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Investigating Microbiome-Based Countermeasures to Promote Resilience to Sleep Disruption and Stress

A DISSERTATION

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Abstract

Nearly all animals exhibit behaviors that can be classified as sleep. The distinctly disadvantageous nature of the asleep state, evolutionarily speaking, accentuates its role as a critical physiological process, yet chronic inadequate sleep is prevalent in today's society. Among the multitude of health problems that have been linked to chronic sleep disruption, increased inflammation and alterations to the responsiveness of the hypothalamic-pituitary-adrenal (HPA) axis indicate that the sleep-deprived state may result in a vulnerability to a second hit of an acute stressor. The project described in this dissertation examines this relationship between sleep disruption and stress vulnerability and investigates novel approaches to increase stress robustness in the context of disrupted sleep, focusing on the role of a newly characterized system: the gut microbiome. Our intestinal tracts are home to trillions of microbes with a collective genetic diversity that dwarfs our own and which strongly influence our gastrointestinal, metabolic, endocrine, immune, and nervous systems. These discoveries have opened the door for research into microbiome-based interventions such as vaccination with immunomodulatory bacteria and manipulation of the microbiome with prebiotic diets that aim to improve health.

This dissertation begins with a chapter reviewing our current understanding of sleep, stress responses, and the microbiome, with an emphasis on how sleep disruption may interact with both the microbiome and stress vulnerability. The second chapter describes results from a study in mice characterizing the effect of repeated sleep disruption on the fecal microbiome and fecal

metabolome. Next, the third chapter reports findings from a larger mouse study investigating the impact of repeated sleep disruption on vulnerability to a secondary acute stressor, and the ability of immunization with a heat-killed bacterium called *Mycobacterium vaccae* NCTC 11659 to improve resilience to these manipulations. The fourth chapter reports a detailed characterization of the gut microbiomes and metabolomes from mice studied in Chapter III, identifying candidate molecules that may be contributing to stress vulnerability/resilience in the context of sleep disruption. Then, the Chapter V discusses the findings from a study in rats that used a similar protocol of combined repeated sleep disruption and acute stress, but half of these animals were instead fed a microbiome-modifying prebiotic diet. Finally, the entirety of these results are discussed in relation to the project goals and current/future research directions are outlined in Chapter VI. Overall, the series of experiments and analyses contained in this dissertation contribute to our understanding of the relationship between sleep and stress vulnerability, and provide evidence supporting microbiome-based interventions such as *M. vaccae* immunization and prebiotic diets as intriguing new countermeasures to improve resilience to sleep disruption.

There were 4 supplemental files submitted with this dissertation. Supplemental File 2.1 is a spreadsheet depicting complete statistical results for the PERMANOVA, ANOVA, and mixed-effects models that are reported in Chapter II. Supplemental File 2.2 is also a spreadsheet that reports the results of the PICRUSt2 analysis that was run in Chapter II and reported in **Figure 2.7i**. Supplemental File 3.1 is a spreadsheet reporting complete statistical results for the linear mixed models and post-hoc comparisons reported in figures of Chapter III. Supplemental File

3.2 is a spreadsheet containing the details of the correlational analyses reported in **Figures 3.10 and 3.11**.

Acknowledgements

First I would like to acknowledge and thank Dr. Fred Turek and Dr. Martha Vitaterna, whose guidance and mentorship were paramount to the completion of my PhD dissertation work. Dr. Turek interviewed me during my MD/PhD interview in the fall of 2013, which led me to pursue a rotation in his lab the next year. Dr. Turek provided me with knowledge regarding the science of sleep and circadian rhythms, but his mentorship extended far beyond just scientific facts in that he taught me about the non-scientific aspects of being an independent principal investigator. Much of this stemmed from the trust and respect he gives his trainees. I am a better teacher because he trusted me to create and give two full length lectures and write the tests for his undergraduate class on the biology of clocks and sleep (I have returned to give one of those lectures as a guest speaker for the past 4 years). I am a better presenter because he trusted me to submit new, unpublished findings at multiple international conferences throughout my training and trusted me to give presentations to Office of Naval Research (ONR) site visit review panels. I am a better writer because he read and gave timely feedback on every piece of scientific writing I wrote, and encouraged me to publish multiple times instead of hoarding data into one massive paper. I know more people in the field of sleep research because he actively introduced me to other researchers at conferences. Dr. Turek is particularly adept at establishing productive collaboration with people outside his field of expertise. This, I believe, requires a certain amount of humility, understanding what you know and do not know, and willingness to learn new things from everyone around you. I do not believe it is a coincidence such a high proportion of his trainees have remained in academia and are now running their own laboratories.

I can honestly say that were it not for Dr. Vitaterna, the laboratory's day to day operations would not occur with the same efficiency or productivity. She can manage the intellectual load of many distinct projects at once with precision and poise. Whenever I was stumped or anxious about something regarding experimental design, animal work, or data analysis/presentation, her door was open and I always left feeling better about where things stood. Her leadership and caring attitude has shone particularly brightly in the past few months, when the global COVID-19 pandemic has changed the way research is performed. She and Dr. Turek quickly had the lab adapt smartly and safely, allowing us to conclude ongoing experiments with minimal risk to lab personnel. Together, I believe Dr. Turek and Dr. Vitaterna have given me the tools to confidently enter the next stage of my training.

I would also like to acknowledge the rest of the ONR Multiple University Research Initiative (MURI) team (see Preface for description of the MURI): Dr. Ken Wright (CU Boulder), Dr. Monika Fleshner (CU Boulder), Dr. Chris Lowry (CU Boulder), Dr. Pieter Dorrestein (UCSD), and Dr. Rob Knight (UCSD). Due to the videoconference meetings on a consistent, biweekly basis throughout my 4 years of PhD training, I essentially received mentorship from 7 principal investigators. At each meeting, the teams would share new data and help each other with analyses, so I had regular input from world experts in the microbiome, metabolome, sleep physiology, and stress physiology. I would like to specifically thank the other non-PI team leads who helped me immensely with my work. Dr. Antonio Gonzalez of the Knight laboratory taught me nearly everything I know about how to analyze microbiome datasets. Dr. Fernando Vargas

performed mass spectroscopy on all of the samples we sent him, and he taught me about metabolomics and how to begin analysis of the datasets. Christine Foxx of the Lowry laboratory, Dr. Robert Thompson of the Fleshner laboratory, and Dr. Kate Sprecher of the Wright laboratory were great sources of collaboration in that we shared ideas and approaches throughout my PhD training.

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The work in this dissertation could not have happened without a tremendous amount of help from the other members of the Turek/Vitaterna laboratory. Chris Olker and Eun Joo Song are indispensable members of the laboratory that taught me how to perform nearly every experimental technique involved in the animal studies of this dissertation. They also helped me perform those experiments, from performing EEG/EMG implant surgeries to helping with

injections to helping with tissue collection. They also taught me how to score sleep, one of the most useful skills I have gained during my training, and shouldered a tremendous amount of the sleep scoring burden for these studies. Dr. Peng Jiang was also indispensable to my training, as he taught me how to perform innumerable computational analyses using R. He also provided mentorship regarding manuscript writing, statistical analysis, EEG/EMG implant surgery, just to name a few. Dr. Kazu Shimomura provided helpful discussion regarding the interactions between the microbiome and physiology. Katrina Campbell and Vance Gao are/were fellow PhD students in the lab, and their help throughout many of my experiments exemplified the collaborative environment of the Turek/Vitaterna laboratory. Sophie Lambert, Michelle Ren, Shannon He, Jenny Yim, Christine ChaeJung Lee, Jill Hsu, Chris Lee, and Sarah Kim are all masters students and undergraduates who helped throughout my time in the laboratory as well (see Section I.D.2 for detailed contributions). I would also like to thank Sue Hall-Perdomo, Helen Considine, Chad Gilliland, and Katie Lind for providing administrative assistance and always being warm and kind to me. Finally I would like to thank Joe Girten and George Harris from the Center for Comparative Medicine for assistance with all things related to animal acquisition and husbandry.

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Preface

The experiments of this dissertation operate within a multiple university research initiative (MURI) funded by the Office of Naval Research (ONR) Grant N00014-15-1-2809 entitled "The microbiome and responsiveness to stress: Countermeasure strategies for improving resilience to sleep and circadian disruption." My advisors Dr. Fred Turek and Dr. Martha Vitaterna are two of seven CO-I's on the project, which consists of experts in the fields of microbiome research, metabolomics, stress physiology, and sleep/circadian biology representing the University of California, San Diego and the University of Colorado Boulder along with Northwestern. They include Dr. Rob Knight (UCSD), Dr. Pieter Dorrestein (UCSD), Dr. Monika Fleshner (UCB), Dr. Christopher Lowry (UCB), and Dr. Kenneth Wright (UCB). The research project investigates the gut microbiome, fecal metabolome, and microbiome-based countermeasures in the context of sleep and circadian disruption, using mice, rats, and humans. Dr. Lowry's laboratory studied the impact of *Mycobacterium vaccae* NCTC 11659 immunization on the fecal microbiome and metabolome during and after 8 weeks of repeated environmental circadian disruption in mice. Dr. Fleshner's laboratory studied the impact of a prebiotic diet on the fecal microbiome and metabolome during and after the same circadian disruption protocol, but in rats. Dr. Wright studied the impact of combined sleep and circadian disruption on the microbiome and metabolome in humans. Due to the capacity of the Turek/Vitaterna laboratory to quantify sleep in many rodents at once, we were responsible for studying analogous experiments to those performed in the Lowry *and* Fleshner labs, but using repeated sleep restriction instead of circadian disruption.

The MURI project was a highly collaborative environment, with biweekly videoconference calls in which we all shared our data as it was collected and helped each other with interpretation. Sharing data during the analysis informed each other's analyses and allowed us to identify consistencies across species. We presented our results to the ONR at annual site visits. In this Preface, wish to emphasize that while the general goals of the Northwestern-based studies, i.e. to study the microbiome, the metabolome, and stress vulnerability in the context of repeated sleep disruption after *M. vaccae* treatment in mice and a prebiotic diet in rats, were laid out in the grant proposal, I made significant intellectual contributions to the science that is reported in this document.

Upon arrival at Northwestern University in summer of 2014 to begin the MD/PhD program, I performed my first laboratory rotation with Dr. Turek. The MURI had not been submitted at this time, but the laboratory's interest in the microbiome had begun to emerge, and I expressed interest in performing work relating to sleep and the microbiome at that time. As I completed my first two years of medical school, I kept in touch with the Turek group, and learned of the details of the MURI grant while it was in the conception and writing stage. In the summer of 2016, I joined the Turek laboratory as a PhD candidate in the Northwestern University Interdepartmental Neuroscience Program (NUIN). At this time, the MURI grant had recently been awarded to the group, so the general goals had been decided upon, but our group's principal experiments had not been designed or performed. From day one I took on the lead on designing and performing

the experiments, along with analyzing all of the data, for the Northwestern branch of the MURI. This included many preliminary and pilot experiments (most not shown in this document) to explore *M. vaccae* immunization in the context of other stressors such as restraint stress, optimize a social defeat protocol in the mice and rats, and validate the object location memory task. Furthermore, the exact approach we planned to take regarding the "big data" analyses was not outlined in the grant. At the time of these experiments, very few studies existed in the published literature that examined the microbiome and the metabolome using complex, long-term experimental designs with multiple other physiological outcome measures. Quantifying change in the microbiome over time, along with integration with other metrics such as sleep, required innovation and creativity on the part of myself, Dr. Antonio Gonzalez of the Knight laboratory, and Dr. Fernando Vargas of the Dorrestein laboratory. Finally, I conceptualized, designed, and performed multiple follow-up studies based on the results of the experiments originally outlined in the proposal (see Chapter VI), which will be the basis of future work coming out of the Turek/Vitaterna laboratory.

List of Abbreviations

5-HIAA	5-hydroxyindoleacetic acid
5HT	serotonin (5-hydroxytryptamine)
AAS	ascending arousal system
Ach	acetylcholine
ACTH	adrenocorticotropic releasing hormone
AD	Alzheimer's Disease
ANCOM	analysis of the composition of microbiomes
ANOVA	analysis of variance
ANS	autonomic nervous system
ATP	adenosine triphosphate
BF	basal forebrain
BLA	basolateral amygdala
BSH	bile salt hydrolase
clr	centered log ratio
CNS	central nervous system
CRH	corticotropin releasing hormone
CRP	C-reactive protein
CSF	cerebrospinal fluid
DA	dopamine
DCA	deoxycholic acid

DR	dorsal raphae
EEG	electroencephalogram
EMG	electromyogram
HDCA	hyodeoxycholic acid
FFAR2	free fatty acid receptor 2
FGF	fibroblast growth factor
FOS	fructooligosaccharide
FXR	farsenoid X receptor
GABA	gamma-aminobutyric acid
GOS	galactooligosaccharide
GLP	glucagon-like peptide
HPA	hypothalamus-pituitary-adrenal axis
IL	interleukin
IFN	interferon
LC	locus coeruleus
LCA	lithocholic acid
LXR	liver x receptor
MCI	mild cognitive impairment
MDD	major depressive disorders
MnPO	median preoptic nucleus of the hypothalamus
MRI	magnetic resonance imaging

MURI	multiple university research initiative
MV	*Mycobacterium vaccae*
LDT	laterodorsal tegmental nucleus
LPS	lipopolysaccharide
NAc	nucleus accumbens
NE	norepinephrine
NO	nitric oxide
NREM	non-rapid eye movement
OTU	operational taxonomic unit
PDX	polydextrose
PFC	prefrontal cortex
PPT	pedunculopontine nucleus
PTSD	posttraumatic stress disorder
PVN	paraventricular nucleus
PVT	psychomotor vigilance task
REM	rapid eye movement
RNA	ribonucleic acid
SAM	sympathetic-adrenal-medullary system
SCFA	short chain fatty acid
SCN	suprachiasmatic nucleus
SLD	sublateral dorsal nucleus

SWA	slow wave activity
TGR5	takeda G protein-coupled receptor 5
TNF	tumor necrosis factor
UDCA	ursodeoxycholic acid
vlPAG	ventrolateral periaqueductal grey
VLPO	ventrolateral preoptic area of the hypothalamus
vM	ventral medulla
VSURF	variable selection using random forests
VTA	ventral tegmental area
WASO	wake after sleep onset
ZT	zeitgeber time

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I. CHAPTER I: INTRODUCTION

This introductory chapter will begin with background information about the physiology of sleep, and then will review published literature concerning the health consequences of sleep deprivation. The next section will provide background on stress and stress resilience in humans and rodent models, and discuss the role sleep has in vulnerability or resilience to stressors. Finally, this chapter will describe the microbiota-gut-brain axis, and the use of microbe-based countermeasures to improve health.

A. Sleep Physiology

1. The Definition of Sleep

Humans spend, on average, nearly one third of their lives asleep. From an evolutionary perspective, this is not only a waste of valuable time that could be spent acquiring energy or spreading genes, it appears to greatly increase the risk of having these endeavors thwarted by an untimely death. Yet we sleep nearly every day from birth onward, as do all other animals. The ubiquity of sleep in the natural world has been observed and documented from at least the time of Aristotle, who wrote in his 350 B.C.E work, *On Sleep and Sleeplessness*:

> …[I)f waking is the contrary of sleeping, and one of these two must be present to every animal: it must follow that the state of sleeping is necessary… Accordingly, almost all other animals are clearly observed to partake in sleep, whether they are aquatic, aerial, or terrestrial, since fishes of all kinds, and mollusks, as well as all others which have eyes, have been seen sleeping. ‘Hard-eyed’ creatures and

> insects manifestly assume the posture of sleep…Of testaceous animals, on the contrary, no direct sensible evidence is as yet forthcoming to determine whether they sleep, but if the above reasoning be convincing to anyone, he who follows it will admit this [viz. that they do so.) [Aristotle et al., 1908]

Aristotle, 2,209 years before Darwin's *Origin of Species*, recognized that because sleep appears to be conserved in animals ranging from humans all the way down to "hard-eyed" insects, it must be functionally important. More concrete criteria defining a sleep-like state have been proposed in the millennia since Aristotle, and have been used to characterize sleep in a multitude of members of the animal kingdom. These criteria include 1) consolidated periods of immobility, 2) a stereotypic, species-specific posture, 3) an increased arousal threshold, and 4) homeostatic regulation of the sleep-like behavior, though multiple pharmacological, molecular, and electrophysiological criteria are used in a species-specific manner as well [Campbell and Tobler, 1984; Hendricks et al., 2000; Allada and Siegel, 2008]. Although we have only characterized sleep in a small percentage of the total known species, sleep or a sleep-like state has been observed in all species studied, including representatives from the phyla Chordata [Zhdanova et al., 2001], Arthropoda [Tobler, 1983], Nematoda [Raizen et al., 2008], Mollusca [Vorster et al., 2014], Platyhelminths [Omond et al., 2017], and Cnidaria [Nath et al., 2017]. Thus, despite being evolutionarily maladaptive in many ways, sleep is a highly conserved phenomenon that must be fundamental to survival. Indeed, total sleep deprivation in the rat results in severe pathology and death within 32 days [Rechtschaffen et al., 1983; Everson et al., 1989], further cementing it as a critical physiological process. The remainder of this section will describe features of sleep,

focusing (unless otherwise stated) on mammalian sleep with the ultimate goal of understanding the role of sleep in human health.

2. Sleep Regulation

Although there is significant variation in amount of time spent asleep per day within mammals, from 2-3 hours in large herbivories to 20 hours in brown bats [Siegel, 2005], sleep is generally consolidated to one phase of the sun's light dark cycle. This is in part achieved by an interaction between two general processes that regulate timing and intensity of sleep. The two-process model of sleep regulation, initially proposed nearly four decades ago [Borbely, 1982; Daan et al., 1984], proposes that sleep-wake cycles are principally regulated by a homeostatic drive for sleep that builds during wake (Process S) and an independent circadian drive for wake that peaks during one phase of the light/dark cycle depending on whether the animal is diurnal or nocturnal (Process C; **Figure 1.1**). When S is low wake is promoted, then S builds while the organism is awake and promotes sleep when near its upper limit. The main marker that has been used to measure process S has been non-rapid eye movement (NREM) sleep electroencephalogram (EEG) slow wave activity (SWA), which increases with length of previous wake and decreases as more sleep is attained. The next section of this chapter will describe NREM sleep and EEG in more detail. Process C is measured using various circadian phase markers, such as melatonin, core body temperature, or rest/activity rhythms [Borbely et al., 2016].

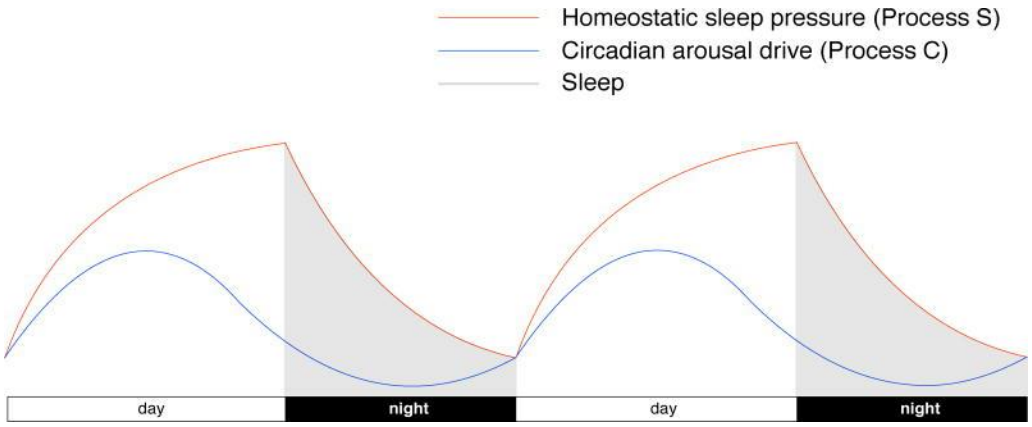


Figure 1.1 The two-process model of sleep regulation. Representation of the model in a diurnal animal. Homeostatic drive to sleep (red line) builds during the daytime and decreases during sleep, while the circadian drive to be awake (blue line) rises during the daytime and falls during the night, independent of sleep. Reprinted from [Oikonomou and Prober, 2017].

While SWA is the well-established phenomenological marker for sleep homeostatic pressure, there has been a quest to determine if a molecular marker for process S exists, otherwise known as a sleep factor. A sleep factor should be higher during wake than during sleep, increase steadily with prolonged wakefulness, and should decrease during sleep. Furthermore, a sleep factor should inhibit neuronal activity and promote sleep when exogenously administered [Porkka-Heiskanen and Kalinchuk, 2011]. A few molecules meet these criteria, but the principal candidate a purported sleep factor is adenosine, the final breakdown product of the energy storage molecule adenosine triphosphate (ATP) [Landolt, 2008]. Adenosine builds up in the extracellular space of the brain with prolonged waking in animal models [Porkka-Heiskanen et al., 1997], and injections of adenosine into the brain promote sleep [Virus et al., 1983], possibly by inhibiting wake-promoting circuitry [Strecker et al., 2000] and disinhibiting sleep-promoting circuitry [Chamberlin et al., 2003]. Caffeine's stimulant action is due to antagonism of the

adenosine A1 receptor [Fredholm et al., 2001]. Nitric oxide (NO), a gaseous neurotransmitter involved in the regulation of circulation, has been suggested as another strong candidate sleep factor. NO increases in a wake-promoting region of the brain during sleep deprivation [Kalinchuk et al., 2006a], and pharmacological increase of NO in the same region promotes sleep [Kalinchuk et al., 2006b]. However, NO and other somnogens like interleukin-1, tumor necrosis factor (TNF) alpha, prostaglandin D2, and growth hormone releasing factor that also meet some of the criteria for a sleep factor may in fact promote sleep via a shared pathway with adenosine (see [Porkka-Heiskanen and Kalinchuk, 2011]).

Lesioning the suprachiasmatic nucleus of the hypothalamus (SCN), the seat of the master circadian pacemaker in humans [Welsh et al., 2010], does not affect sleep homeostasis [Tobler et al., 1983; Trachsel et al., 1992], suggesting that S and C are regulated separately. However, a wide range of modeling data and experimental findings reveal that S and C may also be constantly interacting (reviewed by [Deboer, 2018]). Studying the impact of the two processes on physiological or behavioral outcome measures is therefore difficult, but researchers have devised experimental protocols to attempt to disentangle the two processes. The forced desynchrony protocol enforces a sleep-wake schedule that is outside the range of entrainment of the circadian system (such as a 20 hour day or a 28 hour day in constant dim light, with 8 hours per day to sleep) thus allowing within-subject designs to interrogate the effect of sleep pressure independent of circadian phase, and vice versa [Dijk and Czeisler, 1995; Wyatt et al., 1999]. Overall, the two-process model for sleep regulation has proven to be a useful conceptual

framework that has guided interpretation of findings regarding sleep homeostasis and sleep/circadian interactions for the past few decades.

3. Sleep Architecture and the Role of NREM and REM Sleep

An observer of a sleeping animal would find many reasons to believe that sleep is a state of low brain activity. Indeed, Aristotle described sleep as being "evidently the privation of waking" [Aristotle et al., 1908] and Shakespeare described it as "the death of each day's life" in *Macbeth* (2.2.50). A host of research in the past century has revealed that sleep is much more than simply the absence of wake, and that it consists of two metabolically and electrophysiologically distinct states. These are non-rapid eye movement (NREM) and rapid-eye movement (REM) sleep. As the names imply, REM sleep features quick, saccade-like movements of the eyes while NREM sleep does not, but more than that these are unique brain states controlled by unique brain circuitry and appear to serve unique functions. The temporal and electrophysiological characteristics of NREM sleep and REM sleep make up what is referred to as sleep architecture. Normally, wake, NREM, and REM sleep form their own ultradian rhythm, characterized by an entry into NREM, followed by REM, then wake. This cycle repeats throughout the course of the inactive period, with each cycle lasting around 90 minutes in humans (**Figure 1.2b**). The ratio of NREM:REM sleep in each successive sleep bout changes throughout the inactive period, particularly in humans, with REM sleep becoming more prominent with time. In healthy young and middle aged adult rodents (including both mice and rats), sleep is much less consolidated, with short sleep bouts occurring throughout the 12 hour light phase, and bouts of REM sleep

occurring every 10-20 minutes (**Figure 1.2d**). Although the baseline sleep architecture varies between rodents and humans, there are many core commonalities (particularly sleep EEG) that make animal models fundamental to past and future sleep research [Buzsaki et al., 2013; Toth and Bhargava, 2013]. Although sleep at its core is defined behaviorally (see above), the gold standard method to objectively measure sleep in birds and mammals is the use of EEG paired with electromyogram (EMG). The waveforms are then used to classify a given section of time (often a 10 second epoch) as wake, NREM sleep, or REM. This can be done manually, or through semi-automated software featuring aspects of machine learning [Gao et al., 2016]. Fast Fourier transformation of the EEG waveform is used to characterize which frequency bins are predominant during a particular time bin. These bins are typically (but not always) labelled as follows: delta (0.5-4 Hz), theta (4-8 Hz), alpha or theta2 (8-12 Hz), sigma (12-15 Hz), beta (15-30 Hz), and gamma (30-100 Hz). Changes in the total amount, relative fragmentation, and EEG characteristics of NREM, REM, and wake are the principal outcome measures in most sleep studies, and are the keys to investigating the role sleep plays in physiology and pathophysiology.

Wake

The awake state is examined regularly in sleep research to both aid in quantification of sleep fragmentation as well as to examine factors of wake EEG that may correlate with underlying brain function. Wake is characterized by high EMG tone, along with desynchronized, high frequency oscillations on EEG (**Figure 1.2a,c**), typically featuring predominantly beta oscillations [Fuller et al., 2006]. Though EEG is only a surface recording, reflecting activity of

enormous numbers of neurons across a multitude of circuits, different patterns in the wake EEG are thought to represent various underlying processes. Delta power during wake is thought to be a marker of neurobehavioral impairment and sleepiness, as it is increased after extended wakefulness and correlates negatively with cognitive performance [D'Rozario et al., 2013]. Theta power progressively increases during sustained wakefulness [Cajochen et al., 1995; Aeschbach et al., 1997], which has led some to postulate that theta power during wake can be used to model process S [Borbely et al., 2016]. Alpha power during wake has been affiliated with increased alertness [Wang et al., 2015], increased cognitive and motor performance in patients with ischemic stroke [Dubovik et al., 2013], and is decreased in people with mild cognitive impairment (MCI) [O'Keeffe et al., 2017] and Alzheimer's disease (AD) [Giannitrapani et al., 1991]. This, along with multiple EEG studies in healthy humans, has led to the hypothesis that alpha during wake represents underlying processes governing attention and processing of stimuli [Klimesch, 2012]. Overall, though it may seem paradoxical, the study of wake cannot be neglected as an important part of sleep research.

NREM

NREM sleep in general is characterized by reduced EMG compared to wake and EEG with large amplitude, low frequency oscillations (**Figure 1.2a,c**). In humans, NREM sleep is further divided into four stages which are defined by the American Academy of Sleep Medicine as follows [Berry et al., 2015]. Stage 1 NREM (N1) is the first and lightest stage of sleep, and is always the first epoch of sleep following wake. It is defined by slow eye movements and some

low-amplitude, mixed frequency theta waves. N1 bouts usually only last 1 to 7 minutes, and constitute only 2-5% of total sleep [Institute of Medicine Committee on Sleep Medicine and Research, 2006]. N2 is a slightly deeper (more difficult to awaken) state and lasts around 10-25 minutes on the first NREM-REM cycle, becoming more dominant (up to 45-55%) on subsequent cycles [Institute of Medicine Committee on Sleep Medicine and Research, 2006]. On EEG N2 is characterized by the presence of features called K complexes and sleep spindles. Spindles are short 0.5-2 second bursts of 10-16 Hz activity in EEG leads, are seen in NREM sleep across species, and data from humans and rodents suggest they play a role in memory consolidation (reviewed in [Geva-Sagiv and Nir, 2019]). N3 is a short stage, only lasting a few minutes and constituting 3-8% of sleep, and is the first stage to show dominant high-voltage slow waves in the low end of the delta band. N3 leads into N4, which is present predominantly early in the night, constituting 10-15% of total sleep [Institute of Medicine Committee on Sleep Medicine and Research, 2006]. It was observed as early as 1937 that it is more difficult to awaken a subject the more predominant slow (<4 Hz) waves were on EEG [Blake and Gerard, 1937] , and indeed N4 sleep is the sleep stage with the highest arousal threshold. In mice, NREM sleep is also characterized by high amplitude, low frequency oscillations (**Figure 1.2.c**), but is not subdivided into the 4 substages.

The predominant power band in NREM sleep is the delta band, and the role of delta power in physiology has been extensively studied. As mentioned above, delta power (also labelled as slow wave activity) is homeostatically regulated, and is used as a marker for sleep pressure. In

humans, delta power during sleep is highest early in the night, proportional to length of prior wake, and dissipates across the night [Dijk et al., 1990]. In rodents, the same pattern holds during the inactive phase, with delta being highest at the beginning of the day, and dissipating over time (**Figure 1.3**). As one would expect, many studies have shown that NREM delta power increases in sleep following sleep deprivation in humans and in rodent models, further cementing it as a marker of the sleep homeostat [Leger et al., 2018]. Delta power during NREM sleep generally declines with age in humans, at an estimated rate of about 2% per decade [Astrom and Trojaborg, 1992; Landolt et al., 1996; Carrier et al., 2011; Mander et al., 2017], though it is unclear if the same happens in aging rodents [Panagiotou et al., 2017; McKillop et al., 2018; Soltani et al., 2019]. Reduction of delta power is also observed in humans with MCI [Taillard et al., 2019], humans with AD [Cedernaes et al., 2017], and in mouse models of AD [Kent et al., 2018], indicating an important role for slow wave activity in cognitive processes, and memory in particular (reviewed by [Leger et al., 2018; Geva-Sagiv and Nir, 2019]). Interesting recent research has investigated enhancing slow waves during sleep with in-phase acoustic stimulation as a method of improving cognitive function (reviewed by [Zhang and Gruber, 2019]). Results have shown that this approach can both increase amplitude of slow waves and can improve cognitive function in healthy adults [Ngo et al., 2013; Leminen et al., 2017; Papalambros et al., 2017], and shows promise as a mechanism to improve cognitive function in adults with various forms of cognitive impairment.

Delta power during NREM sleep has also been proposed to be important in processing of stressful events that transpired during the previous wake. In rodents, social interactions and social defeat stress increase NREM delta power in the subsequent sleep period above what would be expected from the sleep deprivation caused by the stressor [Meerlo et al., 2001a; Kamphuis et al., 2015; Henderson et al., 2017]. This may be viewed as an adaptive response by the brain to cope with particularly stimulating experiences during wake. For further discussion of the impact of stressors on sleep, see Section I.B.2.

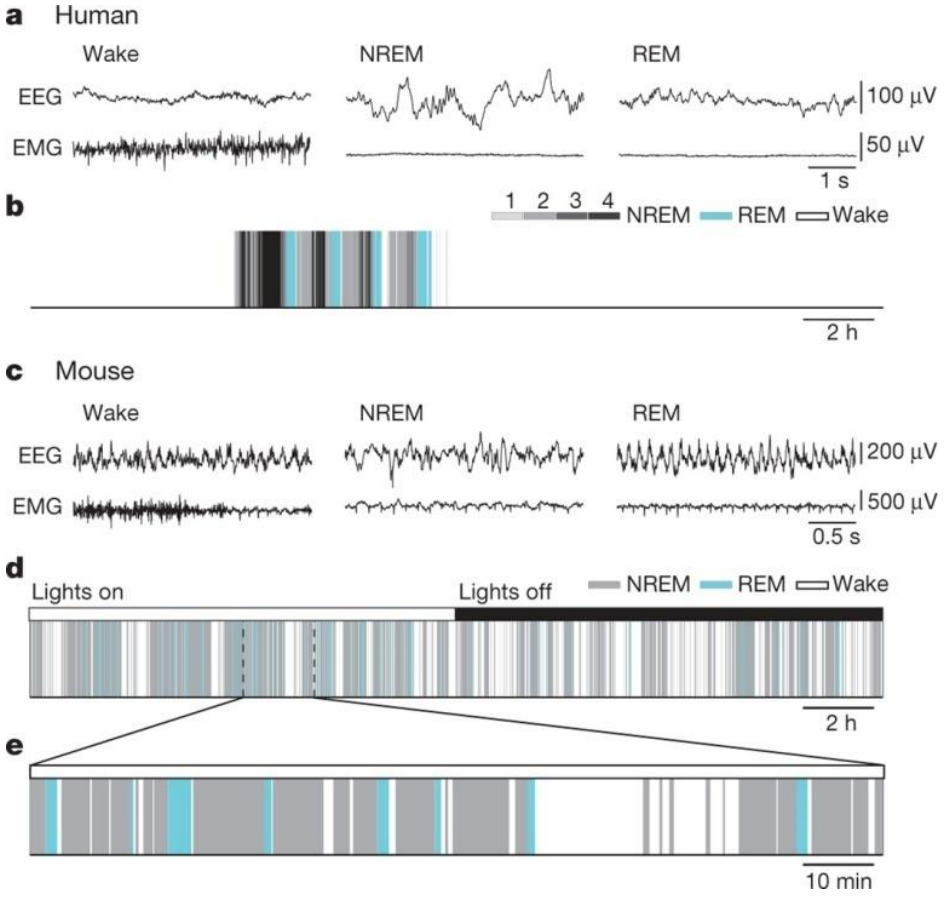


Figure 1.2 Characteristics of Sleep Architecture in Humans and Mice. (A) Representative EEG and EMG tracings depicting wake, NREM sleep, and REM sleep in humans. (B) A temporal layout of brain states (hypnogram) depicting 22 hours of continuous recording in a

human, with grey/black indicating stages of NREM, blue indicating REM, and white indicating wake. (C) Representative EEG and EMG tracings depicting wake, NREM sleep, and REM sleep in the mouse. (D) A hypnogram of a 24 hour recording in a mouse on a 12:12 light:dark cycle, with grey indicating NREM sleep, blue indicating REM sleep, and white indicating wake. (E) Zoom in of a 2 hour segment of the hypnogram in (D). Reprinted from [Weber and Dan, 2016].

Outside the central nervous system (CNS), studies have shown delta power during NREM sleep plays a role in multiple processes, from energy metabolism to hormone release to immune function. Slow wave sleep has always been associated with subjective feelings of being rejuvenated, and a study in rats reported an ATP surge in wake-promoting brain regions during the first hours of sleep that correlated positively with delta power during that sleep [Dworak et al., 2010]. Growth hormone is released during sleep, and the magnitude of that release is correlated with delta power during sleep [Sassin et al., 1969; Gronfier et al., 1996]. A bidirectional relationship between NREM sleep and the immune system has been proposed (see reviews by [Imeri and Opp, 2009; Lange et al., 2010]), as many proinflammatory cytokines enhance slow wave activity [Kapsimalis et al., 2005; Krueger et al., 2011]. However, inconsistencies between animals and humans have been reported, as some proinflammatory cytokines that promote NREM sleep and delta activity in rodents do not do so in humans [Irwin, 2015]. Overall, NREM EEG delta power appears to be one of the most important markers for the physiological function of NREM sleep across multiple systems, and is thus an important outcome measure in many studies that objectively measure sleep.

A shift in the NREM EEG power spectrum away from delta and towards higher frequencies is thought to represent reduced sleep depth, and research has revealed associations of high

frequency power bands with different pathological conditions. Increased NREM beta (15-30 Hz) power has been regularly observed in people with primary insomnia [Freedman, 1986; Merica et al., 1998; Spiegelhalder et al., 2012]. Elevations in NREM beta power are even seen in childhood and adolescence and predict later development of primary insomnia [Fernandez-Mendoza et al., 2016; Fernandez-Mendoza et al., 2019]. These results have led to the hypothesis that inappropriate high frequency EEG during NREM sleep may be a reflection of cortical hyperarousal [Perlis et al., 2001; Bonnet and Arand, 2010]. Recent studies have also observed cortical hyperarousal in the sleep of people with major depression with suicidal ideation [Dolsen et al., 2017], people with posttraumatic stress disorder (PTSD) [Woodward et al., 2000b; Germain et al., 2006; de Boer et al., 2019; Wang et al., 2019a; Blaskovich et al., 2020], as well as in mouse models of chronic stress or PTSD [Wells et al., 2017; Sharma et al., 2018]. It is of note that increase in high frequency power during NREM sleep is often observed in tandem with decreased delta power [Wang et al., 2019a; Blaskovich et al., 2020], which makes it difficult to assess if cortical hyperarousal is itself a pathological process, or if it is the lack of beneficial delta power, or a combination of both.

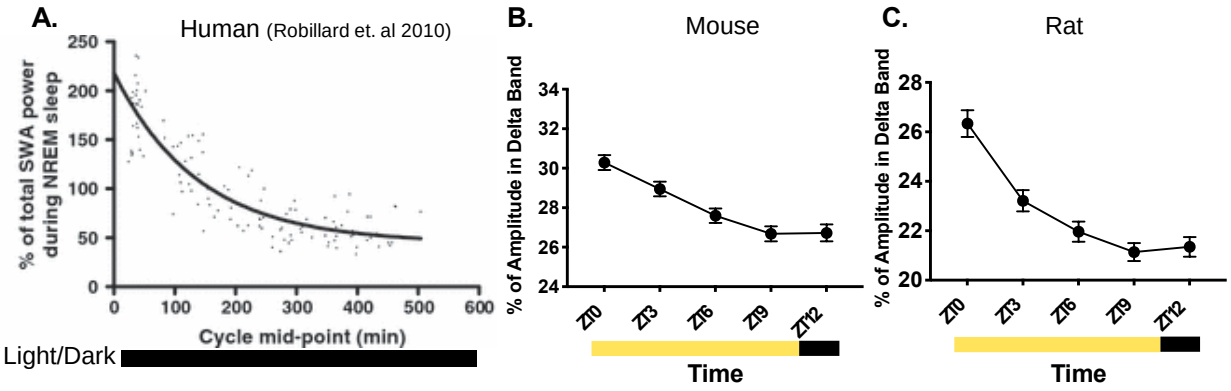


Figure 1.3 The Dissipation of Delta Power During NREM Sleep Across Time in Three Species. (A) Data from a night of sleep in humans, plotting the relative amount of slow wave (0.5-4 Hz) activity during NREM sleep at various times across a night of sleep. Adapted from [Robillard et al., 2010]. (B) Percent of total NREM EEG amplitude in the delta (0.5-4 Hz) band across the light phase and three hours of the dark phase in mice. Data are from baseline sleep recordings in control animals during the experiment in Chapter III, and were collected by the Turek/Vitaterna laboratory. This graph is not reported in Chapter III (n = 52). (C) Percent of total NREM EEG amplitude in the delta (0.5-4 Hz) band across the light phase and three hours of the dark phase in rats. Data are from baseline sleep recordings in control animals during the experiment in Chapter V, and were collected by the Turek/Vitaterna laboratory. This graph is not reported in Chapter V (n = 38). The light/dark conditions of the study are indicated as colored bars below the x-axes of the graphs, with yellow indicating lights on and black indicating lights off. ZT represents zeitgeber time (number of hours since lights on).

REM

REM sleep was discovered in the 1950's by Eugene Aserinsky along with Nathaniel Kleitman, and William Dement [Aserinsky and Kleitman, 1953; Dement and Kleitman, 1957]. This was accomplished in part by performing the first continuous all-night recording of ocular motility in sleep in Dr. Aserinsky's eight year old son, which consumed almost a half-mile of paper [Aserinsky and Kleitman, 1953; Aserinsky, 1996]. This came at a time when many, including his own advisor, believed that sleep was a completely passive phenomenon [Aserinsky, 1996]. Later experiments have confirmed that REM sleep is in fact an incredibly active brain state, as brain metabolism during REM sleep is equivalent to during wake [Madsen and Vorstrup, 1991], and whole-body oxygen consumption during REM sleep is actually higher than during wake [Parmeggiani, 2003]. REM sleep is defined by relatively low voltage mixed frequency EEG oscillations much akin to wake, very low EMG tone, and irregular fast (<500 ms) eye movements (**Figure 1.2a,c**). In rodents, REM sleep is classified by a characteristic sawtooth-like pattern of sharp waves oscillating at around 4-9 Hz (**Figure 1.2c**) and low EMG tone; eye

movements are not traditionally monitored in rodent sleep experiments. Since REM sleep EEG looks similar to wake, but with muscle atonia, REM sleep has also been labeled “paradoxical sleep” by some scientists [Peever and Fuller, 2017]. Other features of REM sleep include muscle twitches, increased sympathetic nervous system activity compared to wake, and variable breathing depth and rhythm [Institute of Medicine Committee on Sleep Medicine and Research, 2006]. States that can be classified as REM sleep have now been identified in many species of bird, mammal, and even reptiles [Shein-Idelson et al., 2016]. In humans, REM sleep bouts occur every 90-120 minutes and are much less prominent in early sleep cycles, only lasting around 1-5 minutes [Carskadon and Dement, 1980]. However, with subsequent sleep cycles REM sleep becomes more prominent compared to NREM, and ultimately totals about 20-25% of total sleep across the course of the night. In rodents, REM sleep bouts occur every 10-15 minutes, are highly concentrated to the light cycle (**Figure 1.2d,e**), and average ~10-15% of total sleep over a 24 hour period in baseline conditions (Turek lab data).

Mechanisms of REM sleep regulation contain both circadian and homeostatic components. The propensity for REM sleep coincides with the trough of the core body temperature circadian rhythm, indicating that REM sleep timing may be under circadian control [Czeisler et al., 1980; Dijk and Czeisler, 1995]. Examining REM sleep totals in REM-deprived, SCN-lesioned rats revealed that the SCN promotes the transition into REM sleep during the inactive phase, but once REM sleep is initiated the quantity of the subsequent REM sleep depends on prior REM sleep amounts and was thus homeostatically regulated [Wurts and Edgar, 2000]. The REM sleep

homeostat appears to be slightly more resistant to changes in total sleep time than the NREM sleep homeostat, as ~40 hours of total sleep deprivation significantly increases NREM sleep without significant changes to REM sleep time [Borbely et al., 1981]. However, selective REM sleep deprivation or sleep disruption protocols that result in more loss of REM sleep than NREM sleep (see **Chapters III** and **V**) cause a REM sleep rebound, confirming that there are homeostatic mechanisms at play in REM sleep [Dement, 1960; Agnew et al., 1967; Beersma et al., 1990]. There is also evidence that the REM sleep recovery process may take longer than the NREM sleep process [Le Bon, 2020], though this may be in part due to the role REM sleep has in recovering from stressful stimuli [Nollet et al., 2019], and sleep deprivation can be considered a stressful event.

Because most studied mammals and birds display something resembling REM sleep, it is reasonable to believe REM sleep must serve an important fundamental function. What exactly that function is remains unclear, but researchers have accumulated sufficient evidence to make a few informed hypotheses (see [Peever and Fuller, 2017] for a thorough review). Infant mammals spend a majority of their sleep in a REM-like state called active sleep, which prompted the hypothesis that some process REM sleep was responsible for some process crucial to brain maturation and development [Marks et al., 1995]. The muscle twitches observed during REM, particularly prominent during early development, have been shown to activate the hippocampus, cerebellar cortex, and red nucleus, which together support the idea that REM sleep may contribute to sensorimotor system development [Blumberg et al., 2013; Sokoloff et al., 2015a;

Sokoloff et al., 2015b]. Indeed, REM sleep appears to impact multiple processes like sensorimotor development that involve plasticity of neuronal circuits in mammals.

The largest volume of data supporting a physiological function for REM sleep comes from studies linking REM sleep to the formation and consolidation of certain types of memory (reviewed by [Boyce et al., 2017]). Studies examining sleep in the hours after the training phase of a learning task provide circumstantial and correlational evidence for REM's role in learning and memory consolidation. For example, an increase in REM sleep was observed after appetitive [Smith and Lapp, 1991] and aversive [Smith et al., 1980] learning tasks in rodents, and after a variety of tasks including studying [Smith and Lapp, 1991], motor skill learning [Buchegger and Meier-Koll, 1988], and foreign language learning [De Koninck et al., 1989] in humans. Furthermore, the character of REM sleep can change after learning a task, as REM EEG theta power (the dominant band in the REM sleep EEG power spectrum) increased after fear conditioning in rats [Fogel et al., 2009], which could be considered an increased "intensity" of REM. A host of studies have found that selectively depriving REM sleep in humans and rodents impairs learning of complex tasks such as detailed maze navigation and two-way aversive conditioning (see [Boyce et al., 2017]. In rodents, extended periods of REM sleep deprivation results in a reduction in long-term potentiation in the hippocampus [Davis et al., 2003; Ravassard et al., 2009], which may explain the memory impairments seen in REM sleep deprivation experiments. However, other studies have reported no effect of REM sleep deprivation on memory consolidation, particularly with simple tasks such as shuttle box avoidance or simple

maze navigation [Sloan, 1972; Van Hulzen and Coenen, 1979]. Overall, though, the evidence from REM sleep deprivation studies is difficult to interpret with full confidence, as it is difficult to deprive REM sleep without any impact on NREM sleep [Benington and Heller, 1994] and many REM sleep deprivation protocols are inherently stressful (see Section 1.A.6).

More recent studies using molecular and cellular techniques have illuminated potential mechanisms by which REM sleep could be modulating memory in the mammalian brain at the regional, neuronal, and synaptic levels. Theta oscillations, which are evident on surface EEG during REM sleep, are also found at the circuit level, as coherence of theta oscillations between the prefrontal cortex and the amygdala are correlated with fear memory consolidation in [Popa et al., 2010]. Furthermore, theta oscillations are found at the neuronal level in the hippocampus, and coordination of theta oscillations across hippocampal networks is posited to facilitate memory consolidation [Montgomery et al., 2008]. Interestingly, a recent study tested this hypothesis directly by using optogenetic techniques to specifically silence GABAergic cells in the medial septum that drive hippocampal theta oscillations [Boyce et al., 2016]. They found that doing so during post-training REM sleep (but not during NREM sleep or wake) resulted in impaired subsequent novel object place recognition and fear-conditioned contextual memory without disturbing sleeping behavior [Boyce et al., 2016]. There is also evidence REM sleep may be impacting learning at the synaptic level. Markers of synaptic plasticity go up during REM sleep after tasks known to require remodeling of the visual cortex [Dumoulin Bridi et al., 2015; Renouard et al., 2018]. Furthermore, synaptic layer 5 cortical dendritic spines showed synaptic

pruning during REM sleep following learning on a sensory motor task [Li et al., 2017]. More discussion on sleep's role in synaptic plasticity will be discussed in the next section.

It is well understood that sleep, and particularly REM sleep, increases compared to baseline after stress, and it has been hypothesized that this is an adaptive coping measure [Suchecki et al., 2012; Sanford et al., 2015]. In fact, there is some evidence that the function of REM sleep is, in part, to consolidate memories of particularly emotionally valent events that occurred during the prior wake. A recent meta-analysis of 8 studies examining the role of REM sleep versus NREM sleep in memory consolidation suggested there is a selective enhancement of emotional over neutral memory after REM sleep [Schafer et al., 2020]. However, while REM sleep rebound immediately after an event may be adaptive, sustained increases in REM sleep are thought to be a sign of sleep pathology and are seen in many psychiatric conditions. For a more in-depth discussion of the role of REM sleep in stress-related psychiatric disorders and in stress responses in general, see Sections I.B.2-3.

It is important to note that the role of REM sleep in learning and memory has been questioned due to the fact that some relatively intelligent species display very little (if any) REM sleep, and some medications (i.e. citalopram and other selective serotonin reuptake inhibitors) suppress REM sleep without any noted decline in memory or cognitive performance in their users [Vertes and Eastman, 2000; Siegel, 2001; Rasch et al., 2009; Horne, 2013]. Large mammals such as giraffes, elephants, and horses spend less than one hour per day in REM sleep [Tobler, 1992;

Tobler and Schwierin, 1996; Gravett et al., 2017], and cetaceans such as dolphins and porpoises appear to have no identifiable REM sleep [Mukhametov, 1987; Lyamin et al., 2008]. One theory that could account for this postulates that REM sleep occurs to warm the brain after NREM sleep (reviewed by [Harding et al., 2019]). Brain and body temperature drop during NREM sleep and rewarm during wake and REM sleep [Alfoldi et al., 1990; Landolt et al., 1995]. In general, the time between REM sleep bouts seems to scale with body mass [Siegel, 2005], so small animals that have a larger surface area to volume ratio would in theory cool faster during NREM sleep and thus would need REM-induced rewarming to occur more frequently. Large animals and cetaceans that display uni-hemispheric sleep (and thus only one side of the brain cools at a time) would then need little or no REM sleep to rewarm the brain. While this theory makes logical sense, I posit that just because a mammal appears to show little or no REM sleep based on EEG, EOG, and behavioral criteria does not mean that the underlying process that occurs during REM sleep is not happening. The criteria which we use to score sleep as REM are based on surface electrodes and behavioral manifestations that may just be epiphenomena of physiological processes that are occurring at different, more difficult to measure scales. One case that supports this hypothesis is the echidna, which does not display most of the classic measured features of REM sleep (EEG pattern, muscle atonia, rapid eye movements), but does have REM-like neuronal activity in the brainstem [Siegel et al., 1996].

Overall, the use of EEG to quantitatively and qualitatively measure sleep architecture is paramount in sleep research, and its use in combination with clever experimental design is

largely responsible for the vast knowledge base scientists have accumulated in the last century regarding the function of sleep in mammals. However, as will be described below, it has taken technological advancements in the last 10-15 years to shed new light on the more fundamental physiological processes going on beneath the surface during sleep.

4. The Fundamental Functions of Sleep

As I mentioned in the previous section, it is important to recognize that surface EEG, EMG, and behavior are simply observable phenotypes of some presumably cellular level process occurring during sleep. This section will briefly review a few of the hypotheses for the fundamental function of sleep, focusing on hypotheses generated due to new data brought to light using recent technological advances in molecular and cellular neuroscience.

The Synaptic Homeostasis Hypothesis

They synaptic homeostasis hypothesis of sleep, first proposed by Giulio Tononi and Chiara Cirelli, states that, due to informational and energetic constraints, learning should occur primarily via the accumulation of new synapses and increased synaptic strength due to experiences had during wake, followed by net downscaling of synaptic strength during sleep, when external input is reduced [Tononi and Cirelli, 2003; Tononi and Cirelli, 2006; Tononi and Cirelli, 2014]. Multiple studies in the years since have utilized techniques such as 3-D electron microscopy [de Vivo et al., 2017] and *in vivo* calcium imaging [Li et al., 2017] to test this hypothesis, and have reported results generally supporting reduced synaptic strength after sleep

(reviewed in detail by [Tononi and Cirelli, 2014; Cirelli, 2017]). In apparent opposition to the synaptic homeostasis hypothesis, multiple studies have shown that synaptic downscaling and synaptic upscaling may be occurring in parallel during sleep, and that regional specificity may be at play [Chauvette et al., 2012; Abel et al., 2013; Frank, 2013; Diering et al., 2017]. Furthermore, if you use firing rate as a proxy for synaptic strength, a line of thinking which itself has been questioned [Cirelli, 2017], a period of sleep appears to increase or decrease firing rate depending on the pre-sleep state of the neuron [Puentes-Mestril et al., 2019]. I don't particularly believe these findings are mutually exclusive. There may be a complexity to the upscaling and downscaling of synaptic strength during sleep that we do not fully understand, but whose ultimate outcome is brain-wide synaptic remodeling resulting in maintenance of circuits and consolidation of memories.

Increase in Glymphatic Flow and Waste Clearance

New data from the past 10 years has suggested that one of the root functions of sleep is to clear the brain interstitial space of metabolites that have built up during the high neural activity of prior wakefulness. While the brain does not have a designated tubule based lymphatic system to drain intercellular waste like the rest of the body, it has been posited in recent years that this process is accomplished via bulk flow of cerebrospinal fluid (CSF) across the brain, propelled along penetrating vasculature [Abbott, 2004; Iliff et al., 2012; Iliff et al., 2013a; Iliff et al., 2013b]. It was then discovered that this process, sometimes termed the glymphatic system, is significantly more active during sleep compared to wake, and that exogenous amyloid beta (one

of the hallmarks of AD pathology) is cleared more quickly during sleep [Xie et al., 2013]. In fact, failure to adequately clear amyloid beta due to chronic inadequate sleep has been hypothesized to contribute to the pathophysiology of AD [Cedernaes et al., 2017; Boespflug and Iliff, 2018]. More recent studies have confirmed that tau protein (involved with Parkinson's disease pathology) is also cleared more quickly during sleep [Holth et al., 2019], and that the rate of glymphatic flow correlates positively with amplitude of delta waves [Hablitz et al., 2019]. Finally, an interesting study in humans used combined magnetic resonance imaging (MRI) and EEG techniques to discover that there is a temporal coupling between delta waves, hemodynamics, and CSF flow during sleep [Fultz et al., 2019]. These data suggested that peak synchronization of cortical neurons during the apex of a delta wave induces a strong oxygen demand, and during the inactivity-induced trough of the delta wave a surge in blood flow occurs to deliver oxygen. Then, utilizing the perivascular glymphatic system, the CSF flow increases, clearing the interstitial space of metabolic waste products. One interpretation of this model is that the EEG tracings we use to delineate sleep stages are just an epiphenomenon, and that the synchronized rhythmic neuronal activity that makes up the slow waves we all know and love is just a carrot leading the true workhorse process of sleep: glymphatic clearance of metabolites. It will be interesting in the coming years to see whether hemodynamic and glymphatic coupling during sleep will prove to play a role in the many other functions of sleep, such as learning and memory.

Energy Conservation Related Theories

The aforementioned lines of evidence are convincing, but rely on the presence of neurons and a central nervous system. However, we know a sleep-like state exists across at least six different phyla, many of whom do not have a CNS [Anafi et al., 2019]. A few theories for the fundamental evolutionary reason for sleep avoid this problem by focusing on temporal separation of energetic processes. The energy allocation hypothesis states that species have evolved to perform unique and essential biological processes during sleep so as to repartition energy resources across behavioral states and increase overall evolutionary fitness [Schmidt, 2014]. By doing this, organisms are balancing waking effort during wake with biological investment during sleep. Theories like the energy allocation hypothesis have been espoused as potential unifying theories of sleep that are not mutually exclusive with CNS-centric theories and can be applied broadly to the many members of the animal kingdom that partake in sleep [Anafi et al., 2019].

5. The Neuroanatomy of Sleep

In the 1910's and 1920's, an outbreak of a unique form of viral encephalitis emerged and swept across Europe and North America. Two groups of patients with this disease emerged: one that displayed prolonged insomnia, and another with prolonged sleepiness. One Viennese physician named Baron Constantin Von Economo treated many patients with this newly termed encephalitis lethargica, and discovered that the brains of people with the insomnia phenotype had lesions in their anterior hypothalamus, while those with the sleepiness phenotype had lesions at junction of the brainstem and forebrain [Von Economo, 1930]. Decades of research, much of which has occurred in the past 20 years, have revealed that the insights provided by Von

Economo were accurate, as the ventrolateral area of the preoptic nucleus (VLPO) of the hypothalamus is one of the key sleep promoting nuclei in the brain, and many of the wake promoting centers of the ascending arousal system (AAS) lie in the midbrain. Due to the recent discoveries and applications of tools involving mouse genetics, single cell gene expression, viral genomics, and optogenetics, the past decade has witnessed an incredible expansion in our knowledge base surrounding the neural circuitry driving sleep and wakefulness. This section will briefly summarize what is currently known about the neuronal populations governing wake, NREM and REM sleep (reviewed by [Weber and Dan, 2016; Scammell et al., 2017]).

Populations of neurons promoting wakefulness exist in the pons, midbrain, hypothalamus, and forebrain, and use a variety of neurotransmitters, including acetylcholine (Ach), norepinephrine (NE), serotonin (5HT), dopamine (DA), histamine, glutamate, and gamma-amino butyric acid (GABA) [Saper et al., 2005; Scammell et al., 2017]. Nearly all of these regions send projections to the cortex or thalamus, and provide unique, yet often redundant and overlapping, contributions to establishing and maintaining wakefulness (**Figure 1.4a**).

Researchers have now established that neuronal populations in the VLPO and median preoptic nucleus (MnPO), along with some basal forebrain (BF) neurons [Hassani et al., 2009], are essential for promoting NREM sleep, though these nuclei appear to be heterogeneous in that they also may contain wake-promoting neurons [Scammell et al., 2017]. The VLPO appears to promote transition to NREM sleep primarily by sending inhibitory GABAergic or galanergic

projections to wake-promoting brain areas such as the BF, tuberomammillary nucleus, dorsal raphae (DR), and locus coeruleus (LC) [Sherin et al., 1998; Uschakov et al., 2007]. The VLPO may also aid in mediating the homeostatic regulation of sleep, as the firing rate of sleep-active VLPO neurons rises with increased sleep pressure [Alam et al., 2014]. Interestingly, these sleep-promoting brain regions also receive inhibitory input from wake-promoting brain regions. This reciprocal inhibition may generate a flip-flop switch of sorts that allows for rapid transition from one brain state to another [Saper et al., 2005; Saper et al., 2010] (**Figure 1.4b**).

While early studies localized REM sleep to the general area of the pons [Jouvet, 1962; Siegel et al., 1984], the details of the circuit that generates and maintains REM sleep were not understood until recently, due to sophisticated cellular marker and optogenetic studies that identified populations of REM-ON and REM-OFF neurons in the midbrain, pons, and medulla [Weber and Dan, 2016; Weber et al., 2018]. REM-ON neurons are those that promote REM sleep, include populations in the sublateral dorsal nucleus of the pons (SLD) ventral medulla (vM) that indirectly and directly (respectively) inhibit spinal motor neurons to promote REM sleep atonia.

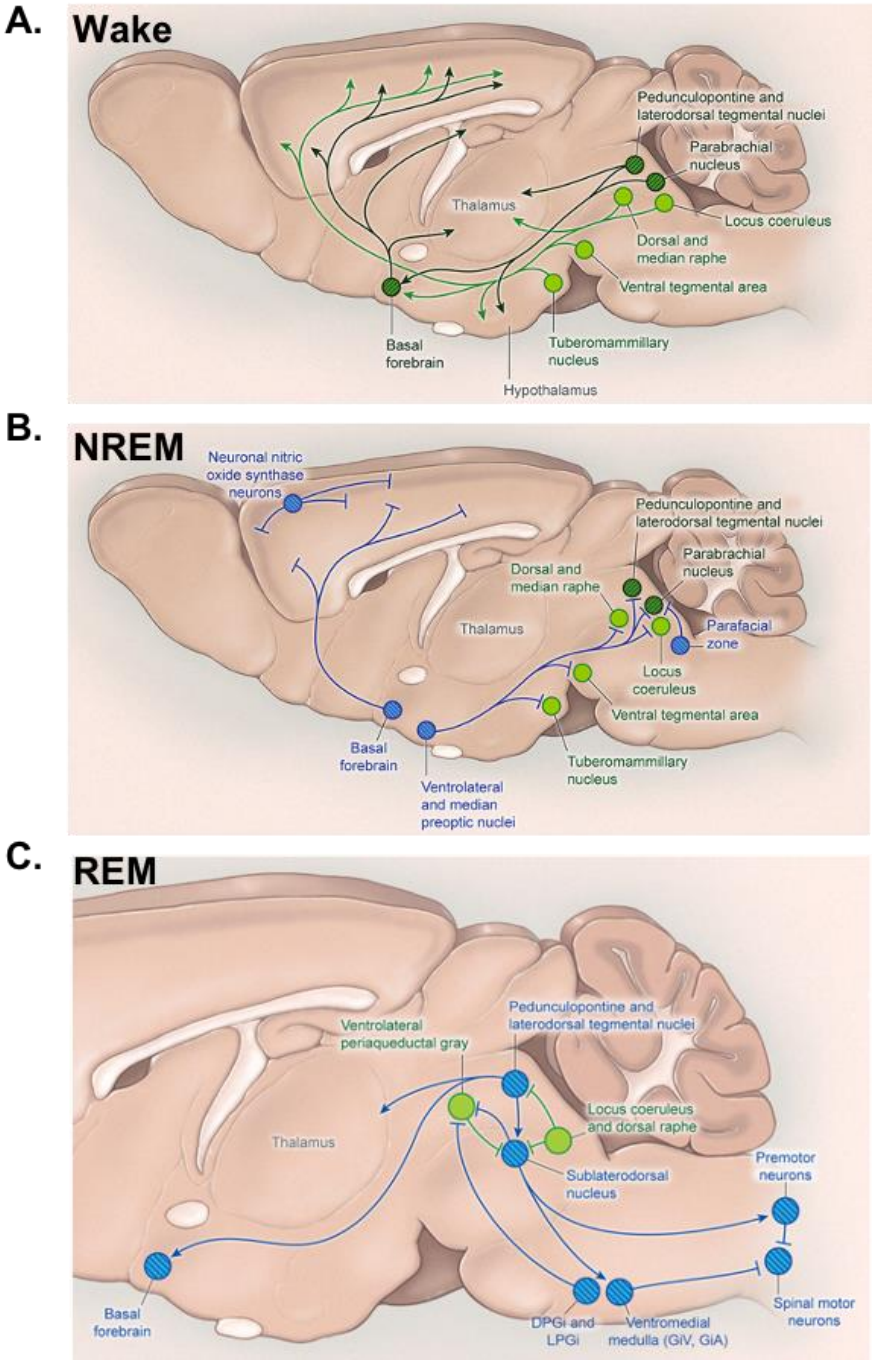


Figure 1.4 Wake, NREM, and REM Sleep Neural Circuitry. (A) Wake-promoting monoaminergic (light green) neurons and their projections. Dark green, hatched symbols represent wake-promoting neurons that use acetylcholine or other neurotransmitters. (B) NREM sleep promoting neurons and their projections are represented by dark blue/hatched symbols, and wake-promoting nuclei that are inhibited by NREM-promoting nuclei are in green. (C) REM

sleep promoting neurons (REM-ON) are depicted with blue/hatched symbols, and REM-suppressing neurons (REM-OFF) are depicted with green symbols. Excitatory projections are represented by arrows, and inhibitory projections are represented by flat lines. Adapted from [Scammell et al., 2017].

Another group of REM-ON neurons exist in the pedunculopontine (PPT) and laterodorsal tegmental (LDT) nuclei of the pons, and they send excitatory projections to the SLD and to the thalamus and BF, which may result in the high frequency EEG oscillations observed during REM sleep. REM-OFF neurons exist in the ventrolateral periaqueductal grey (vlPAG), LC, and DR, and actively inhibit REM sleep during wake (LC, DR, vlPAG) and NREM (vlPAG) by sending inhibitory projections to the REM-ON neurons in the SLD and PPT/LDT [Scammell et al., 2017]. In turn, REM-ON neurons in the SLD and vM send inhibitory projections to REM-OFF neurons in the vlPAG, thus creating another flip-flop switch between NREM sleep and REM sleep allowing rapid transition from NREM sleep to REM sleep [Weber et al., 2015; Weber et al., 2018]. Degeneration of aspects of this circuit is thought to lead to REM sleep behavioral disorder (RBD), which itself may be a prodrome for Parkinson's disease and other synucleinopathies [St Louis et al., 2017] (**Figure 1.4c**).

Neurons that produce the neuropeptide orexin (also known as hypocretin) are scattered across the lateral hypothalamus and act to stimulate all wake promoting brain regions discussed above, as well as some REM-OFF populations. Sleep-promoting neurons from the VLPO also directly inhibit orexin neurons [Saito et al., 2013]. Together, these actions principally stabilize consolidated periods of wake and sleep, while strongly inhibiting wake to REM sleep transitions

[Sasaki et al., 2011]. Narcolepsy is a common sleep disorder in which there is selective loss of orexin-producing neurons. Without orexin to 'stabilize' the flip-flop switches governing wake/NREM/REM state changes, people with narcolepsy and animals without orexin neurons display a phenotype characterized by an inherently sleepy and unstable wake state, with occasional intrusion of REM sleep into wake [Lin et al., 1999; Branch et al., 2016]. This can manifest as cataplexy, or spontaneous loss of muscle tone.

6. Sleep Disruption and Health

As outlined in the above sections, sleep serves a fundamental physiological role that cannot be ignored, yet sleep disruption is extraordinarily common in western society. It is estimated that 50-70 million Americans have a sleep disorder [Institute of Medicine Committee on Sleep Medicine and Research, 2006], and the Centers for Disease Control and Prevention (CDC) estimates that 35.2% of adults in the United States regularly have a short (<7 hours) sleep duration [Centers for Disease Control and Prevention, 2014]. Furthermore, among adults with regular short sleep, these ten health conditions were significantly more likely to be reported: heart attack, coronary heart disease, stroke, asthma, chronic obstructive pulmonary disease, cancer, arthritis, depression, chronic kidney disease, and diabetes [Centers for Disease Control and Prevention, 2014]. Although clearly these data do not indicate that sleeping a less than 7 hours per day causes all of these conditions, sleep deprivation should be considered a risk factor for poor health. Research in rodents and humans has revealed the health impacts of sleep deprivation to be wide ranging, affecting multiple physiological systems. This section will

examine some of the models used to study sleep disruption, then will summarize findings from human and rodent studies regarding the impact of sleep disruption on general physiological processes such as metabolism, cognition, and inflammation, along with some associated disease states.

Models of Sleep Disruption

Researchers investigating the toll of sleep disruption on health have used a wide range of experimental designs, both using human subjects and animal models. When designing a sleep disruption experiment, there are a few factors that need to be considered, depending on the goals of the research. The first factor is the model organism used. Most sleep research has been done in humans or rodents, but in the past few decades less complex organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans* have been studied in order to take advantage of their well understood genetics and neural circuitry [Freeman et al., 2013; Kayser and Biron, 2016]. The next factor is the desired tempo of the disruption. Models of 1-2 nights of short sleep [Benedict et al., 2016], all the way thorough 32 days of total sleep deprivation (which ultimately resulted in the death of the experimental animals [Rechtschaffen et al., 1983; Everson et al., 1989]) have been examined. The third factor is the character of the disruption. Sleep may be just shortened, or deprived entirely. It may be interrupted periodically, causing fragmentation, or it may be misaligned with external or internal circadian cues. It may be disrupted by externally derived stimulation (i.e. mechanical manipulation to prevent sleep), self-motivated stimulation (i.e. being told to be awake or having a novel environment to interact with), pharmacological

stimulation, or even by exogenous stimulation of wake-promoting neural circuitry. In this document, I will use the term “sleep disruption” to refer more generally to any manipulation that changes the sleep architecture away from baseline values, while “sleep deprivation” usually refers to the near complete elimination of a stage of sleep (NREM, REM, both) for a period of time. In humans these protocols typically involve asking patients to stay awake or follow a certain sleep/wake schedule, engaging them in a variety of activities that help them maintain sufficient vigilance levels, using polysomnography to wake patients up during certain sleep stages. However, the techniques researchers have used to achieve these goals in model organisms are variable, and some of the most commonly used paradigms will be discussed below.

Control over the experimental conditions and access to genetic and neuroscientific tools, along with ethical considerations, make rodent models of sleep disruption invaluable. However, as rodents lack a fundamental understanding of the experimental protocol (you cannot ask a rodent to do their best stay awake for 20 hours), any sleep manipulation will be inherently stressful to the animal. Therefore, most commonly used protocols are designed to alter sleep patterns while minimizing the stress to the animal. Rodents generally respond to CNS stimulants such as caffeine and modafinil, and caffeine has been used as a model of insomnia in rats [Paterson et al., 2007]. However, it becomes difficult to standardize the amount of sleep loss in these experiments, and it is difficult to control for off-target effects of stimulants. There are multiple commonly used non-pharmacological methods to achieve total sleep deprivation in rodents. Another method that is much more common, and does not require concomitant EEG/EMG

recording is the gentle handling procedure. This method involves close monitoring of the animals in their home cages by experimenters, and when the animals enter sleeping posture or demonstrate behavioral drowsiness, experimenters manually manipulate the animal or its cage just enough to keep the animal awake. This method can also be achieved by monitoring EEG/EMG for signs of entry into sleep [Alam et al., 2014], but is less common. Advantages of this method include limiting exposure to stressors (it is important to acclimate the animals to handling in the days/weeks prior to the experiment), and limiting confounding variables associated with increased or forced locomotion, though gentle handling does not completely eliminate either [Meerlo et al., 2008; Longordo et al., 2011]. Gentle handling also can be very effective at reducing sleep, as one study reported 92% reduction of NREM sleep and 100% reduction of REM sleep in rats [Franken et al., 1991]. Drawbacks to this method include the fact that it is labor intensive for the experimenter which makes it difficult to achieve long periods of sleep deprivation in large cohorts of animals, and the fact that it can be difficult to standardize the amount of manipulation each animal receives within an experiment and across laboratories.

One strategy some have used to attempt to circumvent the issue that rodent sleep deprivation usually achieved by externally enforced wakefulness is to utilize the inherent curiosity of rodents. By placing a mouse or rat in an enriched environment, with novel objects, littermates, and a varied and expanded cage layout, mice will naturally keep themselves awake to explore [Hairston et al., 2005; Achariyar et al., 2016]. However, this method may only work for 3-8

hours of reduced sleep and can confound later behavioral or cognitive tasks [Colavito et al., 2013].

Automated disruption methods have been developed to reduce manipulation variability between animals and to expand the temporal and replicate capacity of experimental protocols. Continuously moving treadmills or rotating wheels have been used to achieve total sleep deprivation, though it is difficult to control for confounding factors of increased locomotion, increased fatigue, and even the benefits of increased exercise with these protocols [Colavito et al., 2013]. Rechstchaffen and colleagues attempted to improve on the treadmill method by developing the "disk-over-water" apparatus. One experimental and one control animal are housed on each side of a cage with a divider in the middle and the floor replaced by a rotating disk over water. As soon as the experimental rodent enters a the vigilance state that is being deprived, the disk will begin to rotate, forcing the animal to wake up and walk against the rotation direction to avoid falling in the water. The yoked control animal is able to sleep whenever the experimental animal is awake, but still receives a comparable locomotor and stress load. This reduced sleep time in the experimental animal by 91% and the yoked control animal by 28% in one study [Everson et al., 1989]. Drawbacks of this method include an imperfect sleep-disruption control (they are still getting mild sleep deprivation), as well as high levels of stress induced every time the animal falls in the water. A method that has been used recently employ the use of a slowly moving bar at the bottom of the cage [Poroyko et al., 2016; Zhang et al., 2017; Bowers et al., 2020]. This method is only mildly stressful, as it does not involve any

water, allows the animals to stay in their home cage, and can be performed for extended periods of time. The periodicity of bar passes can be adjusted based on the goals of the experiment, resulting in sleep apnea-esque fragmentation [Poroyko et al., 2016] as well as a combination of short sleep and sleep fragmentation [Bowers et al., 2020].

New discoveries regarding sleep circuitry and optogenetics have made it possible to stimulate wake without any physical manipulation of the animal. Increased wake and sleep fragmentation has been achieved using optogenetic stimulation of cholinergic neurons in the BF and orexin neurons, respectively [Rolls et al., 2011; Xu et al., 2015]. While this is an exciting opportunity to achieve sleep disruption without many of the aforementioned confounding variables, a new set of hurdles arises. First, we do not know optogenetic stimulation of wake or inhibition of sleep can be performed chronically without neuronal plasticity or unintended changes to other behaviors. Second, we are limited to surface EEG to score sleep in these animals. It is possible stimulation of BF neurons generates behaviors and EEG that look like wake, but sleep-dependent processes are still occurring locally throughout the brain.

Methods of selective REM sleep deprivation in humans usually involve an experimenter watching the polysomnography recordings and waking the subject when he or she enters REM. In mice and rats, researchers will often take advantage of the atonia that accompanies REM sleep. Known as the platform method or the “flower pot method”, it consists of animals being placed on a small platform (or in a cage of multiple platforms) above water. The platforms are

large enough for the animal to achieve NREM sleep while crouching, but when the atonia of REM sleep sets in they slip off the platform and wake up [Jouvet et al., 1964; Abel et al., 1983]. There are significant drawbacks to this method, as it is difficult to establish control conditions, involves certain amounts of NREM sleep deprivation as well [Machado et al., 2004], and falling into water is moderately to severely stressful to the animal [Revel et al., 2009].

Sleep-like states have been described in *Drosophila* and *C. elegans*, and sleep disruption experiments have been crucial in determining the function of sleep in these organisms. Sleep in *Drosophila* has been deprived traditionally using manual stimulation (tapping or nudging the dish containing the flies) or machines that rotate the dish [Hendricks et al., 2000]. Total rest time can be reduced by pharmacological stimulants like modafinil [Hendricks et al., 2003] as well. In *C. elegans*, sleep occurs in a larval stage called lethargus, a 2-3 hour long period at the end of each larval stage [Raizen et al., 2008]. Mechanical sleep disruption in *C. elegans* is achieved by touching it on the posterior end of its body with an eyelash tethered to the end of a Pasteur pipette until it moves [Raizen et al., 2008]. Sleep has also been reduced by shaking the dish in which the animal resides [Nagy et al., 2014], stimulating the organism with blue light pulses [Nagy et al., 2014], and optogenetic stimulation of neural circuits [Cho and Sternberg, 2014], all of which cause a homeostatic rebound in sleep.

The use of sleep deprivation as a method to study the fundamental function of sleep in healthy conditions has been criticized due to some evidence that rebound sleep may not be an identical

process to normal sleep [Anafi et al., 2019]. However, studying the physiological toll of sleep disruption itself is still valuable, as this is an extremely common phenomenon with significant health implications, as will be discussed in the below subsections.

Sleep Disruption and Metabolism

The link between inadequate sleep and metabolic disorders such as type 2 diabetes (T2D), obesity, hypertension, heart disease, stroke is evident in many epidemiological studies [Anothaisintawee et al., 2016; Cappuccio and Miller, 2017]. Laboratory studies of sleep deprivation in humans and rodent models have provided insights into the possible mechanisms behind these associations. In one of the first studies to establish the link between insufficient sleep and alterations in glucose metabolism, restriction to 4 hours of time in bed for 6 nights caused a decrease in glucose tolerance (measured by the intravenous glucose tolerance test) in adult men [Spiegel et al., 1999]. Insulin insensitivity also decreased in *ex vivo* adipose tissue after 4 days of restricted sleep in humans [Broussard et al., 2012]. Sleep fragmentation for two nights also decreased insulin sensitivity in healthy adults [Stamatakis and Punjabi, 2010], indicating sleep quality is also important to metabolism. Recently, it was shown that a single night of total sleep deprivation not only causes impaired insulin sensitivity, tissue-specific changes in transcriptomic, epigenetic, proteomic and metabolomic markers were observed, thus capturing the breadth of changes that can occur even after one night of lost sleep [Cedernaes et al., 2018].

Lipid metabolism is altered after sleep disruption as well. A recent study revealed that a single 6 hour sleep deprivation via gentle handling increased propensity for hepatic steatosis via upregulation of hepatic lipogenic enzymes in mice [Shigiyama et al., 2018]. 24 hour sleep deprivation also increased the presence of multiple acetylcarnitines and sphingolipids in the plasma, indicating alterations in lipid metabolism [Davies et al., 2014]. These metabolic changes, combined with changes to endothelial function and immune regulation combine to increase the risk for atherosclerosis and cardiovascular disease [Sauvet et al., 2010; Liu and Chen, 2019; McAlpine et al., 2019]. Overall, proper sleep appears to be crucial for normal metabolism, and countermeasures to improve resilience to sleep disruption are warranted.

Sleep Disruption and Cognition

J.A. Hobson wrote in 2005: "Sleep is of the brain, by the brain and for the brain" [Hobson, 2005]. If taken at face value, there are a long line of counterarguments to this statement, from the wealth of metabolic/immunologic impacts of sleep, to the observation that restoration of the clock gene *Bmal1* in muscle, but not brain, restores sleep amounts in a *Bmal1* knockout mouse [Ehlen et al., 2017], to the fact that some animals that do not have central nervous systems sleep [Anafi et al., 2019]. However, one cannot argue that sleep has evolved to serve a crucial role in normal brain function in animals with brains (see previous sections of this chapter), and studies of sleep deprivation on cognition and other brain processes support this.

Studies in humans have established that sleep restriction impairs higher cognitive functions like attention and executive function, and these findings have implications for public health. Sustained attention (also called vigilant attention) is often measured by assessing detection of a simple stimulus in tasks such as the psychomotor vigilance test (PVT) [Dinges and Powell, 1985]. Repeated nights of sleep restriction (to 4 or 5 hours per night) reliably reduces performance on the PVT [Dinges et al., 1997; Wu et al., 2010; Pejovic et al., 2013]. Interestingly, one study examined five nights of sleep restriction (6 hours sleep opportunity per night) followed by two nights of recovery sleep (10 hours opportunity per night) and found that although the "weekend" of recovery sleep resulted in recovery of sleep restriction-induced elevations in IL-6 and subjective sleepiness, performance on the PVT did not recover to baseline levels [Pejovic et al., 2013]. This indicates that the impact of sleep disruption on attention may outlast subjective feelings of being rested, which could result in dangerous lapses during the day. Other studies have shown that performance on attentional tasks deteriorates in a dose-dependent manner with increased sleep debt [Belenky et al., 2003; Van Dongen et al., 2003]. These lapses in attention have been proposed to be brief periods of sleep-like brain activity, or 'microsleeps' [Poudel et al., 2009]. One study examined a visual-motor tracking task over 28 hours of wakefulness and compared it to various levels of blood alcohol concentration, and found that performance declined linearly across the 28 hours, and at the 24th hour performance was roughly equivalent to a blood alcohol concentration of 0.10%, a level exceeding legal intoxication limits [Dawson and Reid, 1997]. Indeed the attentional impairments that come with extended

wakefulness are estimated to be among the main causative factors in transportation and workplace accidents [Philip and Akerstedt, 2006; Lahti et al., 2011].

Sleep disruption also has a negative impact on executive function, including emotional regulation [Walker and van der Helm, 2009; Goldstein and Walker, 2014; Lowe et al., 2017]. This has been observed in subjective self-report studies where even a single night of sleep deprivation leads to increases in perceived negative moods and reduced ability to cope with frustrating social situations [Tempesta et al., 2010]. Objective evaluations of emotional perception were also altered, as subjects rated neutral images significantly more negatively following sleep disruption, independent of self-reported mood [Tempesta et al., 2010]. Furthermore, studies have shown that sleep deprivation results in a hyperactive response of the amygdala to emotional stimuli, and it has been posited that this is due to a decrease in executive inhibition of emotional circuitry [Thomas et al., 2000; Yoo et al., 2007a]. The implications of hyperactive emotional arousal systems and stress vulnerability will be discussed in the next section.

Sleep deprivation studies have been crucial in establishing the link between sleep and memory, which has ballooned into an enormous field of research (see [Abel et al., 2013; Rasch and Born, 2013; Boyce et al., 2017] for thorough reviews). Sleep disruption has been experimentally shown to disrupt both encoding and consolidation of memory. Human studies and animal models have shown sleep disruption prior to learning a task reduces the ability of the subject to encode the

new memory. For example, 35 hours of total sleep deprivation before the learning phase of a word-association task reduced recognition scores two days later by 19% compared to subjects who slept normally prior to encoding [Yoo et al., 2007b], and a similar deficit in encoding trace-conditioned memory after short (6-hour) sleep deprivation was observed in rats [Chowdhury et al., 2011]. It is possible these findings support the synaptic homeostasis hypothesis of sleep, in that sleep deprivation may increase the density of synaptic spines and thus reduce the capacity to learn new tasks [Rasch and Born, 2013].

Lack of sleep in the time period between learning a task and being tested impairs performance, thus indicating sleep (and particularly SWA during sleep) is important for memory consolidation as well [Scullin, 2013]. Sleep disruption appears to have a particularly strong impact on hippocampal-dependent memory [Killgore, 2010; Abel et al., 2013; Krause et al., 2017]. Indeed, multiple studies demonstrate impaired function of the hippocampus after sleep restriction [Kreutzmann et al., 2015; Havekes and Abel, 2017]. 72 hours of sleep disruption substantially reduced the ability to induce hippocampal long term potentiation in hippocampal neurons [McDermott et al., 2003]. This reduction in plasticity is accompanied by reduced hippocampal synaptic connectivity [Havekes et al., 2016; Havekes and Abel, 2017; Raven et al., 2019] and neurogenesis [Fernandes et al., 2015]. Furthermore, sleep disruption reduces synthesis of proteins associated with neuroplasticity [Fernandes et al., 2015] and mTOR-mediated protein synthesis [Tudor et al., 2016]. Together, hippocampal functioning is impaired down to the

molecular level in the sleep-deprived state, and this manifests in impairment in many hippocampal-dependent tasks including memory encoding and consolidation.

Alterations in normal sleep architecture are a hallmark of many neurological disease states that are characterized by cognitive decline. There is evidence for sleep disturbances or elevated risk for a comorbid sleep disorder in an extraordinarily wide ranging set of disorders, from genetic metabolic brain diseases like Wilson disease [Xu et al., 2020], to acquired states like traumatic brain injury [Grima et al., 2016], to chronic neurodegenerative [Moran et al., 2005; Palma et al., 2013; Veauthier and Paul, 2014] and psychiatric conditions [Baglioni et al., 2016]. In some conditions, sleep disruption has been proposed as a risk factor for development or progression of disease. Among the conditions with the most evidence that sleep disruption may be involved with the pathogenesis of disease are MCI and AD (reviewed by [Yaffe et al., 2014; Cedernaes et al., 2017; Boespflug and Iliff, 2018]). Insomnia diagnosis [Osorio et al., 2011], self-reported sleep problems [Benedict et al., 2015], and lower sleep duration [Hahn et al., 2014] have all been associated with an increased risk of AD. Prospective studies indicate that sleep disturbance may be a risk factor for development of AD or MCI early on, even before onset of cognitive symptoms. In a prospective cohort study of older adults without dementia, those who were in the 90^{th} percentile or above in actigraphy measured sleep fragmentation had a 1.5 fold risk of developing AD compared to the 10^{th} percentile [Lim et al., 2013]. Poor sleep quality and alterations in sleep EEG predicted subsequent onset of MCI in a cohort of older adults as well [Taillard et al., 2019]. The mechanism by which sleep disruption promotes MCI and AD

pathology has been proposed to involve reduced clearance of the AD-promoting proteins tau and amyloid beta in the brain, increased oxidative stress, and reduced integrity of the blood-brain barrier [Cedernaes et al., 2017; Boespflug and Iliff, 2018].

Experimental sleep deprivation studies support these hypothesized mechanisms. Recent experiments examining the glymphatic clearance of metabolites have observed that amyloid beta and tau protein are cleared from the brain during normal sleep [Xie et al., 2013; Holth et al., 2019]. Sleep deprivation in rats accelerated cognitive impairment and increased generation of amyloid beta, suggesting sleep deprivation in early stages of AD may in fact accelerate its progression [Chen et al., 2017]. In one of the few such studies using human subjects, one night of sleep deprivation prevented the usual dip in CSF amyloid beta-42 levels, indicating chronic insufficient sleep could result in increased amyloid beta in the brain [Ooms et al., 2014]. A subsequent study in humans found that depriving slow wave sleep was sufficient to increase CSF amyloid beta, and poor sleep efficiency over multiple nights correlated loosely with increased CSF tau [Ju et al., 2017]. One week of sleep disruption in mice increased permeability of the blood-brain barrier in mice, indicating a route other oxidative or neurotoxic metabolites could enter the brain in neurodegenerative disease as well [He et al., 2014].

Thus, sleep disruption studies indicate as little as a single night of sleep deprivation can alter cognition and brain function, and chronic sleep disruption may have dire consequences ranging from functional impairment to increased risk of neurodegenerative disease.

Sleep Disruption and Inflammation

Sleep and the immune system are bidirectionally linked, as changes in immune activation alter sleep patterns, while sleep disruption changes the function of the immune system (reviewed by [Kinnucan et al., 2013; Besedovsky et al., 2019]. Many immune system effector molecules have been found to alter sleep (usually NREM sleep) in some way. Prostaglandins, particularly prostaglandin D2, appear to increase sleep in rodents [Urade and Hayaishi, 2011], though they have rarely been studied as somnogens in humans [Besedovsky et al., 2019]. In rodent studies, proinflammatory cytokines such as TNFalpha, interleukin (IL)-1, interferon (IFN), IL-2, IL-6, IL-15, and IL-18 all have evidence supporting roles as NREM sleep-promoting molecules [Opp, 2005; Imeri and Opp, 2009; Besedovsky et al., 2019]. Anti-inflammatory cytokines IL-4 and IL-10 have been shown to attenuate NREM sleep in rabbits [Kushikata et al., 1998; Kushikata et al., 1999]. In humans, less research has been done regarding the effect of cytokines on sleep, but IL-6 has been shown to reduce REM sleep in one study [Spath-Schwalbe et al., 1998] and increase SWA in the second half of the night in another [Benedict et al., 2009]. Thus, more research on the impact of cytokine administration needs to be done in humans.

In general, sleep disruption increases the inflammatory state of the organism. One night of partial sleep deprivation increases plasma C-reactive protein (CRP), a general marker for inflammation [Irwin et al., 2016]. Consistent with this, many studies examining blood leukocytes and lymphocytes during sleep disruption and during recovery sleep have shown an increase in levels

of both due to sleep disruption, with a subsequent decrease with sleep, though some studies failed to replicate this finding (see [Besedovsky et al., 2019]). This indicates a general mobilization of white blood cells during states of deprived sleep. Immune effector molecules, especially cytokines, are have been shown to be impacted by sleep disruption as well. While IL-6 levels in plasma or saliva have been to go up [Thimgan et al., 2013], go down [Frey et al., 2007], or remain unchanged [Chennaoui et al., 2011] after shorter duration sleep deprivation protocols, longer term protocols tend to show increases in IL-6 in both rodents and humans [Hu et al., 2003; Vgontzas et al., 2004; Haack et al., 2007]. TNFalpha, another proinflammatory cytokine, has been shown to increase after 50 hours of total sleep deprivation in humans, and after 36 hours of total sleep deprivation in mice [Hu et al., 2003; Chennaoui et al., 2011], although again this finding is sometimes inconsistent [Thimgan et al., 2013]. Similarly, a preponderance of the evidence in rodents and humans indicate IL-1 is increased in the circulation after sleep disruption, especially after prolonged sleep disruption (see [Besedovsky et al., 2019]). A study examining 5 nights of short (4 hours per night) sleep in humans found leukocyte gene expression profiles shift to a more proinflammatory, activated state [Aho et al., 2013], supporting general claims that the sleep-deprived state is a proinflammatory state. In fact, a study following over 3,000 older adults over a 9 year period found that proinflammatory markers such as IL-6, TNF, and CRP were one of the key factors explaining the association between short sleep duration and increased mortality [Hall et al., 2015].

Consistent with this, inadequate sleep has been linked with a host of disease states that are characterized by increased inflammation [Ranjbaran et al., 2007]. One such disease is inflammatory bowel disease (IBD) [Kinnucan et al., 2013]. The IBD patient population not only has a high likelihood of having concomitant sleep disturbances [Zimmerman, 2003; Burgess et al., 2010], recent poor sleep quality is associated with activation or relapse of disease [Ali et al., 2013; Ananthakrishnan et al., 2013]. This association of sleep deprivation with earlier onset or increased severity of chronic inflammatory conditions has been observed in disease models as well. In an animal model of systemic lupus erythematosus, sleep deprivation using the two platform method resulted in an earlier onset of the disease [Palma et al., 2006]. Large longitudinal cohort studies in people with disrupted sleep due to obstructive sleep apnea [Kang and Lin, 2012]or due to a non-sleep apnea sleep disorder [Hsiao et al., 2015] have reported increased risk of developing autoimmune diseases. Thus, while definitive mechanisms are not yet understood, sleep disruption appears to increase the risk of developing chronic inflammatory conditions, or to worsen preexisting chronic inflammatory conditions.

Some psychiatric conditions are thought to be tightly linked to increased inflammation, and are sometimes considered conditions of chronic low level inflammation [Jones and Thomsen, 2013; Daniels et al., 2017; Michopoulos et al., 2017]. The link between sleep and psychiatric disease appears to be strong, with sleep disruption being a hallmark phenotype of many conditions, particularly depression [Meerlo et al., 2015; Steiger and Pawlowski, 2019], PTSD [Ross et al., 1989; Germain, 2013; Miller et al., 2017], and anxiety disorders [Baglioni et al., 2016]. The

sleep phenotypes of these disorders, and their association with sleep disruption, will be discussed more thoroughly in the context of stress vulnerability in Sections I.B.2 and I.B.3.

Overall, our understanding of sleep and its function have come a long way since the writings of Aristotle that attributed sleep to the necessary risings and fallings of heated substances in our bodies. The near century of sleep research since the development of EEG recording by Hans Berger in 1924 has revealed that sleep is a nearly ubiquitous, active, and dynamic process that serves multiple functions within the organism. Despite this new understanding, repeated inadequate sleep is prevalent in our society, and has an immense potential health impact, with increased risk of metabolic, neurologic, and inflammatory disorders. Furthermore, the vulnerability to sleep deprivation is trait-like [Rupp et al., 2012; Sprecher et al., 2019], thus finding countermeasures to improve resilience to sleep disruption is vital, particularly in those who are more susceptible. The sleep-deprived state can also be thought of as a vulnerable health state, where metabolic dysregulation, reduced cognitive capacity, and increased proinflammatory tone could act as catalysts in multiple medical, neurologic, and psychiatric disease processes. This idea forms the basis of the project described in this dissertation, and will be discussed in detail in the next sections.

B. Sleep and Stress

The stress response consists of a series of physiological changes that are meant to protect the organism from a perceived physiological threat. It evolved to turn on during times of danger, and turn off when it was no longer needed. However, a large body of evidence has shown that

persistent activation of this stress response is deleterious to health and survival (see reviews by [McEwen, 2007; McEwen et al., 2015c; Slavich, 2016]). This could be due to repeated exposure to stressful environments, the pathological inability to shut the stress response off after an acute event, or, as is particularly relevant to the human species, could be a result of self-generating psychological stress. In the 2004 edition of his 1994 book *Why Zebra's Don't Get Ulcers*, Dr. Robert Sapolsky addresses this latter phenomenon thusly: "Essentially, we humans live well enough and long enough, and are smart enough, to generate all sorts of stressful events purely in our heads" [Sapolsky, 2004]. This section will outline the physiological processes of the stress response and will introduce the concept of stress vulnerability and resilience. It will then characterize the close interaction between stress and sleep and will examine sleep phenotypes of stress-related psychiatric disorders. Finally, this section will take a detailed look at the evidence supporting the idea that sleep disruption is a factor that increases stress vulnerability.

1. Stress and Stressors

Stress Physiology

The stress response is the physiological reaction to a stressful sensory input (a stressor), and involves coordinated multisystem responses that occur over multiple time scales. The first stage of the stress response is the perception of a stressor. Stressors represent an extremely wide range of stimuli, but have been classified into the general categories of physical stressors and psychological stressors [Godoy et al., 2018]. Physical stressors typically involve actual disturbances to physiological systems that are kept in strict homeostasis and include events like

hemorrhage, injury, or infection. These stressors are typically perceived by brainstem and hypothalamic structures that maintain day-to-day homeostasis such as the nucleus of the solitary tract (NTS) in the brainstem [Gaillet et al., 1991], but can also involve limbic forebrain structures such as the hippocampus and prefrontal cortex (PFC) that receive input from sensory subcortical or cortical areas [Dayas et al., 2001; Ulrich-Lai and Herman, 2009]. Psychological stressors are classified as perceived threats to the current state, and are often anticipatory in nature [Godoy et al., 2018]. The brain regions involved in perception of psychological stressors feature limbic forebrain structures as well, with an emphasis on multiple structures that govern emotional salience of psychological stimuli, such as the amygdala, ventral tegmental area (VTA), and nucleus accumbens (NAc) [Ulrich-Lai and Herman, 2009; Russo and Nestler, 2013; Godoy et al., 2018]. Physical and psychological stressors both ultimately activate preganglionic autonomic and hypophysiotropic neurons in the paraventricular nucleus of the hypothalamus (PVN), which activates the two main arms of the stress response, the sympathetic-adrenal-medullary (SAM) system and the HPA axis [Ulrich-Lai and Herman, 2009; Godoy et al., 2018], eventually resulting in metabolic, immune, and neurologic changes (**Figure 1.5**).

The fastest acting branch of the stress response is the sympathetic nervous system (SNS) and the SAM axis. Preganglionic cholinergic neurons of the SNS lie mostly in the thoracolumbar region of the spinal cord (with the exception of the cervical chain, which provides sympathetic innervation of the head), projecting to paravertebral ganglia. The postganglionic neurons project around the body to target organs, using norepinephrine to activate adrenergic receptors [Buijs,

2013; Goldstein, 2013; Godoy et al., 2018]. The notable exception to this organization are the preganglionic neurons that do not synapse with postganglionic neurons but directly project to the chromaffin cells of the adrenal medulla. These cells produce norepinephrine and epinephrine and secrete them into circulation. Catecholamines like norepinephrine and epinephrine are responsible for many of the "fight or flight" responses of the sympathetic nervous system, such as increased heart rate and blood pressure, pupillary dilation, increased alertness, thermogenesis, glycogenolysis and gluconeogenesis, immune activation, and neuromodulation in the CNS [Buijs, 2013; Tank and Lee Wong, 2015; Fleshner and Crane, 2017] (see **Figure 1.5**).

Activation of the HPA axis is slightly slower than the synaptic action of the SAM axis, but results in the release of one of the crucial mediators of the stress response: glucocorticoids. Activated neurons in the PVN release corticotropin releasing hormone (CRH) into the hypophyseal portal system of the anterior pituitary, where cell are stimulated to release adrenocorticotropic releasing hormone (ACTH) into systemic circulation. ACTH then stimulates the release of glucocorticoids such as corticosterone and cortisol from the adrenal cortex [Balbo et al., 2010; Godoy et al., 2018]. Glucocorticoids are steroid hormones that have cytosolic receptors in nearly all nucleated cells, though the function of that receptor may vary based on cell type [Cain and Cidlowski, 2017]. Activated glucocorticoid receptors can then activate or repress gene transcription (up to 20% of the genome is regulated by glucocorticoid receptors [Galon et al., 2002]), but recent studies have revealed that glucocorticoids can have multiple non-genomic effects as well [Cain and Cidlowski, 2017]. Together the SAM and HPA axes form

the two main arms of the stress response, though the relative balance between the two depends on the nature of the stressor [Skoluda et al., 2015]. The physiological effects of these two branches and their effector molecules are wide ranging and have significant relevance to health and disease, some of which will be described below.

The interaction between the stress response and immune activation has become well characterized in the past few decades, and involves complex interactions between immune cells and the SNS, glucocorticoids, and other immune activating sequelae [Cain and Cidlowski, 2017; Fleshner and Crane, 2017]. The impact of the SAM axis on the immune system is, generally, to activate proinflammatory pathways, increasing peripheral and central levels of cytokines/chemokines in the absence of pathogens. This process has been labeled sterile inflammation, and is thought to have evolved to prime the host for possible infections as a result of the perceived threat or to improve tissue repair/wound healing [Rock et al., 2010; Fleshner and Crane, 2017]. However, repeated activation of sterile inflammation, or chronic low level activation with impaired ability to shut the response off can be maladaptive and is thought to be the source of many disease states, including cardiovascular [Golia et al., 2014], metabolic [Lontchi-Yimagou et al., 2013], and psychiatric disorders [Audet et al., 2014; Passos et al., 2015; Hori and Kim, 2019]. The mechanisms of sterile inflammation are driven via catecholamine-induced increases in damage associated molecular patterns [Cox et al., 2014], though there is evidence to support that stress-induced translocation of immunogenic microbial associated molecular patterns from the gut microbiota work in concert with DAMPs in generating sterile

inflammation [Bailey et al., 2006; Maslanik et al., 2012]. Glucocorticoids from the HPA axis have a complex interaction with inflammation. Classically, steroids such as cortisol are known for their immunosuppressive properties, and are widely used clinically to suppress the immune system. In fact, the 1950 Nobel Prize in Physiology or Medicine was awarded to Hench, Kendall, and Reichstein for discovering these properties, and the mechanisms behind glucocorticoid-induced immunosuppression have been well characterized [Cain and Cidlowski, 2017]. However, there is evidence to support the notion that glucocorticoids also can stimulate the immune system, particularly at lower concentrations [Galon et al., 2002; Lim et al., 2007; van de Garde et al., 2014]. Thus, chronic low level stress may result in chronic inflammation via a synergy of SNS tone and low level glucocorticoids.

The brain is one of the main targets of stressful experiences, and glucocorticoids have been shown to alter plasticity and function, particularly limbic structures such as the hippocampus, amygdala, and PFC (reviewed by [McEwen et al., 2015a; McEwen et al., 2016]). Glucocorticoids exert their actions via glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs), and both were first discovered in the extra-hypothalamic brain in the hippocampus [McEwen et al., 1968], but have since been found in structures throughout the limbic system. Accordingly, exposure to acute stress changes the gene expression profiles of neurons in the hippocampus, though a history of chronic stress exposure alters that effect [Datson et al., 2013; Gray et al., 2014]. In general, chronic stress causes different changes in the plasticity of these three brain regions. In the hippocampus, neurogenesis is inhibited, spines are

lost, and dendrites shrink due to glucocorticoids [Vyas et al., 2002]. In the basolateral amygdala (BLA) the opposite is observed [Vyas et al., 2002; McEwen et al., 2015a]. This is thought to be mediated by epigenetic factors [McEwen et al., 2015a], along with the observation that brain-derived neurotropic factor is downregulated in the hippocampus but upregulated in the BLA after chronic stress [Lakshminarasimhan and Chattarji, 2012]. In the PFC, chronic stress causes debranching and shrinkage of dendrites, which is associated with cognitive rigidity, while orbitofrontal neurons expand dendrites, which is associated with increased vigilance (a common finding in PTSD) [Radley et al., 2004; Liston et al., 2006; Radley et al., 2006].

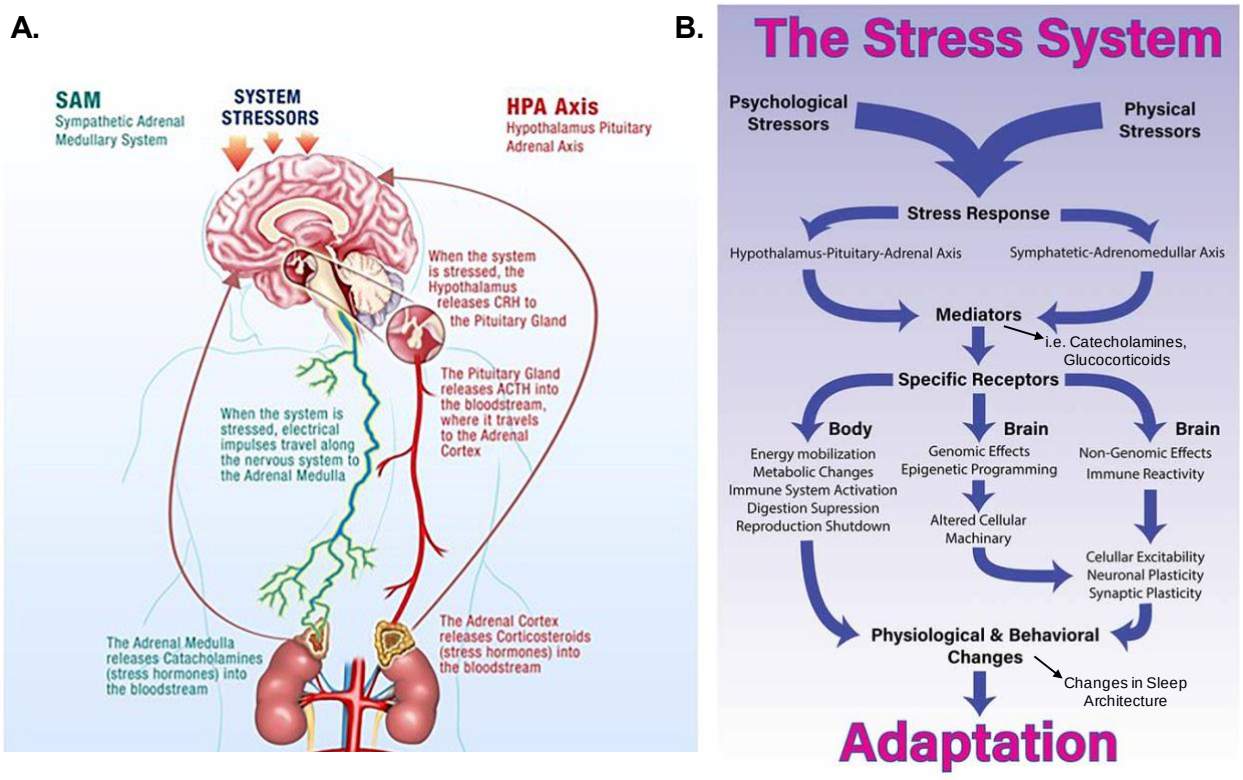


Figure 1.5 Schematics of the Stress Response. (A) Simplified schematic representing the two main arms of the initial stress response, the sympathetic-adrenal-medullary (SAM) axis, and the hypothalamic-pituitary-adrenal (HPA) axis. SAM activation is relatively fast acting, and the resultant effector molecules (mainly catecholamines like norepinephrine and epinephrine) impact tissues across the brain and body. Neuroendocrine system activation via the HPA axis is on a

slower timescale, and the ultimate release of glucocorticoids results in similarly pan-tissue changes. Adapted from [Rooney et al., 2019]. (B) Simplified representation of the impact of stress response activation on various systems. Adapted from [Godoy et al., 2018].

Rodent Models of Stress

As many forms of stress are psychological in nature, it is inherently difficult to develop protocols in rodents that accurately model human stressors. Furthermore, since it is know that the exact nature and duration (acute vs chronic, e.g. [Keeney et al., 2006]) of the stressor can alter how the stress response is activated and thus the downstream physiological effects, many rodent models of stress have been developed (see review by [Schoner et al., 2017]). This subsection will review the pros and cons of some of the more prominent models, arranging them from more "physical" in nature to more "psychological".

Multiple commonly used stress paradigms utilize foot or tail shock to create a high amplitude acute stress response. For instance, inescapable tail shock paradigms involve restraining the animal in a chamber of restricted size and administering uncomfortable shocks to their tail, resulting in SNS activation, a robust corticosterone response, and activation of sterile inflammation (e.g. [Maslanik et al., 2012; Cox et al., 2014; Greenwood et al., 2014]). Inescapable and unpredictable foot shock also elicits long-lasting behavioral and physiological effects [Van Dijken et al., 1992], and usually consists of placing the animal in a chamber with a wire grid floor that can have current pass through it at controllable intensities and intervals. Foot shock can be used as an acute stressful event alone, or, importantly, it can be paired with a contextual stimulus to create a contextual, fear-associated memory. These protocols, called fear

conditioning protocols, have been instrumental in the study of stress responses and of emotional memory processes in mammals [Maren, 2001; Izquierdo et al., 2016].

Restraint is extremely stressful to rodents. As such, many experimental protocols attempt to minimize procedures whereby the conscious rodent has to be physically restrained (e.g. to do an injection or draw blood). However, many stress protocols utilize 1-6+ hours of restraint stress because it initiates an acute stress response while minimizing pain or injury [Buynitsky and Mostofsky, 2009]. Furthermore, chronic exposure to restraint stress (6 hours per day for 28 days) results in increased proinflammatory cytokines and depressive-like behaviors [Voorhees et al., 2013]. However, restraint stress is not particularly ethologically relevant, and still requires physical discomfort of the animal. Stress paradigms have been developed to utilize predator odors to attempt to activate ethologically relevant fear pathways, and single exposures cause activation of fear neurological pathways as well as cause behavioral changes, some of which last up to 3 weeks after a single exposure [Adamec and Shallow, 1993; House et al., 2011]. One criticism of predator odor paradigms is that while they are ethologically relevant to the rodent, predatory fear pathways are difficult to translate to human stress and health.

Social stress paradigms are common in stress research, and attempt to create psychological stress in rodents in a way that is (on the surface) relatable to human psychosocial stress. One type is social defeat, which typically involves exposing an experimental mouse to a trained aggressor mouse and allowing the experimental mouse to be defeated, then quickly separating it from the

aggressor to avoid serious injury [Golden et al., 2011]. Social defeat has been studied in acute and chronic settings, and has been shown to activate stress responses, resulting in changes such as induced sterile inflammation [Audet et al., 2011], depressive-like behaviors [Iniguez et al., 2014]. Another chronic social stressor paradigms have been developed that do not involve trained aggressor mice, but instead revolve around multiple hostile cage mates for extended periods of time [Reber et al., 2006]. Social isolation [Hatch et al., 1965; Pibiri et al., 2008] and maternal separation [Kalinichev et al., 2002] are other forms of social stress that have been used in rodents to study chronic stress effects as well as early life stress effects.

Experiments also sometimes combine these paradigms to create models of chronic repeated stress (e.g. [Nollet et al., 2019]), or chronic-on-acute stress (e.g. [Greenwood et al., 2014]) do answer diverse experimental questions. One such protocol is the single prolonged stress paradigm. In this paradigm, rats or mice are subjected to two hours of restraint stress, followed immediately by a forced swim in water (without a platform) for 20 minutes, followed by induced unconsciousness via ether [Liberzon et al., 1997]. Single prolonged stress was the first experimental paradigm to reproduce PTSD-like changes to HPA axis activity, though there have been many others that mimic other features of the disorder more accurately [Schoner et al., 2017]. It is of note that the stress response is under tight circadian control (reviewed by [Dumbell et al., 2016], and thus the time of day of the stressor must be considered and kept constant between groups when designing animal experiments.

Stress Vulnerability and Resilience

It has been observed that individuals vary in their physiological reaction to stressful events. For example, while 70% of the adults worldwide will experience a traumatic event at some point in their lives, and 31% of those will experience four or more, the lifetime prevalence of PTSD is only estimated at 1.3-12.2% [Karam et al., 2014; Benjet et al., 2016]. This concept, that there are some features of individuals that allow them to endure stressful events with fewer lasting repercussions, has been generally labelled stress resilience. The converse, that some people are primed for a more maladaptive, lasting response, is labelled stress vulnerability.

Laboratory research has observed multiple individual differences in stress responses that could explain differences in stress vulnerability (reviewed by [McEwen et al., 2015b; Osorio et al., 2017; Walker et al., 2017; Carnevali et al., 2018; Faye et al., 2018]). Genetic factors may play a role [Feder et al., 2009], as monozygotic twin studies have revealed a heritability rate of 32%-38% for PTSD [Southwick and Charney, 2012]. Furthermore, alterations in HPA axis function have been associated with vulnerability. Studies in humans with major depressive disorder (MDD) suggest a reduced sensitivity to glucocorticoids [Juruena et al., 2006], and mice underexpressing the glucocorticoid receptor show vulnerability to a 30 minute restraint stress [Ridder et al., 2005]. Altered autonomic regulation is thought to be involved with vulnerability to stress as well, and is often measured by heart rate variability, with low variability associated with vulnerability [Sgoifo et al., 2015], and vice versa [Souza et al., 2013]. Furthermore, differences in the proinflammatory response to a stressor, or the inflammatory state before a stressor, have

been implicated in vulnerability and resilience [Walker et al., 2017]. For instance, children who had higher plasma IL-6 in the 24 hours after a motor vehicle accident were more likely to go on to develop PTSD [Pervanidou et al., 2007], and individual differences in stress-induced cytokine reactivity had increased stress-induced behavioral abnormalities [Hodes et al., 2014]. Thus, conditions such as chronic stress that increase basal inflammatory reactivity, particularly in the brain [Tynan et al., 2010], could contribute to subsequent vulnerability to a secondary stressor [Greenwood et al., 2014; Thompson et al., 2014]. Differences in neural circuitry have also been associated with resilience. In mice, knockdown of cholinergic signaling in the hippocampus resulted in increased vulnerability to social defeat [Mineur et al., 2013]. Conversely, reduction of nicotinic cholinergic signaling in the amygdala increased resilience to social defeat stress, indicating differential impacts of these structures [Mineur et al., 2016]. Indeed, in humans, individual differences in amygdala reaction to threatening facial expressions predict later development of depression and anxiety symptoms in humans [Swartz et al., 2015].

The impetus for studying mechanisms stress vulnerability is both to discover biomarkers of vulnerability in order to identify individuals who would benefit from more aggressive intervention, as well as to develop countermeasures to improve stress robustness. Stress robustness can be thought of as the combination of stress resistance (ability to withstand stressors without significant negative physiological response) and stress resilience (the ability to quickly recover from stressors without lasting maladaptive changes). As will be discussed in a

forthcoming subsection, repeated sleep deprivation may be a model for stress vulnerability and an opportunity to test novel countermeasures to improve stress robustness.

2. Impact of Stress on Sleep

Sleep and stress form a bidirectional relationship in that stressful events result in changes to sleep architecture, while sleep disruptions can impact stress responses. This section address the former, and will discuss the impact of different stressors on sleep in human and rodent models. While stressful events usually cause an increase in wakefulness during the event itself and sometimes in the immediate aftermath, the loss of sleep itself cannot account for the changes in sleep architecture seen afterwards. In general, the character of the stressor (e.g. predictable/unpredictable, physical/psychological), time course of the stressor, and vulnerability of the individual all influence the nature of the subsequent sleep changes (reviewed by [Sanford et al., 2015]).

Several models of acute stress alter subsequent sleep in rodents, though in different ways. Short term (1-2 hours) restraint stress has been shown to be initially suppress REM, followed by an increase in REM sleep during the subsequent recovery period above the amount that was lost [Rampin et al., 1991; Meerlo et al., 2001b]. This “REM rebound” has been observed in acute social defeat as well [Henderson et al., 2017; Feng et al., 2020], and has been proposed to represent adaptive coping mechanisms [Suchecki et al., 2012]. However, a REM sleep rebound was not observed in various models of inescapable foot shock stress [Sanford et al., 2003;

Sanford et al., 2010], indicating heterogeneity in the sleep response. Another feature of post-acute stress sleep that has been observed in multiple models is an increase in NREM delta power. This effect has mostly been reported in acute social defeat models [Meerlo et al., 1997; Meerlo et al., 2001a; Meerlo and Turek, 2001; Kamphuis et al., 2015; Henderson et al., 2017; Feng et al., 2020], but was observed after acute exposure to predator odors as well [Sharma et al., 2018]. As described in Section I.A.3, delta power during NREM sleep plays a large role in homeostasis, memory, and other physiological functions. Interestingly, both the winner and loser of a social conflict displayed delta power rebounds during subsequent sleep, indicating NREM delta power increases may not be specific to aversive stimuli [Kamphuis et al., 2015].

Studies examining chronic models of stress have revealed repeated generation of the acute stress response can result in changes to sleep architecture that last well beyond the acute phase. Long term sleep difficulties in humans have been attributed to chronic stress in multiple contexts, including high-stress workplaces and early life stressful experiences [Armon et al., 2008; Charuvastra and Cloitre, 2009; Nomura et al., 2009]. Furthermore, in humans an acute traumatic event can result in intrusive memories of the event, which evoke stress responses and essentially turn the trauma into a chronic stressor [Sanford et al., 2015]. In rodent models, chronic exposure to stressors can result in the loss of delta power rebound, along with a gradual increase in the amount of REM, number of REM sleep bouts, or frequency of REM sleep [Cheeta et al., 1997; Gronli et al., 2004; Nollet et al., 2019], indicating a cumulative effect of the stressors that differs from the acute response. It should be noted, though, that this cumulative effect of increased REM

sleep was not observed in another study of 10 consecutive days of social defeat in mice [Henderson et al., 2017]. Another sleep phenotype that has been observed during chronic stress in rodents is an increase in sleep fragmentation [Gronli et al., 2004; Henderson et al., 2017]. Finally, an interesting phenomenon that has been observed to emerge after multiple days of stress is an increase in high frequency EEG power in NREM sleep [Henderson et al., 2017; Sharma et al., 2018]. This phenotype is observed in many pathological states including insomnia and PTSD.

The mechanisms by which stress modulates sleep are unknown, but may involve both effector molecules and neural circuitry of the stress response. There is evidence the HPA axis may modulate sleep architecture. For example, mice that overexpress CRH receptor in the brain display increased baseline REM sleep compared to wild-type, indicating the REM rebounds observed in many stress protocols may be mediated by HPA axis activation [Kimura et al., 2010]. However, administration of CRH directly to the brain during REM sleep deprivation inhibited the expected REM rebound [Machado et al., 2010], so the link between CRH and REM sleep is still unclear. Interestingly, though, daily administration of corticosterone to rats for 14 or 21 days (but not 7 days), resulted in increased REM sleep and shortened REM sleep latency [Cui et al., 2018]. Brain regions such as the amygdala and PFC that are activated during the stress response have also been implicated in sleep regulation via lesion and activation studies [Sanford et al., 2002; Sanford et al., 2015], so sleep may be influenced by direct synaptic stimulation/inhibition as well.

Overall, the changes in sleep post-stress are diverse and dependent of the prior stress, but in general are thought to represent adaptive coping mechanism in the acute setting [Suchecki et al., 2012; Sanford et al., 2015]. It follows, then, that impairments in that post-stress sleep may inhibit some of the coping mechanisms at play and result in vulnerability. Indeed, lines of stress-vulnerable mice show differential sleep changes after stress [Fenzl et al., 2011]. The role of disturbed sleep and stress vulnerability will be discussed in detail in Section I.B.4.

3. Sleep in Stress-Related Psychiatric Disorders

The link between stress responses and sleep is clear, and, as would be expected, there is also a role for sleep in stress-related psychiatric disorders. Conditions such as MDD, anxiety disorders, and PTSD are all characterized by sleep disturbances. Whether these disturbances are part of the pathogenesis of the disease, are a symptom of different underlying disease mechanisms, or a combination of the two, is unclear. This section will describe the sleep architecture changes typically seen in stress-related psychiatric disorders (see **Figure 1.6**).

Major Depressive Disorder

Major depressive disorder affects up to 16% of Americans at some point during their lifetimes, and in any given year can impact 7% [Kessler et al., 2003]. Depression is also tightly linked with chronic stress [Belmaker and Agam, 2008]. One of the most commonly reported symptoms of depression is sleep disruption, with 80% of patients reporting insomnia and 15-35% reporting

hypersomnia [Hawkins et al., 1985; Steiger and Pawlowski, 2019]. Objective changes in sleep architecture, particularly REM sleep, have been reported as an objective marker for depression for nearly 50 years, when researchers observed a decreased REM sleep latency in patients with depression [Kupfer and Foster, 1972]. Since that time, many studies have replicated the general finding of increased REM sleep in patients with depression, and two large-scale meta-analyses have concluded that increased REM sleep density is a hallmark of disease and is sometimes even seen in remission [Pillai et al., 2011; Baglioni et al., 2016]. Another commonly observed change to sleep architecture include impaired continuity and fragmentation [Baglioni et al., 2016; Steiger and Pawlowski, 2019]. Interestingly, reductions in slow-wave sleep amount and delta power during sleep have been frequently observed as well, and this feature may also persist during remission, being labelled a biological scar of the disorder [Pillai et al., 2011; Steiger and Pawlowski, 2019]. However, one meta-analysis did not observe decreased SWS in patients with affective disorders (though they found other evidence of decreased sleep depth), perhaps because they excluded EEG findings from the first night of observation in their analysis [Baglioni et al., 2016].

Anxiety

The 5th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) lists 10 different conditions under the "Disorders of Anxiety" category. Anxiety disorders have been considered the most frequently occurring category of mental disorder in the general population, with a 10-25% lifetime prevalence [Staner, 2003]. While the feeling of anxiety is a common part

of the acute stress response, persistent anxiety or inappropriate anxiety can be crippling and is considered a sign of dysregulated stress physiology. Furthermore, sleep complaints are common in anxiety disorders [Breslau et al., 1996]. There are fewer studies studying objective EEG sleep parameters in anxiety disorders than in depression or PTSD, but those that have quantified sleep have found changes in sleep continuity, NREM, and REM, though the results have been inconsistent, possibly due to high rates of comorbidity with other psychiatric disorders [Staner, 2003]. Generalized anxiety disorder is mostly associated with issues with sleep continuity, including fragmentation and sleep maintenance insomnia [Monti and Monti, 2000]. Differences in NREM sleep and REM sleep are inconsistent across studies, but some show a reduction in stage 4 NREM sleep [Monti and Monti, 2000; Staner, 2003].

PTSD

PTSD affects an estimated 8 million Americans in any given year, as reported by the US Department of Veteran Affairs [2016]. PTSD, and other disorders classified together as "Trauma- and Stressor-Related Disorders" by the latest edition of the DSM V, are often comorbid with sleep disturbances as well as mood, anxiety, or substance-use disorders [Pietrzak et al., 2011], and are associated with significant disability, medical illness, and premature death [Schlenger et al., 2015]. Despite many years of research, finding successful treatments has been a challenge, with inconsistent response rates, large inter-individual variability, and little evidence of remission [Shalev et al., 2017]. The predominance of intrusive, highly stressful nightmares in PTSD initially led Ross and colleagues to propose that REM sleep disturbances were the

hallmark of PTSD [Ross et al., 1989]. Polysomnographic studies in people with PTSD conducted since then have revealed that the relationship between PTSD and sleep is a bit more complex, but some major patterns have emerged.

Due to REM sleep's involvement in vivid dreaming along with its role in stress responses and emotional memory consolidation, it is logical to hypothesize REM sleep disturbances will be seen in PTSD. Indeed, multiple studies have observed increases in REM sleep density, whether that be via increase in total REM, decreased REM sleep latency, or increased number of REM sleep bouts [Ross et al., 1994; Mellman et al., 1995; Mellman et al., 1997; Woodward et al., 2000a; Woodward et al., 2000b]. However, there have been multiple studies that have reported no changes in REM sleep in PTSD vs control [Woodward et al., 2002; de Boer et al., 2019; Wang et al., 2019a], raising doubt about REM sleep being the "hallmark" of PTSD [Germain, 2013]. A meta-analysis of studies in combat veterans as well as civilians found overall there was greater REM sleep density in PTSD patients, particularly in studies with a low rate of comorbid depression [Kobayashi et al., 2007], and this finding was replicated in a later meta-analysis [Baglioni et al., 2016]. One study that controlled for comorbid depression found a decrease in REM sleep in the early post-trauma time periods, followed by an increase in REM sleep in chronic PTSD, indicating proximity of measurement to the traumatic event may also have led to mixed results regarding PTSD and REM sleep [Mellman et al., 2014].

There are other changes to sleep architecture that are more regularly observed in PTSD. The first is increased fragmentation [Mellman et al., 1997; Woodward et al., 2000a; Germain et al., 2006; Baglioni et al., 2016; de Boer et al., 2019; Wang et al., 2019a]. Furthermore, changes in EEG spectra during sleep are seen in PTSD. An increase in high frequency oscillations during sleep is thought to be an indication of cortical hyperarousal, and has been associated with insomnia [Merica et al., 1998; Spiegelhalder et al., 2012; Fernandez-Mendoza et al., 2016], suicidal ideation [Dolsen et al., 2017]. It is also seen in PTSD [Woodward et al., 2000b; Germain et al., 2006; Sharma et al., 2018]. Furthermore, two recent studies examining sleep architecture and EEG activity in individuals with PTSD reported the combination of reduced NREM delta power along with increased frontal high frequency power in both NREM sleep [de Boer et al., 2019; Wang et al., 2019a] and REM sleep [Wang et al., 2019a] sleep.

Overall, PTSD appears to have a sleep phenotype that is characterized by increased REM, increased fragmentation, and EEG changes involving a decrease in NREM delta power and signs of cortical hyperarousal during sleep. Interestingly, a recent study found associations between genes involved in sleep disturbances and genes associated with PTSD risk [Lind et al., 2019], indicating the link between sleep changes and PTSD may have shared genetic etiologies, though this does not confirm causation and more research needs to be done.

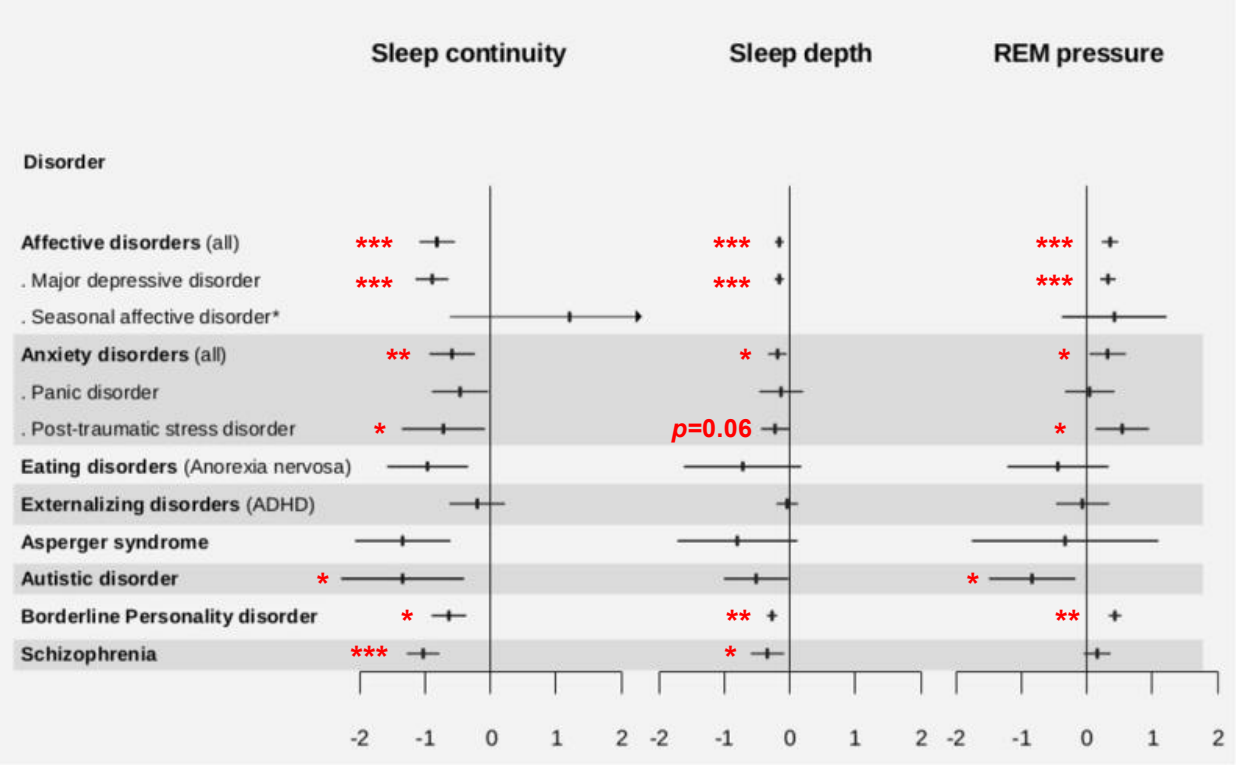


Figure 1.6 Sleep Features of Various Psychiatric Disorders. Results from a meta-analysis of 91 studies of polysomnographic findings in various psychiatric disorders performed by [Baglioni et al., 2016]. Sleep disturbances are binned into 3 categories. "Sleep continuity" was defined by higher sleep efficiency, shorter sleep onset latency, and reduced number of awakenings. "Sleep depth" was defined by shorter duration of stage 1 sleep, and longer duration of stage 2 and slow wave sleep. "REM pressure" was defined by shorter REM sleep latency, increased REM sleep density, and longer duration of REM sleep. Asterisks indicate findings that were significant, with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Adapted from [Baglioni et al., 2016].

4. Sleep Disruption as a Stress-Vulnerable State

Along with sleep being a well-documented outcome of stress related psychiatric disorders, there is growing evidence that sleep disruption may increase stress vulnerability as well. As outlined in sections above, inadequate sleep has been shown to lead to metabolic [Spiegel et al., 1999], immunologic [Irwin, 2015], and cognitive [Krause et al., 2017] deficits. These effects, along with direct changes to the HPA axis and neural circuitry involved in stress responses, have led to

the hypothesis that sleep disruption increases stress vulnerability and may be involved in the pathophysiology of stress related psychiatric disorders [Meerlo et al., 2015]. Indeed, there is now clinical and experimental evidence that this is the case, which will be discussed in this section.

Multiple studies show sleep disruption alters the HPA axis, but the exact phenotype of that change appears to be complex [Balbo et al., 2010]. Sleep disruption has been shown to increase circulating and salivary cortisol levels, but that may depend on the duration and intensity of the disruption. For instance, one night of sleep deprivation increases saliva cortisol levels [Leproult et al., 1997; Schwarz et al., 2018], while 7 days of short (6 hours/night) sleep did not change cortisol levels [Pejovic et al., 2013]. However, another protocol of examining 6 days of 4 hours time in bed per day did see significant cortisol increases [Spiegel et al., 1999], and a large scale study of chronic insomnia concluded repeated short sleep increases cortisol levels [Abell et al., 2016], so the severity of the disruption may play a role. Disruption of sleep quality via sleep fragmentation for two nights led to increase in morning cortisol levels [Stamatakis and Punjabi, 2010] as well. Overall, sleep deprivation appears to activate the HPA axis, even if it is only brief and only after severe disruption.

Significant attention has been paid to examining HPA axis *reactivity* in the context of sleep disruption, as changes in stress reactivity have been associated with conditions such as PTSD and depression (albeit the character of HPA axis functionality changes have been inconsistent) [Cohen et al., 2006; Juruena et al., 2018; Dunlop and Wong, 2019; Schumacher et al., 2019]. In

general, most studies in rodents and humans have implied blunted stress-induced activation of the HPA axis after sleep deprivation, but whether this is due to blunted activation or enhanced feedback remains unclear. Self-reported low sleep quality, but not duration, correlated with reduced cortisol response to social stress in college students [Bassett et al., 2015]. Consistent with this, a laboratory study of short sleep in humans has also reported blunted ACTH responses to exogenous CRH the day after a 4-hour sleep restriction [Guyon et al., 2017], though a single night of total sleep deprivation did not alter salivary cortisol response to the same social stressor in another study [Schwarz et al., 2018]. The evidence for a blunting of the HPA axis response to stressors is more clear in longer term disruption protocols. For example, 48 hours of sleep deprivation blunted HPA response to restraint in rats [Sgoifo et al., 2006]. Also, while one day of sleep restriction (4 hours) resulted in no change in the ACTH response to acute stress, the response was blunted after 6 days of sleep disruption in rats [Novati et al., 2008]. In another rodent study, repeated sleep deprivation blunted ACTH response to restraint stress [Meerlo et al., 2002]. Furthermore, as mentioned above, variability in inflammatory status is thought to account for differences in stress vulnerability [Hodes et al., 2014]. Sleep disruption results in increased inflammation (e.g. [Vgontzas et al., 2004; Aho et al., 2013]), so has been postulated to increase risk of psychiatric disorders that are also characterized by increased inflammation [Meerlo et al., 2015; Michopoulos et al., 2017].

There are also signs in humans that sleep deprivation alters the neurological aversive stimulus response, thus potentiating vulnerability to stressors. The amygdala is important in the regulation

of behavioral, physiological, and neuroendocrine responses to emotionally salient stimuli, including stressors [Roozendaal et al., 1991; Bohus et al., 1996]. One night of SD resulted in a 60% increase in amygdala reactivity to negative images such as weapons, snakes, spiders, and mutilations. [Yoo et al., 2007a]. A similar response has been demonstrated in the lab after 5 nights of short sleep [Motomura et al., 2013], and in individuals who report regular poor sleep quality [Prather et al., 2013]. Amygdala hyperreactivity is seen in trauma and stressor related disorders such as PTSD [Rauch et al., 2000; Stevens et al., 2013] and has been proposed as a causal factor in stress related psychiatric disorders [Admon et al., 2009]. Another imaging study in humans found that sleep loss resulted in heightened activity of the insula in anticipation of aversive images [Goldstein et al., 2013], which has been seen in PTSD and anxiety disorder patients as well [Etkin and Wager, 2007].

While most of the aforementioned research has focused on circumstantial relationships between sleep deprivation and vulnerability to stress and its related psychiatric disorders, a few studies have observed sleep disruptions prior to stress do predict incidence of mood changes or psychiatric disorder. In one study, researchers assessed subjective recent sleep quality of healthy adults and examined whether preexisting sleep disturbances would predict subsequent endotoxin-mediated increase in depressed mood in a placebo-controlled and double-blinded study [Cho et al., 2016]. They found that inflammation-induced depressed mood was of significantly larger magnitude in female subjects with prior sleep disturbance, while sleep disturbances did not alter outcomes in males. In another study, a total of 1033 traumatically injured patients who were

admitted to the hospital were asked about sleep disturbance in the 2 weeks prior to the incident, and then were followed for 3 months to assess incidence of new psychiatric disorder [Bryant et al., 2010]. They found that patients who displayed sleep disturbance prior to the injury, and had no history of prior disorder, were more likely to develop any psychiatric disorder at 3 months. This held particularly strong for PTSD, agoraphobia, obsessive compulsive disorder, anxiety disorders, major depression, and substance use disorder (**Figure 1.7**). Unfortunately, professions with high rates of PTSD such as warfighters, law enforcement, and emergency workers also often find it difficult to get adequate sleep due to the nature of their jobs. A sleep deprived state, therefore, could act as the first hit in a two-hit model of trauma and stressor-related disorders [Suchecki et al., 2002]. In this model, genetic or environmental factors (chronic sleep disruption in this case) prime an individual for lasting psychological and physiological effects of a secondary traumatic event. This project studies chronic sleep disruption as a model for a stress-vulnerable state to understand the mechanisms behind stress vulnerability and to evaluate microbial-based countermeasures to improve stress robustness.

Including Prior Psychiatric Disorder (n = 898)	Psychiatric Disorder		% with Sleep Disturbance		Association with sleep disturbance	
	N	%	Diagnosis	No Diagnosis	OR	95% CI
PTSD	67	7.5	28.4	14.1	2.56**	1.46–4.94
Panic disorder	46	5.1	34.8	14.0	4.07**	2.04–8.12
Agoraphobia	95	10.5	32.6	13.0	3.40**	2.02–5.73
Social phobia	43	4.8	32.6	14.2	2.19*	1.01–4.76
OCD	21	2.3	42.9	14.4	5.05**	2.01–12.68
GAD	78	8.7	26.9	14.0	1.97*	1.08–3.58
Any anxiety disorder	181	20.2	26.5	12.3	2.47**	1.59–3.84
Major depression	147	16.3	28.6	12.5	2.81**	1.78–4.44
Substance use disorder	56	6.2	25.0	14.4	2.05*	1.01–4.24
Any psychiatric disorder	255	28.4	24.7	11.4	2.44**	1.62–3.69
Excluding Prior Psychiatric Disorder (n = 324)						
PTSD	34	10.5	18.8	9.1	2.89**	2.06–9.79
Panic disorder	16	4.9	8.3	4.3	2.17	0.65–7.24
Agoraphobia	41	12.7	27.1	10.1	3.48**	1.57–7.71
Social phobia	10	3.1	0.0	3.6	0.01	0.01–0.02
OCD	10	3.1	12.5	1.4	11.44**	2.90–45.16
GAD	29	9.0	12.5	8.3	1.07	0.34–3.36
Any anxiety disorder	72	22.2	37.5	19.6	2.50*	1.23–5.09
Major depression	50	15.4	35.4	12.0	3.98**	1.87–6.25
Substance use disorder	19	5.9	14.6	4.3	3.90**	1.31–11.59
Any psychiatric disorder	96	29.6	52.1	29.7	3.16**	1.59–4.75

Figure 1.7 Sleep Disruption in the Weeks Prior to Trauma Increases Risk of Psychiatric Disorder at 3 Months. Results from [Bryant et al., 2010]. A total of 1083 traumatically injured patients were assessed at initial hospital admission. Sleep disturbance in the 2 weeks prior to trauma was assessed using the Sleep Impairment Index, and incidence of psychiatric disorder was assessed 3 months later. Odds ratios for development of various disorders in patients with, and without prior psychiatric disorder are reported above. From the text: "For cases excluding prior psychiatric disorder, odds ratios calculated after controlling for gender, mechanism of injury, age, and injury severity score. For cases including prior psychiatric disorder, odds ratios

calculated after controlling for gender, mechanism of injury, age, injury severity score, and prior psychiatric disorder." * $p < 0.05$, ** $p < 0.01$. Reprinted from [Bryant et al., 2010].

C. The Microbiome-Gut-Brain Axis

Our intestinal tract is colonized by bacteria soon after birth, and plays host to an entire microbial ecosystem throughout the rest of our lives. It has been long understood that these microbes live parsimoniously in our intestines and play important roles in certain metabolic processes, such as heme and bile acid metabolism. However, recent technological advances in genetic sequencing and information processing have allowed scientists to begin to appreciate the scope of the complexity and diversity of the ecosystem of microbes that live in us and on us. Distinct ecosystems of bacteria live on the different environmentally-exposed surfaces of our body, including our skin, mouth, nose, vaginal tracts, and intestines. These microbiomes are highly specialized to each niche, and are incredibly diverse. This project, as much of the research to date on microbiomes, focuses on the microbes of the intestinal tract. The understanding has emerged that these microbes (collectively termed the gut microbiome) play a role in a diverse set of physiological functions outside of the gut [Gilbert et al., 2018]. This includes a bidirectional relationship between the gut microbiota and the brain, labeled the gut-brain-axis or the microbiome-gut-brain axis [Bercik, 2011; Cryan and Dinan, 2012; Foster and McVey Neufeld, 2013; Bienenstock et al., 2015; Mu et al., 2016; Molina-Torres et al., 2019]. Importantly, recent evidence has implicated the gut microbiome and host-microbe interactions in stress vulnerability and associated psychiatric disorders [Rook et al., 2014b; Leclercq et al., 2016; Lowry et al., 2016]. In this section I will characterize the gut microbiome and discuss some of the most

important findings regarding its newly appreciated role in mammalian health, emphasizing studies involving stress, psychiatric disorders, and sleep. Furthermore, I will introduce the concept of using microbial-based countermeasures to improve health in general, and improve stress robustness in particular.

1. The Gut Microbiome

Definitions

The gut microbiota consists of trillions of microbes whose genetic diversity dwarfs our own [Xiao et al., 2015] and who influence our gastrointestinal, metabolic, endocrine, immune, and nervous systems. As we learn more about the microbiome, we can ask ourselves: "How human are we?" If you consider our cells to be our "humanness", then we are less than 50% human, as it is estimated that a 70 kg man has approximately $38x10^{12}$ bacterial cells and only $30x10^{12}$ human cells (although the bacterial cells are log units smaller and only weigh ~0.2 kg) [Sender et al., 2016]. If you only count nucleated cells, we are even less human, as ~80% of the cells in our bodies are anucleate erythrocytes. If you reasonably assume that it is our genes that make us human, then we are less human still, as while humans have about 20,000 genes, surveys of mouse and human microbiomes estimate between 2-3 million non-redundant genes [Qin et al., 2010; Xiao et al., 2015]. These genes are spread among 500-1,000 different species within 6 phyla (though ~90% lie within *Firmicutes* or *Bacteriodetes*) [Turnbaugh et al., 2007; Rinninella et al., 2019], and even more subspecies and strains [Poyet et al., 2019]. They thus represent a genetic diversity and functional metabolic flexibility that is well beyond our own. We have coevolved with these microbes [Ley et al., 2008], and it should come as no surprise that they

play a role in our physiology. The community structure of the microbiome is sensitive to changes in physiology/diet/environment but its core structure is robust within an individual human [Gilbert et al., 2018], and this idea of a "personalized" microbiome may even have forensic applications [Fierer et al., 2010]. The deviation of this community structure from one seen in a "control" or "healthy" condition is often called dysbiosis. Most of the studies to date associating the microbiome with disease have simply observed dysbiosis in different disease states, though mechanistic studies are appearing. The next subsection will discuss how the microbiome is measured, and how dysbiosis is quantified.

Measuring the Gut Microbiome

There are multiple methods commonly used to measure the microbiome, as well as various approaches to statistical analysis of the data, which will be briefly summarized here. Since the large majority of the species of bacteria living in the gut are extraordinarily difficult to culture [Poyet et al., 2019], marker gene or metagenome sequencing of fecal samples are the best methods to survey the microbial community. The most common is amplification and sequencing of part of the prokaryotic 16S ribosomal RNA gene. The region of the gene that is typically used is highly variable among bacteria, and can thus be used to map taxonomy on to the groups of 150 base pair features, also called sub-operational taxonomic units (OTU). This method is useful because it is fast, affordable, and its common use allows for easy comparisons with other studies. Drawbacks include low taxonomic resolution (typically down to the genus level, but only rarely to the species level), the choice of the particular variable region of the 16 rRNA gene can

introduce bias, and information about functional gene content is more difficult to obtain [Knight et al., 2018]. However, there are tools such as Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) that use predicted taxonomy of OTU and publicly available sequences of species within that taxon to predict functional gene content of a sample [Langille et al., 2013]. Metagenomics involves sequencing all microbial genomes within a sample. This results in higher taxonomic resolution and direct inference of functional gene content within the sample. Drawbacks to shotgun metagenomics include the fact that it is relatively expensive and laborious, is liable to contamination from eukaryotic host-derived DNA, and often requires deep sequencing depths [Knight et al., 2018].

The above methods usually generate a matrix with individual counts of different taxa (or OTU if taxonomy was not assigned) for each sample. This is used to quantify metrics of community structure including beta diversity, alpha diversity, and relative abundance of different taxa. Beta diversity tells you how similar or dissimilar the overall community structure is between two samples. A pairwise distance matrix is created using one of multiple distance measures, which should be carefully chosen as it can change the results [Barwell et al., 2015; Knight et al., 2018], and is usually graphed using a method of dimensionality reduction such as principal coordinates (PCoA). Alpha diversity is the measure of the taxonomic diversity within a sample (such as overall number of OTU) and can be compared across experimental groups. The count table is generally high dimensional (hundreds of taxa), very sparse (many zeros), and compositional (all

counts within a sample add up to a certain number of total sequences observed in the sample), which greatly influences the methodology appropriate when testing for differential abundance of a single feature between two or more groups [Gloor et al., 2017; Weiss et al., 2017]. Using standard methods such as ANOVA, Student's t-test, and Spearman's correlation can result in false discovery rates as high as 90% [Mandal et al., 2015; Weiss et al., 2017]. Tools such as DESEq2 [Love et al., 2014] and ANCOM [Mandal et al., 2015] have been used to identify differentially abundant taxa with much more acceptable sensitivity and specificity [Weiss et al., 2017]. Overall, the study of the microbiome in biomedical research is still quite new, and the measurement and analytical techniques are rapidly changing and improving. It is important for researchers to be aware of the analytical methodology of these datasets, along with their caveats and limitations, as we continue to use them to draw conclusions about the microbiome and health.

2. The Gut Microbiome and Health

Links between the gut microbiome and health have been observed in a plethora of disease states by using multiple experimental approaches. Many studies simply look for differences in the microbiome in individuals with their disease state (or disease model) of interest compared to healthy controls, often concluding that "patients with X disease have dysbiosis". Other experiments study germ free mice that have been raised without gut microbes to determine the necessity of the microbiome for some physiological process. In order to demonstrate a degree of causality, others transplant the microbiome or individual microbes from one group of rodents to another, and look for transfer of phenotype or improvement of phenotype (though true causality

is difficult to prove in these experiments [Walter et al., 2020]). Some of the relevant evidence from these studies will be outlined below.

Metabolic and Immunologic Disorders

Some of the first studies to implicate an important role for the gut microbiota did so in models of metabolic disease and obesity. Individuals with obesity have different microbiomes compared to lean controls (particularly changes in the ratio of the two most common phyla: *Firmicutes* and *Bacteroidetes*) [Ley et al., 2005; Turnbaugh et al., 2006; Yun et al., 2017], and one study observed that a transfer of microbiota from human twins discordant for obesity to mice resulted in transfer of the obese phenotype [Ridaura et al., 2013]. The microbiome of obese individuals was noted to have increased capacity for energy harvest and increase insulin resistance in recipient mice. Indeed, adults with type 2 diabetes display differences in the microbiome as well [Larsen et al., 2010; Qin et al., 2012]. Mechanisms by which certain microbiomes may promote metabolic disease and obesity may include different microbially-produced metabolites, decreased gut barrier integrity that leads to systemic inflammation, or direct activation of inflammation at the mucosal immune system (reviewed by [Hur and Lee, 2015]).

One of the principal functions of the microbiome is thought to be development and regulation of immune function [Round and Mazmanian, 2009; Honda and Littman, 2016; Lazar et al., 2018]. The immune systems of germ free mice do not develop correctly, with immature lymphoid tissues, fewer intestinal lymphocytes, and reduced levels of mucosal immune effector molecules

such as antimicrobial peptides and immunoglobulin A [Macpherson and Harris, 2004; Bouskra et al., 2008; Nishida et al., 2018]. The microbiome is thought to be particularly important to promoting the balance between immune activation and immune tolerance [Honda and Littman, 2016]. For instance, regulatory T cells (T_{reg}) that produce anti-inflammatory cytokines and act as the "breaks" on the immune response [Bilate and Lafaille, 2012; Josefowicz et al., 2012] have receptors specific for many commensal species [Lathrop et al., 2011], and multiple experiments have shown that the gut microbiota are responsible for induction of stable T_{reg} lineages in the gut [Ohnmacht et al., 2015; Sefik et al., 2015]. Alterations in immune regulation in dysbiotic states has been implicated in the disease process of inflammatory conditions such as allergy [Cao et al., 2014; Arrieta et al., 2015], autoimmune disease [de Oliveira et al., 2017; Lazar et al., 2018], and inflammatory bowel disease [Nishida et al., 2018]. Thus, conditions that cause dysbiosis, particularly changes that promote proinflammatory conditions or reduce immune tolerance, could create a proinflammatory environment that may result in the pathogenesis or exacerbation of inflammatory disorders.

An interesting body of evidence is emerging that supports the role for the microbiome in pathogenesis of oral and gastrointestinal cancers [Lazar et al., 2018]. Furthermore, multiple studies have reported that patients who respond to anti programmed death receptor (PD-1) immunotherapy for melanoma had higher measures of gut microbiome alpha diversity [Gopalakrishnan et al., 2018b; Matson et al., 2018]. The impact of the microbiome on cancer and cancer therapy is outside the scope of this document, but is a promising field of research that

requires mechanistic studies to further evaluate its clinical potential (reviewed by [Gopalakrishnan et al., 2018a; Routy et al., 2018]).

Stress

There exists a bidirectional relationship between stress responses and the gut microbiome in that psychological stressors alter the composition of the microbiome, and differences in the microbiome can impact stress responses (see reviews by [Tetel et al., 2018; Molina-Torres et al., 2019]). Experiments in rodents reveal have observed that many of the stress protocols described in previous sections result in a change to the beta diversity, alpha diversity, and relative abundances of various taxa in the microbiome. For instance, stress early in life (even prenatally) has been shown to influence gut microbial composition later in life [De Palma et al., 2015; Golubeva et al., 2015]. Furthermore, a two-hour acute social stressor in mice was shown to alter the overall community structure of the microbiome, as well as change a few individual taxa (an increase in the genus *Rosburia*, and a decrease in the genus *Parabacteroides*) [Bailey et al., 2011].

There is more evidence of the impact of subacute and chronic stressors on the microbiome. Ten days of a social stressor in mice caused a change in beta diversity, along with an increase in the ratio of the phyla *Firmicutes:Bacteroidetes* ratio and reductions in the relative abundance of the genera *Lactobacillus* and *Oscillospora* [Gautam et al., 2018]. In another study, a similar protocol of chronic social defeat (10d) changed certain taxa (reduced genus *Parabacteroides*,

Oscillospira, Turicibacter, among others) [Szyszkowicz et al., 2017]. A third study of 10 days of social defeat stress found a decrease in alpha diversity over time and changes in beta diversity, but few changes in relative abundance of different taxa met statistical significance [Bharwani et al., 2016]. A study examining subchronic mild social defeat stress found significant changes to the microbiome and fecal metabolome in stressed mice, with notable decreases in bacteria from the class *Clostridia* [Aoki-Yoshida et al., 2016]. The results of these studies were similar but by no means identical, and serve as a small example of the problem of heterogeneity of findings in this field. This is why mechanistic studies, and studies that take multiple samples across time need to be performed [Poyet et al., 2019].

Studies experimentally testing the effect of different stressors on the microbiome in humans are relatively lacking, but one in military personnel found that a 4-day cross country ski march (a mixture between a physical and a psychological stressor) altered the gut microbial population and increased intestinal permeability [Karl et al., 2017]. Interestingly, individuals with increased alpha diversity and increased relative abundance of the phylum *Actinobacteria* before the march were the most resilient to the stress-induced intestinal hyperpermeability, indicating the microbes that maintain gut barrier integrity may promote resilience to stressors. Indeed, studies have shown that microbial associated molecular patterns (MAMPs) that translocate across the intestinal barrier during stress may be involved with the sterile inflammatory response [Bailey et al., 2006; Maslanik et al., 2012]. Consistent with this, the gut microbiota have been shown to have an impact on the stress response. Germ free mice have an exaggerated HPA axis response

to stress [Sudo et al., 2004; Crumeyrolle-Arias et al., 2014]. Furthermore, transfer of the microbiomes of stress-exposed mice to germ free mice resulted in a hyper-inflammatory response to infection with a gastrointestinal bacterium [Willing et al., 2011]. The ability of the microbiota to influence stress responses and inflammatory responses has led to the hypothesis that stress related psychiatric disorders that are characterized by heightened stress reactivity and increased inflammation such as depression and PTSD, which will be reviewed in the next subsection.

Stress Related Psychiatric Disorders

A host of recent evidence has established a bidirectional communication system between the central nervous system and the gut microbiota, labeled the microbiome-gut-brain axis [Cryan and Dinan, 2012; Mu et al., 2016]. Studies in germ free rodents have revealed a role for intestinal microbes in normal expression of anxiety-like behaviors, with some demonstrating reduced anxiety in germ free mice [Diaz Heijtz et al., 2011; Neufeld et al., 2011; Clarke et al., 2013] and others demonstrating increased anxiety in rats [Crumeyrolle-Arias et al., 2014] and mice [Nishino et al., 2013]. Strain differences in susceptibility to anxiety-like behavior have been proposed to explain the discrepancies between these studies [Mu et al., 2016]. There is also evidence particular microbial populations can impact host stress behavior. Transfer of the microbiota of stress prone Balb/C mice to germ free Swiss Webster mice increased anxiety-like behavior above that of normal Swiss Webster mice, and transfer of Swiss Webster microbiota to germ free Balb/C mice had an anxiolytic effect [Bercik et al., 2011a]. Thus, it is clear the

microbiome can influence stress-related behaviors. Indeed, studies in patients and rodent models of psychiatric disorders support this link further.

In major depression, multiple studies of patient populations have observed changes in the microbiome, though conflicting results exist. For instance, one study found an increase in alpha diversity along with an increase in *Bacteroidetes* and *Actinobacteria* with a reduction of *Firmicutes* in patients with MDD [Jiang et al., 2015]. Another study also found a significant shift in the microbiome in MDD patients and an increase in *Actinobacteria*, but saw a decrease in *Bacteroidetes,* and no change in *Firmicutes* [Zheng et al., 2016]. A third study found no change in alpha diversity between control subjects and subjects with MDD, along with changes in multiple genera that do not perfectly match the other two studies [Naseribafrouei et al., 2014]. Nevertheless, a role for microbes in the pathogenesis of depressive behavior has been hypothesized. In rodents, transplant of microbes from depressed human to germ free rats or mice increases anxiety like behaviors and anhedonia [Kelly et al., 2016; Zheng et al., 2016], lending support to this hypothesis. Furthermore, as outlined above the microbiome plays a significant role in inflammation and stress responses, which could be mechanisms by which the microbiome impacts vulnerability to depression [Daniels et al., 2017; Foster et al., 2017].

The aforementioned studies in human subjects may have had conflicting results due in part to different use of control groups or limited power [Foster et al., 2017]. Recently, one group attempted to get around these problems by utilizing advanced computational techniques to

survey the microbiomes of thousands of people and associate them with quality of life depression measures [Valles-Colomer et al., 2019]. They found certain taxa (*Faecalibacterium* and *Coprococcus*) were consistently associated with higher quality of life and were reduced in people with depression. Overall, the microbiota is altered in patients with MDD, though more research and further mechanistic studies are needed to help determine whether the microbiome can be a therapeutic target for depression.

Based on the evidence linking the microbiome to the HPA axis, inflammation, and stress-related behaviors, the hypothesis that the microbiome is involved in the pathogenesis of PTSD has been proposed [Leclercq et al., 2016]. Indeed, there is evidence the microbiomes of PTSD patients show some differences compared to controls. A recent study examined the gut microbiomes of PTSD patients versus controls in the setting of liver cirrhosis, and found reduced alpha diversity in PTSD groups, along with increases in proinflammatory genera *Enterococcus* and *Escherichia/Shigella*, and decreases in the families *Lachnospiraceaeae* and *Ruminococcaceae* [Bajaj et al., 2019]. Another compared of DSM V PTSD diagnosed patients to trauma exposed controls without PTSD and found no differences in alpha or beta diversity between the groups [Hemmings et al., 2017]. However, they did find that the combination of the relatively low abundance phyla *Actinobacteria*, *Lentisphaerae*, and *Verrucomicrobia* were lower in PTSD patients and correlated negatively with PTSD symptom severity [Hemmings et al., 2017]. Interestingly, *Actinobacteria* pre-stress were also correlated with increased resilience to a 4-day cross country ski march in a study of military personnel [Karl et al., 2017]. Together, these data

suggest the microbiome may play a role in stress related psychiatric disorders like MDD and PTSD, possibly via modulation of stress reactivity.

3. Sleep and the Gut Microbiome

Many of the pathological states that arise from sleep disruption also occur in conjunction with gut dysbiosis. This includes metabolic disease [Larsen et al., 2010; Vijay-Kumar et al., 2010; Ridaura et al., 2013] and cognitive impairment [Bercik et al., 2011a; Cryan and Dinan, 2012] as well as other proinflammatory and neuro-behavioral disorders such as multiple sclerosis [Colpitts et al., 2017], depression [Daniels et al., 2017], anxiety [Bercik et al., 2011a; Crumeyrolle-Arias et al., 2014], and posttraumatic stress disorder [Hemmings et al., 2017] (see previous sections for a more thorough discussion). This has led to the hypothesis that there is a relationship between inadequate sleep and the gut microbiome. Only a small number of studies have directly tested this hypothesis using heterogeneous sleep disruption protocols, and have found variable results. Studies in short term sleep loss have revealed a subtle, but detectable, change in the microbiome. A study of acute sleep loss in mice (5-hour sleep deprivation) found no changes in alpha or beta diversity, and only slight alterations in the relative abundances of some taxa [El Aidy et al., 2019]. Two nights of short sleep opportunity (4.25 hours per night) in humans resulted in a more substantial effect, with no overall changes in beta diversity but an increase in the *Firmicutes:Bacteroidetes* ratio, along with increases in the families *Coriobacteriaceae* and *Erysipelotrichaceae* and a decrease in *Tenericutes* [Benedict et al., 2016]. Repeated sleep fragmentation (4 weeks) in mice, on the other hand, led to overall changes in beta

diversity with increases in the families *Lachnospiraceae* and *Ruminococcaceae* and a decrease in *Lactobacillaceae* [Poroyko et al., 2016]. Furthermore, glucometabolic derangements induced by the sleep fragmentation were partially replicated by transfer of a sleep-fragmented microbiome to a germ free mouse [Poroyko et al., 2016]. Conversely, one study studying sleep disruption (4 hour sleep opportunity per day) for 7 days in rats and 5 days in humans found no changes to the gut microbiome [Zhang et al., 2017]. These discrepancies may involve differences in control conditions, or dominant individual differences within humans (see **Chapter II** for further discussion).

Outside the context of sleep disruption, there is evidence for a role in the microbiota in normal physiological sleep. A recent study found an association between alpha diversity and actigraphy-assessed sleep quality in humans [Smith et al., 2019b]. Similarly, links between multiple taxa, including genus *Odoribacter*, and different sleep phenotypes were found in a recent study in mice using a systems genetic analysis approach [Bubier et al., 2020]. Multiple probiotic and prebiotic studies show individual microbes and microbial modifying diets can impact sleep as well (see **Section I.C.5** for further discussion of these studies).

While the results of the studies outlined in the previous few sections are heterogeneous, one could say a preponderance of the evidence supports the idea that changes such as an increase in the *Firmicutes:Bacteroidetes* ratio, reductions in *Actinobacteria*, and deviations in alpha diversity appear to be caused by stress and sleep disruption and are also prominent in stress

vulnerable states. The inability to find a definitive "vulnerable" microbiome may have to do with the variability in the microbiota from site to site, along with many other factors that make inter-study replicability difficult in microbiome research [Franklin and Ericsson, 2017; Schloss, 2018; Walter et al., 2020]. Perhaps, since the physiological outcomes of microbiome deviations are much more replicable than the deviations themselves, there may be convergence in the mechanisms by which the microbes affect physiology irrespective of their actual taxonomic classification. This is why it is important to assess different measures of the function of the microbiome alongside the content of the microbiome.

4. The Fecal Metabolome as a Mechanistic Link Between the Microbiota and Physiology

Despite the mounting evidence supporting an important role for the gut microbiota in normal physiology, the mechanism by which they influence the host are still unclear. Proposed mechanisms include direct interactions with the enteric nervous system [Ray, 2015], interactions with toll-like receptors in the intestinal epithelium [Mukherji et al., 2013], regulation of the immune system [Lowry et al., 2016], and signaling of microbially-modified metabolites including those originating from food sources and host bile acids [Furusawa et al., 2013; De Vadder et al., 2014; Kuipers et al., 2014; Govindarajan et al., 2016; Stilling et al., 2016; Yanguas-Casas et al., 2017]. In fact, the fecal metabolome has been proposed to be a functional readout of the microbiome, as a study of 786 individuals from a population-based twin study found that the fecal metabolome was only modestly influenced by host genetics (18% heritability) but explained a significant proportion of the variance in the host microbiome

(~68%) [Zierer et al., 2018]. Another multi-omic study of human fecal samples also found that the fecal metabolome changed over time with the microbiome and, like the microbiome, is highly individualized [Poyet et al., 2019]. Interestingly, this study found that fecal amino acid variability best explained the within-individual variance over time, while fecal bile acid composition best explained the inter-individual variance [Poyet et al., 2019].

Microbially produced short chain fatty acids (SCFA) such as butyrate and propionate have been shown to have strong impacts on host metabolism, immunity, and even behavior. Butyrate has been shown to be a promoter of gut mucosal immunoregulation, potentially via a combination of histone deacetylase inhibition and free fatty acid receptor 2 (FFAR2) receptor activation [Furusawa et al., 2013; Smith et al., 2013]. Multiple studies have shown systemic administration of butyrate to have antidepressant/anxiolytic effects in rodents [Han et al., 2014; Wei et al., 2014], but it is worth noting that these paradigms induced a supraphysiological serum concentration of butyrate [Stilling et al., 2016].

Another class of microbially derived molecules that have the potential to influence host peripheral and central physiology are the bile acids. Primary bile acids are cholesterol derivatives that are synthesized in the mammalian liver and excreted into the intestinal lumen to aid in lipid emulsification and absorption. In the intestine, primary bile acids are dehydroxylated and deconjugated by the gut microbiota, creating secondary bile acids and greatly enhancing the diversity of the bile acid pool [Thomas et al., 2008]. Mounting evidence in the past decade has

described bile acids as versatile signaling molecules, with receptors throughout the mammalian organism [Thomas et al., 2008; Kuipers et al., 2014; Mertens et al., 2017]. Signaling through the bile acid receptors of farnesoid X receptor (FXR) and Takeda G protein-coupled receptor 5 (TGR5), some bile acids can act as anti-inflammatory and immunoregulatory agents in the intestinal tract and the central nervous system [Vavassori et al., 2009; Yanguas-Casas et al., 2017]. These receptors, along with both conjugated and unconjugated bile acids, are found in the mammalian brain [Mano et al., 2004; McMillin and DeMorrow, 2016; McMillin et al., 2016; Yanguas-Casas et al., 2017], but their role in normal neuronal signaling is still unclear [Mertens et al., 2017]. Bile acids may also impact brain function indirectly via intermediate signaling molecules like fibroblast growth factor (FGF) 15/19 [Marcelin et al., 2014] or glucagon like peptide (GLP)-1 [Ullmer et al., 2013; Punjabi et al., 2014]. The role of the bile acid-brain axis in health and disease is an exciting new field that has the potential to reveal mechanistic links between the gut microbiota and host physiology. Furthermore, studies investigating the microbiome's impact on physiology should also investigate the metabolome to glean possible mechanistic insights.

5. Microbiome-Based Countermeasures to Improve Health

As our knowledge of the role of non-pathogenic microorganisms in our physiology has ballooned in the past two decades, multiple approaches to utilize these microbes to improve health have been developed. The first is the use of probiotics. A probiotic is defined as a live bacteria deliberately introduced to the animal to produce a therapeutic effect, and it is usually

delivered orally [Butel, 2014; Gilbert et al., 2018]. In order to be considered a probiotic, the bacteria must be able to survive the physiological stresses that are associated with transit through the esophagus and stomach into the intestine and must confer some physiological benefit to the host [Butel, 2014]. An overlapping but slightly different approach is the use of immunomodulatory bacteria to change the immune system and confer health benefits [Rook et al., 2014b; Rook et al., 2014a; Lowry et al., 2016]. This may consist of intranasal, oral, or subcutaneous administration of live or dead bacteria. The concept revolves around exposure to specific antigens on the surface of the bacterium that causes an increase in anti-inflammatory tone in the immune system. The third approach that will be described in this section is the use of prebiotics. Prebiotics are dietary molecules (usually fibers and oligosaccharides) that are not metabolized by mammalian enzymes but are utilized by the commensal microbes to alter the composition of the gut microbiome with subsequent benefit to host physiology [Gibson and Roberfroid, 1995; Manning and Gibson, 2004; do Carmo et al., 2016]. This approach is useful as it alters the microbiome at the community level, which avoids many of the complex and unpredictable effects introducing a single species via probiotic supplementation can have. Some of the evidence of the benefits of these microbiome-based countermeasures will be described below, with an emphasis on studies relating to sleep, stress, and psychiatric disorders.

Probiotics

There has now been a substantial number of studies examining various probiotics in the context of gut [Wilkins and Sequoia, 2017], metabolic [Yoo and Kim, 2016], immune [de Oliveira et al.,

2017], and neurological [Fond et al., 2020; Sivamaruthi et al., 2020] health. Experiments using probiotics to impact stress behaviors, inflammation, and sleep have been essential to the establishment of the concept of the microbiome-gut-brain axis and have resulted in multiple clinical trials for probiotics as treatments for mental health disorders.

Probiotics have been investigated in various stress models [Molina-Torres et al., 2019]. Oral administration of *Lactobacillus rhamnosus* for 28 days prevented some, but not all, behavioral and inflammatory effects of chronic social defeat in mice [Bharwani et al., 2017]. Consistent with this, other studies have shown that supplementation with *Lactobacillus rhamnosus* and *Bifidobacterium longum* have been shown to be anxiolytic in rodents [Bercik et al., 2011b; Bravo et al., 2011] and have positive psychological effects in humans [Messaoudi et al., 2011; Steenbergen et al., 2015]. *Lactobacillus* and *Bifidobacterium* species have also been shown to induce T_{reg} populations and improve outcomes in a model of autoimmune encephalomyelitis [Lavasani et al., 2010] and a chronic social defeat protocol [Bharwani et al., 2017], which may be the mechanism by which they reduce anxiety-like behavior after stress. Probiotic species may also reduce inflammation by preventing stress-induced increases in intestinal permeability [Ait-Belgnaoui et al., 2012; Bron et al., 2017].

As stress and inflammation are known to impact sleep, a few studies have examined the impact of probiotics on sleep. Four weeks of dietary supplementation of a heat-killed strain of *Lactobacillus brevis* in mice did not change 24-hour totals of sleep, but increased the diurnal

amplitude of the sleep rhythm, which is thought to be adaptive [Miyazaki et al., 2014]. Another species of *Lactobacillus, L. casei* strain Shirota, was administered to 4th year medical students during stressful exam preparation and was found to increase sleep length and NREM delta power early in the night, and prevent stress-induced increases in sleep latency [Takada et al., 2017]. Conversely, *Lactobacillus rhamnosus* for 8 weeks in a randomized, placebo-controlled, cross over designed study healthy adults showed no impact on sleep [Kelly et al., 2017].

The aforementioned experiments have shown probiotics to be beneficial for stress, inflammation, and sleep, all factors in the pathogenesis of various stress-related disorders, leading to the hypothesis that probiotics could be beneficial in clinical treatment of these disorders. Clinical trials of various probiotics as treatment for depression and anxiety have been performed to investigate this hypothesis. While results have varied, a recent meta-analysis of 34 controlled clinical trials found small but significant effects of probiotics (mostly species of *Lactobacillus* and *Bifidobacterium*) in the treatment of depression and anxiety [Liu et al., 2019]. Thus, while there is some therapeutic potential for oral probiotics, more research is required to attempt to overcome particular hurdles such as heterogeneity of response and unclear effects after supplementation has ended.

Immunomodulatory Bacteria and the Old Friends Hypothesis

Since at least 1873, when it was observed that children of wealthy townsfolk were more likely to develop hay fever than children of rural farmers [Blackley, 1873], we have understood that the

environment plays a role in inflammatory processes, though we did not understand the mechanisms until much later. This protective "farm effect" has since been rigorously studied, and is believed to be mostly explained by the fact that children living in rural areas tend to get exposed to a much more increased microbial biodiversity [Ege et al., 2011]. Indeed, it has been observed that as a country's income increases, rates of allergy, autoimmune diseases, and inflammatory bowel diseases increase in unison [von Hertzen et al., 2011; Rook et al., 2014b]. The Old Friends hypothesis states that mammals co-evolved with a host of different microbes in our environment with which we were in daily contact. Since immune tolerance of these organisms was crucial for their and our survival, some took on a role of inducers of immunoregulatory circuits [Rook, 2010]. According to this hypothesis, one of the reasons rates of chronic inflammatory conditions rise as areas industrialize is because the built environment, and all of the cleaning that comes with it, results in a massive loss of immunomodulatory microbes, our "old friends." These microbes increase tolerance by stimulating regulatory dendritic cells, regulatory macrophages, and regulatory B cells, ultimately increasing the total number of peripheral T_{reg} cells [Rook et al., 2014b; Lowry et al., 2016; Langgartner et al., 2019]. Use of these immunomodulatory bacteria has therefore been proposed as tools to suppress autoimmunity [Dwivedi et al., 2016], and other proinflammatory medical conditions such as inflammatory bowel disease and type 1 diabetes [Rook, 2010]. Importantly, since psychiatric disorders such as PTSD [Sommershof et al., 2009] and depression [Li et al., 2010] are associated with decreased T_{reg} cell numbers and accompanying proinflammatory tone, the hypothesis that

immunomodulatory old friends could help prevent these disorders has been suggested [Rook and Lowry, 2008; Rook et al., 2014b; Lowry et al., 2016; Langgartner et al., 2019].

Studies have examined multiple old friends in the context of immune regulation, including *Bacteroides fragilis* [Round and Mazmanian, 2010], *Bifidobacterium infantis* , *Lactobacillus rhamnosus [Konieczna et al., 2015],* and *Mycobacterium vaccae*, among others [Lowry et al., 2016; Langgartner et al., 2019]. I will focus on *M. vaccae*, at it has been studied particularly in the context of stress resilience. *M. vaccae* is a non-pathogenic saprophyte and known immunomodulator [Zuany-Amorim et al., 2002] that is prevalent in the environment. It is a pseudo-commensal bacterium in that it does not colonize mammalian intestinal tracts but is present in food and drinking water and thus is regularly exposed to the mammalian immune system via the intestinal lumen. *M. vaccae*'s immunoregulatory properties and abundance in the environment make it a great example of an "old friend", and has been studied in past few years in the context of stress and stress resilience. *M. vaccae's* immunoregulatory properties were observed and found to attenuate allergic airway inflammation in mice [Zuany-Amorim et al., 2002; Hunt et al., 2005], but the observation that intradermal *M. vaccae* administration with standard chemotherapeutics improved quality of life measures in cancer patients began the investigation of *M. vaccae* in the context of stress and anxiety [O'Brien et al., 2000; O'Brien et al., 2004]. In one study in mice, subcutaneous injections with a heat-killed preparation of *M. vaccae* reduced depressive-like behavior in the forced swim test and altered serotonergic neuronal activity [Siebler et al., 2018]. Importantly, recent studies have demonstrated that

immunization with a heat-killed preparation of *M. vaccae* protects against stressor-induced changes in the gut microbiota [Reber et al., 2016], blunts stressor-induced potentiation of chemically-induced colitis [Reber et al., 2016; Amoroso et al., 2019], prevents stress-induced neuroinflammation and anxiety-like defensive behavioral responses [Frank et al., 2018; Frank et al., 2019], enhances fear extinction when given before or after fear conditioning [Hassell et al., 2019; Loupy et al., 2019], and prevents a PTSD-like syndrome in a mouse model of chronic psychosocial stress [Reber et al., 2016]. Follow-up experiments have suggested peripheral administration of *M. vaccae* may be exerting its stress-protective effects via production of T_{reg} peripherally [Reber et al., 2016] and promotion of an anti-inflammatory state in the brain that includes prevention of stress-induced microglial priming [Fonken et al., 2018; Frank et al., 2018]. The exact mechanisms of *M. vaccae's* stress protective properties are unknown, but recent work supports the hypothesis a unique anti-inflammatory triglyceride, 1,2,3-tri (Z-10-hexadecenoyl) glycerol, found on the surface of *M. vaccae,* is part of the antigen repertoire that stimulates immune tolerance pathways [Smith et al., 2019a]. Sleep, which plays a crucial role in stress responses and stress resilience, has never been studied in the context of *M. vaccae* administration.

Prebiotics

The use of dietary prebiotics is thought to impact physiology via selective utilization and growth of probiotic species (such as *Bifidobacterium* or *Lactobacillus* species) that themselves provide benefit to the host, and/or via the provision of substrates that are metabolized by the gut bacteria

into beneficial metabolites such as SCFA's. Prebiotics have been studied in the context of gastrointestinal health [Clark et al., 2012; Slavin, 2013; Goulet, 2015], metabolic and cardiovascular disorders [Yoo and Kim, 2016], autoimmune disease [Dwivedi et al., 2016], and stress-related disorders [Mika et al., 2016; Liu et al., 2019]. Prebiotics such as polydextrose (PDX), galactooligosaccharide (GOS), and fructooligosaccharides (FOS), have been shown to reduce inflammation in a mouse model of inflammatory bowel disease [Bassaganya-Riera et al., 2011], in infants with atopic dermatitis [Moro et al., 2006], and in healthy humans [Vulevic et al., 2008]. This attenuation of inflammation has also been shown to translate to inflammation-associated behavior, as GOS attenuated post-inflammatory anxiety in mice [Savignac et al., 2016]. Furthermore, prebiotics have been shown to influence stress responses as well. In a study of healthy human volunteers, 3 weeks of daily GOS reduced the cortisol awakening response [Schmidt et al., 2015]. A combination of dietary GOS, PDX, and the glycoprotein lactoferrin (LAC), when administered to juvenile rats, protected against inescapable stress-induced learned helplessness and altered serotonergic gene expression in the prefrontal cortex, indicating these diets may be modulating stress behavior via suppression of inflammation and ultimately modulation of brain circuitry [Mika et al., 2017]. Another study examined a test diet of GOS, PDX, LAC, and milk fat globule membrane in rats and found that daily consumption of the test diet reduced the effect of acute tail shock stress on diurnal rhythmicity of body temperature and enhanced REM rebound and NREM sleep consolidation in sleep after the stressor [Thompson et al., 2016; Thompson et al., 2020]. This was the first sign that a crucial stress coping process, sleep, is impacted by prebiotic diet.

Due to the stress-protective and anti-inflammatory effects of prebiotic diets in preclinical studies, along with their innocuous safety profile, it is reasonable to hypothesize that they have promise as therapeutics in stress-related psychiatric disease. A recent meta-analysis examined 7 studies of prebiotic diets in patients with depression or anxiety and found that none found significant symptom improvement in the prebiotic diet group, though there was significant variability in the specific prebiotics used, duration of treatment, and clinical measures used [Liu et al., 2019]. Though more research is needed to examine the effect of prebiotics in the clinical setting, and should be expanded to include patients with other proinflammatory psychiatric disorders like PTSD. Furthermore, no published studies in humans or rodents have investigated these diets in the context of sleep disruption, an important modulator of stress vulnerability.

Overall, the microbiome in relation to health is a burgeoning new field with potential to inform an entirely new area of therapeutics. While the nature of these datasets make interpretation of the results complex and nuanced, the fact still remains that the trillions of microbes that live in us and on us strongly influence our physiology. Continuing research to understand these relationships will fuel development of microbiome-based interventions that can improve health, such as the ones investigated in this dissertation.

D. Summary

For thousands of years sleep has been regarded as a universal, essential, yet mysterious phenomenon. The past decades of research have revealed that sleep is a dynamic, active, and fundamental physiological process that is important for metabolic, immune, and neurobehavioral functionality. It functions from the microscopic scale of proteins and metabolites to the macroscopic scale of higher level cognitive function. It should be of no surprise then that sleep disruption has a wide ranging set of health consequences, causing an estimated $411 billion in economic losses per year due to disease burden, lost productivity, accidents, etc. [Hafner et al., 2017]. Among these consequences are dysregulated metabolic processes, reduced memory and cognitive capacity, and increased proinflammatory state. These effects together may result increased vulnerability to stressors and the host of disease states associated with a dysregulated stress response. Unfortunately, loss of stress resilience poses a particularly potent threat to members of society whose professions involve high rates of acute stressor exposure (warfighters, emergency responders, etc.) because these jobs are often also associated with sleep disruptions. Therefore, identification of markers of stress vulnerability in the context of repeated sleep disruption, and investigation into potential novel countermeasures to improve stress resilience in this context, is warranted.

The gut microbiome consists of trillions of genetically diverse microorganisms which have coevolved with their hosts to influence host physiology. Research in the last two decades has revealed the microbiome influences host metabolism, immune function, and neurobiology, the

latter via a bidirectional relationship termed the microbiome-gut-brain axis. These microbes may directly interact with these systems, and/or may be doing so via the library of microbially-modified metabolites they produce, which is why it is important to assess the fecal metabolome in parallel with the microbiome. Researchers and clinicians have begun to take advantage of these complex microbe-host interactions to engineer microbiome-based therapies to improve health. Approaches to do this include feeding of live, probiotic species, modulating immune responses with immunoregulatory bacteria such as *M. vaccae*, and providing substrates to promote growth of probiotic species as well as generation of helpful microbial metabolites using prebiotic diets. Multiple studies have found that sleep disruption changes the microbiome, features of the microbiome may influence stress vulnerability, and different microbiome-based interventions can improve stress resilience in some contexts. Thus, the gut microbiota, and the metabolites they produce, may be mechanistic links between sleep disruption and stress vulnerability. Within this framework, microbiome-based countermeasures such as immunomodulatory bacteria and prebiotic diets provide an excellent opportunity to promote resilience to sleep disruption and acute stressors.

1. Scope and Goals of this Project

The project described in this dissertation hypothesizes that repeated sleep disruption increases vulnerability to lasting physiological effects of an acute stressor and that one of the mechanisms by which this occurs is via sleep disruption-induced changes to the gut microbiome and metabolome (**Figure 1.8a**). Furthermore, it hypothesizes that microbiome-based

countermeasures such as *M. vaccae* immunization and dietary prebiotics can improve stress robustness in the context of sleep disruption (**Figure 1.8b**). The forthcoming chapters will attempt to test these hypotheses by addressing the following experimental questions: Chapter II - Does 5 days of repeated severe sleep disruption impact the fecal microbiome and the fecal metabolome in mice? Chapter III - Does this 5-day sleep disruption protocol increase the vulnerability to a "second hit" of an acute social defeat stressor? If so, what sleep and physiological markers predict subsequent vulnerability? Does preimmunization with the immunomodulatory bacterium *M. vaccae* NCTC11659 prevent sleep any disruption-induced stress vulnerability? Chapter IV – What changes in the fecal microbiome, fecal metabolome, and serum metabolome were induced by the double hit of sleep disruption plus acute social defeat in the mice from Chapter III? Were these different in *M. vaccae*-treated mice? Chapter V – What impact does a prebiotic diet containing GOS and PDX have on the fecal microbiome, fecal metabolome, and serum metabolome in rats? Does this diet alter sleep? Does this diet improve resilience to sleep disruption, acute social defeat, or both? Chapter VI – Is dietary supplementation with any microbially-derived metabolites discovered in Chapters III-V sufficient to influence sleep or promote stress resilience?

Overall, the goal of these experiments is to generate new knowledge about the role of sleep disruption in stress vulnerability/resilience by approaching the problem from the angle of the newly characterized system of the microbiome. It is my hope that these results will promote the study of sleep disturbance as both a symptom of pathology and as a risk factor for future health

problems. Furthermore, I hope to add to the growing evidence base advocating clinical investigation of microbiome-based interventions to promote health and prevent disease.

2. Contributions and Overview of Data Chapters

As described in the Preface, the experiments of this dissertation operate within a multiple university research initiative (MURI) funded by the Office of Naval Research Grant N00014-15-1-2809 entitled "The microbiome and responsiveness to stress: Countermeasure strategies for improving resilience to sleep and circadian disruption." My specific contributions, along with the contributions of the host of labmates and collaborators who helped me along the way, are outlined below.

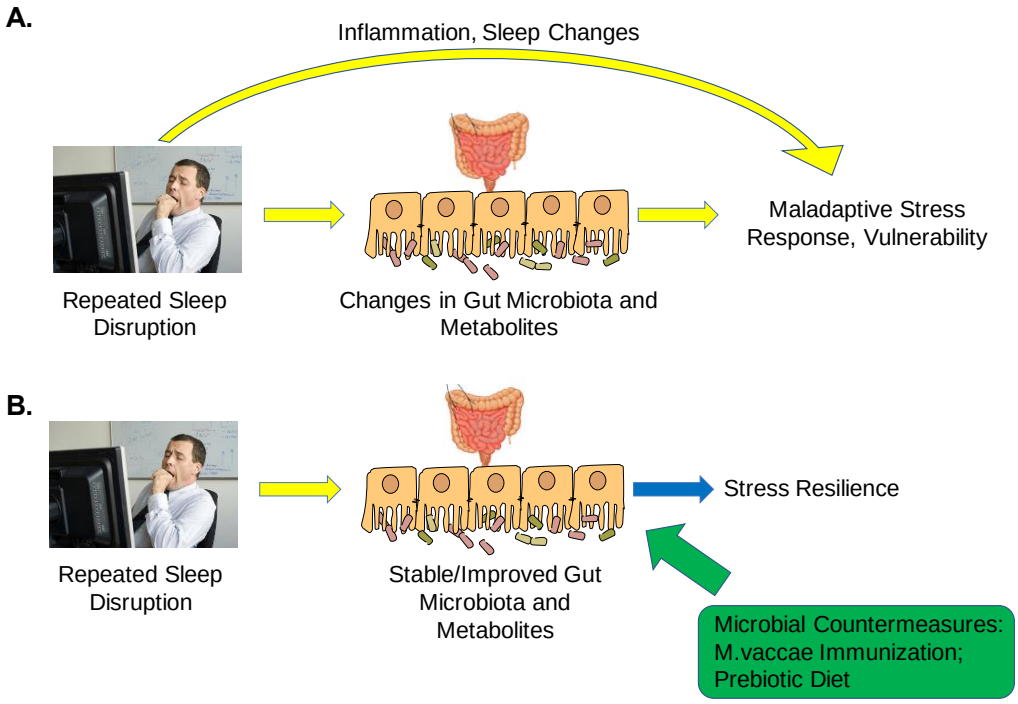


Figure 1.8: Schematic of Project Hypotheses. (A) This project operates under the hypothesis that sleep disruption increases vulnerability to secondary acute stressors ("the double hit") in part by changing the fecal microbiome and metabolome. (B) It also hypothesizes that microbiome-based countermeasures such as immunization with heat-killed *Mycobacterium vaccae* NCTC11659 and prebiotic diets can improve stress resilience.

In all of the experiments described in this dissertation, the raw data collection for the microbiome was performed by members of Dr. Rob Knight's laboratory at UCSD and led by Dr. Antonio Gonzalez. All raw data collection for the metabolomics was collected by Dr. Fernando Vargas, then a graduate student in Dr. Pieter Dorrestein's laboratory at UCSD. They would receive samples from Northwestern, generate raw data tables, and send them back to me to perform secondary analyses and integration into other physiological datasets. I learned how to interpret these raw data tables, and learned about how to analyze them, from Dr. Gonzalez and Dr. Vargas, which is reflected in the authorship of publications resulting from this work.

Chapter II describes the impact of a 5-day sleep disruption protocol on the fecal microbiome and fecal metabolome in mice. The animals were run through this sleep disruption protocol and had fecal samples collected just before I arrived in the laboratory by laboratory members Chris Olker, Shannon He, and Eun Joo Song. I then prepared the samples for microbiome and metabolomics data collection that was performed by the Knight laboratory and Dorrestein laboratory as outlined above. I performed subsequent analyses, with help from Dr. Peng Jiang from the Turek laboratory , who performed the PICRUSt2 analysis and returned raw data tables to me. This chapter is a modified version of a 2020 publication in *PLoS One* (see citation below). It includes extended introduction and discussion to put the experiment into the context of the

overarching goals of the project, and incorporates supplemental figures/tables into the main body of the text. Some large supplemental tables from the publication are included as .csv files as Supplemental Files. See below for citation and author contributions:

Bowers SJ, Vargas F, González A, He S, Jiang P, Dorrestein PC, Knight R, Wright KP Jr, Lowry CA, Fleshner M, Vitaterna MH, Turek FW. Repeated sleep disruption in mice leads to persistent shifts in the fecal microbiome and metabolome. PLoS One. 2020 Feb 20;15(2):e0229001. doi: 10.1371/journal.pone.0229001. eCollection 2020. PubMed PMID: 32078624; PubMed Central PMCID: PMC7032712.

Author Contributions

Conceptualization: Samuel J. Bowers, Pieter C. Dorrestein, Rob Knight, Kenneth P. Wright, Jr, Christopher A. Lowry, Monika Fleshner, Martha H. Vitaterna, Fred W. Turek.

Data curation: Samuel J. Bowers, Fernando Vargas, Antonio González.

Formal analysis: Samuel J. Bowers, Fernando Vargas, Antonio González, Peng Jiang.

Funding acquisition: Pieter C. Dorrestein, Rob Knight, Kenneth P. Wright, Jr, Christopher A. Lowry, Monika Fleshner, Martha H. Vitaterna, Fred W. Turek.

Investigation: Samuel J. Bowers, Fernando Vargas, Antonio González, Shannon He, Peng Jiang.

Methodology: Antonio González.

Project administration: Pieter C. Dorrestein, Martha H. Vitaterna, Fred W. Turek.

Resources: Samuel J. Bowers, Fernando Vargas, Antonio González, Pieter C. Dorrestein, Rob Knight, Martha H. Vitaterna, Fred W. Turek.

Software: Samuel J. Bowers, Fernando Vargas, Antonio González, Peng Jiang.

Supervision: Pieter C. Dorrestein, Rob Knight, Kenneth P. Wright, Jr, Christopher A. Lowry, Monika Fleshner, Martha H. Vitaterna, Fred W. Turek.

Visualization: Samuel J. Bowers.

Writing – original draft: Samuel J. Bowers.

Chapter III depicts results from the first major study of the Northwestern branch of the MURI project, whereby mice were subjected to sleep disruption followed by acute social defeat stress ("the double hit") and pretreated with either *M. vaccae* or vehicle. The impact of the double hit on sleep, behavior, and immune function was investigated, along with whether or not *M. vaccae*

prevented such effects. This experiment was conceptualized by the entire team of MURI CO-I's along with myself. The exact protocols were designed by me, Dr. Vitaterna, and Dr. Turek. Data collection was performed by me, along with lab technicians Christopher Olker and Eun Joo Song, and MS student Sophia Lambert. Data analysis was performed by me, with help scoring sleep from Christopher Olker and Eun Joo Song and help scoring behavioral videos from undergraduate Shannon He. I also generated the figures and wrote the manuscript that was submitted for publication in April of 2020. That manuscript is presented in Chapter III as a modified version, with an extended introduction and discussion to place the results into context of the overarching goals of the project. Furthermore, supplemental figures were incorporated into the text body, and supplemental tables were submitted with this dissertation as Supplemental Files. See citation below.

Bowers SJ, Lambert S, He S, Lowry CA, Fleshner M, Wright KP Jr, Turek FW, Vitaterna MH. Immunization with a soil-derived, non-pathogenic bacterium *Mycobacterium vaccae* NCTC 11659 prevents the development of cortical hyperarousal and a PTSD-like sleep phenotype after sleep disruption followed by acute stress in mice. 2020. In Review.

Chapter IV depicts the microbiome and metabolome findings from the experiment in Chapter III. Fecal samples were taken throughout the experiment by me with help from Eun Joo Song, Chris Olker, Shannon He, and Sophie Lambert. These findings were not in the submitted manuscript, but will be included in a later manuscript to come after my graduation from the laboratory. The author contributions were the same as in Chapter III, with the addition of contributions to raw

data generation by the Dorrestein and Knight laboratories as described above. Christine Lee helped with annotation of the pathways and modules shotgun microbiome functional analysis. Bowers SJ, Vargas F, González A, Lee C, Dorrestein PC, Knight R, Wright KP Jr, Lowry CA, Fleshner M, Vitaterna MH, Turek FW. The gut microbiome and fecal metabolome in sleep disruption, social defeat, and the combination of the two: stabilizing effects of *Mycobacterium vaccae* NCTC11659 and the role of hyodeoxycholic acid. In preparation.

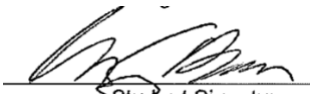
Chapter V reports unpublished findings from a study investigating the impact of a prebiotic diet on the microbiome and metabolome, sleep, behavior, and physiology in the context of sleep disruption plus acute social defeat stress in rats. This study was conceptualized by the CO-I's of the MURI team and myself, and the detailed protocols were designed by me, Dr. Turek, and Dr. Vitaterna. Data collection was performed by me, Chris Olker, and Eun Joo Song. Data were analyzed by me, with help scoring sleep from Chris Olker and Eun Joo Song. Microbiome and metabolome raw data were generated and analyzed with the help of the Knight and Dorrestein laboratories as described above. PICRUSt2 analysis was performed with the help of Dr. Peng Jiang. Object location memory behavioral testing was scored primarily by Chris Olker and an undergraduate Jenny Yim. Data were interpreted and visualized by me, and written into the document presented in Chapter V by me, which will be used as the basis for a manuscript for publication.

Bowers SJ, Vargas F, González A, Olker C, Jiang P, Dorrestein PC, Knight R, Wright KP Jr, Lowry CA, Fleshner M, Vitaterna MH, Turek FW. A prebiotic diet alters the microbiome and metabolome and promotes resilience to sleep disruption in rats. In preparation.

Chapter VI is a discussion and future directions chapter that includes some preliminary unpublished findings of a study examining the impact of dietary supplementation of the microbially modified bile acid hyodeoxycholic acid on physiology in mice exposed to the double hit of sleep disruption plus acute social defeat stress. This experiment was conceptualized and designed by me, Dr. Vitaterna, and Dr. Turek based on findings from Chapters III and IV. The data collection was performed by me, with help from Chris Olker, Eun Joo Song, and an undergraduate Christine Lee. Data were analyzed and visualized by me.

This statement confirms that Samuel J Bowers is the primary author of the first author manuscripts that are included as chapters.

Sincerely,



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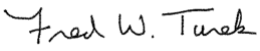

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II. CHAPTER II: REPEATED SLEEP DISRUPTION IN MICE LEADS TO PERSISTENT SHIFTS IN THE FECAL MICROBIOME AND METABOLOME

The project described in this dissertation hypothesizes that repeated sleep disruption increases vulnerability to lasting physiological effects of an acute stressor and that one of the mechanisms

by which this occurs is via sleep disruption-induced changes to the gut microbiome and metabolome. As the literature investigating sleep deprivation and the microbiome has been limited, utilizing various disruption protocols with variable results, the first goal of testing this hypothesis is to investigate whether or not sleep disruption indeed changes the microbiome, and, importantly, the molecules they produce as measured by the metabolome. This chapter introduces the sleep disruption protocol that is used throughout this dissertation, and provides an in-depth characterization of the fecal microbiome and fecal metabolome before, 2 days after, and 4 days after the disruption protocol.

A. Introduction

Inadequate sleep can lead to metabolic[Spiegel et al., 1999], immunologic[Mullington et al., 2009; Irwin, 2015], and cognitive deficits[Krause et al., 2017]. Many of the pathological states that arise from sleep disruption also occur in conjunction with gut dysbiosis, defined as a disruption of the community structure of the gut microbiome. This includes metabolic disease[Larsen et al., 2010; Vijay-Kumar et al., 2010; Ridaura et al., 2013] and cognitive impairment[Bercik et al., 2011a; Cryan and Dinan, 2012] as well as other proinflammatory and neuro-behavioral disorders such as multiple sclerosis[Colpitts et al., 2017], depression[Daniels et al., 2017], anxiety[Bercik et al., 2011a; Crumeyrolle-Arias et al., 2014], and posttraumatic stress disorder[Hemmings et al., 2017]. This has led to the hypothesis that there is a relationship between inadequate sleep and the gut microbiome. Only a small number of studies have tested this hypothesis, using heterogeneous sleep disruption protocols (e.g., acute sleep

restriction[Benedict et al., 2016; El Aidy et al., 2019], chronic sleep fragmentation[Poroyko et al., 2016]), in humans[Benedict et al., 2016; Zhang et al., 2017], mice[Poroyko et al., 2016; El Aidy et al., 2019], and rats[Zhang et al., 2017], and have yielded mixed results[Zhang et al., 2017]. More research is therefore required to explore the relationship between sleep, the gut microbiome, and potential mediators of microbe-host interactions.

Despite the mounting evidence that supports an important role for the gut microbiome in normal physiology, the mechanisms by which commensal microorganisms influence the host are still unclear. Proposed mechanisms include direct interactions with the enteric nervous system[Ray, 2015], interactions with toll-like receptors in the intestinal epithelium[Mukherji et al., 2013], regulation of the immune system[Lowry et al., 2016], and signaling of microbially-modified metabolites including those originating from food sources and host bile acids[Furusawa et al., 2013; De Vadder et al., 2014; Kuipers et al., 2014; Govindarajan et al., 2016; Stilling et al., 2016; Yanguas-Casas et al., 2017]. These metabolites serve as a functional measure of microbial activity, and the fecal metabolome closely reflects the composition of the fecal microbiome[Zierer et al., 2018]. Therefore, to understand the impacts of the microbiome on the host, it is crucial to study not only the microbes, but also to examine the molecules that they produce and that are present in their microenvironment. However, there have been no studies to date examining the effects of sleep disruption on the fecal metabolome using untargeted metabolomics.

We thus investigated the impact of a sub-chronic, five-day sleep disruption protocol on the fecal microbiome and fecal metabolome in mice. Assessment of the fecal microbiome using 16S rRNA gene amplicons and of the fecal metabolome using untargeted LC-MS/MS mass spectrometry revealed a global shift in both the microbiome and metabolome after sleep disruption, and aspects of these changes persisted through the fourth day after returning to *ad libitum* sleep. Furthermore, microbial differential abundance testing and utilization of Global Natural Products Social Molecular Networking[Wang et al., 2016] (GNPS) allowed us to identify specific taxa of bacteria and families of metabolites that change in response to five days of sleep disruption, many of which have known physiological relevance. These findings support the hypothesis that gut dysbiosis, and changes in the fecal metabolome, after sleep disruption may contribute to some of the health problems long known to be associated with inadequate sleep and that these changes may be present even after the sleep-wake state is normalized.

B. Materials and Methods

Animals

Seven-week old male C57BL/6N mice (Experiment 1, $N = 7$; Experiment 2, $N = 20$; Charles River Laboratories, USA) were used in these experiments. Mice were group-housed upon arrival for one week until surgery (Experiment 1) or until being placed into individual sleep disruption chambers (Experiment 2). Mice were maintained on a 12:12 L:D cycle at room temperature (23 °C $\pm$ 2 °C) with food and water available *ad libitum* throughout the experiment. The light source was two 14 W fluorescent tubes (soft white, 3000 K), resulting in an average light intensity of

~500 lux inside the cylindrical sleep disruption cage. Zeitgeber Time (ZT) is defined as the number of hours after the onset of the light period (light onset = ZT0). All mice were housed and handled according to the Federal Animal Welfare guidelines, and all studies were approved in advance by the Institutional Animal Care and Use Committee at Northwestern University (Assurance Number: A3283-01; Protocol Number IS00001718).

Sleep disruption protocol

The same sleep disruption protocol was used in Experiment 1 and Experiment 2. Prior to the sleep disruption protocol, all mice were transferred from their home cages into individual sleep disruption cylindrical cages. Cages had corncob bedding and food/water available *ad libitum*. Mice were allowed to acclimate to the chambers for 7 days before beginning the sleep disruption protocol. Sleep disruption was achieved using a commercially available system integrated into the chambers (Pinnacle Technology, Lawrence, KS, USA), which simulates the gentle handling technique via a rotating metal bar (22 cm in length) attached to a post at the center of the cage. For the sleep disruption period, the bar's rotation speed was set at seven rotations per minute with reversals of rotation direction (i.e., clockwise vs. counterclockwise) set to occur at semi-random intervals of 10 ± 10 seconds. The bar was programmed to rotate for 20 hours per day (ZT6-ZT2), and was stationary from ZT2-ZT6, for 5 days. Experimenters visually inspected mice at regular intervals during the sleep disruption windows to ensure that the bar mechanism was functioning properly and that the sleep-disrupted mice were awake. Control animals were placed in identical cages with bars that remained stationary throughout the experiment. At ZT2

of the fifth sleep disruption day, the motorized bars were stopped, and mice were allowed to sleep *ad libitum* for the remainder of the experiment.

Sleep recording and analysis

One week after arrival, mice for Experiment 1 were implanted with electroencephalographic/electromyographic (EEG/EMG) sleep recording devices (Pinnacle Technologies, Lawrence, KS, USA). Surgical procedures were performed using a mouse stereotaxic apparatus with standard aseptic techniques in a ventilated, specially-equipped surgical suite. Anesthesia was induced by IP injection of cocktail of ketamine HCl (98 mg/kg; Vedco Inc, St. Joseph, MO, USA) and xylazine (10 mg/kg; Akorn Inc, Lake Forest, IL, USA) before surgical implantation of a headmount, which consisted of a plastic 6-pin connector connected to four EEG electrodes and two EMG electrodes. Four stainless steel screws serving as anchors for the EEG leads and grounds were screwed into the skull with one screw located 1 mm anterior to bregma and 2 mm lateral to the central suture, and the other at 1 mm anterior to lambda and 2.5 mm lateral to the central suture. The exposed ends of two stainless steel Teflon-coated wires (0.002 in. in diameter) serving as EMG leads were then inserted into the nuchal muscles using a pair of forceps. The headmount was then sealed by dental acrylic and a single suture at the front and back of the implant was given to close the skin. Subcutaneous injection of analgesic meloxicam (2 mg/kg; Norbrook Laboratories, Newry, Northern Ireland) was given to the animals immediately after the surgery while the animals were still under anesthesia and once more on the following day.

One week after implant surgery, mice were moved into cylindrical sleep recording cages (25 cm in diameter and 20 cm tall, Pinnacle Technologies) within individual acoustically isolated chambers and the headmount was connected to the transmission tether. Cages had corncob bedding and food/water available *ad libitum*. Two days were allowed for acclimation to the tether before baseline sleep was recorded. Sleep was recorded continuously throughout the remainder of the experiment. Data were collected using Pinnacle Acquisition software (Pinnacle Technologies), then scored as non-rapid eye movement sleep (NREM), rapid eye movement sleep (REM), or Wake in 10 second epochs using machine learning-assisted sleep scoring software developed in the Turek/Vitaterna laboratory[Gao et al., 2016]. The initiation of a bout of NREM, REM, or Wake was defined by the occurrence of two consecutive epochs of NREM, REM, or Wake (respectively). A bout was terminated when a bout of another state occurred. Sleep bouts were initiated by two consecutive epochs of a sleep state (NREM or REM) and were only terminated when a wake bout occurred. The delta power band was defined as 0.5-4 Hz. Relative power was calculated as the raw power (uV^2) in a particular band divided by the total power in all bands. Power was then reported as a percent of baseline to reduce inter-individual variability.

Fecal sample collection

In Experiment 2, fecal samples were collected at 3 different timepoints: 1) after mice were transferred to sleep disruption cages but before starting sleep disruption (BL); 2) on the second

afternoon (~30 h) after the sleep disruption protocol was ended (R2); and 3) on the fourth afternoon after the sleep disruption protocol was ended (R4). At each collection, mice were placed into a clean sleep disruption chamber with fresh bedding and food and monitored closely until two fresh fecal pellets from each mouse were collected. Only spontaneously voided pellets were collected, so not every animal produced fecal pellets at every timepoint. Samples were placed into individual 1.5 mL microfuge tubes, and frozen at -80 ºC until microbiome/metabolome analysis. All fecal pellets were collected between ZT8 and ZT12.

Microbiome analysis

Microbiome data were generally analyzed using the Quantitative Insights Into Microbial Ecology 2 (QIIME2, version 2018.2) bioinformatics software package[Caporaso et al., 2010; Bolyen et al., 2019]. A total of 56 fecal samples (BL: *n* = 10/10 Control/Sleep Disruption; R2: *n* = 8/8; R4: *n* = 10/10) were processed for microbiome analyses. DNA was extracted from fecal samples and the V4 region of the 16S rRNA gene was amplified using the 515f/806rB primer pair with the barcode on the forward read[Apprill et al., 2015] and sequenced as previously described[Caporaso et al., 2012] using an Illumina MiSeq. Sequence data were processed using Deblur v1.0.2[Amir et al., 2017], trimming to 150 nucleotides to create sub-operational-taxonomic-units (sOTUs). These were then inserted into the Greengenes 13_8[McDonald et al., 2012] 99% reference tree using SATe-enabled Phylogenetic Placement (SEPP)[Mirarab et al., 2012]. SEPP uses a simultaneous alignment and tree estimation strategy[Liu et al., 2009] to identify placements for sequence fragments within an existing phylogeny and alignment.

Taxonomy was assigned using an implementation of the Ribosomal Database Project (RDP) classifier[Wang et al., 2007] as implemented in QIIME2[Caporaso et al., 2010].

The OTU feature table was filtered to remove any features present in three or fewer samples (out of the 56 original samples), and alpha and beta diversity metrics were performed at a rarefied depth of 8431 reads, resulting in the removal of five samples from the dataset (final *n* for diversity metrics: Control/Sleep disruption - BL: *n* = 8/8; R2: *n* = 8/8; R4: *n* = 10/9). Beta diversity was assessed using weighted UniFrac distance[Lozupone et al., 2011] matrices, which were used to generate PCoA plots and to perform PERMANOVA in QIIME2. Within-group distance was calculated from distance matrices by averaging the weighted UniFrac distance from an individual sample to all other samples in the same group (Control vs Sleep Disruption) at the same timepoint. Distance from baseline was calculated by averaging the distance from an individual sample at R2 or R4 to all samples in the same group at the BL timepoint. Alpha diversity metrics were calculated using scikit-bio 0.5.1 as implemented by QIIME2. To test for differentially abundant taxa between control and sleep-disrupted groups, samples with less than 8000 reads were removed (final *n* for differential abundance testing: Control/Sleep Disruption - BL: *n* = 8/8; R2: *n* = 8/8; R4: *n* = 10/9), and DESeq2 (version 1.14.1) was performed on the non-rarefied dataset at each timepoint and at each taxonomic level using the Bioconductor R package in RStudio (version 1.0.136, RStudio Inc). This was used in favor of techniques that more adequately account for the compositionality of microbiome datasets[Gloor et al., 2017] such as

Analysis of the Composition of Microbiomes (ANCOM) due to the extremely low sensitivity of ANCOM when sample size is less than 20 per group[Weiss et al., 2017].

PICRUSt2 analysis of 16S rRNA gene data

We inferred the microbial gene content from the taxa abundance using the software package Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2; https://github.com/picrust/picrust2; v2.1.4-b)[Langille et al., 2013]. This tool allows assessment of functional capacity of a microbiome using 16S rRNA sequencing data. We then used DESeq2 to identify genes that were differentially abundant between control and sleep-disrupted groups (notated with Enzyme Commission numbers).

Metabolomic analysis

A total of 56 fecal samples (BL: n = 10/10 Control/Sleep Disruption; R2: n = 8/8; R4: n = 10/10) were processed for analysis of the fecal metabolome. Fecal samples were analyzed using an ultra-high performance liquid chromatography system coupled to a quadrupole-Orbitrap mass spectrometer (Q Exactive, Thermo Scientific, Waltham, MA, USA). Chromatographic separation was accomplished using a Kinetex C18 1.7 µm, 100 Å pore size, 2.1 mm (internal diameter) x 50 mm (length) column (Phenomenx, Torrance, CA, USA). The column was maintained at 40 °C during chromatographic separation. 5.0 µL of extract was injected per sample. Mobile phase composition was (A) water with 0.1 % formic acid (v/v) and (B) acetonitrile with 0.1 % formic acid (v/v) with a flow rate of 0.5 mL/min. Chromatographic elution was performed as follows: 0.00-0.50 min, 5% B; 0.50 - 4.00 min, 50% B; 4.00 - 5.00 min, 99% B; 5.00 - 7.00 min, 99% B;

7.00 -7.10 min, 5% B; 7.10 - 9.00 min, 5% B. Positive mode electrospray ionization was performed using a heated electrospray ionization source using the following source parameters: spray voltage, 3500 V; capillary temperature, 268.75 °C; sheath gas flow rate, 52.50 (arb. units); auxiliary gas flow rate, 13.75 (arb. units); probe heater temperature, 437.50 °C; and S-lens RF level, 50 (arb. units). Mass spectrometry data were collected using data-dependent acquisition. The MS1 scan range was set to 150-1,500 *m/z* with a resolution of 17,500 at 200 *m/z*. MS2 scans of the five most abundant ions in the previous MS1 scan, acquired in a data-dependent manner, were collected at a resolution of 17,500 at 200 *m/z*. MS1/MS2 automatic gain control target and maximum ion injection time were set to 5.0 E5 and 100 ms respectively. Higher-energy collision-induced dissociation was performed with a normalized collision energy stepped from 20, 30, to 40%.

The LC/MS/MS feature table, generated using Optimus[Protsyuk et al., 2018] peak detection, was normalized to an internal standard followed by a row sum (total ion count) normalization and filtered to remove features present in less than two samples. The resulting table contained 1124 metabolites. PCoA plots were then generated using Bray-Curtis distance, and PERMANOVA was performed at each timepoint on the normalized feature table using the Vegan package (version 2.5-5) in RStudio. In order to identify metabolites that were different between sleep-disrupted and control groups, we used a multiple-method approach that included machine learning and nonparametric hypothesis testing. In order to first identify the group of metabolites that were the key drivers of differences between groups at each timepoint, Variable

Selection Using Random Forests (VSURF, version 1.0.3)[Genuer et al., 2010; Genuer et al., 2015] analysis was performed using the VSURF.R package in RStudio. Briefly, this protocol uses multiple iterations of the random forest supervised machine learning technique to isolate the most important drivers of separation between two groups by defining a threshold variable importance. Taking this list of suprathreshold features, we then performed Wilcoxon Rank Sum tests at each timepoint as a form of a 'post hoc' test to confirm differences between groups.

Features of interest were annotated using GNPS (version 1.3.0) [Wang et al., 2016], which allows MS1 and MS2 spectra to be shared between researchers, forming a large database. By matching an unknown spectrum to one or more in the database, and examining similarity to others within a molecular network, GNPS can be used to identify purported molecular structures of features from untargeted metabolomics. A molecular network was created using the online workflow at GNPS. The data were filtered by removing all MS/MS peaks within +/- 17 Da of the precursor *m/z*. MS/MS spectra were window-filtered by choosing only the top six peaks in the +/- 50 Da window throughout the spectrum. The data were then clustered with MS-Cluster with a parent mass tolerance of 0.1 Da and a MS/MS fragment ion tolerance of 0.1 Da to create consensus spectra. Further, consensus spectra that contained less than two spectra were discarded. A network was then created where edges were filtered to have a cosine score above 0.6 and more than four matched peaks. Further edges between two nodes were kept in the network if and only if each of the nodes appeared in each other's respective top ten most similar nodes. The spectra in the network were then searched against GNPS' spectral libraries. The

library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.6 and at least four matched peaks. Results can be found at https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=6fb1d63a51764c7ea75a4e7256b6936a

Individual features of interest from the feature table were then matched to nodes (clusters) in the network whose average *m/z* and RT were within 0.025 and 30 s, respectively, of the feature of interest. Features that were matched to multiple clusters using the aforementioned criteria were assigned to the cluster with the closest average *m/z* and RT.

Statistical analysis and software

All graphs depict the mean +/- SEM unless otherwise stated. All PCoA plots were generated using the EMPeror visualization tool as implemented in QIIME2[Vazquez-Baeza et al., 2013]. Microbiome data processing and analysis, including PERMANOVA, were performed in QIIME2 as outlined above. Wilcoxon Rank-Sum tests, Kruskal-Wallace tests, VSURF, DESeq2 (with Benjamini Hochberg adjustment), heatmaps, and boxplots/scatterplots were performed or generated in RStudio (version 1.0.136, RStudio Inc). Two way ANOVA and mixed-effects models with Bonferroni post hoc testing of sleep, alpha diversity, and beta diversity measures, along with generation of all other graphs/figures, was performed using GraphPad PRISM (version 8.2.1; GraphPad Inc, San Diego, CA, USA). Test statistics generated by PERMANOVA, ANOVA, and mixed-effects models are reported in **Supplemental File 2.1**.

Data Availability

Sequencing data and metadata are available on Qiita[Gonzalez et al., 2018] under study ID 10777 and on EBI-ENA with accession number EBI: ERP113564[Bowers, 2019]. The metabolomics dataset is publicly available in the MassIVE database under accession number MSV000080630 (https://massive.ucsd.edu/ProteoSAFe/dataset.jsp?task=8f3141b17a1e4b5886df0d4c515f2a16)[Vitaterna, 2019].

C. Results

The five-day sleep disruption protocol significantly reduces and fragments sleep

In Experiment 1, we performed a detailed analysis of sleep before, during, and after the sleep disruption protocol that was used in Experiment 2 (see **Methods** and **Figure 2.1a**). Compared to control animals, sleep-disrupted animals had significantly less total sleep, NREM sleep, and REM sleep per 24 hours during the protocol (**Figure 2.2a-c**, F statistics can be found in **Supplemental File 2.1**). While the amount of 24-hour NREM sleep recovered to the level of controls within the first day of recovery sleep (**Figure 2.2b**), there was a significant rebound in the amount of REM sleep on the first day of recovery sleep (**Figure 2.2c**). In order to observe sleep with greater resolution, we examined the fifth day of the sleep disruption protocol and the beginning of the first day of recovery sleep using two-hour time bins. It was evident that the majority of this 24-hour sleep loss occurred during the hours of the light period in which the motorized sleep disruption bar was moving (ZT0-ZT2 and ZT6-ZT12, **Figure 2.2e-g**). REM

sleep was reduced to nearly zero percent while the motorized bar was moving, and this resulted in strong REM rebounds during the first two hours of the *ad libitum* recovery windows (**Figure 2.2g**). Sleep disruption also resulted in more fragmented sleep. During the five days of sleep disruption, there was a significantly higher number of state changes in sleep-disrupted mice compared to controls (**Figure 2.2d,h**). Furthermore, there was an increase in the number of bouts of sleep and bouts of NREM in the sleep disruption group, accompanied by a significant decrease in the bout length, further suggesting fragmentation (**Figure 2.3**). The number of REM bouts per 24 hours was significantly decreased on days the motorized bar was on, was significantly increased on the first recovery day, but no longer significantly different from control by the second recovery day (**Figure 2.3**).

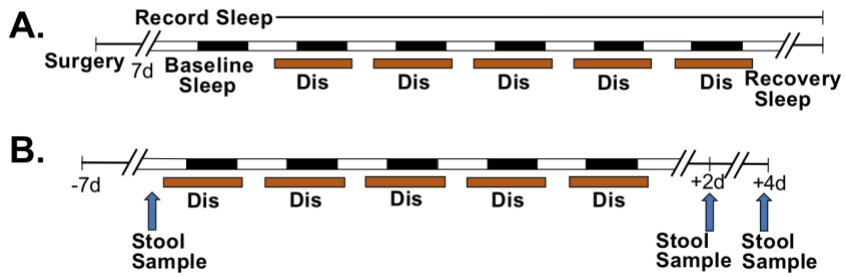

Figure 2.1: Experimental Timelines. A) Experiment 1. Adult male C57BL/6N mice (*n* = 3, Control; *n* = 4, Sleep Disruption) received surgical implants of sleep recording devices. After recovery from surgery, mice were subjected to five days of repeated sleep disruption and two days of *ad libitum* recovery sleep. Sleep was disrupted for 20 h/day, with an *ad libitum* sleep window between ZT2-ZT6. Sleep was recorded throughout the experiment. B) Experiment 2. Non-instrumented adult male C57BL/6N mice (*n* = 10/group) were subjected to the same sleep disruption protocol, but with four days of recovery sleep. Stool samples were collected before sleep disruption, on day 2 post-sleep disruption and on day 4 post-sleep disruption (arrows). Abbreviations: Dis, Sleep Disruption.

Five days of sleep disruption creates changes in the fecal microbiome that last at least four days after disruption has ended

In Experiment 2, fecal samples were collected before sleep disruption (BL), at day two post-sleep disruption (R2), and at day four post-sleep disruption (R4) (see **Methods** and **Figure 2.1b**) to assess the fecal microbiome and fecal metabolome. Beta diversity, or the difference in diversity between two or more communities, was assessed at each experimental time point with weighted UniFrac distance, which takes into account both the abundances and phylogenetic relatedness of two communities[Lozupone and Knight, 2005; Lozupone et al., 2011]. Principal coordinates analysis (PCoA) revealed no difference between control and sleep-disrupted groups at baseline, as expected (p = 0.877, PERMANOVA; **Figure 2.4a**), but significant clustering of control mice and sleep-disrupted mice indicated a global difference in community structure at R2 (p = 0.018, PERMANOVA; **Figure 2.4b**) that was gone by R4 (p = 0.663, PERMANOVA; **Figure 2.4c**). The distance from baseline, the average weighted UniFrac distance between an individual post-sleep disruption and all individuals within the same group at BL, was increased at R2 and R4 (**Figure 2.4d, Right panel**). Furthermore, sleep disruption significantly increased the dissimilarity between individuals within the sleep-disrupted group at R2 and R4 (**Figure 2.4d, Left panel**). Therefore, five days of repeated sleep disruption had a "destabilizing" effect in that it not only shifted microbial communities away from controls, it increased dissimilarity within the group, and this effect lasted at least four days after return to *ad libitum* recovery sleep.

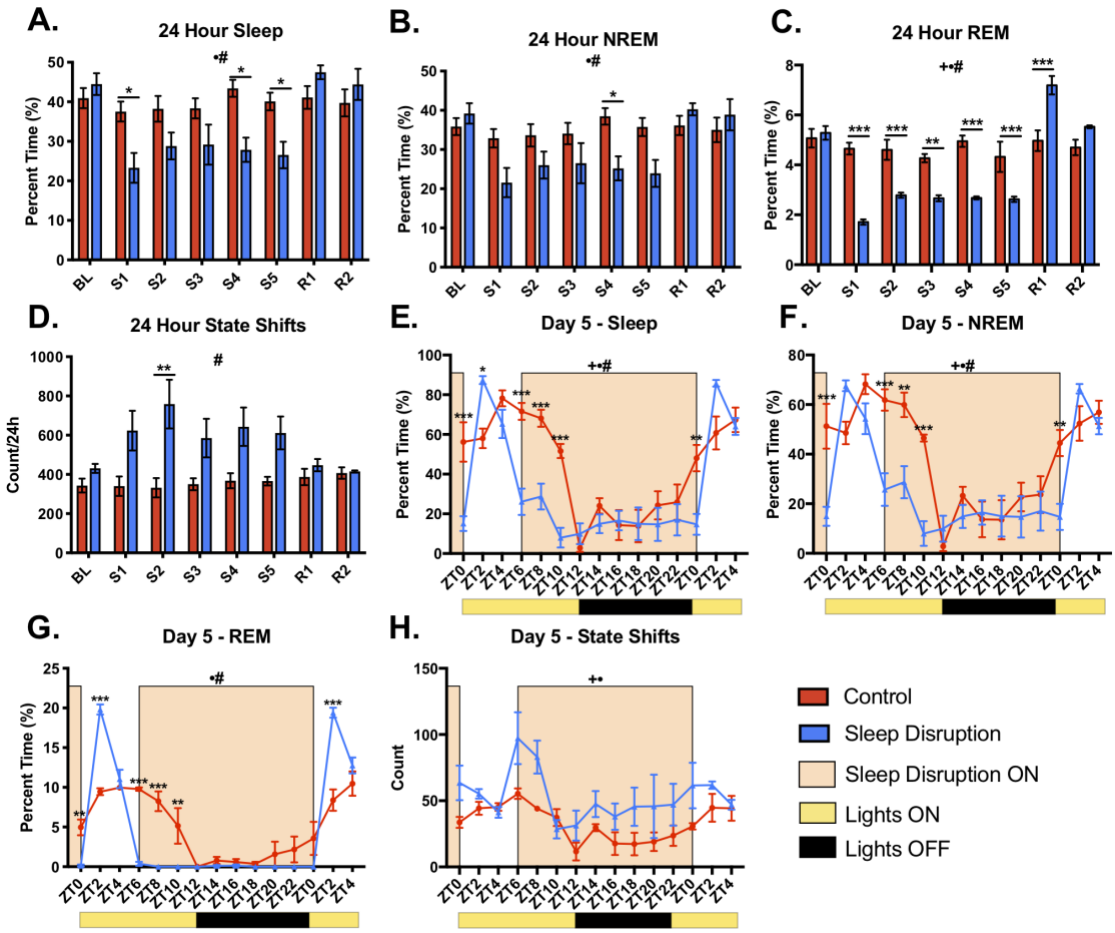

Figure 2.2: Effect of Sleep Disruption Protocol on Sleep Measures. A-D) 24-hour totals of total sleep, non-rapid eye movement sleep (NREM), rapid eye movement sleep (REM), and state shifts. There was a significant decrease in sleep, NREM, and REM during the sleep disruption protocol, and an increase in state shifts. E-H) Two-hour bins of total sleep, NREM, REM, and state shifts from the fifth day of the sleep disruption protocol through ZT4 of the first day of recovery sleep. Yellow bars under the x axis indicate the lights being on, while black bars indicate the lights being off. Abbreviations: BL, baseline; S, sleep disruption; R, recovery; ZT, zeitgeber time. n = 3-4/group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Bonferroni post hoc test); +$p < 0.05$ (overall effect of sleep disruption over entire time interval, Mixed-effects model); •$p<0.05$ (overall effect of Time over entire time interval, Mixed-effects model); #$p < 0.05$ (Sleep Disruption x Time interaction over entire time interval, Mixed-effects model).

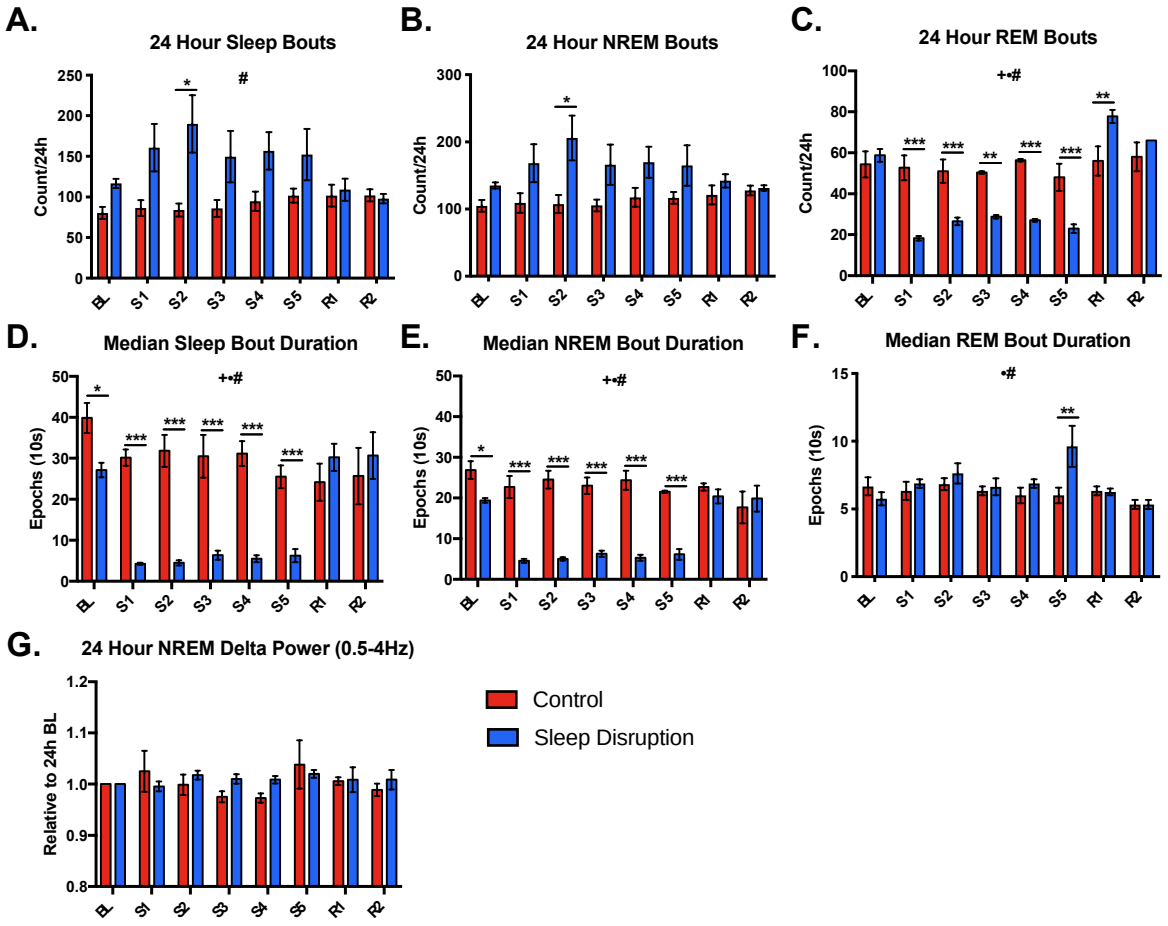

Figure 2.3: Effect of Sleep Disruption Protocol on Sleep Fragmentation Measures and Delta Power. A,B) There was a significant increase in the 24-hour totals of total sleep bouts (A) and non-rapid eye movement sleep (NREM) bouts (B) during the sleep disruption protocol in the sleep disruption group. C) Rapid eye movement (REM) bouts were decreased during the sleep disruption protocol, and increased on the first day of recovery in the sleep-disrupted group. D,E,F) Median sleep bout duration (D) and NREM bout duration (E) were significantly decreased in the sleep-disrupted group during the protocol, while the median REM bout duration (F) was unaffected in all days except for on S5. G) There was no change in 24-hour NREM delta power due to sleep disruption. Abbreviations: BL, baseline; S, sleep disruption; R, recovery; ZT, zeitgeber time. n = 3-4/group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Bonferroni post hoc test); +$p < 0.05$ (overall effect of sleep disruption, Mixed-effects model); •$p < 0.05$ (overall effect of Time, Mixed-effects model); #$p < 0.05$ (Sleep DisruptionxTime interaction, Mixed-effects model).

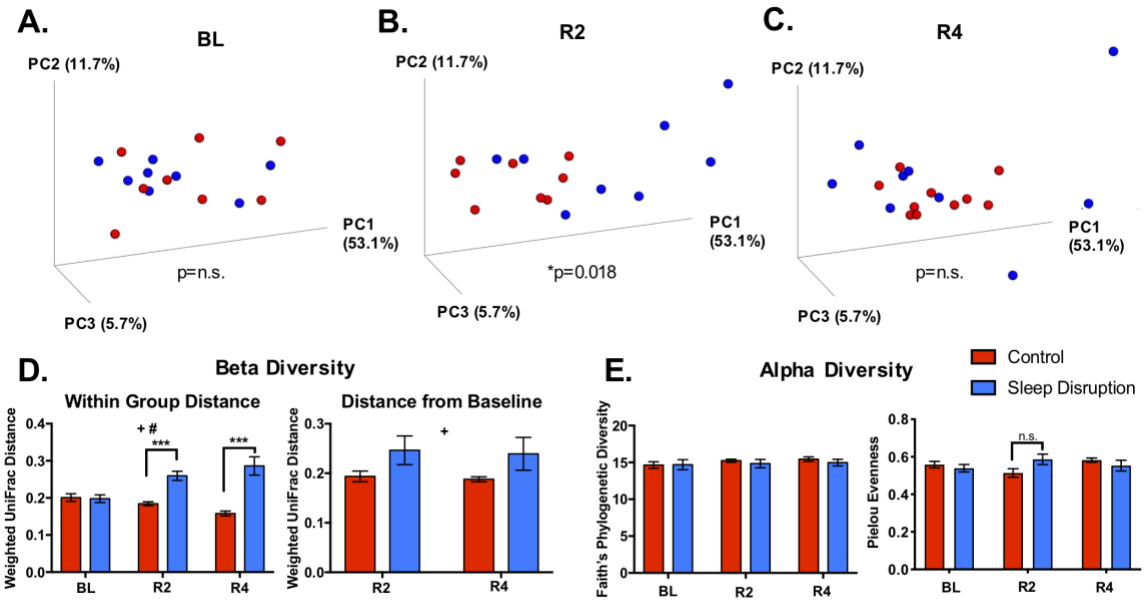

Figure 2.4: Effect of Sleep Disruption on Microbiome Beta and Alpha Diversity. A-C) Principal coordinates analysis (PCoA) plots using weighted UniFrac distance. A significant difference between sleep disruption and control groups at day 2 post-sleep disruption was detected using PERMANOVA. D) Average weighted UniFrac distance from an individual to all individuals within the same group (left) and from an individual post-sleep disruption to each individual pre-sleep disruption (right) is increased at both day 2 and day 4 post-sleep disruption. E) Faith's Phylogenetic Diversity (left) and Pielou Evenness (right) were unchanged throughout the experiment. Abbreviations: BL, baseline; R2, day 2 post-sleep disruption; R4, day 4 post-sleep disruption. n = 8-10/group. *$p < 0.05$ (PERMANOVA); **$p < 0.01$, ***$p < 0.001$ (Bonferroni post hoc test); +$p < 0.05$ (Overall effect of Sleep Disruption, Mixed-effects model); #$p < 0.05$ (Sleep Disruption x Time interaction, Mixed-effects model).

Multiple measures of alpha diversity, the microbial diversity within an individual community, were also examined because reductions in alpha diversity have been associated with pathological states such as inflammatory bowel syndrome[Codling et al., 2010], chronic stress[Reber et al., 2016], and obesity[Yun et al., 2017]. Faith's phylogenetic diversity index was not affected by sleep disruption (**Figure 2.4e, Left**), consistent with results in different sleep disruption

models[Poroyko et al., 2016]. Pielou evenness[Pielou, 1969] was also unaffected by sleep disruption (**Figure 2.4e, Right**).

Multiple bacterial taxa are differentially abundant in the sleep-disrupted group

We tested for differential abundance between control and sleep disruption groups at each taxonomic level, at each timepoint. Of the 142 originally identified taxa (includes all levels), 0, 16, and 6 were significantly different at the BL, R2, and R4 timepoints, respectively (FDR < 0.1, **Figure 2.5a**; **Table 2.1**). The ratio of the two most prevalent phyla in the mammalian gut, the *Firmicutes*:*Bacteroidetes* (F:B) ratio, is a blunt measure of community shift. An increase in the F:B ratio has been seen in obesity[Ley et al., 2005; Koliada et al., 2017], stress[Gautam et al., 2018], as well as models of acute[Benedict et al., 2016] and chronic[Poroyko et al., 2016] sleep disruption. We found a significant sleep disruption-induced increase in the F:B ratio (**Figure 2.5b**) that was significant at R2 but not at R4.

The increase in the F:B ratio was due to a significant increase in the relative abundance of *Firmicutes* at R2 (**Figure 2.5c**). Within the *Firmicutes* phylum, two major classes changed in different directions at R2. *Bacilli* were significantly decreased in sleep-disrupted mice (**Figure 2.5d**), while *Clostridia* were significantly increased (**Figure 2.5g**). The decrease in the class *Bacilli* appeared to mostly be due to significant decreases in the genus *Lactobacillus* (**Figure 2.5e**) and genus *Turicibacter* (**Figure 2.5f**). Within the *Clostridia* class, one unknown genus within the *Clostridiaceae* family was significantly decreased at R2 (**Figure 2.5h**), while other

taxa within class *Clostridia* were significantly increased (**Table 2.1**). The low abundance phylum *Actinobacteria* (**Figure 2.5i**) was significantly decreased in the sleep-disrupted group at both R2 and R4. This decrease was evident in the genus *Bifidobacterium* within the *Actinobacteria* phylum. These results parallel the beta diversity findings in that the greatest magnitude of shift in the fecal microbiome was at R2, and while some measures recover, others persist into R4.

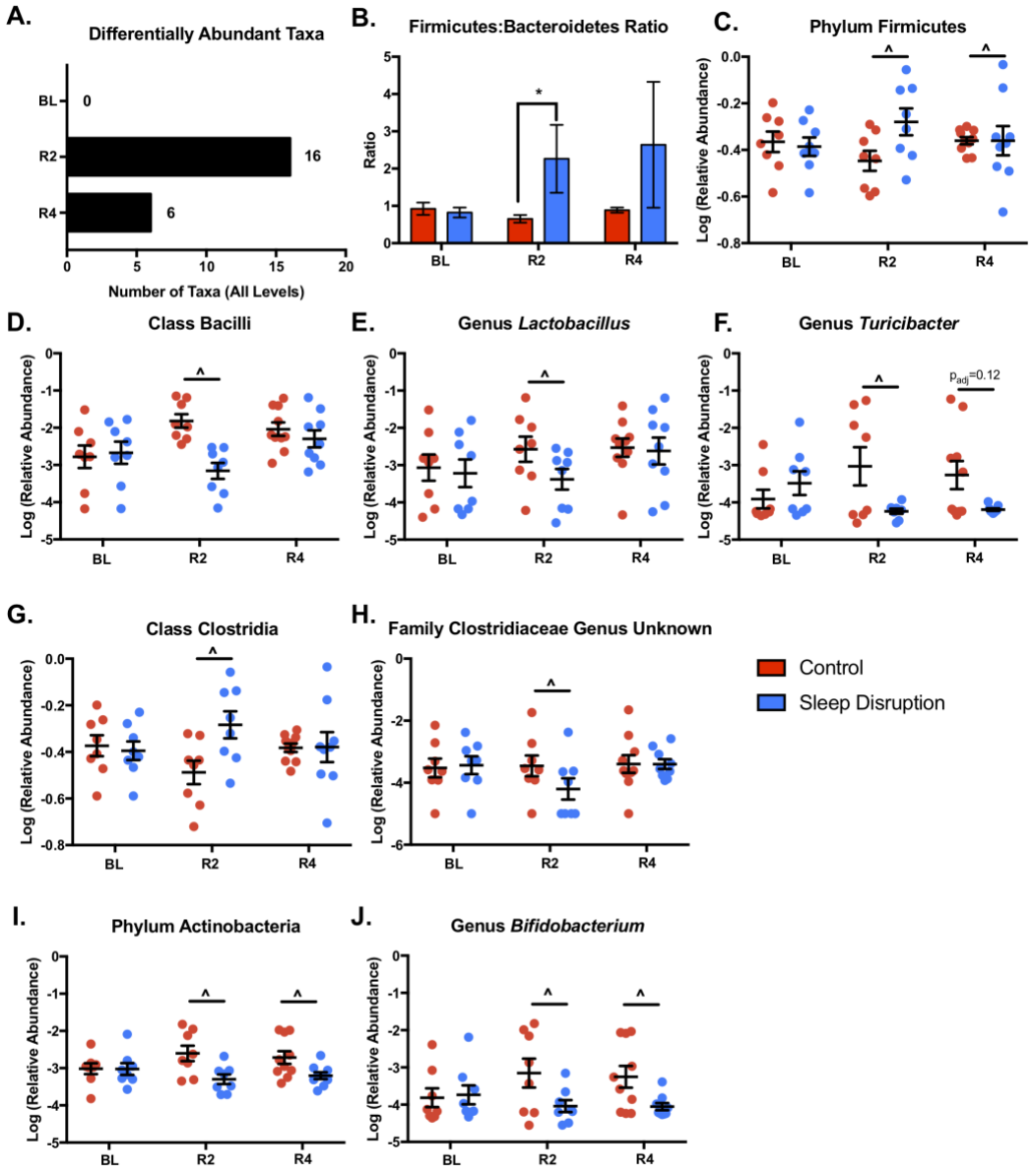

Figure 2.5: Effect of Sleep Disruption on Individual Microbial Taxa. At each timepoint, DESeq2 was performed at each taxonomic level to identify taxa differentially abundant between sleep disrupted and control groups. A) Summary of significantly different taxa by timepoint. B) The ratio of relative abundances of the phyla *Firmicutes* to *Bacteroidetes* was significantly increased at day 2 post-sleep disruption in sleep-disrupted animals. This increase was mostly driven by a significant increase in *Firmicutes* (C). Within the *Firmicutes* phylum, the class *Bacilli* (D), genus *Lactobacillus* (E), and genus *Turicibacter* (F) were reduced at day 2 post-sleep disruption. The class *Clostridia* was increased (G) and an unknown genus within the *Clostridiaceae* family (H) was decreased in sleep-disrupted animals at day 2 post-sleep

disruption. Both the phylum *Actinobacteria* (I), and the genus *Bifidobacterium* (J) were decreased at day 2 and day 4 post-sleep disruption in the sleep disrupted group. Abbreviations: BL, baseline; R2, day 2 post-sleep disruption; R4, day 4 post-sleep disruption. n = 8-10/group. Data represent means ± SEM. *$p < 0.05$ (Wilcoxon Rank-Sum Test); ^FDR < 0.1 (DESeq2).

Five days of sleep disruption changes the fecal metabolome

Due to the increasing evidence supporting the role microbes play in generating or altering physiologically active metabolites, we examined the impact of sleep disruption on the fecal metabolome. Normalized feature tables containing 1124 features were used for PCoA analysis at each timepoint to assess global changes due to sleep disruption. No separation was observed at BL ($p = 0.881$, PERMANOVA; **Figure 2.6a**), but a clear separation between sleep-disrupted and control mice was seen at R2 ($p = 0.007$, PERMANOVA; **Figure 2.6b**). This separation was no longer present at R4 ($p = 0.381$, PERMANOVA; **Figure 2.6c**). Of the 1124 molecular features assayed, 250 were identified as significantly changing over time, relative to BL, in either the control group, the sleep-disrupted group, or both (Kruskal-Wallace FDR < 0.1, **Figure 2.6d**). Many features (101/250) significantly changed only in control animals, suggesting sleep disruption prevented a naturally occurring change. Conversely, 57/250 features significantly changed over time in the sleep-disrupted mice but not in the non-sleep-disrupted mice. We also compared sleep disruption to control groups at each timepoint individually to assess the relative amount of differentially abundant features at each stage of the experiment, and the majority of significantly differentially abundant features (142/204; Wilcoxon Rank-Sum, $p < 0.05$) were found at R2 (**Figure 2.6e**), with 57 of those 142 decreased and 85 of the 142 increased in the sleep-disrupted group. Only 20/204 (13 decreased, 7 increased in the sleep disruption group) significantly differentially abundant features were found at BL, whereas 42/204 (29 decreased,

13 increased in the sleep disruption group) significantly differentially abundant features were found at R4. Overall, these results indicate that five days of sleep disruption results in a global shift in the fecal metabolome, both preventing naturally occurring shifts in the abundances of some metabolites and creating changes in others. While this global shift is present only at R2, some metabolites remained altered on the 4th day of *ad libitum* recovery sleep.

A subset of metabolites drive separation between sleep-disrupted and control groups at day two post-sleep disruption

Variable Selection Using Random Forests (VSURF)[Genuer et al., 2010; Genuer et al., 2015] was used to identify features that were important drivers of separation between sleep disruption and control groups at R2. VSURF identified 98 features that were above the threshold variable importance (suprathreshold) and that successfully distinguished the two groups on a heatmap (**Figure 2.7a-b; Table 2.2**).

From here we sought to learn about the possible identities of these features of interest using GNPS[Wang et al., 2016]. By matching an unknown spectrum or cluster of spectra to spectra in a large database, and examining their similarity to others within a molecular network, GNPS can be used to identify molecular classes and annotate purported molecular structures of features from untargeted metabolomic datasets. This is a level 2 or 3 metabolite identification according to the 2007 Metabolomics Standards Initiative[Sumner et al., 2007], where level 1 is considered a high confidence identification. Using GNPS to generate a molecular network for this dataset,

the MS2 spectra of 21/98 suprathreshold R2 features were matched to annotated spectra, including 4 of the top 25 drivers identified by VSURF (**Figure 2.7b**). Examining only the top annotated features (**Figure 2.7c**), many features with spectral matches to di- and tripeptides, along with the lysine degradation product L-saccharopine, were significantly increased in the sleep-disrupted group (**Figure 2.7d-f; Figure 2.8**).

Table 2.1. Differentially Abundant Bacterial Taxa Post-Sleep Disruption

Day 2 Post-Sleep Disruption

Taxon	Control Relative Abundance	Sleep Disrupted Relative Abundance	Fold Difference (Dis vs Con)	Adjusted p
Phylum Actinobacteria	0.0050 ± 0.0020	0.0006 ± 0.0002	-0.8800	0.0004
Class Actinobacteria	0.0044 ± 0.0020	0.0001 ± 0.0001	-0.9773	0.012
Order Bifidobacteriales	0.0042 ± 0.0021	0.0001 ± 0.0001	-0.9762	0.028
Family Bifidobacteriaceae	0.0042 ± 0.0021	0.0001 ± 0.0001	-0.9762	0.032
Genus Bifidobacterium	0.0042 ± 0.0021	0.0001 ± 0.0001	-0.9762	0.046
Phylum Firmicutes	0.370 ± 0.036	0.558 ± 0.072	0.508	0.002
Class Bacilli	0.027 ± 0.010	0.001 ± 4.2e-4	-0.963	1.9e-6
Order Lactobacillales	0.013 ± 0.008	0.001 ± 4.2e-4	-0.923	0.003
Family Lactobacillaceae	0.013 ± 0.008	0.001 ± 3.7e-4	-0.923	0.023
Genus Lactobacillus	0.012 ± 0.008	0.001 ± 3.7e-4	-0.917	0.047
Order Turicibacterales	0.014 ± 0.008	0.000 ± 0.000	-1.000	0.015
Family Turicibacteraceae	0.014 ± 0.008	0.000 ± 0.000	-1.000	0.003
Genus Turicibacter	0.014 ± 0.008	0.000 ± 0.000	-1.000	0.047
Class Clostridia	0.340 ± 0.037	0.553 ± 0.072	0.627	0.051
Order Clostridiales	0.340 ± 0.037	0.553 ± 0.072	0.627	0.028
Family Clostridiaceae, Genus unknown	0.003 ± 0.002	0.001 ± 0.0005	-0.667	0.047

Day 4 Post-Sleep Disruption

Taxon	Control Relative Abundance	Sleep Disrupted Relative Abundance	Fold Difference (Dis vs Con)	Adjusted p
Phylum Actinobacteria	0.0038 ± 0.0014	0.0006 ± 0.0002	-0.8421	0.004
Class Actinobacteria	0.0029 ± 0.0014	4.9e-5 ± 3.8e-5	-0.9831	0.005
Order Bifidobacteriales	0.0028 ± 0.0013	4.9e-5 ± 3.8e-5	-0.9825	0.002

Family Bifidobacteriaceae	0.0028 ± 0.0013	4.9e-5 ± 3.8e-5	-0.9825	0.015
Genus Bifidobacterium	0.0028 ± 0.0013	4.9e-5 ± 3.8e-5	-0.9825	0.005
Phylum Firmicutes	0.439 ± 0.015	0.475 ± 0.073	0.082	0.045
Order Turicibacterales	0.010 ± 0.007	0.000 ± 0.000	-1.00	0.087

Table 2.1: Differentially Abundant Bacterial Taxa Post-Sleep Disruption. DESeq2 was performed at each taxonomic level to determine taxa differentially abundant between sleep disrupted and control groups. Taxa significant at an FDR < 0.1 are listed below as the mean relative abundance ± SEM, along with Benjamini Hochberg-adjusted *p* values. Fold difference: (Sleep Disruption-Control)/Control. Abbreviations: Dis, Sleep Disruption; Con, Control. *n* = 8-10/group.

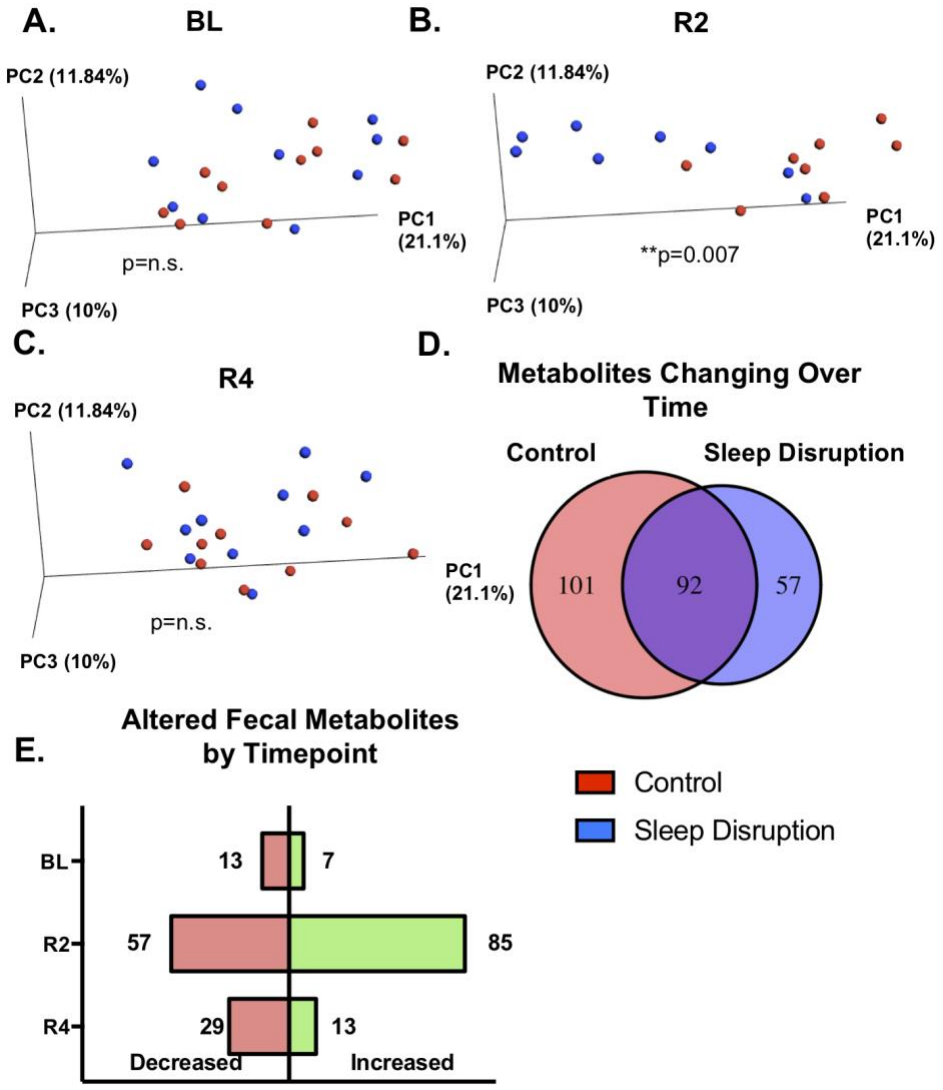

Figure 2.6: Effect of Sleep Disruption on the Fecal Metabolome. A,B,C) Principal coordinates analysis (PCoA) plots using Bray Curtis distance. PERMANOVA detected a significant difference between sleep disruption and control groups day 2 post-sleep disruption, but not BL or at day 4 post-sleep disruption. D) Kruskal-Wallace tests were run within the control group and within the sleep disruption group to determine metabolites significantly changing over the course of the experiment (FDR < 0.1). The number of metabolites found to have an effect of time only in the control group (left number), an effect of time only in the sleep-disrupted group (right number), or in both groups (middle number) is depicted in the Venn diagram. E) Wilcoxon Rank-Sum tests were performed at each timepoint to quantify the number of metabolites increased (right, green bars) or decreased (left, pink bars) in the sleep disruption group at each timepoint (uncorrected $p < 0.05$). Abbreviations: BL, baseline; R2, day 2 post-sleep disruption; R4, day 4 post-sleep disruption. n = 8-10/group. **$p < 0.01$ (PERMANOVA).

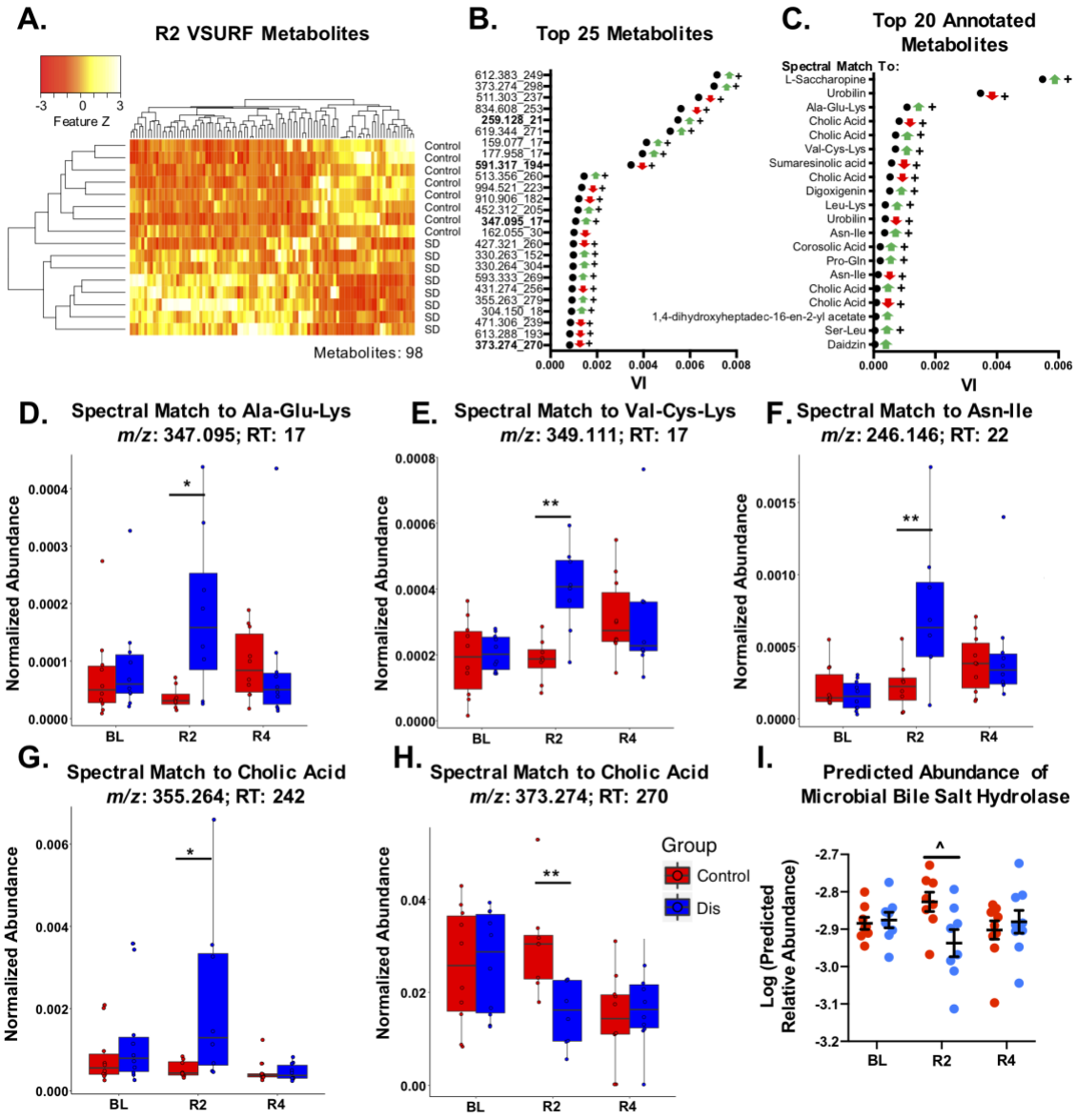

Figure 2.7: Metabolites That Drive Changes Seen at Day 2 Post-Sleep Disruption. Variable Selection Using Random Forests (VSURF) was performed at the second day post-sleep disruption (R2) to identify metabolites that are the most important drivers (above a threshold variable importance) of separation between sleep-disrupted and control groups. A) Heatmap of the 98 suprathreshold metabolites. B) Variable importance scores of the top 25 suprathreshold metabolites (*m/z*_RT), along with direction of change (arrows, green/up = increased in sleep disruption group, red/down = decreased in sleep disruption group). Bold indicates metabolites that were annotated using Global Natural Products Social Molecular Networking (GNPS). C) Top 20 annotated metabolites. Normalized abundance (peak intensity normalized to total ion count) of metabolites with spectral matches to D) Ala-Glu-Lys, E) Val-Cys-Lys, and F) Asn-Ile

were increased at R2 but not at day R4. G,H) One metabolite with a spectral match to cholic acid was increased (G) and another was decreased (H) at R2. I) Inferred abundance of the microbial bile salt hydrolase gene (EC:3.5.1.24) was also reduced at R2. For (D-H), boxes indicate median, 25_{th} and 75_{th} quantiles; whiskers indicate 2*IQR from edges of box. For (I), data represent mean ± SEM. Abbreviations: BL, baseline; R2, day 2 post-sleep disruption; R4, day 4 post-sleep disruption; Dis, Sleep Disruption; VI, variable importance; *m/z*, mass to charge ratio; RT, retention time (seconds). n = 8-10/group. +FDR < 0.1, *p < 0.05, **p < 0.01 (Wilcoxon-Rank Sum test); ^FDR < 0.1 (DESeq2).

We also noticed sleep disruption-induced changes in features with spectral matches to bacterially modified molecules including bile acids and urobilin. Two suprathreshold features with spectral matches to the bile acid cholic acid were significantly reduced, and two were significantly increased in sleep-disrupted mice at R2 (**Figure 2.7g-h**). Furthermore, two unannotated features with structural similarity to bile acids, as indicated by their presence in the same molecular networks as primary and secondary bile acids, were also significantly reduced (**Figure 2.9**), indicating structural similarity to bile acids. Bile acids are commonly modified by bacteria in the gut lumen by the enzyme encoded by the gene *bile salt hydrolase* (BSH)[Russell and Setchell, 1992; Foley et al., 2019], and have diverse signaling properties that involve the immune[Vavassori et al., 2009] and nervous systems[Mertens et al., 2017; Yanguas-Casas et al., 2017]. Thus, we used the software package PICRUSt2 to infer microbial gene content from the 16S rRNA gene data and assess inferred abundance of microbial BSH in our fecal samples. We found the inferred abundance of BSH (EC:3.5.1.24) was significantly reduced in the sleep-disrupted group at R2 (**Figure 2.7i, Supplemental File 2.2**). These results provide evidence that

microbially modified, physiologically active classes of molecules are impacted by five days of sleep disruption, and that the microbiome has an altered functional capacity to produce them.

Table 2.2: VSURF Suprathreshold Metabolites
Day 2 Post-Sleep Disruption

VSURF Rank	Feature ID	M/Z	RT (sec)	Annotated Name	Fold Difference (Dis vs Con)	P Value	Adj. P Value
1	964	612.383	249	N/A	9638.33	0.0025	**0.0218**
2	83	373.274	298	N/A	3.80	0.0030	**0.0218**
3	908	511.303	237	N/A	-0.54	0.0011	**0.0218**
4	516	834.608	253	N/A	-0.99	0.0037	**0.0218**
5	79	259.128	21	L-Saccharopine	0.80	0.0006	**0.0218**
6	998	619.344	271	N/A	4.32	0.0019	**0.0218**
7	780	159.077	17	N/A	2.69	0.0047	**0.0218**
8	809	177.958	17	N/A	6.24	0.0019	**0.0218**
9	13	591.317	194	Urobilin	-0.38	0.0104	**0.0262**
10	727	513.356	260	N/A	2.97	0.0030	**0.0218**
11	351	994.521	223	N/A	-0.63	0.0235	**0.0391**
12	1029	910.906	182	N/A	-0.91	0.0159	**0.0331**
13	65	452.312	205	N/A	0.51	0.0047	**0.0218**
14	582	347.095	17	Ala-Glu-Lys	4.08	0.0207	**0.0349**
15	653	162.055	30	N/A	-0.16	0.1049	0.1105
16	1075	427.321	260	N/A	-0.43	0.0047	**0.0218**
17	467	330.263	152	N/A	0.58	0.0499	**0.0596**
18	939	330.264	304	N/A	0.62	0.0499	**0.0596**
19	41	593.333	269	N/A	17.86	0.0047	**0.0218**
20	1014	431.274	256	N/A	-0.45	0.0047	**0.0218**
21	52	355.263	279	N/A	2.50	0.0207	**0.0349**
22	503	304.15	18	N/A	1.74	0.0070	**0.0236**
23	889	471.306	239	N/A	-0.42	0.0104	**0.0262**
24	589	613.288	193	N/A	-0.46	0.0104	**0.0262**

25	564	373.274	270	Cholic Acid	-0.48	0.0070	**0.0236**
26	622	417.336	230	N/A	0.53	0.0104	**0.0262**
27	906	491.279	156	N/A	-0.39	0.0379	**0.0489**
28	243	176.103	17	N/A	1.87	0.0047	**0.0218**
29	207	355.264	223	Cholic Acid	1.67	0.0650	**0.0758**
30	533	349.111	17	Val-Cys-Lys	1.17	0.0070	**0.0236**
31	1111	559.308	134	N/A	1.99	0.0104	**0.0262**
32	790	231.19	19	N/A	1.06	0.0148	**0.0315**
33	515	351.252	309	N/A	1.52	0.0148	**0.0315**
34	140	437.3	256	N/A	1.11	0.0148	**0.0315**
35	683	265.022	16	N/A	7.78	0.0263	**0.0418**
36	44	595.348	268	N/A	0.41	0.0207	**0.0349**
37	897	473.326	308	Sumaresinolic acid	-0.79	0.0030	**0.0218**
38	984	458.371	308	N/A	2.27	0.0047	**0.0218**
39	960	176.105	18	N/A	2.44	0.0070	**0.0236**
40	501	471.309	254	N/A	-0.61	0.0104	**0.0262**
41	1079	373.273	289	Cholic Acid	-0.50	0.0047	**0.0218**
42	927	451.317	283	N/A	1.31	0.0499	**0.0596**
43	51	408.311	286	Digoxigenin	1.79	0.0281	**0.0418**
44	1126	399.145	16	N/A	4.29	0.0499	**0.0596**
45	1140	887.444	149	N/A	1.46	0.0148	**0.0315**
46	969	440.36	307	N/A	1.11	0.0047	**0.0218**
47	69	367.209	179	N/A	0.55	0.0499	**0.0596**
48	477	369.261	271	N/A	1.58	0.0207	**0.0349**
49	1004	592.319	231	N/A	-0.44	0.0207	**0.0349**
50	729	490.324	239	N/A	-0.75	0.0148	**0.0315**
51	715	614.292	194	N/A	-0.49	0.0281	**0.0418**
52	685	592.321	195	N/A	-0.44	0.0207	**0.0349**
53	484	591.317	267	N/A	-0.45	0.0207	**0.0349**
54	734	260.196	20	Leu-Lys	1.13	0.0104	**0.0262**
55	338	591.317	232	Urobilin	-0.43	0.0207	**0.0349**
56	1110	246.146	22	Asn-Ile	2.21	0.0104	**0.0262**
57	71	365.107	17	N/A	0.87	0.0047	**0.0218**
58	288	293.21	183	N/A	0.33	0.0379	**0.0489**
59	898	474.33	274	N/A	-0.70	0.0047	**0.0218**
60	1119	732.384	165	N/A	17.55	0.0070	**0.0236**

61	365	202.071	18	N/A	0.60	0.0379	**0.0489**
62	204	485.327	238	N/A	4.84	0.0281	**0.0418**
63	266	455.356	262	Corosolic Acid	1.80	0.0047	**0.0218**
64	97	244.079	17	Pro-Gln	0.85	0.0070	**0.0236**
65	273	473.367	261	N/A	1.90	0.0070	**0.0236**
66	78	151.035	14	N/A	1.36	0.0281	**0.0418**
67	133	519.332	227	N/A	0.42	0.0379	**0.0489**
68	1124	495.787	126	N/A	112.84	0.0298	**0.0434**
69	594	246.133	25	Asn-Ile	-0.68	0.0659	**0.0760**
70	965	656.409	250	N/A	Inf	0.0128	**0.0313**
71	899	260.185	262	N/A	1.66	0.2345	0.2369
72	656	851.397	290	N/A	-0.99	0.0752	**0.0847**
73	197	485.326	293	N/A	1.84	0.0281	**0.0418**
74	1002	632.912	233	N/A	-0.75	0.0301	**0.0434**
75	871	421.346	308	N/A	1.01	0.0104	**0.0262**
76	59	355.264	242	Cholic Acid	3.15	0.0148	**0.0315**
77	945	414.269	283	N/A	-0.62	0.0207	**0.0349**
78	740	817.582	257	Cholic Acid	-0.65	0.0379	**0.0489**
79	393	375.28	230	N/A	-0.49	0.0379	**0.0489**
80	935	311.257	309	1,4-dihydroxyheptadec-16-en-2-yl acetate	1.33	0.1049	0.1105
81	893	457.33	305	N/A	-0.45	0.0499	**0.0596**
82	1011	303.133	182	N/A	-0.32	0.0830	**0.0924**
83	19	434.189	26	N/A	1.47	0.0207	**0.0349**
84	233	491.246	21	N/A	1.61	0.0740	**0.0843**
85	645	489.32	214	N/A	-0.63	0.0070	**0.0236**
86	1104	219.134	32	Ser-Leu	0.93	0.0104	**0.0262**
87	85	255.065	186	Daidzin	0.28	0.1049	0.1105
88	402	469.293	233	N/A	-0.47	0.0379	**0.0489**
89	612	367.246	294	N/A	1.64	0.0207	**0.0349**
90	36	230.186	17	Milnacipran	0.89	0.0650	**0.0758**
91	363	577.29	132	N/A	1.91	0.1049	0.1105
92	244	506.265	151	N/A	1.25	0.1949	0.1989
93	188	257.081	186	N/A	-0.42	0.1889	0.1949
94	220	217.068	17	N/A	0.77	0.0281	**0.0418**
95	206	466.291	196	N/A	1.37	0.1049	0.1105

96	1135	628.81	114	N/A	1.61	0.3717	0.3717
97	1093	473.369	262	N/A	1.39	0.0379	**0.0489**
98	332	568.224	223	N/A	-0.28	0.1559	0.1626

Day 4 Post-Sleep Disruption

VSURF Rank	Feature ID	M/Z	RT (sec)	Annotated Name	Fold Difference (Dis vs Con)	P Value	Adj. P Value
1	238	621.293	189	N/A	-0.22	0.0039	**0.0622**
2	697	343.175	181	N/A	-0.58	0.0039	**0.0622**
3	894	437.304	280	Hederagenin or N/A	-0.43	0.0005	**0.0312**
4	586	455.314	292	Wilforlide A	-0.43	0.0011	**0.0336**
5	241	434.227	17	N/A	3.18	0.0101	**0.0803**
6	70	520.339	303	Methyl 3-acetoxy-16-hydroxy-4,4,8,12,16-pentamethyl-15,17,19-trioxoandrost-11-ene-14-carboxylate	-0.47	0.0892	0.1903
7	62	412.236	196	N/A	-0.20	0.1051	0.1922
8	524	293.21	220	trans-EKODE-(E)-Ib	-0.26	0.0288	0.1317
9	739	391.245	305	N/A	-0.78	0.0191	0.1109
10	579	456.318	306	N/A	-0.24	0.0147	**0.0940**
11	980	990.591	118	N/A	99.00	0.0423	0.1457
12	522	594.401	222	N/A	-0.31	0.0232	0.1144
13	638	391.215	271	N/A	-0.07	0.1207	0.2019
14	661	287.244	16	N/A	5.39	0.0082	**0.0803**
15	1096	686.356	120	N/A	4.40	0.0752	0.1852
16	187	385.143	161	N/A	-0.25	0.7913	0.8583
17	659	463.305	266	N/A	-0.26	0.0892	0.1903
18	838	407.239	102	N/A	-0.77	0.1028	0.1922
19	910	879.381	228	N/A	-0.70	0.1655	0.2522
20	540	221.639	136	N/A	5.11	0.0535	0.1632
21	170	442.27	136	N/A	2.84	0.1230	0.2019
22	915	843.226	233	N/A	-0.87	0.0968	0.1922
23	45	441.201	191	N/A	-0.60	0.0753	0.1852
24	558	989.584	118	N/A	73.92	0.0518	0.1632
25	376	174.076	18	N/A	1.85	0.0355	0.1335
26	306	248.152	119	N/A	7.57	0.0113	**0.0803**

27	118	495.296	120	N/A	5.26	0.0892	0.1903
28	500	903.378	190	N/A	-0.87	0.0355	0.1335
29	966	248.653	118	N/A	16.15	0.0695	0.1852
30	744	330.263	294	N/A	-0.37	0.1051	0.1922
31	37	1191.503	184	N/A	0.08	0.4359	0.5166
32	754	455.311	306	N/A	-0.22	0.0433	0.1457
33	557	883.532	135	N/A	26.43	0.0752	0.1852
34	912	281.104	189	N/A	-0.84	0.0089	**0.0803**
35	716	470.34	289	N/A	0.15	0.4359	0.5166
36	928	364.27	191	N/A	0.39	0.4359	0.5166
37	1021	618.333	268	N/A	-0.05	0.4813	0.5404
38	407	860.468	186	N/A	-0.19	0.0632	0.1839
39	1047	279.1	31	N/A	13.66	0.1012	0.1922
40	284	333.203	308	N/A	-0.54	0.0089	**0.0803**
41	275	457.329	283	N/A	-0.09	0.8534	0.8810
42	532	295.226	225	13-Keto-9Z,11E-octadecadienoic acid	-0.13	0.0892	0.1903
43	889	471.306	239	N/A	-0.28	0.1230	0.2019
44	509	692.341	182	N/A	-0.29	0.1431	0.2290
45	251	215.139	22	Pro-Val	-0.44	0.1230	0.2019
46	724	473.364	263	N/A	-0.16	1.0000	1.0000
47	855	502.324	280	N/A	-0.27	0.1655	0.2522
48	899	260.185	262	N/A	0.84	0.8534	0.8810
49	964	612.383	249	N/A	Inf	0.0350	0.1335
50	58	374.277	232	N/A	-0.22	0.2799	0.3894
51	405	412.305	288	N/A	-0.04	0.4813	0.5404
52	459	311.182	129	N/A	0.53	0.3930	0.5031
53	155	303.169	160	N/A	-0.51	0.0232	0.1144
54	451	521.344	303	N/A	-0.23	0.3930	0.5031
55	914	440.194	228	N/A	-0.73	0.3068	0.4177
56	1103	378.239	160	Val Leu Phe	0.57	0.3527	0.4702
57	411	330.119	107	N/A	0.36	0.4359	0.5166
58	962	542.271	135	N/A	0.96	0.1903	0.2833
59	137	406.295	288	N/A	-0.03	0.7394	0.8159
60	512	514.283	181	N/A	0.12	0.8534	0.8810
61	422	378.212	182	N/A	4.07	0.2083	0.3030

62	1079	373.273	289	Cholic Acid	0.07	0.4813	0.5404
63	116	379.295	236	N/A	-0.17	0.2475	0.3519
64	1026	862.64	256	N/A	5.39	0.8815	0.8955

Table 2.2: VSURF Suprathreshold Metabolites: Variable Selection Using Random Forests (VSURF) was performed at day 2 post-sleep disruption (R2) and day 4 post-sleep disruption (R4) to identify top drivers of separation between sleep-disrupted and control groups. Metabolites that were above the VSURF threshold variable importance ('suprathreshold') from each timepoint are listed above, along with the feature's ID, m/z ratio, retention time, annotated name (if any), fold difference in the sleep-disrupted group compared to the control group, unadjusted Wilcoxon Rank-Sum *p* value, and Benjamini Hochberg (FDR)-adjusted *p* value. Bold indicates adjusted *p* values that are below the FDR of 0.1. Fold difference: (Sleep Disruption-Control)/Control. Abbreviations: Dis, sleep disruption; Con, control; MZ, mass to charge ratio; RT, retention time; N/A, not annotated; Inf, infinity. *n* = 8-10/group.

Another class of molecules that was affected by sleep disruption was dietary-derived pentacyclic triterpenoids. Triterpenoids are plant-derived molecules, and some have been shown to have anti-inflammatory properties[Banno et al., 2004]. We identified a molecular network containing 43 clusters, 12 of which were annotated as pentacyclic triterpenoids or close derivatives (**Figure 2.10**). Of the 43 clusters in the network, 9 were matched to VSURF suprathreshold features. This includes seven that were suprathreshold at R2 (**Figure 2.10, b-h**) and two that were suprathreshold at R4 (**Figure 2.10, i,j**). A feature with a spectral match to sumaresinolic acid (**Figure 2.10, b**), along with unannotated feature 645 (**Figure 2.10, g**) were significantly reduced at R2. A feature matching corosolic acid (**Figure 2.10, c**), along with unannotated features with the ID's 871, 204, 133 and 273 (**Figure 2.10, d-f,h**) were significantly increased at R2. Another molecular network of interest contained two unannotated ions that only appeared in sleep-disrupted groups (**Figure 2.11**). These metabolites therefore hold potential to act as markers for recent sleep disruption, and future additions to the GNPS database may result in level 2 or 3 annotation of clusters in the network.

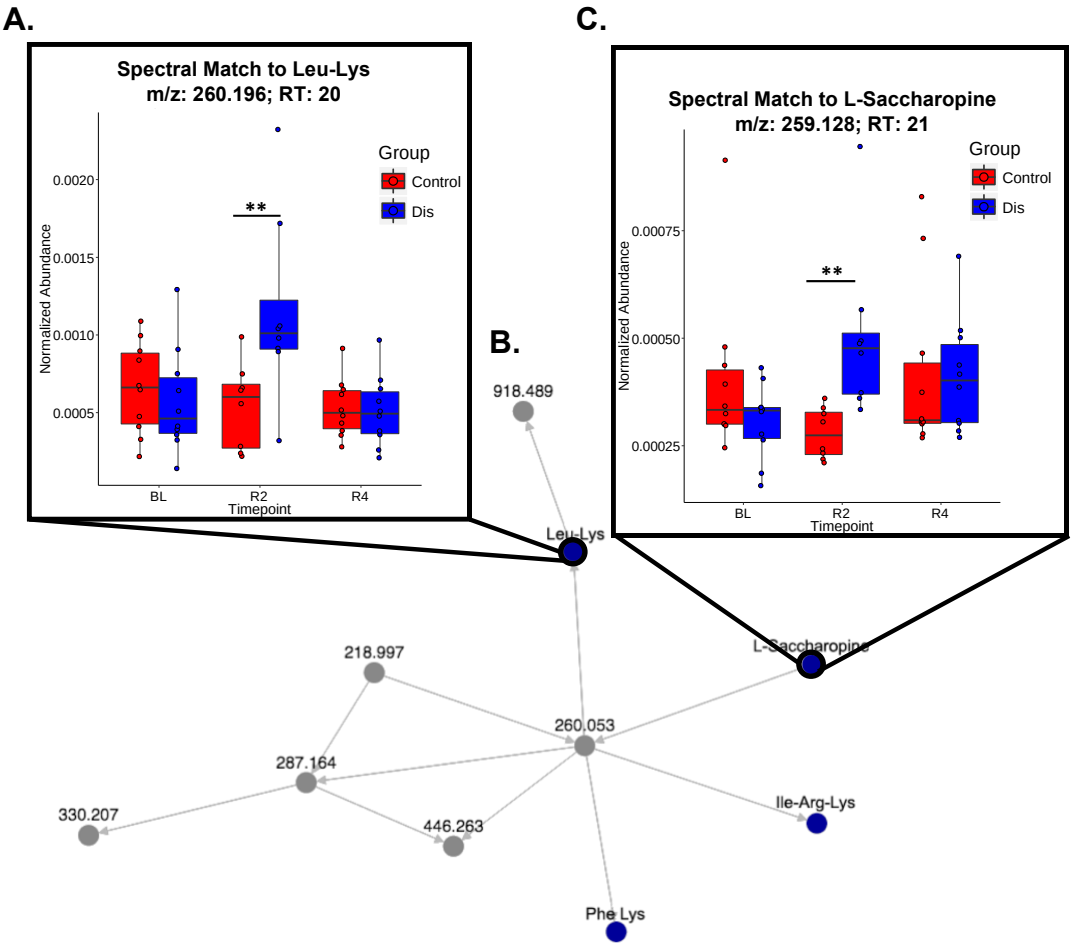

Figure 2.8: Sleep Disruption Changes Fecal Levels of Molecules Related to Protein Metabolism. A) Normalized abundance (peak intensity normalized to total ion count) of a metabolite with a spectral match to Leu-Lys was increased at day 2 post-sleep disruption. B) Global Natural Products Social Molecular Networking (GNPS)-generated molecular network containing multiple annotated (blue) and unannotated (grey) clusters. Numbers next to grey clusters indicate average parent mass of the spectra in the cluster. Metabolites that were above the threshold variable importance in Variable Selection Using Random Forests (VSURF) analysis are outlined with a black circle. Length of grey lines connecting clusters indicates relative similarity of the MS2 spectra. Arrowheads point towards clusters with a larger *m/z*. C) Normalized abundance of a metabolite with a spectral match to L-saccharopine was increased at day 2 post-sleep disruption (R2). Boxes indicate median, 25th and 75th quantiles; whiskers indicate 2*IQR from edges of box. Abbreviations: BL, baseline; R2, day 2 post-sleep disruption; R4, day 4 post-sleep disruption; Dis, Sleep Disruption; VI, variable importance; *m/z*, mass to charge ratio; RT, retention time (seconds). n = 8-10/group. *$p < 0.05$, **$p < 0.01$ (Wilcoxon-Rank Sum test).

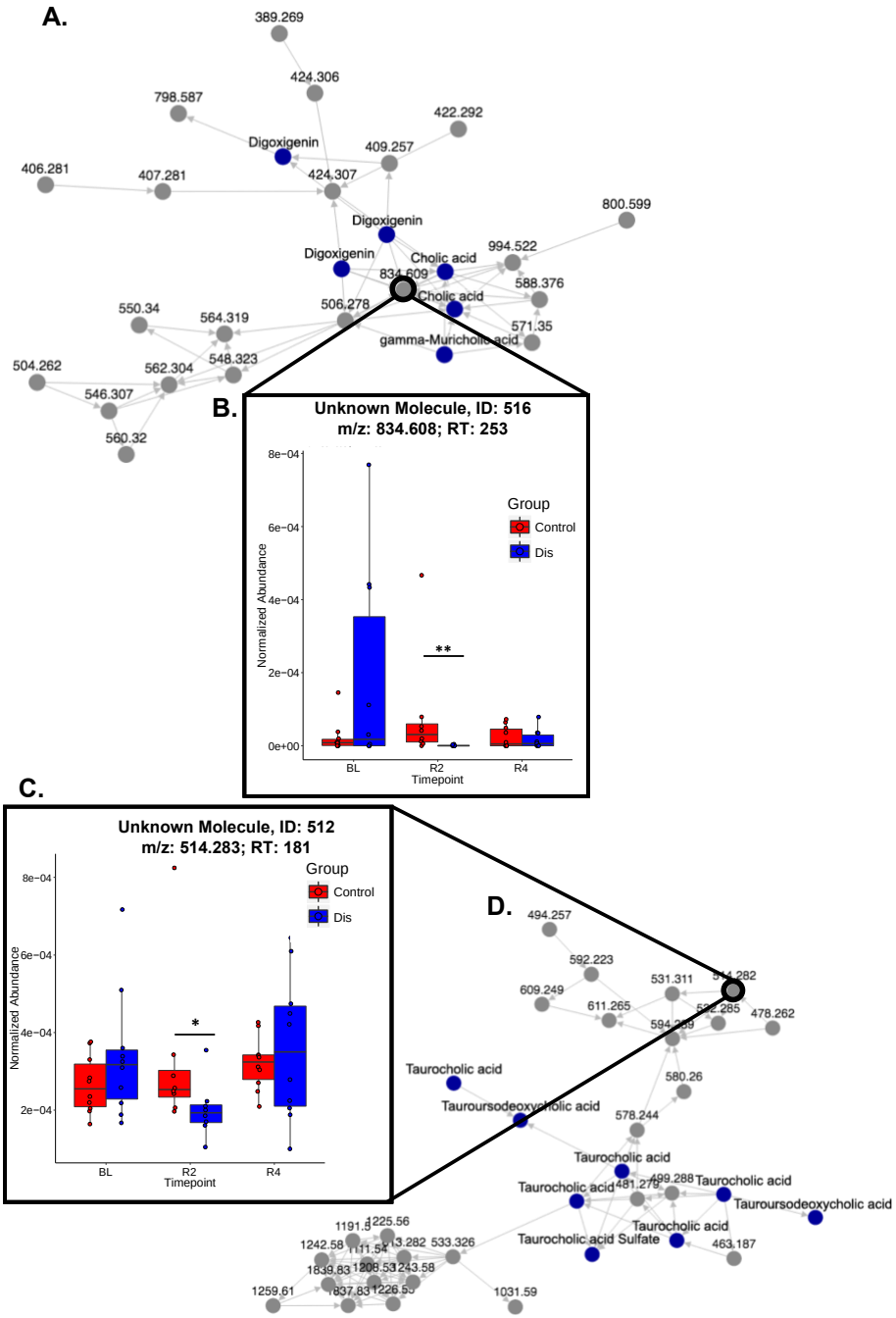

Figure 2.9: Sleep Disruption Decreases Fecal Levels of Unknown Molecules in Networks with Bile Acids. A,D) Global Natural Products Social Molecular Networking (GNPS)-generated molecular networks containing multiple annotated (blue) and unannotated (grey) clusters. Numbers next to grey clusters indicate average parent mass of the spectra in the cluster. Metabolites that were above the threshold variable importance in Variable Selection Using

Random Forests (VSURF) analysis are outlined with a black circle. Length of grey lines connecting clusters indicates relative similarity of the MS2 spectra. Arrowheads point towards clusters with a larger *m/z*. B) Normalized abundance (peak intensity normalized to total ion count) of an unannotated metabolite with ID 516 was decreased at day 2 post-sleep disruption (R2). C) Normalized abundance (peak intensity normalized to total ion count) of an unannotated metabolite with ID 512 was decreased at R2. Boxes indicate median, 25th and 75th quantiles; whiskers indicate 2*IQR from edges of box. Abbreviations: BL, baseline; R2, day 2 post-sleep disruption; R4, day 4 post-sleep disruption; Dis, Sleep Disruption; VI, variable importance; *m/z*, mass to charge ratio; RT, retention time (seconds). n = 8-10/group. *$p < 0.05$, **$p < 0.01$ (Wilcoxon-Rank Sum test).

Some changes to fecal metabolites are present at day four post-sleep disruption

Although no global change was seen on PCoA, we also ran VSURF analysis on the R4 feature table and identified 64 suprathreshold metabolites that were able to separate the control and sleep-disrupted groups (**Figure 2.12, a-b; Table 2.2**). Seven of these metabolites had MS2 spectra that matched reference spectral libraries in GNPS. Among the annotated features were molecules with spectral matches to hederagenin and wilforlide A (**Figure 2.10, i,j**), which were significantly reduced compared to controls at R4, and fell into the same molecular network as multiple metabolites that were suprathreshold at R2. Others that were significantly increased or decreased at R2 compared to controls did not recover by R4 (e.g. **Figure 2.12, d-f**). This indicates that, while no global changes were evident by day four of recovery sleep, sleep disruption did have an impact on some individual metabolites that persisted for at least four days.

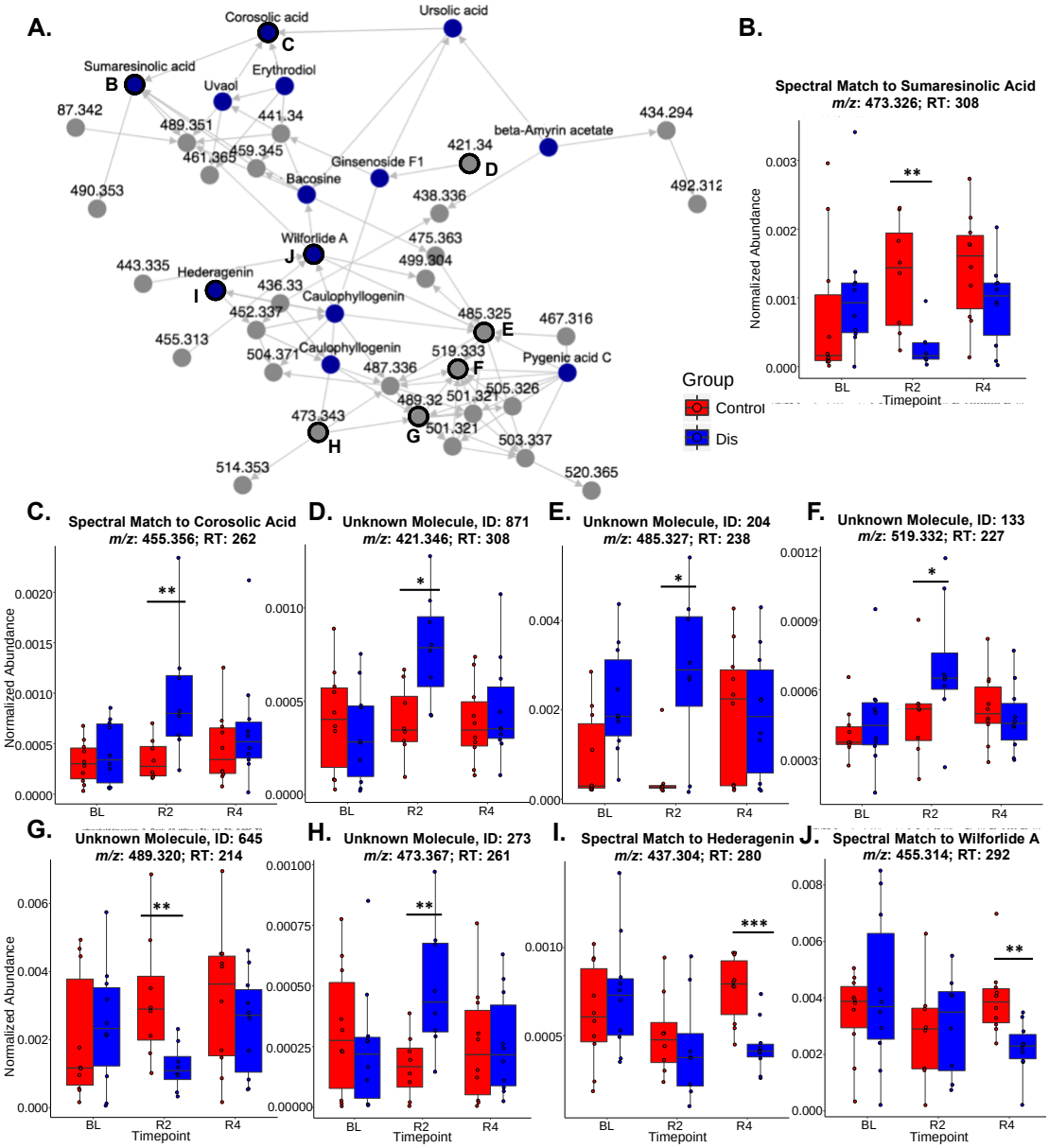

Figure 2.10: Sleep Disruption Changes Fecal Levels of Molecules Related to Pentacyclic Triterpenoids. A) Global Natural Products Social Molecular Networking (GNPS)-generated molecular network containing multiple annotated (blue) and unannotated (grey) clusters. Numbers next to grey clusters indicate average parent mass of the spectra in the cluster. Metabolites that were above the threshold variable importance in Variable Selection Using Random Forests (VSURF) analysis are outlined with a black circle. Letters next to the black circles indicate the panel of the figure corresponding to the cluster. Length of grey lines connecting clusters indicates relative similarity of the MS2 spectra. Arrowheads point towards clusters with a larger *m/z*. B,C) Normalized abundance (peak intensity normalized to total ion count) of a metabolite with a spectral match to sumaresinolic acid (B) was decreased at day 2

post-sleep disruption, while a spectral match to corosolic acid (C) was increased at R2. D,E,F,H) Unannotated molecules with ID 871 (D), 204 (E), 133 (F), and 273 (H) were increased at R2. G,I,J) Unannotated molecule ID 645 (G) was decreased at R2. Metabolites matching hederagenin (I), and wilforlide A (J) were decreased at R4. Boxes indicate median, 25th and 75th quantiles; whiskers indicate points within 2*IQR from edges of box. Abbreviations: BL, baseline; R2, day 2 post-sleep disruption; R4, day 4 post-sleep disruption; Dis, Sleep Disruption; VI, variable importance; *m/z*, mass to charge ratio; RT, retention time (seconds). n = 8-10/group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Wilcoxon-Rank Sum test).

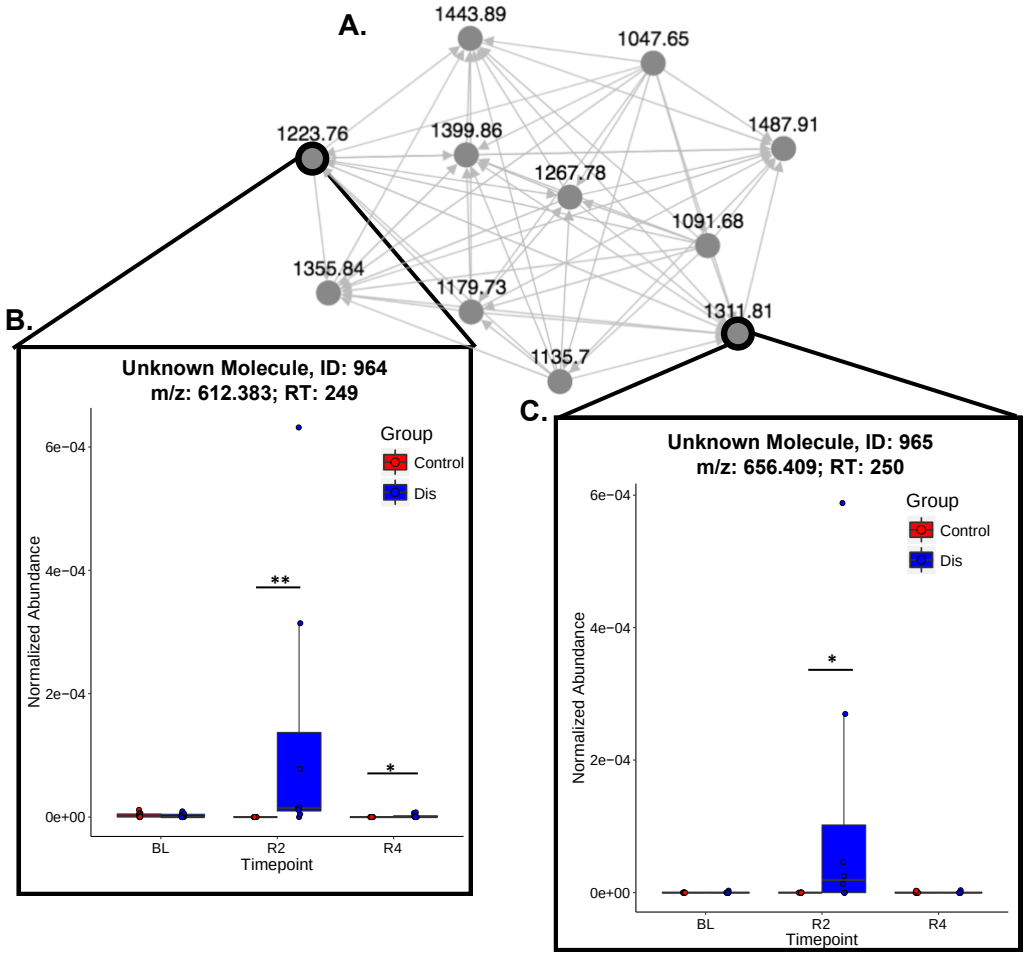

Figure 2.11: Two Unknown Fecal Metabolites are Present Only in Sleep-Disrupted Mice. A) Global Natural Products Social Molecular Networking (GNPS)-generated molecular network containing multiple unannotated (grey) clusters. Numbers next to grey clusters indicate average parent mass of the spectra in the cluster. Metabolites that were above the threshold variable importance in Variable Selection Using Random Forests (VSURF) analysis are outlined with a black circle. Length of grey lines connecting clusters indicates relative similarity of the MS2 spectra. Arrowheads point towards clusters with a larger *m/z*. B) Normalized abundance (peak intensity normalized to total ion count) of an unannotated metabolite with ID 964 was increased

at day 2 post-sleep disruption and at day 4 post-sleep disruption. C) Normalized abundance (peak intensity normalized to total ion count) of an unannotated metabolite with ID 965 was increased at day 2 post-sleep disruption but not day 4 post-sleep disruption. Boxes indicate median, 25th and 75th quantiles; whiskers indicate points within 2*IQR from edges of box. Abbreviations: BL, baseline; R2, day 2 post-sleep disruption; R4, day 4 post-sleep disruption; Dis, Sleep Disruption; VI, variable importance; *m/z*, mass to charge ratio; RT, retention time (seconds). *n* = 8-10/group. *$p < 0.05$, **$p < 0.01$ (Wilcoxon-Rank Sum test).

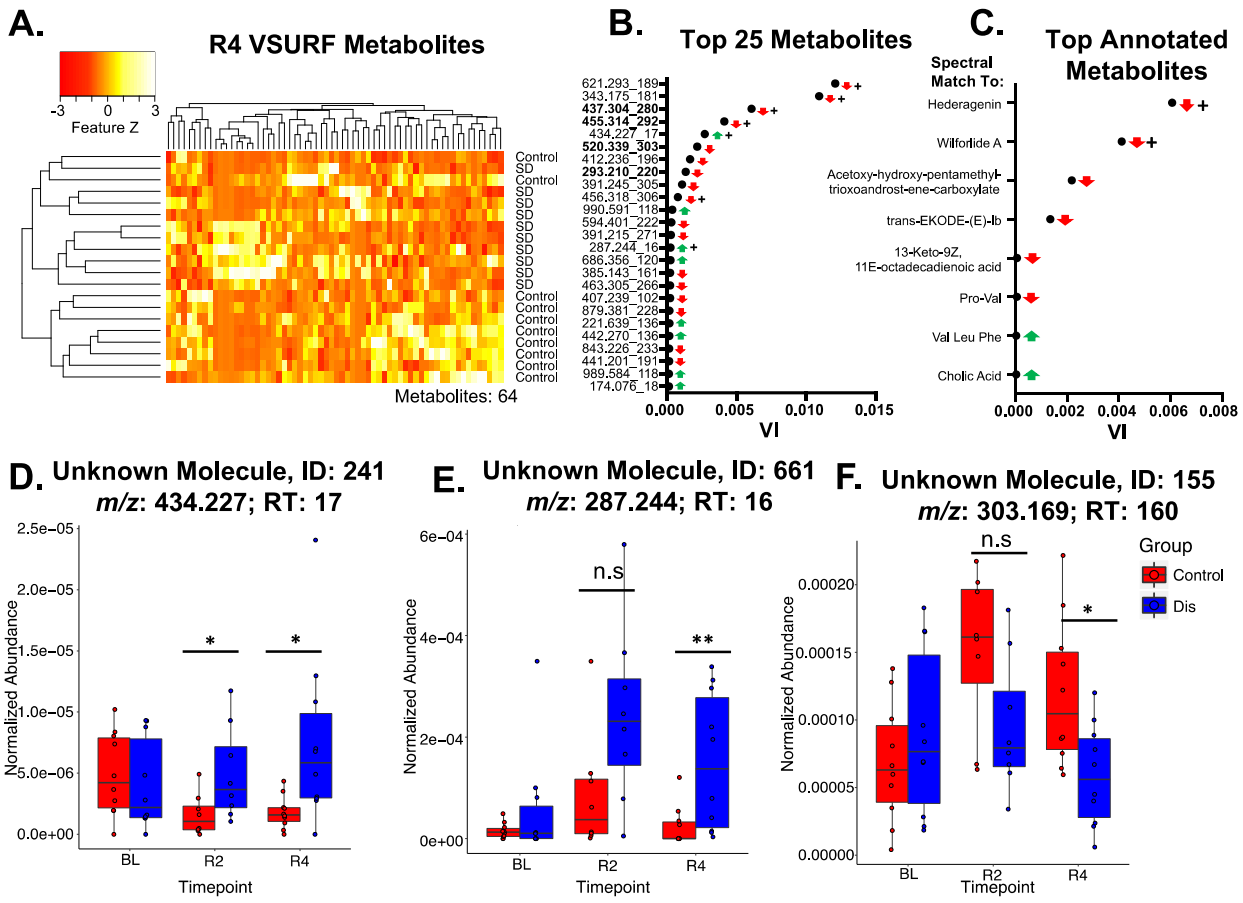

Figure 2.12: Metabolites that are Changed at Day 4 Post-Sleep Disruption. A) Heatmap of the 64 metabolites that were above threshold variable importance in Variable Selection Using Random Forests (VSURF) analysis. B) Variable importance (VI) scores of the top 25 suprathreshold metabolites (*m/z*_RT). Bold indicates metabolites that were annotated using Global Natural Products Social Molecular Networking (GNPS). C) VI scores of annotated metabolites. D) Normalized abundance (peak intensity normalized to total ion count) of an unannotated metabolite with ID 241 was increased in sleep-disrupted relative to control mice at both day 2 post-sleep disruption (R2) and day 4 post-sleep disruption (R4). E) An unannotated metabolite with ID 661 was trending towards an increase in sleep-disrupted compared to control

at R2 and was increased at R4. F) An unannotated metabolite with ID 155 was trending towards a decrease in sleep-disrupted mice compared to control mice at R2 and was decreased at R4. Boxes indicate median, 25th and 75th quantiles; whiskers indicate 2*IQR from edges of box. Abbreviations: BL, baseline; R2, day 2 post-sleep disruption; R4, day 4 post-sleep disruption; VI, variable importance; Dis, sleep disruption; *m/z*, mass to charge ratio; RT, retention time (seconds). n = 8-10/group. *$p < 0.05$, **$p < 0.01$ (Wilcoxon-Rank Sum test).

D. Discussion

Repeated sleep disruption is ubiquitous in modern society and has been linked to a multitude of health problems. Recent lines of scientific inquiry have established an important role for the gut microbiome in multiple facets of mammalian health and disease, many of which are also affected by sleep disruption. The present study took a detailed look at the impact of a sub-chronic, five-day sleep disruption protocol on the fecal microbiome as well as the fecal metabolome in mice, and found that repeated exposure to inadequate sleep had an impact on the microbiome and metabolome that lasted at least four days after the sleep disruption had ended. Importantly, sleep disruption reduced levels of beneficial bacterial genera, altered the metabolic function of the microbiome, and changed fecal levels of bacterially modified metabolites such as bile acids. These results can provide insights into possible mechanisms by which sleep disruption may impact host physiology.

The protocol used in this study resulted in severely disrupted sleep for five days, characterized by an decrease in sleep amounts, and an increase in fragmentation. This pattern was fairly stable across the five days, indicating that the animals were unable to adapt to the paradigm or find strategies to improve sleep as the protocol went on. This is a relevant model because repeated nights of inadequate sleep followed by a few days of recovery sleep is a common schedule in society today, and short sleep mixed with fragmentation is particularly prominent among groups with demanding work schedules such as on-call physicians[Wali et al., 2013] and active duty military personnel[Peterson et al., 2008]. By the second day of recovery sleep, nearly all sleep

parameters had returned to control levels. Whether the specific characteristics of sleep disruption determine how the microbiome or metabolome changes is unknown and warrants further investigation.

Previous work has demonstrated chronic (four week) sleep fragmentation (short disruption every two minutes) in mice alters the gut microbiome[Poroyko et al., 2016]. Our results suggest that a sub-chronic, more severe sleep disruption paradigm also results in significant shifts in the microbial community structure, without large variation in alpha diversity. This sleep disruption protocol also increased the dissimilarity of the fecal microbiome between animals within the sleep disruption group (within group distance) at both R2 and R4, indicating a "destabilizing" effect that persisted long into recovery sleep. The sleep-wake pattern had normalized by R2, suggesting that recovery of the microbiome may be a slower process than sleep homeostatic mechanisms.

Changes to particular taxa observed in sleep-disrupted mice suggest the dysbiosis induced by repeated inadequate sleep may have a detrimental physiological impact. Differential abundance analysis of individual taxa revealed multiple bacterial taxa that were increased or decreased in the sleep-disrupted group compared to controls, including an increase in the *Firmicutes*:*Bacteroidetes* (F:B) ratio and a decrease in *Lactobacillus*, *Actinobacteria*, and *Bifidobacterium*, all of which have established physiological impacts. An increase in the F:B ratio is a blunt measure of community shift and has been seen in many pathological states

including obesity[Ley et al., 2005; Koliada et al., 2017], chronic stress[Gautam et al., 2018], as well as an acute short sleep paradigm in humans[Benedict et al., 2016] and a chronic sleep fragmentation paradigm in rodents [Poroyko et al., 2016]. The phylum *Actinobacteria,* genus *Bifidobacterium,* and genus *Lactobacillus* were all low in sleep-disrupted mice. Previous studies in rodents and humans support a positive role for these taxa in stress resilience [Hemmings et al., 2017; Karl et al., 2017] and anxiety-like behavior [Bercik et al., 2011b; Messaoudi et al., 2011; Steenbergen et al., 2015]. Therefore, an increased F:B ratio, along with reduced *Actinobacteria, Bifidobacterium,* and *Lactobacillus* indicates a state whereby ability to cope with a proinflammatory or anxiety-inducing stimulus may be reduced. Indeed, sleep deprivation results in altered responsiveness of the hypothalamic-pituitary-adrenal (HPA) axis[Suchecki et al., 2002; Bassett et al., 2015; Guyon et al., 2017], increased inflammation[Vgontzas et al., 2004; Aho et al., 2013], and potentiated effects of a chemical stressor in a model of colitis in mice[Tang et al., 2009], indicating that sleep deprivation may be a factor promoting stress vulnerability. This is supported by a human study that observed that preexisting complaints of poor sleep increased the risk of posttraumatic stress disorder (PTSD) and other stress-related psychiatric disorders following trauma exposure[Bryant et al., 2010].

As one of the principle mechanisms by which a change in the fecal microbiome can impact host physiology is via change in the molecules they produce, we also examined the fecal metabolome in this study. An untargeted metabolomic approach allows for wide surveillance of the molecular environment as well as discovery of new molecular classes of interest[Schrimpe-Rutledge et al.,

2016]. Although untargeted mass spectrometry cannot confirm exact structures of metabolites of interest without secondary targeted standard assays, GNPS[Wang et al., 2016] allows us to infer the general class of many interesting features based on spectral matches and molecular networking. Using this approach, we identified multiple classes of molecules significantly impacted by sleep disruption, including bile acids, which are microbially modified and physiologically relevant.

Multiple results from this study suggest microbiome-influenced bile acid metabolism was impacted by sleep disruption. Primary bile acids are cholesterol derivatives that are synthesized in the mammalian liver and excreted into the intestinal lumen to aid in lipid emulsification and absorption. In the intestine, primary bile acids are dehydroxylated and deconjugated by the gut microbiome, creating secondary bile acids and greatly enhancing the diversity of the bile acid pool[Thomas et al., 2008]. Mounting evidence in the past decade has described bile acids as versatile signaling molecules, with receptors throughout the mammalian organism[Thomas et al., 2008; Kuipers et al., 2014; Mertens et al., 2017]. Some bile acids can act as anti-inflammatory and immunoregulatory agents in the intestinal tract and the central nervous system by activating the bile acid receptors FXR (farnesoid X receptor) and TGR5 (Takeda G protein-coupled receptor 5)[Vavassori et al., 2009; Yanguas-Casas et al., 2017]. Furthermore, bile acid receptors play a role in glucose, lipid, and cholesterol metabolism[Li et al., 2013a; Parseus et al., 2017]. Two molecules with spectral matches to cholic acid and two unannotated molecules within

molecular networks that contained multiple primary and secondary bile acids were decreased in the sleep-disrupted group at R2.

Importantly, analysis of the inferred gene content also revealed a reduction in the abundance of the microbial *bile salt hydrolase* (BSH) gene in the microbiomes of sleep-disrupted mice at R2. BSH catalyzes the critical first step in microbial bile acid metabolism, and multiple lines of evidence suggest these enzymes may be the "gatekeepers" of host-microbiome crosstalk[Foley et al., 2019]. In a previous experiment, feeding *Escherichia coli* engineered to overexpress *Lactobacillus* BSH to mice protected them from weight gain, and curbed lipid and cholesterol metabolism[Joyce et al., 2014]. A reduction in the fecal bile acid pool due to a reduction in microbial BSH, therefore, could result in a proinflammatory, metabolically dysregulated state in the host. Indeed, increased inflammatory markers have been observed in a sub-chronic short sleep (10 nights of 4 hours of sleep opportunity per night) model in humans [Haack et al., 2007], while a similar protocol (6 nights of 4 hours of sleep opportunity per night; also in humans) reduced glucose tolerance and increased sympathetic nervous system activity [Spiegel et al., 1999].

We also noticed a general increase in the abundance of metabolites with spectral matches to tripeptides and dipeptides. This could indicate an increase in host mucosal proteolysis or in microbial proteolysis[Antalis et al., 2007]. Host and microbial proteolytic enzymes play a role in gastrointestinal physiology, including activating signaling pathways (e.g. protease-activated

receptors or PAR's) controlling inflammation and gut barrier function[Antalis et al., 2007] as well as modulation of dorsal root ganglion neuron excitability[Sessenwein et al., 2017]. Furthermore, commensal bacteria have been shown to create molecules with potent protease inhibitory activity[Guo et al., 2017], so a shift in microbial community structure could have a direct impact on host proteolysis and physiology.

A third class of molecules that was impacted by sleep disruption was pentacyclic triterpenoids and close derivatives. The molecular family of dihydroxylated pentacyclic triterpenoids, judged by spectral matches to sumaresinolic acid and corosolic acid, were decreased and increased, respectively, at R2, while the dihydroxylated and monohydroxylated spectral features, with spectral matches to hederagenin and wilforlide A, were significantly decreased at R4. Also, four unannotated spectra within the network were significantly increased, and one was decreased, at R2. Triterpenoids are a class of diverse, plant-derived molecules that have been traditionally studied for their anti-tumor or anti-inflammatory properties[Banno et al., 2004; Banno et al., 2005; Wang et al., 2006]. Shifts in the balance of this molecular network could therefore have impacts on host physiology. While it has been shown that administration of triterpenoid molecules can modulate the microbiome[Chen et al., 2016], and that certain pentacyclic triterpenoids are metabolized by the microbiome[Li et al., 2013b], it is unclear whether the changes seen in this family of molecules due to sleep disruption were due to changes in the microbiome. In order to evaluate the biological impacts and therapeutic potential of the

molecules discovered in this study, follow-up studies will need to be done to verify the structures of the features discovered here as well as to quantify their concentrations in the gut.

Our results are consistent with a study by Poroyko *et al.* investigating the gut microbiome in a mouse model of obstructive sleep apnea[Poroyko et al., 2016]. In that study, four weeks of chronic sleep fragmentation caused global shifts in the fecal microbiome, as well as an increase in the F:B ratio, similar to this study. Furthermore, a study of acute sleep loss (four-hour sleep opportunity) in humans also observed an increase in the F:B ratio, but not a global shift in beta diversity[Benedict et al., 2016]. Recently, a study in rats found marked shifts in the fecal microbiome and urinary metabolites after a seven day severe stress/REM deprivation protocol[Ma et al., 2019]. Taken with the present results, a link between inadequate sleep and the fecal microbiome appears to be present across species and sleep disruption protocols. Importantly, the present study expands on these findings to include the fecal metabolome, which has important implications as an effector system of microbe-host interactions[De Vadder et al., 2014].

It is of note that a study published by Zhang et al. [Zhang et al., 2017] used a similar sleep restriction protocol (20h/day sleep disruption using a rotating bar for 7 days) but found no changes in the fecal microbiome. There are a few potential reasons for this discrepancy. First, the study by Zhang and colleagues used rats while ours used mice. Second, the rats in both the sleep restriction and control groups were manipulated every day to collect body weight measurements

and fecal pellets. We chose to leave the animals relatively undisturbed throughout the sleep disruption protocol. Our automated protocol allowed mice to remain in the same home cage to have undisturbed sleep opportunity and to have limited contact with human researchers, which can affect the microbiome[Ma et al., 2012; Franklin and Ericsson, 2017]. However, this approach did introduce some limitations to the experiment because it did not allow for constant monitoring of food intake or fecal microbiome/metabolome during the sleep disruption period.

Overall, this study characterizes the impact of inadequate sleep on fecal microbiome as well as the fecal metabolome, a potential effector system in microbe-host interactions. The changes to microbiome and metabolome were present on the second day of recovery sleep, and some changes persisted until at least the fourth day of recovery sleep, despite the recovery of most sleep within two days of the cessation of sleep disruption. This is particularly interesting in view of the observation that some of the neurobehavioral impairments observed after a week of short sleep do not recover after a 'weekend' of recovery sleep despite reduction of subjective sleepiness[Belenky et al., 2003; Pejovic et al., 2013]. Our findings also suggest that changes seen in particular bacteria and bacterially-influenced signaling molecules such as bile acids suggest a proinflammatory, metabolically dysregulated state in the days following a five-day sleep disruption protocol. Interventions designed to maintain the fecal microbiome and metabolome, or to proactively offset the negative impacts of dysbiosis, should be investigated to promote resilience to repeated sleep disruption, a problem that is ubiquitous in modern society.

E. Conclusions

Five days of intermittent sleep disruption (20 hours per day) not only severely disrupted sleep on each day of the protocol, it significantly altered the makeup of the microbiome and metabolome. This result supports the working hypothesis of this project in the following ways. First, it establishes a subacute sleep disruption protocol that results in severe sleep disruption. This tempo and severity of disruption which reduces sleep amounts to fragmented sleep over a 20 hour window, is analogous to situations of high stress, including those experienced by warfighters in the field. Second, the character of the microbiome and metabolome changes induced by this protocol suggested a dysbiotic, proinflammatory state, which supports the hypothesis that subacute sleep disruption is a stress-vulnerable state. This includes reductions in probiotic, anti-inflammatory *Lactobacillus* and *Bifidobacterium*, as well as reductions in bile acids, as discussed above. While the level of annotation of these metabolites was not optimal in this study (Level II), these findings inspired a closer look at bile acids in subsequent experiments, including using purified standards to verify the identity of different bile acids. Third, this chapter established a change in the microbiome and metabolome that lasted for days after the sleep disruption, even beyond when objective sleep measures returned to normal. So, any changes that contribute to stress vulnerability may be longer lasting than previously thought. The next chapter utilizes the same protocol in a new set of mice, but directly interrogates the question of stress vulnerability.

III. CHAPTER III: IMMUNIZATION WITH A HEAT-KILLED BACTERIUM, *MYCOBACTERIUM VACCAE* NCTC 11659, PREVENTS THE DEVELOPMENT OF PTSD-LIKE SLEEP AND BEHAVIORAL PHENOTYPES AFTER SLEEP DISRUPTION AND ACUTE STRESS IN MICE

As discussed in Chapters I and II, there is ample evidence repeated sleep disruption can alter responses to stressors and may confer stress vulnerability. A sleep deprived state, therefore, could act as the first hit in a two-hit model of trauma and stressor-related disorders[Suchecki et al., 2002]. In this model, genetic or environmental factors (repeated sleep disruption in this case) prime an individual for lasting psychological and physiological effects of a secondary traumatic event. The concept of the "double hit" of sleep disruption plus acute stress is explored in this chapter, and the double hit phenotype in mice is characterized. Furthermore, this chapter explores *M. vaccae* as an immunomodulatory microbial countermeasure to improve resilience to sleep disruption, acute stress, and the double hit.

A. Introduction

A pattern of repeated nights of inadequate sleep is common in Western, urban societies, and has been shown to lead to a host of physiological problems including metabolic[Spiegel et al., 1999], immunologic[Irwin, 2015], and cognitive[Krause et al., 2017] deficits. Disrupted sleep also alters the responsiveness of the hypothalamic-pituitary-adrenal (HPA) axis[Suchecki et al., 2002; Bassett et al., 2015; Guyon et al., 2017], increases inflammation[Vgontzas et al., 2004; Aho et al., 2013] and potentiates the effects of a chemical stressor in a model of colitis in mice[Tang et al., 2009], indicating that the sleep-deprived state may be a stress-vulnerable state. Indeed, there is evidence that short sleep duration is associated with stress-related psychiatric disorders such as depression/anxiety[van Mill et al., 2014] as well as posttraumatic stress disorder (PTSD)

following trauma exposure[Bryant et al., 2010]. Unfortunately, professions with high rates of PTSD such as warfighters, law enforcement, and emergency workers also often find it difficult to get adequate sleep due to the nature of their jobs. There is a need, therefore, to study the relationship between sleep disruption and stress vulnerability and to investigate potential countermeasures to increase resilience to the "double hit" of repeated sleep disruption plus acute stress.

Mycobacterium vaccae (strain NCTC 11659) is a non-pathogenic saprophytic bacterium with immunoregulatory and anti-inflammatory properties[Zuany-Amorim et al., 2002] and is prevalent in the environment. Bacteria like *M. vaccae* have been labeled "Old Friends" because mammals evolved with frequent exposure to these bacteria, which are thought to play a beneficial role in health (including mental health) by promoting differentiation of regulatory T cells and thereby immunoregulation[Rook et al., 2014b]. Importantly, recent studies have demonstrated that immunization with a heat-killed preparation of *M. vaccae* protects against stressor-induced changes in the gut microbiota[Reber et al., 2016], blunts stressor-induced potentiation of chemically-induced colitis[Reber et al., 2016; Amoroso et al., 2019], prevents stress-induced neuroinflammation and anxiety-like defensive behavioral responses[Frank et al., 2018; Frank et al., 2019], enhances fear extinction when given before or after fear conditioning[Hassell et al., 2019; Loupy et al., 2019], and prevents a PTSD-like behavioral syndrome in a mouse model of chronic psychosocial stress[Reber et al., 2016]. Follow-up studies suggested peripheral administration of *M. vaccae* may be exerting its stress-protective effects via

promotion of an anti-inflammatory state in the brain and prevention of stress-induced microglial priming[Fonken et al., 2018; Frank et al., 2018]. It remains unknown, however, what effect *M. vaccae* has on stress-induced changes in sleep, or whether *M. vaccae* immunization can confer stress-protective effects in the context of sleep disruption. The goal of this study was to characterize the impact of repeated sleep disruption, acute stress, and sleep disruption plus acute stress (the double hit) on sleep, physiology, and behavior in mice, and to determine if *M. vaccae* immunization can ameliorate these effects.

B. Materials and Methods

Animals and Experimental Design

Five cohorts of 24 seven-week-old male C57BL/6N mice (Charles River Laboratories, USA; n = 24 from Charlotte, NC; n = 96 from Kingston, NY) were used for this experiment (N = 120). A total of 5 animals did not complete the experimental protocol and were thus eliminated from all analyses, for a total N = 115. No explicit power analysis was used, the sample size was determined in to ensure adequate number of biological replicates of the main outcome measure: sleep recordings (target of n = 10-12). The experiment consisted of 8 experimental groups in a 2 x 2 x 2 design (Vehicle vs *M. vaccae* injection, *ad libitum* sleep vs sleep disruption, no social defeat vs social defeat). Experimental groups were balanced in each cohort. Mice were group housed until EEG/EMG implant surgery, after which they were individually housed until the end of the experiment. After EEG/EMG surgery, mice were assigned to experimental groups randomly, but effort was made to ensure prior cagemates were in different groups. Male CD1

retired breeder mice (Charles River Laboratories, USA) were used as aggressor mice in the social defeat model (see below). All mice were maintained on a 12:12 L:D cycle at room temperature (23 ± 2 ^{o}C) with food and water available *ad libitum* throughout the experiment. All protocols were approved by the Northwestern Institutional Animal Care and Use Committee. The light source in the sleep recording chambers was two 14 W fluorescent bulbs (soft white, 3000 K), resulting in an average light intensity of ~500 lux inside the cylindrical sleep disruption cage. Zeitgeber Time (ZT) is defined as the number of hours after the onset of the light period (light onset = ZT0).

EEG/EMG Implantation Surgery

One week after arrival, and 5 days prior to the first injection (i.e., on day –22), mice were implanted with electroencephalographic/electromyographic (EEG/EMG) sleep recording devices (Pinnacle Technologies, Lawrence, KS, USA). Surgical procedures were performed using a mouse stereotaxic apparatus with standard aseptic techniques in a ventilated, specially-equipped surgical suite. Anesthesia was induced by intraperitoneal (i.p.) injection of a cocktail of ketamine HCl (98 mg/kg; Vedco Inc, St. Joseph, MO, USA) and xylazine (10 mg/kg; Akorn Inc, Lake Forest, IL, USA) before surgical implantation of a headmount, which consisted of a plastic 6-pin connector connected to four EEG electrodes and two EMG electrodes. Four stainless steel screws serving as 2 EEG leads and grounds were screwed into the skull with one EEG lead located 1 mm anterior to bregma and 2 mm lateral to the central suture, and the other at 1 mm anterior to lambda and 2.5 mm lateral to the central suture. The exposed ends of two stainless steel Teflon-

coated wires (0.002 in. in diameter) serving as EMG leads were then inserted into the nuchal muscles using a pair of forceps. The entire headmount was then sealed by dental acrylic and a single skin suture at the front and the back of the implant was placed to close the incision. Heat support was provided until recovery from anesthetic by placing animals on a circulating water blanket. Subcutaneous injection of analgesic meloxicam (2 mg/kg; Norbrook Laboratories, Northern Ireland) was given to the animals at the time of surgery and once more on the following day.

Mycobacterium vaccae Administration

Heat-killed preparations of *M. vaccae* NCTC 11659 were provided by BioElpida (Lyon, France; batch ENG#1). Injections of *M. vaccae* (0.1 mg, 0.1 mL) or vehicle (sterile borate-buffered saline) were administered subcutaneously to the rear-left flank once per week for three weeks. The first injection was given 5 days after EEG/EMG implant surgery, and the third injection was given 3 days before baseline sleep recordings (i.e., days –17, –10, and –3; **Figure 3.1a**).

Sleep Recording and Analysis

One week prior to the sleep disruption protocol (day –6), mice were moved into cylindrical sleep recording cages (25 cm in diameter and 20 cm tall, Pinnacle Technologies , Lawrence, KS, USA) within individual acoustically-isolated and Faraday-shielded chambers and the headmount was connected to the transmission tether. Cages had corncob bedding and food/water available *ad libitum*. Five days were allowed for acclimation to the tether before baseline sleep was recorded

(i.e., starting at ZT0 on day 0). The sleep recording windows were divided into the following segments (see **Figure 3.1a,b**): "**Baseline**" was the 24 h period beginning 30 h prior to the sleep disruption protocol (i.e., starting at ZT0 on day 0). During the sleep disruption protocol, 24 h bins (ZT6-ZT6) were reported as "**Day 1-Day 4**", and contained the 20 h disruption (**Dis:** ZT6-ZT2) followed by the 4 h recovery (ZT2-ZT6) window. This time period was further divided into the 20 h windows during which the motorized bars were on ("**Dis1-Dis5**") and the 4 h recovery windows ("**R1-R4**"). At the end of the fifth 20 h sleep disruption session, all mice were unplugged from their EEG/EMG tethers for social defeat (or control manipulation, see below). Upon return to home cages, sleep recording resumed for ~20 h (~ZT7-ZT3) until mice were unplugged from their EEG/EMG tethers for testing in the OLM test. This segment was labeled "**Day 6**". After OLM behavior testing was complete, EEG/EMG tethers were again plugged in and sleep was recorded for 6 days until the end of the experiment ("**Day 7-Day 12**").

Data were collected using Pinnacle Acquisition software (Pinnacle Technologies), then scored as non-rapid eye movement sleep (NREM), rapid eye movement sleep (REM), or wake in 10 second epochs using machine learning-assisted sleep scoring software developed in the Turek/Vitaterna laboratory[Gao et al., 2016]. The initiation of a bout of NREM, REM, or wake was defined by the occurrence of two consecutive epochs of NREM, REM, or wake (respectively). A bout was terminated when a bout of another state occurred. Sleep bouts were initiated by two consecutive epochs of a sleep state (NREM or REM) and were only terminated when a wake bout occurred. A brief arousal was defined as a single epoch of wake within a sleep

bout. The average NREM to REM duration was defined as the average number of epochs of NREM preceding each REM bout. The delta power band was defined as 0.5-4 Hz, theta1 as 4-8 Hz, theta2 as 8-11 Hz, sigma as 11-15 Hz, and beta as 15-30 Hz. Relative power was calculated as the raw power (uV^2) in a particular band divided by the total power in all bands. Power was then reported as a percent of baseline to reduce inter-individual variability.

Sleep Disruption Protocol

After 7 days of acclimation to the sleep chambers and baseline sleep recordings, half of the mice entered the sleep disruption protocol. All cages had corncob bedding and food/water available *ad libitum* throughout the protocol. Sleep disruption was achieved using a commercially available system integrated into the chambers (Pinnacle Technology), which simulates the gentle handling technique via a rotating metal bar (22 cm in length) attached to a post at the center of the cage. For the sleep disruption period, the bar's rotation speed was set at 7 rotations per minute with reversals of rotation direction (i.e., clockwise vs. counterclockwise) set to occur at semi-random intervals of 10 ± 10 seconds. The bar was programmed to rotate for 20 hours per day (ZT6-ZT2), and was stationary from ZT2-ZT6, for 5 days. Experimenters visually inspected mice at regular intervals during the sleep disruption windows to ensure that the bar mechanism was functioning properly and that the sleep-disrupted mice were awake. Control animals were placed in identical cages with bars that remained stationary throughout the experiment.

Social Defeat Protocol

Male CD1 retired breeder mice were singly housed in large (44 cm x 24 cm x 21 cm) polycarbonate cages and were screened for aggressive behavior before the experiment. Mice that started to injure their opponents by harmful bites during screening were not used for the social defeat procedure. On the day of the social defeat stressor (between ZT2-ZT4), each experimental mouse was introduced into the cage of an aggressor. As soon as the aggressor mouse attacked and defeated the intruder mouse (upper limit of attack latancy was 30 seconds), the intruder was covered with a 10.2 cm x 12.7 cm x 10.2 cm metal mesh cage while still inside the aggressor cage, and left for 1 h. Control animals not receiving social defeat were also unplugged, and placed in a new cage with clean woodchip bedding for one hour (novel cage as a control manipulation").

Object Location Memory Task

The object location memory (OLM) task is a hippocampal-dependent memory task[Murai et al., 2007] that is sensitive to stress[Cazakoff et al., 2010; Howland and Cazakoff, 2010]. All groups of mice underwent testing in the OLM task the day after social defeat (or control manipulation), beginning at ZT4. Mice were placed in a dimly lit (~50 lux) 53 cm x 53 cm x 30 cm arena containing two identical cylindrical black caps on the same side of the arena. Mice were allowed to explore the arena for 5 minutes and after 90 minutes in their home cage were allowed to explore the arena again, with one object moved. Exploration of the moved object more than the non-moved object is considered evidence of successful acquisition of contextual memory, and is denoted by a "location index" (100 x {time exploring moved object/total time exploring either

object}) significantly different from 50%. In this experiment, location index was quantified during the first 3 minutes of the testing session. LimeLight (Actimetrics, Wilmette, IL, USA) behavioral software was used to track the animal's path traveled within the open field over time. De-identified video files were scored by two experimenters blind to treatment or stress manipulation and average location indices were reported. Location index inter-scorer reliability was high ($rho = 0.96$, $p < 0.0001$).

Tissue Collection

Six days after OLM testing (day 13), mice were euthanized via rapid decapitation between ZT4-ZT6 and trunk blood was collected in Z-gel serum tubes (Sarstedt AG & Co, Nümbrecht, Germany). The blood was centrifuged at 7500 rpm for 5 min, and serum was collected and stored at –80 ºC until analysis. Spleens were dissected and weighed. Mesenteric lymph nodes were collected and quickly frozen at –80 ºC until processing for cytokine analysis (see below).

Cytokines

Serum cytokines and chemokines were assessed using a customized magnetic bead multiplex (Millipore-Sigma, Burlington, MA, USA) containing: 1) interleukin 6 (IL-6); 2) monocyte chemoattractant protein 1 (MCP-1); and 3) cytokine-induced neutrophil chemoattractant-1 (CINC-1/CXCL-1). Serum samples were diluted 1:2 per the manufacturer's instructions and assayed in duplicate. Cytokines were also measured in mesenteric lymph node extract. Lymph nodes were homogenized in tissue extraction reagent (ThermoFisher, Waltham, MA, USA) using

a glass bead sonicator (Diagenode, Denville, NJ, USA). Tubes were centrifuged and the protein extract was aliquoted for multiplex analysis. Total protein was quantified in the extracts (Pierce BCA, ThermoFisher) and used to normalize results. Extracts were diluted 1:5 and cytokine concentrations were determined using a customized magnetic bead multiplex (Millipore-Sigma) containing: 1) IL-6; 2) IL-10; 3) IL-4; 4) interferon gamma (IFNγ); 5) IL-1β; and 6) IL-17A. Assays were conducted using duplicate samples (technical replicates).

Statistical Analysis and Software

All detailed statistical results can be found in **Supplementary Files 3.1 and 3.2**. All graphs depict the mean ± SEM. Sleep, behavior, and cytokine data were analyzed using linear mixed effects models using the lmer() and anova() functions in the lme4 package of RStudio (version 1.0.136, RStudio Inc, Boston, MA, USA). Models were tested for effects of treatment, sleep disruption, and social defeat, accounting for random effects of cohort (and plate number for cytokine data). Where appropriate, an effect of time was added to the model, along with terms accounting for repeated measures (see **Supplementary File 3.1**). When an overall effect on LMM was detected, post hoc comparisons were performed using the emmeans() function, with the Tukey or Dunnett correction for multiple comparisons when noted in the text. Two-sided Wilcoxon Rank-Sum testing was performed in RStudio. Spearman's Rank Based correlation analyses were performed using the rcorr() function within the Hmisc package in RStudio, and visualized using the corrplot package. *P* values from the correlation analyses were adjusted for multiple comparisons using the qvalue package in RStudio (see **Supplementary File 3.2** for rho,

p, and *q* matrices). All other graphs/figures were generated using GraphPad PRISM (version 8.2.1; GraphPad Inc., San Diego, CA, USA).

Outlier testing was performed on all datasets in GraphPad PRISM. For the sleep dataset, when individual observations were detected as extreme outliers using the Grubbs' test[Grubbs, 1969] (two-tailed alpha = 0.05), the EEG/EMG raw data files were visually inspected, and those observations were excluded from analysis if signal noise was deemed to interfere with accurate stage scoring or power analysis. This amounted to 0.62% of the sleep-related datapoints across the entire experiment (117/18,865). Extreme outliers detected in the behavioral datasets with the Grubbs' test and verified to be due to software tracking errors were excluded (n =2/115 mice, 6/460 datapoints). Cytokine and chemokine multiplex datasets contained multiple extreme outliers, and those identified using the ROUT test (Q = 1%) were removed from analysis: 1) serum IL-6, n = 7; 2) serum CINC-1, n = 1; 3) serum MCP-1, n = 2; 4) MLN IL-6, n = 6; 5) MLN IL-17A, n = 8; 6) MLN IL-1β, n = 2; 7) MLN IFNγ, n = 3; 8) MLN IL-4, n = 4; 9) MLN IL-10, n = 8.

C. Results

Male C57BL/6N mice (N = 115) were randomly assigned to one of eight experimental groups. The experiment followed a 2 x 2 x 2 design (**Figure 3.1a,b,** see **Methods**). Mice received subcutaneous injections once per week for three weeks with either a heat-killed preparation of *M. vaccae* (NCTC 11659) or vehicle (borate-buffered saline). After baseline sleep recording, groups

were then exposed to a five-day sleep disruption protocol or allowed to sleep *ad libitum*. Immediately after the fifth sleep disruption period, groups were exposed to a 1-hour social defeat or a control manipulation (1-hour novel cage). Behavior in the OLM test was assessed the day after social defeat stress, sleep was recorded throughout the experiment, and tissues were collected at the end of the experiment for analyses (**Figure 3.1a,b**).

M. vaccae immunization has minimal impact on baseline sleep measured three days after the final injection

Electroencephalogram (EEG) and electromyogram (EMG) recording devices were implanted before the first injection of *M. vaccae* or vehicle (i.e., on day –22), and 24 h baseline sleep was recorded starting three days after the third injection (i.e., on day 0). We observed no group differences between the vehicle- and *M. vaccae*-treated groups in the amount of time spent in non-rapid eye movement (NREM) sleep, rapid eye movement (REM) sleep, the REM:Sleep ratio, or the number of state shifts (**Figure 3.2a-d**). For *F* statistics and *p* values for all LMM analysis, see **Supplemental File 3.1**.There were no differences between groups in the NREM EEG power spectra or the REM EEG power spectra (**Figure 3.2e,f**). Overall these results suggest that the three immunizations with *M. vaccae* did not have an appreciable impact on sleep measures under basal conditions.

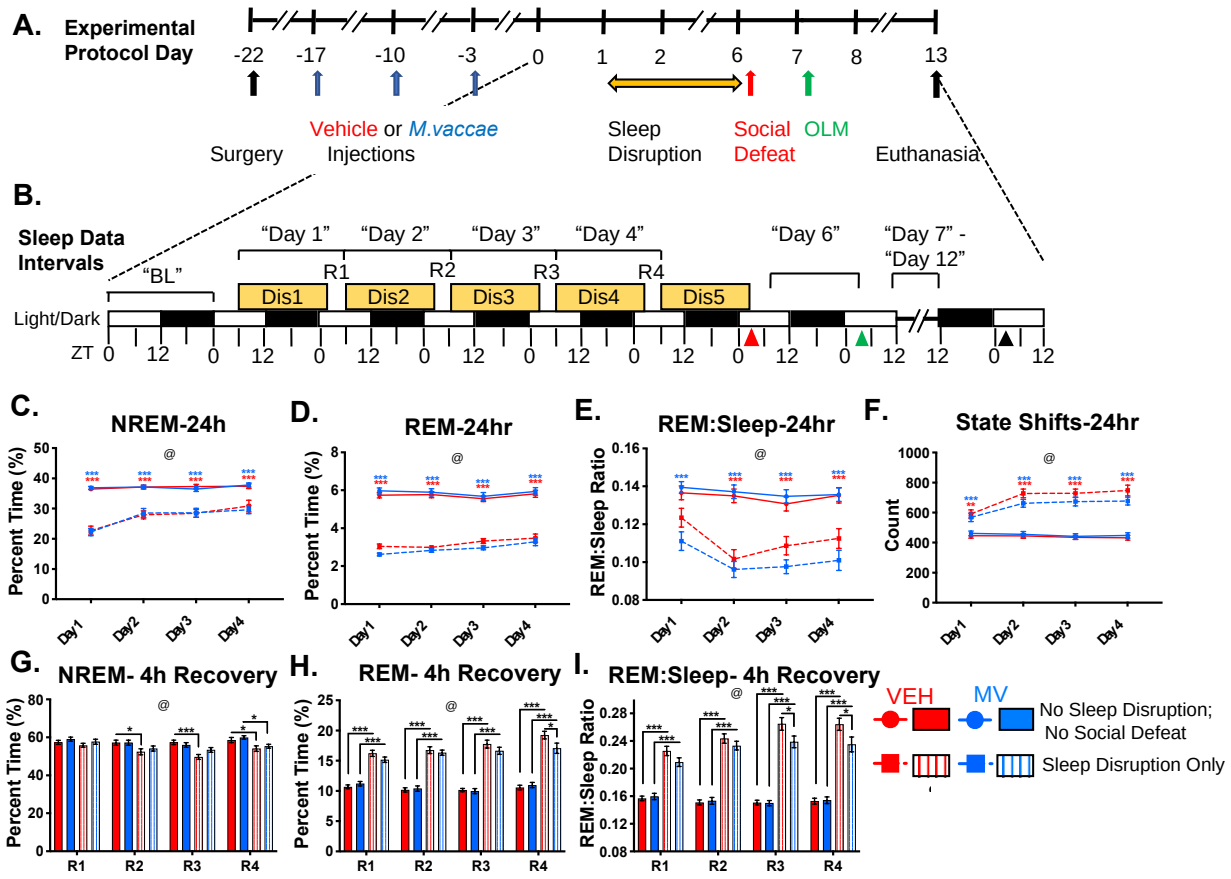

Figure 3.1: Experimental Timeline and Effect of Sleep Disruption on Sleep in Vehicle-Treated and *M. vaccae*-Treated Mice. (A) Overview of the experimental protocol. Experimental protocol days are depicted along the axis of the timeline, with tick marks indicating ZT0 of that experimental day. (B) Detail of indicated time period that includes the sleep disruption protocol, social defeat stress (red arrow), object location memory (green arrow), and terminal sample collection (black arrow). Tick marks along the bottom indicate zeitgeber time on that day, white/black rectangles indicate 12 h light/dark phases, and yellow rectangles indicated time periods of sleep disruption for the sleep-disrupted groups (ZT6-ZT2). EEG/EMG recording intervals, as used in the forthcoming figures, are indicated above the timeline in quotation marks. (C) NREM, (D) REM, (E) REM:Sleep ratio, and (F) state shifts per 24 h (ZT6-ZT6) during the sleep disruption. (G) NREM, (H) REM, and (I) REM:Sleep ratio during the four hour (ZT2-ZT6) *ad libitum* sleep opportunities. Data are mean ± SEM. Symbols: @ $p < 1.0 \times 10^{-4}$ (overall effect of "Sleep Disruption"), linear mixed effects model; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Tukey's post hoc test). Red asterisks indicate vehicle-injected control group vs vehicle-injected sleep-disrupted group at that timepoint. Blue asterisks indicate MV injected control group vs MV injected, sleep-disrupted group at that time point. n = 29-30/group. Abbreviations: BL, baseline; Dis, sleep disruption; EEG, electroencephalography; EMG, electromyography; MV, *Mycobacterium vaccae* NCTC 11659 injection; NREM, non-rapid eye movement; OLM,

object location memory; R, recovery; REM, rapid eye movement; VEH, vehicle injection; ZT, zeitgeber.

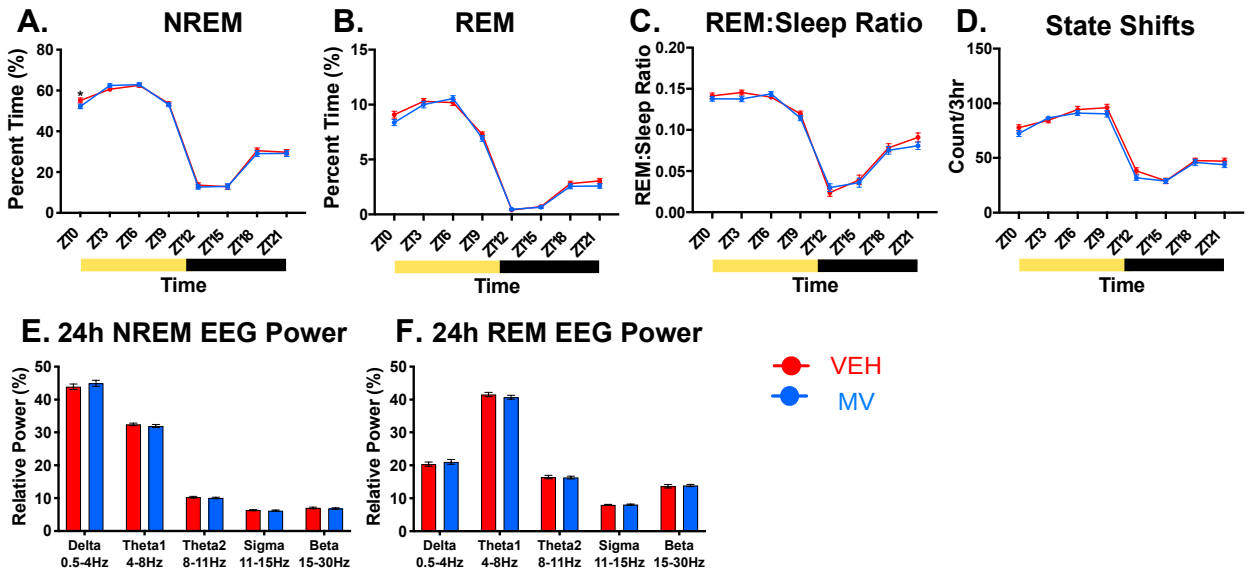

Figure 3.2: *M. vaccae* Alone Does Not Impact Baseline Sleep. Three days after the third injection of *M. vaccae* or vehicle, 24 h of baseline sleep was recorded. (A) NREM, (B) REM, (C) REM:Sleep, and (D) state shifts are reported in 3 hour bins. Yellow bars below the x axes represent times where the lights were on, while black bars represent times the lights were off. (E) 24 hour NREM and (F) 24 hour REM EEG power bands are expressed as a percent of total power. Data are mean ± SEM. Symbols: **p* < 0.05 (Tukey's post hoc test). *n* = 52-53/group. Abbreviations: EEG, electroencephalography; MV, *Mycobacterium vaccae* NCTC 11659 injection; NREM, non-rapid eye movement; REM, rapid eye movement; VEH, vehicle injection; ZT, zeitgeber time.

The 5-day sleep disruption protocol significantly reduces NREM and REM sleep, and increases sleep fragmentation in both vehicle-injected and M. vaccae-injected mice.

The sleep disruption protocol used in this study consisted of 5 days of disruption during which a motorized bar at the bottom of the cage disrupted sleep for 20 h/day (ZT6-ZT2), followed by a 4 hour *ad libitum* recovery window (ZT2-ZT6; see **Methods**). EEG/EMG recording during the sleep disruption protocol revealed a significant reduction in 24 h NREM and REM sleep, along

with a significant increase in the number of state shifts in sleep-disrupted mice (**Figure 3.1c-f)**. A close inspection of sleep measures during the 20 h disruption windows showed that the NREM sleep was severely disrupted while the motorized bar was turning. This was characterized by a greater number of bouts of NREM and a reduced median bout duration, along with a reduction of NREM delta (0.5-4 Hz) EEG power and increase in NREM beta (15-30 Hz) EEG power (**Figure 3.3f-m)**. The mice reached REM sleep extremely rarely during the 20 h sleep disruption window (**Figure 3.3g**).

Examining the 4 h recovery windows (ZT2-ZT6), sleep-disrupted mice showed a large rebound in the amount of REM sleep during recovery compared to controls (**Figure 3.1h**). This rebound was similar in magnitude in vehicle-treated and *M. vaccae*-treated groups until recovery window R4, when the percent of time spent in REM was higher in vehicle-treated, sleep-disrupted mice than their *M. vaccae*-treated counterparts (**Figure 3.1h**). The recovery sleep was REM-dominant, as indicated by a significant increase in the REM:Sleep ratio (**Figure 3.1i**). Vehicle-injected, sleep-disrupted mice exhibited a greater increase in REM:Sleep than did *M. vaccae*-injected, sleep-disrupted mice during R3 and R4 (**Figure 3.1i**). This recovery sleep was so REM dominant that it resulted in a reduction in the amount of NREM sleep in the sleep-disrupted groups compared to controls, an effect that increased over time, beginning on R2 in vehicle-treated mice, but not until R4 in *M. vaccae*-treated mice (**Figure 3.1g)**. NREM sleep during recovery sleep, though reduced in quantity, often featured increased EEG delta (0.5-4 Hz) power (**Figure 3.3a**). The REM rebound in sleep-disrupted groups during the recovery windows was

accompanied by an increase in REM theta2 (8-12Hz) EEG power in vehicle-treated mice at R2-R4 and *M. vaccae*-treated mice at R3-R4 (**Figure 3.3d**). The sleep disruption protocol also resulted in a plateau in the growth curves of both vehicle-treated and *M. vaccae*-treated mice, with body weights promptly recovering after return to *ad libitum* sleep (**Figure 3.4**).

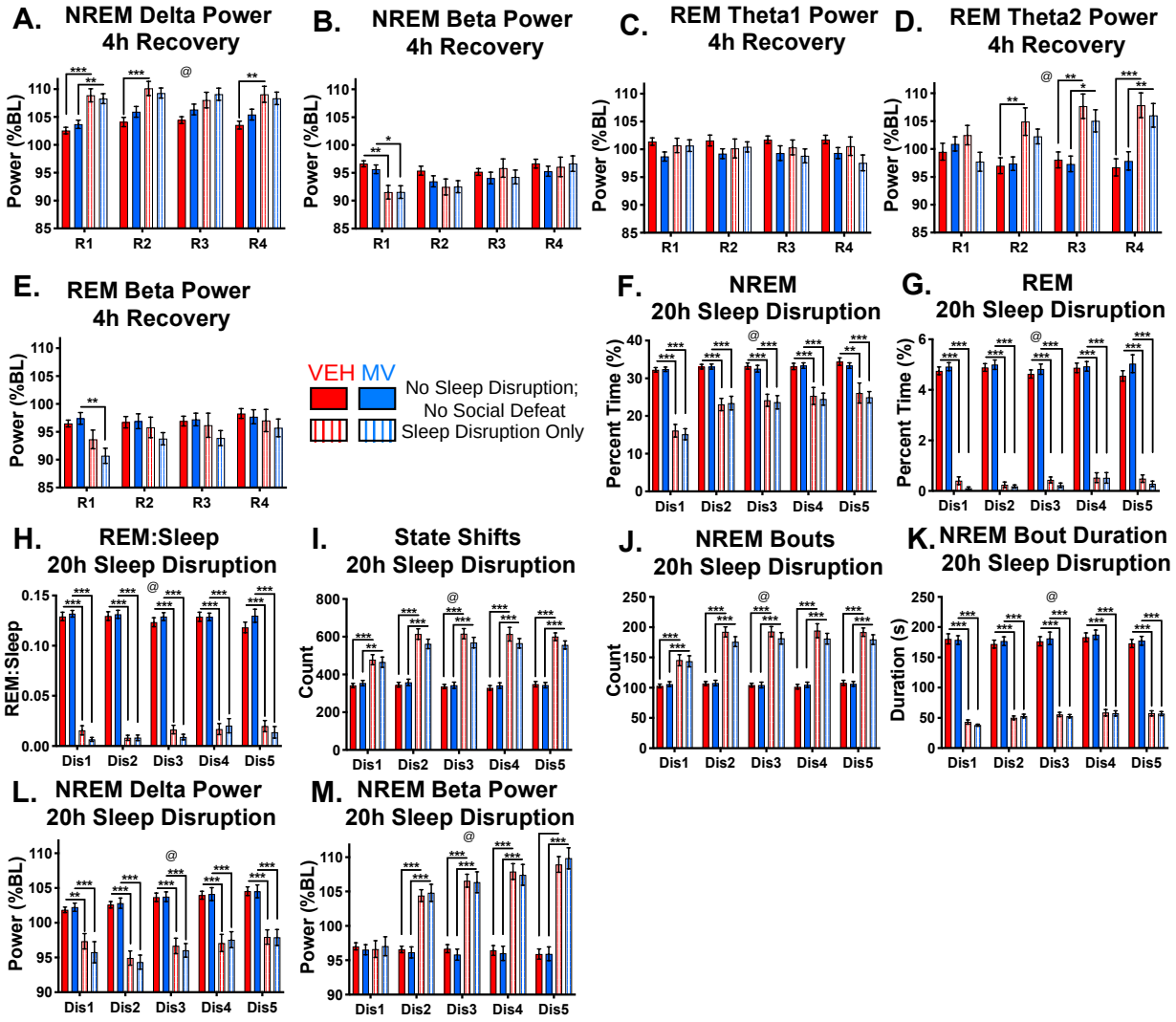

Figure 3.3: Other Sleep Measures During the Sleep Disruption Protocol. Continuation of Figure 3.1. (A) NREM EEG delta (0.5-4 Hz) power, (B) NREM EEG beta (15-30 Hz) power, (C) REM theta1 (4-8 Hz) power, (D) REM theta2 (8-12 Hz) power, and (E) REM beta power during the 4 hour (ZT2-ZT6) *ad libitum* sleep recovery windows during the sleep disruption protocol. EEG power is reported as a percent of baseline values between ZT2-ZT6. (F) NREM, (G) REM, (H) REM:Sleep ratio, (I) state shifts, (J) NREM bout count, (K) median NREM bout duration, (L) NREM EEG delta power, and (M) NREM EEG beta power during the daily 20-hour window (ZT6-ZT2) that the automated sleep disruption units were functioning. Data are means ± SEM. Symbols: @ $p < 1.0 \times 10^{-4}$ (overall effect of "Sleep Disruption"), linear mixed effects model; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Tukey's post hoc test). n = 29-30/group. Abbreviations: BL, baseline; Dis, sleep disruption; EEG, electroencephalography; MV, *Mycobacterium vaccae* NCTC 11659 injection; NREM, non-rapid eye movement; REM, rapid eye movement; VEH, vehicle injection; ZT, zeitgeber time.

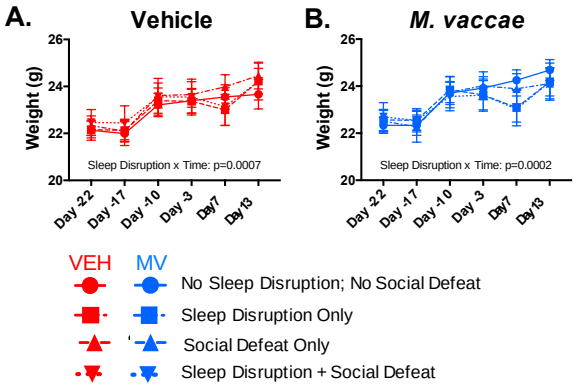

Figure 3.4: Body Weights Across the Experiment. Body weight data for all (A) vehicle and (B) *M. vaccae*-treated groups. Body weights were measured on the day of EEG/EMG surgery, on each injection day, on the day of OLM testing, and at the end of the experiment. Linear mixed effects model testing was performed for each treatment condition. Data are mean ± SEM. *n* = 14-15/group. Abbreviations: EEG, electroencephalography; EMG, electromyography; MV, *Mycobacterium vaccae* NCTC 11659 injection; OLM, object location memory; VEH, vehicle injection.

The double hit causes a maladaptive sleep phenotype acutely after social defeat that is prevented by M. vaccae immunization

After the fifth 20 h sleep disruption period, half of the mice were exposed to a 1 h social defeat or control manipulation (see **Methods**). Sleep in the immediate aftermath of traumatic events is thought to be important to processing and coping with the negative experience[Meerlo et al., 2001a; Suchecki et al., 2012; Kamphuis et al., 2015]. Therefore, upon return to their home cage, EEG/EMG signals were recorded for the next ~20 hours to examine post-stress sleep (~ZT7-ZT3, "Day 6", see **Figure 3.1a,b**). In vehicle-treated animals, NREM sleep was relatively unchanged in all groups (**Figure 3.5a**), while the sleep disruption only and double hit groups showed large increases in REM sleep and the REM:Sleep ratio compared to controls (**Figure**

3.5b,c). Social defeat increased the number of brief arousals per hour in vehicle-treated animals (overall effect of social defeat, $p = 0.004$), especially in those receiving the double hit (**Figure 3.5d**). *M. vaccae* immunization altered this phenotype. Among *M. vaccae*-treated mice, there was an increase in NREM after the double hit, relative to mice in control conditions, that was not present in vehicle-treated mice (**Figure 3.5a**). Furthermore, *M. vaccae* immunization prevented the double-hit induced increase in brief arousals that was seen in vehicle-treated mice (**Figure 3.5d**).

In vehicle-treated mice, the control manipulation, sleep disruption alone, and social defeat alone resulted in an increase in NREM EEG delta power relative to the same time window at baseline (i.e., greater than 100%), accompanied by either a reduction or no change in theta1(4-8 Hz), theta2 (8-12 Hz), sigma (11-15 Hz), or beta (15-30 Hz) power (**Figure 3.5e**). In vehicle-treated mice receiving the double hit, however, this rebound in NREM delta power, thought to be an adaptive phenomenon[Meerlo et al., 2001a; Kamphuis et al., 2015], was absent (**Figure 3.5e**). Instead, a significant increase above baseline in NREM beta power was observed (**Figure 3.5e**). In REM sleep, there was an increase in theta2 EEG power due to sleep disruption, though only the vehicle-injected, double hit group was significantly higher than the control group (**Figure 3.5g**). Similar to findings in NREM sleep, an increase in REM beta EEG power relative to baseline was only seen in the double hit group (**Figure 3.5g**).

M. vaccae immunization prevented the double hit-induced loss of NREM delta power rebound that was seen in vehicle-injected mice (**Figure 3.5f**). The increase in NREM and REM beta power relative to baseline that was seen in vehicle-injected, double hit-exposed mice was also not present in *M. vaccae*-treated mice (**Figure 3.5f,h**). *M. vaccae* groups exhibited largely the same phenotype of NREM theta1, theta2, and sigma power bands as did their vehicle-injected counterparts (**Figure 3.5f**). Overall, sleep in the immediate aftermath of the double hit ("Day 6") was characterized by increased brief arousals, a lack of NREM delta power rebound, and increased NREM and REM beta power in vehicle-treated mice, and this phenotype was prevented by *M. vaccae* immunization.

The double hit causes lasting changes in sleep that are prevented by M. vaccae immunization

In order to assess the lasting impact of sleep disruption, social defeat, and the double hit on sleep, *ad libitum* sleep was recorded for six days (day 7-12). Social defeat alone did not cause any lasting changes in sleep measures (**Figure 3.6**). In vehicle-treated mice, sleep disruption alone and the double hit did not result in a lasting change to NREM sleep compared to control (**Figure 3.7a**), but REM remained elevated above controls for multiple days (**Figure 3.7b**). There was not a sustained overall effect of sleep disruption alone on the REM:Sleep ratio in vehicle-treated mice (**Figure 3.7c**)**.** In the vehicle-treated double hit group, however, the REM:Sleep ratio was elevated compared to control until Day 11 and there was an overall effect of the double hit based on mixed effects linear modeling (**Figure 3.7c**). This lasting increase in REM:Sleep in the vehicle-injected double hit group was accompanied by a trend ($p = 0.057$) towards a decrease in

the average number of epochs of NREM preceding a REM bout (average NREM to REM duration) (**Figure 3.7d**). Furthermore, the vehicle-injected double hit group displayed a lasting elevation in the number of state shifts per 24 h that was not present in the sleep disruption only group (**Figure 3.7e**). The NREM EEG beta power was elevated in the double hit group compared to control on Day 7 but returned to control levels thereafter (**Figure 3.7f**). Importantly, *M. vaccae* immunization prevented all of the aforementioned sleep alterations due to sleep disruption alone or the double hit (**Figure 3.7**).

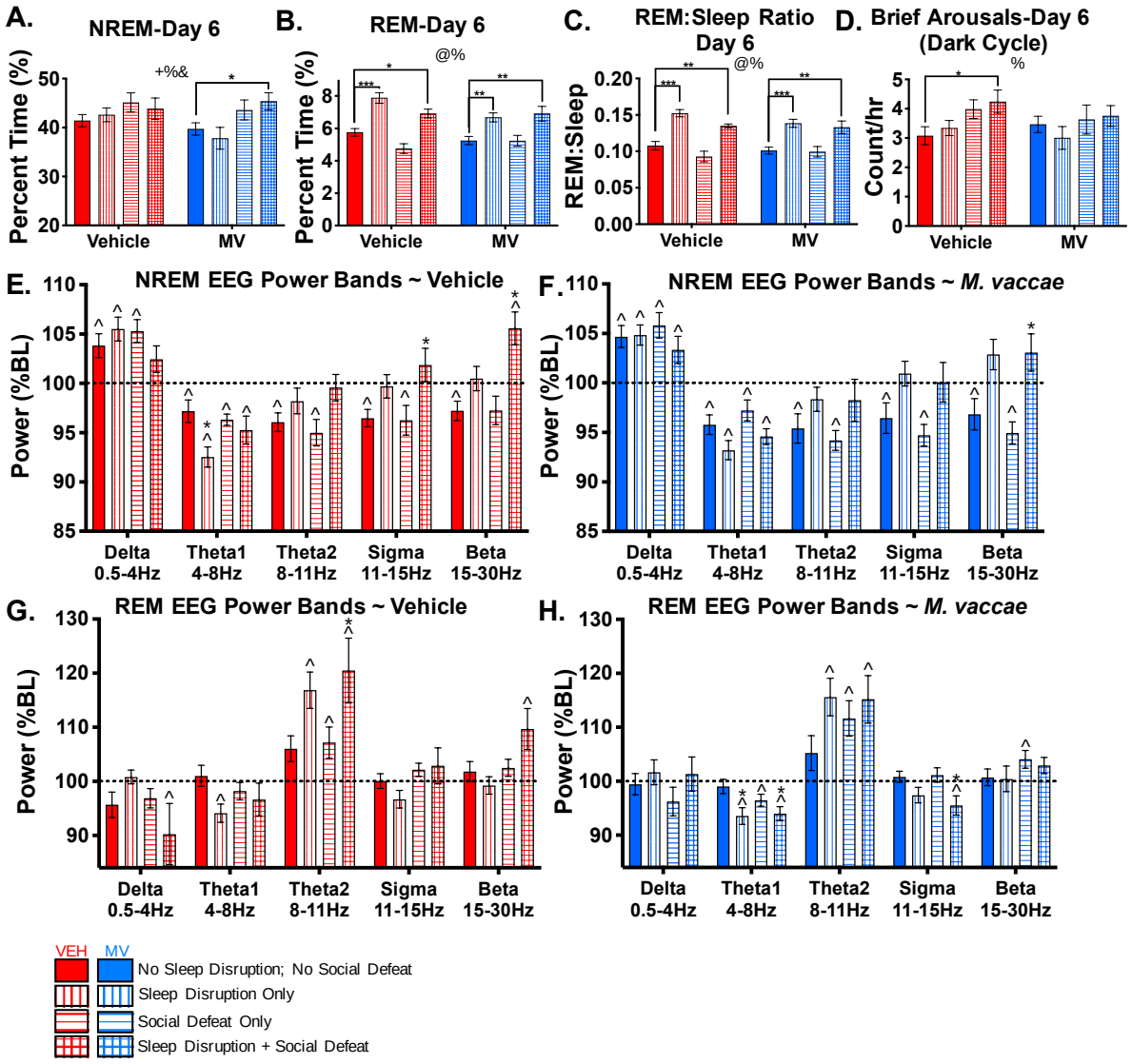

Figure 3.5: The Double Hit Causes a Maladaptive Sleep Phenotype Acutely After Social Defeat, Which is Prevented by *M. vaccae* Immunization. EEG/EMG sleep recordings were started after social defeat or control manipulation (~ZT7) and continued until object location memory (OLM) testing began the next morning (ZT3), for a ~20h window labeled "Day 6". (A) NREM, (B) REM, (C) REM:Sleep ratio during Day 6. (D) Brief arousals during the dark cycle of Day 6. (E-F) NREM EEG spectral power and (G-H) REM EEG spectral power during Day 6, expressed as a percent of the power during the identical ZT time window during baseline recording. Data are mean ± SEM. Symbols in panels (A-D): + $p < 0.05$ (overall effect of "Treatment"); @ $p < 0.05$ (overall effect of "Sleep Disruption"); % $p < 0.05$ (overall effect of "Social Defeat"); & $p < 0.05$ ("Treatment" x "Sleep Disruption" x "Social Defeat" interaction), linear mixed effects model; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Tukey's post hoc test). Symbols in panels (E-H): ^$p < 0.05$ vs 100% (Wilcoxon Rank-Sum test), *$p < 0.05$ vs Control

(Tukey's post hoc test). *n* = 10-14/group. Abbreviations: BL, baseline; EEG, electroencephalography; EMG, electromyography; OLM, object location memory; MV, *Mycobacterium vaccae* NCTC 11659 injection; NREM, non-rapid eye movement; REM, rapid eye movement; VEH, vehicle injection; ZT, zeitgeber time.

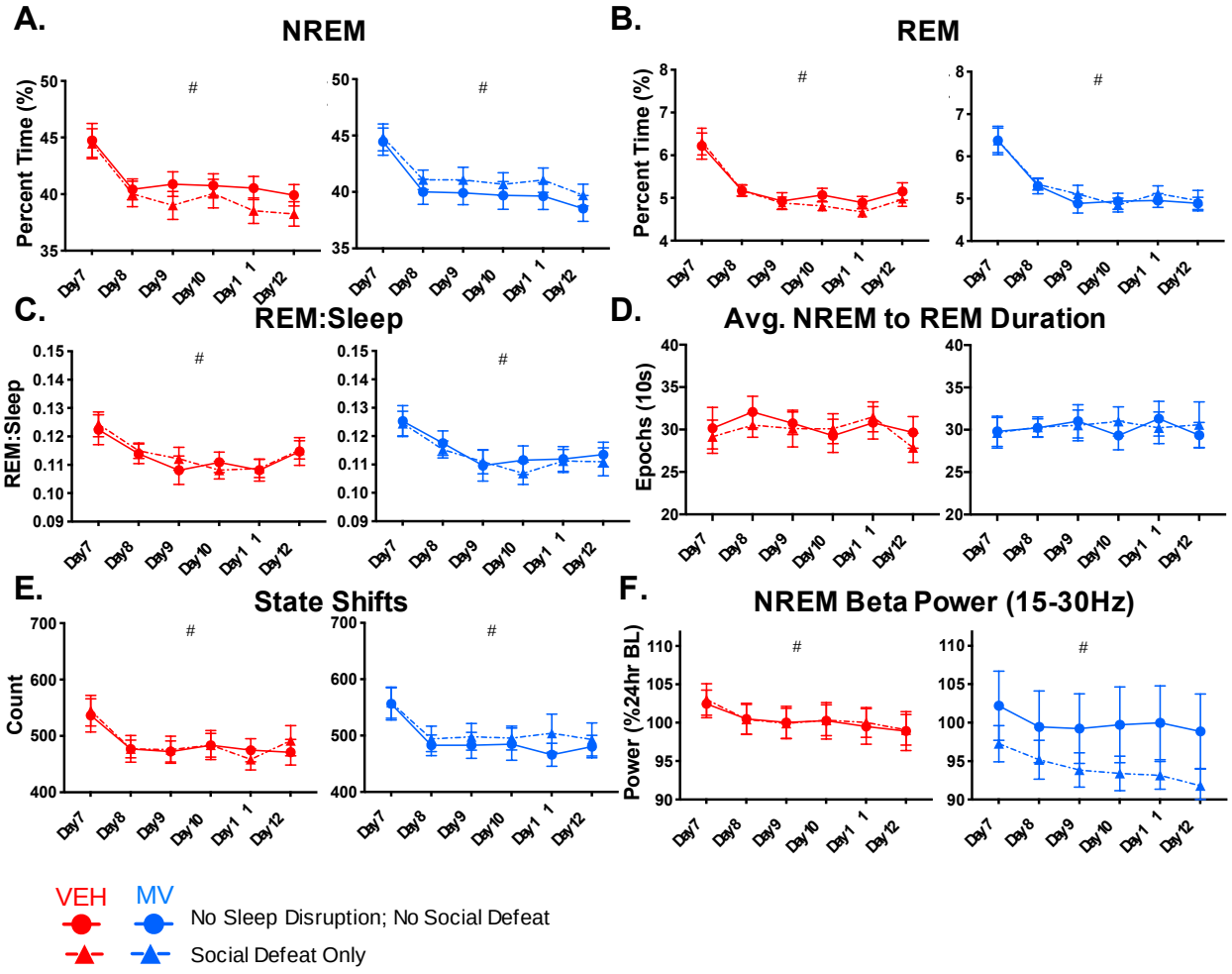

Figure 3.6: Social Defeat Alone Did Not Result in Lasting Sleep Changes. Continuation of Figure 3.7. (A) NREM, (B) REM, (C) REM:Sleep ratio, (D) average number of epochs of NREM preceding a REM bout, (E) state shifts, and NREM EEG beta (15-30 Hz) power is reported for control and social defeat alone groups. Symbols: # $p < 0.05$ (overall effect of time), linear mixed effects model. Data are mean ± SEM. *n* = 11-14/group. Abbreviations: MV, *Mycobacterium vaccae* NCTC 11659 injection; NREM, non-rapid eye movement; REM, rapid eye movement; VEH, vehicle injection.

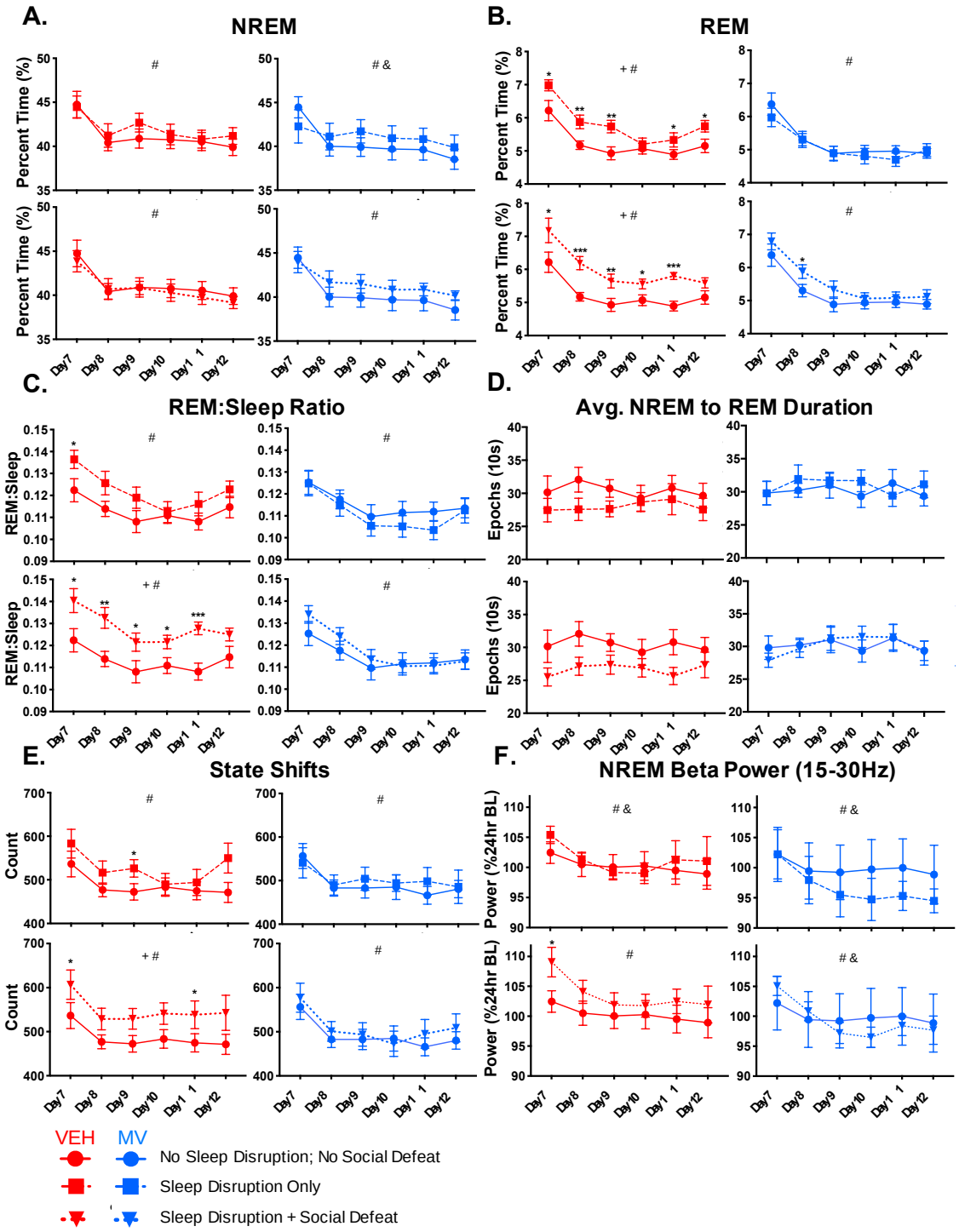

Figure 3.7: The Double Hit Causes Lasting Changes in Sleep That Are Prevented by *M. vaccae* Immunization. *Ad libitum* recovery sleep was recorded from Day 7 (after object location memory testing) until the end of the experiment (Day 12). 24-h bins of (A) NREM, (B) REM,

(C) REM:Sleep ratio, (D) average number of epochs of NREM preceding a REM bout, (E) state shifts, and (F) NREM EEG beta (15-30 Hz) power is reported for control, sleep disruption only, and sleep disruption plus social defeat (double hit) groups. Beta power is reported as a percent of 24-h baseline. Data are mean ± SEM. Symbols: + $p < 0.05$ (overall effect of sleep disruption or double hit), # $p < 0.05$ (overall effect of time), & $p < 0.05$ (group x time interaction), linear mixed effects model; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Tukey's post hoc testing between groups at individual timepoints). n = 10-14/group. Abbreviations: BL, baseline; MV, *Mycobacterium vaccae* NCTC 11659 injection; NREM, non-rapid eye movement; REM, rapid eye movement; VEH, vehicle injection.

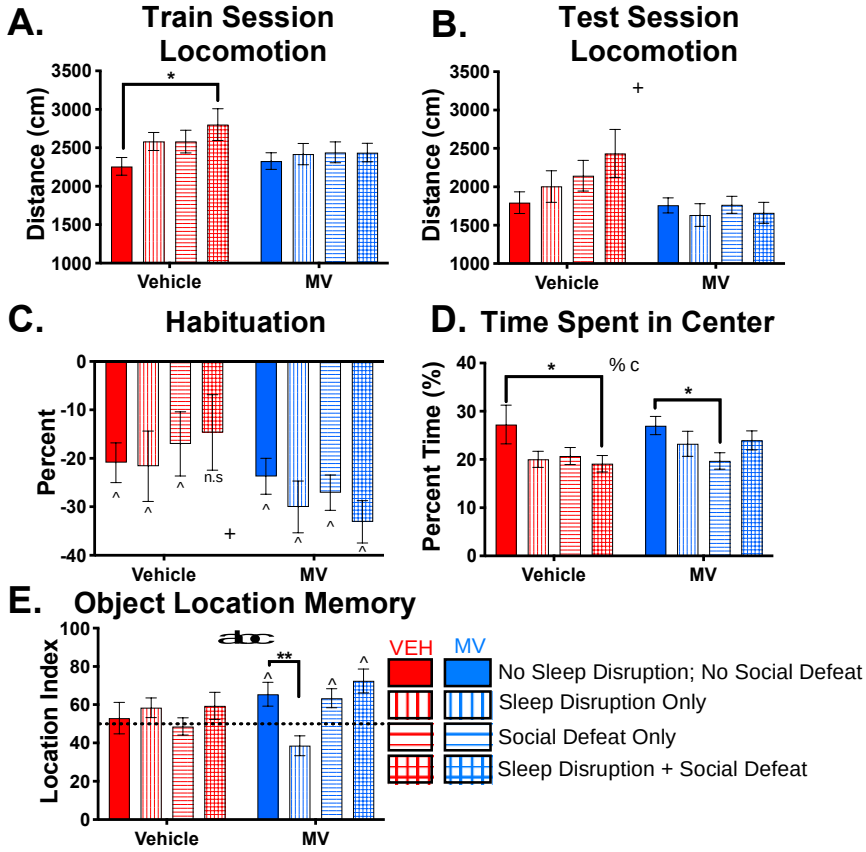

Figure 3.8: The Double Hit Causes a Behavioral Phenotype That is Prevented by *M. vaccae* Immunization. Approximately 24 h after acute social defeat (or control manipulation consisting of being moved to a clean cage in a quiet room for 1 h), mice were exposed to the object location memory (OLM) task, which consisted of a five-minute training session and a five-minute testing session 90 min later. (A) Total locomotion during the training session and (B) total locomotion during the testing session. (C) Habituation to the testing arena over the two sessions as measured by the percent change in locomotion from the training to the testing session. (D) Percent of the five-minute training session spent in the center of the arena. (E) Object location memory, measured as percent of exploration time devoted to exploring the displaced object during the testing session (Location Index). Significant deviations from 50% indicate learning. Data are

mean ± SEM. Symbols: + $p < 0.05$ (overall effect of "Treatment"); % $p < 0.05$ (overall effect of "Social Defeat"); a $p < 0.05$ ("Treatment" x "Sleep Disruption" interaction); b $p < 0.05$ ("Treatment" x "Social Defeat" interaction); c $p < 0.05$ ("Social Defeat" x "Sleep Disruption" interaction), linear mixed effects model; *$p < 0.05$, ** $p < 0.01$ (Dunnett's post hoc test). n = 13-15/group. Abbreviations: BL, baseline; MV, *Mycobacterium vaccae* NCTC 11659 injection; NREM, non-rapid eye movement; OLM, object location memory; REM, rapid eye movement; VEH, vehicle injection.

The double hit causes hyperlocomotion and anxiety-like behavioral responses that are prevented by M. vaccae immunization

The day after social defeat stress, behavior was assessed using the object location memory (OLM) task, which consists of a training session followed by a testing session 90 min later (see **Methods**). Vehicle-treated mice receiving the double hit were hyperlocomotive in the training session compared to controls (**Figure 3.8a**). Habituation to the testing arena was measured by the percent change in locomotion from one session to the next. While control, sleep disruption only, and social defeat only groups displayed significant reductions in locomotion, mice receiving the double hit did not (**Figure 3.8c**). *M. vaccae* treatment prevented the double hit-induced hyperlocomotion in the training session (**Figure 3.8a**), reduced locomotion in the testing session (**Figure 3.8b**), and prevented the double hit-induced loss of habituation (**Figure 3.8c**). Anxiety-like defensive behavioral responses during the initial training session were assessed by measuring the percent of time avoiding the center of the arena (see **Methods**). There was an overall effect of social defeat and a sleep disruption x social defeat interaction based on linear mixed effect modeling, as well as a significant reduction in time spent in the center due to the double hit compared to controls in vehicle-injected mice (**Figure 3.8d**). *M. vaccae*-treated mice showed a reduction in time spent in the center in the social defeat alone group compared to *M.*

vaccae-treated controls, but the sleep disruption and double hit groups were unchanged relative to controls (**Figure 3.8d**). Object location memory is a hippocampal-dependent learning task that is sensitive to stress and anxiety[Murai et al., 2007]. We found a treatment by sleep disruption interaction, treatment by social defeat interaction, and social defeat by sleep disruption interaction in object location memory using linear mixed effects modeling (**Figure 3.8e**). While no vehicle-treated groups had location indices significantly different from 50%, *M. vaccae*-treated mice receiving control manipulations, social defeat alone, or the double hit learned the task (**Figure 3.8e**). *M. vaccae*-treated mice receiving sleep disruption alone showed a *decrease* in location index below 50%, that trended towards statistical significance (one sample Wilcoxon Rank-Sum test vs 50% $p = 0.08$; **Figure 3.8e**). In summary, the double hit caused a behavioral phenotype consisting of hyperlocomotion and increased anxiety-like behavior in vehicle-treated mice, and *M. vaccae* immunization largely prevented this phenotype and enhanced learning in the OLM task.

Sleep disruption, social defeat, and the double hit impacted multiple inflammatory markers. M. vaccae treatment ameliorated some, but not all, of these effects

Serum, mesenteric lymph nodes (MLN), and spleen were collected at the end of the experiment, after the 7 days of recovery sleep, to investigate the inflammatory impact of the sleep disruption, social defeat, and the double hit in both vehicle-treated and *M. vaccae*-treated mice (see **Figure 3.1a,b**). There were no overall effects of treatment, sleep disruption, or social defeat on serum IL-6 or serum monocyte chemoattractant protein (MCP-1; i.e., CCL2) (**Figure 3.9a,b**). There

was an overall effect of social defeat on serum cytokine-induced neutrophil chemoattractant-1 (CINC-1 i.e., CXCL-1) based on linear mixed effect modeling, and post hoc testing revealed a significant increase in CINC-1 due to the double hit compared to controls in vehicle-treated mice (**Figure 3.9c**). Serum CINC-1 was not increased in the double hit group compared to controls in *M. vaccae*-treated mice (**Figure 3.9c**). Although linear mixed effect modeling did not reveal an overall effect of treatment, *a priori* comparison of vehicle-treated and *M. vaccae*-treated control groups revealed an increase of serum CINC-1 in the *M. vaccae* group (**Figure 3.9c**). There was an overall effect of sleep disruption and an overall effect of social defeat on spleen weight based on linear mixed effects modeling, and post hoc testing indicated that the double hit resulted in an increase in spleen weight compared to controls in the *M. vaccae*-treated mice (**Figure 3.9d**). The sleep disruption protocol had an impact on multiple cytokines measured in the MLN's. While there were no overall effects of treatment, sleep disruption, or social defeat on MLN IL-17A or IL-1B (**Figure 3.9e,f**), there was an overall effect of sleep disruption based on linear mixed effect modeling on MLN IL-6, IFNγ, IL-4, and IL-10 (**Figure 3.9g-j**). Within these data, the only post hoc comparison to reach statistical significance was an increase in MLN IFNγ seen in the *M. vaccae*-treated sleep-disrupted group compared to *M. vaccae*-treated controls (**Figure 3.9h**).

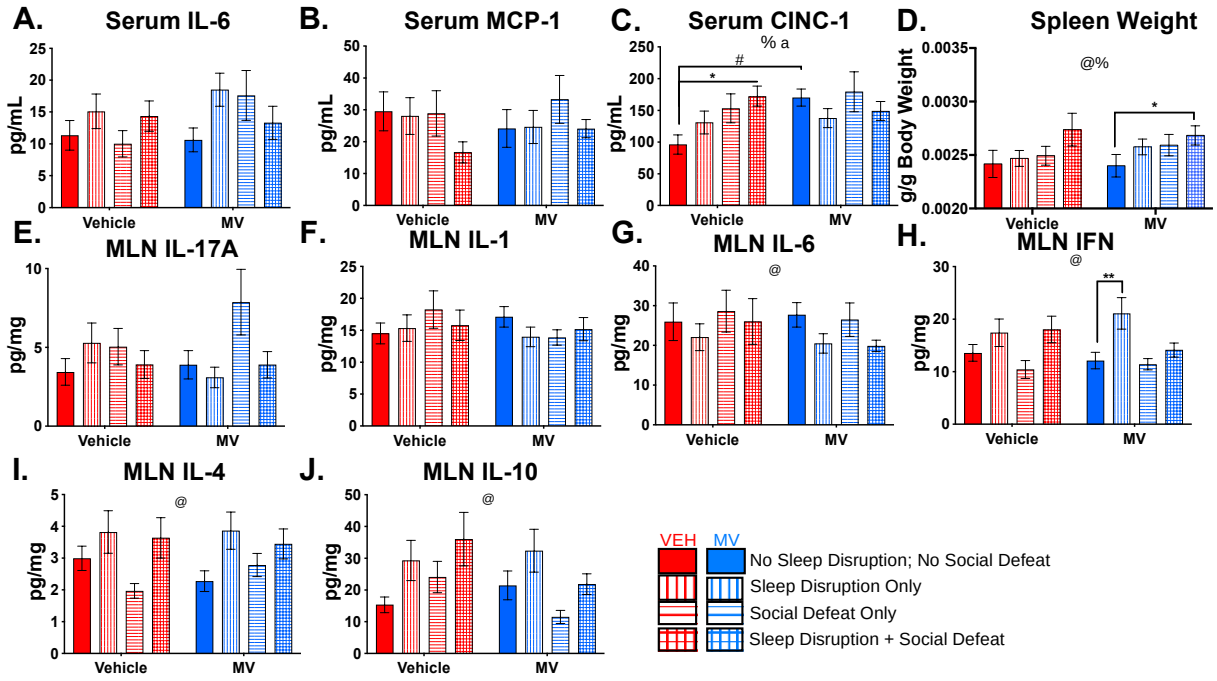

Figure 3.9: Impact of the Double Hit and *M. vaccae* on Serum Cytokines, Mesenteric Lymph Node Cytokines, and Spleen Weight. Serum levels of (A) IL-6, (B) MCP-1 (i.e., CCL2), and (C) CINC-1 (i.e., CXCL-1) were assessed using a magnetic bead multiplex. (D) Spleens were weighed and normalized to body weight. Mesenteric lymph nodes were homogenized and concentrations of (E) IL-17A, (F) IL-1beta (G) IL-6, (H) IFNγ, (I) IL-4, and (J) IL-10 were assessed using a magnetic bead multiplex and normalized to total protein concentration. Data are mean ± SEM. Symbols: @ $p < 0.05$ (overall effect of "Sleep Disruption"); % $p < 0.05$ (overall effect of "Social Defeat"); a $p < 0.05$ ("Treatment" x "Sleep Disruption" interaction), linear mixed effects model; *$p < 0.05$, ** $p < 0.01$ (Dunnett's post hoc test); # $p < 0.01$ (*a priori* Wilcoxon Rank-Sum test). n = 11-15/group. Abbreviations: IL, interleukin; CINC-1, cytokine-induced neutrophil chemoattractant-1; MCP-1, monocyte chemoattractant protein-1; MLN, mesenteric lymph node; MV, *Mycobacterium vaccae* NCTC 11659 injection.

Multiple characteristics of post-double hit sleep predict later changes in sleep, physiology, and behavior

We then performed pairwise Spearman's ranked correlation testing of 32 selected variables to examine the relationships among post-social defeat (Day 6) sleep changes, lasting sleep changes (Day 11), and behavioral/physiological changes induced by the double hit (**Figure 3.10, Figure**

3.11, Supplemental File 3.2). In vehicle-injected mice, we found 54 associations that were significant after correcting for multiple comparisons (**Figure 3.10a**). Interestingly, multiple sleep features immediately after social defeat stress (Day 6) correlated with behavioral, sleep, or physiological outcomes days later in vehicle-treated mice. NREM EEG beta power on Day 6 was positively correlated with the REM:Sleep ratio on Day 11 (**Figure 3.10b**). Other NREM EEG power bands on Day 6 also correlated with outcome measures later in the experiment. NREM delta power on Day 6 correlated negatively with NREM beta power on Day 11 (**Figure 3.11a**), while NREM theta2 and sigma power both correlated positively with NREM beta power on Day 11 (**Figure 3.11b,c**). NREM theta2 on Day 6 also correlated negatively with time spent in the center of the arena during the OLM task (**Figure 3.11d**) and positively with spleen weight at the end of the experiment (**Figure 3.11e**). Brief arousals on Day 6 were positively correlated with spleen weight at the end of the experiment (**Figure 3.10c**). Additionally, the REM:Sleep ratio on Day 6 correlated negatively with time spent in the center of the arena during the OLM task (**Figure 3.10d**) and correlated positively with spleen weight at the end of the experiment (**Figure 3.11f**).

In *M. vaccae*-treated mice, there were 42 significant ($q < 0.05$) correlations between the 32 variables (**Figure 3.10e**), but the average strength of each tested correlation was significantly weaker than in vehicle-injected groups, as the average Spearman's rho was significantly smaller (**Figure 3.11g**). Unlike in vehicle-treated mice, NREM EEG beta power was not significantly associated with the REM:Sleep ratio on Day 11, brief arousals on Day 6 were not associated with spleen weight, and the REM:Sleep ratio on Day 6 was not correlated with behavior the next day

(**Figure 3.10f-h**). In fact, of the aforementioned associations between sleep on Day 6 and subsequent behavior or sleep in vehicle-treated mice, only the negative correlation between NREM delta power on Day 6 and NREM beta power on Day 11 was maintained in *M. vaccae*-treated mice (**Figure 3.11h**). Instead, there were many associations between EEG power on Day 6 and MLN cytokines. NREM theta2, sigma, and beta power were all positively correlated with both MLN IFNγ and MLN IL-10 (**Figure 3.11i-n**). NREM theta2 and NREM sigma power on Day 6 were also positively associated with locomotion during the OLM training session in *M. vaccae*-treated mice (**Figure 3.11o,p**). Finally, while REM beta power was not significantly correlated with any of the other 31 measures after correction for multiple comparisons in vehicle-treated mice, it was positively correlated with the REM:Sleep ratio at Day 11 (**Figure 3.11q**), and negatively correlated with MLN IL-17 (**Figure 3.11r**) in *M. vaccae*-treated mice. In summary, multiple features of post-social defeat sleep (including increased NREM beta power, fragmentation, and the REM:Sleep ratio) predicted double hit-induced changes in sleep, behavior, and physiology in vehicle-injected mice. In *M. vaccae*-treated mice, many of these associations were not present, and instead a set of associations between Day 6 sleep EEG power and MLN cytokines emerged.

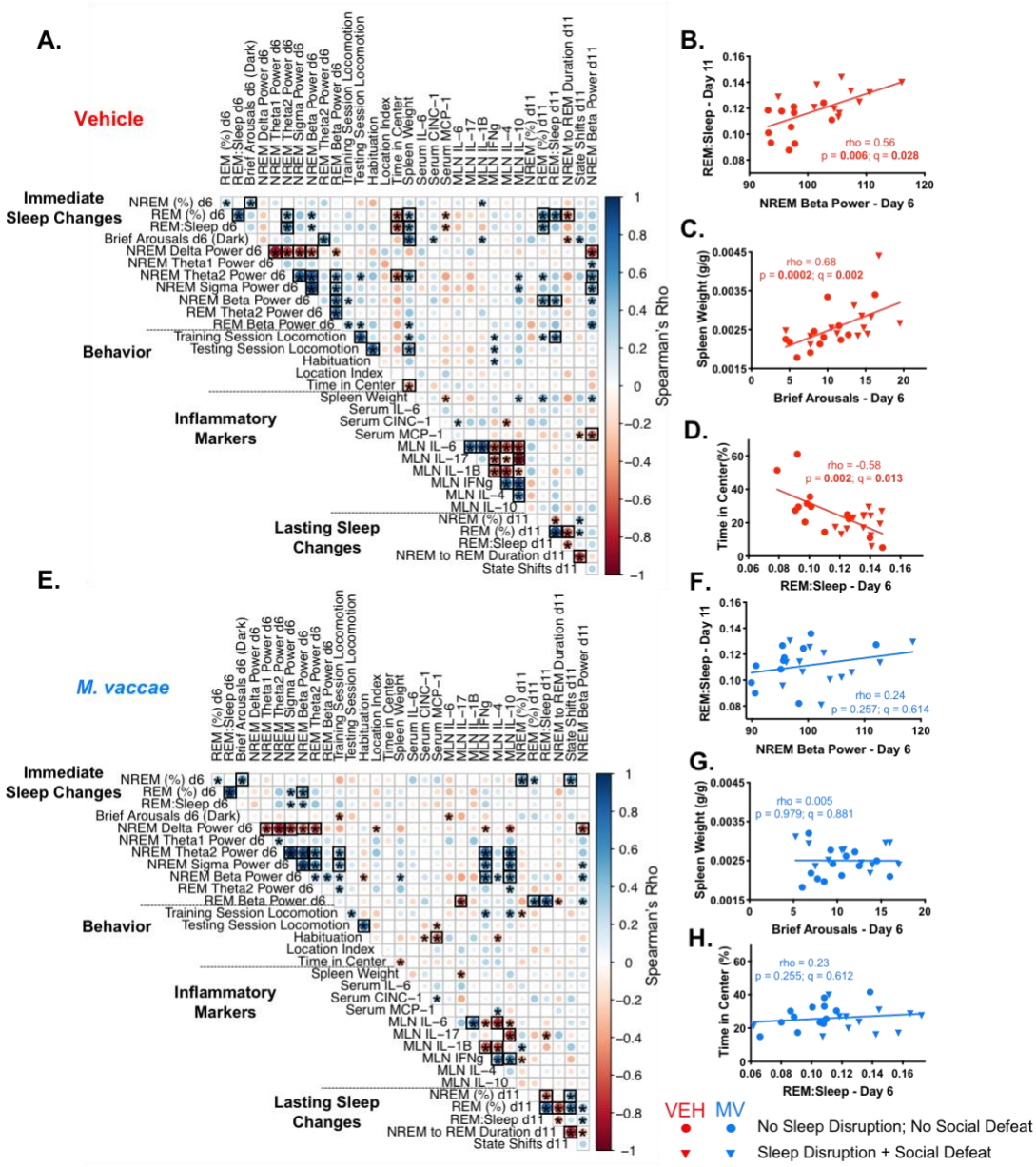

Figure 3.10: Associations Among Immediate Sleep Changes, Lasting Sleep Changes, Behavior, and Physiological Measures in Vehicle-Treated and *M. vaccae*-Treated Mice. Pairwise Spearman's rank order correlations of 32 measures of interest from throughout the experiment were computed for (A) vehicle-treated and (E) *M. vaccae* (MV)-treated mice that received either control manipulations or sleep disruption plus social defeat (double hit). The value of the Spearman's *rho* for each comparison is represented by the size and shade of circle in each square of the correlation plot, with darker blue meaning more positive *rho* and darker red meaning more negative *rho*. Asterisks indicate uncorrected $p < 0.05$, while black boxes indicate $q < 0.05$. REM:Sleep ratio on Day 11 vs NREM EEG beta (15-30 Hz) power on Day 6, spleen

weight per gram of body weight at the end of the experiment vs brief arousals during the dark phase of Day 6, and time spent in the center of the arena during the OLM training session vs REM:Sleep on Day 6 are presented in (B-D) vehicle-treated and (F-H) MV-treated mice. *n* = 9-12/group. Abbreviations: EEG, electroencephalography; IFNg, interferon gamma; IL, interleukin; CINC-1, cytokine-induced neutrophil chemoattractant-1; MCP-1, monocyte chemoattractant protein-1; MLN, mesenteric lymph node; MV, *Mycobacterium vaccae* NCTC 11659 injection; NREM, non-rapid eye movement; OLM, object location memory; REM, rapid eye movement; VEH, vehicle injection.

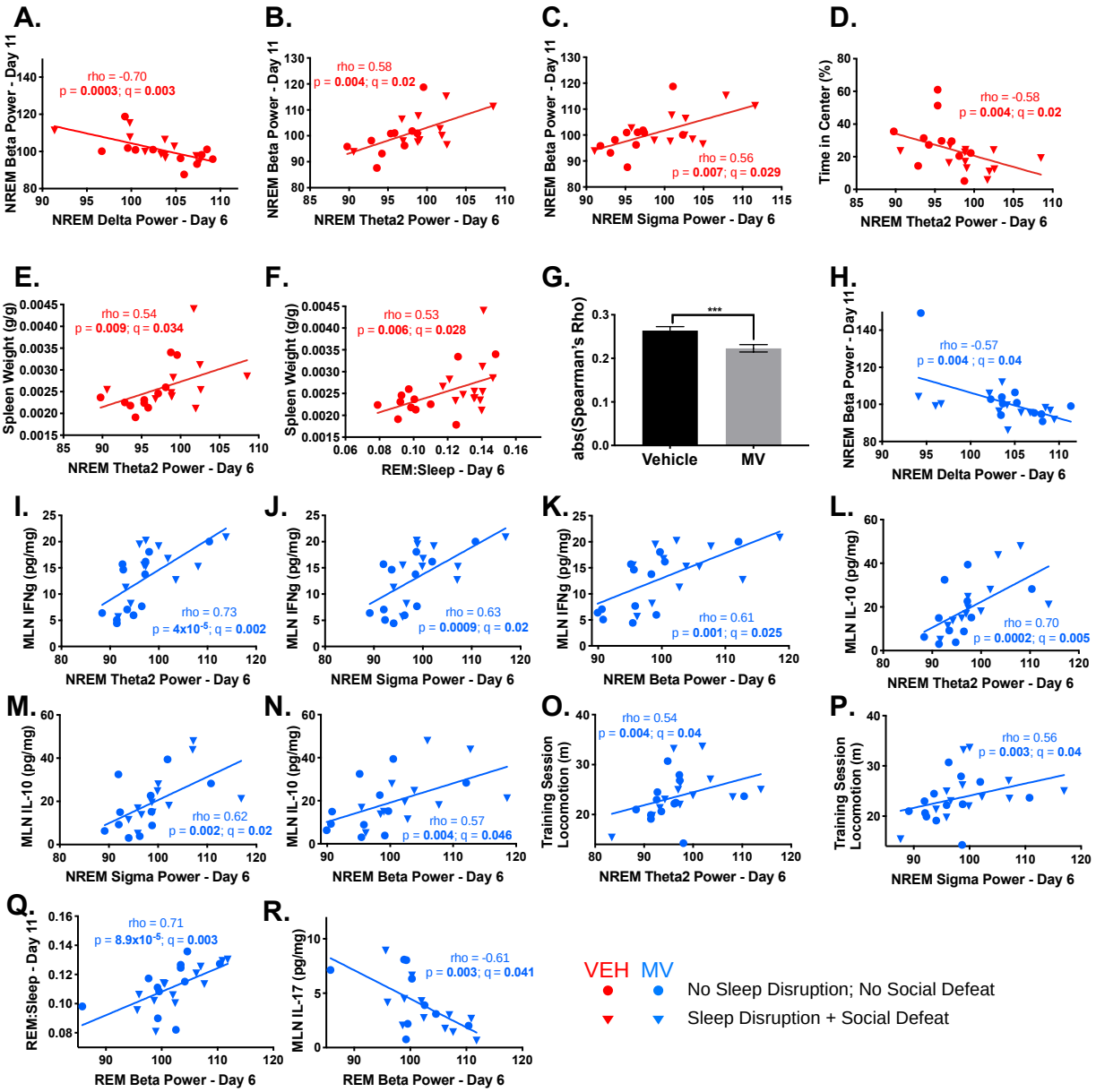

Figure 3.11: Selected Associations Between Acute Sleep Changes, Lasting Sleep Changes, Behavior, and Physiological Measures. Continuation of Figure 3.10. Pairwise Spearman's rank order correlations of 32 measures of interest from throughout the experiment were computed for vehicle-treated and *M. vaccae* (MV)-treated mice that received either control manipulations or sleep disruption plus social defeat (double hit). NREM beta (15-30 Hz) EEG power on Day 11 (percent of baseline) vs (A) NREM delta (0.5-4 Hz) power (B) NREM theta2 (8-12 Hz) power, and (C) NREM sigma (12-15 Hz) power on Day 6 in vehicle-treated mice. (D) Time spent in the center of the arena during the object location memory (OLM) training session vs NREM theta2 power on Day 6 in vehicle-treated mice. Spleen weight at the end of the experiment vs (E)

NREM theta2 power on Day 6 and (F) REM:Sleep ratio on Day 6 in vehicle-treated mice. (G) The average of the absolute value of all pairwise Spearman's Rho (n = 512) values for the vehicle and MV treatment groups. (H) NREM beta power on Day 11 vs NREM delta power on Day 6 in MV-treated mice. Mesenteric lymph node (MLN) interferon gamma (IFNγ) vs (I) NREM theta2 power on Day 6, (J) NREM sigma power on Day 6, and (K) NREM beta power on Day 6 in MV-treated mice. MLN interleukin (IL) 10 vs (L) NREM theta2 power on Day 6, (M) NREM sigma power on Day 6, and (N) NREM beta power on Day 6 in MV-treated mice. Locomotion during the training session of the OLM task vs (O) NREM theta2 power on Day 6, and (P) NREM sigma power on Day 6 in MV-treated mice. (Q) REM:Sleep ratio on Day 11 vs REM beta power on Day 6 in MV-treated mice. (R) MLN IL-17 vs REM beta power on Day 6 in MV-treated mice. Symbols: ***$p < 0.001$, Wilcoxon Rank-Sum test. n = 9-12/group. Abbreviations: IL, interleukin; MLN, mesenteric lymph node; MV, *Mycobacterium vaccae* NCTC 11659 injection; NREM, non-rapid eye movement; OLM, object location memory; REM, rapid eye movement; VEH, vehicle injection.

D. Discussion

In this we study tested the hypothesis that sleep disruption induces vulnerability to lasting changes in inflammatory state, sleep, and behavior brought after a "second hit" of acute social defeat stress in mice (the double hit). Furthermore, we investigated immunization with heat-killed *Mycobacterium vaccae* NCTC 11659, a nonpathogenic, soil-derived bacterium with immunoregulatory and anti-inflammatory properties, as a countermeasure to improve resilience to sleep disruption, acute social defeat stress, and the double hit. We found that the double hit resulted in sleep and behavioral phenotypes that were longer lasting and more severe than was seen after sleep disruption or acute social defeat alone. In particular, a maladaptive sleep phenotype immediately after social defeat stress was present only in the vehicle-injected double hit group, multiple features of which predicted changes in behavior, physiology, and sleep disturbances nearly one week later. *M. vaccae* immunization prevented nearly all of the

components of the double hit phenotype. Together, these results suggest sleep disruption is a factor promoting vulnerability to lasting effects of acute stress, that inappropriate high frequency EEG power during post-stress sleep is a marker predicting future stress-related sleep impairments, and that immunization with *M. vaccae* is a promising avenue to effectively prevent the development of many of the aspects of the double hit phenotype.

In vehicle-treated mice, five days of repeated sleep disruption potentiated the response to subsequent acute social defeat, resulting in a maladaptive sleep phenotype that was evident within the first 20 hours after social defeat. This phenotype was characterized by increased brief arousals during sleep compared to control mice along with changes to NREM and REM EEG power spectra. Vehicle-treated mice that were mildly stressed by the control manipulation (unplugging from EEG/EMG recording devices and moved in a new cage to a quiet room), those that experienced the sleep disruption protocol alone, and those that experienced 1 h of social defeat alone all displayed rebounds in NREM delta power on Day 6, but the vehicle-treated double hit group did not. NREM delta power is an accepted marker for the homeostatic drive for sleep[Leger et al., 2018], and is thought to be important for coping with stressful events[Meerlo et al., 2001a; Kamphuis et al., 2015; Henderson et al., 2017; Feng et al., 2020]. Thus, we interpret an absence of a rebound in delta power after the double hit to be part of a maladaptive change in sleep EEG. The vehicle-treated mice receiving the double hit instead displayed increases in NREM and REM beta power. An increase in high frequency oscillations during sleep is thought to be an indication of cortical hyperarousal, and has been associated with

insomnia[Merica et al., 1998; Spiegelhalder et al., 2012; Fernandez-Mendoza et al., 2016], suicidal ideation[Dolsen et al., 2017], and PTSD[Woodward et al., 2000b; Germain et al., 2006; Sharma et al., 2018]. Furthermore, two recent studies examining sleep architecture and EEG activity in individuals with PTSD reported the combination of reduced NREM delta power along with increased frontal high frequency power in both NREM[de Boer et al., 2019; Wang et al., 2019a] and REM[Wang et al., 2019a] sleep, similar to the pattern seen in this study.

Importantly, along with the maladaptive acute post-stress sleep phenotype, sleep during the 7 days of *ad libitum* recovery in vehicle-injected, double hit mice was altered as well. While increases in NREM and REM sleep in the immediate aftermath of a stressful event are thought to be adaptive[Mellman et al., 2002; Mellman et al., 2007; Suchecki et al., 2012; Cowdin et al., 2014], alterations in sleep that persist long after a traumatic event are thought to be pathological and are commonly observed in trauma- and stressor-related disorders in humans[Ross et al., 1989; Germain, 2013]. Vehicle-injected mice exposed to the double hit exhibited an increase in sleep fragmentation (as illustrated by an increase in the total number of state shifts), REM sleep, and the REM:Sleep ratio many days after return to *ad libitum* sleep, and these sleep disturbances have been seen in models of highly stress-reactive mice[Fenzl et al., 2011] and in studies of humans with a diagnosis of PTSD. While the exact sleep disturbances seen in PTSD vary[Ross et al., 1994; Mellman et al., 1995; Mellman et al., 1997; Woodward et al., 2002], a meta-analysis of polysomnographic studies conducted with military veterans and civilians with PTSD found modest changes in multiple sleep parameters including an increase in REM density long after the

traumatic event compared to individuals without PTSD[Kobayashi et al., 2007]. In the present study, the REM:Sleep ratio and measures of sleep fragmentation were increased up to six days into recovery sleep after the double hit in vehicle-injected mice, long after they had recovered to control levels in the sleep disruption alone group. A recent study in humans did not find signs of increased responsivity to acute psychosocial challenge after a single night of sleep deprivation[Schwarz et al., 2018], so it is possible only more protracted or severe disruption protocols like the one in this study increase stress vulnerability. The mechanism by which this repeated sleep disruption increases stress vulnerability warrants further study, and may involve systemic changes that promote a proinflammatory state[Vgontzas et al., 2004; Aho et al., 2013], including changes to the fecal microbiome and metabolome[Bowers et al., 2020].

We identified multiple features of post-social defeat sleep in vehicle-injected mice that predicted double hit-induced changes to behavior, physiology, and sleep 1-7 days later. These included increased NREM beta power, decreased NREM delta power, increased brief arousals, and increased REM:Sleep ratio, which predicted increases in the REM:Sleep ratio on Day 11, increased NREM beta power on Day 11, increased spleen weight at the end of the experiment, and increased anxiety-like defensive behavioral responses during OLM testing on Day 7. These results suggest features of the first sleep period in the immediate aftermath of a traumatic event (such as signs of cortical hyperarousal paired with lack of delta power rebound) may have utility as biomarkers that can be used to identify individuals with an increased risk of developing lasting trauma-related sleep pathology. However, longitudinal studies in rodent models of PTSD

and in humans are required to further evaluate the predictive power of markers during post-trauma sleep and to potentially elucidate the mechanisms involved.

Importantly, *M. vaccae* immunization completely prevented the development of the double hit sleep phenotype in this study (see **Table 3.1**). The earliest signs of protection in *M. vaccae*-treated mice started during the five-day sleep disruption protocol. These early effects may identify sleep changes conferring stress vulnerability. In vehicle-treated mice, although the amount of REM sleep lost during the 20-hour sleep disruption windows did not change substantially from one day to the next, the subsequent REM rebounds seen during the four-hour recovery windows increased in magnitude across the days of the protocol. This increased REM:Sleep ratio persisted for two days after the sleep disruption protocol in mice who did not receive the second hit of social defeat. A recent study in mice demonstrated that chronic low level stress gradually led to an increase in the REM drive, manifested by an increase in the REM:Sleep ratio, that correlated with measures of HPA axis function[Nollet et al., 2019]. Therefore, the gradual increase in REM during recovery sleep seen in vehicle-injected mice may be indicative of a cumulative stress response process driving REM sleep on top of the homeostatic drive to recover the REM lost in the previous 20 hours. Importantly, this cumulative increase in REM sleep did not occur in *M. vaccae*-treated, sleep-disrupted mice. So, even though the sleep disruption was equally effective in reducing sleep totals in *M. vaccae*-treated mice, their sleep appeared to recover more quickly than in their sleep-disrupted, vehicle-treated counterparts, without any cumulative effects of the five-day protocol. Therefore, *M. vaccae*

immunization may modulate the response to the sleep disruption itself, thereby preventing both double hit-induced cortical hyperarousal/sleep fragmentation as well as subsequent protracted increases in sleep fragmentation and the REM:Sleep ratio.

Another aspect of the double hit phenotype that was prevented by *M. vaccae* was a change in behavior observed during OLM testing. *M. vaccae* prevented the double hit-induced increase in locomotion in both the training and testing sessions. Hyperlocomotion has been observed in models of chronic mild stress[Harris et al., 1997; Spasojevic et al., 2016], and, along with the signs of cortical hyperarousal during sleep, may be indicative of increased central arousal and hypervigilance, both of which are seen in PTSD. *M. vaccae* also enhanced two measures of memory during OLM testing. First, it maintained habituation to the OLM chamber that was lost after the double hit in vehicle-treated mice, again indicating prevention of a persistent state of arousal and hypervigilance. Second, *M. vaccae*-treated mice successfully distinguished between the moved and not-moved objects in the OLM task while no vehicle-injected groups were able to do so. Object location memory is particularly sensitive to anxiety and prior stress[Murai et al., 2007], so it is possible that any experimental manipulation, even the control manipulation used in the place of social defeat, was stressful enough to block learning in the vehicle-treated mice. It is unclear from these data alone whether the improvement in OLM memory seen in *M. vaccae*-treated groups was due to resilience to stressful experimental manipulations or due to enhancement of hippocampal memory *per se*. Recent studies have demonstrated that *M. vaccae* enhanced fear extinction in the fear-potentiated startle paradigm in rats whether administered

before[Fox et al., 2017; Loupy et al., 2019] or after[Hassell et al., 2019] fear conditioning, and that it prevented memory impairments in a model of post-operative cognitive dysfunction[Fonken et al., 2018]. However, the impact of *M. vaccae* immunization on various forms of memory in a stressor-independent paradigm has not been explicitly studied and deserves follow-up investigation.

The exact mechanisms of *M. vaccae's* stress protective properties are unknown, but recent work supports the hypothesis that the anti-inflammatory and immunoregulatory properties of the bacterium are key contributors, both centrally and peripherally[Reber et al., 2016; Smith et al., 2019a]. This study offers some data to support this hypothesis as *M. vaccae* immunization modified the effect of sleep disruption on serum CINC-1 seven days after the sleep disruption ended. However, *M. vaccae* treatment did not attenuate the sleep disruption-induced increases in serum IL-6, MLN IFNγ, MLN IL-4, or double hit-induced increased spleen size seen at the end of the experiment, indicating that some of the inflammatory impact of the double hit was still present. A recent study in mice observed increases in serotonergic activation in the dorsal raphe nucleus acutely after *M. vaccae* injection [Siebler et al., 2018]. Another study in rats observed increases in anti-inflammatory IL-4 expression in the hippocampus eight days after *M. vaccae* administration, along with a reduction in measures of microglial priming[Frank et al., 2018]. Thus, modulation of serotonergic networks and reduced neuroinflammation may be mechanisms by which *M. vaccae* prevents double hit-induced changes in sleep and behavior, but further studies are required to test this hypothesis directly.

Some aspects of the study may limit interpretation of some results. First, the measures of inflammatory response were taken at the end of the experiment, a full seven days after social defeat. This makes measurement of markers of the inflammatory response to the double hit difficult to interpret. Follow-up studies examining physiological changes directly after the social defeat stress, when cortical hyperarousal was at its peak in vehicle-treated, double hit-exposed mice, could help elucidate the mechanism by which *M. vaccae* prevents the development of the double hit phenotype. Second, the OLM task alone does not fully characterize the behavioral impact of the double hit. Performing multiple behavioral assessments would have prevented the acquisition of undisturbed sleep, one of the most important outcome measures of this study. Evidence of the sleep loss induced by behavioral testing can be seen in this dataset, as the OLM resulted in an increase in sleep the next day in non-sleep disrupted, non-socially defeated control mice on Day 7 compared to the other days of recovery sleep. Third, this study does not address questions about the duration of the double hit phenotype or the duration of *M. vaccae*-induced protection. Follow up experiments that include a time series study will have to be performed before exploring the use of *M. vaccae* in humans.

Taken together, these results indicate that the sleep-disrupted state can be a stress vulnerable state whereby physiological and psychological effects of acute stress may be more severe and longer lasting. In this context, our data support the hypothesis that cortical hyperarousal during post-stress sleep may be an early marker of long-lasting sleep disturbances after a traumatic

event, which potentially would be useful criteria for initiation of sleep-targeted interventions before they become severe. Follow-up studies investigating the predictive potential of increased beta power during sleep are warranted. While simply avoiding repeated bouts of sleep disruption would be one approach to improve stress resilience, there are many professions, such as warfighters in combat and emergency responders, which, by the nature of their work, will unavoidably be exposed to intermittent periods of sleep disruption. Therefore, another important conclusion from this study is that immunomodulatory bacteria, and *M. vaccae* NCTC 11659 in particular, have therapeutic potential to improve health in the context of sleep disruption, a common problem in Western urban societies today.

Table 3.1: Similarities Between the Double Hit Phenotype and Features of Human Disorders

Double Hit Phenotype[1] (Figures 2-5)	Analogous Features of Stress-Related Disorders in Humans	Impact of *M. vaccae*
Increased brief arousals and state shifts (Figure 3.5d, 3e)	➢ Sleep continuity problems have been observed in many psychiatric disorders[Baglioni et al., 2016], including PTSD[Mellman et al., 1995; Baglioni et al., 2016], depression[Baglioni et al., 2016], and anxiety disorder[Baglioni et al., 2016]	**Prevented**
Lack of post-stress NREM delta power rebound (Figure 3.5e)	➢ Reduced NREM delta power has been measured in individuals with PTSD[de Boer et al., 2019; Wang et al., 2019a]	**Prevented**

Increased NREM beta power (Figure 3.5d, 3e)	➤ Increased sleep EEG beta (15-30 Hz) power has been noted in individuals with insomnia[Merica et al., 1998; Spiegelhalder et al., 2012; Fernandez-Mendoza et al., 2016], major depression with suicidal ideation[Dolsen et al., 2017], and PTSD[Woodward et al., 2000b; Germain et al., 2006; de Boer et al., 2019; Wang et al., 2019a]	**Prevented**
Long term increase in REM (Figure 3.7b-d)	➤ Many[Kobayashi et al., 2007; Mellman et al., 2014], but not all[Germain, 2013], studies in individuals with PTSD show increased REM amounts or REM density ➤ REM sleep elevation has been commonly observed in major depression[Pillai et al., 2011; Baglioni et al., 2016]	**Prevented**
Hyperlocomotion during OLM, reduced habituation, reduced time in the center of the training arena (Figure 3.8a-d)	➤ Psychomotor agitation and/or hypervigilance is common in individuals with PTSD[Shalev et al., 2017]	**Prevented**
Increased serum CINC-1 (Figure 3.9c)	➤ Increased proinflammatory cytokines have been observed in individuals with insomnia[Fernandez-Mendoza et al., 2017], anxiety disorders[Michopoulos et al., 2017], depression[Jones and Thomsen, 2013], and PTSD[von Kanel et al., 2007; Gill et al., 2009; Eraly et al., 2014; Michopoulos et al., 2017], but CINC-1 in particular has not been affiliated with any of these disorders in humans[Stuart and Baune, 2014].	**Unclear** CINC-1 was not increased compared to control in MV-double hit group, but there was an overall increase in CINC-1 due to MV. Further, many proinflammatory changes due to sleep disruption were seen in both treatment groups.

ıThe 'double hit phenotype' was defined as the collection of measures that were altered in sleep-disrupted, socially-defeated (double hit) vehicle-injected mice compared to controls (left column). Many features of this phenotype are similar to those seen in various human disorders, particularly those that are often affiliated with stress or trauma, including insomnia, depressive disorders, anxiety disorders, and PTSD. The third column indicates which of these phenotypic features was prevented by immunization with MV. Abbreviations: CINC-1, cytokine-induced neutrophil chemoattractant 1; EEG, electroencephalography; MV, *Mycobacterium vaccae* NCTC 11659; NREM, non-rapid eye movement sleep; OLM, object location memory; PTSD, posttraumatic stress disorder; REM, rapid eye movement sleep.

E. Conclusions

Within the framework of this dissertation, this study confirmed that the sleep disruption protocol used in Chapter II created a state of stress vulnerability, whereby a second hit of acute social defeat stress resulted in a lasting sleep and behavioral phenotype that was not present after sleep

disruption or social defeat alone. However, this chapter did not address the microbiome or metabolome, so multiple questions remain: Does *M. vaccae* itself modulate the fecal microbiome or metabolome? What changes to the microbiome or metabolome in the sleep-deprived, stress-vulnerable state may be influencing the organism's response to acute stress? These questions will be addressed in Chapter IV.

IV. CHAPTER IV - THE GUT MICROBIOME AND FECAL METABOLOME IN SLEEP DISRUPTION, SOCIAL DEFEAT, AND THE DOUBLE HIT: STABILIZING EFFECTS OF *MYCOBACTERIUM VACCAE* AND THE ROLE OF HYODEOXYCHOLIC ACID

A. Introduction

One of the goals of this project is to better understand the relationship between the microbiota, the molecules they produce, and stress vulnerability. In Chapter II we demonstrated that a five day sleep disruption protocol resulted in changes in the fecal microbiome and metabolome that suggested a proinflammatory, stress-vulnerable state. In Chapter III we directly tested whether this sleep disruption protocol increased vulnerability to the second hit of an acute stressor (the double hit). We found that the double hit induced a lasting sleep and behavioral phenotype that is characterized by maladaptive sleep changes immediately after the acute stressor, altered behavior and cytokine profiles, and lasting changes in sleep that resemble some of those seen in human PTSD. Importantly, immunization with a heat-killed preparation of *Mycobacterium vaccae* NCTC 11659 before beginning the protocol prevented the development of nearly all of these phenotypes. It remains unclear, however, whether any changes to the gut microbiome or metabolome that may occur during sleep disruption are linked to measures of stress vulnerability. It is also unknown what role *M. vaccae* may play in the actions of the gut-brain-axis during the double hit protocol.

To the best of our knowledge, the gut microbiome has only been measured in one publication studying *M. vaccae*. Reber and colleagues investigated the impact of *M. vaccae* in the context of

a chronic subordinate colony psychosocial stress paradigm [Reber et al., 2016]. They found that chronic stress altered the composition of the microbiome, resulting in a gradual reduction in alpha diversity along with changes in bacteria such as *Helicobacter* and *Proteobacteria*, which have all been reported in different stress paradigms [Guo et al., 2009; Bailey et al., 2011; Bailey, 2014]. *M. vaccae* immunization prevented some changes in alpha diversity, and stabilized the relative abundance of some, but not all, stress-sensitive bacterial taxa [Reber et al., 2016]. However, the impact of *M. vaccae* on changes to the gut microbiome in the context of acute stress or sleep disruption have never been studied, nor has the fecal metabolome been examined in any *M. vaccae* studies.

As one of the most likely mechanisms by which the microbiota impact host physiology is via microbially-modified metabolites [Zierer et al., 2018], we also investigated the fecal metabolome in this study. There is evidence that microbially-influenced metabolites play a role in inflammatory processes, and thus may modulate stress resilience. The study reported in Chapter II revealed that sleep disruption impacts many metabolites, particularly bile acids. Mounting evidence in the past decade has described bile acids as versatile signaling molecules, with receptors throughout the mammalian organism [Thomas et al., 2008; Kuipers et al., 2014; Mertens et al., 2017]. Signaling through the bile acid receptors of farnesoid X receptor (FXR) and Takeda G protein-coupled receptor 5 (TGR5), some bile acids can act as anti-inflammatory and immunoregulatory agents in the intestinal tract and the central nervous system [Vavassori et al., 2009; Yanguas-Casas et al., 2017].

Thus, we collected fecal samples throughout the experiment depicted in Chapter III for 16S rRNA microbiome analysis and untargeted LS/MS/MS metabolomics. The goal of these analyses was to investigate the impact of *M. vaccae* immunization on any sleep disruption-, social defeat-, or double hit-induced changes in the fecal microbiome and fecal metabolome in mice. Furthermore, since *M. vaccae* immunization created a stress-resilient state, we hypothesized that differential effects of sleep disruption, social defeat, or the double hit on the microbiome or metabolome could be used to discover candidate effector microbes or molecules that may promote stress vulnerability or resilience.

B. Materials and Methods

The animals used in this study were the same as those described in Chapter III. See section III.B for full description of methods, including all behavioral manipulations and physiological outcome measurements. Below is an abridged version of relevant methods, along with methods describing microbiome and metabolome analyses.

Animals and experimental design

Five cohorts of 24 seven-week-old male C57BL/6N mice (Charles River Laboratories, USA; n = 24 from Charlotte, NC; n = 96 from Kingston, NY) were used for the experiment, though only the cohorts from Kingston, NY (n = 96) were used for microbiome and metabolome analysis due to the known impact of different lab facilities on the microbiome (reviewed by [Franklin and

Ericsson, 2017]). A total of 4 of these animals did not complete the experimental protocol and were thus eliminated from all analyses, for a total $N = 92$. The experiment consisted of 8 experimental groups in a 2 x 2 x 2 design (Vehicle vs *M. vaccae* injection, *ad libitum* sleep vs sleep disruption, no social defeat vs social defeat). Experimental groups were balanced in each cohort. Mice were group housed until EEG/EMG implant surgery, after which they were individually housed until the end of the experiment in sleep recording chambers that had corncob bedding and food/water available *ad libitum*. After EEG/EMG surgery, mice were assigned to experimental groups randomly, but effort was made to ensure prior cagemates were in different groups. All mice were maintained on a 12:12 L:D cycle at room temperature ($23 \pm 2\ ^{o}C$) with food and water available *ad libitum* throughout the experiment. All protocols were approved by the Northwestern Institutional Animal Care and Use Committee.

Fecal Sample Collection

Fecal samples were collected at 4 different timepoints: 1) on the day of surgery; 2) after mice were transferred to sleep disruption cages but before starting sleep disruption (BL); 3) during object location memory testing ~24 hours after conclusion of sleep disruption (or *ad libitum* sleep) and subsequent social defeat (or control manipulation) (SD+2); and 4) at the end of the experiment, after 5 days of recovery *ad libitum* sleep (SD+7). Only the latter three timepoints were used for analysis in this chapter, as the data collected from the surgery timepoint had multiple factors including having been group housed, being of younger age, and being naïve to anesthesia that confounded comparison to the other timepoints. At each collection, mice were

placed into a clean sleep disruption chamber with fresh bedding and food and monitored closely until two fresh fecal pellets from each mouse were collected. Only spontaneously voided pellets were collected. Samples were placed into individual 1.5 mL microfuge tubes, and frozen at -80 ºC until microbiome/metabolome analysis. All fecal pellets were collected between Zeitgeber Time (ZT)6 and ZT8 (light onset = ZT0). At each collection timepoint, duplicate samples of bedding, water, food, and blank tubes were also collected.

Microbiome analysis

Microbiome data were generally analyzed using the Quantitative Insights Into Microbial Ecology 2 (QIIME2, version 2018.4) bioinformatics software package[Caporaso et al., 2010; Bolyen et al., 2019]. A total of 368 fecal and 64 environmental samples were processed for microbiome analyses. DNA was extracted from fecal samples and the V4 region of the 16S rRNA gene was amplified using the 515f/806rB primer pair with the barcode on the forward read[Apprill et al., 2015] and sequenced as previously described[Caporaso et al., 2012] using an Illumina MiSeq. Sequence data were processed using Deblur v1.1.0[Amir et al., 2017], trimming to 150 nucleotides to create sub-operational-taxonomic-units (sOTUs). These were then inserted into the Greengenes 13_8[McDonald et al., 2012] 99% reference tree using SATe-enabled Phylogenetic Placement (SEPP)[Mirarab et al., 2012]. SEPP uses a simultaneous alignment and tree estimation strategy[Liu et al., 2009] to identify placements for sequence fragments within an existing phylogeny and alignment. Taxonomy was assigned using an implementation of the

Ribosomal Database Project (RDP) classifier[Wang et al., 2007] as implemented in QIIME2[Caporaso et al., 2010].

Beta diversity metrics were performed at a rarefied depth of 10,000 reads, resulting in the removal of 12 fecal samples from the dataset, bringing the total samples analyzed for beta diversity across BL, SD+2, and SD+4 to $n = 265$. The rarefaction depth of 10,000 was made with guidance of jackknifed beta rarefaction analysis at 10 iterations. Beta diversity was assessed using weighted and unweighted UniFrac distance[Lozupone et al., 2011] matrices, which were used to generate PCoA plots and to perform PERMANOVA in QIIME2. Within-group distance was calculated from distance matrices by averaging the unweighted UniFrac distance from an individual sample to all other samples in the same group (Control vs Sleep Disruption) at the same timepoint. Alpha diversity metrics were calculated using scikit-bio 0.5.1 as implemented by QIIME2.

A subset of samples used in16S rRNA analysis were also used to perform shotgun metagenomic analysis ($n = 94$). Metagenomic sequence analysis was performed using Shogun with default parameters as described in [Hillmann et al., 2018]. In short, adapter and host removal were performed using Atropos [Didion et al., 2017] then used UTree to generate alignments against rep82, to finally generate taxonomic, module, and pathway tables using the redistribute commands within Shogun. To identify differentially abundant functional pathways and modules, DESeq2 (version 1.14.1) was performed using the Bioconductor R package in RStudio (version

1.2.1335, RStudio Inc). This was used in favor of techniques that more adequately account for the compositionality of microbiome datasets[Gloor et al., 2017] such as Analysis of the Composition of Microbiomes (ANCOM) due to the extremely low sensitivity of ANCOM when sample size is less than 20 per group [Weiss et al., 2017]. Modules were annotated using the following database: https://github.com/biocore/American-Gut/blob/master/data/AG/BeyondBacteria/PathwayTable.names.txt

Metabolome Analysis

A total of 368 fecal and 64 environmental samples were processed for fecal metabolome analyses. A clean stainless-steel bead (Qiagen Catalog# 69989) and 1.5 mL chilled extraction solvent (50% MeOH) was added to each sample. The samples were then homogenized for 5 min at 25 Hz using a TissueLyser II system (Qiagen Catalog# 85300) and allowed to incubate for 20 min at −20 °C. The fecal homogenates were then centrifuged at 14000 rpm for 15 min at 4 °C. 1.2 mL aliquots were then transferred into Nunc 2.0 mL DeepWell plate (Thermo Catalog# 278743) and frozen at −80 °C prior to lyophilization using a FreeZone 4.5 L Benchtop Freeze Dryer with Centrivap Concentrator (Labconco). Wells were resuspended with 200 µL of resuspension solvent (50% MeOH spiked with 2.0 µM sulfadimethoxine), vortexed for 30 secs, and centrifuged at 2000 rpm for 15 min at 4 °C. 150 µL of the supernatant was transferred into a 96-well plate and maintained at 4 °C prior to LC-MS analysis. A resuspension solvent QC and a six standard mix QC (50% MeOH spiked with 1.0 µM Sulfamethazine, 1.0 µM Sulfamethizole, 1.0 µM Sulfachloropyridazine, 1.0 µM Amitrypline, and 1.0 µM Coumarin 314) was run

every12th sample to assess sample background, carry over, chromatography behavior, peak picking and plate effects.

Fecal extracts were analyzed using an ultra-high performance liquid chromatography system (Vanquish, Thermo) coupled to a hybrid quadrupole-Orbitrap mass spectrometer (Q-Exactive, Thermo) fitted with a HESI probe. Reverse phase chromatographic separation was achieved using a Kinetex C18 1.7 µm, 100 Å, 50 × 2.1 mm column (Phenomenex) held at 40 °C with a flow rate of 0.5 mL/min. 5.0 µL aliquots were injected per sample/QC. The mobile phase used was (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. The elution gradient was: 5% B for 1 min, increased to 100% B in the next 8 min, held at 100% B for two min, returned to 5.0% B in 0.5 min, equilibrated at 5.0% B for two min. Positive electrospray ionization parameters were: sheath gas flow rate of 52 (arb. units), aux gas flow rate of 14 (arb. units), sweep gas flow rate of 3 (arb. units), spray voltage of 3.5 kV, capillary temperature of 270 °C, S-Lens RF level of 50 (arb. units), and aux gas heater temperature of 435 °C. Negative electrospray ionization parameters were: sheath gas flow rate of 52 (arb. units), aux gas flow rate of 14 (arb. units), sweep gas flow rate of 3 (arb. units), spray voltage of 2.5 kV, capillary temperature of 270 °C, S-Lens RF level of 50 (arb. units), and aux gas heater temperature of 435 °C. MS data was acquired using a data dependent acquisition method with a resolution of 35,000 in MS_1 and 17,000 in MS_2. An MS_1 scan from 100–1500 *m/z* was followed by an MS_2 scan, produced by collision induced disassociation, of the five most abundant ions from the prior MS_1 scan.

The orbitrap files (.raw) were exported to mzXML files using MSConvert [Chambers et al., 2012]. Feature detection of the MS_1 data was performed using MZmine2 [Pluskal et al., 2010]. The resultant feature table was normalized to an internal standard followed by a row sum (total ion count) normalization and contained 2785 features. PCoA plots were then generated using Canberra distance, and PERMANOVA was performed at each timepoint on the normalized feature table using the Vegan package (version 2.5-5) in RStudio. PERMANOVA was performed after the full feature table was filtered down to the groups of interest and filtered to remove features present in fewer samples than one half of the *n* of one experimental group. In order to identify metabolites that were different between groups of interest, we used a multiple-method approach that included machine learning and nonparametric hypothesis testing. In order to first identify the group of metabolites that were the key drivers of differences between groups at each timepoint, Variable Selection Using Random Forests (VSURF, version 1.0.3) [Genuer et al., 2010; Genuer et al., 2015] analysis was performed using the VSURF.R package in RStudio. Briefly, this protocol uses multiple iterations of the random forest supervised machine learning technique to isolate the most important drivers of separation between two groups by defining a threshold variable importance. Taking this list of suprathreshold features, we then performed Wilcoxon Rank-Sum tests at each timepoint as a form of a 'post hoc' test to confirm differences between groups.

Features of interest were annotated using GNPS (version 1.2.3) [Wang et al., 2016], which allows MS1 and MS2 spectra to be shared between researchers, forming a large database. By matching an unknown spectrum to one or more in the database, and examining similarity to

others within a molecular network, GNPS can be used to identify purported molecular structures of features from untargeted metabolomics. A molecular network was created using the online workflow at GNPS. The data were filtered by removing all MS/MS peaks within +/- 17 Da of the precursor *m/z*. MS/MS spectra were window-filtered by choosing only the top six peaks in the +/- 50 Da window throughout the spectrum. The data were then clustered with MS-Cluster with a parent mass tolerance of 0.1 Da and a MS/MS fragment ion tolerance of 0.1 Da to create consensus spectra. Further, consensus spectra that contained less than two spectra were discarded. A network was then created where edges were filtered to have a cosine score above 0.7 and more than four matched peaks. Further edges between two nodes were kept in the network if and only if each of the nodes appeared in each other's respective top ten most similar nodes. The spectra in the network were then searched against GNPS' spectral libraries. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.7 and at least four matched peaks. Results can be found at https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=17ff5c89186d4fa3af75d0f33173a4d6 Individual features of interest from the feature table were then matched to nodes (clusters) in the network whose average *m/z* and RT were within 0.01 and 0.5 min, respectively, of the feature of interest. Features that were matched to multiple clusters using the aforementioned criteria were assigned to the cluster with the closest average *m/z* and RT. Primary, secondary, conjugated and unconjugated bile acids were purchased and used for level 1 identification of some of our unknown molecules. Standards were solubilized to a final concentration of 10uM in 50% MeOH

prior to LC-MS/MS injection. Accurate mass with retention time alignment and MS/MS fragmentation pattern between a metabolite of interest and a chemical reference standard was used for all MSI level 1 annotations. Accurate mass and MS/MS fragmentation pattern between a metabolite of interest and a reference library, via GNPS, was used for all MSI level 2 annotations.

Statistical Analyses and Software

All graphs depict the mean +/- SEM unless otherwise stated. All PCoA plots were generated using the EMPeror visualization tool as implemented in QIIME2[Vazquez-Baeza et al., 2013]. Microbiome data processing and analysis, including microbiome PERMANOVA, were performed in QIIME2 as outlined above. Wilcoxon Rank-Sum tests, VSURF, DESeq2 (with Benjamini Hochberg adjustment), linear mixed effects modeling for alpha diversity measures, and correlation analysis and plots, were performed or generated in RStudio (version 1.0.136, RStudio Inc, Boston, MA, USA). Spearman's Rank Based correlation analyses were performed using the rcorr() function within the Hmisc package in RStudio, and visualized using the corrplot package. *P* values from the correlation analyses were adjusted for multiple comparisons using the qvalue package in RStudio. Mixed-effects models with Tukey post hoc testing of alpha diversity and beta diversity measures, along with generation of all other graphs/figures, was performed using GraphPad PRISM (version 8.2.1; GraphPad Inc, San Diego, CA, USA).

C. Results

M. vaccae immunization alone does not alter the gut microbiome or fecal metabolome

Fecal samples were collected at baseline (3 days after the third and final injection with *M. vaccae* or vehicle), on the second say after the sleep disruption protocol (or *ad libitum* sleep) ended, and at the end of the experiment (SD+7) and 16S rRNA microbiome analysis was performed (**Figure 4.1a**). We first investigated whether the three *M. vaccae* immunizations alone altered the fecal microbiome or metabolome *per se*, before any other experimental manipulations. *M. vaccae* did not alter alpha diversity, as measured by Faith's phylogenetic diversity ($p = 0.32$, Wilcoxon Rank-Sum test), the total number of observed OTU ($p = 0.36$, Wilcoxon Rank-Sum test), or the Pielou evenness ($p = 0.90$, Wilcoxon Rank-Sum test), (**Figure 4.1b-d**). Beta diversity, measured with unweighted ($p = 0.36$, PERMANVOA) or weighted ($p = 0.77$, PERMANOVA) UniFrac, was also not altered by *M. vaccae* (**Figure 4.1e,f**). Furthermore, *M. vaccae* did not alter the fecal metabolome at baseline ($p = 0.27$, PERMANOVA, **Figure 4.1g**).

M. vaccae immunization has a stabilizing effect on the alpha and beta diversity of the microbiome

Since *M. vaccae* prevented changes to physiology after sleep disruption, social defeat, and the double hit (Chapter III), we next sought to determine if *M. vaccae* altered the impact of these manipulations on the fecal microbiome. 16S rRNA microbiome analysis was performed on fecal samples collected at baseline, SD+2, and SD+7, and measures of alpha diversity and beta diversity were assessed. We noticed all vehicle-treated, non-control groups showed a general

decrease in Faith's Phylogenetic diversity index and the total number of OTU over the course of the experiment, while *M. vaccae*-treated groups remained consistent across time, and linear

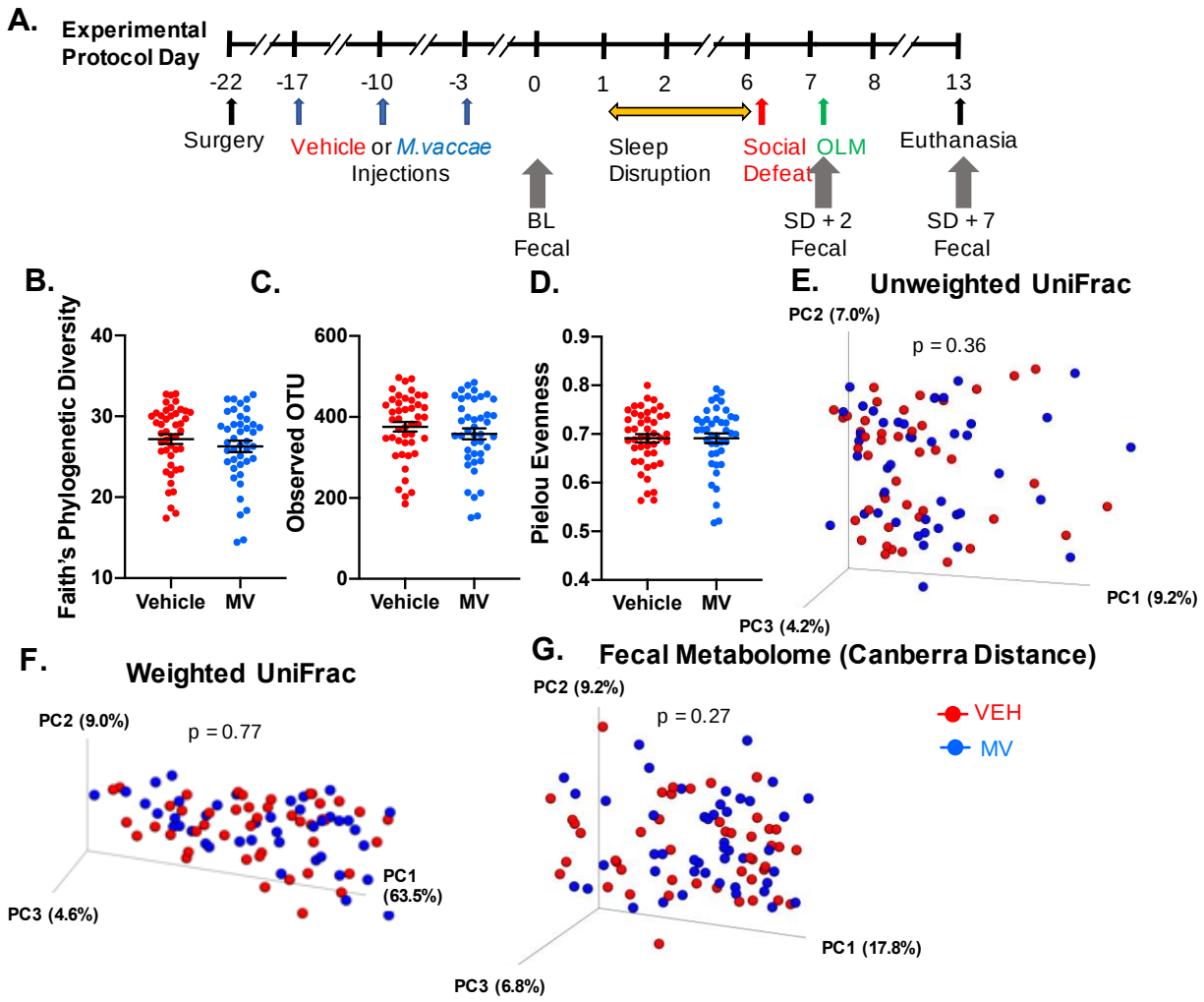

Figure 4.1: *M. vaccae* Immunization Does Not Impact the Fecal Microbiome or Fecal Metabolome *per se*. (A) Schematic of experimental design (the same experiment as in Chapter III). Fecal samples were collected throughout the experiment for 16S rRNA microbiome and untargeted LC/MS/MS metabolome analysis. Baseline samples were collected before baseline sleep was recorded, 3 days after the third and final injection with vehicle or *M. vaccae*. Samples were also collected on the second day post-sleep disruption (or *ad libitum*) sleep during OLM testing, and at the end of the experiment. Microbiome alpha diversity was measured using (B) Faith's Phylogenetic diversity index, (C) overall number of observed OTU, and (D) the Pielou evenness measure at the BL timepoint, comparing vehicle-injected and *M. vaccae*-injected mice. Beta diversity was measured using (E) unweighted UniFrac and (F) weighted UniFrac distance

measures. (G) Untargeted LC/MS/MS metabolomics was performed on separate fecal samples taken at the same time as those used for microbiome analyses and is reported using Canberra distance. *P* values for (E-G) correspond to PERMANOVA analysis for effect of treatment. Abbreviations: OLM, object location memory; SD, sleep disruption; VEH, vehicle injection; MV, *M. vaccae* injection; PC, principal coordinate. n = 43-45/group.

mixed modeling confirmed treatment x time interactions (**Figure 4.2a,b**). A similar effect was observed in Pielou evenness, a measure of how close in abundance different taxa within an environment are [Pielou, 1969], with the exception that the vehicle-injected double hit group did not see the same magnitude of reduction as did sleep disruption alone and social defeat alone groups (**Figure 4.2c**).

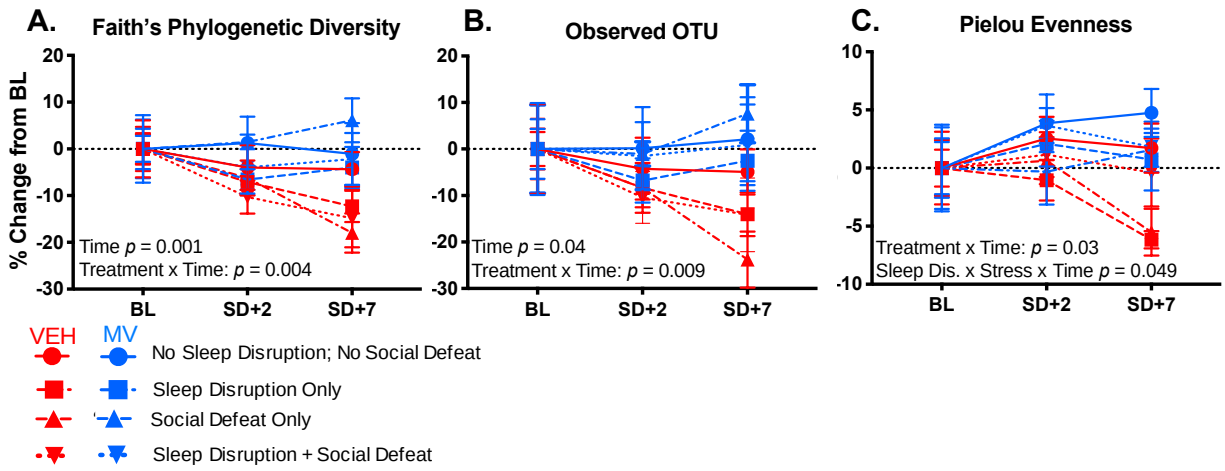

Figure 4.2: *M. vaccae* Prevents Sleep Disruption- and Stress-Induced Reductions in Alpha Diversity. Fecal samples were collected at baseline, day two post-sleep disruption (24 hours after social defeat or control manipulation), and at the end of the experiment. (A) Faith's phylogenetic diversity, (B) the total number of observed OTUs, and (C) the Pielou evenness are reported for the control, sleep disruption only, social defeat only, and sleep disruption plus social defeat (double hit) groups. Data are reported as a percent change from the average for their group at baseline. Mixed effect linear modeling was performed for each measure. Abbreviations: OTU, operational taxonomic unit; BL, baseline; SD, sleep disruption; VEH, vehicle injection; MV, *M. vaccae* injection. *n* = 11-12/group.

We then investigated the impact of sleep disruption, social defeat, and the double hit on measures of microbiome beta diversity. We did not find a significant effect of sleep disruption, social defeat, or the double hit compared to control at day 2 post-sleep disruption or day 7 post-sleep disruption (**Table 4.1**). However, we did notice that an overall effect of cohort was

prevalent (**Table 4.1**). Thus, to attempt to reduce the magnitude of this effect, we decided to examine individual groups over time. Doing this, we found subtle effects of sleep disruption, social defeat, and the double hit in vehicle-treated groups. In vehicle-treated groups, there was a shift in weighted UniFrac distance at SD+7 due to sleep disruption alone ($p = 0.029$, PERMANOVA), and social defeat alone ($p = 0.024$, PERMANOVA) compared to baseline (**Figure 4.3b,e**). Furthermore, both social defeat alone and the double hit had a destabilizing effect over time on the microbiome, as measured by an increase in the within-group unweighted UniFrac distance over time (**Figure 4.3g,h**). Similarly to what was seen in alpha diversity measures, *M. vaccae* had a stabilizing effect on the microbiome community structure, as no effects of sleep disruption, social defeat, or the double hit were observed in weighted UniFrac PERMANOVA or unweighted UniFrac within group distance (**Figure 4.4**).

Measure	Timepoint	Comparison	Group Effect (*p*)	Cohort Effect (*p*)
Unweighted UniFrac	SD+2	Vehicle, Control vs Sleep Disruption Only	0.203	**0.001**
Unweighted UniFrac	SD+2	Vehicle, Control vs Social Defeat Only	0.997	**0.001**
Unweighted UniFrac	SD+2	Vehicle, Control vs Double Hit	0.923	**0.002**
Unweighted UniFrac	SD+2	MV, Control vs Sleep Disruption Only	0.776	**0.002**
Unweighted UniFrac	SD+2	MV, Control vs Social Defeat Only	0.990	**0.001**
Unweighted UniFrac	SD+2	MV, Control vs Double Hit	0.716	**0.002**
Unweighted UniFrac	SD+7	Vehicle, Control vs Sleep Disruption Only	0.456	**0.001**
Unweighted UniFrac	SD+7	Vehicle, Control vs Social Defeat Only	0.858	**0.001**
Unweighted UniFrac	SD+7	Vehicle, Control vs Double Hit	0.926	**0.001**
Unweighted UniFrac	SD+7	MV, Control vs Sleep Disruption Only	0.938	**0.001**
Unweighted UniFrac	SD+7	MV, Control vs Social Defeat Only	0.920	**0.001**
Unweighted UniFrac	SD+7	MV, Control vs Double Hit	0.152	**0.003**
Weighted UniFrac	SD+2	Vehicle, Control vs Sleep Disruption Only	0.350	0.695
Weighted UniFrac	SD+2	Vehicle, Control vs Social Defeat Only	0.994	0.129
Weighted UniFrac	SD+2	Vehicle, Control vs Double Hit	0.999	0.146
Weighted UniFrac	SD+2	MV, Control vs Sleep Disruption Only	0.669	0.204
Weighted UniFrac	SD+2	MV, Control vs Social Defeat Only	0.632	0.056
Weighted UniFrac	SD+2	MV, Control vs Double Hit	0.588	0.785
Weighted UniFrac	SD+7	Vehicle, Control vs Sleep Disruption Only	0.883	0.410
Weighted UniFrac	SD+7	Vehicle, Control vs Social Defeat Only	0.158	**0.003**
Weighted UniFrac	SD+7	Vehicle, Control vs Double Hit	0.645	**0.023**
Weighted UniFrac	SD+7	MV, Control vs Sleep Disruption Only	0.640	0.089

Weighted UniFrac	SD+7	MV, Control vs Social Defeat Only	0.900	0.487
Weighted UniFrac	SD+7	MV, Control vs Double Hit	0.401	0.421

Table 4.1: Within Timepoint Beta Diversity Comparisons. PERMANOVA was performed in Qiime for unweighted and weighted UniFrac measures of beta diversity. Individual comparisons of experimental groups vs control groups within timepoint and within treatment groups were performed, testing for an effect of overall group and cohort. Abbreviations: SD, sleep disruption; MV, *M. vaccae* injection.

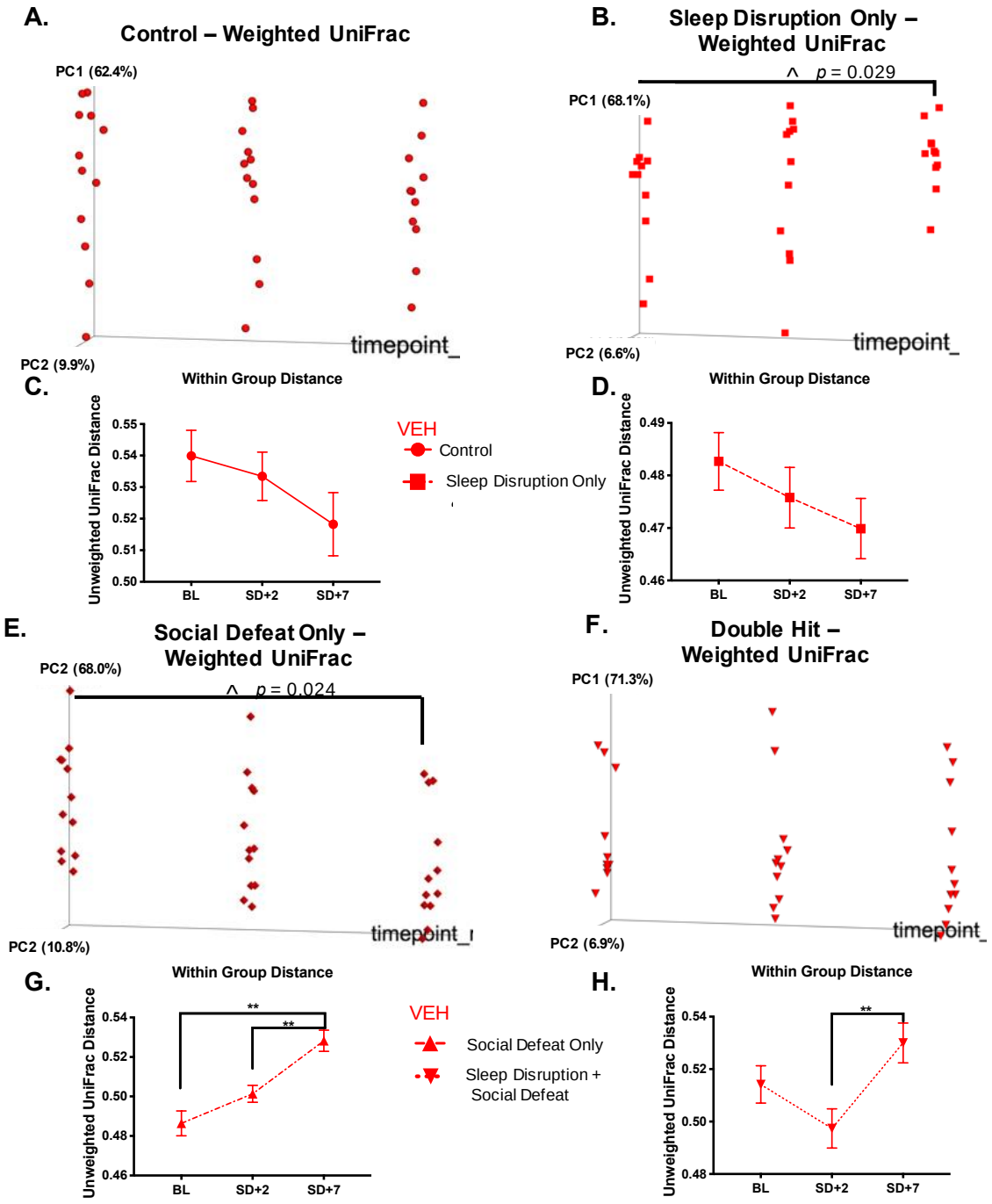

Figure 4.3: Impact of Sleep Disruption, Social Defeat, and the Double Hit on Microbiome Beta Diversity in Vehicle-Treated Mice. We examined microbiome beta diversity measures across the experiment in individual groups. PCoA of weighted UniFrac distance, with timepoint as one axis (BL, SD+2, SD+7; left to right) are depicted for vehicle-treated (A) control, (B) sleep disruption only, (E) social defeat only, and (F) double hit groups. Unweighted UniFrac within

group distance, calculated as the average distance of each sample to other samples in that group at that timepoint, is depicted for these groups as well, in (C,D,G,H). Symbols: ^ $p < 0.05$, PERMANOVA; ** $p < 0.01$, Tukey's post-hoc test. Abbreviations: BL, baseline; SD, sleep disruption; VEH, vehicle injection; PC, principal coordinate. n = 10-12/group.

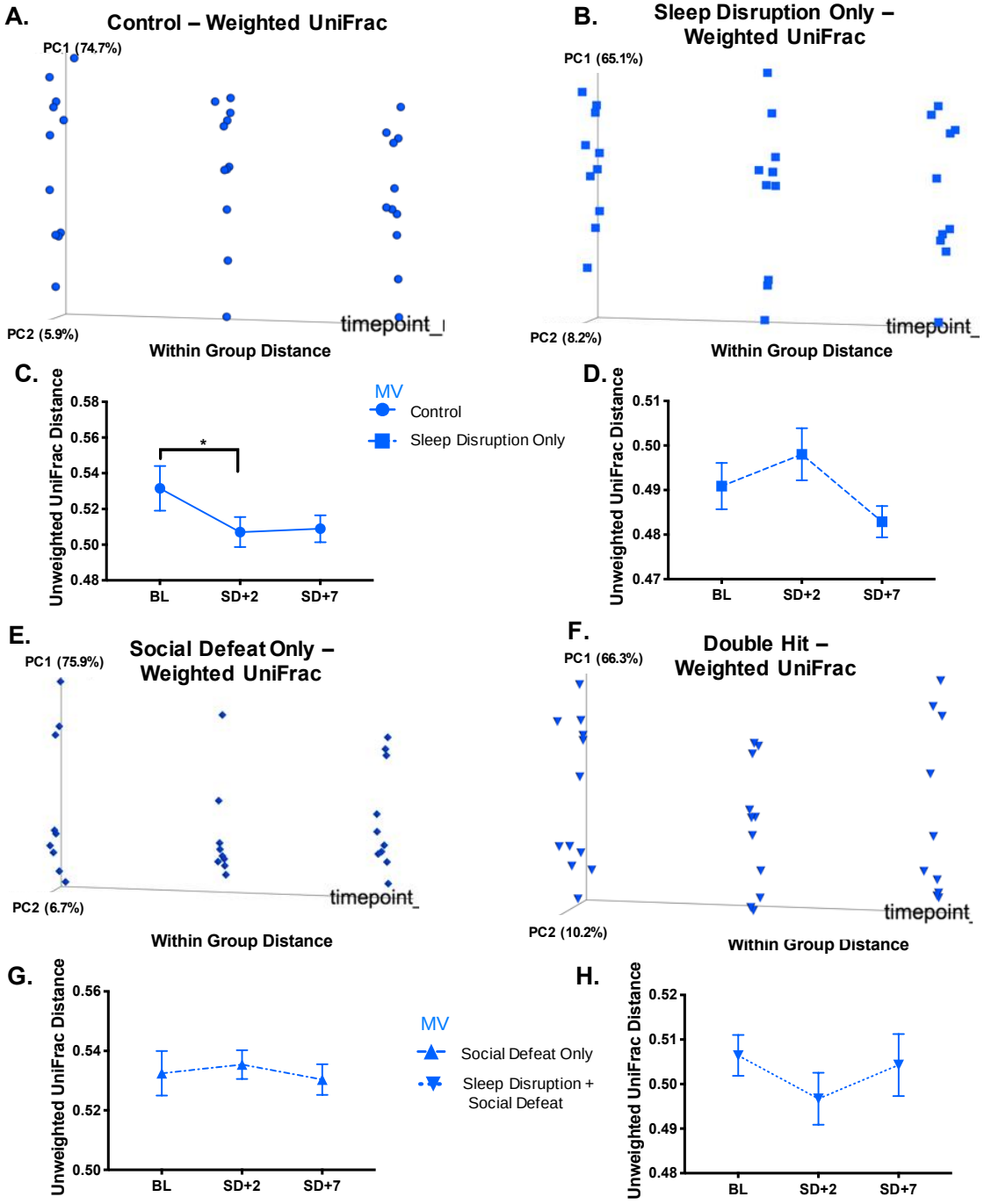

Figure 4.4: Impact of Sleep Disruption, Social Defeat, and the Double Hit on Microbiome Beta Diversity in *M. vaccae*-Treated Mice. We examined microbiome beta diversity measures across the experiment in individual groups. PCoA of weighted UniFrac distance, with timepoint as one axis (BL, SD+2, SD+7; left to right) are depicted for MV-treated (A) control, (B) sleep disruption only, (E) social defeat only, and (F) double hit groups. Unweighted UniFrac within group distance, calculated as the average distance of each sample to other samples in that group at that timepoint, is depicted for these groups as well, in (C,D,G,H). Symbols: ^ $p < 0.05$, PERMANOVA; ** $p < 0.01$, Tukey's post-hoc test. Abbreviations: BL, baseline; SD, sleep disruption; MV, *M. vaccae* injection; PC, principal coordinate. n = 10-12/group.

The Double Hit has a Greater Impact on the Function of the Microbiome in M. vaccae-Treated Mice

In order to assess the functional capacity of the gut microbiome, we performed shotgun metagenomic sequencing on samples taken at BL and at SD+2 from vehicle-treated and *M. vaccae*-treated mice from the control and double hit groups. Comparing baseline to SD+2 within groups revealed a modest impact of the double hit on functional pathways and modules in vehicle-treated mice. One pathway was significantly decreased (FDR < 0.1, **Figure 4.5a**), and 5 modules were significantly decreased (FDR < 0.1, **Figure 4.5b**). However, in *M. vaccae*-treated mice the double hit had an impact of a larger magnitude, with 42 pathways decreased, 23 pathways increased (FDR < 0.1, **Figure 4.5a**), 60 modules decreased, and 56 modules increased (FDR < 0.1, **Figure 4.5b**). Of these altered modules that were significant at a level of FDR < 0.1, 5 were significant at the FDR < 0.05 level in the vehicle, double hit group, and 4 were significant at the FDR < 0.05 level in the *M. vaccae*-treated, double hit group. These are depicted in **Table 4.2**.

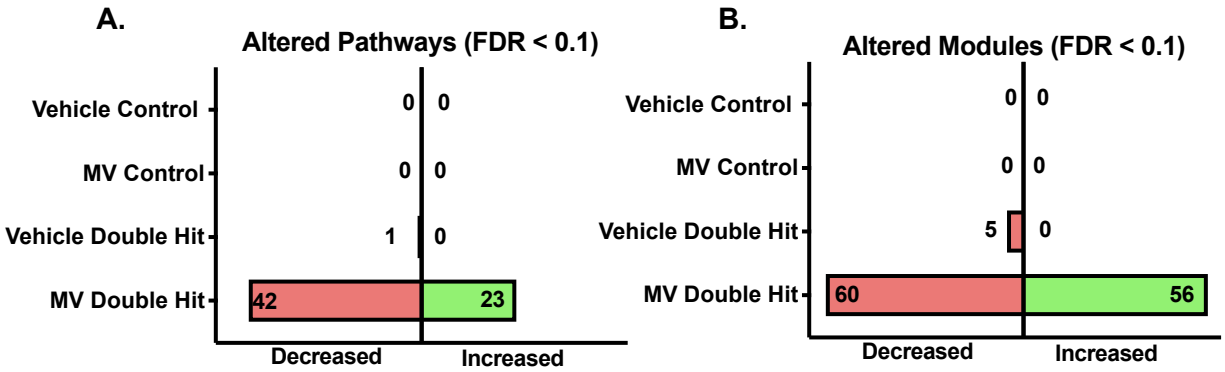

Figure 4.5: The Double Hit Results in a Larger Change to the Function of the Microbiome in *M. vaccae*-Treated Mice. Shotgun metagenomic sequencing was performed on samples from control and double hit groups at baseline and at SD+2, and functional analysis of the gene content was performed. DESEq2 was performed within each individual group comparing baseline to SD+2 to identify metabolic pathways and modules that change from one timepoint to the other. The number of significant (FDR < 0.1) (A) pathways and (B) modules are depicted above. Abbreviations: FDR, false discovery rate; MV, *M. vaccae* injection. *n* = 11-12/group.

Group	Module ID	Description	Fold Change	*p* value	*q* value
Vehicle, Double Hit	M00150	Fumarate reductase, prokaryotes	-0.62	3.6×10^{-6}	0.001
	M00224	Fluoroquinolones transport system	-0.59	5.1×10^{-5}	0.007
	M00234	Cystine transport system	-0.56	0.0001	0.007
	M00443	SenX3-RegX3 (phosphate starvation response), 2 component regulatory system	-0.51	8.7×10^{-5}	0.007
	M00047	Creatine pathway	-0.65	0.0003	0.016
***M. vaccae*, Double Hit**	M00443	SenX3-RegX3 (phosphate starvation response), 2 component regulatory system	-0.54	0.0001	0.042
	M00244	Putative zinc-manganese transport system	-0.53	0.0005	0.042
	M00244	Sugar transport system	-0.50	0.0005	0.042
	M00365	C10-C20 isoprenoid biosynthsis (Terpenoid backbone biosynthesis)	-0.32	0.0005	0.042

Table 4.2: Most Significantly Altered Functional Modules After the Double Hit. Functional analysis of shotgun metagenomic sequencing was performed on samples from baseline and SD+2. DESEq2 was performed comparing SD+2 to baseline for the vehicle, double hit group and the *M. vaccae*, double hit group. Modules that had an FDR (*q* value) < 0.05 are reported

above, along with their description, fold change from baseline, uncorrected *p* value, and *q* value. Descriptions of modules were found at https://github.com/biocore/American-Gut/blob/master/data/AG/BeyondBacteria/PathwayTable.names.txt.

M. vaccae Immunization Alters the Response of the Fecal Metabolome to Sleep Disruption and the Double Hit

In order to assess the fecal metabolome, untargeted LC/MS/MS metabolomics was performed on fecal samples taken at the same timepoints as the samples used for microbiome analysis. Again tracing individual groups across time, we found that in vehicle-treated mice, sleep disruption resulted in a change from baseline in the fecal metabolome at SD+2 ($p = 0.001$, PERMANOVA, Canberra distance), which was no longer present by SD+7 (**Figure 4.6b**). There were no global changes compared to baseline at either SD+2 or SD+7 in the vehicle-treated control, social defeat alone, or double hit groups (**Figure 4.6a,c,d**). While *M. vaccae* immunization prevented the shift in the fecal metabolome due to sleep disruption alone (**Figure 4.7b**), there was a significant shift compared to baseline at SD+2 due to the double hit ($p = 0.001$, PERMANOVA; **Figure 4.7d**). This finding is consistent with the functional analysis of the microbiome, suggesting that although the community structure of the microbiome was unchanged, the metabolism of these microbes was altered.

We were then interested in determining which fecal metabolites were changed due to sleep disruption in the stress-vulnerable, vehicle-injected mice with the goal of identifying potential candidate molecules that may increase stress-vulnerability after sleep disruption. To do this, we performed Variable Selection Using Random Forests (VSURF) [Genuer et al., 2015] analysis comparing control and sleep-disrupted mice at SD+2 (**Figure 4.8a**). Multiple bile acids were in the top 25 suprathreshold metabolites, and all were decreased due to sleep disruption. VSURF

comparing *M. vaccae*-treated control vs sleep-disrupted groups at SD+2 depicted reductions in two bile acids, as well as changes in other annotated metabolites, but many of these suprathreshold metabolites did not meet statistical significance upon follow-up Wilcoxon Rank-Sum testing (**Figure 4.8b**). This is consistent with the PERMANOVA results that suggested the impact of sleep disruption was greater in vehicle-treated than in *M. vaccae*-treated mice. We also performed VSURF comparing control and double hit groups at SD+2. The top drivers of separation between the groups were different for the vehicle-treated and *M. vaccae*-treated mice (**Figure 4.8c,d**). The double hit appeared to impact more metabolites with spectral matches to amino acids and dipeptides in the vehicle-treated group, while in the *M. vaccae*-treated mice the double hit had an effect on primary bile acids such as muricholic acid.

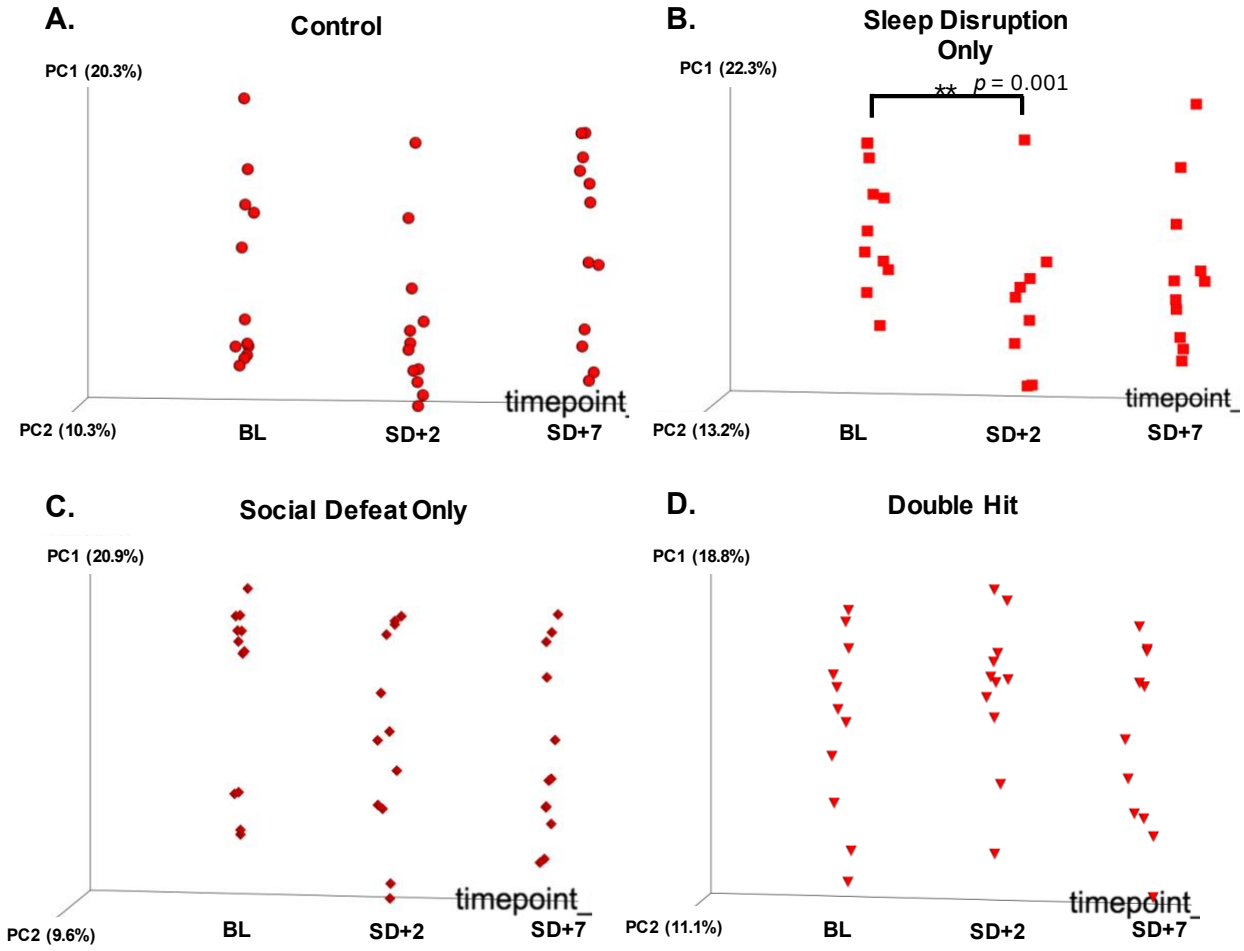

Figure 4.6: Sleep Disruption Results in a Shift in the Fecal Metabolome in Vehicle-Treated Mice. We assessed the fecal metabolome using untargeted LC/MS/MS. PCoA of Canberra distance, with timepoint as one axis (BL, SD+2, SD+7; left to right) are depicted for vehicle-treated (A) control, (B) sleep disruption only, (C) social defeat only, and (D) double hit groups. PERMANOVA was performed within each group, comparing SD+2 and SD+7 to baseline. Symbols: * $p < 0.05$, PERMANOVA. Abbreviations: BL, baseline; SD, sleep disruption; PC, principal coordinate. n = 11-12/group

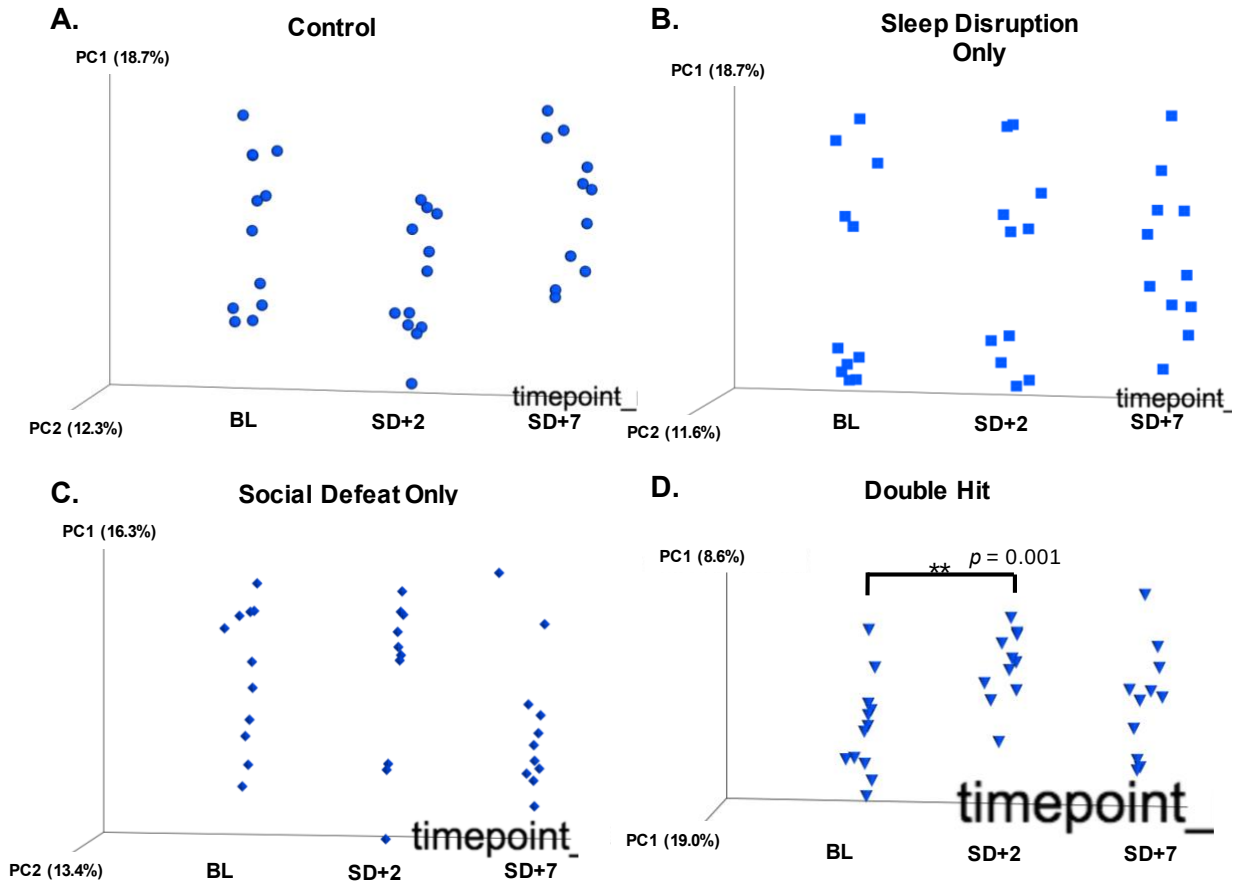

Figure 4.7: The Double Hit, but Not Sleep Disruption Alone, Changes the Fecal Metabolome in *M. vaccae*-Treated Mice. We assessed the fecal metabolome using untargeted LC/MS/MS. PCoA of Canberra distance, with timepoint as one axis (BL, SD+2, SD+7; left to right) are depicted for MV-treated (A) control, (B) sleep disruption only, (C) social defeat only, and (D) double hit groups. PERMANOVA was performed within each group, comparing SD+2 and SD+7 to baseline. Symbols: ** $p < 0.01$, PERMANOVA. Abbreviations: BL, baseline; SD, sleep disruption; PC, principal coordinate. n = 10-12/group

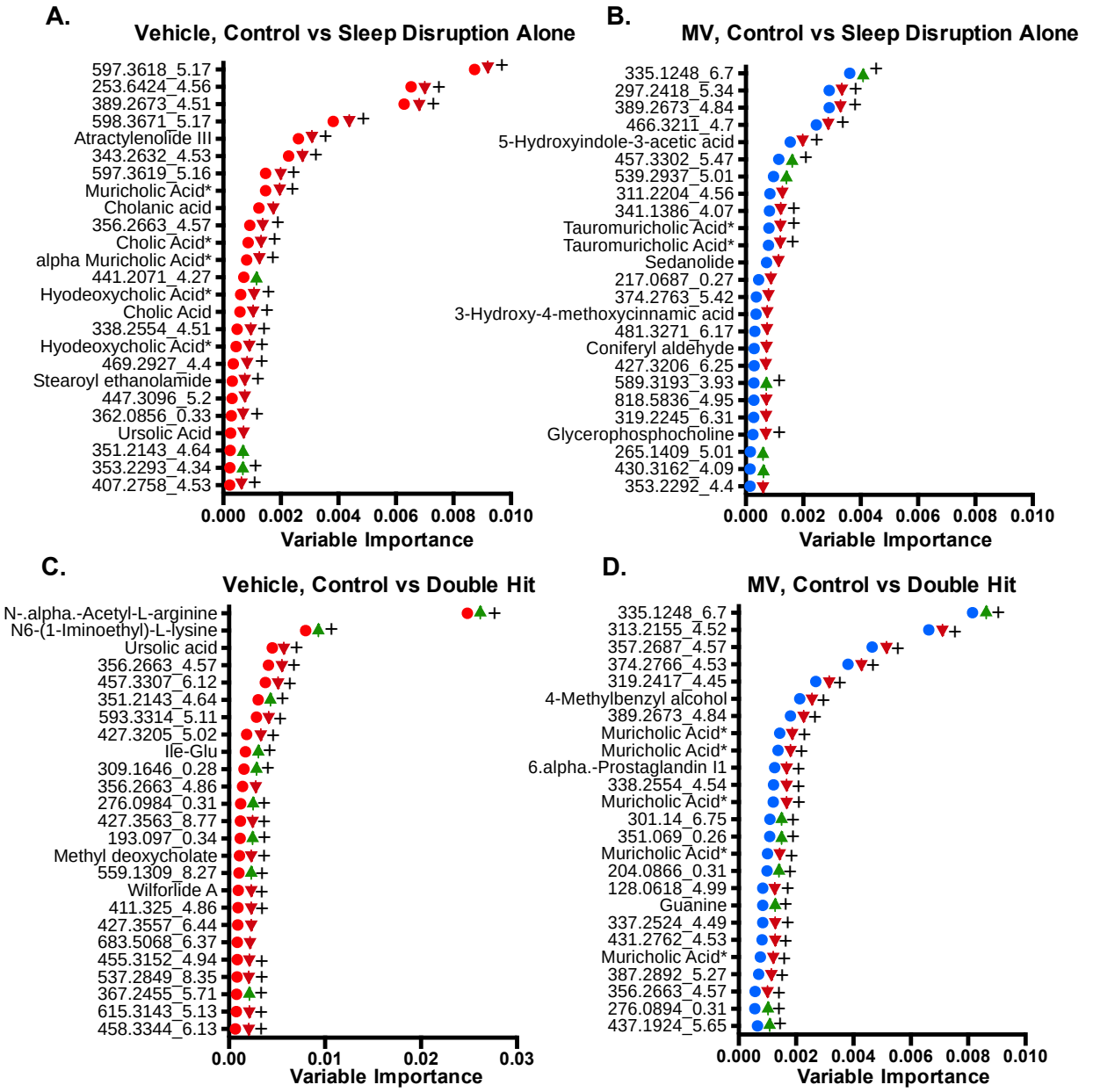

Figure 4.8: Top Metabolites Changed by Sleep Disruption and the Double Hit. Variable Selection Using Random Forests (VSURF) was performed at the second day post-sleep disruption (SD+2) to identify metabolites that are drivers (above a threshold variable importance) of separation between (A,B) sleep-disrupted vs control groups and (C,D) double hit vs control groups for vehicle-treated (A,C) and MV-treated (B,D) mice. Variable importance scores of the top 25 suprathreshold metabolites (*m/z*_RT), along with direction of change (arrows, green/up = increased in sleep disruption or double hit group, red/down = decreased in sleep disruption or double hit group). Metabolites that were annotated using Global Natural Products Social

Molecular Networking (GNPS) are labelled with their annotated names. Metabolite names with an asterisk are those that were later verified using purified standards. Symbols: * metabolites which have been verified with purified standards; + $p < 0.05$ (Wilcoxon Rank-Sum test). Abbreviations: MV, *M. vaccae* injection; SD, sleep disruption; RT, retention time (min).

The Secondary Bile Acid Hyodeoxycholic Acid is Decreased Due to Sleep Disruption, and Correlates with Multiple Physiological Phenotypes of the Double Hit

After performing VSURF, we noticed that features with spectral matches to various bile acids were commonly altered due to sleep disruption and the double hit in all groups, but two features with spectral matches to the secondary bile acid hyodeoxycholic acid (HDCA) were only present among the suprathreshold metabolites in vehicle-treated mice (e.g. **Figure 4.8a**). This observation, paired with previous results using the same sleep disruption protocol that implicated an impact of sleep disruption on fecal bile acid content (see Chapter II), led us to use purified standards to verify the identities of features of interest that had a spectral match to some form of bile acid in GNPS. Using this approach, we were able to annotate 49 features as bile acids at the level 1 identification standard [Sumner et al., 2007]. The direction of change of each of the identified bile acids due to sleep disruption or the double hit, in vehicle-treated or *M. vaccae*-treated mice, at SD+2 is reported in **Table 4.3**. Many primary bile acids, particularly muricholic acid, were reduced by sleep disruption or the double hit at SD+2 in both vehicle-treated and *M. vaccae*-treated groups.

However, we were intrigued to find that four features identified as HDCA, two of which were suprathreshold on VSURF analysis (HDCA1: 375.2841_5.15; *m/z*_RT and HDCA2: 375.2836_5.14; *m/z*_RT), were decreased primarily in the sleep-disrupted, vehicle-treated group. HDCA is a microbially modified bile acid that, unlike some of its fellow secondary bile acids such as lithocholic acid or deoxycholic acid, has been shown to have health benefits in mouse

models [Sehayek et al., 2001; Shih et al., 2013]. Therefore, we decided to investigate these features further. Indeed, 3/4 features identified as HDCA were reduced due to sleep disruption alone compared to controls at SD+2 in vehicle-treated mice, and this relationship held up upon summing the 4 individual metabolites (**Figure4.9a,c,e,g,i**). Furthermore, 2/4 HDCA metabolites were also reduced due to the double hit at SD+2 in vehicle-treated mice (**Figure 4.9a,c**). In *M. vaccae*-treated mice, no HDCA metabolites were reduced by sleep disruption, but 2/4 were reduced at SD+2 in the *M. vaccae*-treated, double hit group compared to control (**Figure 4.9b,d,f,h,j**). Thus, HDCA is an interesting secondary bile acid that is sensitive to sleep disruption, and *M. vaccae* blunts that response.

Based on the above, we postulated that levels of HDCA could correlate with vulnerability or resilience to acute social defeat. Therefore, we performed Spearman's correlation testing of the SD+2 normalized abundances of the 4 standard-verified HDCA metabolites plus their sum versus 32 sleep, behavioral, and physiological measures (see Chapter III for full reporting of these measures) in all groups that received social defeat (**Figure 4.10**). This included vehicle-treated and *M. vaccae*-treated mice receiving social defeat alone, or the double hit. We found 49 significant correlations ($p < 0.05$), and 29 of those remained significant after correcting for multiple comparisons ($q < 0.05$). Fecal HDCA levels appeared to be tightly linked to cytokine levels in the mesenteric lymph nodes (measured 5 days later). Multiple HDCA metabolites correlated positively with the proinflammatory cytokines IL-6 and IL-1beta, and negatively with IFNgamma, IL-4, and to a lesser extent, IL-10. Interestingly, HDCA metabolites correlated with

multiple sleep measures at different times of the experiment as well. HDCA correlated negatively with NREM sleep EEG beta power measured immediately after social defeat (starting ~20 hours before collection of the SD+2 fecal sample) and measured 4 days later (Day 11). Furthermore, HDCA metabolites correlated positively with NREM sleep and state shifts, and weakly negatively with the average NREM sleep to REM sleep duration measured on Day 11. Thus, a microbially modified secondary bile acid measured 24 hours after acute social defeat or the double hit appears to be linked to stress-sensitive physiology.

Primary/ Secondary Bile Acid	**Name**	***m/z***	**RT**	**Vehicle, Sleep Disruption**	**Vehicle, Double Hit**	***M. vaccae*, Sleep Disruption**	***M. vaccae*, Double Hit**
Primary	Cholic acid	373.2736	4.99	No Change	No Change	No Change	decreased
	Cholic acid	373.2736	4.9	**decreased**	No Change	decreased	No Change
	Cholic acid	817.5822	4.95	No Change	No Change	decreased	No Change
	Cholic acid	355.2628	4.99	No Change	No Change	decreased	No Change
	Muricholic acid	391.2832	4.67	No Change	No Change	decreased	**decreased**
	Muricholic acid	839.5639	4.65	No Change	No Change	No Change	**decreased**
	Muricholic acid	391.2827	4.66	**decreased**	**decreased**	No Change	No Change
	Muricholic acid, alpha	373.2736	4.56	**decreased**	**decreased**	No Change	**decreased**
	Muricholic acid, alpha	817.5814	4.53	**decreased**	decreased	No Change	**decreased**
	Muricholic acid, alpha	817.5829	4.53	**decreased**	decreased	No Change	**decreased**
	Muricholic acid, alpha	355.2628	4.52	**decreased**	No Change	No Change	**decreased**
	Muricholic acid, alpha	373.2736	4.52	**decreased**	No Change	No Change	**decreased**
	Muricholic acid, alpha	839.5614	4.54	decreased	No Change	No Change	**decreased**
	Muricholic acid, alpha	355.2628	4.59	No Change	No Change	No Change	**decreased**
	Muricholic acid, alpha	391.283	4.63	**decreased**	decreased	No Change	No Change
	Muricholic acid, gamma (Hyocholic acid)	355.2628	4.89	decreased	No Change	No Change	No Change
	Muricholic acid, gamma (Hyocholic acid)	839.5621	4.83	No Change	No Change	No Change	No Change
	Muricholic acid, omega	391.2829	4.46	**decreased**	No Change	No Change	**decreased**
	Muricholic acid, omega	373.2737	4.08	decreased	No Change	No Change	No Change
	Muricholic acid, omega	355.2628	4.4	No Change	No Change	No Change	No Change

	Muricholic acid, omega	373.2737	4.28	No Change	No Change	No Change	No Change
	Muricholic acid, omega	391.2827	4.31	No Change	No Change	No Change	No Change
Primary, Conjugated	Taurochenodeoxycholic acid or tauromuricholic acid	498.2869	4.25	No Change	No Change	**decreased**	No Change
	Taurochenodeoxycholic acid or tauromuricholic acid	498.2869	4.25	No Change	No Change	**decreased**	No Change
	Taurocholic acid	480.2758	4.14	No Change	No Change	decreased	No Change
	Taurocholic acid	462.2657	4.13	No Change	No Change	decreased	No Change
	Taurocholic acid	538.2799	4.13	No Change	No Change	No Change	No Change
	Tauromuricholic acid	538.2791	3.67	No Change	No Change	**decreased**	**decreased**
	Tauromuricholic acid	480.2763	3.67	No Change	No Change	decreased	No Change
	Tauromuricholic acid	480.2764	3.67	No Change	No Change	**decreased**	No Change
	Tauromuricholic acid	462.2654	3.64	No Change	No Change	decreased	No Change
Secondary	Deoxycholic acid	357.2784	5.77	No Change	No Change	No Change	**decreased**
	Deoxycholic acid	357.2785	5.77	No Change	No Change	No Change	**decreased**
	Deoxycholic acid	785.5924	5.76	No Change	No Change	No Change	**decreased**
	Deoxycholic acid	375.2888	5.76	No Change	No Change	No Change	**decreased**
	Deoxycholic acid	375.2888	5.76	No Change	No Change	No Change	**decreased**
	Hyodeoxycholic acid	375.2841	5.15	**decreased**	**decreased**	No Change	**decreased**
	Hyodeoxycholic acid	375.2836	5.14	**decreased**	**decreased**	No Change	**decreased**
	Hyodeoxycholic acid	357.2783	5.23	**decreased**	decreased	No Change	No Change
	Hyodeoxycholic acid	357.2783	5.23	decreased	No Change	No Change	No Change
	Lithocholic acid	359.2941	6.74	decreased	No Change	No Change	No Change
	Lithocholic acid	359.2941	6.74	No Change	No Change	No Change	No Change
	Ursodeoxycholic acid or hyodeoxycholic acid	357.2785	5.1	No Change	No Change	No Change	No Change
	Ursodeoxycholic acid or hyodeoxycholic acid	357.2786	5.09	No Change	No Change	No Change	No Change
	Ursodeoxycholic acid or hyodeoxycholic acid	339.2681	5.18	No Change	No Change	No Change	No Change
	Ursodeoxycholic acid or hyodeoxycholic acid	391.2819	5.18	No Change	No Change	No Change	No Change
Secondary, Conjugated	Taurohyodeoxycholic acid	464.2815	4.64	No Change	No Change	No Change	**decreased**
	Taurohyodeoxycholic acid	464.2813	4.64	No Change	No Change	No Change	**decreased**
	Taurohyodeoxycholic acid	464.281	4.64	No Change	No Change	No Change	**decreased**

Table 4.3 Effect of Sleep Disruption and the Double Hit on Standard-Verified Bile Acids. Purified standards of various primary and secondary bile acids were run to identify the identities of 49 features from the untargeted LC/MS/MS analysis. This table reports the whether or not the bile acid is primary or secondary (secondary bile acids are modified by the microbiota), if it is conjugated to an amino acid, and direction of change compared to vehicle-treated or *M. vaccae*-

treated controls for the sleep-disruption only or double hit groups at SD+2. Bold terms indicate $p < 0.05$ for Wilcoxon-Rank Sum tests vs control conditions. Non-bolded terms signify trends ($p < 0.1$) in the indicated direction. Any p value > 0.1 is indicated with “No Change”. Abbreviations: *m/z*, mass to charge ratio; RT, retention time (minutes).

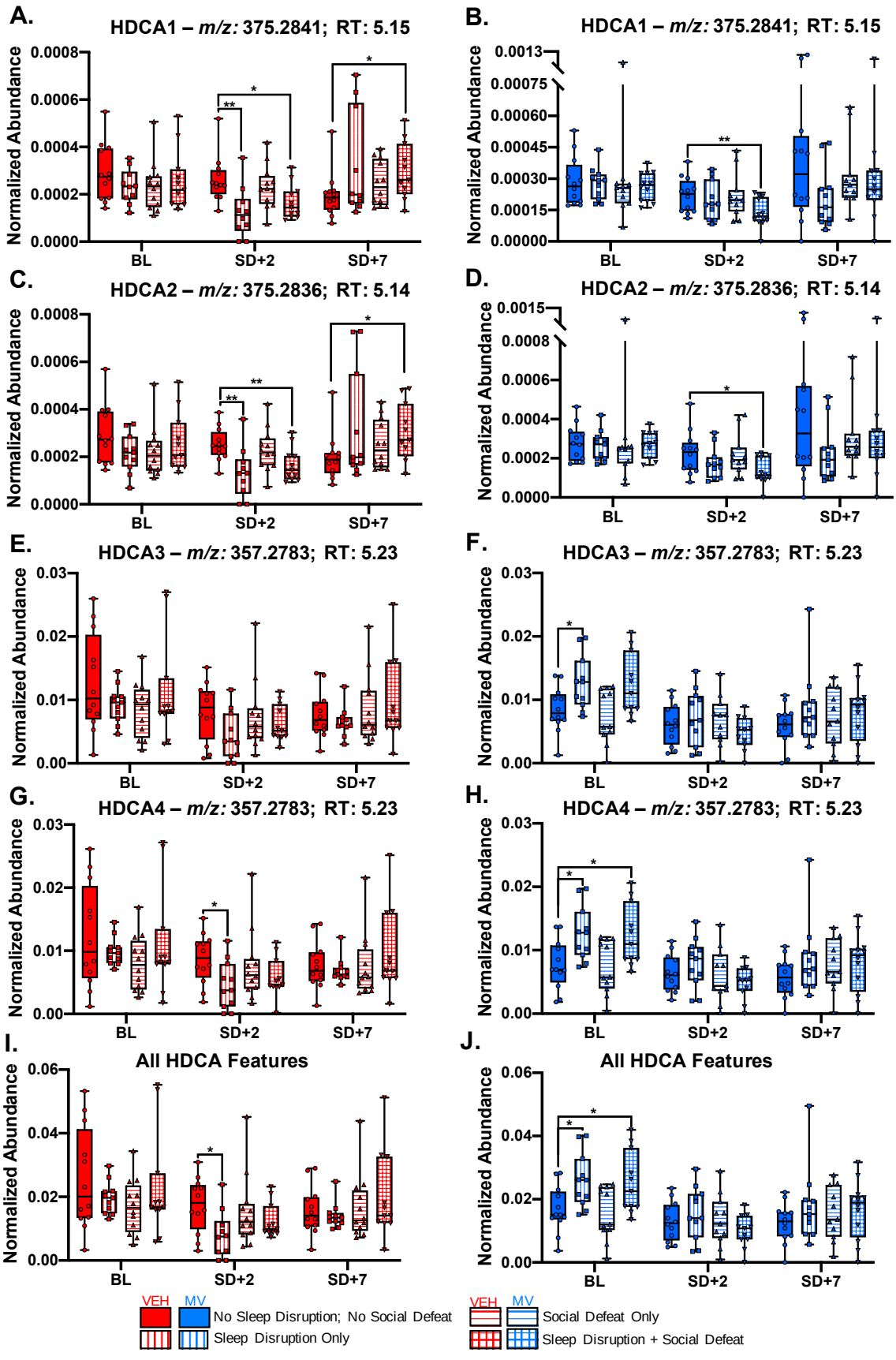
A.
HDCA1 – m/z: 375.2841; RT: 5.15
Normalized Abundance
0.0008
0.0006
0.0004
0.0002
0.0000
BL
SD+2
SD+7
B.
HDCA1 – m/z: 375.2841; RT: 5.15
Normalized Abundance
0.0013
0.00075
0.00060
0.00045
0.00030
0.00015
0.00000
BL
SD+2
SD+7
C.
HDCA2 – m/z: 375.2836; RT: 5.14
Normalized Abundance
0.0008
0.0006
0.0004
0.0002
0.0000
BL
SD+2
SD+7
D.
HDCA2 – m/z: 375.2836; RT: 5.14
Normalized Abundance
0.0015
0.0008
0.0006
0.0004
0.0002
0.0000
BL
SD+2
SD+7
E.
HDCA3 – m/z: 357.2783; RT: 5.23
Normalized Abundance
0.03
0.02
0.01
0.00
BL
SD+2
SD+7
F.
HDCA3 – m/z: 357.2783; RT: 5.23
Normalized Abundance
0.03
0.02
0.01
0.00
BL
SD+2
SD+7
G.
HDCA4 – m/z: 357.2783; RT: 5.23
Normalized Abundance
0.03
0.02
0.01
0.00
BL
SD+2
SD+7
H.
HDCA4 – m/z: 357.2783; RT: 5.23
Normalized Abundance
0.03
0.02
0.01
0.00
BL
SD+2
SD+7
I.
All HDCA Features
Normalized Abundance
0.06
0.04
0.02
0.00
BL
SD+2
SD+7
J.
All HDCA Features
Normalized Abundance
0.06
0.04
0.02
0.00
BL
SD+2
SD+7
VEH
MV
No Sleep Disruption; No Social Defeat
Sleep Disruption Only
VEH
MV
Social Defeat Only
Sleep Disruption + Social Defeat

Figure 4.9: *M. vaccae* Immunization Blunts Sleep Disruption-Induced Decreases in Fecal Hyodeoxycholic Acid. Four features from the untargeted LC/MS/MS dataset that were annotated as bile acids from GNPS were confirmed to be hyodeoxycholic acid (HDCA) using purified standards. The normalized abundance of (A-G) these 4 metabolites, and (H,I) the sum of all 4 are reported at baseline, SD+2, and SD+7. Boxes indicate median, 25th and 75th quantiles; whiskers indicate full range of individual values. Symbols: * $p < 0.05$, ** $p < 0.01$, Wilcoxon Rank-Sum test. Abbreviations: BL, baseline; SD, sleep disruption; *m/z*, mass to charge ratio; RT, retention time (minutes); VEH, vehicle injection; MV, *Mycobacterium vaccae* NCTC 11659 injection. n = 10-12/group

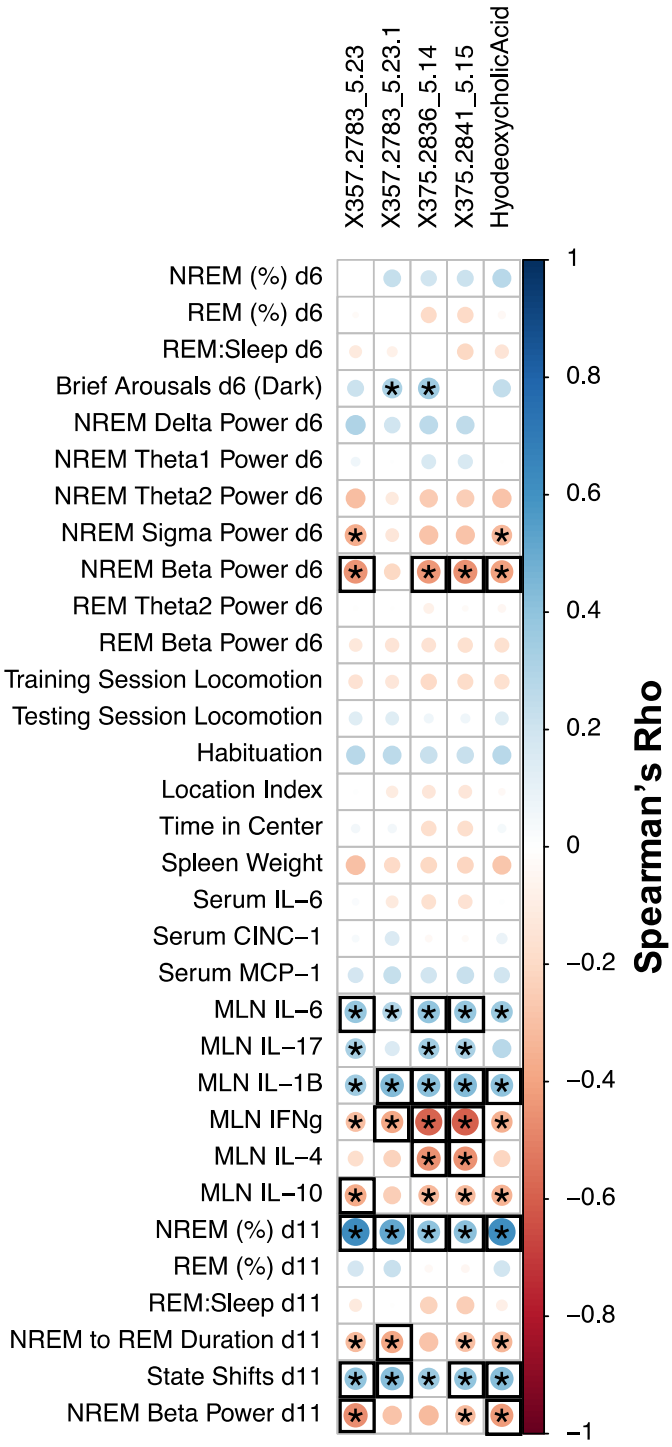

Figure 4.10: HDCA Correlates With Multiple Measures of Sleep, Behavior, and Physiology. Spearman's rank order correlations of the SD+2 values of the 4 HDCA metabolites, along with their sum ("HyodeoxycholicAcid"), vs 32 measures of interest from throughout the experiment were computed for vehicle-treated and *M. vaccae* (MV)-treated mice that received either social defeat alone or sleep disruption plus social defeat (double hit). Metabolites are labeled as their *m/z*_RT. Physiological measures are organized by sleep measures on Day 6 (the first ~20 hours immediately post-social defeat), behavior in the OLM task on Day 7, serum and MLN cytokines at the end of the experiment, and sleep on Day 11. The value of the Spearman's *rho* for each comparison is represented by the size and shade of circle in each square of the correlation plot, with darker blue meaning more positive *rho* and darker red meaning more negative *rho*. Asterisks indicate uncorrected $p < 0.05$, while black boxes indicate $q < 0.05$. Abbreviations: d6, Day 6 of the experiment; d11, Day 11 of the experiment; EEG, electroencephalography; IFNg, interferon gamma; IL, interleukin; CINC-1, cytokine-induced neutrophil chemoattractant-1; MCP-1, monocyte chemoattractant protein-1; MLN, mesenteric lymph node; NREM, non-rapid eye movement; OLM, object location memory; REM, rapid eye movement.

D. Discussion

In Chapter III, we exposed groups of mice to five days of sleep disruption, one hour of acute social defeat stress, sleep disruption followed immediately by social defeat (the double hit), or no sleep disruption followed by a control manipulation (control). Results showed that sleep disruption increased vulnerability to a second hit of acute stress, as sleep and behavioral changes were observed that were not seen in sleep disruption or social defeat alone groups. Furthermore, immunization with *M. vaccae* prevented nearly all of the effects of the double hit. In this chapter, we performed 16S rRNA microbiome and untargeted LC/MS/MS metabolome analyses on fecal samples taken from these mice. The goals of these experiments were 1) to further characterize the double hit phenotype by measuring changes to the fecal microbiome and metabolome, and 2) to utilize the fact that vehicle injection plus sleep disruption is a stress-vulnerable state and *M. vaccae* immunization creates a stress-resilient state to test the hypothesis that microbes, or the molecules they produce, may play a role in stress vulnerability. We found that while the impact of the double hit on global measures of the microbiome were minor, *M. vaccae* stabilized the microbiome. Furthermore, we found that sleep disruption shifted the fecal metabolome in vehicle-treated mice, and that this effect was also blocked by *M. vaccae*. However, the double hit did change the metabolome in *M. vaccae*-treated mice, the implications of which will be explored below. Importantly, we also found that the microbially modified secondary bile acid hyodeoxycholic acid (HDCA) was reduced by sleep disruption in vehicle-injected, but not *M.*

vaccae-injected mice. The abundance of HDCA also correlated with many of the sleep measures that were altered in vehicle-injected, double hit mice, suggesting that reductions in this molecule in the context of sleep disruption may increase stress vulnerability and warrant further investigation.

Microbiome Findings

We found that three injections of *M. vaccae* did not change the microbiome or metabolome measured at baseline. This is consistent with the findings of [Reber et al., 2016], which did not note any overall effects of *M. vaccae* treatment in alpha diversity or beta diversity after 3 weekly injections of the same heat-killed preparation at the same dose used in this study. This finding is not surprising, as *M. vaccae* immunization does not alter baseline or control group sleep (**Figure 3.2**), locomotion (**Figure 3.8**), or elevated plus maze behavior [Reber et al., 2016], indicating the phenotypic changes due to *M. vaccae* may be best observed during or after perturbations to the system in the form of a stressor. Whether *M. vaccae* immunization acts principally to maintain stability in the face of stress, or whether it can also promote active and dynamic adaptive changes, will be discussed in the context of other findings later in this section.

In vehicle-treated mice, sleep disruption alone, social defeat alone, and the double hit resulted in reductions in alpha diversity over time, with the largest decrease observed a full week after the end of the experimental manipulations. In Chapter II, no effects of sleep disruption on microbiome alpha diversity were observed, but samples were only taken at SD+2 and SD+4.

Therefore it is possible some of the changes to alpha diversity induced by sleep disruption take nearly one week to develop. Alpha diversity reductions have been observed at the end of a 10-day chronic social defeat protocol, [Bharwani et al., 2016], but the timeline of when these changes developed was not measured. An acute social stressor was shown to change the community structure of the cecal microbiome, even when measured immediately after stress [Bailey et al., 2011], but there are multiple differences between that study and this study in the nature of the stressor and the method by which the microbiome was assayed, which may explain why we did not see social defeat-induced changes to the microbiome at early timepoints. *M. vaccae* immunization prevented reductions in alpha diversity over time in all experimental groups. This is also consistent with what was reported in [Reber et al., 2016], where *M. vaccae* also prevented alpha diversity reductions over time.

In Chapter II, the same sleep disruption protocol as the one used in this study resulted in a significant shift in the gut microbiome beta diversity at day 2 post-sleep disruption. However, when comparing sleep-disrupted vs control groups at SD+2 in this study, we found no differences in global beta diversity (see **Table 4.1**). In fact, no within-timepoint comparisons of beta diversity versus control groups were significant using PERMANOVA. There may be a few reasons for this discrepancy. First, there were strong cohort effects in most comparisons. While the experimental groups were balanced within each cohort, the significant portion of overall variance that was explained by cohort effects may have masked changes occurring as a result of the experimental manipulations (see Chapter VI for discussion of the cohort effect problem).

Another possibility is that direct comparisons between control groups and experimental groups at each timepoint were confounded by the fact that the control manipulations may have acted as a form of mild stress which could have mildly altered the microbiome, thus making further changes induced by sleep disruption or social defeat difficult to detect at the community level. This notion is supported by some of the findings of Chapter III, where changes and sleep and inability to perform the OLM task suggested that the combination of EEG/EMG surgery, vehicle injections, and the control manipulation of being moved into a quiet novel cage acted as mild stressor that affected physiology for a short time before recovery to baseline levels.

Thus, we chose to look within groups, comparing the SD+2 and SD+7 timepoints to their own baseline. We found a global shift in the microbiome (measured by weighted UniFrac) due to sleep disruption alone and social defeat alone at SD+7, similar to the alpha diversity findings. The double hit, however, did not cause a global change in weighted UniFrac at any timepoint, but did exhibit a significant increase in the within group unweighted UniFrac distance, indicating an overall destabilization of the microbiome. Therefore, it is possible that an insignificant PERMANOVA comparing double hit to baseline was caused by changes of individual microbiomes in inconsistent directions in principal coordinate space and not due to a true lack of shift in the microbiome. *M. vaccae* prevented these changes, again similar to alpha diversity findings and other physiological findings (see Chapter III).

Metabolome Findings

Again examining individual groups over time, we found that sleep disruption caused a shift in the fecal metabolome at SD+2. This observation is much more consistent with the findings of Chapter II, which also saw the greatest sleep disruption-induced shift in the metabolome at SD+2. Furthermore, some of the VSURF suprathreshold metabolites were similar to those seen in Chapter II, such as reductions in bile acids and triterpenoids such as ursolic acid. Furthermore, in this study a feature with a spectral match to atractylenolide III was reduced due to sleep disruption. Atractylenolide III is a sesquiterpenoid molecule shown to have anti-inflammatory properties *in vitro* and *in vivo* [Li et al., 2007a; Li et al., 2007b]. This is further evidence that the changes in the fecal metabolome after sleep disruption could in theory promote a proinflammatory, stress-vulnerable state. The discrepancy between the timing of the microbiome and metabolome findings in sleep-disrupted animals is mildly perplexing. Perhaps changes to the microbiome at the earlier (SD+2) timepoint were subtle, outside of the resolution of global 16S rRNA analysis, but their functional effect (approximated by the metabolome) was already evident. *M. vaccae* prevented a global change in the fecal metabolome due to sleep disruption, consistent with microbiome and physiology findings. The top 25 VSURF suprathreshold metabolites comparing control to the sleep-disrupted group had lower variable importance scores, and fewer of them reached statistical significance on follow-up Wilcoxon Rank-Sum testing compared to their vehicle-treated counterparts, as would be expected. Besides reductions in two primary bile acids, the few changes in the metabolome observed in *M. vaccae*-treated, sleep-disrupted mice were different than those seen in vehicle-treated, sleep-disrupted mice. One intriguing annotated suprathreshold feature was one with a spectral match to 5-hydroxyindole-3-

acetic acid (5-HIAA), which is the main metabolite of the neurotransmitter serotonin. This, along with a reduction in glycerophosphocholine, which is a precursor of choline, another neuroactive compound, suggests that perhaps molecules that alter brain function are changing due to sleep disruption in *M. vaccae*-treated mice.

Does M. vaccae Promote Adaptive Shifts in Microbiome Function After the Double Hit?

One interesting observation in this chapter was that *M. vaccae*-treated, double hit mice displayed a large magnitude shift in microbiome function, as measured by metabolic pathway/module analysis and by global assessment of the fecal metabolome, yet were protected from developing most features of the double hit phenotype (Chapter III). One interpretation of this discrepancy would be that the fecal metabolome has no link to physiology in this context. However, the evidence tying the fecal microbiome and metabolome to stress-related physiological measures is strong enough (see Section I.C) that I find this conclusion to be less likely.

Another possible explanation is that the double hit does alter the fecal metabolome equally in vehicle-treated and *M. vaccae*-treated groups, but the difference from baseline is only detectable in the *M. vaccae* group because the baseline samples are more "normal" than the vehicle-treated mice. This stems from the discussion above, suggesting that the control manipulations (even those occurring before baseline fecal collection such as surgery and vehicle injections) acted as a form of mild stress that made stress- and sleep disruption-induced changes to the microbiome difficult to measure. If *M. vaccae*-treated control groups were more resilient to those mild

stressors (which is evident in the OLM data depicted in **Figure 3.8e**), then a strong stressor such as the double hit may have changed the metabolome enough to note a discrepancy compared to baseline while this discrepancy was not discernable in vehicle-injected mice.

A third, more intriguing interpretation is that *M. vaccae* immunization results in microbiome/metabolomic stability due to mild stressors such as sleep disruption, but when much stronger stressors (such as the double hit) occur, dynamic changes occur that result in *adaptive* maintenance of phenotypic homeostasis. Indeed, one of the bile acids that was decreased due to the double hit uniquely in *M. vaccae*-treated mice was the secondary bile acid deoxycholic acid (see **Table 4.3**), which is has been shown to be proinflammatory, to impair glucose homeostasis [Zaborska et al., 2018], and promote colon carcinogenesis [Bernstein et al., 2011; Prasad et al., 2014] in animal studies. Perhaps the double hit initiates a modulated response to the double hit in the mucosal immune system of *M. vaccae*-treated mice that promotes subtle shifts in the function of the microbiome, preventing production of proinflammatory or harmful metabolites. Indeed, the mucosal immune system impacts the microbiota and vice versa (see [Lazar et al., 2018]), so this pathway is feasible. Follow up experiments should be done to test this hypothesis. For example, one could perform sufficiency experiments involving fecal transplant from *M. vaccae*-injected mice after the double hit to vehicle-injected mice to observe if stress resilience is also transplanted. Another experiment that would test necessity of immunomodulation for this effect to occur would be to use anti-CD25 antibodies to reduce the T_{reg} populations, perform the double hit protocol on *M. vaccae*-treated mice, and examine the fecal microbiome and metabolome.

At first glance the idea that *M. vaccae*-treated mice incur a larger magnitude change in the fecal metabolome after the double hit appears to contradict the narrative that *M. vaccae* is the "great stabilizer" that protects homeostasis and creates stress resistance by preventing change. However, close examination of immune function both peripherally and centrally after *M. vaccae* immunization reveals that there are multiple active changes occurring that may prime adaptive responses to stress. Mesenteric lymph node T_{reg} populations are elevated at least 20 days after *M. vaccae* immunization in control mice [Reber et al., 2016], and an increase in anti-inflammatory cytokine gene expression, including IL-4, can be measured in the brain 8 days after *M. vaccae* injection [Frank et al., 2018]. Furthermore, *M. vaccae*-treated mice display increased proportions of active coping behaviors in a chronic subordinate colony housing protocol [Reber et al., 2016]. Thus, while the outward phenotypic impression of multiple stress-reactivity measures may be that of stability, the action of *M. vaccae* immunization to create that impression may be an active process.

HDCA as a Potential Modulator of Stress Reactivity

One of the most important findings of this study was the observation that standard-verified HDCA was one of the top drivers of separation between vehicle-injected control and sleep-deprived groups at SD+2, while remaining unchanged by sleep disruption in *M. vaccae*-treated

mice. We were intrigued by this finding, as HDCA both a secondary bile acid that is generated by the gut microbiota and a bioactive molecule that has been shown to have health benefits in multiple animal models. HDCA is formed from muricholic acid and hyocholic acid by bacteria in the intestine [Eyssen et al., 1999]. It has agonist activity at both the liver X receptor (LXR) [Song et al., 2000] and at TGR5 receptors [Sato et al., 2008]. LXR subtypes are expressed throughout the body, particularly in liver, intestine, adipose tissue, and macrophages [De Marino et al., 2017], and have been shown to exert anti-inflammatory activity [Joseph et al., 2004; N et al., 2009] and improve glucose tolerance in rodents [Laffitte et al., 2003], which has led to the hypothesis that LXR's are druggable targets that might be useful in the treatment of diseases like obesity, diabetes, neurodegenerative disease, and chronic inflammatory diseases [Hong and Tontonoz, 2014]. Unfortunately, high levels of LXR activation results in activation of lipogenic enzymes and increased triglyceride synthesis and accumulation [Grefhorst et al., 2002]. HDCA is only a partial agonist of LXR, much weaker than other bile acids [Song et al., 2000], making it an interesting candidate for therapeutic effects [De Marino et al., 2017]. Indeed, feeding rodents a diet enriched with HDCA results has hypolipidemic effects [Sehayek et al., 2001; Wang et al., 2003; Shih et al., 2013; Watanabe and Fujita, 2014], reduces atherosclerotic plaque formation in multiple animal models [Sehayek et al., 2001; Shih et al., 2013], and reduced fasting glucose [Shih et al., 2013]. Interestingly, APP/PS1 mice (a model for AD) demonstrate reduced plasma HDCA, and both mouse models and humans with AD appear to have altered bile acid pools in the serum and the brain [Pan et al., 2017]. For these reasons, we focused on this molecule for further analyses as a potential modulator of stress vulnerability.

Not only were multiple features identified as HDCA reduced in vehicle-treated, sleep-disrupted mice, the abundance of these metabolites in the feces at SD+2 correlated with multiple sleep measures and MLN cytokines in mice exposed to social defeat or the double hit. The more HDCA that was present in the feces at SD+2, the less cortical hyperarousal was observed in post-social defeat sleep and on Day 11. This is noteworthy, because cortical hyperarousal is thought to be maladaptive, and post-stress cortical hyperarousal correlated positively with REM sleep disturbances nearly one week later (see Chapter III). Furthermore, a high degree of correlation was observed between fecal HDCA and different MLN cytokines, which further solidifies this molecule as one that has potential to modulate sterile immune responses in the context of stress.

Although these results are encouraging, an alternative interpretation of the bile acid data would be that since the sleep disruption protocol results in a slowing of body weight gain (**Figure 3.4**), perhaps the mice are eating less and thus releasing less bile into the intestine. This would mean HDCA dropped simply due to a lack of precursor primary bile acids. One argument against this scenario revolves around the fact that although multiple standard-verified primary bile acids were reduced, very few other secondary bile acids were reduced after sleep disruption. Thus, perhaps only bacteria that prefer to generate HDCA were altered in some way, resulting in a semi-selective reduction of the beneficial HDCA.

One fact that limits the interpretation of these results is that measurement of HDCA in the intestine does not necessarily reflect levels in the blood or brain. Serum samples were taken in this experiment, and untargeted LC/MS/MS was run. Exploration of these results, with specific targeting of bile acids in the blood, is indicated.

E. Conclusions

The findings of this chapter integrate into the overall goals of the project in a few ways. First of all, these data demonstrate that 5-days of sleep disruption impact the microbiome, but failed to exactly replicate the findings of Chapter II. Reasons for this discrepancy could include cohort effects and the stress of this experimental protocol versus the one in Chapter II, but together provide a good example of one of the issues of microbiome research, that a host of experimental conditions can impact the microbiome, making replication studies difficult. However, the metabolomic changes induced by sleep disruption alone were much more comparable to Chapter II in both timing and character, perhaps indicating that while the taxonomy of the microbiome may be heterogeneous, metabolic function and thus physiological impact of that microbiome may be more stable/reproducible. Furthermore, these results demonstrated that *M. vaccae* has a stabilizing effect on the microbiome in the context of sleep disruption alone, which is consistent with the physiological findings in Chapter III.

It is worth noting that although the largest shift in sleep, behavior, and physiology came after the double hit in vehicle-treated mice in Chapter III, there did not seem to be proportional shifts in

the fecal microbiome and metabolome in this chapter. At face value this weakens the central hypothesis of this project, that changes in the microbiome and metabolome contribute mechanistically to stress vulnerability. However, we believe this hypothesis may still be true, as changes in the sleep disruption-alone group consistently support it. It is possible the double hit of sleep disruption plus social defeat results in heterogeneous changes in the fecal microbiome and metabolome that increases noise in the data, resulting in insignificant analyses at the "-omic" level. The changes that result in stress-vulnerability at the time of social defeat may only be subtle shifts in a few microbes or metabolites, such as HDCA.

Importantly, this chapter identified a microbially modified candidate molecule, HDCA, that may contribute to stress vulnerability after sleep disruption. This molecule has been fed to rodents in preclinical studies, and thus has exciting potential as a countermeasure to improve stress resilience. The concept of using bacterially-derived compounds, either metabolites they produce or components of their external structure, for health benefit has been termed "postbiotics", and has been proposed as an alternative to other microbiome-targeting therapies such as probiotics that have multiple limitations [Tsilingiri and Rescigno, 2013; Suez and Elinav, 2017].

V. CHAPTER V – A PREBIOTIC DIET ALTERS THE FECAL MICROBIOME AND METABOLOME, IMPROVES NREM CONSOLIDATION, AND PROMOTES STRESS RESILIENCE FOLLOWING SLEEP DISRUPTION IN RATS

A. Introduction

The main hypothesis of this dissertation is that sleep disruption is a stress vulnerable state, in part due to changes in the gut microbiome and fecal metabolome, and that microbial-based countermeasures therefore have potential to increase stress resilience in the face of sleep disruption. Thus far we have demonstrated that repeated sleep disruption can alter the fecal microbiome and fecal metabolome, and indeed creates vulnerability to a secondary acute stressor in mice. Furthermore, Chapter III reported data suggesting immunomodulatory bacteria like *M. vaccae* do increase stress resilience. However, subcutaneous immunization with heat-killed *M. vaccae* understandably did not alter the fecal microbiome under basal conditions (Chapter IV), and *M. vaccae* does not normally colonize the intestinal tract, instead acting as a pseudocommensal that passes through after oral ingestion in the drinking water or on food. Therefore, the experiment depicted in Chapters III and IV does not shed as much light on the role of the microbiota themselves in responses to sleep disruption and acute stress as would an experiment examining an intervention that more directly modulates the microbiome. One such intervention is a prebiotic diet.

As outlined in Chapter I, a prebiotic is a dietary compound that must meet a few criteria [Manning and Gibson, 2004; do Carmo et al., 2016]. The first is that it must not be hydrolyzed or absorbed by the host in the stomach or small intestine. The second is that it must be a selective substrate for 'beneficial' bacteria in the gastrointestinal tract. The third is that fermentation of the compound by the bacteria should result in some physiological benefit to the host. Galactooligosaccharides (GOS) are an example of a prebiotic compound that is found in many animal milks, including human milk at ~1g/L [Angus et al., 2005]. GOS has very low nutritional value (1.7 kcal/g) and is not hydrolyzed directly by host enzymes [Macfarlane et al., 2008]. The health benefits of dietary supplementation with GOS have been studied in multiple contexts. It was found to reduce plasma levels of C-reactive protein, insulin, total cholesterol, and triglycerides in overweight humans [Vulevic et al., 2013]. Furthermore, GOS supplementation has been shown to improve immune function in elderly adults [Vulevic et al., 2008], reduce stress-induced neuroendocrine responses in adult volunteers [Schmidt et al., 2015], and attenuate post-inflammatory anxiety in mice [Savignac et al., 2016]. Together, these data suggest GOS supplementation may impact stress resilience in the context of sleep disruption. However, this has never been investigated.

Another example of a prebiotic is polydextrose (PDX), a highly branched polymer of 6 carbon sugars that is often used as a filler substance in the food industry [do Carmo et al., 2016]. The bonds of PDX are not hydrolyzed by mammalian enzymes, and thus it also has very low caloric value (1 kcal/g) [do Carmo et al., 2016]. Studies of dietary supplementation of PDX have shown

that it can attenuate triglycerides and cholesterol in mice fed a Western diet [Raza et al., 2017], and that it can reduce intestinal inflammation in various animal models (see [do Carmo et al., 2016] for review).

A few studies have examined prebiotic diets that utilize both GOS and PDX (along with other supplements such as lactoferrin), with encouraging results. An experimental diet of GOS, PDX, and bioactive milk fractions fed early in life attenuated learned helplessness behaviors after acute stress and modified gene expression in multiple stress-responsive brain areas in rats [Mika et al., 2017; Mika et al., 2018]. Another study in rats found that the same diet of GOS/PDX and bioactive milk fractions found that rats fed the test diet displayed a larger REM sleep rebound after a severe tailshock stressor [Thompson et al., 2016]. No studies to date, however, have examined GOS and PDX in the context of sleep deprivation.

Therefore, we fed rats a prebiotic diet containing GOS/PDX (hereafter "the prebiotic diet") in the context of sleep disruption followed by acute social defeat stress, similar to the protocol used in Chapter III. The purpose of this study was to perform a detailed evaluation of the impact of the prebiotic diet on the fecal microbiome, fecal metabolome, and serum metabolome over time, as well as to test the hypothesis that microbiome-modifying countermeasures can improve resilience to sleep disruption plus acute stress.

B. Materials and Methods

Animals and Experimental Design

Twelve cohorts of eight 32-day old male Sprague Dawley (Envigo Laboratories, USA) were used for this experiment ($N = 96$). A total of 13 animals did not complete the experimental protocol and were thus eliminated from all analyses, for a total $N = 83$. No explicit power analysis was used, the sample size was determined in to ensure adequate number of biological replicates of the main outcome measure: sleep recordings (target of $n = 10\text{-}12$). The experiment consisted of 8 experimental groups in a 2 x 2 x 2 design (control diet vs prebiotic diet, *ad libitum* sleep vs sleep disruption, no social defeat vs social defeat). Rats were co-housed until EEG/EMG implant surgery, after which they were individually housed until the end of the experiment. Diet groups were randomly assigned to cages on the day of arrival. After EEG/EMG surgery, rats were assigned to further experimental groups randomly, but effort was made to ensure prior cagemates were in different groups. Male Long Evans rats (Envigo Laboratories, USA) were used as aggressors in the social defeat model (see below). All rats were maintained on a 12:12 L:D cycle at room temperature ($23 \pm 2\ ^{o}C$) with food and water available *ad libitum* throughout the experiment. All protocols were approved by the Northwestern Institutional Animal Care and Use Committee. Zeitgeber Time (ZT) is defined as the number of hours after the onset of the light period (light onset = ZT0).

Experimental Diets

Rats were started on *Ad libitum* experimental or control diet upon arrival at the facility. The control diet was Envigo Teklad diet TD.110883 (Envigo Teklad, Madison WI, USA). The prebiotic diet consisted of the control diet supplemented by galactooligosaccharides (GOS 21.23 total g/kg (7.00 active g/kg); FrieslandCampina, Zwolle, Netherlands) and polydextrose (PDX 7.69 total g/kg (7.00 active g/kg); Danisco, Terre Haute, IN, USA). The prebiotic diet was custom made by Envigo Teklad (TD.110889).

EEG/EMG Implantation Surgery

Four weeks after arrival, and 14 days prior to baseline sleep (i.e., on day –14), rats were implanted with electroencephalographic/electromyographic (EEG/EMG) sleep recording devices (Pinnacle Technologies, Lawrence, KS, USA). Surgical procedures were performed using a rat stereotaxic apparatus with standard aseptic techniques in a ventilated, specially-equipped surgical suite. Anesthesia was induced by isoflurane gas. The EEG/EMG headmount consisted of a plastic 6-pin connector connected to four EEG electrodes and two EMG electrodes. Four stainless steel screws serving as 2 EEG leads and grounds were screwed into the skull with one lead located 5 mm anterior to bregma and 2 mm lateral to the central suture, another 1mm anterior to bregma, 2 mm lateral to the central suture, and the other two at 1 mm anterior to lambda and 2.5 mm lateral to each side of the central suture. The exposed ends of two stainless steel Teflon-coated wires serving as EMG leads were then inserted into the nuchal muscles using a pair of forceps. The entire headmount was then sealed by dental acrylic and a skin staple at the front and the back of the implant was placed to close the incision. Heat support was provided

until recovery from anesthetic by placing animals on a circulating water blanket. Subcutaneous injection of analgesic meloxicam (2 mg/kg; Norbrook Laboratories, Northern Ireland) was given to the animals at the time of surgery and once more on the following day.

Sleep Recording and Analysis

After surgery, rats were moved into cylindrical sleep recording cages (Pinnacle Technologies , Lawrence, KS, USA) within individual acoustically-isolated and Faraday-shielded chambers. Two days before baseline sleep, the headmount was connected to the transmission tether. Cages had corncob bedding and food/water available *ad libitum*. Sleep was recorded for a 24 hour baseline, then recordings were begun at the start of sleep disruption (ZT6). At the end of the fifth 20 h sleep disruption session, rats were unplugged from their EEG/EMG tethers for social defeat (see below). Upon return to home cages, sleep recording resumed for 24 h (ZT7-ZT7) until rats were unplugged from their EEG/EMG tethers for testing in the OLM test. Data were collected using Pinnacle Acquisition software (Pinnacle Technologies), then scored as described in Chapter III.

Sleep Disruption Protocol

After baseline sleep recordings, half of the rats entered the sleep disruption protocol. All cages had corncob bedding and food/water available *ad libitum* throughout the protocol. Sleep disruption was achieved as described in Chapter III.

Social Defeat Protocol

Male Long Evans rats were singly housed in large (44 cm x 24 cm x 21 cm) polycarbonate cages and were screened for aggressive behavior before the experiment. Rats that began to injure their opponents by harmful bites during screening were not used for the social defeat procedure. On the day of the social defeat stressor (at ~ZT6), half of the experimental rats were introduced into the cage of an aggressor. As soon as the aggressor rat attacked and defeated the intruder rat, the intruder was covered with a 25 cm x 15 cm x 15 cm metal mesh cage while still inside the aggressor cage, and left for 1 h. Control animals not receiving social defeat were unplugged from the recording tether and placed in a clean cage in a quiet room.

Object Location Memory Task

The object location memory (OLM) task is a hippocampal-dependent memory task[Murai et al., 2007] that is sensitive to stress[Cazakoff et al., 2010; Howland and Cazakoff, 2010]. All groups of rats underwent testing in the OLM task the day after social defeat, beginning at ZT7. Rats were placed in a dimly lit (~50 lux) 53 cm x 53 cm x 30 cm arena with no objects and allowed to explore for 5 minutes. Ten minutes later, they were returned to the chamber, this time containing two identical cylindrical objects (100 mL pyrex bottles with caps) on the same side of the arena. Rats were allowed to explore the arena for 5 minutes and after 90 minutes in their home cage were allowed to explore the arena again, with one object moved. Exploration of the moved object more than the non-moved object is considered evidence of successful acquisition of contextual memory, and is denoted by a “location index” (100 x {time exploring moved

object/total time exploring either object}) significantly different from 50%. In this experiment, location index was quantified during the 5 minutes of the testing session. LimeLight (Actimetrics, Wilmette, IL, USA) behavioral software was used to track the animal's path traveled within the open field over time. De-identified video files were scored by two experimenters and average location indices were reported.

Serum Collection

Six days after OLM testing (day 13), rats were euthanized via rapid decapitation between ZT4-ZT6 and trunk blood was collected in Z-gel serum tubes (Sarstedt AG & Co, Nümbrecht, Germany). The blood was centrifuged at 7500 rpm for 5 min, and serum was collected and stored at –80 ºC until analysis.

Fecal Sample Collection

Fecal samples were collected at 4 different timepoints: T1) on the day of arrival; T2) one to two days before surgery (4 weeks on diet); T3) at baseline sleep (6 weeks on diet); and T4) at the end of the experiment (see **Figure 5.1a**). Each collection occurred on days where clean cages were provided, so rats were placed into a clean chamber with fresh bedding and food and monitored closely until at least two fresh fecal pellets from each cage were collected. Only spontaneously voided pellets were collected. Samples were placed into individual 1.5 mL microfuge tubes, and frozen at -80 ºC until microbiome/metabolome analysis, at which point one sample from each animal (or two from each cage for timepoints T1 and T2) was cut in half. One half was analyzed

for microbiome the other for metabolome. At each collection timepoint, duplicate samples of bedding, water, food, and blank tubes were also collected.

Microbiome analysis

Microbiome data were generally analyzed using the Quantitative Insights Into Microbial Ecology 2 (QIIME2, version 2018.4) bioinformatics software package[Caporaso et al., 2010; Bolyen et al., 2019]. A total of 334 fecal and 63 environmental samples were processed for 16S rRNA microbiome analyses as described in Chapter IV.

Beta diversity metrics were performed at a rarefied depth of 9,000 reads, resulting in the removal of 21 fecal samples from the dataset. Beta diversity was assessed using weighted and unweighted UniFrac distance [Lozupone et al., 2011] matrices, which were used to generate PCoA plots and to perform PERMANOVA in QIIME2. Alpha diversity metrics were calculated using scikit-bio 0.5.1 as implemented by QIIME2. Differential abundance was assessed at the OTU and at the genus levels using analysis of the composition of microbiomes (ANCOM) [Mandal et al., 2015] as implemented in QIIME2.

PICRUSt2 analysis of 16S rRNA gene data

We inferred the microbial gene content from the taxa abundance using the software package Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2; https://github.com/picrust/picrust2; v2.1.4-b) [Langille et al., 2013]. This tool allows assessment

of functional capacity of a microbiome using 16S rRNA sequencing data. To identify differentially abundant functional pathways and enzymes, DESeq2 (version 1.14.1) was performed using the Bioconductor R package in RStudio (version 1.2.1335, RStudio Inc).

Metabolome Analysis

A total of 334 fecal, 63 environmental, and 83 serum samples were processed for fecal metabolome analyses. Fecal samples were extracted using the protocol described in Chapter IV. Fecal extracts were analyzed using an ultra-high performance liquid chromatography system (Vanquish, Thermo) coupled to a hybrid quadrupole-Orbitrap mass spectrometer (Q-Exactive, Thermo) fitted with a HESI probe as described in Chapter IV for fecal samples.

Serum samples were extracted by adding 100% methanol spiked with 2 µM sulfamethazine to a final concentration of 80%. The samples were vortexed for two minutes and then placed in -20°C for 20 min to aid in protein precipitation. After, they were centrifuged at 15,000 rpm and 80% of the solvent volume was placed into a 96 Well Plate. The plates were then lyophilized using a CentriVap Benchtop Vacuum Concentrator (Labconco) and stored at -80°C. Upon time for analysis, the dried samples were resuspended in 50% methanol spiked with 1µM sulfadimethoxine. The metabolomic extracts were analyzed using an ultra-high performance liquid chromatography system (Vanquish, Thermo Scientific) coupled to a quadrupole-Orbitrap mass spectrometer (Q Exactive, Thermo Scientific). Chromatographic separation was performed using a Kinetex C18 1.7 µm, 2.1 mm x 50 mm column (Phenomenex) at a flow rate of 0.5

mL/min and held at a temperature of 40 °C. The mobile phase consisted of solvent A, 100% LC-MS grade water with 0.1% formic acid (v/v) and solvent B, 100% LC-MS grade acetonitrile with 0.1% formic acid (v/v). The chromatographic elution gradient was: 0.0–1.0 min, 5% B; 1.0–9.0 min, 5–100% B; 9.0-11.0 min, 100% B; 11.0-11.5 min, 100-5% B; 11.5-12.5 min, 5% B. Heated electrospray ionization parameters were: spray voltage, 3.5 kV; capillary temperature, 268.0 °C; sheath gas flow rate, 52.0 (arb. units); auxiliary gas flow rate, 14.0 (arb. units); auxiliary gas heater temperature, 435.0 °C; and S-lens RF, 50 (arb. units). MS data was acquired in positive mode using a data dependent method with a resolution of 35,000 in MS1 and a resolution of 17,000 in MS2. An MS1 scan from 100-1500 m/z was followed by an MS2 scan, using collision induced dissociation, of the five most abundant ions from the prior MS1 scan.

The orbitrap files (.raw) were exported to mzXML files using MSConvert [Chambers et al., 2012]. Feature detection of the MS_1 data was performed using MZmine2 [Pluskal et al., 2010]. The resultant feature tables were normalized to an internal standard followed by a row sum (total ion count) normalization and contained 12570 features (fecal) and 2301 features (serum). Feature tables were also generated for samples of the control and prebiotic diets, containing 2379 features. All of these features were removed from the fecal feature, resulting in a table of 10229 non-dietary fecal metabolites, which was used for most analyses (except where indicated). PCoA plots were then generated using Canberra distance, and PERMANOVA was performed at each timepoint on the normalized feature table using the Vegan package (version 2.5-5) in RStudio. PERMANOVA was performed after the full feature table was filtered down to the groups of interest and filtered to remove features present in fewer samples than one half of the *n* of one

experimental group. In order to identify metabolites that were different between groups of interest, we used a multiple-method approach that included machine learning and nonparametric hypothesis testing. In order to first identify the group of metabolites that were the key drivers of differences between groups at each timepoint, Variable Selection Using Random Forests (VSURF, version 1.1.0) [Genuer et al., 2010; Genuer et al., 2015] analysis was performed using the VSURF.R package in RStudio. Briefly, this protocol uses multiple iterations of the random forest supervised machine learning technique to isolate the most important drivers of separation between two groups by defining a threshold variable importance. Taking this list of suprathreshold features, we then performed Wilcoxon Rank-Sum tests at each timepoint as a form of a 'post hoc' test to confirm differences between groups.

Features of interest for the fecal and serum feature tables were annotated using feature-based molecular networks using the online workflow at GNPS (version 1.2.3) [Wang et al., 2016]. The data was filtered by removing all MS/MS fragment ions within +/- 17 Da of the precursor m/z. MS/MS spectra were window filtered by choosing only the top 6 fragment ions in the +/- 50Da window throughout the spectrum. The precursor ion mass tolerance was set to 0.02 Da and a MS/MS fragment ion tolerance of 0.02 Da. Networks were then created where edges were filtered to have a cosine score above 0.50 and more than 8 matched peaks (fecal) and a cosine score above 0.58 and more than 6 matched peaks (serum). Further, edges between two nodes were kept in the network if and only if each of the nodes appeared in each other's respective top 10 most similar nodes. Finally, the maximum size of a molecular family was set to 100, and the lowest scoring edges were removed from molecular families until the molecular family size was

below this threshold. The spectra in the networks were then searched against GNPS' spectral libraries. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.50 and at least 8 matched peaks (fecal) and above 0.68 and at least 6 matched peaks (serum).

Results can be found at (fecal):

https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=11444f62790649a19ce64fb7798edaf8

Serum: https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=e3ce12fc10204ae9b05c41539f08b673

Accurate mass and MS/MS fragmentation pattern between a metabolite of interest and a reference library, via GNPS, was used for all metabolite standard initiative (MSI) level 2 annotations. Accurate mass with retention time alignment and MS/MS fragmentation pattern between a metabolite of interest and a chemical reference standard was used for all MSI level 1 annotations.

Statistical Analyses and Software

All graphs depict the mean +/- SEM unless otherwise stated. All PCoA plots were generated using the EMPeror visualization tool as implemented in QIIME2 [Vazquez-Baeza et al., 2013]. Microbiome data processing and analysis, including microbiome PERMANOVA, were performed in QIIME2 as outlined above. Wilcoxon Rank-Sum tests, VSURF, and DESeq2 (with Benjamini Hochberg adjustment) were performed or generated in RStudio (version 1.0.136, RStudio Inc, Boston, MA, USA). Mixed-effects models and ANOVA with Bonferroni post hoc

testing, along with generation of all other graphs/figures, was performed using GraphPad PRISM (version 8.2.1; GraphPad Inc, San Diego, CA, USA).

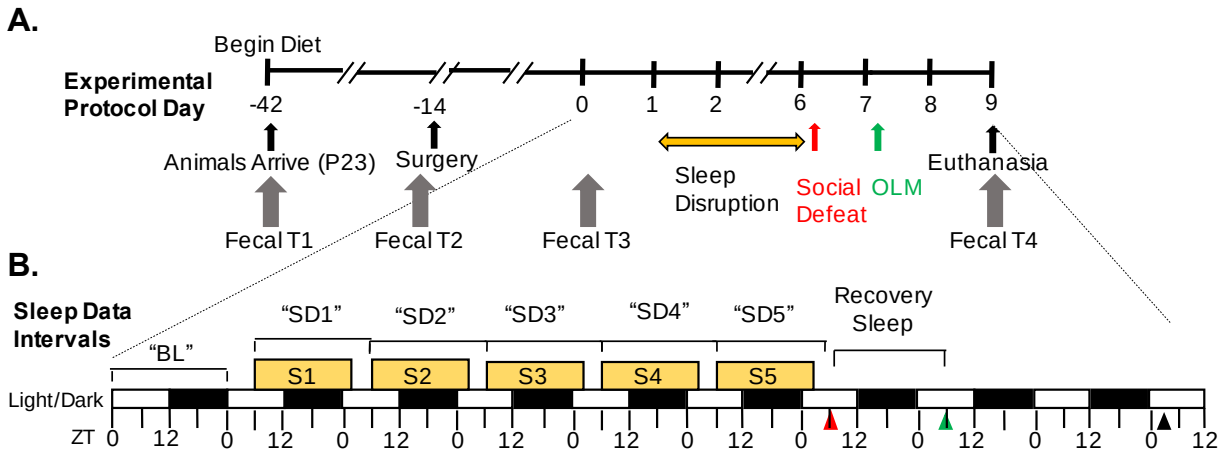

Figure 5.1: Experimental Timeline. (A) Overview of the experimental protocol. Experimental protocol days are depicted along the axis of the timeline, with tick marks indicating ZT0 of that experimental day. Days of fecal samples are indicated with grey arrows. (B) Detail of indicated time period that includes the sleep disruption protocol, social defeat stress (red arrow), object location memory (green arrow), and terminal sample collection (black arrow). Tick marks along the bottom indicate zeitgeber time on that day, white/black rectangles indicate 12 h light/dark phases, and yellow rectangles indicated time periods of sleep disruption for the sleep-disrupted groups (ZT6-ZT2). EEG/EMG recording intervals, as used in the forthcoming figures, are indicated above the timeline. Abbreviations: BL, baseline; OLM, object location memory; P, postnatal day; S, 20-hour sleep disruption window; SD, 24-hour sleep disruption day.

C. Results

The Prebiotic Diet Alters the Structure and Function of the Fecal Microbiome

We first sought to characterize the effect of the prebiotic diet on the fecal microbiome over time. Rats arrived at postnatal day 23, at which time a baseline fecal sample was collected (timepoint T1). Samples were also collected at the start of the fourth week on diet (T2), the sixth week on

diet (T3), and at the end of the experiment or seventh week on diet (T4, see **Figure 5.1a**), and processed for 16S rRNA microbiome analysis. Unweighted and weighted UniFrac revealed a significant shift in the microbiome due to the diet at T2, T3, and T4 (**Figure 5.2a,b**). This was accompanied by a reduction in alpha diversity in the prebiotic diet-fed rats compared to control diet-fed rats. Both Faith's phylogenetic diversity (**Figure 5.2c**) and the total number of observed OTU (**Figure 5.2d**) were reduced at T2 and T3 in the prebiotic diet group compared to control, but this difference was no longer present at T4. Furthermore, the pielou evenness was unchanged throughout the experiment (**Figure 5.2e**).

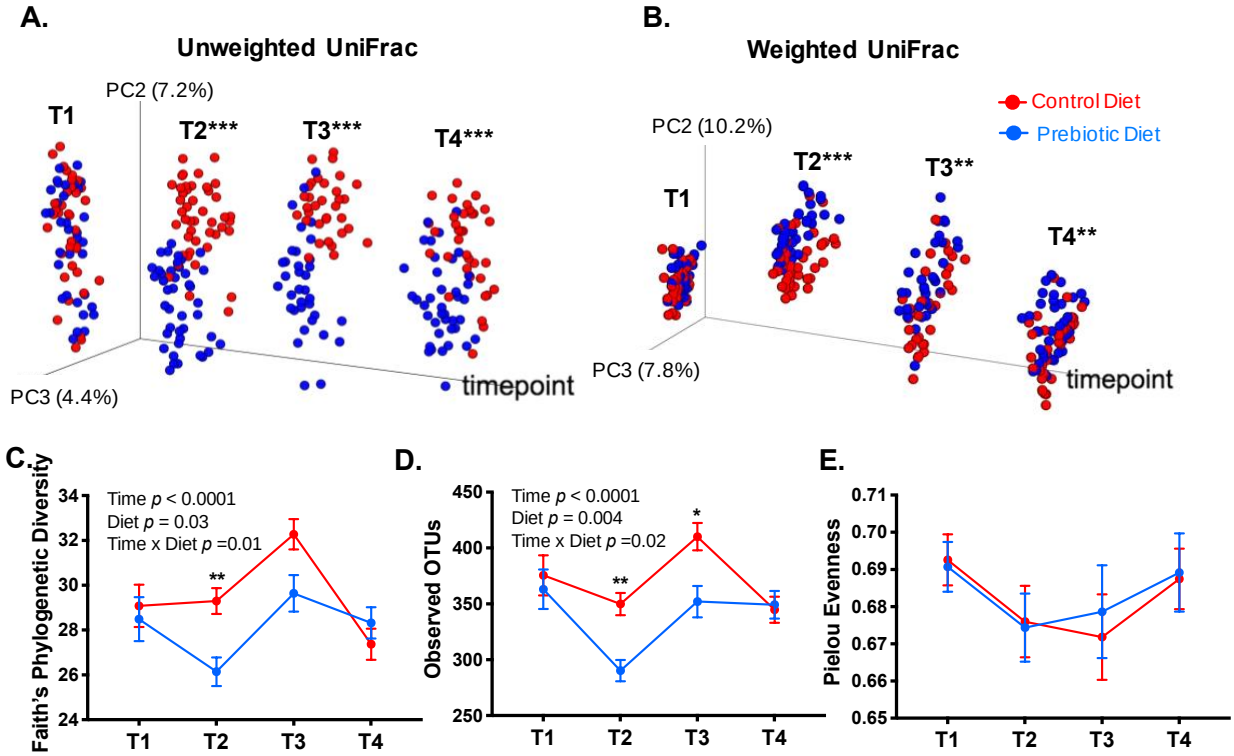

Figure 5.2: The Prebiotic Diet Causes Changes to Fecal Microbiome Beta and Alpha Diversity. Fecal samples were collected on the day of arrival to the facility (T1), after 4 weeks on diet (T2), at baseline sleep (T3), and at the end of the experiment (T4) and 16S rRNA microbiome analyses were performed. PERMANOVA testing for an effect of diet was performed at each timepoint, and PCoA depicting (A) unweighted UniFrac and (B) weighted

UniFrac analysis of beta diversity, with one axis representing timepoint, are reported. Alpha diversity was measured at each timepoint using (C) Faith's phylogenetic diversity index, (D) the total number of OTU, and (E) Pielou evenness metric. Mixed-effect modeling testing for an effect of timepoint, diet, and interactions was performed for each, and significant results are reported in the figure. Data are mean ± SEM. Symbols: (A,B) ***$p < 0.001$, **$p < 0.01$, PERMANOVA; (C-E)**$p < 0.01$, *$p < 0.05$, Bonferroni *post hoc*. Abbreviations: OTU, operational taxonomic analysis; PC, principal coordinate. n = 33-45/group.

In order to characterize the prebiotic diet-induced changes to the microbiome, we performed ANCOM at each timepoint to determine differentially abundant taxa. At the OTU level, ANCOM detected 0 differentially abundant features at T1 (as expected), 55 OTU at T2, 32 OTU at T3, and 30 at T4 (**Figure 5.3a**). Due to the fact that 16S rRNA microbiome analysis is generally more reliable at accurate taxonomic prediction at the genus level than the species level [Gilbert et al., 2018; Knight et al., 2018], we then performed ANCOM on features that had taxonomic assignment at the genus level. We found 4 genera to be differentially abundant due to diet at one or more timepoints (**Figure 5.3b-e**). *Ruminococcus* was increased at T2 in the prebiotic diet group, but not at the other timepoints (**Figure 5.3b**). The genus *Coprobacillus* was also increased due to the prebiotic diet, but only at T3 and T4 (**Figure 5.3c**). Conversely, an unknown genus within the class *Bacteroidales* was lower compared to control diet-fed rats at T2, T3, and T4 (**Figure 5.3d**). Interestingly, the genus *Parabacteroides* was greatly increased at all non-baseline timepoints, by a factor of 6.5 T2, a factor of 7 at T3, and a factor of 5 at T4 (**Figure 5.3e**). Due to the particularly marked increase in genus *Parabacteroides* due to the diet, and the fact that there is a growing body of literature describing the role of a particular species within *Parabacteroides* (*Parabacteroides distasonis*) in host physiology [Lathrop et al., 2011; Dziarski et al., 2016; Valles-Colomer et al., 2019; Wang et al., 2019b], we then performed ANCOM again

at each timepoint at the species level to investigate whether *P. distasonis* was the driving factor behind the increase in genus *Parabacteroides*. Indeed, we found that *P. distasonis* was the dominant species within the genus, and that is was significantly increased at T2, T3, and T4 (**Figure 5.3f**). We are aware of the limited resolution of taxonomic assignment using 16S rRNA analysis, but we are confident this taxonomic classification is accurate. There were 26 OTU that were assigned to *P. distasonis*, and the average confidence score of the assignments was 0.9755 (95% CI: 0.9459-1.005).

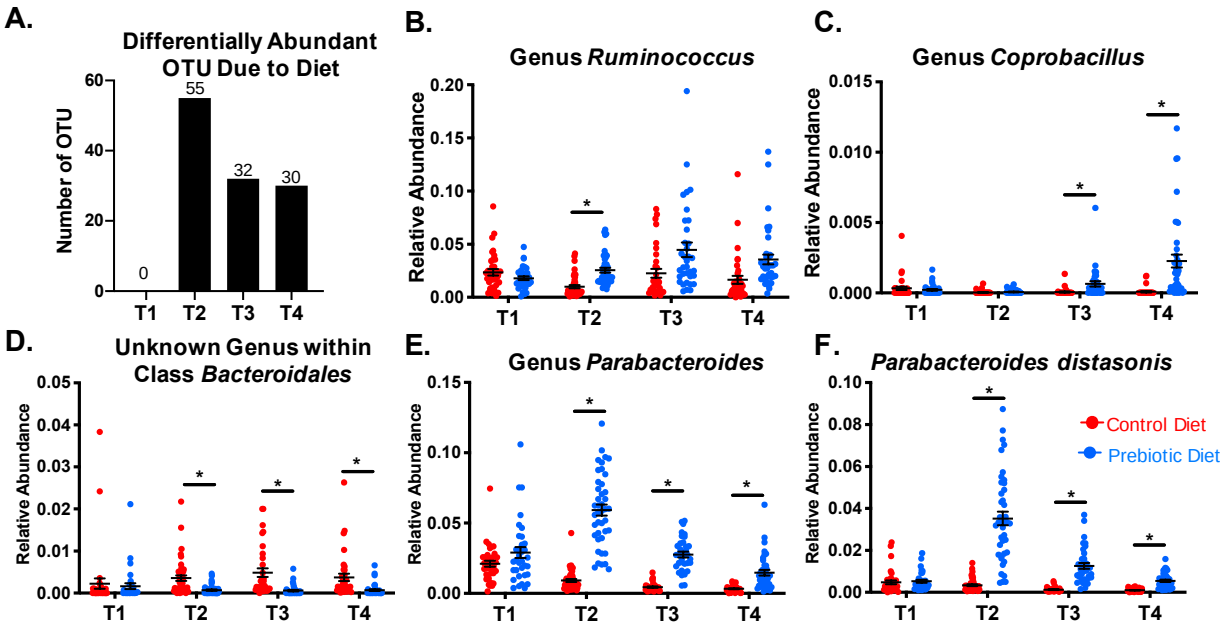

Figure 5.3: The Prebiotic Diet Results in Large Changes in the Relative Abundance of Several Taxa. Fecal samples were collected on the day of arrival to the facility (T1), after 4 weeks on diet (T2), at baseline sleep (T3), and at the end of the experiment (T4) and 16S rRNA microbiome analyses were performed. ANCOM was performed at each timepoint to assess differentially abundant taxa due to the diet. The number of OTU that were differentially

abundant at each timepoint is reported in (A). (B-E) ANCOM was then performed at the genus level, revealing 4 genera that were differentially abundant at ≥ 1 timepoint. (F) ANCOM performed at the species level confirmed *P. distasonis* was differentially abundant at T2-T4. Data are mean ± SEM. Symbols: *differentially abundant at that timepoint via ANCOM. Abbreviations: ANCOM, analysis of the composition of microbiomes; OTU, operational taxonomic unit. *n* = 33-45/group.

In order to assess whether the diet changed the functional capacity of the microbiome, we performed PICRUSt2 [Langille et al., 2013], which uses taxonomy based on 16S rRNA data to predict functional gene content of the microbiome based on reference sequences of each taxa. Then, we used DESEq2 to identify differentially abundant genes and pathways due to diet at each timepoint. We found that, using an FDR cutoff of 0.1, there were 1114 altered genes (525 increased, 589 decreased) due to diet at T2, 882 changed (387 increased, 495 decreased) at T3, and 250 changed (134 increased, 216 decreased) at T4 (**Figure 5.4a**). Altered pathways followed a similar pattern, with 243 changed (113 increased, 130 decreased) at T2, 160 changed (73 increased, 87 decreased) at T3, and 22 (11 increased, 11 decreased) at T4 (**Figure 5.4b**). Most of the altered pathways at T2 and T3 overlapped (144/257; **Figure 5.4c**). However, only 8 pathways were differentially abundant due to diet at all 3 timepoints (**Figure 5.4c, Table 5.1**). The abundance of two pathways involved in lipopolysaccharide (LPS) synthesis were significantly increased with diet, and two pathways relating to metabolism of exogenous molecules such as aromatic amines and nitrates were decreased due to diet at all timepoints (**Table 5.1**). Furthermore, three pathways involved in sugar metabolism were altered either up or down across due to the prebiotic diet (**Table 5.1**). Finally, abundance of a pathway involved in pyrimidine deoxyribonucleotides de novo biosynthesis was increased at all three timepoints

(**Table 5.1**). Together, this set of results show that the GOS/PDX prebiotic diet had a strong impact on the structure and function of fecal microbiome throughout the experiment which was particularly characterized by a 5-7x increase in the bacteria *P. distasonis*.

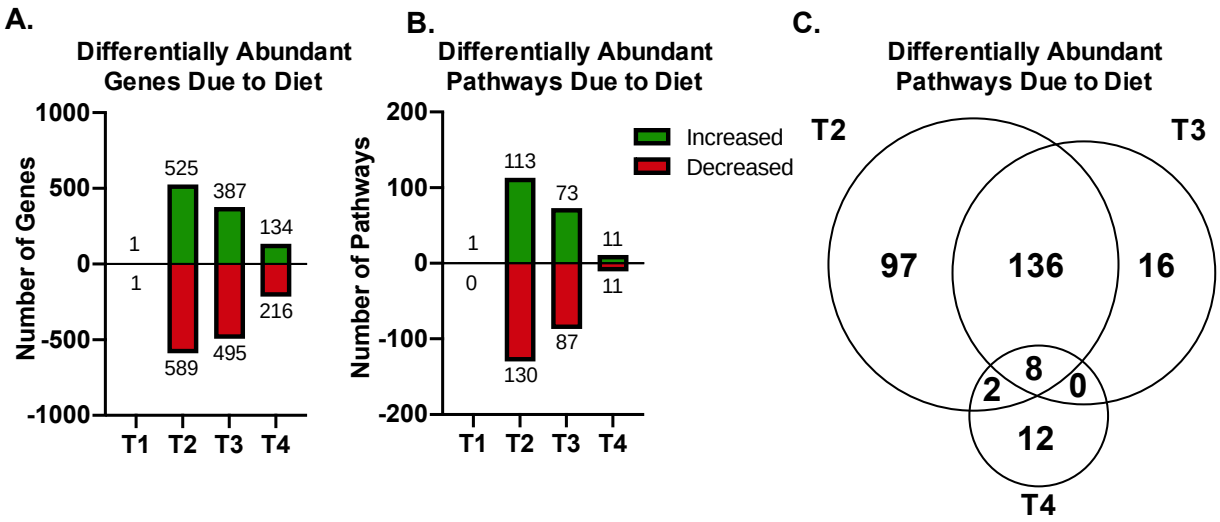

Figure 5.4: The Prebiotic Diet Alters the Predicted Function of the Microbiome. Fecal samples were collected on the day of arrival to the facility (T1), after 4 weeks on diet (T2), at baseline sleep (T3), and at the end of the experiment (T4) and 16S rRNA microbiome analyses were performed. PICRUSt2 was then performed to predict abundance of genes and metabolic pathways based on the 16S rRNA data. DESEq2 was performed at each timepoint to identify differentially abundant (A) genes, and (B) pathways due to diet. (C) a Venn diagram depicting the number of differentially abundant pathways present at each timepoint.

	Pathway	Description	Fold Change T2	Fold Change T3	Fold Change T4
LPS Synthesis	PWY-7332	superpathway of UDP-N-acetylglucosamine-derived O-antigen building blocks biosynthesis	↑ 0.57	↑ 1.11	↑ 4.20
	PWY-7090	UDP-2,3-diacetamido-2,3-dideoxy-α-D-mannuronate biosynthesis	↑ 0.80	↑ 1.21	↑ 1.90
Sugar Metabolism	PWY-621	sucrose degradation III (sucrose invertase)	↓ -0.27	↓ -0.22	↓ -0.004
	FUC-RHAMCAT-PWY	superpathway of fucose and rhamnose degradation	↑ 0.10	↑ 0.11	↑ 1.04
	GLYOXYLATE-BYPASS	Glyoxylate cycle (alternative to TCA cycle that utilizes fatty acid, alcohol, and ester substrates	↓ -0.63	↓ -0.43	↓ -0.46
	PWY-6545	pyrimidine deoxyribonucleotides de novo biosynthesis III	↑ 0.04	↑ 0.02	↑ 0.83
Exogenous Molecule Metabolism	PWY-7431	aromatic biogenic amine degradation (bacteria)	↓ -0.65	↓ -0.53	↓ -0.43
	PWY490-3	nitrate reduction VI (assimilatory)	↓ -0.47	↓ -0.31	↓ -0.35

Table 5.1: Predicted Microbial Metabolic Pathways Altered by the Prebiotic Diet. Fecal samples were collected after 4 weeks on diet (T2), at baseline sleep (T3), and at the end of the experiment (T4) and 16S rRNA microbiome analyses were performed. PICRUSt2 was performed on the 16S rRNA microbiome data to predict genetic content. DESEq2 was then performed at each timepoint to identify predicted pathways that were differentially abundant due to diet. The above reports the pathway ID, description, and fold change/direction of change of the 8 pathways that were significantly altered by the diet at T2, T3, and T4.

The Prebiotic Diet Alters the Fecal and Serum Metabolome

We next examined the effect of the prebiotic diet on the fecal metabolome as measured by untargeted LC/MS/MS. Analysis of the initial fecal metabolomics feature table revealed a significant effect of the diet on the fecal metabolome at T2, T3, and T4 (**Figure 5.5a**). However, we thought it was possible that many of the features driving this separation were molecules deriving from the different diets that were passing unaltered in the feces. In order to control for this, we removed all features from the fecal feature table that were present in the feature table generated by LC/MS/MS of diet samples (see **Methods**). The fecal metabolome was still

significantly shifted due to the diet at T2, T3, and T4 (**Figure 5.5b**). All of the subsequent analyses were performed using the feature table with dietary features removed.

We next performed VSURF to determine the top drivers of separation between the control diet-fed and prebiotic diet-fed rats at T2 and T3 (both before sleep disruption occurred). VSURF identified 301 suprathreshold metabolites at T2, only 6 of which were annotated using GNPS. These 6 all had spectral matches to different saccharides (**Table 5.2**). Furthermore, all 301 of the suprathreshold metabolites had a retention time below 1 minute, indicating a strong preponderance of polar molecules that eluted quickly. VSURF at T3 resulted in 146 suprathreshold metabolites, none of which were annotated with GNPS, and 145 of which had retention times under 1 minute.

Untargeted LC/MS/MS was also performed on serum samples taken at the end of the experiment. We found that the prebiotic diet had an overall effect on the serum metabolome and there was separation between the control diet, no sleep disruption, no social defeat group and the prebiotic diet, no sleep disruption, no social defeat group on PCoA using Canberra distance (**Figure 5.5c**). We then performed VSURF comparing the two aforementioned groups, which identified 541 suprathreshold metabolites. In contrast to the fecal metabolome, examination of the top annotated suprathreshold metabolites revealed changes in multiple types of metabolite, not just saccharides and their derivatives (**Table 5.3**). Interestingly, 7 of the top 15 annotated suprathreshold metabolites turned out to be standard-verified primary and secondary bile acids,

the normalized abundances of which were all reduced in the prebiotic diet group (**Table 5.3**). It should be noted, however, that of the top 100 suprathreshold metabolites (annotated or unannotated), 71 were increased due to the diet.

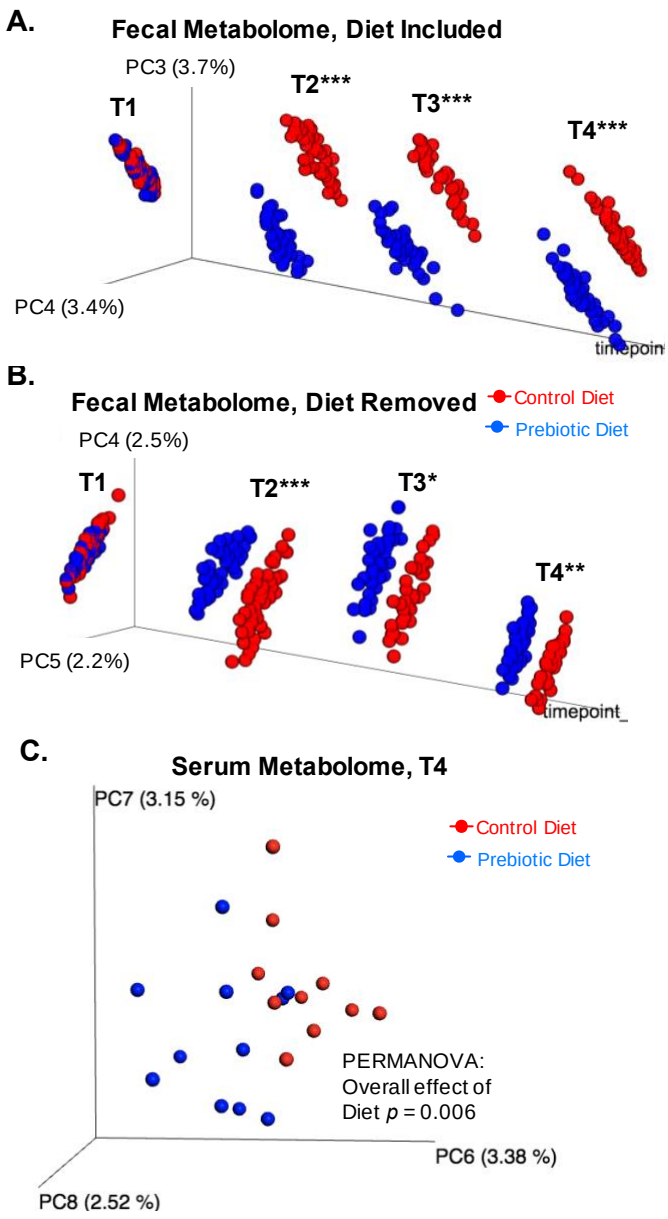

Figure 5.5: The Prebiotic Diet Significantly Shifts the Fecal and Serum Metabolomes. Fecal samples were collected on the day of arrival to the facility (T1), after 4 weeks on diet (T2), at baseline sleep (T3), and at the end of the experiment (T4) and untargeted LC/MS/MS metabolomics were performed. (A) Analysis of the entire feature table is represented by PCoA using Canberra distance, with timepoint as one axis. (B) PCoA using Canberra distance of the fecal metabolite feature table after removal of features that were present in LC/MS/MS of samples of the experimental diets. LC/MS/MS was also performed on serum samples taken from trunk blood at the end of the experiment (T4). (C) PCoA using Canberra distance of the non-sleep disrupted, non-socially defeated rats of each diet group. PERMANOVA was performed on the serum feature table, revealing an overall effect of diet. Symbols: ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, PERMANOVA. Abbreviations: PC, principal coordinate. (A,B) n = 33-45/group; (C) n = 10-11/group.

Annotated Name, Spectral Match to:	*m/z*	RT (min)	VSURF Rank	Direction of Change
Maltotetraose	649.2204	0.37	31	Increased
Alpha-cyclodextrin	973.3213	0.37	35	Increased
4-O-beta-Galactopyranosyl-D-mannopyranose	325.1159	0.36	43	Increased
Maltotriose	487.1663	0.36	48	Increased
Laminatetraose	345.1001	0.29	196	Increased
Laminatetraose	353.091	0.32	205	Increased

Table 5.2 Annotated Suprathreshold Fecal Metabolites Changing Due to Diet at T2. VSURF was performed on the fecal metabolomic dataset at T2 to identify top drivers of separation between the control diet-fed and prebiotic diet-fed rats. Of 301 suprathreshold metabolites, only 6 were annotated using GNPS molecular networking. The annotated names, mass to charge ratio, retention time, VSURF rank, and direction of change of these 6 features is reported above. Abbreviations: m/z, mass to charge ratio; RT, retention time; VSURF, variable selection using random forests.

Name	Standard Verified?	*m/z*	RT (min)	VSURF Rank	Direction of Change	Wilcox *p* value
Nonaethylene Glycol		415.2539	2.63	29	increased	**0.006**
trans-Androsterone		255.2114	5.07	35	decreased	**0.004**
Cholic Acid	Yes	391.2835	4.96	43	decreased	**0.01**
Muricholic Acid	Yes	355.2626	4.57	49	decreased	**0.004**
Hyodeoxycholic Acid	Yes	357.2782	5.14	72	decreased	**0.01**
Cholic Acid	Yes	373.2744	4.96	83	decreased	**0.01**
9-cis-Retinol		269.2261	7.82	93	decreased	0.152
Cholic Acid	Yes	373.2745	4.96	101	decreased	**0.008**
8S-Hydroxy-9E,11Z,14Z-eicosatrienoic Acid		305.2471	6.74	102	decreased	**0.016**
2-Arachidonoylglycerol		287.2371	7.82	108	decreased	0.132
Hyodeoxycholic Acid	Yes	339.2686	5.14	109	decreased	**0.013**
Muricholic Acid	Yes	355.2627	5.08	122	decreased	**0.008**
1-Palmitoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine		476.2743	6.19	130	decreased	0.212
3 beta -Hydroxy-5-cholenoic Acid		321.2575	5.14	131	decreased	**0.029**
cis-5,8,11-Eicosatrienoic Acid		329.2476	7.73	133	decreased	0.085

Table 5.3: Top Annotated Suprathreshold Serum Metabolites Changing due to Prebiotic Diet. VSURF was performed comparing the serum metabolomes of the control diet, no sleep disruption, no social defeat group and the prebiotic diet, no sleep disruption, no social defeat group. The top 15 annotated suprathreshold metabolites, whether they were verified with a purified standard, their mass to charge ratio, retention time, VSURF rank, direction of change, and follow up Wilcoxon Rank-Sum test *p* value are depicted above. Abbreviations: m/z, mass to charge ratio; RT, retention time; VSURF, variable selection using random forests.

The Prebiotic Diet Alters Sleep During the Sleep Disruption Protocol, but Not at Baseline

Baseline sleep was assessed before the sleep disruption protocol began using EEG/EMG recording (see **Methods**, **Figure 5.1**). We found that the prior 6 weeks of consumption of the prebiotic diet had no effect on NREM sleep (**Figure 5.6a**), NREM EEG delta power (**Figure 5.6b**), REM sleep (**Figure 5.6c**), or brief arousals (a measure of sleep fragmentation, **Figure 5.6d**). All of these measures were significantly impacted by time, a clear demonstration of the circadian rhythm of these sleep measures.

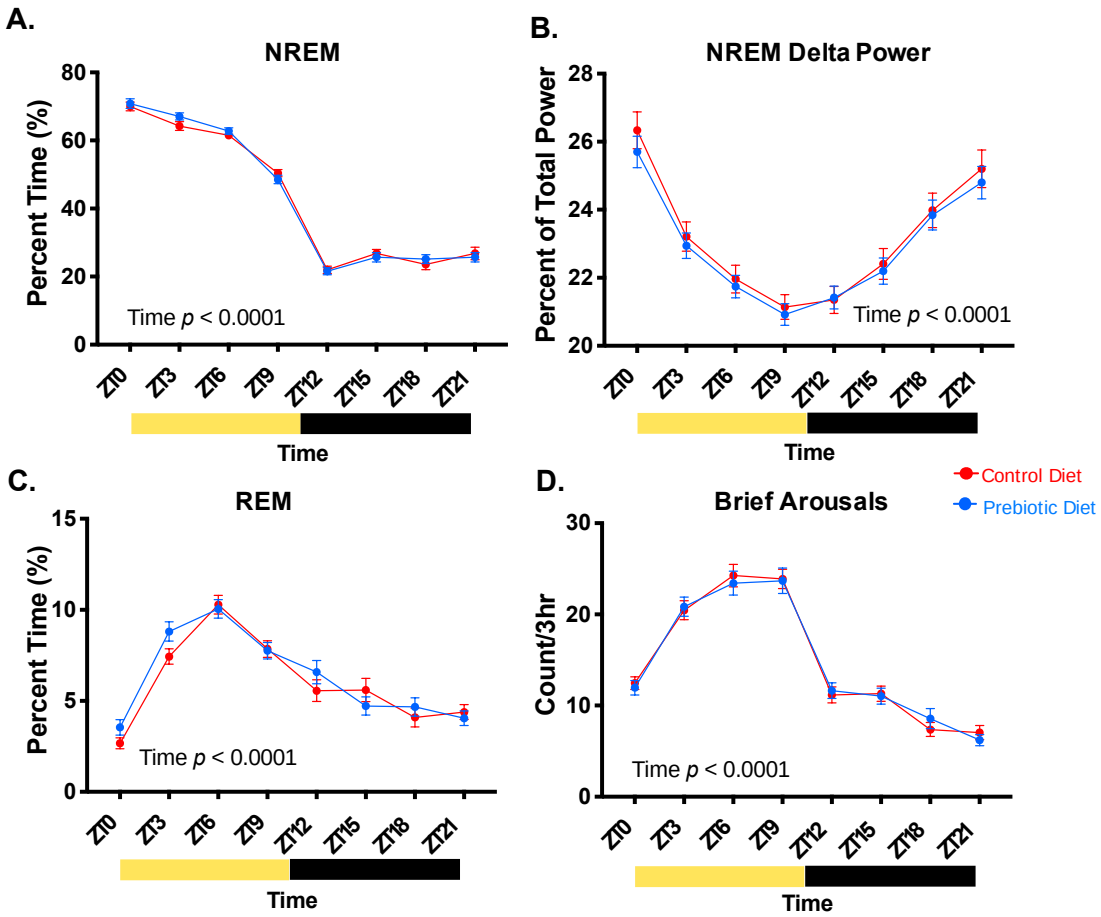

Figure 5.6: The Prebiotic Diet Does Not Impact Baseline Sleep. Two weeks after EEG/EMG surgery, after 6 total weeks on the diets, 24 h of baseline sleep was recorded. (A) NREM sleep, (B) NREM EEG delta power, (C) REM sleep, and (D) brief arousals are reported in 3 hour bins. Yellow bars below the x axes represent times where the lights were on, while black bars represent times the lights were off. Mixed effects modeling was performed to test for effects of

time, diet, and any interactions. Data are mean ± SEM. *n* = 38-40/group. Abbreviations: EEG, electroencephalogram; NREM, non-rapid eye movement; REM, rapid eye movement; ZT, zeitgeber time.

We also measured sleep during the sleep disruption protocol itself to both validate the efficacy of the motorized sleep disruption unit and to examine whether or not the prebiotic diet had an impact. We found that the sleep disruption protocol significantly reduced NREM sleep on all days of the sleep disruption protocol (**Figure 5.7a**), and nearly completely deprived REM sleep, particularly on the first 3 days of the protocol (**Figure 5.7b**). The sleep disruption protocol appeared to become slightly less effective over time, though, as the amount of NREM and REM obtained during the 20 hours of sleep disruption increased over time in both groups (**Figure 5.7a,b**). Interestingly, this process occurred more quickly in the prebiotic diet-fed rats, as on days 2-4 of the sleep disruption protocol, these rats obtained more NREM sleep during the 20h sleep disruption than their control diet-fed counterparts (**Figure 5.7a**). The prebiotic diet-fed rats were even able to obtain more REM sleep during the protocol than control diet-fed rats on days 4 and 5 (**Figure 5.7b**). Thus, while the prebiotic diet does not impact sleep at baseline, it enabled rats to get more sleep *during* the sleep disruption protocol.

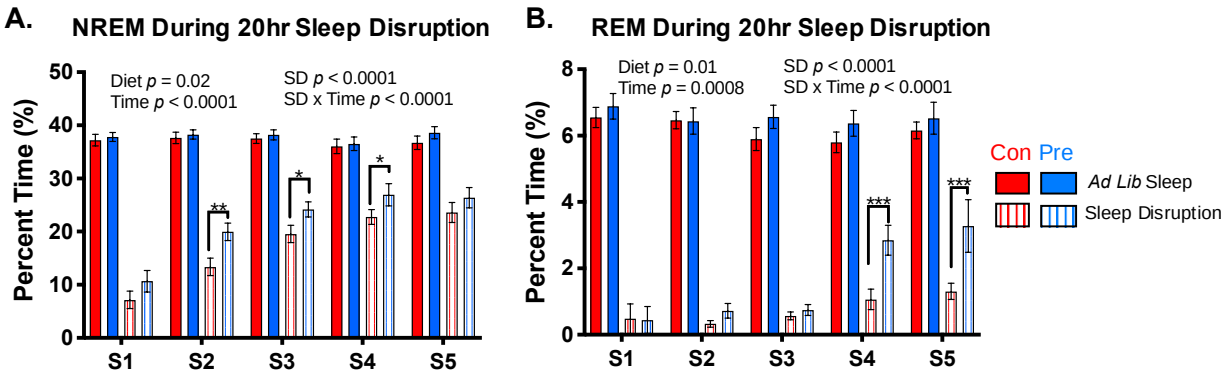

Figure 5.7: The Prebiotic Diet Alters Sleep During the Sleep Disruption Protocol. Rats were exposed to five days of sleep disruption whereby a slowly rotating bar at the bottom of the cage disrupted sleep for 20 h per day (ZT6-ZT2). Sleep was recorded throughout this protocol, and (A) NREM sleep and (B) REM sleep during the 20 h disruption periods is depicted above. Mixed-effect modeling testing for an effect of timepoint, diet, sleep disruption, and interactions was performed for each measure, and significant results are reported in the figure. Data are mean ± SEM. Symbols: $^{**}p < 0.01$, $^{*}p < 0.05$, Fischer's LSD test. Abbreviations: Con, control diet; NREM, non-rapid eye movement sleep; REM, rapid eye movement sleep; Pre, prebiotic diet. n = 19-24/group.

The Prebiotic Diet Enhances Recovery Sleep Post-Sleep Disruption

In order to assess the effect of the prebiotic diet on recovery from the 5-day sleep disruption protocol, we assessed the first 24 hours of recovery sleep. Whereas there was no difference in total sleep due to sleep disruption in the control diet-fed rats, there was a significant increase in total sleep in the prebiotic diet-fed rats (**Figure 5.8a**). However, NREM delta power, a well-accepted measure of sleep intensity and sleep homeostatic drive [Meerlo et al., 2001a; Kamphuis et al., 2015], was not changed in any group (**Figure 5.8b**). Examination of NREM sleep architecture revealed that the prebiotic diet-fed rats had more NREM than their non-sleep disrupted counterparts (**Figure 5.8c**), and that this NREM sleep was more consolidated into longer bouts (**Figure 5.8d,e**). REM sleep was significantly increased during recovery in both the control diet-fed and prebiotic diet-fed groups, and this increase was due to an increase in the number of bouts without an increase in median bout length (**Figure 5.8f-h**). Thus, the prebiotic diet increases total amount of recovery sleep and promotes consolidation of recovery NREM sleep.

Abundance of Fecal P. distasonis is Associated With Measures of Post-Sleep Disruption Sleep

We were then curious to see if any of these prebiotic diet-induced changes to sleep were associated with the observed changes in the fecal microbiome. Normally it is complicated to attempt to correlate microbiome data with physiological data, as the compositional microbiome data are in the simplex space while physiological data are usually in Euclidean space [Gloor et al., 2017]. Mean-centered log ratio (clr) transformation of abundance data puts these data in Euclidean space and allows for correlation with other Euclidean datasets. Using this approach, we found that the clr transformed abundance of *P. distasonis* at T4 correlated positively with the total sleep during the first 24 hours of recovery sleep after sleep disruption in prebiotic diet-fed rats but not in control diet-fed rats (**Figure 5.9a**). *P. distasonis* also positively correlated with REM sleep and the number of REM bouts during recovery sleep (**Figure 5.9b,c**), both of which were sleep measures that were altered by sleep disruption but not by the prebiotic diet (see **Figure 5.8**).

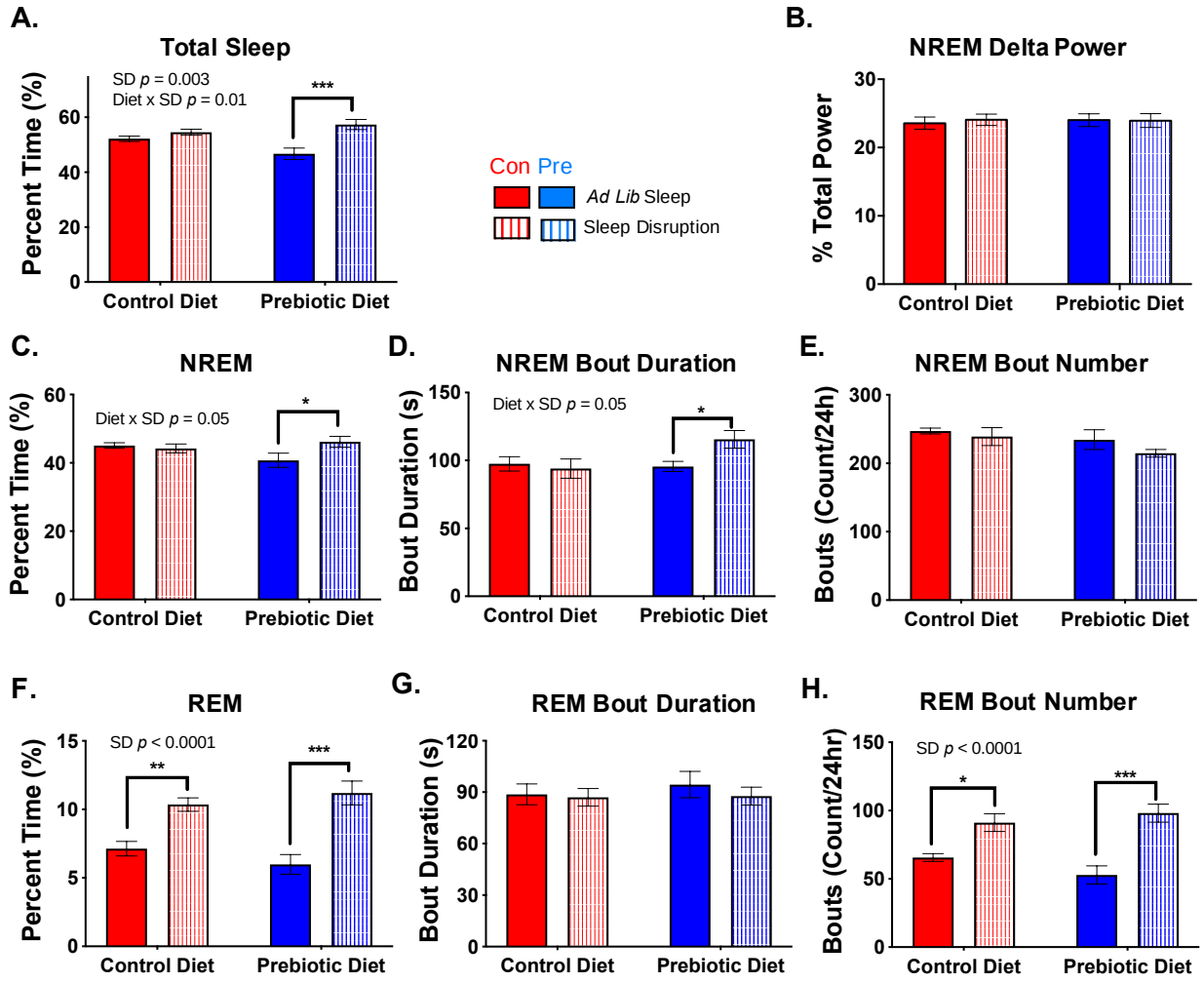

Figure 5.8: The Prebiotic Diet Improves Recovery Sleep Post-Sleep Disruption. 24 hours of *ad libitum* sleep was recorded for 24 hours after the completion of the 5th day of the sleep disruption protocol. (A) total sleep, (B) NREM EEG delta power, (C) NREM sleep, (D) median NREM bout duration, (E) number of NREM bouts in the 24 hours, (F) REM sleep, (G) median REM bout duration, and (H) number of REM bouts in the 24 hours is depicted above for the control (*ad libitum* sleep throughout) and sleep disrupted (with no social defeat) groups. Two-way ANOVA testing for an effect of diet, sleep disruption, and interaction was performed for each measure, and significant or near significant results are reported in the figure. Data are mean ± SEM. Symbols: ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, Bonferroni's *post hoc* test. Abbreviations: Con, control diet; NREM, non-rapid eye movement sleep; REM, rapid eye movement sleep; Pre, prebiotic diet; SD, sleep disruption. n = 8-10/group.

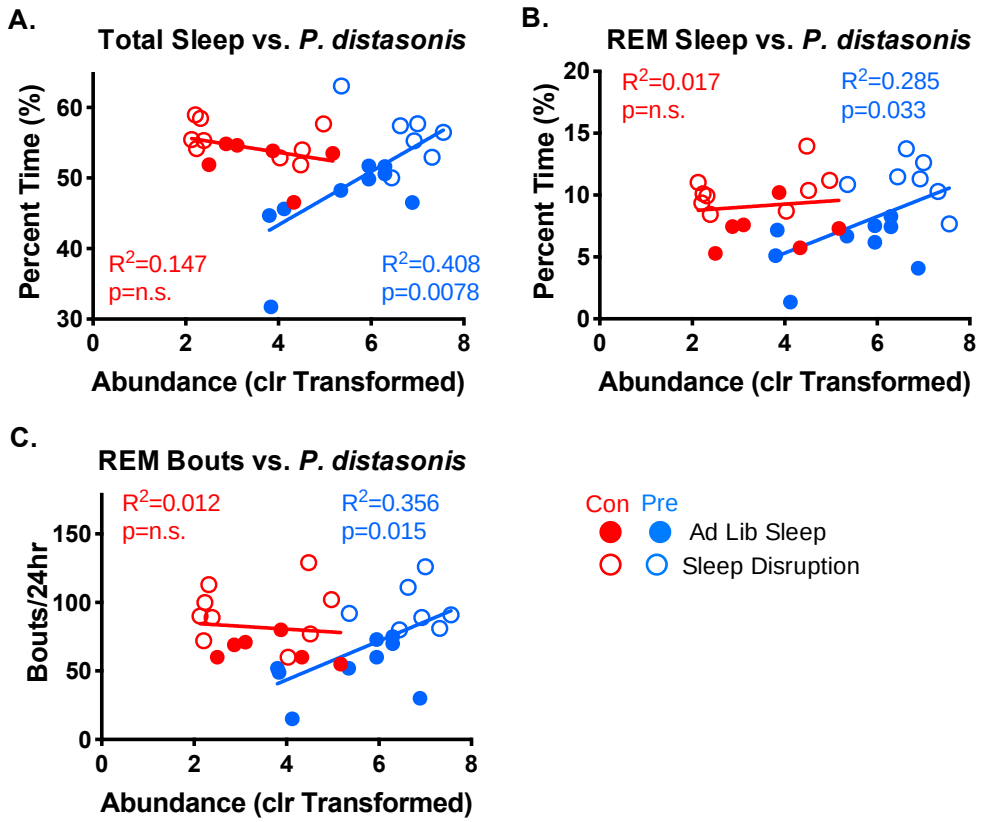

Figure 5.9: *Parabacteroides distasonis* Abundance is Associated with Multiple Measures of Post-Sleep Disruption Recovery Sleep. We performed simple linear regression analysis between the centered log ratio transformed abundance of *P. distasonis* at the end of the experiment (T4) and various measures of recovery sleep during the first 24 hours post-sleep disruption. Regression was performed within diet group for control (no sleep disruption, no social defeat) and sleep disruption only groups. Results for (A) total sleep, (B) REM sleep, and (C) total REM bouts are depicted above. Abbreviations: Con, control diet; clr, centered log ratio; Pre, prebiotic diet; REM, rapid-eye movement sleep. *n* = 6-9/group.

The Prebiotic Diet Alters the Microbiome and Metabolome's Response to Sleep Disruption

Since the prebiotic diet improved sleep disruption-induced changes in sleep, we next explored whether it also altered sleep disruption-induced changes in the microbiome. In control diet-fed rats, there were no changes in beta diversity (weighted or unweighted UniFrac) comparing the

sleep disruption only and control groups at T4 (**Table 5.4**). However, PICRUSt2 analysis of predicted function of the microbiome revealed 8 altered pathways and 19 altered genes due to sleep disruption (FDR < 0.1, DESEq2; **Table 5.4**). In contrast, sleep disruption appeared to have a larger impact on the structure and function of the microbiome in the prebiotic diet-fed rats. There was a significant change in the microbiome as measured by weighted UniFrac, and many more altered predicted functional pathways and genes (40 and 350, respectively, FDR < 0.1, DESEq2; **Table 5.4**).

We also evaluated the effect of sleep disruption on the fecal metabolome in control diet-fed and prebiotic diet-fed rats. While PERMANOVA performed on the entire set of detected features did not detect overall effects of sleep disruption on the fecal metabolome in either diet condition (**Table 5.4**), we performed VSURF to investigate the features that were the top drivers of separation between sleep-disrupted and control groups in each diet condition. VSURF detected 1038 suprathreshold features in the control diet-fed rats, and 998 suprathreshold features in the prebiotic diet-fed rats (**Table 5.4**, **Figure 5.10a**). Interestingly, comparison of these subsets of suprathreshold features revealed little overlap between the two sets (185/2036 total suprathreshold metabolites, **Figure 5.10a**). Upon closer inspection of the suprathreshold metabolites that were annotated using GNPS (see **Methods**), we noticed that a metabolite with a spectral match to the anti-inflammatory dihomo-gamma-linolenic acid (DGLA) was significantly increased due to sleep disruption in the prebiotic diet-fed rats, but not increased due to sleep disruption in control diet-fed rats (**Figure 5.10b**). Conversely, a feature with a spectral match to

the proinflammatory lipid prostaglandin E2 was decreased due to sleep disruption only in prebiotic diet-fed rats (**Figure 5.10c**). Also, standard-verified (MSI level 1) deoxycholic acid, a bacterially modified secondary bile acid, was reduced compared to control due to sleep disruption in control diet-fed rats but not in prebiotic diet-fed rats (**Figure 5.10d**). Together, these results indicate that sleep disruption had a different effect on the fecal metabolome in prebiotic diet-fed rats and control diet-fed rats.

We also assessed the serum metabolome and found that, in contrast to what was observed in the fecal microbiome, sleep disruption had a significant impact on the serum metabolome in control diet-fed rats but not in prebiotic diet-fed rats (**Table 5.4**). Furthermore, VSURF identified 76 suprathreshold metabolites in the control diet-fed rats and 64 suprathreshold metabolites in prebiotic diet-fed rats (**Table 5.4**). Again, these sets of suprathreshold metabolites had very little overlap (5/140, **Figure 5.11a**). We noted that the primary bile acid muricholic acid (standard-verified) and the secondary bile acid deoxycholic acid (standard-verified) were reduced due to sleep disruption in the sera of control diet-fed rats but not in the prebiotic diet-fed rats (**Figure 5.11b,c**). The same pattern was observed in a metabolite with a spectral match to L-tyrosine (**Figure 5.11d**). Together, these results suggest that the sleep disruption protocol caused a change in the serum metabolome that was both of a larger magnitude and of a different character in control diet-fed rats compared to the prebiotic diet-fed rats.

The Prebiotic Diet Prevents Social Defeat-Induced Changes to Behavior

Immediately after the sleep disruption protocol ended, half of the rats were exposed to one hour of social defeat stress (see **Methods**). 24 hours later, all rats were tested in the object location memory task. In the control diet condition, the control group and the sleep disruption only group successfully learned the task, indicated by a learning index significantly above 50% (**Figure 5.12**). The social defeat alone and double hit groups, however, failed to learn significantly above 50% as a group. In contrast, all prebiotic diet-fed groups had learning indices significantly above 50% (**Figure 5.12**). Thus, the prebiotic diet protected rats from one cognitive consequence of acute social defeat stress.

	Control Diet	Prebiotic Diet
Microbiome (PERMANOVA)	**Unweighted UniFrac:** $p = 0.66$ **Weighted UniFrac:** $p = 0.364$	**Unweighted UniFrac:** $p = 0.09$ **Weighted UniFrac:** $\mathbf{p = 0.039}$*****
PICRUSt2 (DESEq2)	**Pathway:** 1 up, 7 down in SD group **Gene:** 4 up, 15 down in SD group (FDR < 0.1)	**Pathway:** 21 up, 19 down in SD group **Gene:** 142 up, 208 down in SD group (FDR < 0.1)
Fecal Metabolome (PERMANOVA)	**Effect of SD:** $p = 0.122$ **VSURF:** 1038 suprathreshold (98 annotated with GNPS)	**Effect of SD:** $p = 0.065$ **VSURF:** 998 suprathreshold (85 annotated with GNPS)
Serum Metabolome (PERMANOVA)	**Effect of SD:** $\mathbf{p = 0.034}$***** **VSURF:** 76 suprathreshold (12 annotated with GNPS)	**Effect SD:** $p = 0.171$ **VSURF:** 64 suprathreshold (4 annotated with GNPS)

Table 5.4: Overall Impact of Sleep Disruption on the Microbiome, Fecal Metabolome, and Serum Metabolome. Fecal samples and serum samples were collected at the end of the experiment (the third day after sleep disruption ended). 16S rRNA fecal microbiome analyses were performed, and unweighted and weighted UniFrac were used to assess beta diversity. PICRUSt2 was performed on the 16S rRNA data to estimate functional pathway and gene content of the microbiome. Untargeted metabolomics was performed on a separate set of fecal samples taken at the same time as those used for microbiome analysis, along with serum samples also taken on the same day. Comparisons of the control manipulation group versus the sleep disruption only group within each diet condition were performed using PERMANOVA (beta

diversity, metabolomics), DESEq2 (functional analysis), and VSURF (metabolomics). Abbreviations: SD, sleep disruption; GNPS, global natural products social molecular networking; VSURF, variable selection using random forests.

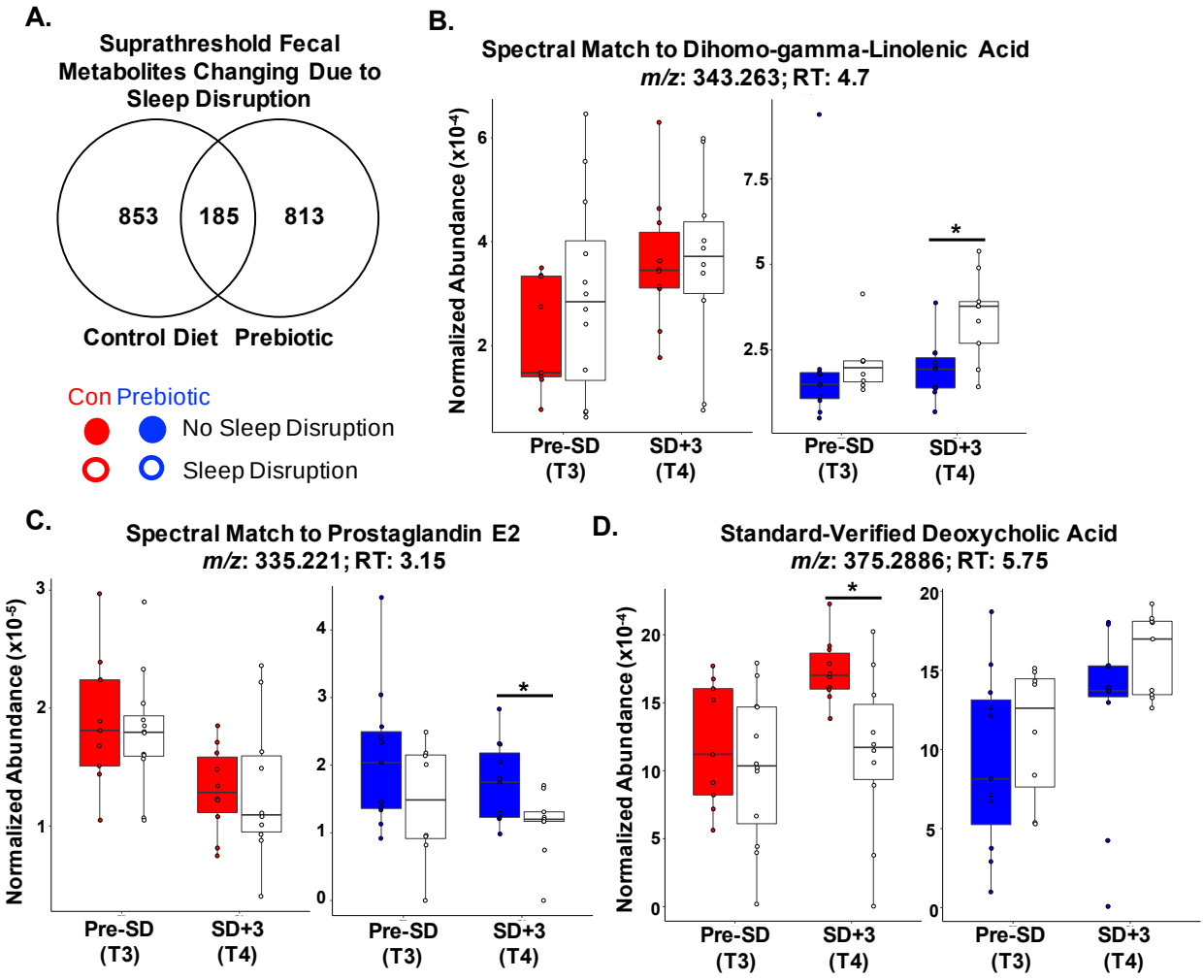

Figure 5.10: Sleep Disruption Alters Different Fecal Metabolites in Prebiotic Diet-Fed Rats. Variable selection using random forests was performed on metabolome feature tables at the end of the experiment (three days after sleep disruption ended, i.e. T4) between control (no sleep disruption) and sleep disruption only groups within each diet. (A) Venn diagram depicting the number of suprathreshold metabolites identified as important drivers of separation between sleep disrupted and control groups within each diet group. Normalized abundance (peak intensity normalized to total ion count) of examples of physiologically interesting metabolites are depicted above. Metabolites with spectral matches to (B) dihomo-gamma-linolenic acid, and (C) prostaglandin E2 were suprathreshold in the prebiotic diet group but not the control diet group. Conversely, (D) a metabolite that was verified to be deoxycholic acid using purified standards was suprathreshold in the control diet group but not the prebiotic diet group. For (B-D), boxes indicate median, 25th and 75th quantiles; whiskers indicate 2*IQR from edges of box. Symbols: *$p < 0.05$, Wilcoxon Rank-Sum test. Abbreviations: Con, control diet; m/z, mass to charge ratio; RT, retention time (minutes); SD, sleep disruption. n = 9-12/group.

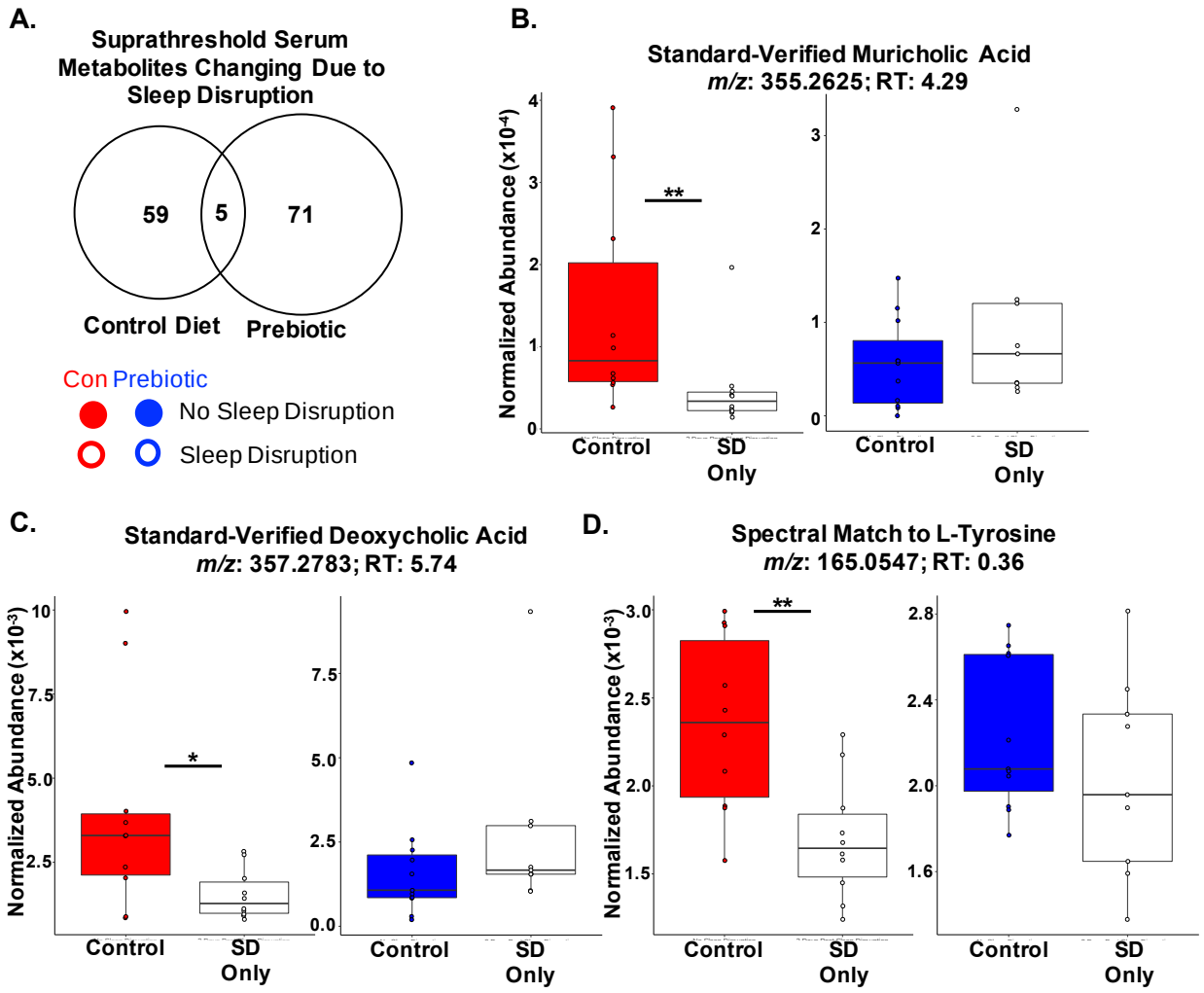

Figure 5.11: Sleep Disruption Alters Different Serum Metabolites in Prebiotic Diet-Fed Rats. Variable selection using random forests was performed on serum metabolome feature tables between control (no sleep disruption) and sleep disruption only groups within each diet. (A) Venn diagram depicting the number of suprathreshold metabolites identified as important drivers of separation between sleep disrupted and control groups within each diet group. Normalized abundance (peak intensity normalized to total ion count) of examples of physiologically interesting metabolites are depicted above. Metabolites that were verified to be (B) muricholic acid and (C) deoxycholic acid using purified standards were suprathreshold in the control diet group but not the prebiotic diet group. Similarly, a feature with a spectral match to L-tyrosine was also suprathreshold in the control group but not in the prebiotic diet group. For

(B-D), boxes indicate median, 25th and 75th quantiles; whiskers indicate 2*IQR from edges of box. Symbols: **$p < 0.01$, *$p < 0.05$, Wilcoxon Rank-Sum test. Abbreviations: Con, control diet; m/z, mass to charge ratio; RT, retention time (minutes); SD, sleep disruption. n = 9-11/group.

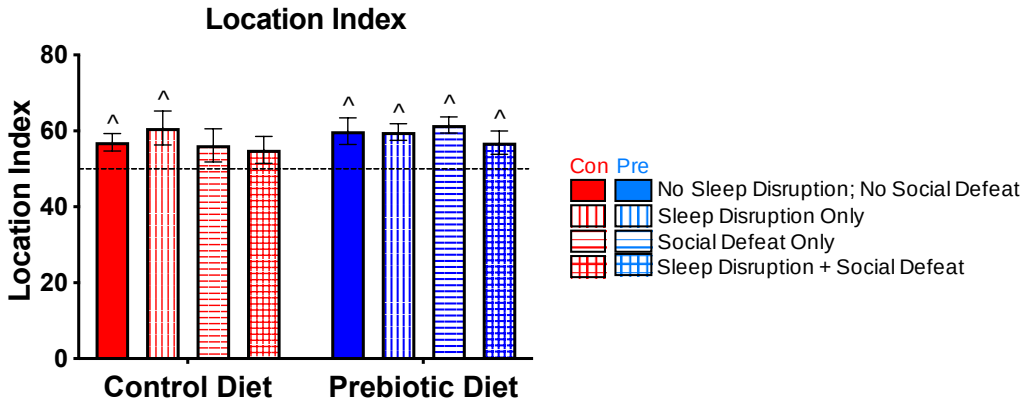

Figure 5.12: The Prebiotic Diet Prevents Social Defeat-Induced Loss of Object Location Memory. Immediately after the end of the last sleep disruption period, half of the rats were exposed to 1 hour of social defeat. 24 hours after that, object location memory was assessed in all rats. Location indices above 50% indicate learning. Data represent mean ± SEM. Symbols: ^$p < 0.05$, one sample Wilcoxon Rank-Sum test vs. 50%. Abbreviations: Con, control diet; Pre, prebiotic diet. n = 9-12/group.

D. Discussion

In this study we administered a prebiotic diet containing the prebiotic compounds GOS and PDX to rats and exposed them to repeated sleep disruption followed by acute stress, examining the impact of the diet on the microbiome, fecal metabolome, serum metabolome, sleep, and behavior. We found that the diet had a large impact on the structure and function of the fecal microbiome, as well as the fecal metabolome and serum metabolome. These changes were highlighted by a significant increase in the relative abundance of the bacteria *P. distasonis*,

which has been shown to have multiple interesting impacts on host physiology (see below). Interestingly, the diet also improved sleep both during the sleep disruption and during recovery, and the improved recovery sleep correlated positively with *P. distasonis* within the prebiotic diet group. The prebiotic diet also influenced the character of sleep disruption-induced changes to the fecal and serum metabolomes, suggesting a more adaptive response. The implications of these findings, along with the follow up work that needs to be done, will be discussed below.

Prebiotic Diet Effects on the Microbiome and P. distasonis as a Probiotic Bacterium

By week 4 of the experiment (T2), there was a large shift in the beta diversity of the microbiome due to diet that remained significant throughout the experiment. This result was expected, as these are accepted prebiotic compounds. We also observed a significant decrease in multiple measures of alpha diversity due to the diet. This is not consistent with [Thompson et al., 2016], but it is noteworthy that GOS/PDX was not the only prebiotic in the test diet. Because alpha diversity is often decreased in conditions such as stress [Bharwani et al., 2016; Thompson et al., 2016] and inflammatory bowel diseases [Nishino et al., 2018], and that increased alpha diversity has been shown to have benefits such as increased likelihood of responding to certain cancer immunotherapies [Gopalakrishnan et al., 2018b; Matson et al., 2018], it has been generally postulated that more alpha diversity is equivalent to a more healthy microbiome and thus a more healthy host. However, this notion presumes that more diversity in the gut means more beneficial bacteria. High alpha diversity simply means more taxonomic representation, and is agnostic to the physiological role of these taxa. A few recent studies illustrate this concept. Examination of

fecal samples of 89 1-year-olds found that those with higher alpha diversity at 1 year of age exhibited lower scores on the Mullen Scale of Early Learning at 2 years of age [Carlson et al., 2018]. In another study, individuals with active major depressive disorder had increased alpha diversity compared to controls [Jiang et al., 2015]. Thus it is possible that although these rats showed reduced alpha diversity at T2 and T3, there may still be a greater abundance of beneficial bacteria and thus a positive physiological state.

The microbial taxon that was most impressively impacted by the prebiotic diet was the genus *Parabacteroides*, particularly its species *P. distasonis*. This was intriguing, as GOS and PDX have often been noted to cause increases mostly in bacteria such as *Lactobacillus* and *Bifidobacterium* [Macfarlane et al., 2008; Thompson et al., 2016], however many of these studies verified these effects through bacterial culture, which has many limitations [Knight et al., 2018]. Nevertheless, *P. distasonis* is an interesting bacteria physiologically. *P. distasonis* was identified as a commensal bacteria with the capacity to induce T_{reg} in the intestine and thus has potential immunomodulating properties [Lathrop et al., 2011]. Oral administration of *P. distasonis* was shown in one study to decrease weight gain, hyperglycemia, and hepatic steatosis in a genetic model of obesity and in high fat diet-fed mice [Wang et al., 2019b]. Interestingly, this study also showed that this effect is at least in part due to *P. distasonis* generation of the short chain fatty acid succinate and the secondary bile acids lithocholic acid and ursodeoxycholic acid, the latter of which is structurally very similar to HDCA. Recall that HDCA was isolated as an potentially important contributor to stress resilience in mice in Chapter IV, and correlated with multiple

sleep measures. In this study, *P. distasonis* also correlated with multiple sleep measures during recovery sleep. Follow up studies directly investigating the link between *P. distasonis*, bile acids, sleep, and stress resilience are necessary (see the next chapter for in depth discussion of this topic).

It is important to note that not all studies of *P. distasonis* have found beneficial effects. One study found that gavage with *P. distasonis* increased the susceptibility to dextran sulfate sodium-induced colitis in mice [Dziarski et al., 2016]. Another study found that individual supplementation of *P. distasonis* into antibiotic-treated mice exacerbated development of a genetic model of amyotrophic lateral sclerosis [Blacher et al., 2019]. This may indicate that what are "beneficial" bacteria may in fact be context dependent, depending on the physiological state of the host. However, these results should be interpreted with caution, as the strain of the bacteria was not quantified in any of them, and strain differences can be quite large at the genetic level [Poyet et al., 2019].

Aside from *P. distasonis*, another bacteria that increased that was interesting was the genus *Ruminococcus*, which is a known butyrate producer and has been observed to increase in humans after PDX administration [Costabile et al., 2012].

The predicted function of the microbiome was impacted by the prebiotic diet as well. PICRUSt2 analysis revealed that many pathways and even more genes were either increased or decreased

across the experiment due to the prebiotic diet, and we looked in detail to the 8 pathways that were altered at all three timepoints (T2, T3, T4). Two pathways that are related to synthesis of different components of LPS were increased due to the diet. This suggests the prebiotic diet increases abundance of gram negative bacteria. Indeed, *P. distasonis* is gram negative, and due to the fact that this species represents 5-10% of the microbiome at T2 in prebiotic diet-fed rats it makes sense that genes that would be contained in gram negative bacteria would be increased. PICRUSt2/DESEq2 also identified three differentially abundant pathways that were involved in microbial metabolism of saccharides. The sucrose degradation pathway III was decreased in abundance due to the diet. Since the prebiotic diet provides carbohydrates in the form of galactose oligomers (GOS) and glucose polymers (PDX), it makes sense that bacteria that have a pathway to utilize sucrose (a disaccharide of glucose and sucrose) would be outcompeted in an environment rich in GOS/PDX. Similarly, the glyoxylate cycle (which is an alternative to the TCA cycle that utilizes non-sugar substrates) was less abundant in the prebiotic diet-fed rats' microbiomes. It is also logical that these bacteria should be outcompeted as well in favor for bacteria that have abundance of pathways such as the fucose and rhamnose degradation pathway. Interestingly, the microbiomes of prebiotic-diet fed rats had reduced abundance of a bacterial aromatic biogenic amine degradation pathway. This pathway has the ability to degrade compounds such as dopamine and Levadopa (L-dopa) via a bacterial monoamine oxidase [Yamada et al., 1967]. L-dopa is a key treatment for Parkinson's disease, and up to 56% of oral L-dopa fails to reach the brain, in part due to metabolism by gut microbes [Maini Rekdal et al.,

2019]. Thus, perhaps coadministration of GOS/PDX and L-dopa could increase the percentage of L-dopa free to perform therapeutic actions.

The Prebiotic Diet and the Metabolome

As with the microbiome, there was a large shift in the fecal metabolome due to the diet. This separation was even evident when removing features found in the undigested food samples that were also run. This confirms that these prebiotics are being metabolized by the microbes. Furthermore, we hypothesize that the main molecules driving the separation between the control and prebiotic diets are direct metabolites of the prebiotics. This is because nearly every suprathreshold metabolite at T2 and T3 had a retention time less than 1 minute (indicating highly polar molecules), and the only features that were annotated using GNPS were spectral matches to different oligosaccharides. This made assessment of molecules not directly related to the prebiotic compounds, such as bile acids that should be altered due to the increased abundance of bacteria like *P. distasonis*, difficult. The prebiotic diet also impacted the serum metabolome at the end of the experiment, and the suprathreshold metabolites in this dataset were not just polar saccharides. Interestingly, multiple standard verified bile acids, including HDCA, were reduced in the serum due to the prebiotic diet. This appears to contradict the developing hypothesis that HDCA is a beneficial microbial metabolite that may be exerting stress-protective effects. However, the regulation of the bile acid pool is dynamically regulated [McMillin and DeMorrow, 2016; Kang et al., 2017], and bile acids in the blood may not correlate with bile

acids in the intestinal lumen or in the portal circulation. The role of serum HDCA in relation to fecal HDCA warrants further investigation.

The Prebiotic Diet and Sleep

We found that rats fed the prebiotic diet and exposed to the sleep disruption protocol slept more during the *Ad libitum* recovery period than did control diet-fed rats. The ability to recover quickly from repeated sleep disruption could be immensely valuable in modern society, especially because we know a single "weekend" of recovery sleep after 5 days of short sleep does not result in a full recovery of cognitive sequelae of the sleep disruption [Pejovic et al., 2013]. While this study does not investigate mechanisms of prebiotic diet-induced changes in sleep homeostatic mechanisms, there is evidence that these compounds can change gene expression in CNS circuits, particularly when the diet is begun early in life. This includes alterations to glutamatergic subunits and BDNF in hippocampus [Williams et al., 2016], as well as changes to stress-induced changes in serotonergic signaling in the DRN [Mika et al., 2017]. However, nobody has examined the impact of prebiotic diets on gene expression or protein levels in sleep promoting areas. It is therefore feasible that the prebiotic diet changes in synaptic protein expression in sleep circuits, particularly those involved with maintenance of NREM sleep, such as the VLPO and BF, and follow up studies are warranted.

During the sleep disruption protocol, a significant amount of NREM sleep was lost over the course of the 5 days. However, in control-diet fed rats, there was no apparent rebound in NREM

sleep amount or in NREM delta power during recovery sleep. This phenomenon has been labelled sleep allostasis [Kim et al., 2007]. This theory postulates that after repeated sleep deprivation, a maladaptive shift in the sleep homeostatic set point develops. Not only does the prebiotic diet improve the amount of sleep rats could get during the sleep disruption protocol, it restored the necessary sleep rebound during recovery. This suggests a diet rich in prebiotics may be helpful in situations where sleep deprivation is inevitable, and rapid recovery is required. However, it is worth noting that one interpretation of the increased NREM and REM obtained by prebiotic diet-fed rats *during* the sleep disruption protocol itself could indicate an overall sleepiness whereby sleep intrudes inappropriately into times where one wants to stay awake. In this case, a prebiotic diet may not actually be beneficial in all situations. However, I argue that because the increase in during-sleep disruption sleep only occurs after a few days of sleep disruption, it is possible the rats have learned that the rotating bar is not dangerous, and thus is willingly transitioning into sleep between bar passes. Follow up studies investigating the role of the prebiotic diet, or *P. distasonis*, in sleep architecture would provide interesting insight into this concept.

The Prebiotic Diet's Impact on Sleep Disruption-Induced Metabolome Changes

Similar to the observation that the prebiotic diet altered the homeostatic sleep response to sleep disruption, it appeared to alter the response of the fecal and serum metabolomes to sleep disruption. A small fraction of the sleep disruption-sensitive fecal metabolites in the control diet groups were also altered in the prebiotic diet groups, and some of the discrepancies had

physiological implications. First, a metabolite with a spectral match to dihomo-gamma-linolenic acid (DGLA) was unchanged by sleep disruption in the control diet group but was increased due to sleep disruption in the prebiotic diet group (see Figure 5.10). DGLA is a polyunsaturated fatty acid, many of which are suggested to be created by, and modified by, gut microbes [Gorissen et al., 2010; Druart et al., 2015]. DGLA specifically has been shown to have anti-inflammatory properties via attenuation of biosynthesis of arachidonic acid metabolites such as prostaglandin E2 [Fan and Chapkin, 1998; Kapoor and Huang, 2006; Wang et al., 2012]. Consistent with this, sleep disruption resulted in a reduction in a metabolite that had a spectral match to prostaglandin E2 in prebiotic diet-fed rats. Prostaglandin E2 is not only a proinflammatory mediator peripherally, its signaling in the brain has been linked to increased vulnerability to chronic social defeat stress [Tanaka et al., 2012] and increased neuropathic pain [Kunori et al., 2011] in mice. Furthermore, prostaglandin E2 has been shown to increase with total sleep deprivation, and is associated with increased pain susceptibility in that context [Haack et al., 2007; Haack et al., 2009].

Thus it is possible the prebiotic diet places the microbiome in a state whereby sleep disruption-induced perturbations in community structure result in increased production of metabolites such as DGLA that then have an anti-inflammatory and protective role against the physiological effects of sleep disruption. This hypothesis is supported by the serum metabolome findings, where changes in the overall metabolome and in individual bile acids were seen due to sleep disruption in the control diet group, but not in the prebiotic diet group. More work needs to be

done to both validate the identities of the aforementioned fecal metabolites, as well as to perform mechanistic studies linking DGLA to inflammatory markers and responses to sleep disruption.

Prebiotic Diet and Stress Resilience

It is important to note that we did not observe the same double hit phenotype in this study as we did in the study using the mice. We did not observe an increase in cortical hyperarousal post-social defeat, lasting increases in sleep fragmentation, or behavioral changes such as hyperlocomotion in the rats receiving both sleep disruption and social defeat (data not shown). There may be a few reasons for this discrepancy. The first is that the social defeat protocol in the rats was much less reliable and consistent than in the mice. Some aggressor rats were much more quick to attack than others, and this response was not reliable. Also, there are inherent differences in the stress response between rats and mice [Schoner et al., 2017]. Perhaps the double hit effect will be observed in rats using a different secondary stressor such as restraint stress or acute tail shock. Nevertheless, the social defeat protocol resulted in a loss of OLM learning in the control diet-fed rats, but not in the prebiotic diet-fed rats, suggesting there are some stress protective effects of the prebiotic diet in our case.

E. Conclusions

Overall, these results support the notion that microbiome-based countermeasures other than *M. vaccae* can have a favorable impact on the response to sleep disruption. Importantly, it also

revealed that *P. distasonis* is a bacterium of interest that may play a role in host sleep regulation and warrants further research. This concept and others will be explored in the next chapter.

VI. CHAPTER VI: DISCUSSION, FUTURE DIRECTIONS, AND CONCLUSIONS

A. Discussion of Research Implications

The experiments in the previous chapters investigated the relationship between repeated sleep disruption, stress vulnerability, and the gut microbiome using rodent models. They sought to test the hypothesis that sleep disruption increases vulnerability to secondary stressors and that microbiome-inspired countermeasures can provide resilience to sleep disruption and the second hit of acute stress. In this final chapter I will attempt to integrate the most significant findings within the framework of that hypothesis and will discuss their potential scientific and clinical implications.

1. Sleep Disruption as a Contributor to Stress Vulnerability

As outlined in Chapter I, there is ample evidence from rodent models as well as prospective human studies suggesting that inadequate sleep may increase stress vulnerability. However, these studies have all either described circumstantial relationships between sleep deprivation and stress responses or related psychiatric disorders (e.g. [Meerlo et al., 2015]), or have associated preexisting sleep disturbances with future development of stress-induced psychiatric symptoms

[Bryant et al., 2010; Cho et al., 2016]. The study described in Chapter III was among the first to experimentally induce repeated sleep disruption and observe the sleep, physiological, and behavioral sequelae of a secondary acute stressor. We found that sleep disruption plus acute social stress resulted in PTSD-like changes in sleep EEG, a protracted increase in REM sleep, sleep fragmentation, along with hyperlocomotive and anxiety-like behavior that was not present after sleep disruption or acute stress alone. This finding was one of the most robust of this dissertation, and contributes to the field by directly demonstrating the physiological manifestations of the synergism between repeated sleep disruption and acute stress. The intent of the study was to investigate stress vulnerability, not to create a rodent model of PTSD. However, we ultimately noted that many aspects of the double hit phenotype were similar to those seen in human PTSD. Rodent models of a PTSD-like state do exist [Schoner et al., 2017; Sillivan et al., 2017], and they often examine a wide range of behaviors such as the social avoidance test that this study did not. Follow up studies need to be done, therefore, to further characterize the behavioral aspects of the double hit phenotype, how long the sleep disturbances last, whether the character of the sleep disruption protocol or secondary stressor matter, and whether the double hit induces neurological changes in sleep or stress circuitry that may be explaining some of the sleep phenotypes.

An interesting question that remains unanswered after this project regards how sleep disruption early in development impacts stress resilience later in life. It is known that sleep architecture changes significantly across the lifespan, yet we still do not clearly understand what functions

sleep has during development. Since it is already understood that childhood stressors, or adverse childhood experiences (ACE), can have damaging physical and psychological effects throughout life, including changes in sleep [Kajeepeta et al., 2015; Sullivan et al., 2019], it would be interesting to determine whether children who do not get sufficient sleep are more vulnerable to lasting effects when an ACE occurs. Furthermore, rodent models of sleep and sleep disruption in juveniles (or earlier) have not been investigated but could provide detailed insight into the neurobehavioral impact of sleep early in life.

2. The Role of Elevated REM Sleep: Adaptive Coping Mechanism or Sign of Pathology?

A slight paradox exists when examining the role of elevated REM sleep in trauma and stressor related psychiatric disorders. There is evidence that REM sleep acts as an adaptive coping mechanism after stressors [Suchecki et al., 2012; Sanford et al., 2015], and that elevated REM sleep immediately after trauma may be protective against future psychiatric symptoms [Mellman et al., 2007; Cowdin et al., 2014]. However, elevated REM sleep has been observed in some studies of patients with chronic PTSD [Kobayashi et al., 2007; Baglioni et al., 2016] and is commonly noted in studies of major depression [Pillai et al., 2011; Baglioni et al., 2016]. Furthermore, REM sleep deprivation has anti-depressive action in humans and rodents, and has been proposed to be one of the mechanisms by which selective serotonin reuptake inhibitors reduce depression symptoms (reviewed by [Riemann et al., 2020]). So, the question becomes: is elevated REM sleep good for mental health or not? It is probable that the answer to this question is that it depends on the context and timing of the REM sleep, with REM sleep rebound

immediately after stress being beneficial, but extended elevations beyond the inciting stressful event are signs of pathology. The results of Chapter III support this hypothesis. All groups showed a rebound in REM sleep after the double hit (though this may have simply been a homeostatic response to the REM loss during sleep disruption), yet only the *M. vaccae*-treated, stress-resilient mice displayed a rapid recovery to control levels of REM in the subsequent days.

It is also possible that REM sleep elevation is a pathological sign in individuals that are more stress vulnerable for other reasons. A recent study demonstrated that people with major depression consolidated more emotionally negative images during REM sleep than NREM sleep compared to healthy controls [Harrington et al., 2018]. Furthermore, more REM sleep per night correlates with reduced fear memory extinction in insomniacs, while in good sleepers more REM sleep appeared to be adaptive, resulting in enhanced fear extinction [Bottary et al., 2020]. This is also consistent with our study, as both people with insomnia and vehicle-injected, double hit mice exhibit increased beta power during NREM EEG, while good sleepers and *M. vaccae*-injected mice do not. Furthermore, more REM:Sleep immediately after acute social defeat correlated with avoidance of the center of the arena the next day and with increased spleen weight at the end of the experiment only in the vehicle-treated mice. Therefore, it is possible that a state such as insomnia or the double hit which creates a maladaptive EEG phenotype is also creating an environment whereby REM sleep is serving a less beneficial role.

3. Biomarkers for Stress Vulnerability Within Post-Trauma Sleep

In Chapter III, we showed that multiple markers during sleep immediately after acute social defeat predicted other phenotypes of the double hit 1-6 days later in vehicle-treated mice. This included NREM EEG beta power and theta2 (i.e. alpha) power, brief arousals, and REM:Sleep ratio. This suggests that EEG changes that have only recently been appreciated as features of sleep in patients with PTSD may be present immediately after trauma and may predict future sleep and behavioral impairments. Objectively measuring sleep in the early aftermath of these events (if possible) may therefore allow clinicians to prioritize individuals for more aggressive treatment early in the disease process. Furthermore, increased high frequency oscillations in sleep EEG may also be a predictor of future sleep impairments independently of trauma, as a recent study found that increased beta power during sleep in childhood (6-11 years old) resulted in increased odds of developing insomnia symptoms in adolescence (13-20 years old) [Fernandez-Mendoza et al., 2019]. More prospective sleep studies like this one could reveal many markers of future disease processes that are hidden in sleep architecture.

4. Microbiome-Based Countermeasures to Improve Health

This study investigated two different types of microbiome-based interventions to improve resilience to sleep disruption, acute stress, and the double hit. Those were immunization with a heat killed immunomodulatory bacterium *M. vaccae*, and feeding of a prebiotic diet consisting of the non-digestible starches GOS and PDX.

Mycobacterium vaccae

We found in Chapters III and IV that *M. vaccae* prevented the development of the double hit phenotype, stabilized microbiome alpha and beta diversity, and prevented some sleep disruption-induced changes to the fecal metabolome. However, as discussed in Chapter IV, we found that the changes to the function of the microbiome and to the fecal metabolome after the double hit were exaggerated in *M. vaccae*-treated mice compared to vehicle-treated mice. These results suggest that immunomodulatory bacteria like *M. vaccae* may be both protecting homeostasis and providing stability *and* creating an environment in which an active coping mechanism can be triggered upon exposure to stress. We hope these studies will provide the groundwork for future experiments evaluating *M. vaccae* as an intervention to improve stress resilience in vulnerable human populations. These include members of professions such as warfighters and emergency workers whose jobs incur the unfortunate combination of regular sleep deprivation and high risk of acute traumatic stress. Strains of heat-killed *M. vaccae* have been administered to humans in clinical trials on multiple occasions, without reports of any major health sequelae [O'Brien et al., 2000; O'Brien et al., 2004]. However, neither of these studies immunized otherwise healthy people, which brings a higher burden of proof regarding safety of the treatment. Overall, though, our findings provide further evidence that immunomodulatory therapies such as *M. vaccae* immunization represent a novel category of preventative measures to improve stress resilience clinically and to understand mechanisms of stress vulnerability in preclinical research.

Prebiotic Diets

In Chapter V, we demonstrated that a diet supplemented with the two prebiotic compounds GOS and PDX significantly altered the fecal microbiome, metabolome, and serum metabolome, and improved recovery sleep after sleep disruption in rats. The bacterial taxon that was most increased by the diet, *P. distasonis*, correlated with this recovery sleep, indicating that it may be a bacteria that is relevant to sleep. These results add to the existing body of literature that prebiotics can have health benefits, and extends those benefits to include resilience to and improved recovery from sleep disruption. Since prebiotics usually have very few (if any) reported side effects [Manning and Gibson, 2004], we believe they should be implemented in human studies in the context of sleep disruption. In fact, one arm of this multi-university research initiative (MURI) is in the process of investigating GOS/PDX in a combined sleep and circadian disruption model in humans. However, we should still proceed with the usual caution before pairing GOS/PDX with every medication regimen or giving it to every soldier during deployment. In chapter IV, rats were able to achieve some sleep during the sleep disruption protocol. While this may be viewed as a positive, it is possible these rats are transitioning to sleep too easily, and giving this to humans may in fact cause sleep to inappropriately intrude into wakefulness after a period of sleep disruption, which of course is unfavorable. However, the fact that the prebiotic diet did not change baseline sleep, and still improved recovery sleep suggests that this may not be the case. Studies in humans are necessary before widespread adoption of GOS/PDX with the purpose of creating resilience to sleep disruption. Dr. Kenneth Wright of the MURI project has begun a study examining GOS/PDX in humans in the context of chronic sleep and circadian disruption, the results of which are forthcoming.

5. Bile Acids as Potential Mechanistic Links Between the Microbiome and Host Physiology

In this project, we observed the fecal microbiome and metabolome in the context of five days of sleep disruption a total of three times in two different model organisms (Chapters II, IV, V). An additional study in humans during sleep/circadian disruption was also performed by collaborators as part of the MURI. While the exact characteristics of the microbiome/ metabolome changes were not identical in any of the these cases, some commonalities emerged. The most prominent of these was that sleep disruption impacted bile acids. A large scale analysis of the standard-verified bile acids in the fecal metabolomes of these three studies found an overall effect of sleep/circadian disruption ($p = 0.01$; PERMANOVA, **Figure 6.1**), indicating cross species consistency in the alteration of these molecules.

In Chapter II, we observed that metabolites with spectral matches to bile acids were reduced due to sleep disruption, and PICRUSt2 analysis of the predicted microbial gene content revealed bile salt hydrolase (BSH) was reduced. BSH is the main microbial enzyme that modifies primary bile acids, creating secondary bile acids and greatly diversifying the bile acid pool [Russell and Setchell, 1992; Foley et al., 2019] and have diverse signaling properties that involve the immune [Vavassori et al., 2009] and nervous systems [Mertens et al., 2017; Yanguas-Casas et al., 2017]. Inspired by this finding, our collaborators in the laboratory of Dr. Pieter Dorrestein acquired purified standards of various bile acids so that we may be able to verify the identities of any other potentially interesting bile acid metabolites in follow-up studies. In Chapter V, sleep

disruption reduced various standard-verified bile acids in the feces *and* in the serum of rats. Most importantly, though, in Chapter IV we found many standard-verified bile acids were reduced due to sleep disruption, but the secondary bile acid hyodeoxycholic acid (HDCA) was unique because it was reduced due to sleep disruption in vehicle-treated (stress-vulnerable) but not *M. vaccae*-treated (stress-resilient) mice. Investigation into HDCA revealed a body of literature describing the potential anti-inflammatory and metabolic benefits of this bile acid (see Section IV.D for discussion), which has led us to the hypothesis that some of the beneficial effects of the microbiome may be mediated, in part, by alterations of the bile acid pool.

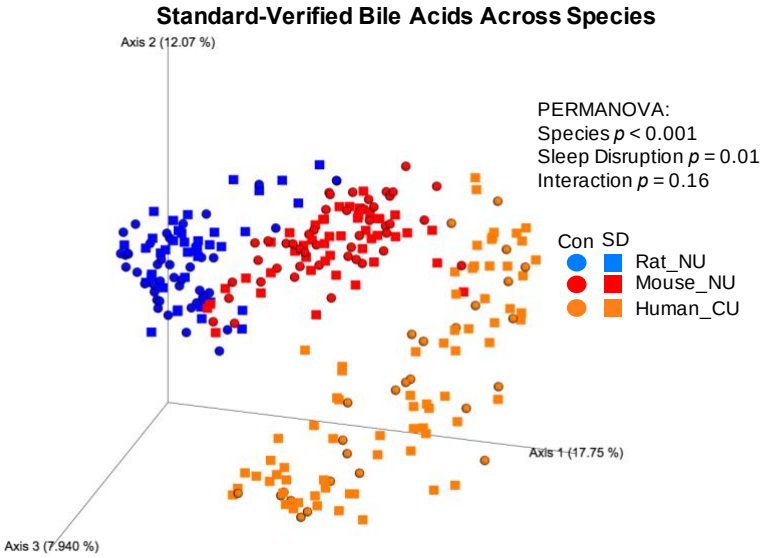

Figure 6.1: Sleep Disruption Alters the Fecal Bile Acid Pool Across Species. Untargeted LC/MS/MS was performed on fecal samples in three studies studying sleep disruption, and features were matched to purified bile acid standards that were also run. The first examined sleep disruption in rats at Northwestern University, the second also examined sleep disruption at Northwestern, but in mice, and the third studied combined sleep and circadian disruption in humans at the University of Colorado. Samples were collected 2 days after a 5-day sleep disruption protocol in the rodent studies. PERMANOVA was performed on all samples shown here, testing for an overall effect of lab/species, sleep (or sleep/circadian) disruption, and interaction. PCoA using Canberra distance is shown above. Abbreviations: Con, control

condition; CU, University of Colorado; NU, Northwestern University; SD, sleep disruption or sleep/circadian disruption.

Secondary bile acids, including lithocholic acid (LCA), deoxycholic acid (DCA), HDCA, and ursodeoxycholic acid (UDCA) (**Figure 6.2a**) all interact with a similar set of receptors (i.e. TGR5, FXR, LXR), but their agonism profiles differ significantly [Song et al., 2000; Sato et al., 2008; De Marino et al., 2017; Hanafi et al., 2018]. This results in differential physiological effects of these bile acids. For instance, feeding DCA or LCA to rodents has been shown to increase colitis [Saracut et al., 2015] and increase colon malignancy burden [Bernstein et al., 2011; Prasad et al., 2014], while UDCA and HDCA have both been shown to do the opposite [Sehayek et al., 2001; Krishna-Subramanian et al., 2012; Hanafi et al., 2018], and this discrepancy has been postulated to have to do with the hydrophobicity index of these bile acids [Hanafi et al., 2018]. In Chapter IV, sleep disruption alone appeared to selectively reduce fecal HDCA, while leaving the other secondary bile acids unchanged in the vehicle-treated mice. This was prevented in the *M. vaccae*-treated mice. Therefore, we propose the following: stimuli that cause changes in microbiome function resulting in an increase in the ratio of hydrophobic (LCA and DCA) to hydrophilic (HDCA and UDCA) secondary bile acids result in aversive inflammatory and metabolic consequences. Under this hypothesis, interventions that reduce the hydrophobic:hydrophilic ratio would promote anti-inflammatory effects and metabolic benefit. This hypothesis is supported by one study that showed that *P. distasonis* exerts positive metabolic changes in part by increasing UDCA (it also increased LCA, but increased UDCA to a much greater extent) [Wang et al., 2019].

Since the prebiotic diet increased *P. distasonis* and has been shown to have many metabolic benefits, we chose to evaluate the above hypothesis using our current dataset. We examined the relative amounts of different standard verified bile acids (normalized to the sum peak area of all bile acids within a sample) at timepoint T2 (4 weeks on diet) in fecal samples from Chapter V. We found that rats fed the prebiotic diet had lower relative amounts of the hydrophobic secondary bile acids (LCA and DCA) than controls (**Figure 6.2b**), while the hydrophilic bile acids (HDCA/UDCA) were unchanged (**Figure 6.2c**). This is preliminary evidence that beneficial microbiome-modulating interventions may also alter the composition of the secondary bile acid pool in a beneficial way. Follow-up studies must be performed to directly test this hypothesis.

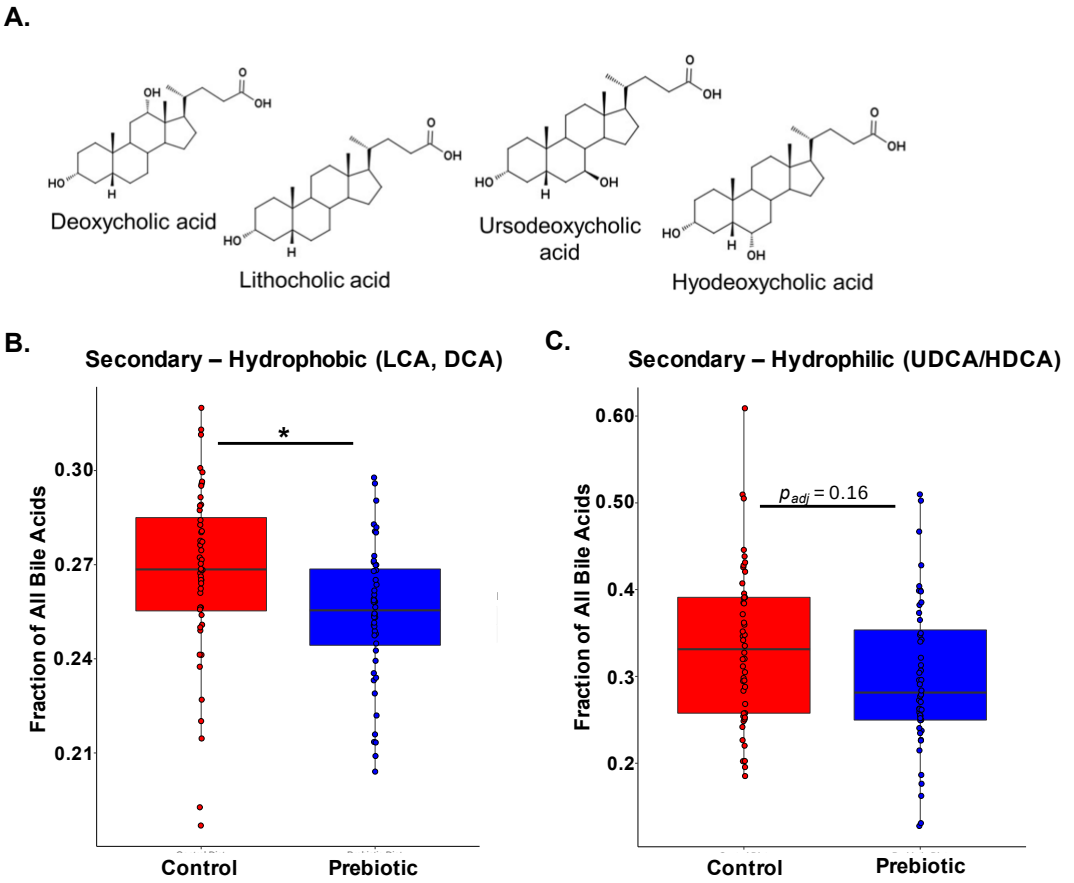

Figure 6.2: The Prebiotic Diet Reduces Only Hydrophobic Secondary Bile Acids. (A) Deoxycholic acid (DCA) and lithocholic acid (LCA) are less hydrophilic than ursodeoxycholic acid (UDCA) and hyodeoxycholic acid (HDCA). Adapted from [Prost et al., 2017]. (B) Relative amounts of secondary, hydrophobic bile acids LCA and DCA are reduced due to the prebiotic diet at four weeks on diet in rats (Chapter V). (C) Relative amounts of secondary, hydrophilic bile acids HDCA and/or UDCA are unchanged by the prebiotic diet. Symbols: *$p_{adj} < 0.05$, Wilcoxon Rank-Sum test with Benjamini Hochberg adjustment. Abbreviations: DCA, deoxycholic acid; HDCA, hyodeoxycholic acid; LCA, lithocholic acid; UDCA, ursodeoxycholic acid.

Together, these studies confirmed that sleep disruption increases stress vulnerability, that both *M. vaccae* and prebiotic diets enhance resilience to sleep disruption and acute stress, and revealed therapeutic potential of the microbe *Parabacteroides distasonis* and the microbial metabolite HDCA (**Figure 6.3**). Follow-up studies investigating HDCA supplementation in the

context of the double hit and investigating the role of *P. distasonis* in non-prebiotic diet contexts are underway (see next section).

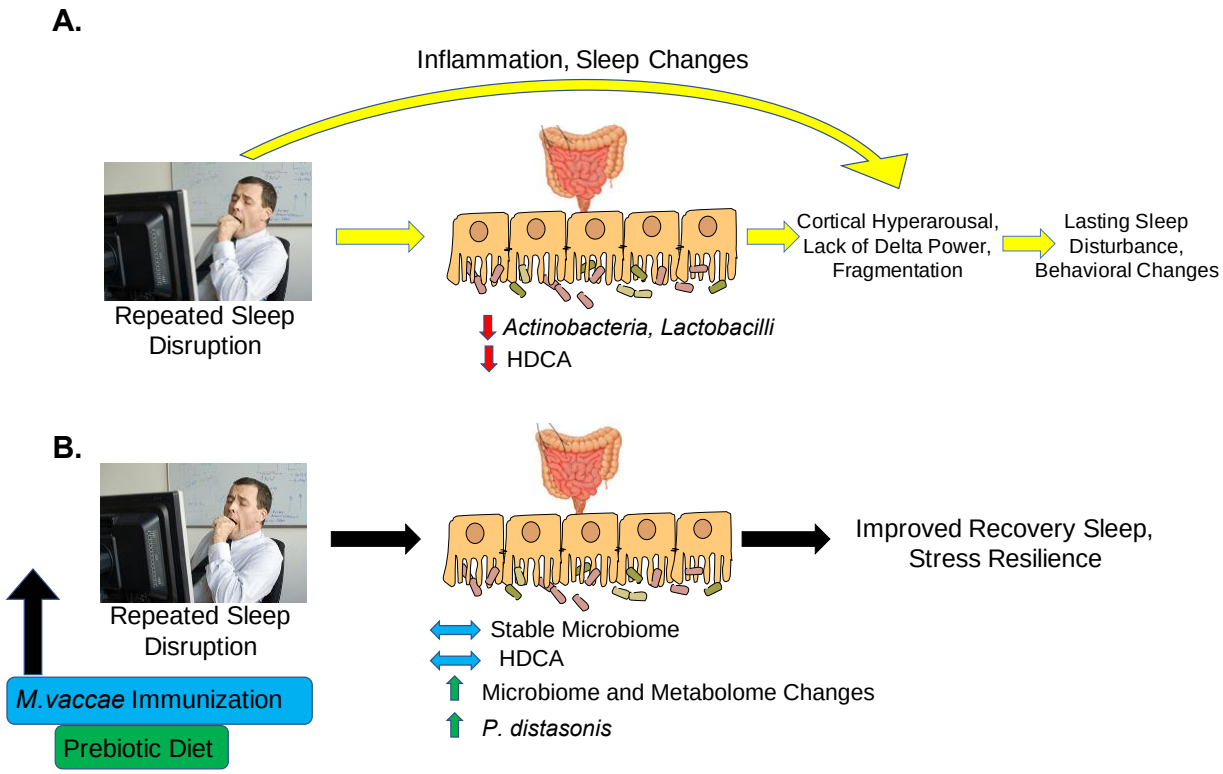

Figure 6.3: Updated Project Model. (A) We found that repeated sleep disruption indeed alters the fecal microbiome and metabolome, involving global shifts in each which are characterized by reductions in beneficial bacteria such as *Actinobacteria* and *Lactobacilli* (Chapter II) and a reduction in the secondary bile acid HDCA (Chapter IV). HDCA correlated with aspects of a maladaptive post-stress sleep phenotype (Chapter IV) featuring increased cortical hyperarousal, reduced NREM EEG delta power, and sleep fragmentation. These measures correlated with later sleep and behavioral disturbances (Chapter III). (B) Microbiome-based countermeasures such as *M. vaccae* (light blue shapes, Chapters III, IV) stabilize the microbiome, including stabilizing HDCA after sleep disruption alone, and prevent sleep disruption-induced stress vulnerability. A prebiotic diet (green shapes) actively altered the microbiome, including increasing *P. distasonis*, and also had beneficial effects after sleep disruption, improving recovery sleep (Chapter V).

B. Current and Future Research Directions

The results of these studies progressed our understanding of the relationship between sleep disruption, stress, and the microbiome. However, more work needs to be done, even within this dataset, to further establish the link between the microbiome, the metabolome, and physiology. This section will describe both ongoing experiments and potential future directions of this research.

1. Continued Analyses of the Present Data

Although we reported many "-omic" level changes to the function of the microbiome and the fecal/serum metabolomes in Chapters IV and V, an in-depth analysis of these data could help us draw more direct lines between microbes, metabolites, and physiology. For instance, the multitude of genes that are changing due to the double hit in *M. vaccae*-treated mice that were identified via shotgun metagenomics may provide insight into specific metabolites to look for in the metabolome. This could be achieved via software systems such as Ingenuity Pathway Analysis that take genetic information and impute chemical and pathway interactions. Furthermore, more purified standards could be run to verify the identities of the many suprathreshold metabolites discovered in Chapter IV and Chapter V. These efforts are ongoing in the Turek laboratory.

2. Follow-Up Studies of the Double Hit Effect

As mentioned in the previous section, the description of the double hit phenotype in Chapter III opened the door for many other experiments characterizing the phenomenon. Christine Lee, and undergraduate student who helped with some of the experiments of this dissertation, is pursuing this line of inquiry. In the experiments described in this dissertation, the recovery sleep window was from ZT2-ZT6. This was designed to fall during the normal circadian peak in amount of sleep and delta power during sleep so the animals could get the most restful recovery sleep possible. However, one could hypothesize that if the recovery window fell during a time where circadian gating of sleep intensity/efficiency was at a trough, the sleep disruption would become more severe, potentially altering the double hit effect. The experiment was run in a 2x2 design (control vs double hit; sleep disruption recovery at ZT2-6 vs recovery at ZT14-18). Outcome measures included sleep, OLM behavior, and fecal samples were collected throughout the experiment. Results are currently being analyzed.

Due to the observation that the peak changes in sleep due to the double hit are observed two days after sleep disruption ends, and that fecal HDCA is reduced due to sleep disruption and the double hit at the same timepoint, I designed and performed another follow-up experiment examining the same double hit protocol, but with the terminal timepoint lying at SD+2 instead of SD+7. The results of this experiment are currently being analyzed.

3. Follow-Up Studies Regarding HDCA

The finding that HDCA was the only secondary bile acid to be reduced due to sleep disruption alone in vehicle-treated mice, but was not reduced in *M. vaccae*-treated mice, paired with the current literature on HDCA prompted the following hypothesis: oral HDCA supplementation is sufficient to improve resilience to sleep disruption plus acute social defeat in mice. To test this hypothesis, we recently fed male C57BL/6 mice (Charles River, $N = 40$) a diet containing 1% HDCA by weight (HDCA from Sigma; Teklad custom diet TD.190295) and subjected them to either *ad libitum* sleep and no social defeat (control) or the double hit (as in Chapter III) (**Figure 6.4a**). This dosage selection was based on the following. First, previous studies using HDCA enriched food at 0.5% [Sehayek et al., 2001] and 1.25% [Shih et al., 2013] demonstrated that these dosages were safe and had metabolic benefit. Second, a rough calculation of the projected concentration of the different dosages in the intestinal tract based on the volume of mouse intestines [Casteleyn et al., 2010] and the normal concentrations and absorption of HDCA across the different sections of the intestine [Yang et al., 2017] indicated that a dose of 1% by weight would result in ~2x the physiological concentration in the colon. This is ideal, as the reduction in HDCA observed in sleep disrupted mice in Chapter III was about 50%.

Results are still being analyzed, but preliminary analysis of body weight data suggests the HDCA diet-fed mice do not gain weight at the same rate across the experiment as do control diet-fed mice, and may lose weight after being plugged into the EEG/EMG tethers (**Figure 6.4b**). Whether this is adaptive or maladaptive will be assessed as more data become available.

Preliminary analysis of the OLM data revealed that while the double hit reduced the percent of time spent in the middle of the arena in the control diet-fed rats, it did not in the HDCA-fed rats (**Figure 6c**). This result is encouraging, but the complete data analysis must be finished before definitive conclusions can be drawn.

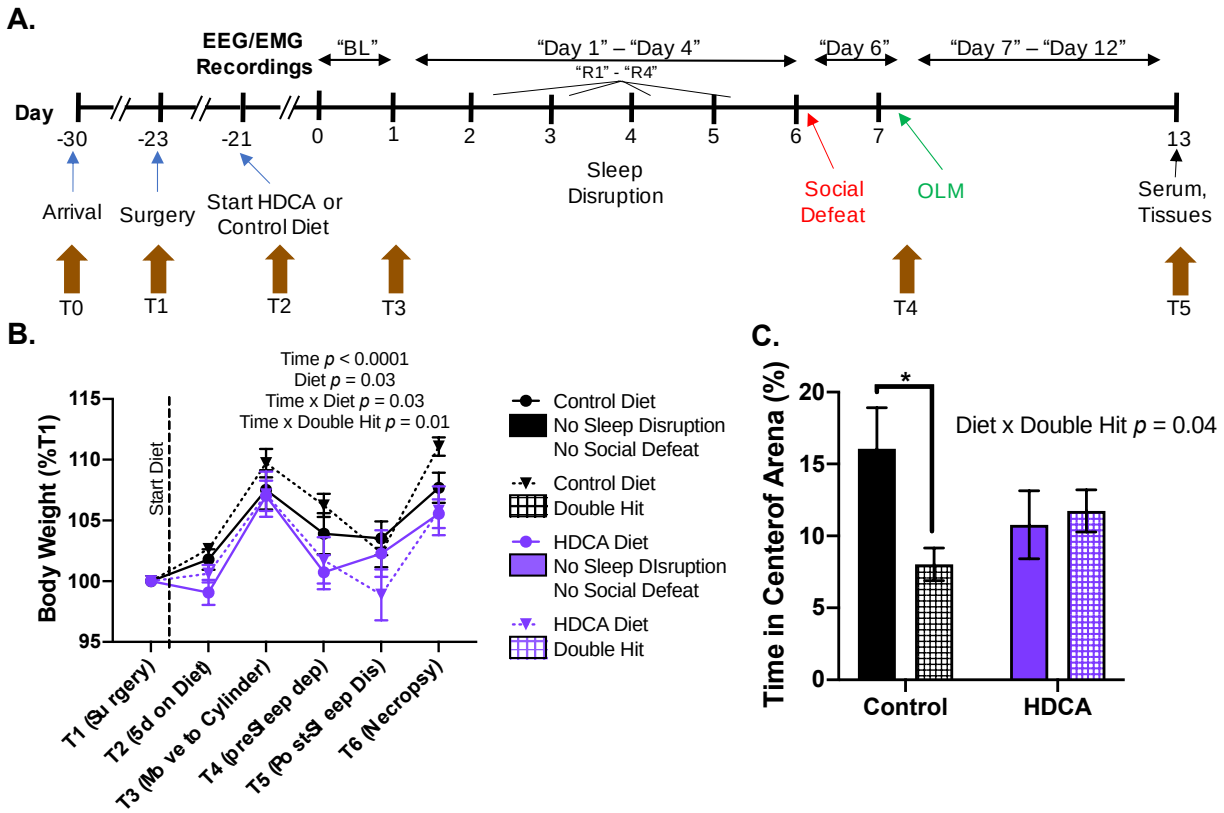

Figure 6.4: Hyodeoxycholic Acid Supplementation Study. (A) Timeline of experiment. One cohort has been completed. (B) Preliminary body weight data suggest the HDCA-fed mice lost weight when being placed in the sleep recording chambers, but eventually gained it back (statistics shown are significant results of linear mixed effects modeling). (C) Percent of time spent in the center of the arena during the OLM testing session was reduced due to the double hit only in the Control diet group. Symbols: *$p < 0.05$ (Bonferroni post hoc test). Abbreviations: BL, baseline; EEG, electroencephalogram; EMG, electromyogram; HDCA, hyodeoxycholic acid; OLM, object location memory; SD, sleep disruption. n = 10/group.

4. Further Examination of *P. distasonis* as a Sleep-Relevant Probiotic Across Species

Another important finding from this project is the identification of the exceptional bloom of fecal *P. distasonis* with the prebiotic diet. As discussed above, there is growing literature surrounding the physiological relevance of this bacterium, and our data from Chapter V suggest it correlates with recovery sleep post-sleep disruption. Furthermore, data from other laboratories within this MURI have found interesting associations with *P. distasonis* across multiple species.

Unpublished data from the laboratory of Dr. Monika Fleshner using the same GOS/PDX formulation in a chronic circadian disruption protocol in rats also found that *P. distasonis* was selectively promoted and that the diet enhanced re-entrainment during circadian disruption. Furthermore, unpublished work from Dr. Ken Wright examining the microbiome in the context of combined sleep and circadian disruption in humans found that *P. distasonis* is more abundant in the colons of humans who have wake after sleep onset (WASO) scores in the bottom 30th percentile of the study participants. Thus, humans (with no prebiotic diet) who have the most consolidated sleep also have the most *P. distasonis* in their microbiomes. Upon learning of these data, we retroactively searched for OTU matching *P. distasonis* in the 16S rRNA data gathered from the experiment described in Chapters III and IV. We found that in vehicle-treated mice, the double hit (which increased sleep fragmentation) resulted in a decrease in the clr transformed abundance of *P. distasonis* compared to controls (**Figure 6.5a**). In *M. vaccae*-treated mice, the double hit (which did not increase sleep fragmentation) did not result in a change in *P. distasonis* (**Figure 6.5b**). This cross site, cross-species consistency is rare in microbiome research, and

provides strong evidence that *P. distasonis* may play a role in sleep continuity. Further research investigating sleep in the context of *P. distasonis* probiotic treatment needs to be done to more directly establish this link, but it is a promising line of inquiry.

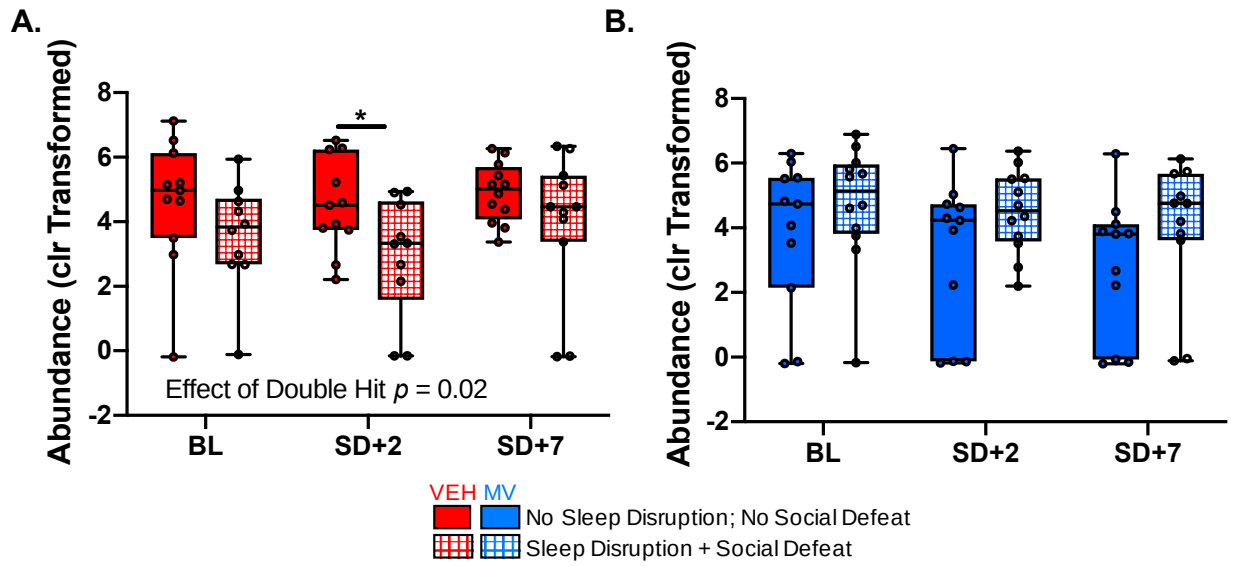

Figure 6.5: *P. distasonis* is Reduced in Vehicle Treated Mice Following the Double Hit. 16S rRNA OTU data from Chapter IV were searched for sequences mapping to *P. distasonis*. The clr-transformed abundances at baseline, 2 days after sleep disruption, and 7 days after sleep disruption are graphed for control and double hit groups within (A) vehicle-injected and (B) *M. vaccae*-treated mice. Mixed effects modeling was performed within treatment group for effects of time, the double hit, and interaction. Boxes indicate median, 25th and 75th quantiles; whiskers indicate full range of individual values. Symbols: *$p < 0.05$, Fischer's LSD test. Abbreviations: BL, baseline; clr, centered log-ratio; MV, *M. vaccae*; SD, sleep disruption; VEH, vehicle. n = 10-12/group.

C. Concluding Remarks

As an undergraduate I took a class on the neurobiology of sleep taught by Dr. Craig Heller. I learned that although sleeping for a significant portion of each and every day appears distinctly unfavorable when viewed through the lens of natural selection, it is nearly universal in the animal kingdom and thus must be of critical importance. Furthermore, I learned that although we

scientists are making progress, we still do not know exactly *why* animals (*Homo sapiens* included) sleep. These two incredibly interesting concepts have served as a driving force behind my research decisions, which have also been guided by my passion for medicine and my desire to become a physician scientist. I therefore chose Northwestern for my MD/PhD training and joined Dr. Fred Turek's laboratory in 2016 with the intent of studying a clinically relevant problem in the field of sleep and circadian rhythms using cutting edge scientific techniques and methods. Four years later, I have come to learn that the measurement of sleep is a powerful clinical tool that contains information regarding the severity of a preexisting disease state and provides biomarkers for vulnerability to subsequent disease processes.

I hope the results of these experiments will inform our understanding of the mechanisms by which sleep disruption increases stress vulnerability, and will add to the growing body of evidence that our interactions with the bacteria that live in our intestinal tract and exist in our environment can be beneficial, providing a wealth of therapeutic promise. Humans evolved among an immense diversity of microorganisms, so it should come as no surprise that host-microbe interactions play a large role in physiology. However, the current focus of medicine often resides on the most negative of those interactions: when pathogens make us sick. While this focus is still extremely important (infections still account for significant morbidity and mortality in our healthcare system), it is also important that we explore the potential benefit microbes can offer. This may be via immunization or supplementation with "beneficial" bacteria like *M. vaccae*, or via dietary supplements to promote the growth of a healthy gut microbiota

such as a prebiotic diet. Furthermore, the mechanistic link between the microbiome and host physiology in large part may revolve around microbial metabolites, so the fecal metabolome should be measured in parallel whenever possible. I believe the broad training I received in the Turek laboratory in the fields of sleep, stress physiology, and the microbiome/metabolome will become of central importance to human health in the coming decades. I hope to be at the forefront of these fields in my career as a physician scientist, using clinical observations to inspire scientific inquiry.

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