-molecule activated receptors along the length of the proximal and medial small intestine.[34] Taken together, these data provide evidence that active signaling from the gut microbiota modulates vagal afferent activity *in vivo*, and that these effects are mediated, at least in part, by microbial small molecules within the lumen of the small intestine and cecum.

Microbiome-dependent bile acids, short-chain fatty acids, and 3-indoxyl sulfate stimulate afferent vagal nerve activity in a receptor-dependent manner

The gut microbiota regulates numerous metabolites within the host.[11,12] However, most characterizations to date have profiled metabolites in fecal or serum samples, excluding signaling molecules localized to the small intestine and cecum that are poised to interact with villus-innervating vagal neurons.[35] To identify candidate microbial metabolites in the small intestine and cecum that may modify vagal afferent activity, we performed liquid chromatography-mass spectrometry (LC-MS/MS) based untargeted metabolomic profiling of lumenal contents from the duodenum and cecum of SPF, GF, ABX, and CONV mice. 931 metabolites were identified from mouse proximal SI and cecal contents (**Tables S1 and S2**). Principal component analysis revealed distinct clustering of GF and ABX samples away from SPF and CONV samples along PC1 (**Figure 2A,** top), indicating that acute ABX depletion of the gut microbiota by weekly treatment with twice-daily oral gavage yields SI and cecal metabolomic profiles that are similar to those seen with GF rearing and that adult inoculation of GF mice with a conventional microbiota induces SI and cecal metabolomic profiles that are similar to those seen with conventional

colonization. This is consistent with our finding that acute depletion and re-introduction of the microbiota or microbial metabolites alters afferent vagal nerve activity (**Figure 1**). However, there were also notable differences within the cecal datasets in particular, with discrimination of ABX from GF profiles and, to a lesser degree, CONV from SPF profiles, along PC2 (**Figure 2A,** bottom). These differences highlight potential developmental influences of microbial colonization on host physiology[36] and/or incomplete depletion and/or restoration of microbial communities within the lower GI tract relative to the proximal small intestine.[37] Based on the ability of both GF status and acute perfusion of nonabsorbable ABX to decrease afferent vagal nerve activity and of CONV to elevate activity toward levels seen in SPF controls (**Figure 1A-1F**), we then filtered the datasets to identify metabolites that were commonly differentially regulated by both microbiota-deficient conditions relative to both colonized conditions. In SI contents, there were 79 shared metabolites that were significantly modulated by both GF and ABX conditions relative to both SPF and CONV conditions, and in cecal contents, there were 521 (**Figure 2B, Table S1 and S2**). Based on the observation that SI/cecal contents and filtrates from SPF mice stimulate afferent vagal nerve activity compared to filtrates from GF controls (**Figure 1G-1I**), we focused in particular on the subset of 49 SI and 335 cecal metabolites that were significantly decreased by microbiota deficiency relative to colonized conditions (**Figure 2B**, **Table S1 and S2**). These included microbial metabolites that were extremely low or undetectable in microbiota-deficient conditions (which we refer to as “microbiome-dependent”), as well as metabolites that were partially downregulated (but still detectable) in microbiota-deficient conditions (which we refer to as “microbially modulated”).

To identify the subset of microbial metabolites that have the potential to signal directly to vagal neurons, we cross-referenced existing bulk and single-cell RNA sequencing datasets[15,16,18,19,38] for reported expression of known or putative receptors for the candidate SI and cecal metabolites. This identified select species of microbiome-dependent bile acids (BAs), a subset of which were

identified as key drivers for classifying microbiota status via random-forest analysis in small-intestinal samples (**Figure 2C**). Additional classes of metabolites that were uncovered included short chain fatty acids (SCFAs) (**Figure 2D-E**), microbially modulated tryptophan derivatives (TRPs, **Figure S1A**), fatty acid ethanolamides (FAEs, **Figure S1B**), monohydroxy fatty acids (MFAs, **Figure S1C**), as well as succinate and glutamate (**Figure S1D-S1E**). To initially assess the ability of these lumenal metabolites to modify vagal activity, we recorded afferent vagal nerve activity while perfusing physiologically relevant concentrations of metabolite pools into the SI. There were no statistically significant changes in afferent vagal nerve activity with SI perfusion of the detected TRPs, FAEs, MFAs, or succinate (**Figure S2A-S2D**). Consistent with existing literature demonstrating vagal responses to gastric delivery of glutamate,[39] SI perfusion of glutamate robustly increased afferent vagal nerve activity (**Figure S2E**). We further observed that SI perfusion of select microbiome-dependent BAs elicited rapid, transient afferent vagal nerve activity (**Figure 3A-3C**), which parallels existing literature on systemic administration of select primary and secondary BAs.[31] In contrast, SI perfusion of microbiome-dependent SCFAs (acetate, propionate, and butyrate) led to slower onset and gradual increases in afferent vagal nerve activity (**Figure 3D-3F**). This latency could be due to metabolite-specific differences in the rate of intestinal absorption,[40] differential spatial localization of metabolite absorption and functional activity along the length of the gastrointestinal tract,[41,42] and/or indirect signaling of the metabolites to non-neuronal mediators.[21,43,44]

Microbiome-dependent BAs and SCFAs in the intestinal lumen have the potential to bind to cognate receptors expressed by various cell types in the gastrointestinal tract (e.g., vagal, enteroendocrine, epithelial, immune)[45,46]. To determine the relative contributions of different cognate GPCRs to vagal responses induced by lumenal microbial metabolites, we perfused select receptor antagonists immediately before and during administration of their corresponding metabolites into the SI lumen of SPF mice. BAs signal through the membrane-bound Takeda G

protein-coupled receptor 5 (TGR5), which is expressed by gut-innervating vagal neurons[31] as well as intestinal epithelial cells and various intestinal innate immune cells .[32,45,47] Intestinal pre- and co-perfusion of the TGR5 antagonist m-tolyl 5-chloro-2-[ethylsulonyl] pyrimidine-4-carboxylate (SBI-115)[48] prevented the initial rapid, transient increases in afferent vagal nerve activity induced by microbiome-dependent BAs (**Figure 3A-3C**). We do not observe a difference in total area under the curve (AUC) across the entire stimulus window, as administration of SBI-115 leads to a delayed rise in vagal activity that was not observed with perfusion of microbiome-dependent BAs alone. This suggests that TGR5 antagonism may elicit compensatory vagal responses to microbiome-dependent BAs through farsenoid X receptor (FXR) or other TGR5-independent signaling mechanisms. SCFAs signal to free fatty acid receptor 2 (FFAR2), which is expressed by intestinal epithelial cells[41] and free fatty acid receptor 3 (FFAR3), which is expressed by gut-innervating vagal neurons.[49] Intestinal pre- and co-perfusion of the FFAR2 antagonist 4-[[(R)-1-(benzo[b]thiophene-3-carbonyl)-2-methyl-azetidine-2-carbonyl]-(3-chloro-benzyl)-amino]-butyric acid 99 (GLPG0974)[50] prevented the increase in afferent vagal nerve activity induced by SCFAs (**Figure 3D-3F**). These results suggest that microbiome-dependent SCFAs likely elevate vagal activity via indirect activation of intestinal epithelial cells or other cellular mediators.

In addition to testing microbial metabolites with reported receptor expression by vagal neurons, we also evaluated effects of select microbiome-dependent metabolites that have as yet unknown signaling mechanisms on vagal activity. We focused in particular on metabolites that i) are reproducibly dependent upon the microbiome across various studies and biological contexts[51] and ii) have been reportedly linked to brain function and/or behavior.[1,52-55] Of these, 3-indoxyl sulfate (3IS), hippurate, and trimethylamine-N-oxide (TMAO) are microbiome-dependent metabolites in SI lumen, imidazole propionate is a microbiome-dependent metabolite in the cecum, and phenethylamine is microbially modulated in the cecum (**Figure 2F**). These metabolites are also reduced in the serum of microbiome-deficient mice,[11] suggesting that they

are typically absorbed from the intestinal lumen and poised to interact with mucosal vagal afferents. Perfusing physiologically relevant concentrations of hippurate, TMAO, imidazole propionate, and phenethylamine individually through the small intestine had no measurable effect on afferent vagal nerve activity (**Figure S2F-S2I**). In contrast, SI perfusion with 3IS elicited rapid and sustained increases in afferent vagal nerve activity relative to vehicle controls (**Figure 3G-3I**). The metabolic precursor to 3IS, indole, and the related tryptophan metabolite indole-3-carboxaldehyde, are reported to activate vagal afferent neurons in zebrafish via indirect stimulation of colonic enteroendocrine cells in a transient receptor potential ankyrin1 (TRPA1)-dependent manner.[43] To evaluate this possible signaling mechanism for 3IS in the small intestine, we pre- and co-perfused the TRPA1 antagonist (1E,3E)-1-(4-Fluorophenyl)-2-methyl-1-penten-3-one oxime (A967079)) with 3IS into the SI lumen, which completely prevented 3IS-induced afferent vagal nerve activity (**Figure 3G-3I**). Overall, these results reveal that microbial BAs, SCFAs, and 3IS promote afferent vagal nerve activity through receptor-dependent signaling from the SI lumen.

Lumenal BAs, SCFAs, and 3IS excite both distinct and shared subsets of afferent vagal neurons with varied temporal responses

Different lumenal stimuli can activate distinct populations of vagal neurons with differing response kinetics. In particular, recent work has identified populations of afferent vagal neurons that respond exclusively to fats versus sugars.[22] Microbiome-dependent BAs, SCFAs, and 3IS are related to dietary metabolism of fats, complex carbohydrates, and proteins, respectively,[30,43,56] raising the question of whether they promote vagal nerve activity via shared vs. distinct vagal afferent neurons. To gain insight, we imaged calcium activity of vagal afferent neurons in response to acute SI perfusion of microbiome-dependent metabolites in mice expressing GCaMP6s in Phox2b+ sensory neurons (**Figure 4A-B**). Microbiome-dependent BAs, SCFAs, and

3IS elicited calcium responses in 57%, 48%, and 58% of detected vagal afferent neurons, respectively (**Figure 4C**). Hierarchical clustering of calcium signals from responsive neurons over time for each metabolite class yielded 2 main clusters of vagal responses to each metabolite class (**Figure 4B**). The latency to maximum calcium response varied within each subclass of microbial metabolite, where SCFAs and 3IS similarly elicited primarily delayed calcium responses, while BAs elicited a bimodal distribution of acute and delayed calcium responses (**Figure 4D**). Upon perfusing pairs of metabolites in sequence, BAs and SCFAs elicited calcium responses in largely distinct afferent vagal neurons, with 43% responsive to BAs only, 38% responsive to SCFAs, and 19% dually responsive to both BAs and SCFAs. In contrast, sequential perfusion of 3IS and SCFAs yielded many shared neuronal responses, where 40% of afferent vagal neurons were dually responsive to sequential perfusion of 3IS and SCFAs, 27% to SCFAs only, and 33% to 3IS only. Similarly, with BAs and 3IS, we observed 43% dual responders, 35% responsive to 3IS only, and 22% responsive to BAs only (**Figure 4E**). Together, these data reveal distinct and shared neuronal populations for sensing different microbiome- and macronutrient- dependent metabolites, with greater distinct neuronal responses to microbial BAs and SCFAs, than either with 3IS.

Receptor-mediated signaling of BAs, SCFAs, and 3IS from the small intestinal lumen activates neurons in the NTS

Changes in the gut microbiota have been associated with altered activation of neurons of the nucleus of the solitary tract (NTS), which receives direct visceral afferents from nodose neurons.[57,58] Consistent with the ability of microbial metabolites in the SI lumen to stimulate afferent vagal neuronal activity (**Figures 3 and 4**), we find that acute lumenal perfusion of microbiome-dependent BAs, SCFAs, and 3IS each increase neuronal expression of the activation marker cFos in the NTS (**Figure 5A-B**), to levels similar to those seen with intestinal perfusion of sucrose.[21,59] As with the afferent vagal nerve and neuronal responses, the microbial metabolite-

driven increases in NTS neuronal activation were prevented by pre- and co-administration of antagonists for TGR5, FFAR2, and TRPA1 with their respective metabolite ligands. Together, these data indicate that microbial BAs, SCFAs, and 3IS in the SI lumen alter brain activity via receptor-mediated modulation of vagal afferent signaling.

DISCUSSION

Results from this study demonstrate that microbial colonization status, as well as acute manipulation of the gut microbiota and microbial metabolites, modulate vagal activity. In particular, we find that the gut microbiota regulates numerous small molecules in the small intestine and cecum. Moreover, administering select microbiome-dependent BAs, SCFAs, and 3IS at physiological concentrations and rates of peristalsis into the lumen of the small intestine stimulates vagal afferent neuronal activity. The vagal responses are elicited within relatively short timescales (<~9 min) and are abrogated by pre- and co-administration of select receptor antagonists, suggesting active signaling between the gut microbiome and vagal afferents via excitatory metabolites.

The functional evidence provided in this study align with prior reports indicating that subdiaphragmatic vagotomy abrogates effects of microbial interventions on behaviors such as anxiety,[7] depression,[57] cognition,[60] feeding,[46] and social behaviors.[2,49] Additionally, a few prior studies have reported that the gut microbiota and various microbial products regulate the transcriptome and excitability of vagal neurons.[58] For instance, nodose neurons from mice reared GF exhibited altered gene expression profiles when compared to those from SPF mice,[61] suggesting that the microbiome modulates the cellular state of vagal afferent neurons. In addition, bacterial supernatants from a cultured microbial community increased the intrinsic excitability of nodose neurons *in vitro*, through a mechanism that implicated a role for bacterial cysteine

proteases.[62] Another study reported that microbial single-stranded RNAs elevated vagal activity via Piezo1-mediated sensing by enterochromaffin cells.[23] Moreover, the tryptophan metabolite indole has been reported to induce serotonin release onto colonic vagal afferents via TRPA1-mediated enteroendocrine cell activation.[43] Together, these findings suggest that there exist multiple signaling factors and pathways by which the host-associated microbiota can impact vagal activity.

Aligning with the complexity of microbial influences on vagal activity, we observed that mice reared as GF exhibited significantly reduced afferent vagal nerve activity relative to mice reared with a conventional SPF microbiota, and while this effect was abrogated by colonizing GF mice with an SPF microbiota during adulthood, it was not fully recapitulated by depletion of the gut bacteria by oral ABX (**Figure 1C**). Many factors could contribute to this discrepancy. First, GF mice lack microbiota across all exposed body sites, whereas oral ABX treatment only partially ablates bacterial members of the oral and gastrointestinal microbiomes.[37] As such, it is possible that the reductions in vagal tone seen in GF mice could be mediated by changes in both intestinal and non-intestinal vagal afferents and/or the presence of residual microbes or microbial products in the intestine of SPF mice treated twice daily with ABX by oral gavage. Moreover, the reported alterations in nodose gene expression in GF mice relative to SPF mice[61] raise the question of whether there are early influences of microbiota status on vagal neuronal development, which are not captured by oral ABX treatment during adulthood. Further studies are needed to uncover the relative contributions of different vagal neuronal subtypes in mediating microbiome-induced alterations in vagal tone, as well as to what degree microbes endogenous to different parts of the gastrointestinal tract contribute to these alterations.

Despite the modest effects of oral ABX treatment in mice on reducing vagal tone (**Figure 1C**), we found that restricted perfusion of non-absorbable ABX through the lumen of the small intestine

acutely reduced afferent vagal nerve activity. We additionally observed that afferent vagal nerve activity was restored by the re-introduction of SPF SI and cecal contents into the small intestine and that these increases in activity were driven, at least in part, by the small molecule fraction. This was not seen with lumenal perfusion of SI and cecal contents from GF mice, indicating a role for small molecules that are modulated by the gut microbiome. Notably, we did not observe an overt vagal nutrient response with perfusion of intestinal contents from GF mice, which could reflects the rapid digestion, absorption, and/or relatively low homeostatic concentration of microbiome-independent nutrients, such as glucose and sucrose, in the SI,[63] as compared to the those exogenously delivered in other studies.[20-22] Overall, these findings reveal that soluble microbiome-dependent metabolites from the lumen of the small intestine can acutely stimulate afferent vagal activity.

By untargeted metabolomic profiling of SI and cecal contents from conventionally colonized (SPF, CONV) and microbiota deficient (ABX, GF) mice, followed by *in vivo* screening of select microbial metabolites with or without pharmacological antagonists, we identified particular subclasses of microbiome-dependent molecules that activate vagal afferent neurons in a receptor-dependent manner upon administration to the small intestine. The metabolites—specific microbial BAs, SCFAs, and 3IS-- promoted afferent vagal nerve activity with differing response kinetics, which could be due to differences in their physiological concentrations, rate of absorption, spatial localization of cognate receptors, and direct vs. indirect action, among other factors. Indeed, neuronal activation via GPCR signaling is dependent upon the concentration of the ligand, whereby differences can induce switching from G-protein coupled to G-protein independent signaling[64] resulting in alterations in the downstream signal transduction pathways engaged during neuronal activation. In addition, microbiome-dependent BAs and SCFAs are reported to be absorbed by the intestinal epithelium, which offers the potential to activate gut-innervating vagal afferents through direct receptor binding,[15,16,18,19] or through indirect interactions with

diverse enteroendocrine cells, subsets of which can synapse directly onto vagal neurons.[65] 3IS, however, has not been shown to be readily re-absorbed following secretion into the intestinal lumen, suggesting this metabolite likely acts through the latter aforementioned pathway in order to activate vagal afferents. It is also possible that select intestinal metabolites may access systemic circulation and act at extra-intestinal sites to modulate vagal activity, presumably with a time delay. In particular, lumenal microbiome-dependent BAs rapidly and transiently increased afferent vagal nerve activity, primarily via TGR5 signaling (**Figure 3A-C**). This may align with prior studies demonstrating that circulating BAs mediate release of cholecystokinin (CCK), and that CCK signaling is dynamic and rapidly desensitizes.[66] In contrast, perfusion of SCFAs into the SI lumen increased afferent vagal nerve activity following a latency period in an FFAR2-dependent manner (**Figure 3D-F**), suggesting indirect activation of FFAR2-expressing epithelial cells and subsequent GLP-1 release.[67] Finally, we found that microbiome-dependent 3IS in the small intestine elicited sustained afferent vagal nerve activity in a TRPA1-dependent manner (**Figure 3G-I**). This may align with a previous study wherein indole stimulated TRPA1+ colonic enteroendocrine cells to release serotonin and activate colon-innervating neurons.[43] Our observations, considered together with existing vagal single cell transcriptomic data, raise the potential for both direct activation of afferent vagal neurons (via TGR5 and TRPA1) and indirect activation of epithelial cells or other cellular mediates (via FFAR2, TGR5, or TRPA1) by lumenal microbial metabolites. Future studies interrogating the cell-type specific role of metabolite receptors expressed on multiple cell types will aid in uncovering the precise differential effects of indirect versus direct signaling on vagal responses to microbial stimuli.

Recent studies demonstrate that lumenal nutrient cues, such as fats and carbohydrates, engage parallel vagal afferent neurons via labeled lines.[20-22] However, further characterization of how different subclasses of diet- and microbiome-dependent small molecules engage gut-brain circuits involved in nutrient sensing is needed. We assessed the effects of acute lumenal

perfusion of select microbial BAs, SCFAs, and 3IS (involved in dietary fat, carbohydrate, and protein metabolism, respectively) on afferent vagal neuronal calcium activity *in vivo*. We found that all three classes of microbial metabolites resulted in increased calcium activity in nodose neurons with varied kinetics (**Figure 4B**)-- BAs elicited a bimodal distribution of immediate vs. delayed responses, whereas SCFAs and 3IS mostly elicited delayed responses (**Figure 4D**), which aligns with the slow, gradual onset of afferent vagal nerve activity in response to SCFA and sustained onset of afferent nerve activity with 3IS perfusion. When assessing single-unit responses to sequential perfusion of two metabolite classes, microbiome-dependent BAs and SCFAs elicited calcium responses via largely non-overlapping subpopulations of afferent vagal neurons, whereas 3IS and BAs or SCFAs acted primarily via shared subpopulations (**Figure 4F**). These findings are supported by previous work demonstrating a shared role for both TGR5- and TRPA1- mediated alterations in digestion and satiety via epithelial CCK signaling,[31,68-71] as well as TRPA1- and FFAR2-mediated alterations in host metabolism and feeding behaviors that have been reported to act via epithelial secretion of GLP-1.[67,72-74] In contrast, previous work demonstrates that BA- and SCFA- mediated alterations in feeding behaviors act via distinct receptor-dependent signaling pathways.[49,75] Future studies utilizing combinatorial functional strategies to elucidate the interplay between distinct metabolite effects on shared host behavioral and physiological processes will aid in uncovering the precise cellular crosstalk that may mediate our observed results.

Despite evidence for vagal chemosensory pathways mediating communication from the intestinal lumen to the brain,[22,58,76] effects of specific microbial metabolites and their associated receptors on CNS neuronal activity remain unclear. We therefore addressed the effects of lumenal perfusion of BAs, SCFAs, and 3IS alongside their respective receptor antagonists for TGR5, FFAR2, and TRPA1 on medial NTS neuronal activation by immunofluorescence detection of the immediate early gene cFos. We found that all metabolite classes significantly increased NTS neuronal

activation, which could be prevented by pre-and co-perfusion of antagonists (**Figure 5**). Together, these data suggest that lumenal metabolites activate CNS neurons via receptor-dependent vagal signaling and may have downstream effects on CNS-associated behaviors. However, more work is needed to determine the downstream targets and differential effects of these gut-to brain signaling pathways on CNS physiology.

Findings from this study highlight lumenal microbial metabolites derived from various sources of dietary macronutrients-- fats (BAs), complex carbohydrates (SCFAs), and proteins (3IS)—that differentially activate vagal afferent neurons via receptor-mediated signaling in order to convey information to the brain. Following the ingestion of dietary fats, BAs are released from the liver into the SI lumen where they undergo chemical transformations, such as deconjugation and dehydroxylation, which are carried out by gut microbes.[77] Enzymes capable of catalyzing such reactions are found across all major bacterial phyla,[78] suggesting a broad role for the microbiota in regulating the lumenal BA pool. Similarly, SCFAs are derived from microbial metabolism of dietary fibers that are otherwise non-digestible by the host, with differential production by bacterial members of the phyla *Bacteroidetes* (acetate, propionate) and *Firmicutes* (butyrate).[79,80] Tryptophan derivatives, such as indole, are produced by pathobionts, such as *Escherichia coli*, *Enterococcus faecalis,* and *Edwardsiella tarda,*[81] before being hydroxylated and sulfated in the liver and secreted as 3IS into the small intestine. As levels of dietary metabolites and microbiome composition can fluctuate depending on meal time[82,83], further examination into circadian effects of diet- and microbiome-dependent metabolites on vagal afferent neuronal activity is warranted.

Understanding the temporal variation in the bioavailability of neuromodulatory microbial metabolites and in vagal activity could reveal important insights into the functional role of vagal interoception of different types of microbial metabolites. As proof of principle, vagal TGR5 signaling mediated the anorexigenic effects of circulating BAs,[31] whereas vagal FFAR3 signaling

mediated the effects of circulating SCFAs on satiety.[49] However, further studies on whether the vagal circuits engaged by lumenal microbial metabolites modulate analogous or additional CNS-associated behaviors remains to be determined. Circuit tracing studies have uncovered polysynaptic connections from gut-innervating vagal afferent neurons to higher order brain regions such as substantia nigra and hippocampus,[84] suggesting the potential for microbial metabolites to impact complex behavioral responses beyond those involved in feeding. BAs, SCFAs, and 3IS have been associated with alterations in behavioral endophenotypes of anxiety and depression,[52,85,86] cognitive impairment,[87] and motor deficits,[3,86] each which has been linked to vagal signaling and alterations in the gut microbiota.[3,9,10] Overall, more research is needed to determine brain and behavioral responses to vagal interoception of lumenal microbial metabolites, and to further evaluate the potential to leverage the microbiome to modify neuronal signaling across the gut-brain axis.

FIGURES AND FIGURE LEGENDS

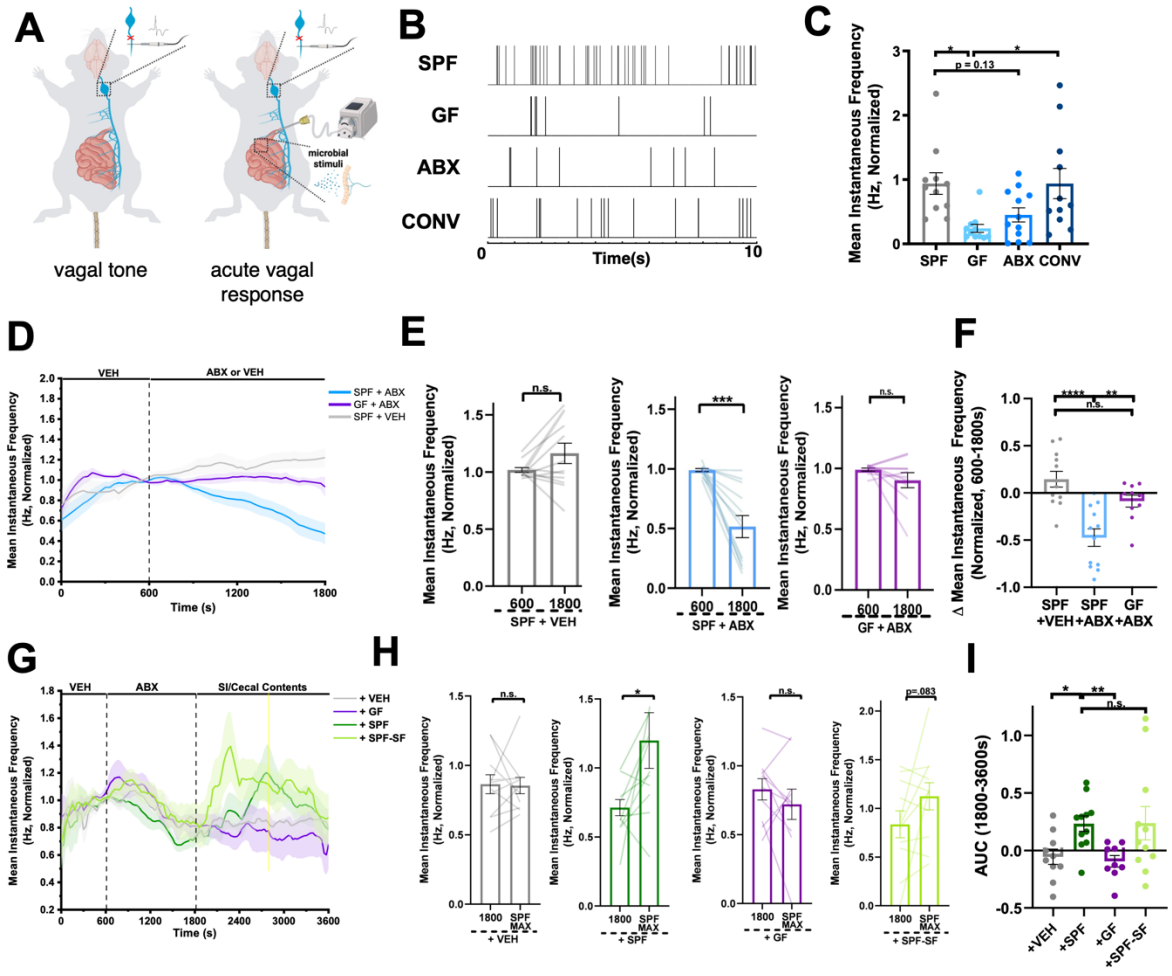


Figure 5.1: The Gut Microbiota and Lumenal Microbial Metabolites Promote Vagal Afferent Activity. A) Diagram of *in vivo* whole nerve vagal electrophysiology for quantification of vagal tone (left, for data in B-C) or acute vagal response to lumenal stimuli (right, for data in D-I) B) Representative images of detected vagal spikes (vertical lines) over 10s in conventionally colonized (SPF), germ-free (GF), antibiotic-treated (ABX), and conventionalized GF (CONV) mice. C) Vagal afferent tone of SPF, GF, ABX, and CONV mice. (SPF, n = 11 mice; GF, n = 11 mice; ABX, n = 12 mice; CONV, n = 11 mice). One-Way ANOVA + Tukey. D) Afferent vagal nerve response in SPF or GF mice perfused intestinally with non-absorbable antibiotics (ABX,

vancomycin/neomycin, 1mg/mL) or vehicle (VEH) (SPF +VEH, n = 12 mice; SPF +ABX, n = 12 mice; GF + ABX, n = 10 mice). E) Afferent vagal nerve firing rates before and after intestinal perfusion of SPF or GF mice with ABX or VEH. (SPF +VEH, n = 12 mice; SPF +ABX, n = 12 mice; GF + ABX, n = 10 mice). Paired t-test. F) Change in afferent vagal nerve activity after intestinal perfusion with ABX or VEH (t=1800) relative to stable baseline (t=600s) (SPF +VEH, n = 12 mice; SPF +ABX, n = 12 mice; GF + ABX, n = 10 mice). One-way ANOVA + Tukey. G) Afferent vagal nerve response in mice intestinally perfused with ABX, followed by re-perfusion with pooled small intestinal (SI) and cecal contents from SPF mice (+SPF) or GF mice (+GF), sterile filtered (SF) SI/cecal contents from SPF mice (+SPF-SF), or VEH (+VEH, n = 10 mice; +SPF, n = 11 mice; +GF, n = 9 mice; +SPF-SF, n = 11 mice). H) Afferent vagal nerve firing rate after intestinal ABX perfusion (t=1800s) and after re-perfusion with VEH, SI/cecal contents from SPF mice, SI/cecal contents from GF mice, or sterile filtered SI/cecal contents from SPF mice, at the time of maximum mean firing rate for perfusion of SPF SI/Cecal contents (SPF MAX, t = 2760s) (+VEH, n = 10 mice; +SPF, n = 11 mice; +GF, n = 9 mice; +SPF-SF, n = 11 mice). Paired t-test. I) Afferent vagal nerve activity as measured by area under the curve (AUC) (from 1800-3600s) in response to intestinal perfusion with VEH (n = 10 mice), SPF SI/cecal contents (n = 11 mice), GF SI/cecal contents (n = 9 mice), and SPF-SF SI/cecal contents (n = 11 mice). Brown-Forsythe and Welch ANOVA + Games-Howell. All data displayed as mean +/- SEM, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$.

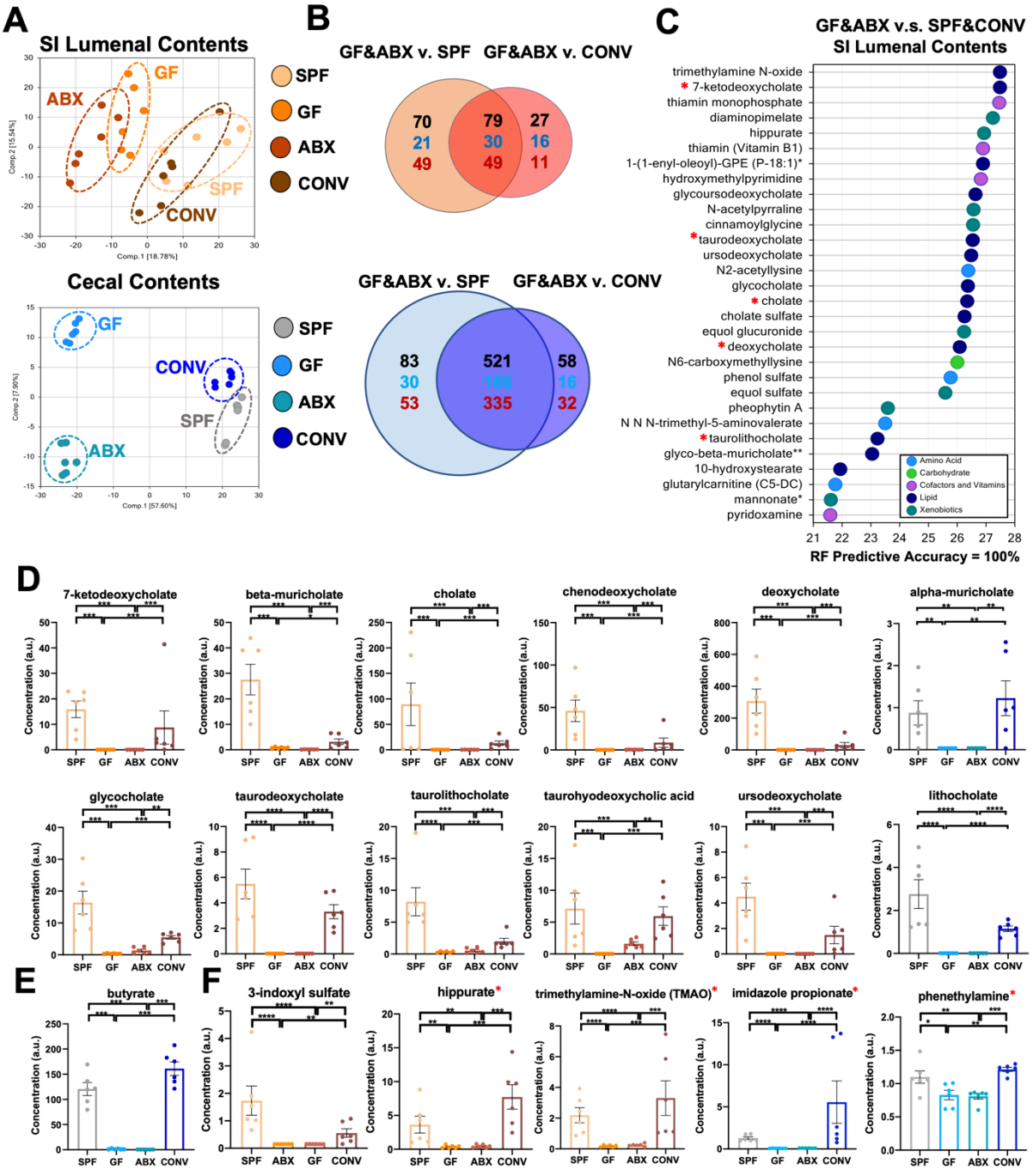


Figure 5.2: The Gut Microbiome Regulates Metabolites in the Lumen of the Small Intestine and Cecum. A) PCA analysis of small intestinal (SI, top) or cecal (bottom) lumenal contents from SPF, GF, ABX, and CONV mice. (n=6 mice for all groups). B) Venn diagram of differentially modulated metabolites in SI (top) and cecal (bottom) lumenal contents. Red numbers indicate

downregulated metabolites and blue numbers indicate upregulated metabolites. (n=6 mice for all groups). C) Random forest (RF) analysis of metabolomic data from SI lumenal contents reveals the top 30 metabolites that distinguish GF/ABX from SPF/CONV samples with 100% predictive accuracy. Red asterisks indicate metabolites tested in Figure 3. (n=6 mice for all groups). D-E) Lumenal levels of microbially modulated bile acids and the short-chain fatty acid butyrate from SI (orange tones) and/or cecum (blue tones) of SPF, GF, ABX, and CONV mice. (n=6 mice for all groups). Welch's t-test. F) Lumenal levels of microbially modulated metabolites with unknown signaling to vagal neurons from SI (orange tones) or cecum (blue tones) of SPF, GF, ABX, and CONV mice. (n=6 mice for all groups). Red asterisks denote metabolites tested in Supplemental Figure 2. Welch's t-test. All data displayed as mean +/- SEM, *p < 0.05, **p < 0.01, ***p < 0.001, ****p<.0001

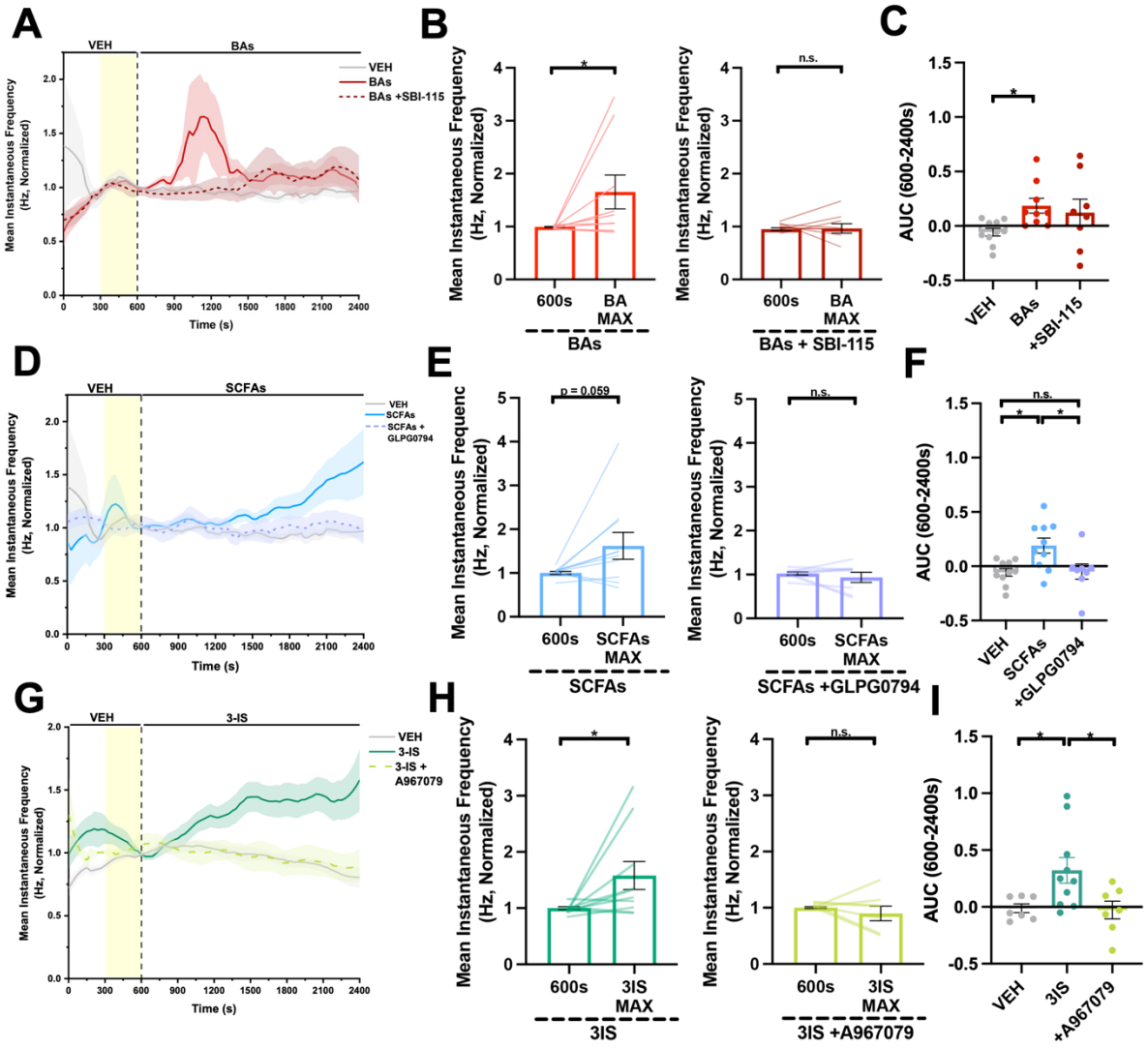


Figure 5.3: Select Lumenal Microbial Metabolites Increase Afferent Vagal Nerve Activity with Varied Response Kinetics. A) Afferent vagal nerve firing rate in SPF mice after intestinal perfusion with vehicle (VEH: PBS, n=10 mice) or pooled bile acids (BAs: cholate, 1240nM; glycocholate, 3.5nM; chenodeoxycholate, 42nM; alpha-muricholate, 142nM; beta-muricholate, 1080nM; deoxycholate, 390nM; taurodeoxycholate, 260nM; ursodeoxycholate, 74nM; taurohyodeoxycholate, 18.8nM; 7-ketodeoxycholate, 100nM; lithocholate, 390nM; taurolithocholate, 0.33nM; n = 9 mice), with or without pre- and co-perfusion with TGR5 antagonist (SBI-115, 200uM, n = 8 mice). Yellow shading indicates perfusion of pure antagonist prior to co-

perfusion of antagonist with metabolites. B) Normalized afferent vagal nerve firing rate before and during treatment with BAs (left, n = 9 mice), or BAs and SBI-115 (right, n = 8 mice). BA max, t = 1114s. Wilcoxon matched-pairs signed rank test. C) Afferent vagal nerve firing rate as measured by area under the curve (AUC) (from 600-2400s) in response to intestinal perfusion with VEH (PBS, n = 10 mice) or pooled BAs (n = 9 mice), with or without pre- and co-perfusion with TGR5 antagonist (SBI-115, 200uM, n = 8 mice). Brown-Forsythe and Welch ANOVA + Games-Howell. D) Afferent vagal nerve firing rate in SPF mice after intestinal perfusion with VEH (PBS, n = 10 mice) or pooled short-chain fatty acids (SCFAs: acetate, 80uM; butyrate, 22uM; propionate, 10uM, pooled, n = 10 mice), with or without pre- and co-perfusion with FFAR2 antagonist (GLPG0794, 10uM, n = 8 mice). Yellow shading indicates perfusion of pure antagonist prior to co-perfusion of antagonist with metabolites. E) Normalized afferent vagal nerve firing rate before and during treatment with SCFAs (left, n = 10 mice), or SCFAs and GLPG0794 (right, n = 8 mice). SCFA max, t = 2400s. Paired t-test. F) Afferent vagal nerve firing rate as measured by AUC (from 600-2400s) in response to intestinal perfusion with VEH (PBS, n = 10 mice) or pooled SCFAs (10uM, n = 10 mice), with or without pre- and co-perfusion with FFAR2 antagonist (GLPG0794, 10uM, n = 8 mice). One-way ANOVA + Tukey. G) Afferent vagal nerve firing rate in SPF mice after intestinal perfusion with VEH (1uM KCl, n = 7 mice) or 3-indoxyl sulfate (3-IS, 1uM, n = 10 mice), with or without pre- and co-perfusion with TRPA1 antagonist (A967079, 10uM, n = 7 mice). Yellow shading indicates perfusion of pure antagonist prior to co-perfusion of antagonist with metabolites. H) Normalized afferent vagal nerve firing rate before and during treatment with 3IS (left, n = 10 mice), or 3IS and A967079 (right, n = 7 mice). 3IS max, t = 2400s. Paired t-test. I) Afferent vagal activity as measured by AUC (from 600-2400s) in response to intestinal perfusion with VEH (1uM KCl, n=7 mice) or 3-indoxyl sulfate (3-IS, 1uM, n = 10 mice), with or without pre- and co-perfusion with TRPA1 antagonist (A967079, 10uM, n = 7 mice). One-way ANOVA + Tukey. All data displayed as mean +/- SEM, *$p < 0.05$.

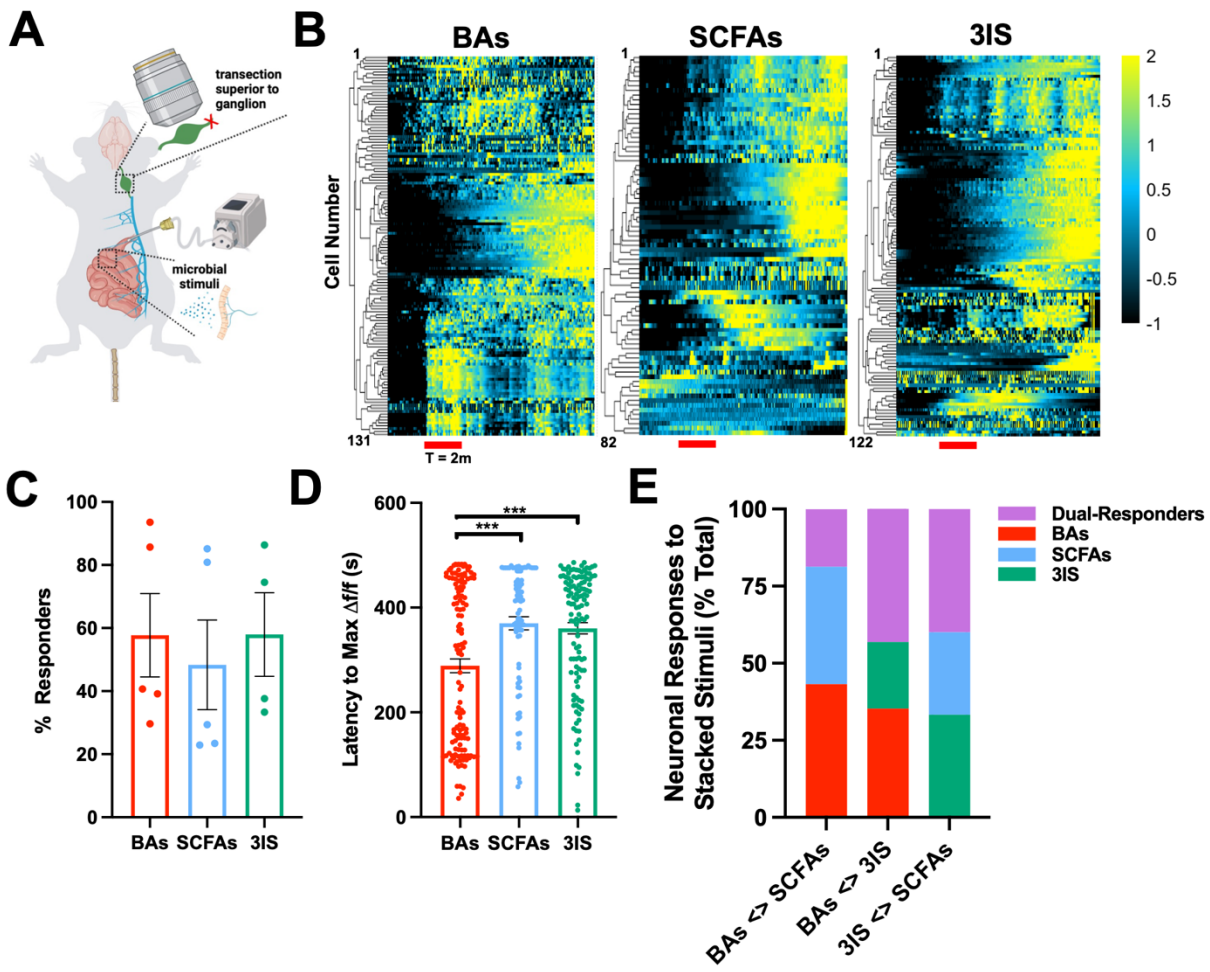


Figure 5.4: Lumenal BAs, SCFAs, and 3IS activate distinct subsets of vagal afferent neurons with heterogeneous kinetics. A) Diagram of experimental setup for *in vivo* calcium imaging. B) Representative heatmaps for cells responding to only pooled bile acids (BAs: cholate, 1240nM; glycocholate, 3.5nM; chenodeoxycholate, 42nM; alpha-muricholate, 142nM; beta-muricholate, 1080nM; deoxycholate, 390nM; taurodeoxycholate, 260nM; ursodeoxycholate, 74nM; taurohyodeoxycholate, 18.8nM; 7-ketodeoxycholate, 100nM; lithocholate, 390nM; taurolithocholate, 0.33nM; n = 4 mice, n = 131 units, right), only pooled short-chain fatty acids (SCFAs: acetate, 80uM; butyrate, 22uM; propionate, 10uM, n = 4 mice, n = 82 cells, middle), or only 3-indoxyl sulfate (3IS, 1uM, n = 4 mice, n = 132 cells, left). Recording duration for all experiments was 10 minutes. C) Percentage of metabolite-responsive neurons out of total

excitable neurons in response to lumenal perfusion of BAs (n = 5 mice), SCFAs (n = 5 mice), or 3IS (n = 4 mice). D) Latency to maximum change in fluorescence for metabolite-responding neurons with lumenal perfusion of BAs (n = 4 mice), SCFAs (n = 4 mice) or 3IS (n = 4 mice). One-way ANOVA + Tukey. E) Percentage of single- or dual- responding neurons following serial perfusion of BAs (n = 5 mice), SCFAs (n = 5 mice), and 3IS (n = 4 mice). Order of perfusion was scrambled between experiments. All data displayed as mean +/- SEM, ***$p < 0.001$

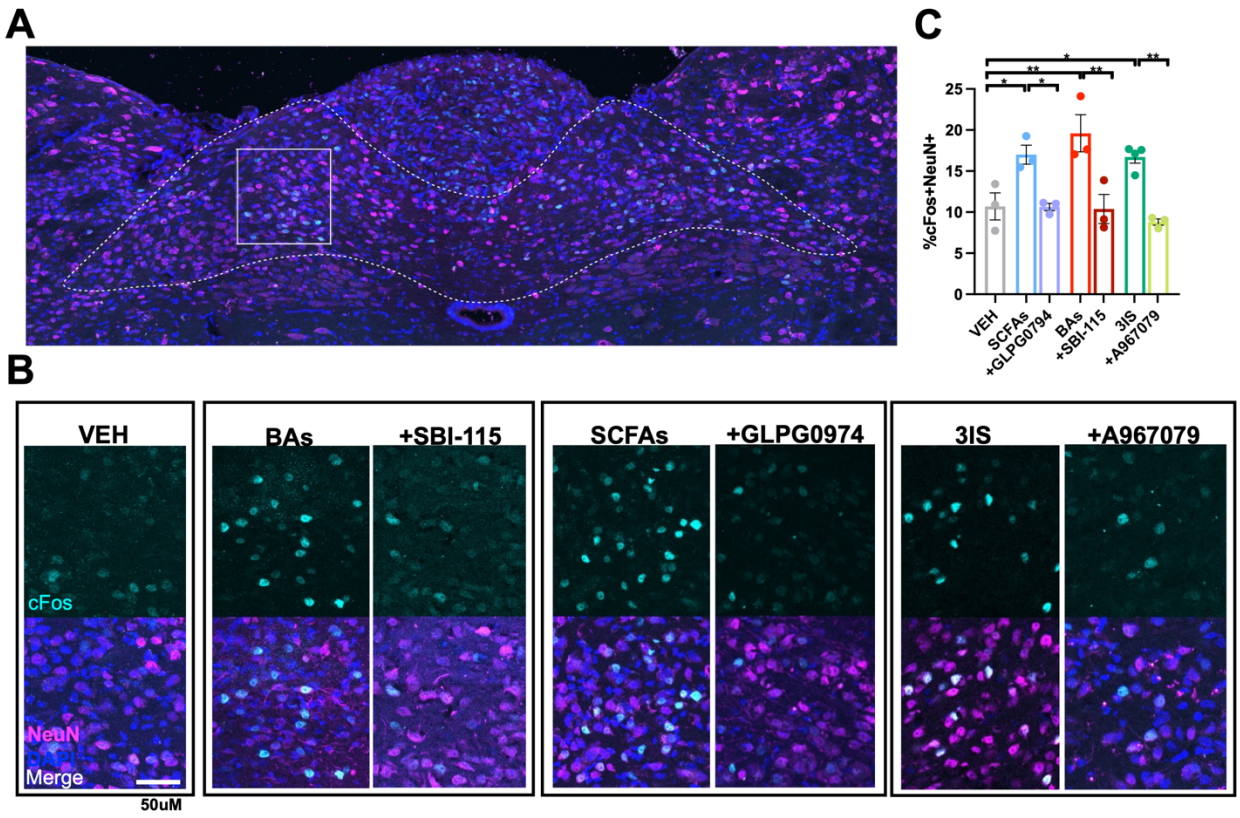
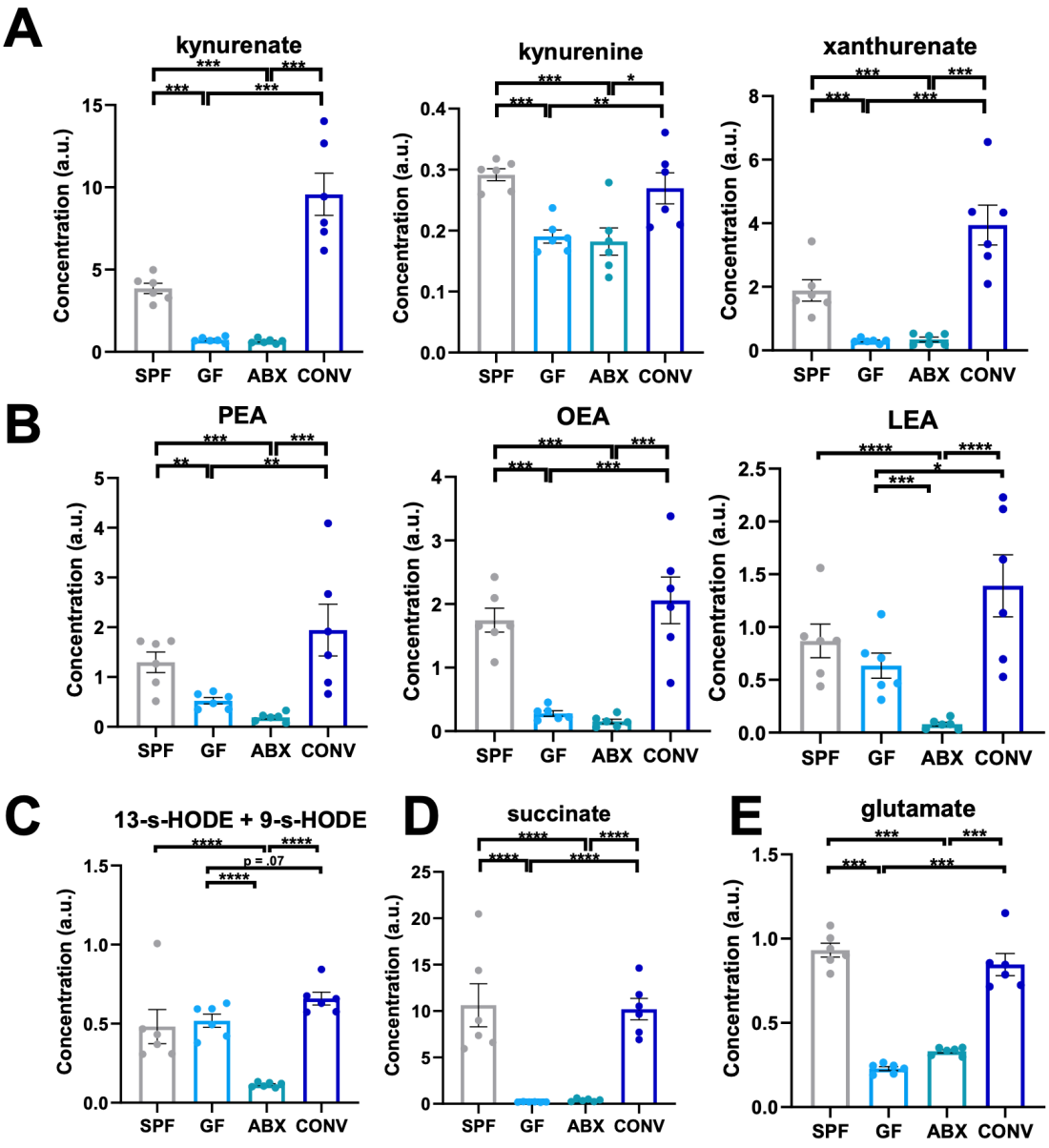
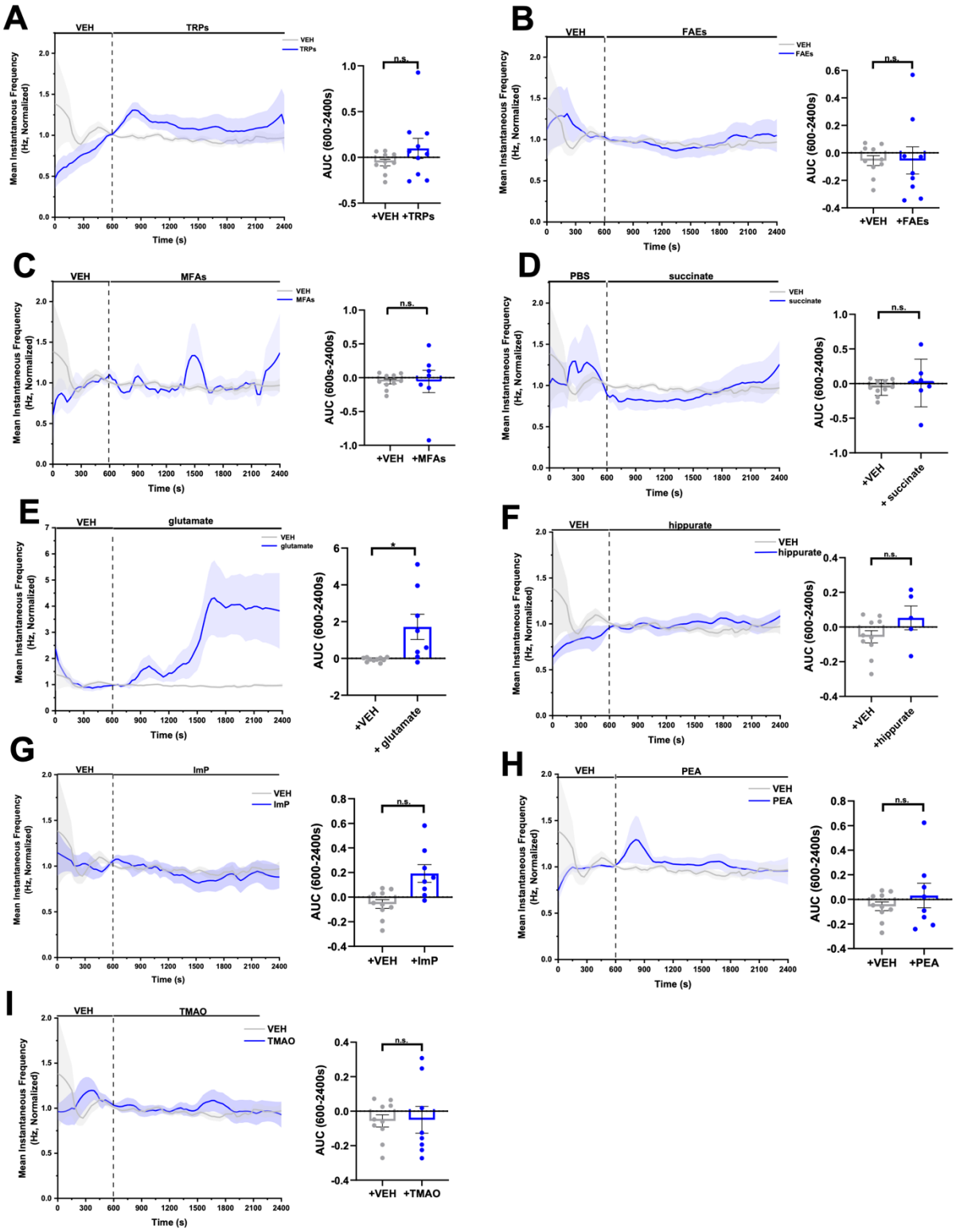


Figure 5.5: Microbial metabolites alter cFos levels in NTS neurons in a receptor-dependent manner. A) Representative image of full ROIs in mNTS for cFos quantification (dotted line) and inset images in **B** (solid line). B-C) Significant increase in the % of cFos+ NTS neurons following lumenal perfusion of BAs (cholate, 1240nM; glycocholate, 3.5nM; chenodeoxycholate, 42nM; alpha-muricholate, 142nM; beta-muricholate, 1080nM; deoxycholate, 390nM; taurodeoxycholate, 260nM; ursodeoxycholate, 74nM; taurohyodeoxycholate, 18.8nM; 7-ketodeoxycholate, 100nM; lithocholate, 390nM; taurolithocholate, 0.33nM, pooled, n = 3 mice), SCFAs (acetate 80uM, butyrate 22uM, propionate 10uM, pooled, n = 3 mice), and 3-IS (1uM, n = 4 mice) compared to vehicle (VEH, n = 3 mice) or corresponding receptor antagonists alongside each respective metabolite class (SBI-115, 200uM, n = 3 mice, GLPG0794, 10uM, n = 3 mice, A967079, 10uM, n = 3 mice). One-way ANOVA + Tukey. All data depicted as mean +/- SEM, *p < 0.05, **p < 0.01.



Supplemental Figure 5.1: Microbially-modulated cecal metabolites that were screened for effects on afferent vagal nerve activity. Lumenal levels of microbially modulated A) tryptophan metabolites, B) Fatty-acid ethanolamides (FAEs) PEA (palmitoyl ethanolmaide), OEA (oleoyl ethanolamide), LEA (linoleoyl ethanolamide), C) monohydroxy fatty acids (MFAs) 9-s- HODE and

13-s- HODE, D) succinate, E) glutamate. Welch's t-test. All data displayed as mean +/- SEM, *p < 0.05, **p < 0.01, ***p < 0.001, ****p<.0001.



Supplemental Figure 5.2: Lumenal perfusion of select microbially-modulated metabolites does not alter vagal afferent nerve activity *in vivo*. A) Afferent vagal nerve firing rate in

response to lumenal perfusion of VEH (PBS, n = 10 mice). Tryptophan metabolites (TRPs) (kynurenine 100uM, kynurenic acid 16.1uM, xanthurenate 1uM, dihydrocaffeate 30nm, tryptamine 0.03uM, pooled, n = 10 mice). Welch's t-test. B) Afferent vagal nerve firing rate in response to VEH (PBS, n = 10 mice) or fatty-acid ethanolamides (FAEs, oleylethanolamide (OEA), palmitoylethanolamide (PEA), linoleylethanolamide (LEA), 10uM, pooled, n = 9 mice). Welch's t-test. C) Afferent vagal nerve firing rate in response to lumenal perfusion of VEH (PBS, n = 10 mice) or monohydroxy fatty acids (MFAs) (9-s- and 13-s- HODE,1uM, pooled, n = 7 mice). Unpaired Welch's t-test. D) Afferent vagal nerve firing rate in response to lumenal perfusion of VEH (PBS, n = 10 mice) or succinate (2mM, n = 7 mice). Welch's t-test. E) Afferent vagal nerve firing rate in response to lumenal perfusion of VEH (PBS, n = 10 mice) or glutamate (50mM, n = 8 mice). Welch's t-test. F) Afferent vagal nerve firing rate in response to lumenal perfusion of VEH (PBS, n = 10 mice) or hippurate (2uM, n = 5 mice). Welch's t-test. G) Afferent vagal nerve firing rate in response to lumenal perfusion of VEH (PBS, n = 10 mice) or imidazole propionate (ImP, 200nM, n = 8 mice). Welch's t-test. H) Afferent vagal nerve firing rate in response to lumenal perfusion of VEH (PBS, n = 10 mice) or phenethylamine (PEA, 100uM, n = 8 mice). Welch's t-test. I) Afferent vagal nerve firing rate in response to lumenal perfusion of VEH (PBS, n = 10 mice) or trimethylamine-N-oxide (TMAO, 3uM, n= 8 mice). Welch's t-test. All data displayed as mean +/- SEM, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Guinea pig anti-NeuN	Sigma	Cat# ABN90
Rabbit anti-cFos	Abcam	Cat# ab209794
Prolong Gold Antifade Mountant with DAPI	ThermoFisher	Cat# P36931
Chemicals and Peptides and Recombinant Proteins		
Ampicillin	Sigma	Cat# A9518
Metronidazole	Sigma	Cat# M1547
Neomycin	Sigma	Cat# N1876
Vancomycin	Chem-Impex International	Cat# 00315
7-Ketodeoxycholic Acid	Sigma	Cat# SMB00806
Alpha-Muricholic Acid	Cayman Chemical	Cat# 20291
Beta-Muricholc Acid	Sigma	Cat# ML2372
Sodium Cholate Hydrate	Sigma	Cat# C9282
Sodium Chenodeoxycholate	Sigma	Cat# C8261
Deoxycholate	Sigma	Cat# D6750
Sodium Glycocholate Hydrate	Sigma	Cat# G7132
Lithocholic Acid	Sigma	Cat# L6250
Sodium Taurodeoxycholate Hydrate	Sigma	Cat# T0557
Sodium Taurohyodeoxycholate hydrate	Sigma	Cat# T0682
Sodium Taurolithocholate	Sigma	Cat# T7515
Ursodeoxycholic Acid	Sigma	Cat# U5127
SBI-115	MedChem Exppress	Cat# HY-111534
Sodium Acetate	Sigma	Cat# S2889
Sodium Butyrate	Sigma	Cat# B5887
Sodium Propionate	Sigma	Cat# P1880
GLPG0974	MedChem Exppress	Cat# HY-12940
Indoxyl Sulfate Potassium Salt	Sigma	Cat# I3875
A967079	MedChem Exppress	Cat# HY-108463
L-Kynurenine	Sigma	Cat# K8625
Kynurenic Acid	Sigma	Cat# K3375
Xanthurenic Acid	Sigma	Cat# D120804
Dihydrocaffeic Acid	Sigma	Cat# 102601
Tryptamine	Sigma	Cat# 193747
Oleylethanolamide	Sigma	Cat# O0383
Palmitoylethanolamide	Sigma	Cat# P0359
Linoleylethanolamide	Sigma	Cat# L1164
9-s-HODE	Sigma	Cat# SML0503

13-s-HODE	Sigma	Cat# H9146
Succinic Acid	Sigma	Cat# S3674
L-Glutamic Acid	Sigma	Cat# G1251
Hippurate	Sigma	Cat# H9380
Imidazole Propionic acid	Sigma	Cat# 77951
Phenethylamine	Sigma	Cat# 241008
Trimethylamine-N-Oxide	Sigma	Cat# 317594
Deposited Data		
Metabolomics Data	This study	N/A
Experimental Models: Organisms/Strains		
Mouse: C57BL/6J	Jackson Laboratories	RRID:IMSR_JAX:000664
Mouse: Phox2b-cre	Jackson Laboratories	RRID:IMSR_JAX:016223
Mouse: Ai96	Jackson Laboratories	RRID:IMSR_JAX:028866
Software and Algorithms		
LabVIEW	National Instruments	www.ni.com
MATLAB	MathWorks	www.mathworks.com
OriginPro	Origin Lab	www.originlab.com
PRISM 6	GraphPad	www.graphpad.com
Minian	Dong et al.	https://minian.readthedocs.io/en/stable/index.html
FIJI	Schindelin et al.	RRID:SCR_002285
Other		
Zeiss LSM780	Zeiss	N/A

EXPERIMENTAL MODELS AND SUBJECT DETAILS

Mice

All experimental procedures were carried out in accordance with US NIH guidelines for the care and use of laboratory animals and approved by the UCLA Institutional Animal Care and Use Committees. Mice used for data collection were males, at least 6 weeks of age. C57BL/6J mice were purchased from Jackson laboratories (stock no. 000664), reared as SPF or rederived as GF and bred in flexible film isolators at the UCLA Center for Health Sciences Barrier Facility. Ai96 (JAX stock no. 028866), *Phox2b-cre* (*Phox2b*$^{cre;}$ JAX stock no.016223), were obtained from Jackson laboratories and bred at the UCLA Biomedical Sciences Research Building barrier facility. Mice were housed on a 12-h light-dark schedule in a temperature-controlled (22-25°C) and humidity-controlled environment with *ad libitum* access to water and standard chow (Lab Diet 5010).

METHOD DETAILS

Antibiotic treatment and conventionalization

Adult SPF mice were gavaged twice daily for 1 week with a cocktail of vancomycin (50mg/kg), neomycin (100mg/kg) and metronidazole (100mg/kg) every 12 hours daily for 7 days. Ampicillin (1mg/mL) was provided *ad libitum* in drinking water. For conventionalization, fecal samples were collected from adult SPF C57BL/6J mice and homogenized in 1mL pre-reduced PBS per pellet. 100uL of the homogenate was administered by oral gavage to recipient GF mice.

Preparation of antibiotics, small-intestinal, and cecal contents

Vancomycin and neomycin were diluted in water to a final concentration of 1mg/mL for all antibiotic perfusion experiments, then sterile filtered. Vehicle for all antibiotic perfusion

experiments was water. For SPF SI and cecal content preparations adult SPF male mice were euthanized, and SI and cecal lumenal contents were snap-frozen in liquid nitrogen. Equal weights of frozen SI and cecal content were then combined and diluted to a concentration of 0.1g/mL wet weight in sterile-filtered PBS. Samples were then centrifuged at 500g for 5 minutes to pellet out any large dietary components, and supernatants were used for lumenal perfusion. GF SI/cecal contents were collected in the same way from donor GF adult male mice. Sterile-filtered SI/cecal contents were prepared by vacuum-filtering SPF SI/cecal content supernatants through a 0.2μm filter. Sterile-filtered PBS was used as vehicle in all SI/cecal perfusion experiments.

***In vivo* vagal electrophysiology**

Baseline vagal tone recordings: Adult SPF, GF, ABX, or CONV male mice were anesthetized with isoflurane (5%) and maintained at 1.8% throughout the experiment. The cervical vagus nerve was exposed, transected inferior to the nodose ganglion and placed across two platinum iridium wires (insulated except for a short segment in contact with the nerve) for recording of baseline vagal tone. Recordings were conducted for 10 minutes. Vagal recordings with lumenal perfusion: the cervical vagus nerve was prepared as above in adult male SPF mice. Additionally, a 20-gauge gavage needle attached to a peristaltic pump (Cole Parmer) with separate tubing for each infusion solution was inserted into the duodenal lumen and secured with sutures. An outflow port was generated by transecting the small intestine ~10cm distal to the inflow site. During recording, vehicle was first perfused through the lumen at a constant rate for 10 minutes to establish a baseline following surgery at a flow rate of 250 ul/minute.[20] Following the baseline period, stimuli were introduced into the small-intestinal lumen and perfused at the same rate for the remainder of the experiment. Data Acquisition: a differential amplifier was used (A-M Systems LLC). The gain was set to 1000x and a bandpass filter was applied (300Hz-5kHz). The signal was digitized at 20kHz using a data acquisition board (National Instruments) under the control of LabView software Data Analysis: Spikes were detected using an SO-CFAR threshold (window duration of

1501/8000, guard duration 10/8000) to generate an adaptive threshold 4SD above RMS noise[88]. Firing rates were calculated by generating 10s (baseline vagal tone) or 30s (perfusion experiments) bins and then applying a Savitszky-Golay filter (OriginPro) of 10 points. Baseline vagal tone was defined as the average of the final 300s of recording. All raw values were normalized to the SPF cohort average for the rig on which the recordings took place. Perfusion experiments: baseline values were defined as the average frequency of the final 60s of recording in the initial baseline period (F_0). Frequency of the recording was then normalized to the baseline value within-subject (F/F_o). Area under the curve was calculated for each stimulus window and defined as the integral of frequencies over the stimulus window.

Metabolomics

Samples were collected from adult SPF, GF, ABX, or CONV mice. Lumenal contents were collected from the first 3cm of small intestine and the entirety of the cecum, then snap frozen in liquid nitrogen and stored at -80°C. Samples were prepared using the automated MicroLab STAR system (Hamilton Company) and analyzed on gas chromatography (GC)-mass spectrometry (MS), liquid chromatography (LC)-MS and LC-MS/MS platforms by Metabolon, inc. Protein fractions were removed by serial extractions with organic aqueous solvents, concentrated using a TurboVap system (Zymark) and vacuum dried. For LC/MS and LC-MS/MS, samples were reconstituted in acidic or basic LC-compatible solvents containing > 11 injection standards and run on a Waters ACQUITY UPLC and Thermo-Finnigan LTQ mass spectrometer, with a linear ion-trap front-end and a Fourier transform ion cyclotron resonance mass spectrometer back-end. For GC/MS, samples were derivatized under dried nitrogen using bistrimethyl-silyl-trifluoroacetamide and analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization. Chemical entities were identified by comparison to metabolomic library entries of purified standards. Following log transformation and imputation with minimum observed values for each compound, data were analyzed using

one-way ANOVA to test for group effects. P and q-values were calculated based on two-way ANOVA contrasts. Principal components analysis was used to visualize variance distributions. Supervised Random Forest analysis was conducted to identify metabolomics prediction accuracies.

Preparation of metabolite pools and single metabolites

All working metabolite solutions were made up in PBS and up to 0.05% DMSO, brought to a pH of 7.3, and sterile filtered. The concentrations for each metabolite pool and individual metabolites were determined from serum metabolomics data in the lab (data not shown) and existing literature and are as follows: Tryptophan metabolites (kynurenine 100uM,[89] kynurenic acid 16.1uM,[90] xanthurenate 1uM,[91] dihydrocaffeate 30nm, tryptamine 0.03uM, pooled), FAEs (oleylethanolamide (OEA), palmitoylethanolamide (PEA), linoleylethanolamide (LEA) 10uM, pooled)[92], HODEs (9-s- and 13-s- HODE,1uM, pooled), succinate (2mM), glutamate (50mM), bile acids (cholate 1240nM, glycocholate 3.5nM, chenodeoxycholate 42nM, alpha-muricholate 142nM, beta-muricholate 1080nM, deoxycholate 390nM, taurodeoxycholate 260nM, ursodeoxycholate 74nM, taurohyodeoxycholate 18.8nM, 7-ketodeoxycholate 100nM, lithocholate 390nM, taurolithocholate .33nM, pooled), short-chain fatty acids (acetate 80uM, butyrate 22uM, propionate 10uM, pooled), hippurate (2uM)[93], Trimethylamine-N-oxide (TMAO, 3uM)[94], imidazole propionate (200nM),[95] phenethylamine (PEA, 100uM)[13] or 3-indoxyl sulfate (1uM).[96] Vehicle for tryptophan metabolites, FAEs, MFAs, succinate, glutamate, SCFAs, TMAO, hippurate, imidazole propionate, and phenethylamine was sterile-filtered PBS. Vehicle for BAs was 0.05% DMSO in PBS, Vehicle for 3-IS was 2μM KCl in PBS.

***In vivo* calcium imaging**

Vagal afferent neuron imaging was performed as previously described.[20] In brief, SPF mice were anesthetized with a cocktail of ketamine and xylazine (100 mg/kg and 10 mg/kg, intraperitoneal),

tracheotomized, and maintained on 1.5% isoflurane for the remainder of the surgery and imaging session via ventilator (Kent Scientific). Body temperature was maintained at 37°C using an electrical heating pad and rectal temperature probe (Kent Scientific). The vagus nerve was transected superior to the jugular ganglion and vagal ganglia were then embedded between two 5mm-diameter glass coverslips (neuVitro) with silicone adhesive (KWIK-SIL, World Precision Instruments). Imaging was conducted on a Zeiss LSM 780 confocal microscope at a frame rate of 1Hz. Analysis of imaging data: Imaging data were analyzed using custom Python scripts based on the Minian pipeline.[97] Imaging data was registered to collect for motion artifacts and denoised using a median filter. A constrained non-negative matrix factorization (CNMF) algorithm was used to identify single cells and extract calcium activity. CNMF output regions of interest were then manually inspected to remove non-neuronal signals. Metabolite stimuli order was randomized to account for order-specific effects on neuronal activity. For single-unit analysis in response to sequential metabolite application, neuronal regions of interest were cross-registered across stimulus runs and compared to determine single- or dual- responsivity to metabolite classes. The baseline period for the calcium signal was calculated as the average of the last 120 seconds before stimulus onset (F_o). Responses were reported in units of baseline fluorescence. Cells were considered responsive to stimuli if the maximal $\Delta F/F$ signal following stimulus onset was i) greater than 2 standard deviations above the baseline fluorescence, and ii) the mean $\Delta F/F$ signal over a 20s window around peak response was >50% of the baseline value. Only units that displayed a $\Delta F/F$ signal >4 standard deviations over the baseline fluorescence in response to an electrical stimulus were included in analysis. For visualization, metabolite-responding units were hierarchically clustered over the entire time-course of each experiment.

Immunohistochemistry for cFos

Lumenal perfusion of stimuli: Mice were anesthetized with 5% isoflurane and maintained at 2% for the entirety of the surgery. To begin intralumenal perfusion, the small intestine was transected at the pyloric sphincter, the junction between the stomach and duodenum, to create an inflow port. Subsequently, a gavage needle attached to a peristaltic pump was inserted into the duodenal lumen. An outflow port was made by transecting the small intestine 3 centimeters distal to the inflow port. All lumenal contents were flushed from the small intestine with PBS. Animals were then continually perfused with PBS at 250uL/min flow rate for a 10-minute baseline period, followed by perfusion of stimuli for 30 minutes at a constant flow rate to account for any mechanical distension. Following stimulus perfusion, mice remained under anesthesia for one hour to allow for cFos induction before tissue harvesting. Following the one-hour rest period, animals were sacrificed, and tissues were harvested and fixed via intracardial perfusion of ice-cold PBS followed by 4% paraformaldehyde (PFA). Brains were then post-fixed in 4% PFA at 4°C for 3 hours followed by an overnight incubation in 30% sucrose at 4°C for cryoprotection. Ganglia were positioned bulb-side down in optimal cutting temperature compound (OCT compound) and frozen for cryostat sectioning. Tissues were then sectioned at 30μm and mounted on a microscope slide for immunohistochemical processing (IHC). Slides were thawed for 10 minutes at room temperature in a humidified chamber to prevent the tissue from drying and then permeabilized in 0.5% Triton/0.05% tween-20 in PBS (PBS-TT). Blocking solution consisting of 5% normal goat serum (NGS) in PBS-TT was applied to the tissue and allowed to incubate at room temperature for two hours to prevent non-specific antibody binding and reduce background staining. The tissue sections were then incubated overnight at 4°C with primary antibodies (rabbit anti-cFos 1:500, and guinea pig anti-NeuN, 1:500) in blocking solution (5% NGS + PBS-TT). The following day, slides were washed three times, five minutes per wash, with PBS-TT before incubating at room temperature for two hours with secondary antibodies (goat anti-rabbit 488 1:1000, goat anti-guinea pig 568 1:1000, and DAPI 1:1000) in blocking solution (5% NGS + PBS-TT). Confocal Imaging and Quantification Analysis: Images were obtained using a 20x air

objective (NA 0.8) on an upright Zeiss LSM 780 confocal microscope. Z-stacks were acquired for three technical replicates of NTS brain tissue and maximum-intensity projections were generated for subsequent analysis in ImageJ. NTS sections were selected and ROI's were drawn based off of the Allen Mouse Brain Atlas. First, NeuN positive (NeuN+) cells that were each confirmed to colocalize with a DAPI nucleus were counted using the multi-point tool to obtain the total number of neurons. Subsequently, cFos+ cells were counted using the multi-point tool by confirming colocalization of a cFos immunofluorescence signal with NeuN and DAPI. For each image, the total number of cFos+ neurons were divided by the total number of NeuN+ cells to obtain the percentage of cFos+ neurons. Finally, the percentage of cFos+ neurons for all technical replicates of NTS slices per animal were averaged to obtain a biological n=1 and find the overall percentage of cFos+ neurons.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was performed using Prism software version 8.2.1 (GraphPad). Data were assessed for normal distribution and plotted in the figures as mean ± SEM. For each figure, *n* = the number of independent biological replicates. Outliers were identified with ROUT using a threshold of q = 2% for all electrophysiological recordings. Differences between two treatment groups were assessed using two-tailed, unpaired Student *t* test with Welch's correction. Differences among >2 groups with only one variable were assessed using one-way ANOVA. If groups were determined to have significantly different variances, groups were assessed with Brown-Forsythe and Welch ANOVA + Games-Howell testing or Wilcoxon matched-pairs signed rank test. Significant differences emerging from the above tests are indicated in the figures by *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Notable non-significant (and non-near significant) differences are indicated in the figures by "n.s.".

REFERENCES

1. Vuong, H.E., Pronovost, G.N., Williams, D.W., Coley, E.J.L., Siegler, E.L., Qiu, A., Kazantsev, M., Wilson, C.J., Rendon, T., and Hsiao, E.Y. (2020). The maternal microbiome modulates fetal neurodevelopment in mice. Nature *586*, 281-286. 10.1038/s41586-020-2745-3.
2. Sgritta, M., Dooling, S.W., Buffington, S.A., Momin, E.N., Francis, M.B., Britton, R.A., and Costa-Mattioli, M. (2019). Mechanisms Underlying Microbial-Mediated Changes in Social Behavior in Mouse Models of Autism Spectrum Disorder. Neuron *101*, 246-259 e246. 10.1016/j.neuron.2018.11.018.
3. Sampson, T.R., Debelius, J.W., Thron, T., Janssen, S., Shastri, G.G., Ilhan, Z.E., Challis, C., Schretter, C.E., Rocha, S., Gradinaru, V., et al. (2016). Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease. Cell *167*, 1469-1480 e1412. 10.1016/j.cell.2016.11.018.
4. Erny, D., Hrabe de Angelis, A.L., Jaitin, D., Wieghofer, P., Staszewski, O., David, E., Keren-Shaul, H., Mahlakoiv, T., Jakobshagen, K., Buch, T., et al. (2015). Host microbiota constantly control maturation and function of microglia in the CNS. Nat Neurosci *18*, 965-977. 10.1038/nn.4030.
5. Singh, V., Roth, S., Llovera, G., Sadler, R., Garzetti, D., Stecher, B., Dichgans, M., and Liesz, A. (2016). Microbiota Dysbiosis Controls the Neuroinflammatory Response after Stroke. J Neurosci *36*, 7428-7440. 10.1523/JNEUROSCI.1114-16.2016.
6. Fulling, C., Dinan, T.G., and Cryan, J.F. (2019). Gut Microbe to Brain Signaling: What Happens in Vagus. Neuron *101*, 998-1002. 10.1016/j.neuron.2019.02.008.
7. Bravo, J.A., Forsythe, P., Chew, M.V., Escaravage, E., Savignac, H.M., Dinan, T.G., Bienenstock, J., and Cryan, J.F. (2011). Ingestion of Lactobacillus strain regulates

emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc Natl Acad Sci U S A *108*, 16050-16055. 10.1073/pnas.1102999108.

8. Bharwani, A., West, C., Champagne-Jorgensen, K., McVey Neufeld, K.A., Ruberto, J., Kunze, W.A., Bienenstock, J., and Forsythe, P. (2020). The vagus nerve is necessary for the rapid and widespread neuronal activation in the brain following oral administration of psychoactive bacteria. Neuropharmacology *170*, 108067. 10.1016/j.neuropharm.2020.108067.
9. Bercik, P., Park, A.J., Sinclair, D., Khoshdel, A., Lu, J., Huang, X., Deng, Y., Blennerhassett, P.A., Fahnestock, M., Moine, D., et al. (2011). The anxiolytic effect of Bifidobacterium longum NCC3001 involves vagal pathways for gut-brain communication. Neurogastroenterol Motil *23*, 1132-1139. 10.1111/j.1365-2982.2011.01796.x.
10. Lee, K.E., Kim, J.K., Han, S.K., Lee, D.Y., Lee, H.J., Yim, S.V., and Kim, D.H. (2020). The extracellular vesicle of gut microbial Paenalcaligenes hominis is a risk factor for vagus nerve-mediated cognitive impairment. Microbiome *8*, 107. 10.1186/s40168-020-00881-2.
11. Wikoff, W.R., Anfora, A.T., Liu, J., Schultz, P.G., Lesley, S.A., Peters, E.C., and Siuzdak, G. (2009). Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. Proc Natl Acad Sci U S A *106*, 3698-3703. 10.1073/pnas.0812874106.
12. Quinn, R.A., Melnik, A.V., Vrbanac, A., Fu, T., Patras, K.A., Christy, M.P., Bodai, Z., Belda-Ferre, P., Tripathi, A., Chung, L.K., et al. (2020). Global chemical effects of the microbiome include new bile-acid conjugations. Nature *579*, 123-129. 10.1038/s41586-020-2047-9.
13. Chen, H., Nwe, P.K., Yang, Y., Rosen, C.E., Bielecka, A.A., Kuchroo, M., Cline, G.W., Kruse, A.C., Ring, A.M., Crawford, J.M., and Palm, N.W. (2019). A Forward Chemical

Genetic Screen Reveals Gut Microbiota Metabolites That Modulate Host Physiology. Cell *177*, 1217-1231 e1218. 10.1016/j.cell.2019.03.036.

14. Cohen, L.J., Esterhazy, D., Kim, S.H., Lemetre, C., Aguilar, R.R., Gordon, E.A., Pickard, A.J., Cross, J.R., Emiliano, A.B., Han, S.M., et al. (2017). Commensal bacteria make GPCR ligands that mimic human signalling molecules. Nature *549*, 48-53. 10.1038/nature23874.
15. Kupari, J., Haring, M., Agirre, E., Castelo-Branco, G., and Ernfors, P. (2019). An Atlas of Vagal Sensory Neurons and Their Molecular Specialization. Cell Rep *27*, 2508-2523 e2504. 10.1016/j.celrep.2019.04.096.
16. Bai, L., Mesgarzadeh, S., Ramesh, K.S., Huey, E.L., Liu, Y., Gray, L.A., Aitken, T.J., Chen, Y., Beutler, L.R., Ahn, J.S., et al. (2019). Genetic Identification of Vagal Sensory Neurons That Control Feeding. Cell *179*, 1129-1143 e1123. 10.1016/j.cell.2019.10.031.
17. Zhao, Q., Yu, C.D., Wang, R., Xu, Q.J., Dai Pra, R., Zhang, L., and Chang, R.B. (2022). A multidimensional coding architecture of the vagal interoceptive system. Nature *603*, 878-884. 10.1038/s41586-022-04515-5.
18. Prescott, S.L., Umans, B.D., Williams, E.K., Brust, R.D., and Liberles, S.D. (2020). An Airway Protection Program Revealed by Sweeping Genetic Control of Vagal Afferents. Cell *181*, 574-589 e514. 10.1016/j.cell.2020.03.004.
19. Tao, J., Campbell, J.N., Tsai, L.T., Wu, C., Liberles, S.D., and Lowell, B.B. (2021). Highly selective brain-to-gut communication via genetically defined vagus neurons. Neuron *109*, 2106-2115 e2104. 10.1016/j.neuron.2021.05.004.
20. Williams, E.K., Chang, R.B., Strochlic, D.E., Umans, B.D., Lowell, B.B., and Liberles, S.D. (2016). Sensory Neurons that Detect Stretch and Nutrients in the Digestive System. Cell *166*, 209-221. 10.1016/j.cell.2016.05.011.
21. Buchanan, K.L., Rupprecht, L.E., Kaelberer, M.M., Sahasrabudhe, A., Klein, M.E., Villalobos, J.A., Liu, W.W., Yang, A., Gelman, J., Park, S., et al. (2022). The preference

for sugar over sweetener depends on a gut sensor cell. Nat Neurosci *25*, 191-200. 10.1038/s41593-021-00982-7.

22. Li, M., Tan, H.E., Lu, Z., Tsang, K.S., Chung, A.J., and Zuker, C.S. (2022). Gut-Brain Circuits for Fat Preference. Nature. 10.1038/s41586-022-05266-z.

23. Sugisawa, E., Takayama, Y., Takemura, N., Kondo, T., Hatakeyama, S., Kumagai, Y., Sunagawa, M., Tominaga, M., and Maruyama, K. (2020). RNA Sensing by Gut Piezo1 Is Essential for Systemic Serotonin Synthesis. Cell *182*, 609-624 e621. 10.1016/j.cell.2020.06.022.

24. Uhlig, F., Grundy, L., Garcia-Caraballo, S., Brierley, S.M., Foster, S.J., and Grundy, D. (2020). Identification of a Quorum Sensing-Dependent Communication Pathway Mediating Bacteria-Gut-Brain Cross Talk. iScience *23*, 101695. 10.1016/j.isci.2020.101695.

25. Blair, J.M., Webber, M.A., Baylay, A.J., Ogbolu, D.O., and Piddock, L.J. (2015). Molecular mechanisms of antibiotic resistance. Nat Rev Microbiol *13*, 42-51. 10.1038/nrmicro3380.

26. Hauser, W.E., Jr., and Remington, J.S. (1982). Effect of antibiotics on the immune response. Am J Med *72*, 711-716. 10.1016/0002-9343(82)90534-4.

27. Liang, G., and Bushman, F.D. (2021). The human virome: assembly, composition and host interactions. Nat Rev Microbiol *19*, 514-527. 10.1038/s41579-021-00536-5.

28. Kennedy, E.A., King, K.Y., and Baldridge, M.T. (2018). Mouse Microbiota Models: Comparing Germ-Free Mice and Antibiotics Treatment as Tools for Modifying Gut Bacteria. Front Physiol *9*, 1534. 10.3389/fphys.2018.01534.

29. Weiner, A., Turjeman, S., and Koren, O. (2023). Gut microbes and host behavior: The forgotten members of the gut-microbiome. Neuropharmacology *227*, 109453. 10.1016/j.neuropharm.2023.109453.

30. Silva, Y.P., Bernardi, A., and Frozza, R.L. (2020). The Role of Short-Chain Fatty Acids From Gut Microbiota in Gut-Brain Communication. Front Endocrinol (Lausanne) *11*, 25. 10.3389/fendo.2020.00025.

31. Wu, X., Li, J.Y., Lee, A., Lu, Y.X., Zhou, S.Y., and Owyang, C. (2020). Satiety induced by bile acids is mediated via vagal afferent pathways. JCI Insight *5*. 10.1172/jci.insight.132400.

32. Thomas, C., Gioiello, A., Noriega, L., Strehle, A., Oury, J., Rizzo, G., Macchiarulo, A., Yamamoto, H., Mataki, C., Pruzanski, M., et al. (2009). TGR5-mediated bile acid sensing controls glucose homeostasis. Cell Metab *10*, 167-177. 10.1016/j.cmet.2009.08.001.

33. Wahlstrom, A., Sayin, S.I., Marschall, H.U., and Backhed, F. (2016). Intestinal Crosstalk between Bile Acids and Microbiota and Its Impact on Host Metabolism. Cell Metab *24*, 41-50. 10.1016/j.cmet.2016.05.005.

34. Haber, A.L., Biton, M., Rogel, N., Herbst, R.H., Shekhar, K., Smillie, C., Burgin, G., Delorey, T.M., Howitt, M.R., Katz, Y., et al. (2017). A single-cell survey of the small intestinal epithelium. Nature *551*, 333-339. 10.1038/nature24489.

35. Zarei, I., Koistinen, V.M., Kokla, M., Klavus, A., Babu, A.F., Lehtonen, M., Auriola, S., and Hanhineva, K. (2022). Tissue-wide metabolomics reveals wide impact of gut microbiota on mice metabolite composition. Sci Rep *12*, 15018. 10.1038/s41598-022-19327-w.

36. Cox, L.M., Yamanishi, S., Sohn, J., Alekseyenko, A.V., Leung, J.M., Cho, I., Kim, S.G., Li, H., Gao, Z., Mahana, D., et al. (2014). Altering the intestinal microbiota during a critical developmental window has lasting metabolic consequences. Cell *158*, 705-721. 10.1016/j.cell.2014.05.052.

37. Tirelle, P., Breton, J., Riou, G., Dechelotte, P., Coeffier, M., and Ribet, D. (2020). Comparison of different modes of antibiotic delivery on gut microbiota depletion

efficiency and body composition in mouse. BMC Microbiol *20*, 340. 10.1186/s12866-020-02018-9.

38. Egerod, K.L., Petersen, N., Timshel, P.N., Rekling, J.C., Wang, Y., Liu, Q., Schwartz, T.W., and Gautron, L. (2018). Profiling of G protein-coupled receptors in vagal afferents reveals novel gut-to-brain sensing mechanisms. Mol Metab *12*, 62-75. 10.1016/j.molmet.2018.03.016.

39. Uneyama, H., Niijima, A., San Gabriel, A., and Torii, K. (2006). Luminal amino acid sensing in the rat gastric mucosa. Am J Physiol Gastrointest Liver Physiol *291*, G1163-1170. 10.1152/ajpgi.00587.2005.

40. Schmitt, M.G., Jr., Soergel, K.H., Wood, C.M., and Steff, J.J. (1977). Absorption of short-chain fatty acids from the human ileum. Am J Dig Dis *22*, 340-347. 10.1007/BF01072192.

41. Karaki, S., Tazoe, H., Hayashi, H., Kashiwabara, H., Tooyama, K., Suzuki, Y., and Kuwahara, A. (2008). Expression of the short-chain fatty acid receptor, GPR43, in the human colon. J Mol Histol *39*, 135-142. 10.1007/s10735-007-9145-y.

42. Tazoe, H., Otomo, Y., Karaki, S., Kato, I., Fukami, Y., Terasaki, M., and Kuwahara, A. (2009). Expression of short-chain fatty acid receptor GPR41 in the human colon. Biomed Res *30*, 149-156. 10.2220/biomedres.30.149.

43. Ye, L., Bae, M., Cassilly, C.D., Jabba, S.V., Thorpe, D.W., Martin, A.M., Lu, H.Y., Wang, J., Thompson, J.D., Lickwar, C.R., et al. (2021). Enteroendocrine cells sense bacterial tryptophan catabolites to activate enteric and vagal neuronal pathways. Cell Host Microbe *29*, 179-196 e179. 10.1016/j.chom.2020.11.011.

44. Bellono, N.W., Bayrer, J.R., Leitch, D.B., Castro, J., Zhang, C., O'Donnell, T.A., Brierley, S.M., Ingraham, H.A., and Julius, D. (2017). Enterochromaffin Cells Are Gut Chemosensors that Couple to Sensory Neural Pathways. Cell *170*, 185-198 e116. 10.1016/j.cell.2017.05.034.

45. Fiorucci, S., Biagioli, M., Zampella, A., and Distrutti, E. (2018). Bile Acids Activated Receptors Regulate Innate Immunity. Front Immunol *9*, 1853. 10.3389/fimmu.2018.01853.

46. Kim, C.H. (2021). Control of lymphocyte functions by gut microbiota-derived short-chain fatty acids. Cell Mol Immunol *18*, 1161-1171. 10.1038/s41423-020-00625-0.

47. Sayin, S.I., Wahlstrom, A., Felin, J., Jantti, S., Marschall, H.U., Bamberg, K., Angelin, B., Hyotylainen, T., Oresic, M., and Backhed, F. (2013). Gut microbiota regulates bile acid metabolism by reducing the levels of tauro-beta-muricholic acid, a naturally occurring FXR antagonist. Cell Metab *17*, 225-235. 10.1016/j.cmet.2013.01.003.

48. Masyuk, T.V., Masyuk, A.I., Lorenzo Pisarello, M., Howard, B.N., Huang, B.Q., Lee, P.Y., Fung, X., Sergienko, E., Ardecky, R.J., Chung, T.D.Y., et al. (2017). TGR5 contributes to hepatic cystogenesis in rodents with polycystic liver diseases through cyclic adenosine monophosphate/Galphas signaling. Hepatology *66*, 1197-1218. 10.1002/hep.29284.

49. Cook, T.M., Gavini, C.K., Jesse, J., Aubert, G., Gornick, E., Bonomo, R., Gautron, L., Layden, B.T., and Mansuy-Aubert, V. (2021). Vagal neuron expression of the microbiota-derived metabolite receptor, free fatty acid receptor (FFAR3), is necessary for normal feeding behavior. Mol Metab *54*, 101350. 10.1016/j.molmet.2021.101350.

50. Pizzonero, M., Dupont, S., Babel, M., Beaumont, S., Bienvenu, N., Blanque, R., Cherel, L., Christophe, T., Crescenzi, B., De Lemos, E., et al. (2014). Discovery and optimization of an azetidine chemical series as a free fatty acid receptor 2 (FFA2) antagonist: from hit to clinic. J Med Chem *57*, 10044-10057. 10.1021/jm5012885.

51. Popkov, V.A., Zharikova, A.A., Demchenko, E.A., Andrianova, N.V., Zorov, D.B., and Plotnikov, E.Y. (2022). Gut Microbiota as a Source of Uremic Toxins. Int J Mol Sci *23*. 10.3390/ijms23010483.

52. Brydges, C.R., Fiehn, O., Mayberg, H.S., Schreiber, H., Dehkordi, S.M., Bhattacharyya, S., Cha, J., Choi, K.S., Craighead, W.E., Krishnan, R.R., et al. (2021). Indoxyl sulfate, a gut microbiome-derived uremic toxin, is associated with psychic anxiety and its functional magnetic resonance imaging-based neurologic signature. Sci Rep *11*, 21011. 10.1038/s41598-021-99845-1.

53. Sun, C.Y., Li, J.R., Wang, Y.Y., Lin, S.Y., Ou, Y.C., Lin, C.J., Wang, J.D., Liao, S.L., and Chen, C.J. (2021). Indoxyl sulfate caused behavioral abnormality and neurodegeneration in mice with unilateral nephrectomy. Aging (Albany NY) *13*, 6681-6701. 10.18632/aging.202523.

54. Le, J., Peng, R., and Li, Y. (2022). Trimethylamine-N-Oxide and Precursors as Novel Potential Biomarkers for Anxiety Disorder. Lab Med *53*, 177-182. 10.1093/labmed/lmab063.

55. Chen, J.J., Bai, S.J., Li, W.W., Zhou, C.J., Zheng, P., Fang, L., Wang, H.Y., Liu, Y.Y., and Xie, P. (2018). Urinary biomarker panel for diagnosing patients with depression and anxiety disorders. Transl Psychiatry *8*, 192. 10.1038/s41398-018-0245-0.

56. Gao, K., Mu, C.L., Farzi, A., and Zhu, W.Y. (2020). Tryptophan Metabolism: A Link Between the Gut Microbiota and Brain. Adv Nutr *11*, 709-723. 10.1093/advances/nmz127.

57. Siopi, E., Galerne, M., Rivagorda, M., Saha, S., Moigneu, C., Moriceau, S., Bigot, M., Oury, F., and Lledo, P.M. (2023). Gut microbiota changes require vagus nerve integrity to promote depressive-like behaviors in mice. Mol Psychiatry. 10.1038/s41380-023-02071-6.

58. Muller, P.A., Schneeberger, M., Matheis, F., Wang, P., Kerner, Z., Ilanges, A., Pellegrino, K., Del Marmol, J., Castro, T.B.R., Furuichi, M., et al. (2020). Microbiota modulate sympathetic neurons via a gut-brain circuit. Nature *583*, 441-446. 10.1038/s41586-020-2474-7.

59. D'Agostino, G., Lyons, D.J., Cristiano, C., Burke, L.K., Madara, J.C., Campbell, J.N., Garcia, A.P., Land, B.B., Lowell, B.B., Dileone, R.J., and Heisler, L.K. (2016). Appetite controlled by a cholecystokinin nucleus of the solitary tract to hypothalamus neurocircuit. Elife *5*. 10.7554/eLife.12225.

60. Vazquez, E., Barranco, A., Ramirez, M., Gruart, A., Delgado-Garcia, J.M., Jimenez, M.L., Buck, R., and Rueda, R. (2016). Dietary 2'-Fucosyllactose Enhances Operant Conditioning and Long-Term Potentiation via Gut-Brain Communication through the Vagus Nerve in Rodents. PLoS One *11*, e0166070. 10.1371/journal.pone.0166070.

61. Yoshioka, Y., Tachibana, Y., Uesaka, T., Hioki, H., Sato, Y., Fukumoto, T., and Enomoto, H. (2022). Uts2b is a microbiota-regulated gene expressed in vagal afferent neurons connected to enteroendocrine cells producing cholecystokinin. Biochem Biophys Res Commun *608*, 66-72. 10.1016/j.bbrc.2022.03.117.

62. Pradhananga, S., Tashtush, A.A., Allen-Vercoe, E., Petrof, E.O., and Lomax, A.E. (2020). Protease-dependent excitation of nodose ganglion neurons by commensal gut bacteria. J Physiol *598*, 2137-2151. 10.1113/JP279075.

63. Ferraris, R.P., Yasharpour, S., Lloyd, K.C., Mirzayan, R., and Diamond, J.M. (1990). Luminal glucose concentrations in the gut under normal conditions. Am J Physiol *259*, G822-837. 10.1152/ajpgi.1990.259.5.G822.

64. Sun, Y., Huang, J., Xiang, Y., Bastepe, M., Juppner, H., Kobilka, B.K., Zhang, J.J., and Huang, X.Y. (2007). Dosage-dependent switch from G protein-coupled to G protein-independent signaling by a GPCR. EMBO J *26*, 53-64. 10.1038/sj.emboj.7601502.

65. Beumer, J., Puschhof, J., Bauza-Martinez, J., Martinez-Silgado, A., Elmentaite, R., James, K.R., Ross, A., Hendriks, D., Artegiani, B., Busslinger, G.A., et al. (2020). High-Resolution mRNA and Secretome Atlas of Human Enteroendocrine Cells. Cell *182*, 1062-1064. 10.1016/j.cell.2020.08.005.

66. Kowalski, C.W., Lindberg, J.E.M., Fowler, D.K., Simasko, S.M., and Peters, J.H. (2020). Contributing mechanisms underlying desensitization of cholecystokinin-induced activation of primary nodose ganglia neurons. Am J Physiol Cell Physiol *318*, C787-C796. 10.1152/ajpcell.00192.2019.

67. Tolhurst, G., Heffron, H., Lam, Y.S., Parker, H.E., Habib, A.M., Diakogiannaki, E., Cameron, J., Grosse, J., Reimann, F., and Gribble, F.M. (2012). Short-chain fatty acids stimulate glucagon-like peptide-1 secretion via the G-protein-coupled receptor FFAR2. Diabetes *61*, 364-371. 10.2337/db11-1019.

68. Cho, H.J., Callaghan, B., Bron, R., Bravo, D.M., and Furness, J.B. (2014). Identification of enteroendocrine cells that express TRPA1 channels in the mouse intestine. Cell Tissue Res *356*, 77-82. 10.1007/s00441-013-1780-x.

69. Fothergill, L.J., Callaghan, B., Rivera, L.R., Lieu, T., Poole, D.P., Cho, H.J., Bravo, D.M., and Furness, J.B. (2016). Effects of Food Components That Activate TRPA1 Receptors on Mucosal Ion Transport in the Mouse Intestine. Nutrients *8*. 10.3390/nu8100623.

70. Nakajima, S., Hira, T., Yahagi, A., Nishiyama, C., Yamashita, T., Imagi, J., and Hara, H. (2014). Unsaturated aldehydes induce CCK secretion via TRPA1 in STC-1 cells. Mol Nutr Food Res *58*, 1042-1051. 10.1002/mnfr.201300412.

71. Purhonen, A.K., Louhivuori, L.M., Kiehne, K., Kerman, K.E., and Herzig, K.H. (2008). TRPA1 channel activation induces cholecystokinin release via extracellular calcium. FEBS Lett *582*, 229-232. 10.1016/j.febslet.2007.12.005.

72. Christiansen, C.B., Gabe, M.B.N., Svendsen, B., Dragsted, L.O., Rosenkilde, M.M., and Holst, J.J. (2018). The impact of short-chain fatty acids on GLP-1 and PYY secretion from the isolated perfused rat colon. Am J Physiol Gastrointest Liver Physiol *315*, G53-G65. 10.1152/ajpgi.00346.2017.

73. Emery, E.C., Diakogiannaki, E., Gentry, C., Psichas, A., Habib, A.M., Bevan, S., Fischer, M.J., Reimann, F., and Gribble, F.M. (2015). Stimulation of GLP-1 secretion downstream of the ligand-gated ion channel TRPA1. Diabetes *64*, 1202-1210. 10.2337/db14-0737.

74. Chepurny, O.G., Holz, G.G., Roe, M.W., and Leech, C.A. (2016). GPR119 Agonist AS1269574 Activates TRPA1 Cation Channels to Stimulate GLP-1 Secretion. Mol Endocrinol *30*, 614-629. 10.1210/me.2015-1306.

75. Perino, A., Velazquez-Villegas, L.A., Bresciani, N., Sun, Y., Huang, Q., Fenelon, V.S., Castellanos-Jankiewicz, A., Zizzari, P., Bruschetta, G., Jin, S., et al. (2021). Central anorexigenic actions of bile acids are mediated by TGR5. Nat Metab *3*, 595-603. 10.1038/s42255-021-00398-4.

76. Fernandes, A.B., Alves da Silva, J., Almeida, J., Cui, G., Gerfen, C.R., Costa, R.M., and Oliveira-Maia, A.J. (2020). Postingestive Modulation of Food Seeking Depends on Vagus-Mediated Dopamine Neuron Activity. Neuron *106*, 778-788 e776. 10.1016/j.neuron.2020.03.009.

77. Chadaideh, K.S., and Carmody, R.N. (2021). Host-microbial interactions in the metabolism of different dietary fats. Cell Metab *33*, 857-872. 10.1016/j.cmet.2021.04.011.

78. Jones, B.V., Begley, M., Hill, C., Gahan, C.G., and Marchesi, J.R. (2008). Functional and comparative metagenomic analysis of bile salt hydrolase activity in the human gut microbiome. Proc Natl Acad Sci U S A *105*, 13580-13585. 10.1073/pnas.0804437105.

79. Louis, P., and Flint, H.J. (2017). Formation of propionate and butyrate by the human colonic microbiota. Environ Microbiol *19*, 29-41. 10.1111/1462-2920.13589.

80. Louis, P., Young, P., Holtrop, G., and Flint, H.J. (2010). Diversity of human colonic butyrate-producing bacteria revealed by analysis of the butyryl-CoA:acetate CoA-transferase gene. Environ Microbiol *12*, 304-314. 10.1111/j.1462-2920.2009.02066.x.

81. Lee, J.H., and Lee, J. (2010). Indole as an intercellular signal in microbial communities. FEMS Microbiol Rev *34*, 426-444. 10.1111/j.1574-6976.2009.00204.x.

82. Zeb, F., Wu, X., Chen, L., Fatima, S., Ijaz Ul, H., Chen, A., Xu, C., Jianglei, R., Feng, Q., and Li, M. (2020). Time-restricted feeding is associated with changes in human gut microbiota related to nutrient intake. Nutrition *78*, 110797. 10.1016/j.nut.2020.110797.

83. Folz, J., Culver, R.N., Morales, J.M., Grembi, J., Triadafilopoulos, G., Relman, D.A., Huang, K.C., Shalon, D., and Fiehn, O. (2023). Human metabolome variation along the upper intestinal tract. Nat Metab *5*, 777-788. 10.1038/s42255-023-00777-z.

84. Han, W., Tellez, L.A., Perkins, M.H., Perez, I.O., Qu, T., Ferreira, J., Ferreira, T.L., Quinn, D., Liu, Z.W., Gao, X.B., et al. (2018). A Neural Circuit for Gut-Induced Reward. Cell *175*, 887-888. 10.1016/j.cell.2018.10.018.

85. van de Wouw, M., Boehme, M., Lyte, J.M., Wiley, N., Strain, C., O'Sullivan, O., Clarke, G., Stanton, C., Dinan, T.G., and Cryan, J.F. (2018). Short-chain fatty acids: microbial metabolites that alleviate stress-induced brain-gut axis alterations. J Physiol *596*, 4923-4944. 10.1113/JP276431.

86. Lirong, W., Mingliang, Z., Mengci, L., Qihao, G., Zhenxing, R., Xiaojiao, Z., and Tianlu, C. (2022). The clinical and mechanistic roles of bile acids in depression, Alzheimer's disease, and stroke. Proteomics *22*, e2100324. 10.1002/pmic.202100324.

87. Val-Laillet, D., Guerin, S., Coquery, N., Nogret, I., Formal, M., Rome, V., Le Normand, L., Meurice, P., Randuineau, G., Guilloteau, P., et al. (2018). Oral sodium butyrate impacts brain metabolism and hippocampal neurogenesis, with limited effects on gut anatomy and function in pigs. FASEB J *32*, 2160-2171. 10.1096/fj.201700547RR.

88. Zanos, T.P., Silverman, H.A., Levy, T., Tsaava, T., Battinelli, E., Lorraine, P.W., Ashe, J.M., Chavan, S.S., Tracey, K.J., and Bouton, C.E. (2018). Identification of cytokine-specific sensory neural signals by decoding murine vagus nerve activity. Proc Natl Acad Sci U S A *115*, E4843-E4852. 10.1073/pnas.1719083115.

89. Refaey, M.E., McGee-Lawrence, M.E., Fulzele, S., Kennedy, E.J., Bollag, W.B., Elsalanty, M., Zhong, Q., Ding, K.H., Bendzunas, N.G., Shi, X.M., et al. (2017). Kynurenine, a Tryptophan Metabolite That Accumulates With Age, Induces Bone Loss. J Bone Miner Res *32*, 2182-2193. 10.1002/jbmr.3224.

90. Wang, J., Simonavicius, N., Wu, X., Swaminath, G., Reagan, J., Tian, H., and Ling, L. (2006). Kynurenic acid as a ligand for orphan G protein-coupled receptor GPR35. J Biol Chem *281*, 22021-22028. 10.1074/jbc.M603503200.

91. Fazio, F., Lionetto, L., Curto, M., Iacovelli, L., Cavallari, M., Zappulla, C., Ulivieri, M., Napoletano, F., Capi, M., Corigliano, V., et al. (2015). Xanthurenic Acid Activates mGlu2/3 Metabotropic Glutamate Receptors and is a Potential Trait Marker for Schizophrenia. Sci Rep *5*, 17799. 10.1038/srep17799.

92. Fu, J., Gaetani, S., Oveisi, F., Lo Verme, J., Serrano, A., Rodriguez De Fonseca, F., Rosengarth, A., Luecke, H., Di Giacomo, B., Tarzia, G., and Piomelli, D. (2003). Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-alpha. Nature *425*, 90-93. 10.1038/nature01921.

93. Huang, M., Wei, R., Wang, Y., Su, T., Li, P., and Chen, X. (2018). The uremic toxin hippurate promotes endothelial dysfunction via the activation of Drp1-mediated mitochondrial fission. Redox Biol *16*, 303-313. 10.1016/j.redox.2018.03.010.

94. Chan, M.M., Yang, X., Wang, H., Saaoud, F., Sun, Y., and Fong, D. (2019). The Microbial Metabolite Trimethylamine N-Oxide Links Vascular Dysfunctions and the Autoimmune Disease Rheumatoid Arthritis. Nutrients *11*. 10.3390/nu11081821.

95. Koh, A., Molinaro, A., Stahlman, M., Khan, M.T., Schmidt, C., Manneras-Holm, L., Wu, H., Carreras, A., Jeong, H., Olofsson, L.E., et al. (2018). Microbially Produced Imidazole Propionate Impairs Insulin Signaling through mTORC1. Cell *175*, 947-961 e917. 10.1016/j.cell.2018.09.055.

96. Schroeder, J.C., Dinatale, B.C., Murray, I.A., Flaveny, C.A., Liu, Q., Laurenzana, E.M., Lin, J.M., Strom, S.C., Omiecinski, C.J., Amin, S., and Perdew, G.H. (2010). The uremic toxin 3-indoxyl sulfate is a potent endogenous agonist for the human aryl hydrocarbon receptor. Biochemistry *49*, 393-400. 10.1021/bi901786x.

97. Dong, Z., Mau, W., Feng, Y., Pennington, Z.T., Chen, L., Zaki, Y., Rajan, K., Shuman, T., Aharoni, D., and Cai, D.J. (2022). Minian, an open-source miniscope analysis pipeline. Elife *11*. 10.7554/eLife.70661.