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Hydrogen-rich water alters sleep and enhances forebrain neuronal activity in mice

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Hydrogen-rich water alters sleep and enhances forebrain neuronal activity in mice

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

Doctor of Philosophy in Neuroscience

by

Scott Michael Vincent

2022

ABSTRACT OF THE DISSERTATION

Hydrogen-rich water alters sleep and enhances forebrain neuronal activity in mice

by

Scott Michael Vincent

Doctor of Philosophy in Neuroscience

University of California, Los Angeles, 2022

Professor Ketema Nnamdi Paul, Chair

Poor sleep is an unfortunate hallmark of modern life, with diverse consequences for the individual and society. Sleep loss is associated with metabolic, immunological and cognitive consequences, with chronic sleep loss believed to reduce life and health span and increase the risk of several chronic complex disorders. In Chapter 1, I discuss classical behavioral markers of sleep pressure and engage in the investigation of two specific cortical oscillations using polysomnography in mice. Several studies demonstrate that low frequency high amplitude cortical oscillations (0.5-4.0Hz, slow wave activity) are a useful biomarker for underlying sleep need/pressure. Recent work suggests that sub-classes of waveforms, slow oscillations (SOs) and delta waves (DWs), within the parameter range of slow wave activity may have distinct roles in sleep-dependent learning and forgetting in rats. We implanted wild type C57BL/6J mice (n=44) with polysomnographic implants for recording of sleep and arousal behavior. Here I demonstrate that SOs and DWs have significantly different temporal dynamics in undisturbed animals and in the response to the acute homeostatic challenge of sleep deprivation by gentle handling.

In Chapter 2, I test the ability of a putative novel hypnotic to alter sleep and the recovery from sleep loss in mice. Hydrogen is an abundant chemical substance that has recently shown promise as a diverse-acting biological signaling molecule with the capacity to influence metabolism, immunity and cognition, as well as known sleep regulatory regions. We hypothesized that the ability of hydrogen-rich water (HRW) to alter sleep processes and behaviors may be a result of its ability to alter sleep itself. We implanted wild type C57BL/6J mice (n=10) with polysomnographic implants for recording of sleep and arousal behavior tested the ability to *ad libitum* access to HRW to influence several well-established behavioral and electrophysiological sleep phenotypes. We report here that HRW is sufficient to alter several behavioral markers of sleep pressure, but not slow wave activity, in adult mice.

The dissertation of Scott Michael Vincent is approved.

Jeffrey Michael Donlea

Sotirios Masmanidis

Christopher S. Colwell

Ketema Nnamdi Paul, Committee Chair

University of California, Los Angeles

2022

DEDICATION

“Don’t aim at success. The more you aim at it and make it a target, the more you are going to miss it. For success, like happiness, cannot be pursued; it must ensue, and it only does so as the unintended side effect of one’s personal dedication to a cause greater.” - Viktor Frankl

My PhD taught me a great deal about the person I want to be. Its challenges, my successes, my failures, and everything in between gave me invaluable clarity on the “how and why” underlying all my decisions, big and small alike. I leave Los Angeles with a greater sense of the man I want to be for myself, my wife, my family and friends. These “life lessons” are more valuable than any scientific successes which may yet emerge from my formal education in neuroscience.

I dedicate this work to the people who made my personal and scientific growth possible:

To my wife Courtney, for challenging the limits of my comfort zone, for seeing the best in me when I couldn’t see it myself, and for laughing with me when things fell apart. I love you endlessly. I am so excited for the next leg of our journey. I could not have done this without you.

To my parents, Jane and Michael, for endless “second changes” given to a kid who just didn’t know better. The patience and love you had for me as a young man were more than I deserved and everything I needed. Thank you for never giving up on me. I love you.

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To my scientific mentors at UCLA and UCSD, Ketema Paul, Chris Colwell, Cristina Ghiani, Tom O’Dell, Jeff Donlea, Sotiris Masmanidis, Gedeon Deák, Robert Clark, and Mary Boyle, thank you for pushing me to dig deeper. Each of you has shaped my ability to ask questions, to suspend disbelief, and to face difficulty with curiosity. Thank you.

To the greatest trainees of all time, Melika Madani, Cameron Jackson, Kyle Golden, Sam Wimmer, Dante Dikeman and Naomi Crocker. I believe in you and look forward to your success.

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ABSTRACTS:

1. **Vincent, S**., Li, A., Makeig, S., Deak G. Expectancy Violation in Dyadic Interaction: EEG Analyses. Annual Research Experience for Undergraduates Symposium, UCSD, San Diego, CA, 2016

2. Chang, L., Williams, M., Colson, D., **Vincent, S**., Deak, G. Patterns of Verbal and Nonverbal Communication and Performance During a Dyadic Turn-Taking Game. Undergraduate Research Symposium, UCSD, 2016

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4. Golden K., **Vincent, S**., Nichols, I.S., Watts, K., Paul, K. Anaplastic Lymphoma Kinase (ALK) Antagonist Alters Sleep Regulatory Regions After Sleep Loss. Brain Research Institute Research Symposium, UCLA, 2019

5. Madani M., **Vincent, S**., Paul, K. Discrete features of Non-REM Slow Wave Activity in Sleeping Mice. Brain Research Institute Research Symposium, UCLA, 2020

6. Okekenwa S., **Vincent, S**., Chiem E., Paul K., Colwell C. Discrete features of Non-REM Slow Wave Activity in a Mouse Model of Huntington's Disease. Brain Research Institute Research Symposium, UCLA, 2020

7. Jackson C., **Vincent, S**., Golden K., Madani M., Paul K. The Effects of Hydrogen Rich Water on Sleep in Mice. Brain Research Institute Undergraduate Research Symposium, UCLA, 2020

8. **Vincent, S**., Golden K., Jackson C., Madani M., Nichols, I.S., Paul K. Molecular hydrogen alters NREM delta oscillations during the recovery from sleep loss. Society for Neuroscience, Chicago, IL, 2021

9. Golden K., **Vincent, S**., Jackson C., Madani M., Nichols, I.S., Paul K. Molecular hydrogen decreases spontaneous sleep latency in mice. Society for Neuroscience, Chicago, IL, 2021

10. Crocker, N., **Vincent S**., Dikeman D., Golden K., Ghiani C., Paul K., Evaluating the hypnotic potential of hydrogen-rich water. Psychology Undergraduate Research Conference; UCLA, 2022

11. Crocker, N., **Vincent S**., Dikeman D., Golden K., Ghiani C., Paul K., Evaluating the hypnotic potential of hydrogen-rich water. Undergraduate Research and Creativity Showcase; UCLA, 2022

12. Golden, K., **Vincent S**., Dikeman D., Paul K., Evaluating the effect of hydrogen-rich water on novel object recognition behavior. Integrative Biology and Physiology Research Conference; UCLA, 2022

CH 1: DYNAMICS OF NREM DELTA BAND OSCILLATIONS

Introduction

Sleep is a highly conserved behavior across species with important roles in maintaining health. Sleep restriction negatively impacts lifespan [1] and cognition [2], and increases risk of disease [3]. The “Two Process Model”, originally described by Alexander Borbély, is a widely accepted framework for understanding sleep regulation. It posits that sleep-wake behavior is largely defined by two processes: the circadian rhythm (Process C) and the sleep homeostat (Process S) [4]. Process C is our “internal clock” and, while it influences the timing of sleep, it is sleep-*independent* [4]. At the molecular level, circadian pace-keeping machinery are complex transcription-translation feedback networks that reliably oscillate with a time constant of about a day, regulating cell- and organ-specific processes, and influencing behavior like sleep [5]. Nearly 50 years since the identification of the first clock genes and their role in sleep, much is known of circadian biology. Process S, which remains enigmatic by contrast, influences sleep behavior and is sleep-*dependent*: that is, prior wake and sleep experience influence how much sleep pressure (aka “sleep drive” or “sleep propensity”) is created [6]. As a species spends more time awake, Process S generates commensurate pressure to sleep; as they sleep, this need for sleep reconciles. While other systems can influence sleep-wake behavior (like those involved in stress, immunity, etc.) Processed C and S play leading roles in the regulation of sleep timing, duration and quality [4].

Distinctly homeostatic regulation of sleep emerged and persists in a variety of species [6], yet relatively little is known of how Process S interprets waking experience or reconciles sleep pressure during sleep. Several studies suggest that some sleep-promoting factor (such as adenosine) accumulates during wake, gradually increasing sleep pressure, and then breaks down during sleep [7]. Others posit that the homeostat, perhaps through the activity of neuronal protein Homer1a, is part and parcel to the behavior of the connectome, the rich complex and highly

dynamic network of relationships among brain cells [9]. Despite debates over a unifying theory for “why we sleep” and the fundamental organizing principles of sleep processes, our field continues to expand understanding of sleep-related physiological processes and behavior.

Sleep is not a unitary state, but an innately complex behavior organized into cycles of two major sleep types: rapid eye movement (REM) sleep and non-rapid eye movement (NREM) sleep. These broad categories can be further classified into substages, each with behavioral and electrophysiological features that distinguish them. Neural oscillations measured from the surface of the skull using electroencephalography (EEG) reflect the dynamic activity of the cortex and provide information about arousal state - an essential element of sleep behavior. Beyond signaling arousal states, specific neural oscillations are often associated with specific physiological processes and may reflect the activity and connectivity of subcortical regions. For instance, optogenetically silencing GABAergic neurons in the mouse medial septum during REM sleep (but not NREM sleep) attenuates theta waves (4.0-8.0Hz) and significantly impairs memory consolidation [9]. Low frequency (delta band, 0.5-4.0Hz), high amplitude oscillations during NREM sleep are a good indicator of sleep pressure in sleeping mice and are at least partially driven by cyclical hyperpolarization of thalamocortical projections [4,10,11]. While this “slow wave activity” (SWA) is most common in NREM sleep and usually associated with unconsciousness, it can be observed during all states [12].

In 2019, Kim et al. demonstrated that distinct categories of low-frequency waveforms during NREM sleep may contribute unequally to two sleep-dependent behaviors: learning (consolidation) and forgetting [13]. The cortical waveforms observed, slow oscillations (SOs) and delta waves (DWs), have similar intra-event duration and features, but are distinguishable from one another by the presence of a preceding peak in slow oscillations before a shared trough (Figure 1). As

these delta band waveforms appear to contribute to separate sleep-dependent processes, we hypothesized that they may be distinct from each other in their activity during normal, undisturbed sleep (henceforth “baseline”) and in their response to the acute homeostatic challenge of sleep deprivation (henceforth “recovery”). Several reports exist on “slow wave activity”, “delta power”, “delta waves” and “Type I and Type II slow oscillations”. Despite semantic differences, these reports largely share the same topic: oscillations occurring within the delta frequency range, and the waveforms in question often have incompletely overlapping intra-event features such as slope, amplitude, source and others. While some feature-specific investigation of heuristically similar waveforms have been previously reported [14], this is the first direct investigation of daily patterns of these high amplitude (≥100mV) low-frequency (0.8-1.2Hz) NREM waveforms **(Figure 1)** during baseline and recovery NREM sleep in mice.

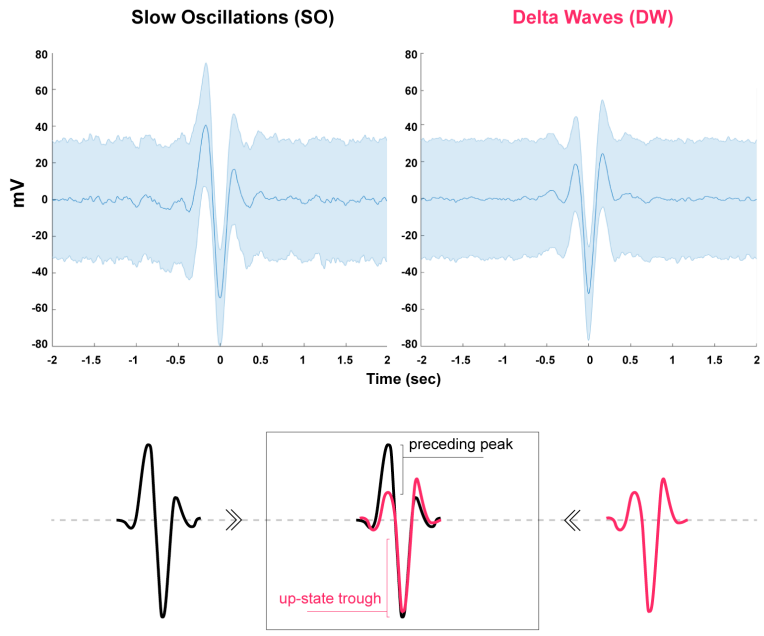


Figure 1. Slow oscillation and delta wave characteristics during NREM sleep. (Top) Representative mean and standard deviation of slow oscillations (SOs) and delta waves (DWs) identified during NREM sleep within a 1-hour sample from a wildtype C57BL/6J mouse. (Bottom) Schematic of SOs and DWs.

Materials and methods

Animals care

Adult C57BL/6J mice (n=44; male=22, female=22) were maintained at the University of California Los Angeles (UCLA) under a 12h-12h light-dark cycle (LD) in a temperature-controlled study area overseen by the university Animal Research Committee and Division of Laboratory Animal Medicine. Food and water were provided *ad libitum*. Experiments were performed using the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.

Polysomnographic implantations

Adult (PNW 12 - PNW 14) C57BL/6J mice were implanted with EEG/EMG headmounts for polysomnographic (PSG) recording. As previously reported [49], mice were implanted with 4x EEG and 2x EMG electrodes under anesthesia. Two electrodes (frontal-parietal and ground) were located 1.5 mm anterior to bregma and 1.5 mm on either side of the central suture. Two additional electrodes (parietal-occipital and common reference) were located 2.5 mm posterior to bregma and 1.5 mm on either side of the central suture **(Figure 2)**. Electrical continuity between the screw electrode and headmount was achieved with silver epoxy. EMG activity was monitored using stainless-steel teflon coated wires inserted into the nuchal muscle. The headmount (2×3 pin grid array) was secured to the skull with dental acrylic. Mice were given 14 days to recover (including a 7-day acclimatization period) before recording.

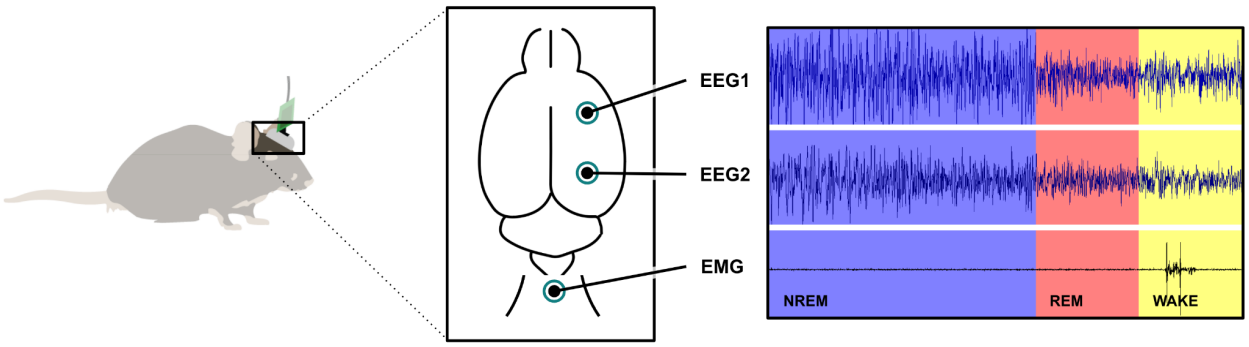


Figure 2. Schematic of polysomnographic implantations and arousal state data.

Total Sleep Deprivation

Following a 24-hour undisturbed baseline recording, PNW 14 - PNW 16 (mean = 15.19 ± 0.59) C57BL/6J mice underwent 6 hours of acute total sleep deprivation by “gentle handling” at the onset of the light phase, ZT 0. For 6 hours, experts blind to the conditions of the experiment delicately touched mice with soft brushes when they displayed behavioral signs of sleep onset (recumbent posture, quiescence, closed eyes). Mice were allowed an undisturbed 18-hour recovery period following sleep deprivation, starting at ZT 6.

Data Acquisition and Processing

Data acquisition was performed on a PC running polysomnographic software (Sirenia Acquisition, Pinnacle Technologies, Lawrence, KS). Signals were amplified and high-pass filtered (0.5 Hz) via a preamplifier. EEG signals were low-pass filtered with a 40Hz cutoff and collected continuously at a sampling rate of 400Hz. After collection, EEG and EMG waveforms were classified in 10-sec epochs as: (1) wake (low-voltage, high-frequency EEG; high amplitude EMG); (2) NREM sleep (high-voltage, mixed-frequency EEG; low-amplitude EMG); (3) rapid-eye movement (REM) sleep (low-voltage EEG with a predominance of 4.0-8.0Hz theta activity; very low amplitude EMG). All

sleep scoring was performed by expert technicians blind to the conditions of the experiment. EEG epochs determined to have artifacts (interference caused by scratching, movement, eating, or drinking) were excluded from analysis. Artifacts comprised less than five percent of all records used for analysis.

Analysis and statistics

56 sleep phenotype covariates were collected for each sample (n=44), resulting in a 57 x 44 dataframe. Chosen covariates include (a) essential animal information (age, sex), (b) established behavioral and electrophysiological phenotypes associated with sleep homeostasis (latency, fragmentation), and (c) strategically chosen single dimension endpoints reflecting temporal and spatial characteristics of DWs and SOs (DW:SO ratio, DW decay slope. For a complete list of included covariates and their definitions, please see **Supplementary Table 1**. Automatic extraction of most sleep features was completed using a proprietary MATLAB function I developed. Mutually exclusive identification of slow oscillations and delta waves was achieved utilizing a MATLAB script provided by Ganguly et al. (University of California, San Francisco) and adapted so that feature distribution and endpoint output could be flexibly controlled as needed for our purposes. Parametric assumption testing and simple linear regressions were completed using GraphPad Prism 9 (details here). Multiple linear regression with backward elimination and fit calculations were completed in RStudio using a built-in LM step function. Analyses of variance and figures were generated using GraphPad Prism 9.

Results

Temporal and spatial dynamics of delta waves and slow oscillations

The reactivation of the parietal cortex during NREM sleep by slow oscillations may be causally linked to learning (memory consolidation), while reactivation by delta waves appears to promote

forgetting [13]. These findings and others [15] demonstrate that neural oscillations may do more than reflect underlying connectivity, with power to alter physiology and behavior. Toward understanding their relationships to NREM sleep and their response to sleep loss, we used polysomnography to assess DWs and SOs throughout 24 hours of undisturbed sleep-wake activity of Adult C57BL/6J mice (n=44; male=22, female=22). Immediately following this baseline recording, we recorded sleep-wake behavior across 6 hours of sleep deprivation by gentle handling and an 18-hour recovery period. The quality and quantity of recovery sleep following 6 hours of acute total sleep deprivation is a commonly used approach to estimate changes in Process S [16]. DWs and SOs were assessed across both recording electrodes: EEG1, frontal-parietal **(Figure 3A-B)**; and EEG2, parietal-occipital **(Figure 3C-D)**. Two-way ANOVA reveals a significant primary effect of waveform and a significant waveform-time interaction across both electrodes and both sleep conditions **(Table 1)**. DWs occur at a significantly greater rate across both conditions, with the greatest delta observed during periods of highest sleep propensity - during the inactive phase and the mid-active “siesta”, ZT 18-22 [17]. We report no effect of sex across conditions **(Supplementary Figure 4)**.

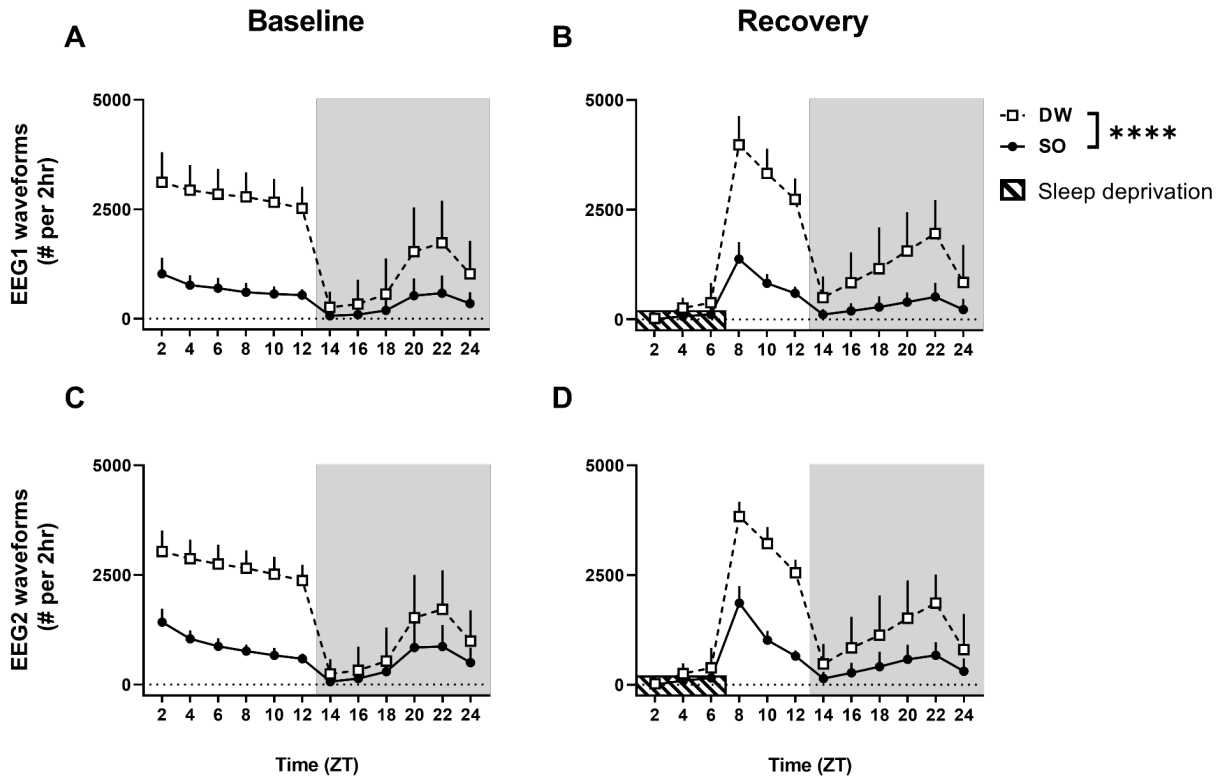


Figure 3. Delta waves are more abundant than slow oscillations during baseline and recovery NREM sleep. Polysomnography was used to record arousal states across baseline, sleep deprivation and recovery conditions of adult C57BL/6J mice. Features of interest from both recording electrodes were binned into 2-hour epochs and their 24-hour distribution plotted for baseline (*left)* and recovery (*right)* conditions. Distribution was analyzed using a two-way ANOVA with waveform type (SO or DW) and time as factors. There are significant differences in waveform distribution during baseline **(A,C)** and recovery **(B,D).** Significant effects of time, waveform type, and time-waveform interaction were observed in both the frontal-parietal (*top*, EEG 1) and the occipital-parietal (*bottom*, EEG 2) electrodes. Data presented as mean+SD. Shaded boxes label the dark phase. **See Table 1 for statistics.**

Previous reports demonstrate spatial differences in DWs and SOs during NREM sleep, with SOs tending to occur more globally across the cortex [13]. We recorded data from two recording electrodes, one over frontal-parietal cortex (EEG1) and one over the parietal-occipital cortex (EEG2). Direct comparison of DWs and SOs across electrodes demonstrates an unequal influence of electrode placement on waveforms. SOs, but not DWs, occur at a higher rate in EEG2 during periods of typically high sleep pressure, independent of sleep condition **(Figure 4)**.

Source of Variation	SS	F(DFn, DFd)	P value	Summary
EEG1 - Baseline				
Time	403896020	***F* (6.483, 505.7) = 176.8**	***P* < 0.0001**	****
Waveform	443277261	***F* (11, 858) = 66.91**	***P* < 0.0001**	****
Time x Waveform	152902665	***F* (11, 858) = 66.91**	***P* < 0.0001**	****
EEG1 - Recovery				
Time	612958102	***F* (5.322, 415.1) = 272.1**	***P* < 0.0001**	****
Waveform	275196671	***F* (1, 78) = 555.7**	***P* < 0.0001**	****
Time x Waveform	182632360	***F* (11, 858) = 81.07**	***P* < 0.0001**	****
EEG2 - Baseline				
Time	427601668	***F* (5.733, 447.1) = 185.7**	***P* < 0.0001**	****
Waveform	302159089	***F* (1, 78) = 712.5**	***P* < 0.0001**	****
Time x Waveform	121635242	***F* (11, 858) = 52.82**	***P* < 0.0001**	****
EEG2 - Recovery				
Time	655615807	***F* (5.061, 394.8) = 317.8**	***P* < 0.0001**	****
Waveform	191432413	***F* (1, 78) = 668.7**	***P* < 0.0001**	****
Time x Waveform	126199139	***F* (11, 858) = 61.17**	***P* < 0.0001**	****

Table 1. Unequal influence of time on NREM delta features during baseline and recovery NREM sleep. Two-way ANOVA with waveform type (SO or DW) and time as factors for each electrode and sleep condition.

Source of Variation	SS	F(DFn, DFd)	P value	Summary
Waveform	15227583455	***F* (1, 340) = 24320**	***P* < 0.0001**	****
Sleep	1244191927	***F* (1, 340) = 198.70**	***P* < 0.0001**	****
Electrode	24432464	***F* (1, 340) = 3.9020**	***P* = 0.0490**	*
Waveform x Sleep	308650576	***F* (1, 340) = 49.300**	***P* < 0.0001**	****
Waveform x Electrode	131497314	***F* (1, 340) = 21.000**	***P* < 0.0001**	****
Sleep x Electrode	2384444	*F* (1, 340) = 0.0381	*P* = 0.8454	ns
Waveform x Sleep x Electrode	385966	*F* (1, 340) = 0.7005	*P* = 0.4032	ns

Table 2. Spatial differences in slow oscillations but not delta waves during NREM sleep. Three-way ANOVA with electrode (EEG1 and EEG2), waveform (DW and SO) and sleep condition (baseline and recovery) as factors.

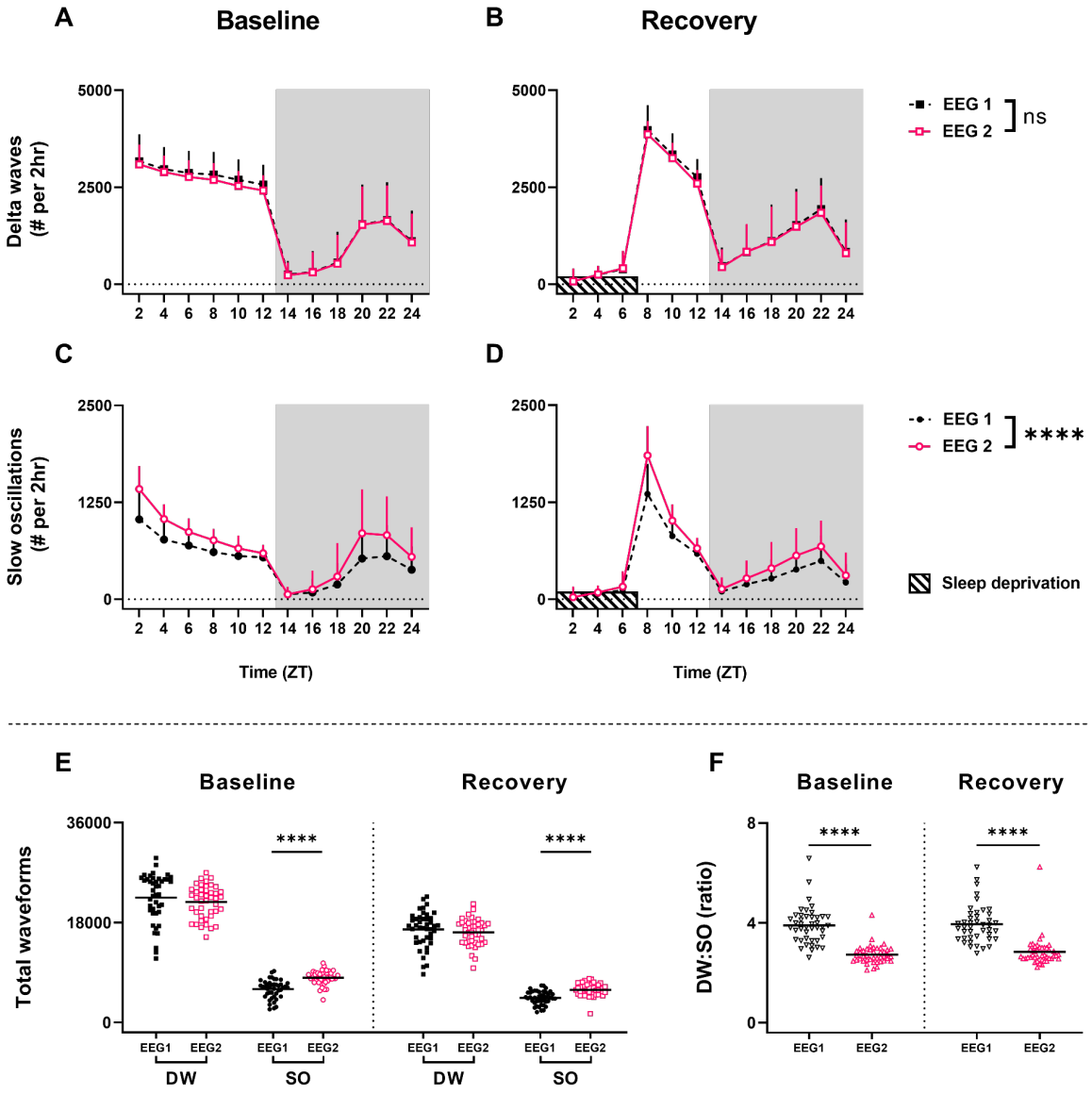


Figure 4. Spatial differences in slow oscillations but not delta waves. Polysomnography was used to record arousal states across baseline, sleep deprivation and recovery conditions of adult C57BL/6J mice. Data was analyzed using a three-way ANOVA with electrode, waveform and sleep condition as factors. **(A-B)** 24-hour distribution of delta waves across EEG1 and EEG2 during baseline and recovery conditions. **(C-D)** 24-hour distribution of slow oscillations, which are significantly different in EEG1 and EEG2 during baseline and recovery. **(E)** Total waveforms identified during 24-hour baseline and 18-hour recovery for EEG1 and EEG2. **(F)** Differences in delta-wave-to-slow-oscillation ratio across electrodes. Red icons are data from EEG2. Data presented in A-D as mean+SD. Shaded boxes label the dark phase. **See Table 2 for statistics**.

We also observe that the ratio of delta-waves-to-slow-oscillations (DW:SO) are lower during baseline and recovery conditions **(Figure 4F)**, that this effect is driven by a relative increase in SOs **(Figure 4E),** and that the ratio of DW:SO changes in similar ways over time during both conditions and in both electrodes **(Supplementary Figure 3)**, suggesting a circadian influence.

Side-by-side-by-side comparison with the field standard: NREM delta power

The standard measurement for Process S (in animals where EEG is available) is NREM SWA. SWA is most abundant at sleep onset following prolonged periods of wakefulness. SWA dissipates across consolidated NREM sleep, is responsive to acute and chronic homeostatic challenges [16], and is sensitive to activity-dependent processing, demonstrating local variation across cortex [17]. Relative delta power (rDelta, rD) is frequently used instead of delta power as it is less sensitive to artifacts. To compare DWs. SOs, and rD during baseline and recovery, we calculated the Z-Score of each feature - DW/2hr, SO/2hr, and mean rD/2hr **(Figure 5A-D)**. A mixed-effects analysis was used with time and feature (DW, SO, rD) as factors. Multiple comparisons with Benjamini and Hochberg correction was used to control false discovery rate. While there was no primary significant effect of feature-type, we report a significant time-feature interaction, and numerous repeated measure discoveries. The overwhelming majority of discoveries are observed during the late inactive phase (ZT 6-12) during baseline and recovery conditions collected from both EEG1 and EEG2 **(Supplementary Table 1).**

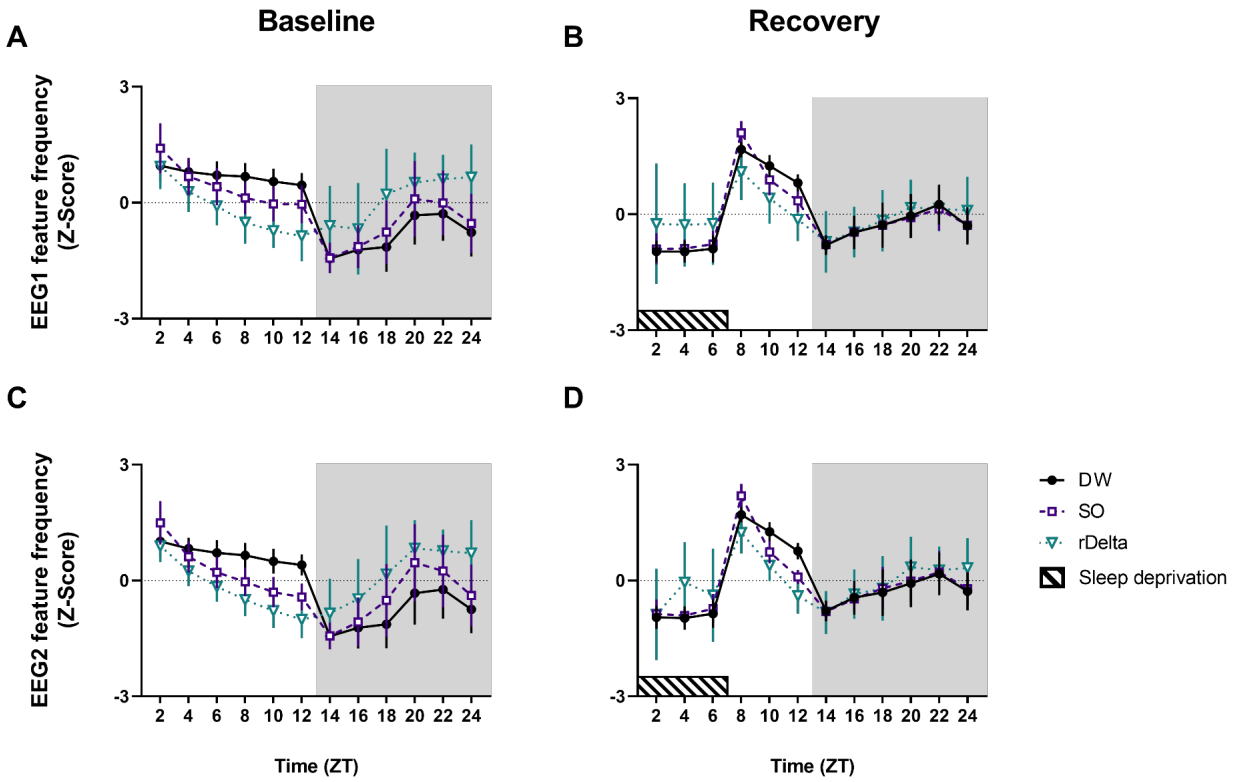


Figure 5. Unequal influence of time on NREM delta features. Polysomnography was used to record arousal states across baseline, sleep deprivation and recovery conditions of adult C57BL/6J mice. Z-Score was calculated for each feature of interest (delta waves, slow oscillations, and relative delta power) across each condition and electrode. A mixed-effects analysis with multiple comparisons and Benjamini and Hochberg correction was used to control false discovery rate. **(A-D)** There is a significant time-feature interaction for both conditions in both EEG1 and EEG2. Repeated measures analysis reveals numerous one-to-one differences across all features, both sleep conditions, and both electrodes. Most repeated measure discoveries were found during ZT 6-12 Data presented as mean ± SD. Shaded boxes label the dark phase. **See Table 3 and Supplementary Table 3 for statistics.**

Fixed effects (type III)	F(DFn, DFd)	P value	Summary
EEG1 - Baseline			
Time	***F* (7.389, 933.8) = 110.7**	***P* < 0.0001**	****
Waveform	*F* (2, 1390) = 2.255	*P* = 0.1053	ns
Time x Waveform	***F* (22, 1390) = 30.45**	***P* < 0.0001**	****
EEG1 - Recovery			
Time	***F* (6.572, 804.8) = 189.5**	***P* < 0.0001**	****
Waveform	*F* (2, 1347) = 1.020	*P* < 0.3609	ns
Time x Waveform	***F* (22, 1347) = 12.37**	***P* < 0.0001**	****
EEG2 - Baseline			
Time	***F* (6.494, 819.4) = 128.3**	***P* < 0.0001**	****
Waveform	*F* (2, 1388) = 2.393	*P* = 0.0917	ns
Time x Waveform	***F* (22, 1388) = 35.85**	***P* < 0.0001**	****
EEG2 - Recovery			
Time	***F* (6.978, 858.4) = 219.3**	***P* < 0.0001**	****
Waveform	*F* (2, 1353) = 0.4909	*P* = 0.6122	ns
Time x Waveform	***F* (22, 1353) = 15.73**	***P* < 0.0001**	****

Table 3. Unequal influence of time on NREM delta features. Mixed-effects analysis with multiple comparisons and Benjamini and Hochberg correction was used to control false discovery rate. **See supplementary Table 3 for detailed results of the repeated measures analysis**

Delta waveform relationships to behavioral markers of sleep pressure

While decades of research support the use of rD as a standard measurement of Process S in mammals, the relationships between DWs, SOs and purely behavioral measurements of sleep pressure (like those used in *Drosophila melanogaster*, NREM latency, NREM amount, and NREM consolidation) are unknown. To assess putative relationships, we began by identifying endpoints that have been robustly associated with sleep pressure and using simple linear regression to test for linear relationships among sleep features. Prior to hypothesis testing, a correlation matrix was generated **(Supplementary Figure 5)** for a high-level assessment of relationships between waveforms (DW, SO, rd) and behavioral endpoints (latency, NREM amount, etc.). Goodness of fit (r^2) and slope differences were calculated for each regression trio.

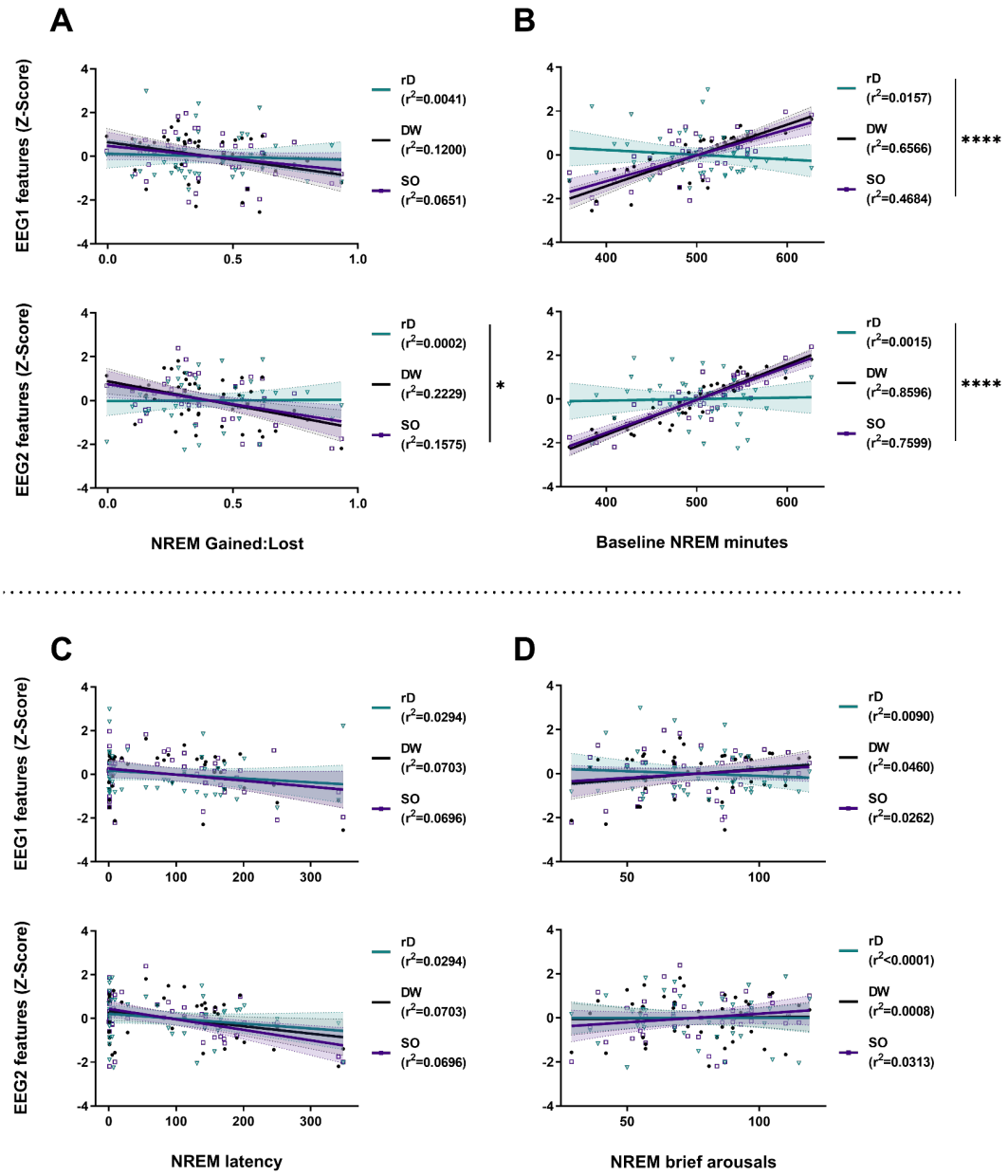


Figure 6. Baseline rD, DWs and SOs do not predict behavioral markers of sleep pressure. (A) Variance in EEG2 mean DWs and SOs accounted for ~22% and ~16% of the variance in NREM gained:lost, respectively. This small effect was likely driven by the collinearity of each SOs and DWs to NREM amount. **(B)** The mean number of DWs and SOs per animal was a strong predictor of total NREM minutes during baseline, as expected, serving as a useful positive control. **(C-D)** rD, DW, nor SO were good predictors of NREM latency or brief arousals. Significance bars reflect differences among features of interest. r^2 represents goodness of fit.

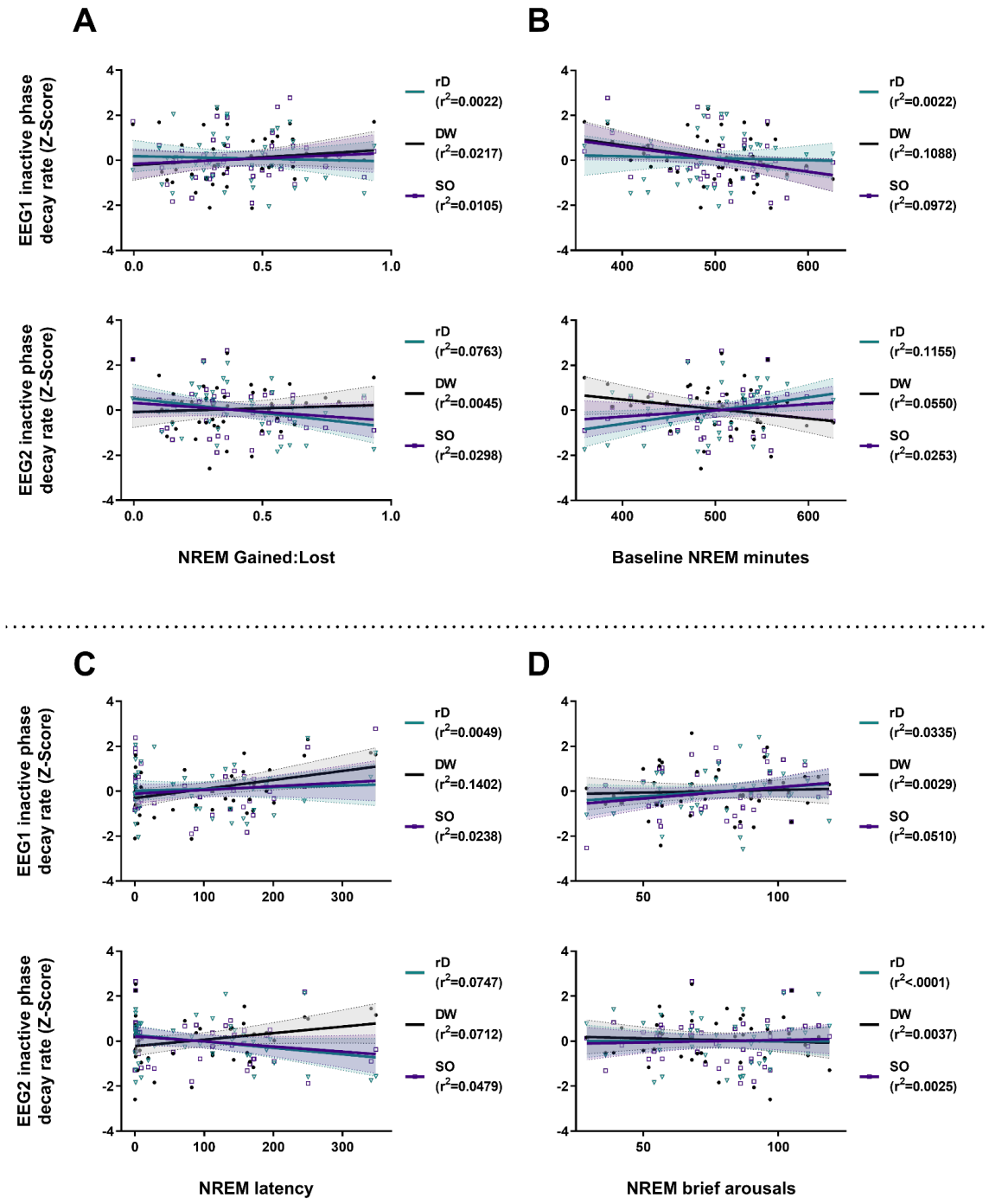


Figure 7. Inactive phase rD, DWs and SOs rates of decay do not predict behavioral markers of sleep pressure. The inactive phase rate of decay (slope) was calculated for each feature of interest for each animal using a simple linear regression (not pictured). **(A-D)** Variance in rD, DW and SO rate of decay did not meaningfully account for any of the variance observed in behavioral markers of sleep pressure.

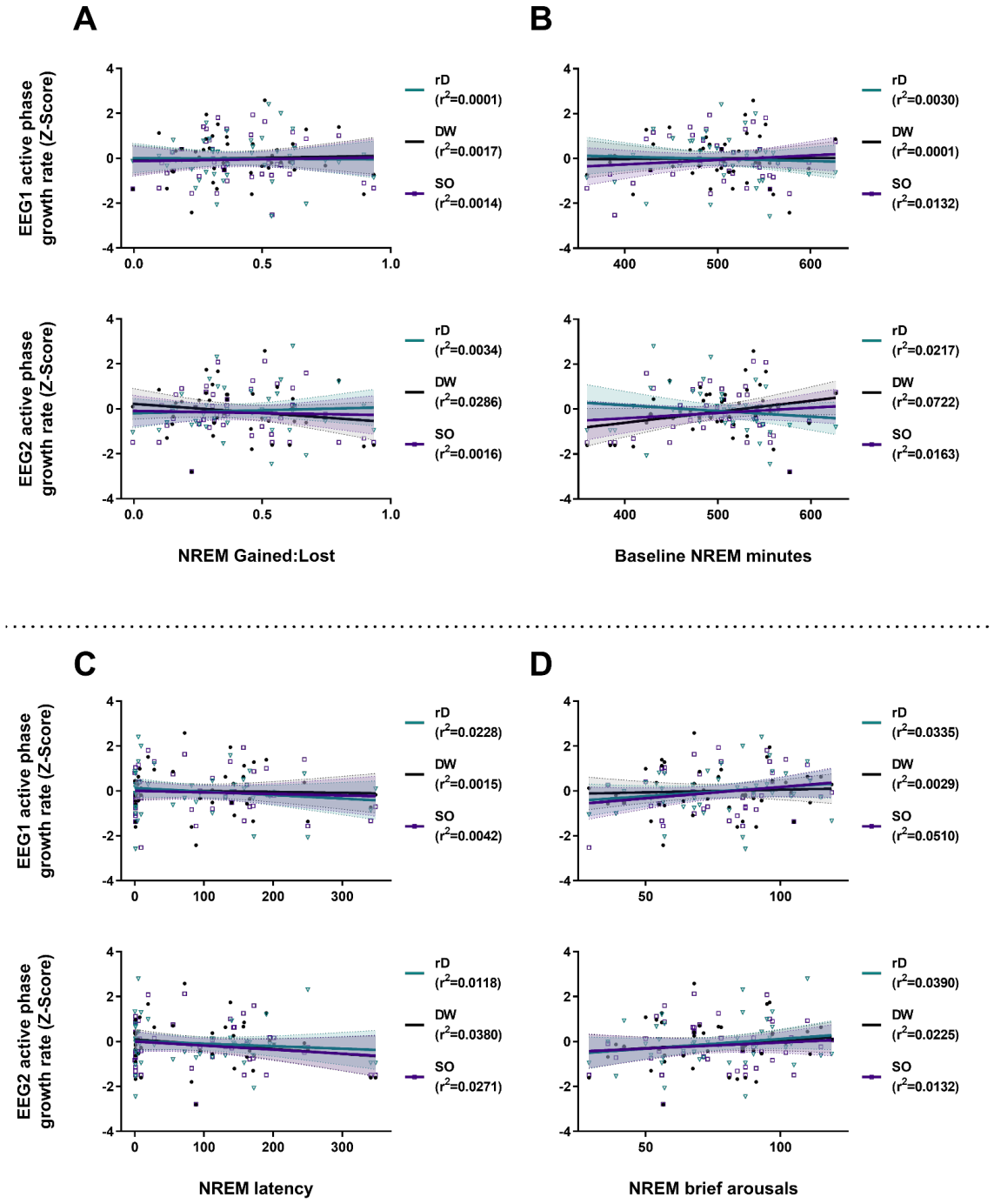


Figure 8. Active phase rD, DWs and SOs rates of growth do not predict behavioral markers of sleep pressure. The active phase rate of growth (slope) was calculated for each feature of interest for each animal using a simple linear regression (not pictured). **(A-D)** Variance in rD, DW and SO rate of growth did not meaningfully account for any of the variance observed in behavioral markers of sleep pressure.

To thoroughly assess potential relationships between features of interest and behavioral markers of sleep pressure, we performed three separate comparisons across data from both electrodes: (1) mean feature amount **(Figure 6)**, (2) inactive phase rate of decay **(Figure 7)** and (3) active phase rate of growth **(Figure 8)**. Rate of decay and rate of growth were evaluated because changes in delta power over time is thought to be a robust indirect reflection of Process S [16] Outside of expected (collinear) relationships among DW, SO and NREM amount **(Figure 6A-B)**, linear regression reveals no significant relationships among these low frequency features of interest and behavioral markers of sleep pressure in wildtype mice.

Discussion

There are numerous established markers of sleep pressure. Behavioral markers include latency to sleep onset, NREM sleep amount, and sleep fragmentation [6]. SWA (0.5-4.0Hz) during NREM sleep is the single electrophysiological (and most widely used) marker of Process S [16], although it has only been validated in models where EEG is available and not, for instance, in *Drosophila melanogaster*, which is becoming an increasingly useful model for assessing sleep and sleep-dependent behavior [6, 20-22]. There have been numerous reports over the last ~20 years, describing different categories of “delta events” and their properties. Until somewhat recently, heuristic properties largely guided the parallel investigation of these features across disciplines. For that reason, there is substantial inconsistency in what precisely constitutes a “slow wave activity”, for instance. Of course this is not absolutely true, as some groups have extensively characterized the properties of “types” of delta waves [14,23]. However, the majority of this work has been completed in humans and, while intra-event properties have been frequently studied, the daily temporal dynamics of these features, nor their response to acute sleep deprivation have not been assessed in mice.

In 2019, Kim et al. observed features they defined as “delta waves (DWs)” and “slow oscillations (SOs)” in the reactivation of the cortex of sleeping rats following the acquisition of a novel neuroprosthetic learning task [13] and demonstrated competing roles of DWs and SOs in task performance using a closed-loop optogenetics system. Inhibition of motor cortex reactivation when DWs were detected lead to increased SO-spindle nesting and improved task recall. Inhibition of motor cortex reactivation when SOs were detected lead to decreased SO-spindle nesting and diminished task recall to below control levels. Importantly, both DWs and SOs exist categorically as part of SWA. This and other studies establish activity-dependent processing in the rodent cortex during sleep and clarify the role of sleep behavior in learning and memory. There is also some evidence that activity-dependent cortical reactivation is homeostatically regulated [18,24] - that is, the synaptic potentiation which occurs in a region-specific manner during intense task learning appears to drive region-specific increases in SWA that decline to pre-training levels in the first 2 hours of sleep. While DWs and SOs during NREM sleep play an important role in sleep-dependent learning and forgetting, it is not clear whether these features, as defined by Kim et al. are under homeostatic or circadian control, in addition to being a function of waking activity.

Our findings demonstrate previously unreported distinct daily rhythms of NREM DWs and SOs during baseline and recovery conditions. DWs are significantly more abundant than SOs during periods of high sleep propensity **(Figure 3)** across undisturbed baseline conditions and following 6-hour of sleep deprivation. We report an unequal influence of time on the activity of these features of interest and their relationship to each other, with the ratio of delta-waves-to-slow oscillations (DW:SO) peaking during times of typically-low sleep pressure **(Figure 3 and Supplementary Figure 1)**, demonstrating unequal relationships between NREM sleep and our features of interest.

We also observe spatial differences in SOs but not DWs, with significantly more SOs observed in EEG2 (parietal-occipital) than in EEG1 (frontal-parietal) during baseline and recovery **(Figure 4)**. This is particularly interesting because previous reports discuss evidence for higher-amplitude slow waves (which share similarities with SOs described here) as more “global” features, broadly reflecting arousal state and more likely to be identified across the entire cortex when high spatial resolution imaging is used [13,23], while demonstrating that lower amplitude slow waves (which share similarities with DWs described here) may be generated locally and likely reflect region specific activity-dependent processing.

These temporal and spatial differences support the idea that these EEG waveforms may have distinct functions. To clarify the role of these features in NREM sleep, we compared their activity to that of a well understood feature of NREM sleep, relative delta (rD), in response to a homeostatic challenge **(Figure 5)**. During the baseline inactive phase, rD and SOs have very similar rates of decay, while the slope of DWs is significantly more linear. Similar trends are present following sleep deprivation, with DWs demonstrating a decidedly more-linear pattern of decay during the inactive phase. In stark contrast, during the baseline active phase, DW and SO behavior is highly similar, with rD the outlier. Interestingly, there are no meaningful differences across all three features during the active phase of recovery sleep **(Figure 5B, D)**, suggesting that differences in feature activity may be phase-dependent and not necessarily homeostatic.

A large body of evidence demonstrates that SWA and several behavioral phenotypes are useful markers of sleep pressure [6]. To clarify the role of DWs and SOs in sleep homeostasis, we implemented a simple linear regression model across numerous combinations of NREM phenotypes, always using well-defined behavioral endpoints as our response/dependent variable and spectral (delta) features as our explanatory/independent variables **(Figures 6-8)**. The only

significant linear relationships we observe are between DWs and NREM sleep amount and SOs and NREM sleep amount **(Figure 6)**. Given the nature of these features, this is an expected relationship due to variable collinearity - DWs and SOs are both intrinsic to NREM sleep. We modeled relationships among mean feature amounts across recordings **(Figure 6)**, the inactive phase rate of decay **(Figure 7)** and the active phase rate of growth **(Figure 8)** as we believed these three variables to best reflect dynamic characteristics of each feature. Overall, we found no meaningful linear relationships between DWs and behavioral markers of sleep pressure, nor any meaningful linear relationships between SOs and behavioral markers of sleep pressure. Variability in these universally-applicable behavioral assessments of sleep pressure cannot be explained/predicted by variability of static (total or mean amount) or dynamic (rate of decay, growth) features of DWs and SOs.

Interestingly, we did not observe an expected linear relationship between SWA (rD) and any behavioral marker of sleep pressure, despite stereotypical NREM sleep rhythms during baseline and recovery sleep **(Supplementary Figure 6)**. This is perhaps because of the dynamic causal interplay between NREM sleep and SWA: elevated SWA increases sleep propensity; increased sleep reconciles sleep pressure and is reflected in decreased SWA, etc. It is unlikely that DWs and SOs share similar, highly-dynamic relationships with NREM sleep and thus, with other behavioral features of Process S in mice, as our positive control demonstrates a highly linear relationship with NREM sleep **(Figure 6)**. This finding supports the idea that DWs and SOs (as they are described and investigated herein) do not reflect the homeostat and, as previously reported [13], likely reflect underlying activity-dependent processing within the cortex - a process which NREM sleep is demonstrably, intimately involved with, but which is not likely regulated by the same systems underlying Process S.

CH 2: HYDROGEN-RICH WATER INCREASES MARKERS OF SLEEP PRESSURE

Introduction

Poor sleep is a hallmark of modern society. Nearly 30% of American adults average ≤ 6 hours of nightly sleep and insomnia conservatively affects >10% of the global population. This widespread problem has severe and complex consequences for individual health. Acute sleep loss generates a proinflammatory response [25], increases physiological stress [26], impairs memory [27], decreases insulin sensitivity [28], and may accelerate the progression of chronic complex disease [29]. Chronic sleep loss is associated with significantly increased all-cause mortality [30] - that is, consistently inadequate sleep increases your likelihood of dying. There are global and local consequences of sleep loss in the brain and body [31,32] and disrupted sleep is a risk factor and consequence of many disorders [33,34]. In instances of comorbid mental health disorders and insomnia, improving sleep is sufficient to improve symptoms of the comorbid disorder [35]. Behavioral interventions for improving sleep can be effective but are often inadequate to resolve the environmental- or disorder-driven sleep disturbances many people face. If behavioral interventions fall short, pharmaceutical hypnotics may be prescribed because they are fast-acting and have a short half-life. However, these drugs are often accompanied by undesirable side-effects and long-term use can lead to drug dependence [35]. Other drugs commonly prescribed to resolve poor sleep are often done so in “off label” fashion and often impact biology and behavior far beyond sleep. The value of an intervention that improves sleep quality or reduces consequences of sleep loss without deleterious side effects cannot be easily overstated.

Over the last decade, molecular hydrogen (H_2) has gained attention as a promising therapeutic with a wide range of potential benefits, including the regulation of proinflammatory mediators [36] and the modulation of insulin sensitivity [37]. H_2 has no cytotoxicity even at high concentrations

and is widely accepted to have no deleterious side effects [38]. H_2 can be administered as a gas, suspended in saline, or suspended in water (hydrogen-rich water, HRW). While there is some evidence that oral ingestion of HRW is unlikely to increase levels of H_2 in the brain [39], high concentrations of HRW appear to increase H_2 centrally in rats [40], and have region-specific influence on brain metabolism in humans [41]. In 2012, administration of HRW demonstrated sufficiency in attenuating dopaminergic cell loss in a mouse model of Parkinson's Disease [42]. Recent work in humans demonstrates that HRW may increase alertness and cognitive function similarly to caffeine, but likely through a different mechanism [41]. In the face of pharmacological or chemical challenges, HRW appears to act as a neuroprotectant in the hippocampus [43]. While numerous reports have demonstrated HRW's sufficiency to alter sleep-related biological processes and behavior, the effect of HRW on sleep itself is unknown.

In this study, we tested the ability of 7 days of *ad libitum* access to hydrogen-rich water (HRW) at 0.7 -1.4 mM to alter baseline sleep-wake architecture and the response to the homeostatic challenge of acute sleep deprivation in wildtype C57BL/6J mice. We use polysomnography to assess several electrophysiological and behavioral markers of circadian activity and sleep pressure in freely moving mice. This randomized, within-subjects investigation of HRW's effect on sleep and wake behavior is the first of its kind in any species. Separately, we performed a between-subjects assessment of neuronal activity in known sleep- and wake-related brain regions following the HRW treatment regimen described above. This assessment of HRW's impact on neuronal activity of sleep regulatory systems is also a first for the field.

Materials and methods

Animal care

Adult C57BL/6J mice were maintained at the University of California Los Angeles (UCLA) under a 12h-12h light-dark cycle (LD) in a light- and temperature-controlled study area overseen by the university Animal Research Committee and Division of Laboratory Animal Medicine. Food and water were provided *ad libitum* except when otherwise described. Sleep deprivation was performed by experts blind to experimental conditions using gentle handling. Experiments were performed using the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.

Polysomnographic implantations

Adult PNW10 C57BL/6J mice (n=10; male = 6, female = 4) mice were implanted with EEG/EMG headmounts for polysomnographic (PSG) recording. As previously reported (Ehlen et al., 2017), mice were implanted with 4x EEG and 2x EMG electrodes under anesthesia. Two electrodes (frontal-parietal and ground) were located 1.5 mm anterior to bregma and 1.5 mm on either side of the central suture. Two additional electrodes (parietal-occipital and common reference) were located 2.5 mm posterior to bregma and 1.5 mm on either side of the central suture. Electrical continuity between the screw electrode and headmount was achieved with silver epoxy. EMG activity was monitored using stainless-steel Teflon coated wires inserted into the nuchal muscle. The headmount (2×3 pin grid array) was secured to the skull with dental acrylic. 7 days after surgery, mice were transferred to sound-proof chambers and connected to the data acquisition system. In the recording chambers, mice acclimated to a lightweight tether attached to a low-resistance commutator mounted above the cage for an additional 7 days before recording. Mice had free range of movement throughout all tethered experiments.

Hydrogen-rich water preparation

HRW was produced by adding magnesium-based tablets to 590ml of deionized water in polycarbonate bottles. Elemental magnesium reacts with water to produce hydrogen by the following reaction: $Mg + H_2O \rightarrow H_2 + Mg(OH)_2$. Bottles were sealed and left overnight at 4°C for next-day administration. H_2 levels of ~2ppm (10 μL/g; 1.0 mM) and 2-hour half-life in glass administration bottles were confirmed by H2Blue titration assay (H2 Sciences; Henderson, NV). Tablets were provided by HRW Natural Health Products (New Westminster, BC, Canada).

Hydrogen-rich water administration

Mice were pseudo-randomly assigned to one of two groups to account for unintended order effects. Group 1 had *ad libitum* access to standard deionized water for 7 days, immediately followed by a 24-hour polysomnographic recording, and subsequent 6 hours of sleep deprivation by gentle handling and 18 hours of recovery sleep. Starting the following day, mice had *ad libitum* access to HRW for 7 days. The half-life of the gas was determined to be approximately two hours, and so the water was replaced every two during their active phase to maintain HRW concentrations of 1.0-2.0ppm throughout the active phase of each day. Immediately following the 7th day of HRW treatment, another 24-hour recording was collected, followed by another 6 hours of sleep deprivation and 18 hours of recovery. Group 2 underwent the opposite schedule to account for any potential order-effects of the treatment condition. While receiving standard deionized water for 7 days, cages of both groups were gently disturbed every two hours during the active phase to control for any unintended effects of repeated bottle changes by the experimenter.

Polysomnographic data acquisition and processing

48-hour continuous PSG recordings consisting of 24-hour baseline, 6-hour sleep deprivation, and 18-hour recovery began at light onset, zeitgeber time (ZT) 0. Data acquisition was performed on a PC running polysomnographic software (Sirenia Acquisition, Pinnacle Technologies, Lawrence, KS). Signals were amplified and high-pass filtered (0.5 Hz) via a preamplifier. EEG signals were low-pass filtered with a 40 Hz cutoff and collected continuously at a sampling rate of 400 Hz. After collection, EEG and EMG waveforms were classified in 10-sec epochs as: 1) wake (low-voltage, high-frequency EEG; high amplitude EMG); 2) NREM sleep (high-voltage, mixed-frequency EEG; low-amplitude EMG); or rapid-eye movement (REM) sleep (low-voltage EEG with a predominance of theta activity (6–10Hz); very low amplitude EMG). All sleep scoring was performed by expert technicians blind to the conditions of the experimental condition. EEG epochs determined to have artifacts (interference caused by scratching, movement, eating, or drinking) were excluded from analysis. Artifacts comprised less than five percent of all records used for analysis. Automatic extraction of sleep features was completed using a proprietary MATLAB script.

Immunohistochemistry

Adult PNW12 C57BL/6J mice (n=33; male=16, female=17) were pseudo-randomly assigned to one of the following groups: (1) undisturbed mice with *ad libitum* access to standard deionized water, CON; (2) sleep deprived mice with *ad libitum* access to standard deionized water, CON+SD; (3) undisturbed mice with *ad libitum* access to HRW, HRW; and (4) sleep deprived mice with *ad libitum* access to HRW, HRW+SD. HRW and HRW+SD mice received 7 days of *ad libitum* access to HRW as described above. CON and CON+SD mice had water bottles disturbed as described above. On day 7 of treatment, CON+UN and HRW+UN groups were allowed to sleep for 6 hours from ZT 0-6, while SD-W and SD-HRW groups were sleep deprived for 6 hours

by gentle handling. All groups were perfused at ZT6 (mean = ZT 6.61 ± 0.2). The difference in mean perfusion time between any two groups was no more than 7 minutes. Mice were euthanized with Euthasol (150 mg/kg), perfused with 10 ml of 1x PBS, and then 10 ml of 4% paraformaldehyde (PFA). Brains were dissected out and post-fixed with 4% PFA at 4°C overnight, then transferred into a solution of 15% sucrose in 1xPBS. Fixed brains are preserved in 15% sucrose in 1xPBS + 0.1% Na azide until ready to cut.

Frozen coronal 50 µm sections were collected into 24-well plates containing 1:1 ice-cold glycerol:PBS and stored at -20ºC in a normal freezer. All tissue was collected by a single person, and equal representation of experimental conditions were represented during any single cryostat session to ensure that tissue collection conditions did not disproportionately influence any group(s). Tissue sections were mounted and stained for cFos protein immunoreactivity (Fos-IR). Regions of interest (ROIs) were defined in ImageJ (National Institutes of Health; LOCI, University of Wisconsin) using the Allen Institute's Mouse Brain Atlas (University of Washington) for reference. 2-4 samples of each ROI were collected for each animal and condition, and Fos-positive cells for each sample were counted in ImageJ by two experts blind to the conditions. Data presented for each observation is the mean of the two experts.

Results

HRW decreases sleep latency in undisturbed mice

Latency to sleep onset is a robust biomarker of sleep quality used across species [15,16,21]. It is used as both a primary indicator of Process S in species where EEG is unavailable, and as part of the diagnostic criteria for sleep disorders like insomnia [44]. Wake duration is inversely correlated with sleep latency and several factors, including age [45], presence or absence of chronic pain [46], alcohol use [47], and exercise [48] can influence sleep latency. Using latency as an important behavioral marker of sleep pressure, we tested our hypothesis that HRW treatment would be sufficient to Process S in mice. During baseline, latency to sleep onset is defined as the amount of time it takes for an animal to accumulate at least one bout (20 seconds or more) of NREM sleep after house lights turn on at ZT 0. Latency to sleep onset during recovery is the amount of time it takes an animal to accumulate at least one bout of NREM sleep following the termination of sleep deprivation at ZT 6. Following 7 days of *ad libitum* access to HRW throughout their active phase, paired t-test reveals a statistically significant difference in latency to sleep onset (P=0.0313, t=2.549, df=9) during the undisturbed, baseline condition, with HRW treatment reducing sleep onset by >50% **(Figure 9A)**. Following 6 hours of acute sleep deprivation by gentle handling, we observe a ceiling effect in latency as a marker for sleep pressure (i.e. mice can't fall asleep faster than "immediately") and thus no effect of HRW during recovery **(Figure 9B)**.

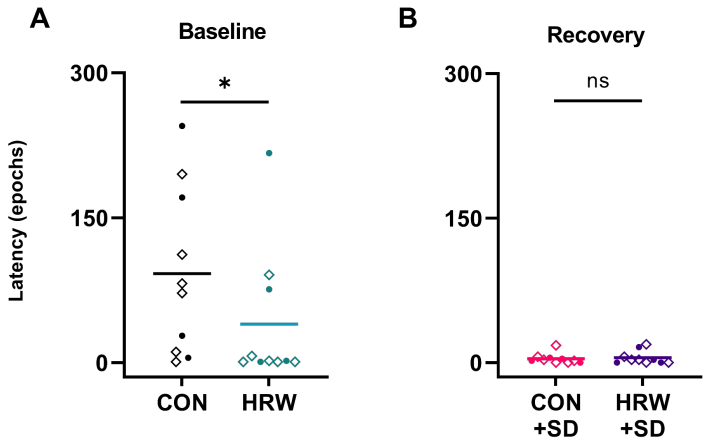


Figure 9. HRW treatment is associated with decreased sleep latency. (A) Paired t-test revealed a significant effect (*P*=0.0313, *t*=2.549, *df*=9) of HRW treatment on sleep latency in undisturbed mice. **(B)** Following sleep deprivation no effect of HRW was observed (*P*=0.5765, *t*=0.5795, *df*=9). Baseline sleep latency is the time it takes an animal to accumulate a bout of NREM sleep following the beginning of the inactive phase (lights on, ZT 0). Recovery sleep latency is the time it takes to accumulate a bout of NREM sleep following the end of sleep deprivation at ZT 6. A NREM bout is two or more adjacent 10-second NREM epochs. Horizontal bars represent mean. Solid circles are female. Diamonds are male..

HRW treatment does not alter normal sleep rhythms

Mice have polyphasic sleep - while they accumulate most of their sleep during the inactive (light) phase, they still achieve significant sleep in cycles through their active (dark phase). Several reports have demonstrated that certain strains of mice, including the C57BL/6J reported here, also have a period of increased sleep propensity during their mid-active phase (typically ZT18-22), known as the “siesta” [17]. The sleep architecture of undisturbed C57BL/6J and their response to acute total sleep deprivation (increased NREM sleep, for instance) are well documented and highly stable [16,49,50]. During HRW treatment, two-way ANOVA reveals no effect of HRW on the robust typical distribution of NREM or REM throughout baseline recording, during sleep deprivation or during the 18-hour recovery period **(Figure 10A-D, Table 4).**

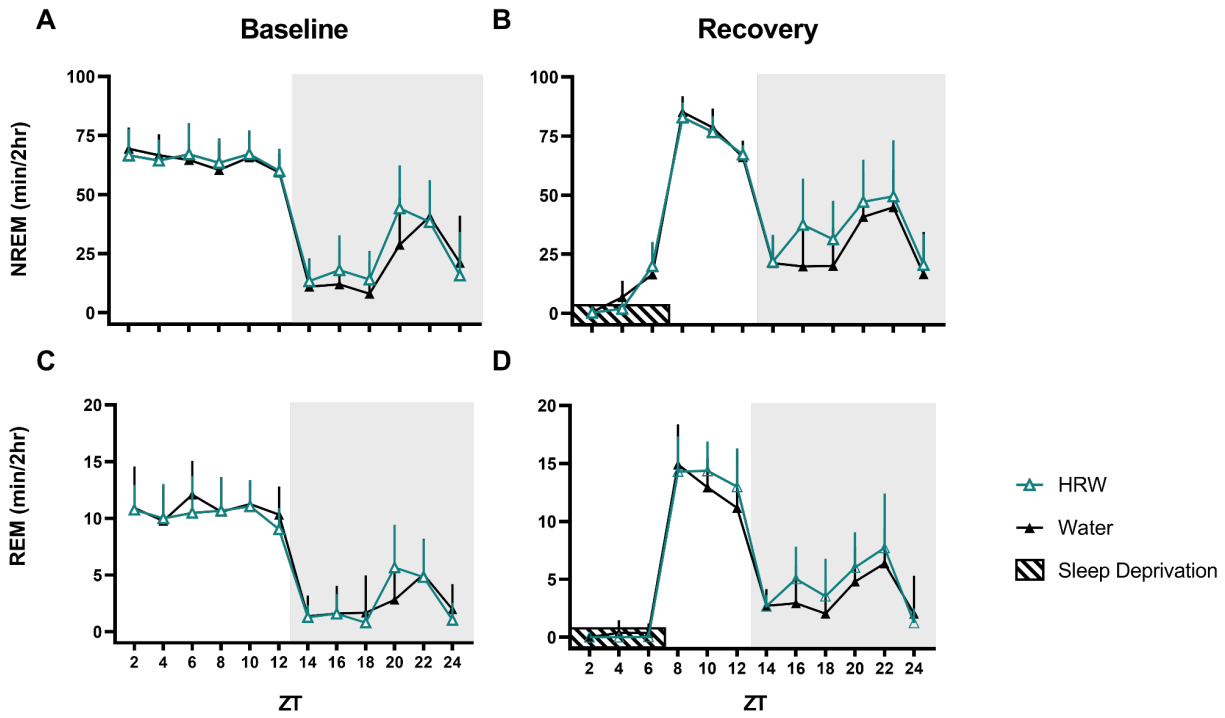


Figure 10. No effect of HRW on organization of sleep behavior. (A-D) Within-subjects comparisons by two-way ANOVA reveal no significant effect of HRW on the distribution of NREM or REM sleep during 24-hour baseline or 18-hour recovery conditions. Icons represent the mean and error bars are standard deviation. Shaded boxes label the dark phase. **See Table 4 for statistics.**

HRW increases sleep consolidation in undisturbed mice

Frequent awakenings and chronically poor sleep consolidation are hallmarks of some neurodegenerative diseases [51,52] and sleep apnea [53]. Previous reports demonstrate that significant sleep fragmentation challenges can impact Process S and sleep-dependent physiological processes without significantly changing daily rhythms [54,55]. To assess changes in fragmentation, we evaluated (1) the number of brief arousals (periods of wake lasting <10 seconds, interrupting NREM), (2) the number of NREM bouts, and (3) the duration of NREM bouts. Paired t-test reveals a significant effect of treatment condition on brief arousals in

undisturbed mice **(Figure 11A)**, with mice experiencing a ~30% reduction in brief arousals following 7 days of *ad libitum* HRW treatment.

Source of Variation	SS	F(DFn, DFd)	P value	Summary
Baseline NREM				
Time	125152	***F* (5.140, 92.52) = 73.25**	***P* < 0.0001**	****
Treatment	251.5	*F* (1, 18) = 0.9161	*P* = 0.3512	ns
Time x Treatment	1652	*F* (11, 198) = 0.9666	*P* = 0.4782	ns
Baseline REM				
Time	6287	***F* (5.078, 91.41) = 86.55**	***P* < 0.0001**	****
Treatment	22.41	*F* (1, 18) = 2.970	*P* = 0.1019	ns
Time x Treatment	60.53	*F* (11, 198) = 0.8333	*P* = 0.6069	ns
Recovery NREM				
Time	168826	***F* (3.997, 71.94) = 96.70**	***P* < 0.0001**	****
Treatment	658.4	*F* (1, 18) = 2.826	*P* = 0.1100	ns
Time x Treatment	2140	*F* (11, 198) = 1.225	*P* = 0.2720	ns
Recovery REM				
Time	4266	***F* (5.508, 462.6) = 334.5**	***P* < 0.0001**	****
Treatment	2.017	*F* (1, 84) = 36.81	*P* = 0.7597	ns
Time x Treatment	68.77	*F* (11, 924) = 7.507	*P* = 0.3353	ns

Table 4. No effect of HRW on organization of sleep behavior. Two-way ANOVA with time and treatment (HRW and control)as factors for NREM and REM sleep during baseline and recovery. The effect of time is a positive control.

We also report a non-significant increase in the duration of NREM bouts **(Figure 11C)**, and a reduction in the number of NREM bouts **(Figure 11B)** of HRW-treated baseline mice. Together, these results suggest that HRW treatment may be meaningfully associated with improved NREM sleep consolidation in undisturbed mice. There is no significant effect of HRW on measures of sleep fragmentation during recovery sleep. Importantly, we do observe a significant, expected effect of sleep deprivation on NREM bouts, with sleep deprived mice experiencing fewer NREM bouts (P<0.0001, t=5.968, df=19) of greater average length (P<0.0443, t=2.154, df=19). This previously known effect of sleep deprivation serves as an important positive control.

HRW increases total NREM and REM sleep amount following sleep deprivation

In adult humans, total sleep has a significant impact on all-cause mortality [30] and is an important clinical endpoint for sleep disorders [44] and several complex chronic conditions [51,52]. Recommendations from the American Academy of Sleep Medicine reflect decades of sleep research and state that fewer than 7 hours of sleep per night is inadequate to sustain health. In models where electrophysiology is not available, behavioral endpoints (quiescence, recumbent posture, increased arousal threshold, etc.) frequently rely on total sleep amount as a primary endpoint to assess sleep quality. In our polysomnographic investigation of mouse sleep, we find that HRW treatment was associated with an increase in NREM (P=0.0003, t=5.767, df=9) and REM (P=0.0045, t=3.757, df=9) sleep amount **(Figure 12B,D)**, despite equivalent sleep deprivation interventions **(Figure 12E)**. While there are no significant differences in the often-used NREM gained-to-lost ratio **(Figure 12F)**, this may be due to the endpoint's insensitivity to phase-specific effects **(Figure 12B,D)**. We observe no significant effect of HRW on REM or NREM sleep amount during baseline conditions; however, a slight increase in NREM sleep during the baseline active phase is near significance (P=0.0524, t=2.233, df=9).

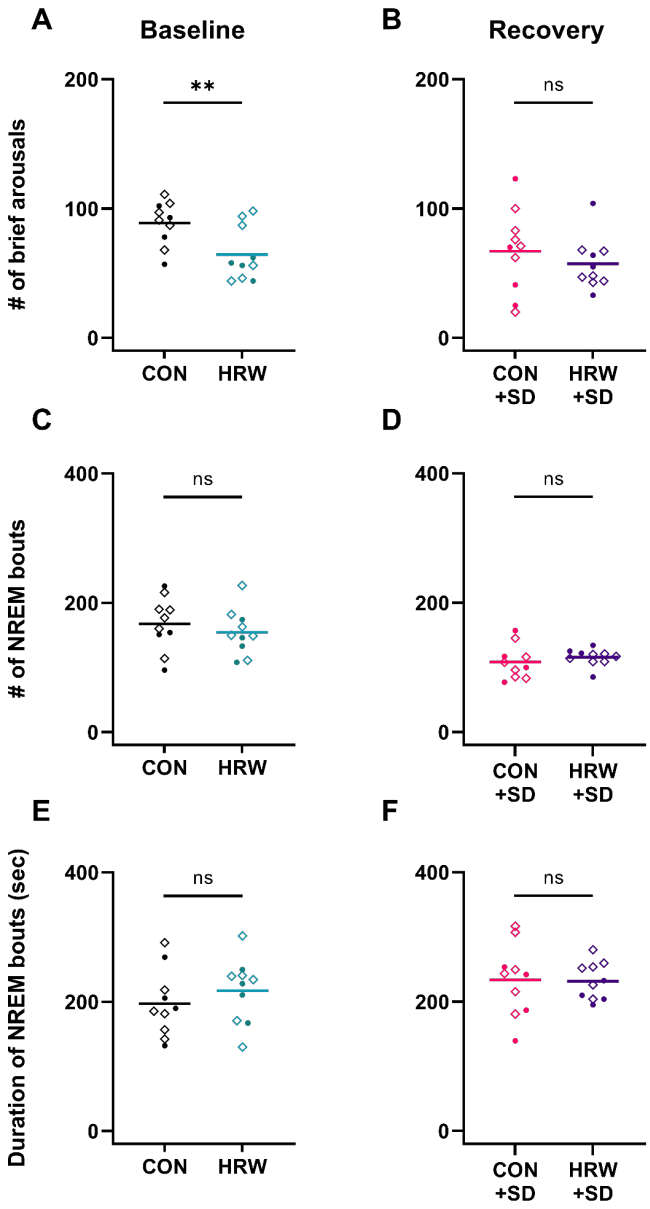


Figure 11. HRW treatment is associated with a significant reduction in brief arousals. (A-B) Paired t-test reveals a significant effect of HRW treatment on brief arousals during 24-hours baseline recording (P=0.0045, t=3.758, df=9) but not in the 18-hour recovery period following sleep deprivation. (C-F) Despite differences in brief arousals, paired t-test reveals no significant differences in the number of bouts each animal experiences or the duration of those bouts across treatment conditions. A brief arousal is counted each time a single 10-second epoch of wake interrupts a bout of NREM sleep. A bout is two or more adjacent 10-second epochs of the same arousal state (NREM, REM, wake). Horizontal bars represent mean. Solid circles are female. Diamonds are male.

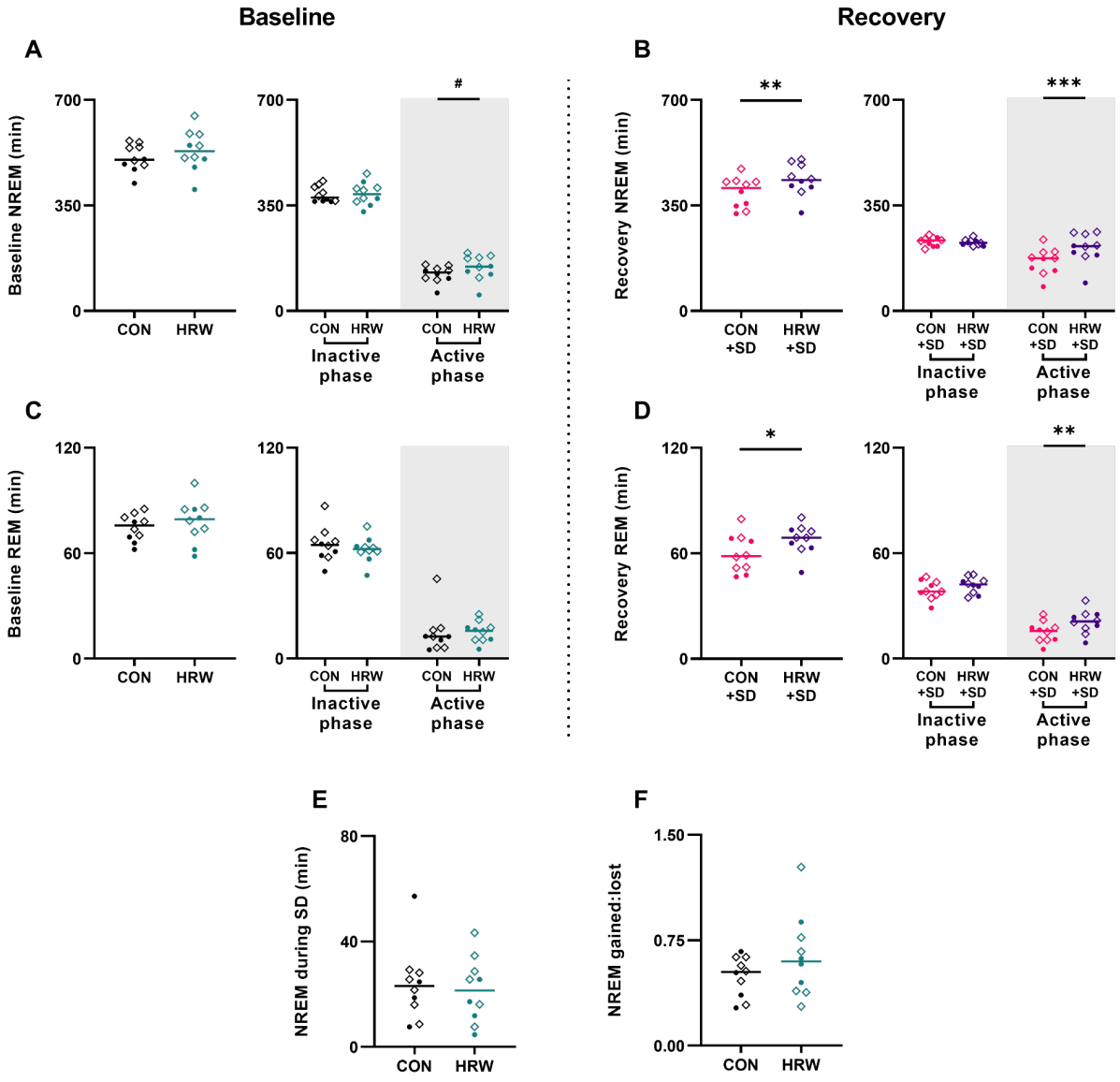


Figure 12. HRW significantly increases NREM and REM sleep following sleep deprivation. (A,C) There is no effect of HRW on total NREM or REM amount in baseline recordings, though NREM active phase differences between treatment groups approaches significance (#; *P*=0.0524). (B,D) Following 6 hours of sleep deprivation, HRW-treated mice accumulated more NREM and REM sleep - this effect is phase-specific. (E) Sleep deprivation was approximately the same across both conditions. (F) Despite differences in recovery sleep and equivalent sleep deprivations, we observe no significant differences in NREM gained-to-lost ratio. Horizontal bars represent mean. Solid circles are female. Diamonds are male. Shaded boxes reflect the dark (active) phase.

HRW treatment does not alter NREM delta features

Slow waves are low frequency (delta band, 0.5-40.Hz), high amplitude oscillations that dominate the deepest stages of NREM sleep, whose changes in power are believed to reflect changes in sleep pressure. Measured in our study using EEG and presented as a fraction of total power, slow wave activity (SWA) is our best measure of homeostatic dynamics in humans and animal models where EEG is currently available. Following sleep deprivation, mice demonstrate an expected rebound in NREM relative delta power, but mixed-effects analysis and multiple comparisons with Benjamini and Hochberg correction reveals no significant differences across treatment conditions **(Figure 13, Table 5)**. We also report no effect of HRW treatment on DWs or SOs **(Figure 14)**.

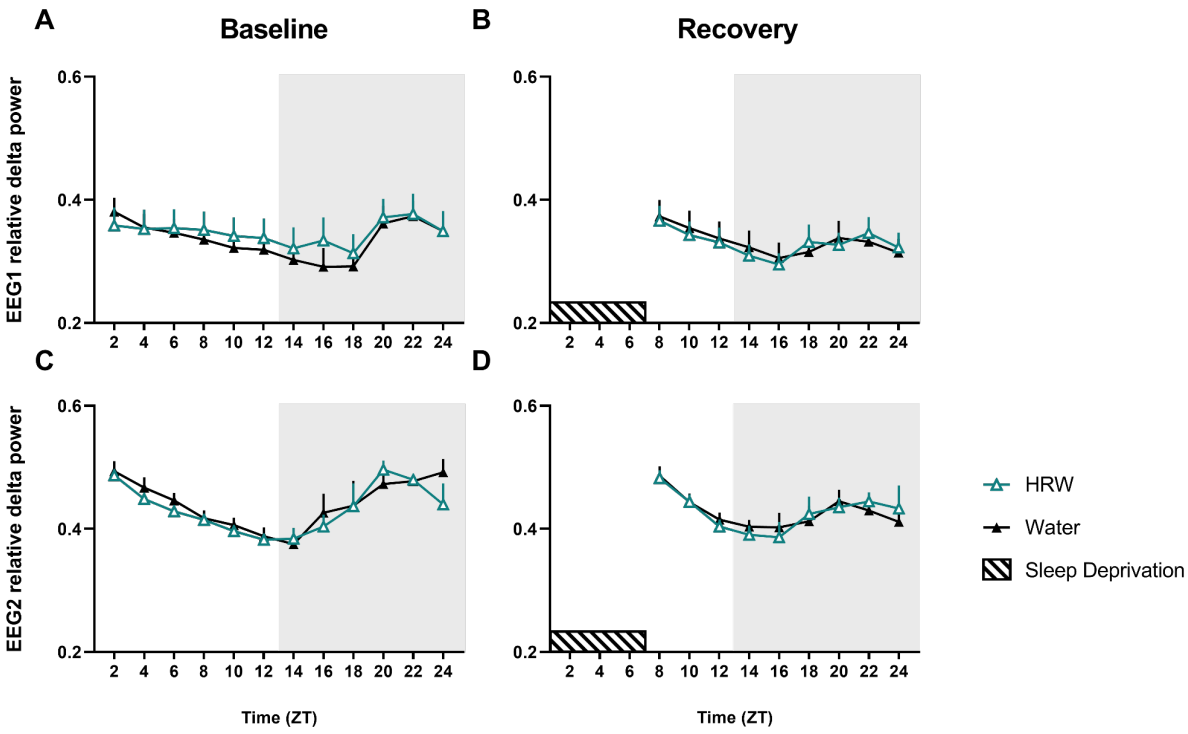


Figure 13. HRW treatment does not alter slow wave activity during baseline or recovery sleep. 24-hour distribution of SWA presented as relative delta power (rD). Icons represent the mean relative delta power for each animal during a 2-hour window, and error bars are standard error (SEM). Shaded boxes label the dark phase.

Fixed effects (type III)	F(DFn, DFd)	P value	Summary
EEG1 - Baseline			
Time	***F* (2.591, 44.29) = 7.513**	***P* = 0.0006**	****
Treatment	*F* (1, 19) = 0.2014	*P* = 0.6587	ns
Time x Treatment	*F (11, 188) = 1.348*	*P* = 0.2008	ns
EEG1 - Recovery			
Time	***F* (3.179, 54.85) = 9.492**	***P* < 0.0001**	****
Treatment	*F* (1, 18) = 0.002776	*P* = 0.9586	ns
Time x Treatment	*F* (8, 138) = 0.8755	*P* = 0.5389	ns
EEG2 - Baseline			
Time	***F* (3.316, 53.96) = 16.91**	***P* < 0.0001**	****
Treatment	*F* (1, 18) = 0.4276	*P* = 0.5214	ns
Time x Treatment	*F* (11, 179) = 0.9781	*P* = 0.4680	ns
EEG2 - Recovery			
Time	***F* (1.291, 22.28) = 9.534**	***P* = 0.0031**	****
Treatment	*F* (1, 18) = 0.0006621	*P* = 0.9798	ns
Time x Treatment	*F* (8, 138) = 0.5450	*P* = 0.8208	ns

Table 5. HRW treatment does not alter slow wave activity during baseline or recovery sleep. Mixed-effects analysis with multiple comparisons and Benjamini and Hochberg correction was used to control false discovery rate.

The effect of HRW on cellular activity in known sleep and arousal nuclei

HRW may have region-specific influence on central processes [43,56] in rodent models, and recent work in humans shows that high-dose HRW can induce regional changes in brain metabolism [41]. It is unclear whether HRW influences the brain by meaningfully increasing H_2 in the brain or through some second messenger [57]. We assessed cFos levels as a proxy for neuronal activity in several known sleep regulatory forebrain structures across 4 conditions: undisturbed, untreated mice (CON); sleep deprived, untreated mice (CON+SD) undisturbed, HRW-treated mice (HRW); and sleep deprived, HRW-treated mice (HRW+SD).

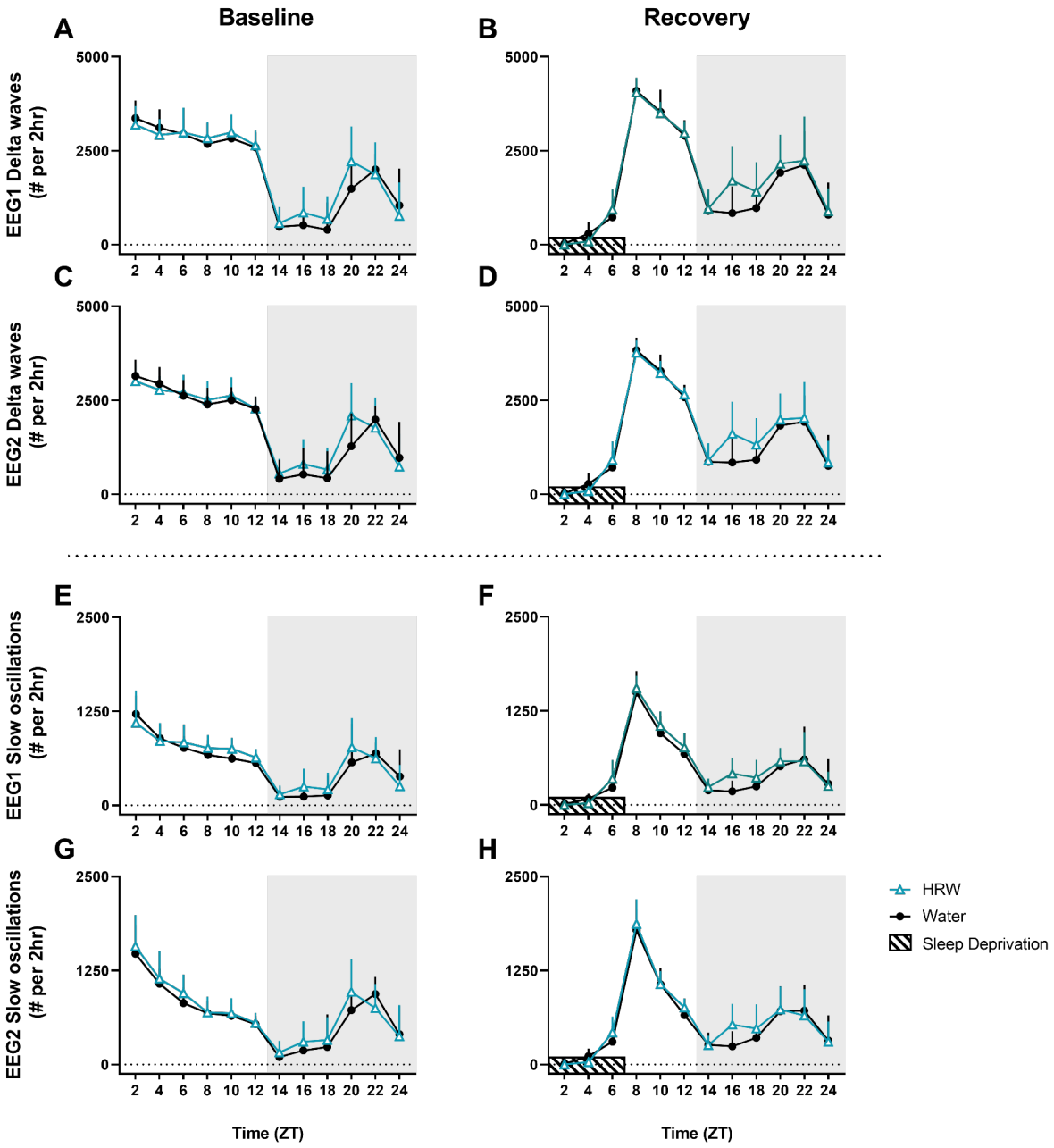


Figure 14. HRW does not meaningfully alter slow oscillations or delta waves. (A-D) Two-way ANOVA reveals no significant effect of HRW on delta waves recorded with EEG1 or EEG2 during baseline or recovery. (E-F) Two-way ANOVA reveals no significant effect of HRW on slow oscillations recorded with EEG1 or EEG2 during baseline or recovery. Icons represent the mean and error bars are standard deviation. Shaded boxes label the dark phase.

We observe a significant effect of HRW treatment in the lateral septum **(LS, Figure 16)**, medial septum **(MS, Figure 16)**, the ventrolateral preoptic area **(VLPO, Figure 17)**, and the median preoptic area **(MnPO, Figure 18)**. There was no effect of HRW on cFos immunoreactivity of forebrain diagonal band neurons **(DB, Figure 15)**. We also observe a significant effect of 6 hours of acute sleep deprivation on the MS, LS, VLPO and MnPO. Results in the MS and LS are consistent with previous reports [69], but the effect of sleep deprivation on VLPO and MnPO on cFos immunoreactivity in mice does not appear to have been previously reported in mice, despite numerous reports of sleep-active neurons in these areas. Finally, two-way ANOVA reveals a significant interaction of treatment and sleep conditions in the MS, LS, VLPO and MnPO; and a significant 3-way interaction of treatment, sleep, and sex in the LS **(Table 6)**.

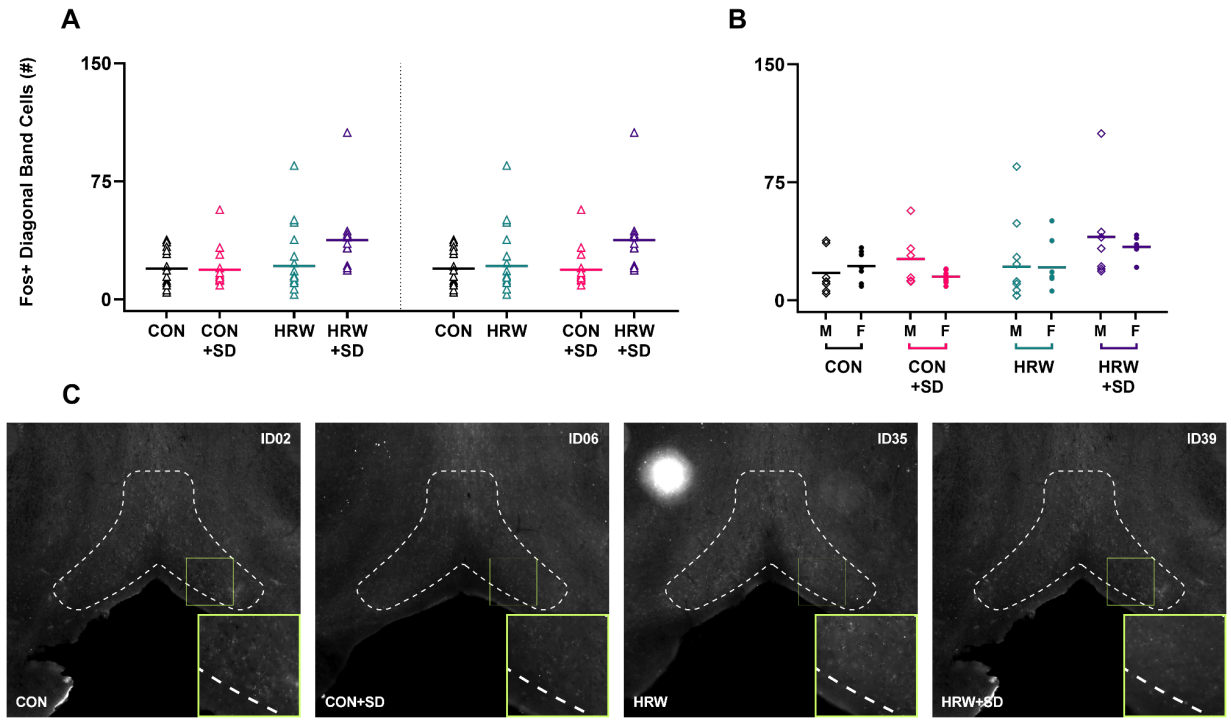


Figure 15. No effect of HRW in the diagonal band highlights regional specificity of treatment. Total cFos+ cells in the diagonal band. 3-way ANOVA reveals no significant direct effect of sex, treatment condition, nor sleep condition; nor any interaction among covariates. **(B)** Full distribution of experimental groups by sex. **(C)** Representative coronal sections of diagonal band, boundaries outlined in dotted white lines. Horizontal bars represent mean. Solid circles are female. Diamonds are male. Triangles are mixed. **See Table 6 for statistics.**

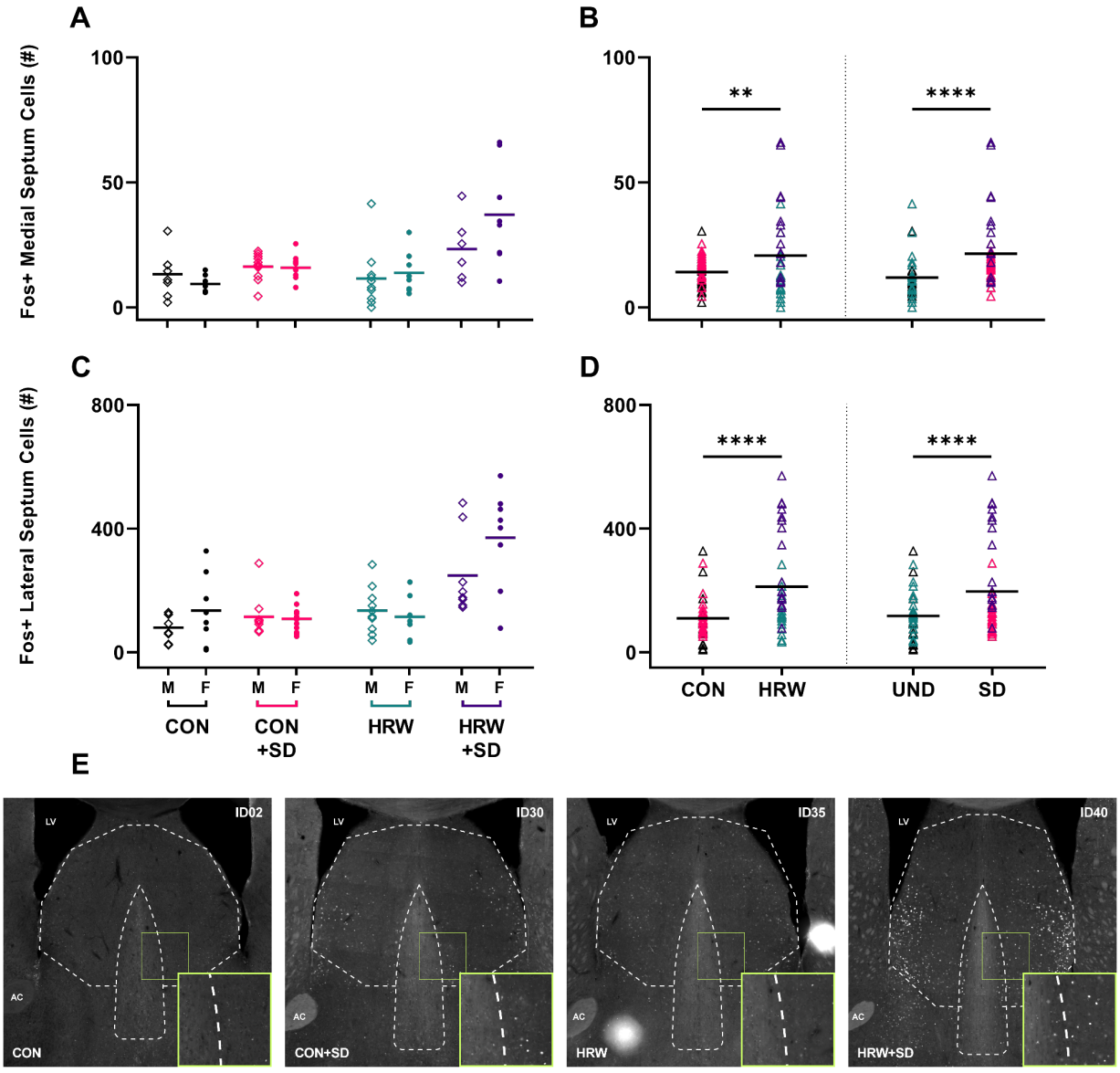


Figure 16. HRW-treated mice have significantly increased cFos activity in septal nuclei. 3-way ANOVA reveals a significant direct effect of HRW treatment and sleep deprivation, as well as a significant interaction of sleep condition and treatment group in both the medial septum and the lateral septum. **(A, C)** Full distribution of all groups. **(B, D)** Primary effect of treatment (left) and sleep deprivation with mixed groups. There is a significant interaction of sex and treatment condition in the medial septum but not the lateral septum, as well as a significant 3-way interaction in the lateral septum, but not the medial septum. No direct of sex is observed in either region. (E) Representative coronal sections of septal nuclei, boundaries outlined in dotted white lines. LV = lateral ventricle. Horizontal bars represent mean. Solid circles are female. Diamonds are male. Triangles are mixed. **See Table 6 for statistics.**

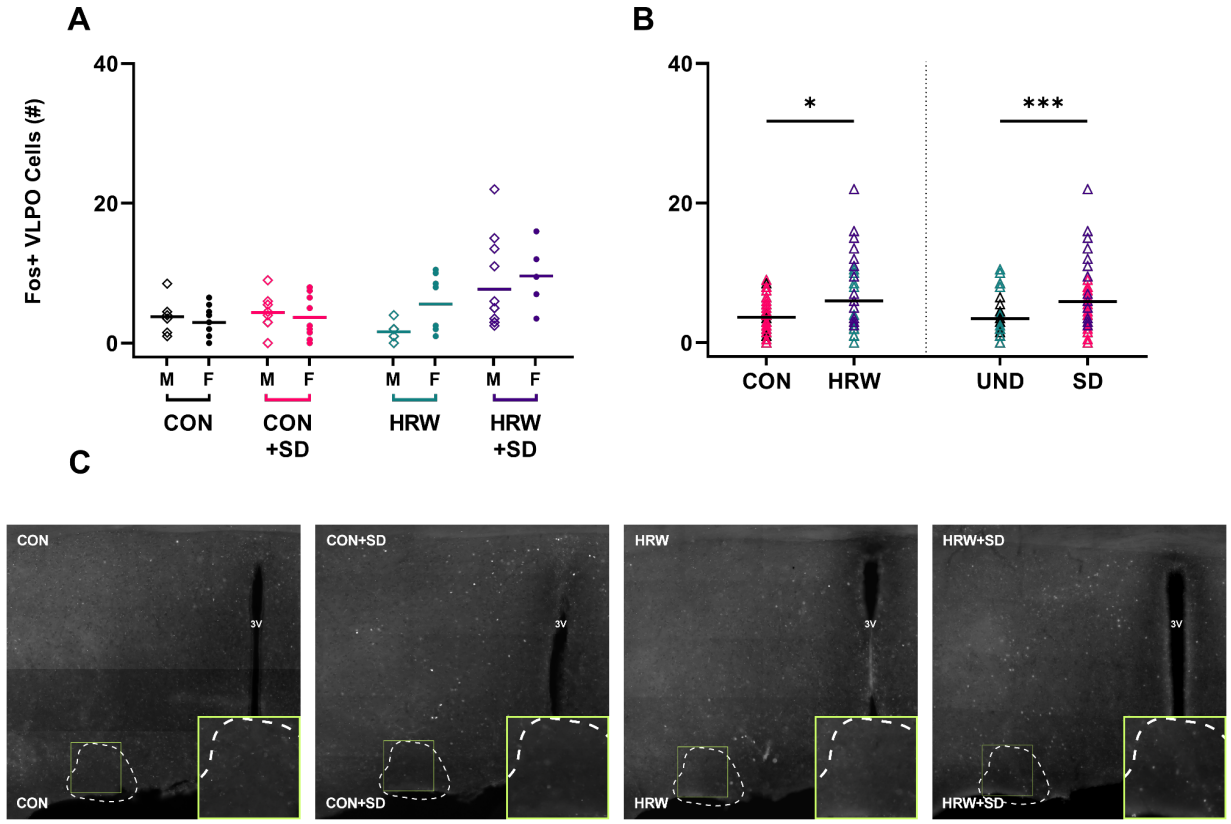


Figure 17. Significant effect of HRW in known sleep-related ventrolateral preoptic area. (A-B) 3-way ANOVA reveals significant direct effects of HRW treatment and sleep deprivation, as well as a significant interaction of sleep condition and treatment group in the VLPO. We observe no effect of sex. We find no evidence of a 3-way interaction. (C) Representative coronal sections of VLPO, boundaries outlined in dotted white lines. 3V = 3rd ventricle. Horizontal bars represent mean. Solid circles are female. Diamonds are male. Triangles are mixed. **See Table 6 for statistics.**

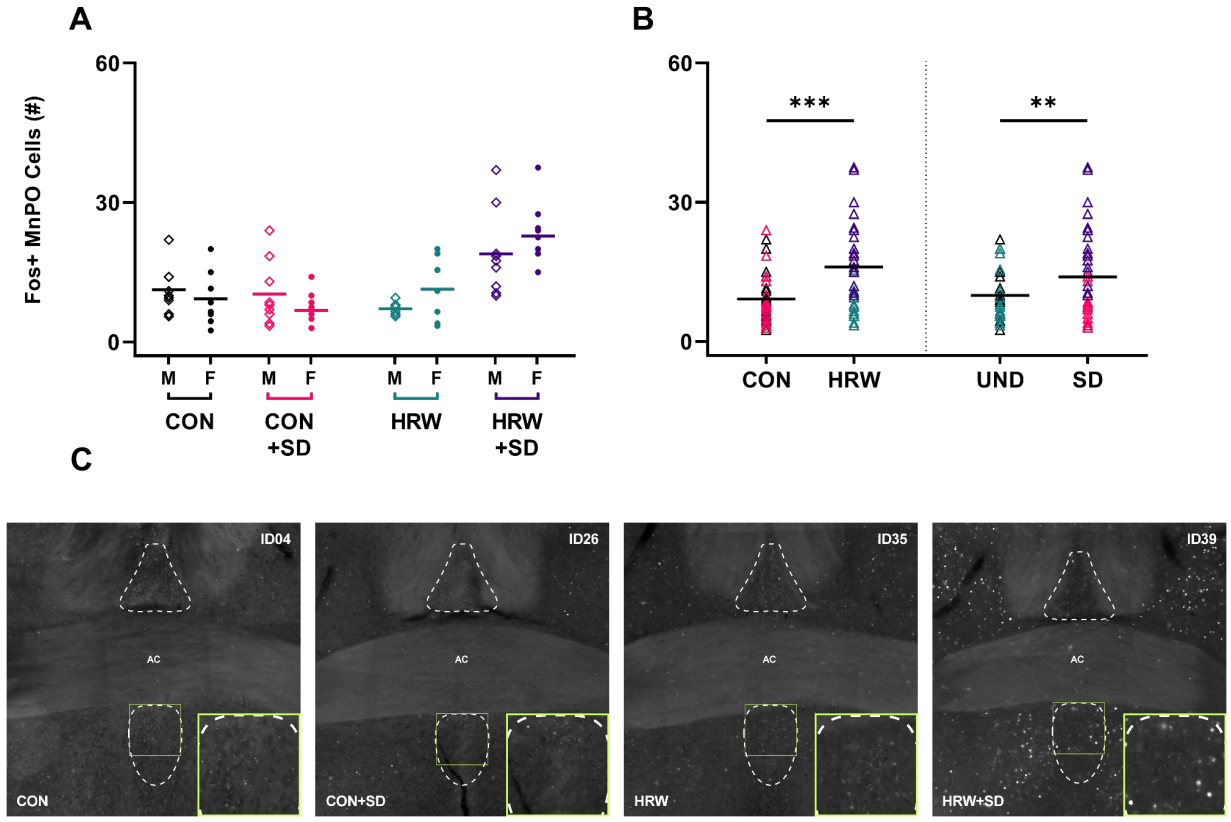


Figure 18. Significant effect of HRW in known sleep-related median preoptic area. (A-B) 3-way ANOVA reveals significant direct effects of HRW treatment and sleep deprivation, as well as a significant interaction of sleep condition and treatment group. We observe no direct effect of sex, but a significant interaction of sex and treatment group. We find no evidence of a 3-way interaction. (C) Representative coronal sections of the MnPO, boundaries outlined in dotted white lines. AC = anterior commissure. Horizontal bars represent mean. Solid circles are female. Diamonds are male. Triangles are mixed. **See Table 6 for statistics.**

Source of Variation	SS	F(DFn, DFd)	P value	Summary
Diagonal Band (DB)				
Sex	162.7	*F* (1, 53) = 0.4967	*P* = 0.4840	ns
Treatment	1175	*F* (1, 53) = 3.5900	*P* = 0.0636	ns
Sleep	1032	*F* (1, 53) = 3.1530	*P* = 0.0815	ns
Sex x Treatment	0.0745	*F* (1, 53) = 0.0002	*P* = 0.9880	ns
Sex x Sleep	421.1	*F* (1, 53) = 1.2860	*P* = 0.2619	ns
Treatment x Sleep	800	*F* (1, 53) = 2.4430	*P* = 0.1240	ns
Sex x Treatment x Sleep	80.53	*F* (1, 53) = 0.2459	*P* = 0.6220	ns
Medial Septum (MS)				
Sex	147.1	*F* (1, 65) = 1.4390	*P* = 0.2347	ns
Treatment	1029	***F* (1, 65) = 10.060**	***P* = 0.0023**	**
Sleep	2131	***F* (1, 65) = 20.840**	***P* < 0.0001**	****
Sex x Treatment	448.7	***F* (1, 65) = 4.3880**	***P* = 0.0401**	*
Sex x Sleep	239.5	*F* (1, 65) = 2.3420	*P* = 0.1308	ns
Treatment x Sleep	697.1	***F* (1, 65) = 6.8160**	***P* = 0.0112**	*
Sex x Treatment x Sleep	67.04	*F* (1, 65) = 0.6556	*P* = 0.4211	ns
Lateral Septum (LS)				
Sex	24594	*F* (1, 63) = 2.8320	*P* = 0.0974	ns
Treatment	202804	***F* (1, 63) = 23.350**	***P* < 0.0001**	****
Sleep	155038	***F* (1, 63) = 17.850**	***P* < 0.0001**	****
Sex x Treatment	3052	*F* (1, 63) = 0.3514	*P* = 0.5554	ns
Sex x Sleep	7031	*F* (1, 63) = 0.8096	*P* = 0.3717	ns
Treatment x Sleep	142362	***F* (1, 63) = 16.390**	***P* = 0.0001**	****
Sex x Treatment x Sleep	45818	***F* (1, 63) = 5.2760**	***P* = 0.0250**	*
Ventrolateral Preoptic Area (VLPO)				
Sex	10.50	*F* (1, 64) = 0.7804	*P* = 0.3803	ns
Treatment	75.67	***F* (1, 64) = 5.6230**	***P* = 0.0207**	*
Sleep	163.10	***F* (1, 64) = 12.120**	***P* = 0.0009**	***
Sex x Treatment	39.90	*F* (1, 64) = 2.9650	*P* = 0.0899	ns
Sex x Sleep	0.6156	*F* (1, 64) = 0.0457	*P* = 0.8313	ns
Treatment x Sleep	101.5	***F* (1, 64) = 7.5440**	***P* = 0.0078**	*
Sex x Treatment x Sleep	1.180	*F* (1, 64) = 0.0877	*P* = 0.7681	ns
Median Preoptic Area (MnPO)				
Sex	7.102	*F* (1, 62) = 0.1920	*P* = 0.6627	ns
Treatment	537.4	***F* (1, 62) = 14.530**	***P* = 0.0003**	***
Sleep	406.4	***F* (1, 62) = 10.990**	***P* = 0.0015**	**
Sex x Treatment	189.0	***F* (1, 62) = 5.1110**	***P* = 0.0273**	*
Sex x Sleep	4.002	*F* (1, 62) = 0.1082	*P* = 0.7433	ns
Treatment x Sleep	745.0	***F* (1, 62) = 20.150**	***P* < 0.0001**	****
Sex x Treatment x Sleep	1.613	*F* (1, 62) = 0.0436	*P* = 0.8353	ns

Table 6. Full three-way ANOVA statistics for brain regions of interest.

Discussion

Inadequate sleep is diversely problematic for individuals and society. Chronic sleep loss shortens life and health span [30-32] and increases risk of motor vehicle accidents [58]. Complex environmental, behavioral and socio-economic factors can make it difficult for people to routinely get sufficient opportunity for rest. Sleep disorders like insomnia make regular sleep difficult, even with sufficient opportunity. Emerging technologies offer individuals personalized insights, and researchers access to ecologically valid sleep assessment [59], yet treatment options for poor and disordered sleep are limited, despite long-time public and scientific interest [35,60].

Hydrogen is the most abundant chemical substance on the planet. As a therapeutic, hydrogen gas (H_2) is non-toxic, has no known lethal dose or deleterious side effects. It has demonstrated the capacity to act as a scavenger of free radicals, to upregulate important immunological pathways, to offset the effects of chemical and physiological challenges, and to improve metabolic condition [for a review, 57]. Significant sleep disturbances are common in patients with Parkinson's Disease (PD), with insomnia and daytime sleepiness frequently reported by patients and bed partners [for a review, 61]. 6-hydroxydopamine (6-OHDA)-induced Parkinson's disease is a frequently used rat model of PD, typified by the selective and rapid destruction of catecholaminergic neurons by administration of a neurotoxin [62]. Acute administration of HRW immediately pre- and post-administration of 6-OHDA is sufficient to both (a) ameliorate the dopaminergic cell loss that typifies this model of PD and (b) alter dopamine-related behavior [42]. The substantia nigra, where dopamine is produced, and whose deterioration is a hallmark of PD, is an important sleep-regulatory region with essential roles in regulating arousal and REM sleep [63]. Together, acute administration of HRW has demonstrated the ability to regulate sleep-dependent processes (immunity, metabolism), and to act as a neuroprotectant in known sleep regulatory regions during a chemical challenge. Hypothesizing that HRW may be sufficient to alter

sleep itself, we conducted a randomized within-subjects investigation of HRW and sleep-wake behavior in adult wildtype C57BL/6J mice.

Our results substantiate the continued investigation of HRW as a novel therapeutic with an extraordinarily uncommon risk profile. Following 7 days of *ad libitum* access to HRW during their active phase, 24-hour baseline polysomnography was collected, followed by 6 hours of sleep deprivation and a subsequent 18-hour recovery period. Mean latency to sleep onset was dramatically reduced by >50% following 7 days of HRW treatment **(Figure 9)** without altering daily rhythms of sleep-wake behavior or the typical structure of recovery sleep **(Figure 10)**. We also observed a significant reduction in brief arousals in undisturbed mice **(Figure 11).** Non-significant changes in NREM bout duration and decreases in NREM bout quantity were consistent with changes in brief arousals **(Figure 11)**. Total NREM and REM sleep amounts were largely unchanged during baseline of each treatment condition **(Figure 11A,C)**.

Following sleep deprivation, mice in the HRW-treatment condition experienced significantly increased NREM **(Figure 12B)** and REM **(Figure 12D)** sleep that appears to have been principally driven by differences in active-phase sleep behavior. It is possible the effect of HRW on recovery sleep is specific to the active phase because sleep-pressure is already near its practical ceiling immediately following 6 hours of sleep deprivation. While we observe significant changes in behavioral markers of sleep pressure (latency, NREM amount and sleep consolidation), a mixed effects model with multiple comparisons reveals no effect of HRW on NREM relative delta power during baseline nor recovery from sleep loss **(SWA, Figure 13).** It may be the case that SWA is not a sensitive enough marker to reflect the subtle changes in sleep pressure induced by HRW. It may also be that the effect of HRW on sleep behavior is driven by

direct activity changes to sleep regulatory systems and these changes influence homeostatic sleep behavior and markers of sleep pressure.

To elucidate the effect of HRW on several sleep-related forebrain nuclei, we performed an extensive between-subjects mapping of cFos protein immunoreactivity in forebrain, hypothalamic, and midbrain structures across 4 groups: undisturbed, untreated mice (CON); sleep deprived, untreated mice (CON+SD) undisturbed, HRW-treated mice (HRW); and sleep deprived, HRW-treated mice (HRW+SD). We report here region-specific changes in sleep- and arousal-related forebrain structures.

Septal nuclei are functionally and chemically heterogeneous forebrain structures often implicated in the regulation of social behaviors, stress, and feeding [64, 70]. It was recently demonstrated to receive sleep-related signals from the hippocampus in rats [65] and chemogenetic activation of GABAergic neurons in the LS is sufficient to significantly increase sleep amount (~2x) at the expense of NREM sleep [66]. The medial septum (MS) is a forebrain structure repeatedly implicated in stress regulation that receives arousal-promoting projections from hypocretin-producing neurons of the lateral hypothalamus [67]. We report a significant increase in Fos-IR of LS and the MS neurons following HRW treatment **(Figure 16, Table 6)**. The theoretical relationship between observed sleep changes in HRW-treated mice and the *increase* in cellular activity the forebrain structures reported here is unclear; though it is possible these cFos changes reflect the activity of local inhibitory interneurons.

Hypothalamic nuclei have long been thought to play diverse, important roles in sleep and arousal [68, 71, 72]. GABAergic and galanergic neurons in the preoptic area send inhibitory projections to important arousal-promoting nuclei, including to the histaminergic neurons of the tuberomammillary nucleus (TMN) [68]. Optogenetic stimulation of these preoptic area neurons

leads to increased SWA in mice. Chemogenetic activation of galanergic neurons in the VLPO significantly reduces sleep latency. For a comprehensive review and as a primary source, I enthusiastically recommend work from Kroeger et al. [71]. This report and others [for a review, 68] suggest that inhibitory neurons in the preoptic area may be central to the homeostatic organization of sleep in mammals. Here we report that HRW is sufficient to increase several important markers of sleep pressure but, importantly, we report no alterations to NREM SWA.

There is significant direct evidence for SWA being a reflection of underlying cortical connectivity [4,10,11] and while some posit a causal role for neural oscillations in sleep behavior, the evidence for this hypothesis is extremely limited. Changes to sleep can occur without changes to SWA, and changes in SWA are demonstrably downstream of region- or cell-type specific modulations, and therefore SWA most likely occurs as a byproduct of the neurochemical changes important for inducing and/or associated with sleep itself. As such, it is theoretically possible that HRW is increasing activity in sleep-promoting preoptic area neurons that are sufficient to alter some markers of sleep pressure, but not SWA. It is also possible that changes to cFos are not occurring in GABAergic or galanergic neurons, explicitly or at all, and instead reflect activity changes in some other population of VLPO and MnPO neurons.

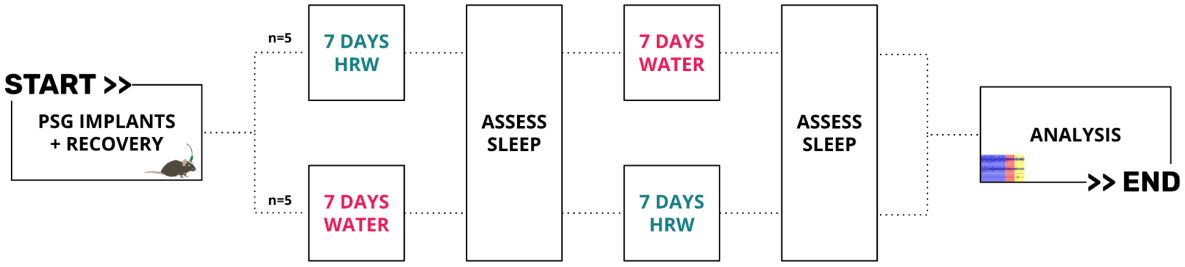
Limitations

This direct investigation HRW and sleep in mice is the first of its kind. Every effort was taken to ensure the responsible assessment of HRW's ability to influence arousal states and the recovery from sleep loss, including clearly defined positive controls for back testing, behavioral (sham) environmental disruptions to avoid unintended consequences of bottle replacements, blinded scoring of arousal behavior, and regular intra-scorer reliability testing to confirm very high agreement (>95%) among sleep scorers (see *Methods*). One limitation of this work is that *ad*

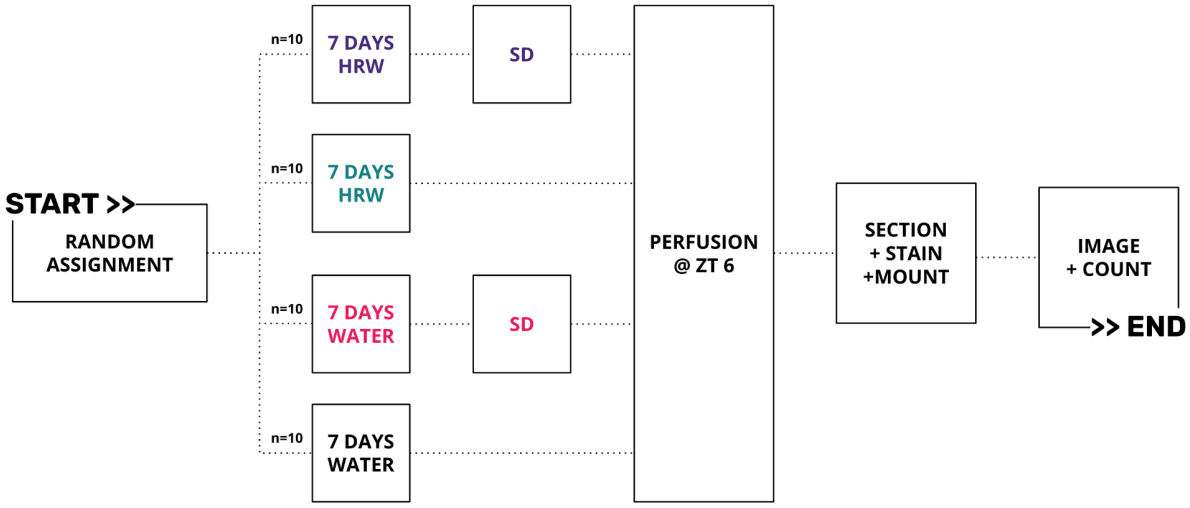
libitum administration of HRW during the sleep assessment experiments. This route of administration was intentionally chosen as alternative administration routes that would provide greater control of dosage and timing (e.g., oral gavage) are highly anxiogenic and would significantly, materially confound our assessment of arousal state by PSG. We believe our implementation of *ad libitum* administration reasonably reflects a possible real-world condition; though, admittedly, it is more likely that HRW would be administered in single doses at one or multiple times per day as has been seen in human trials [73-75].

Oral gavage (and sham gavage) for the IHC study reported here enabled a greater level of temporal control over HRW dosing. However, as there are previously demonstrations of dose-dependent effects of HRW in various models [for a review, 76], it is possible that the regions implicated by acute administration by gavage are wholly separate from the unknown mechanism(s) underlying changes to sleep behavior following *ad libitum* administration. The results of the experiments described above sufficiently justify the use of more invasive *in vivo* tools with cell-type and region specificity (e.g., optogenetics, chemogenetics), employed in parallel with PSG, to clarify the role of sleep regulatory systems in HRW-associated sleep changes. Finally, while immediate early gene activity reported here does not provide cell-type specific information, these results represent an important first step toward elucidating the mechanisms underlying the sleep-promoting effects of HRW in mice.

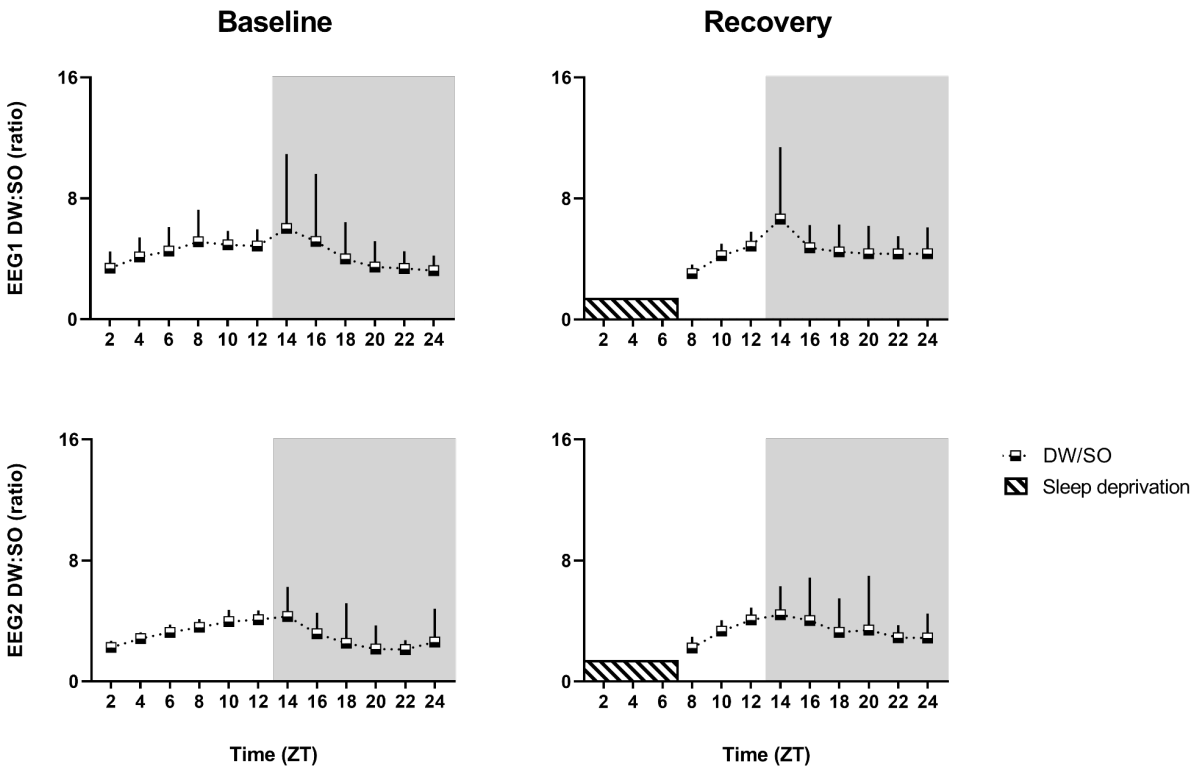
SUPPLEMENTARY INFORMATION



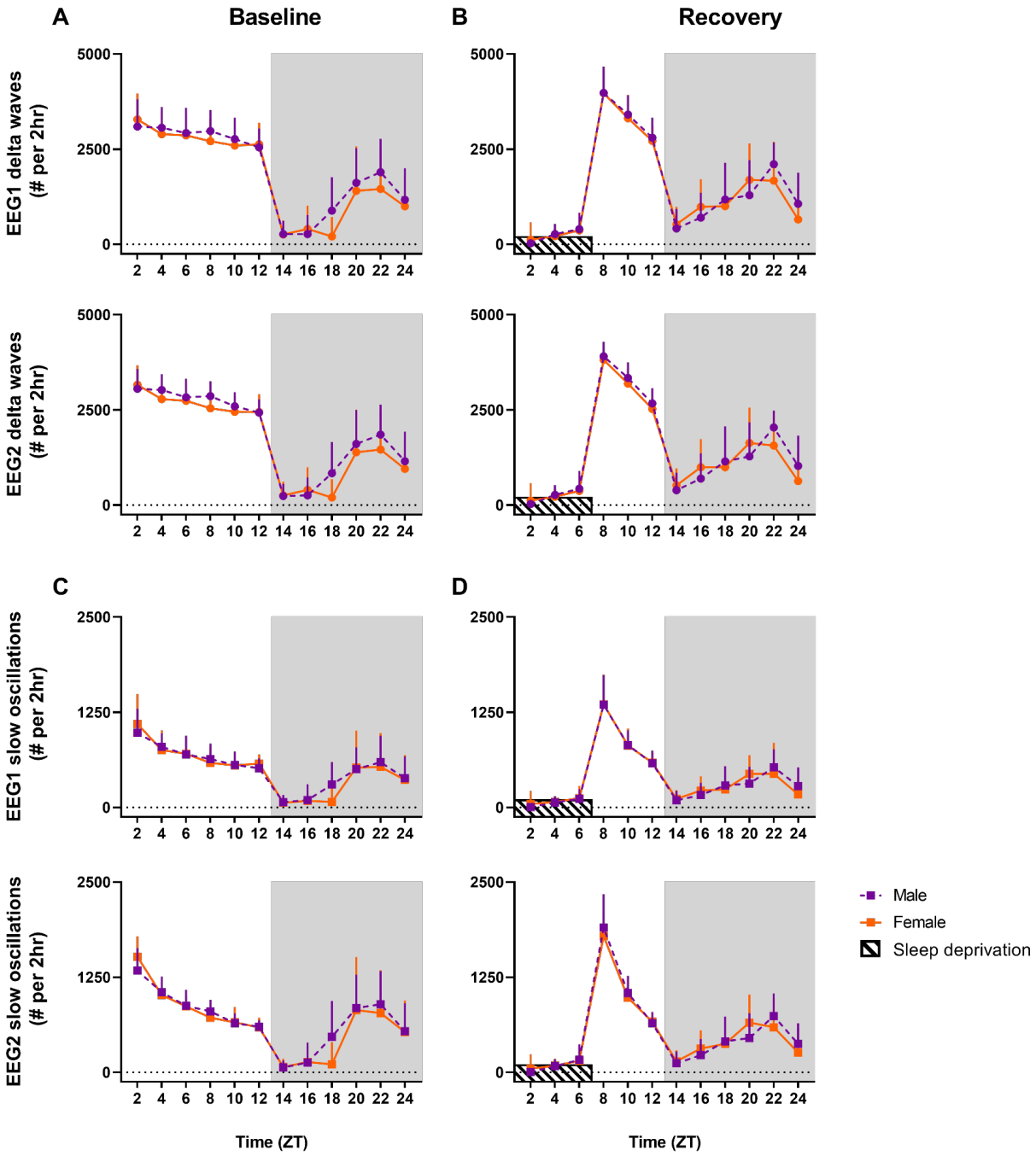
Supplementary Figure 1. Visualization of experimental protocol for Chapter 2 sleep assessment.



Supplementary Figure 2. Visualization of experimental protocol for Chapter 2 IHC experiments



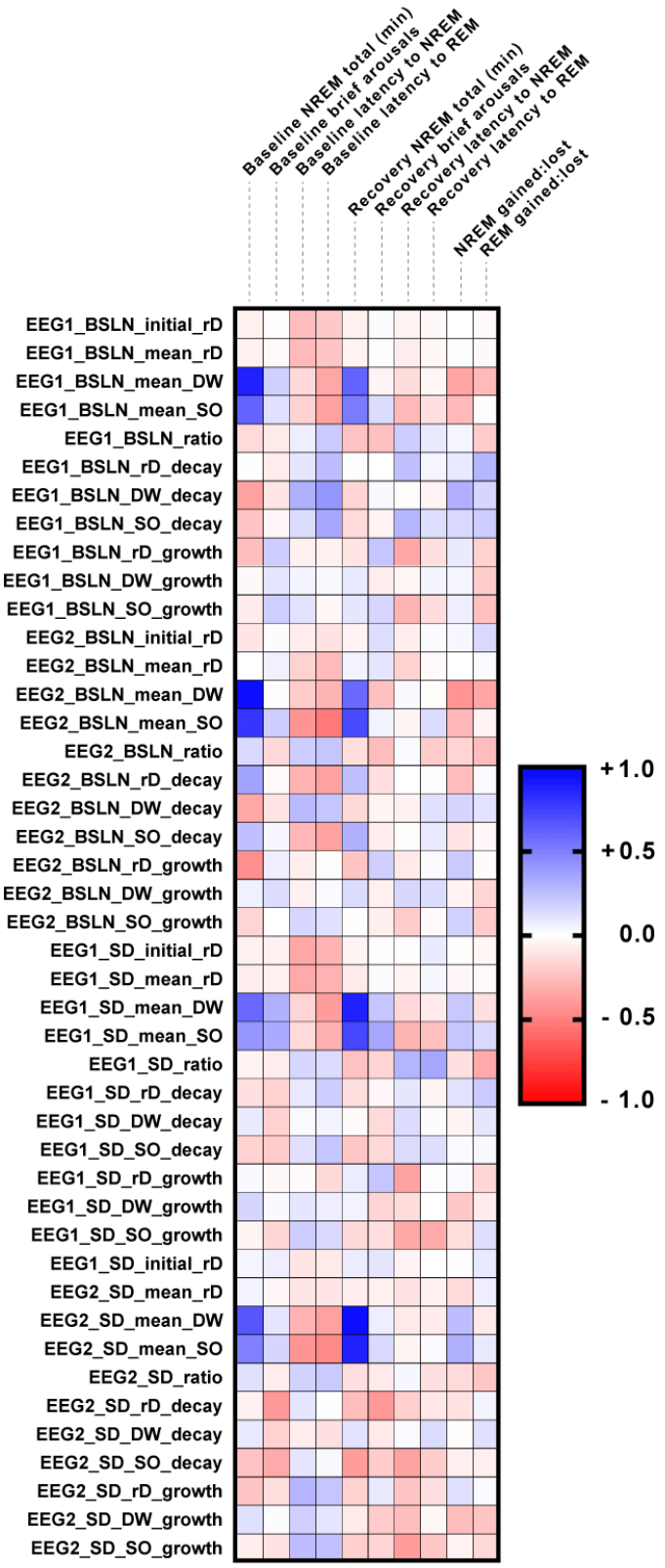
Supplementary Figure 3. Temporal differences in DW:SO ratio across baseline and recovery conditions. Data presented as mean + SD.



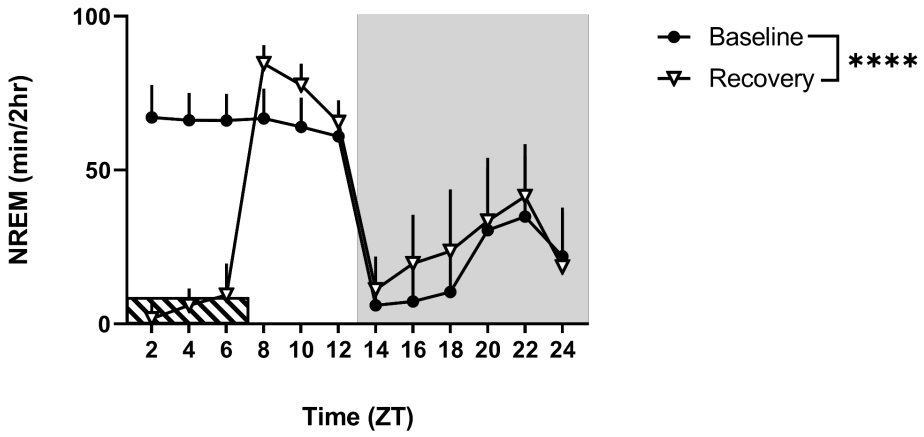
Supplementary Figure 4. Sex comparison across conditions, waveforms, and electrodes. Two-way ANOVA with sex and time for factors reveals no effect of or interaction with sex. Data presented as mean + SD.

	EEG1 Baseline		EEG2 Baseline		EEG1 Recovery		EEG2 Recovery	
Comparison	**Mean Δ**	***P*-value**	**Mean Δ**	***P*-value**	**Mean Δ**	***P*-value**	**Mean Δ**	***P*-value**
ZT 0-2								
DW vs. SO	**-0.4483**	**0.0002**	**-0.4818**	**<0.0001**	-SD-		-SD-	
DW vs. rD	0.0189	0.8609	0.1217	0.1641				
SO vs. rD	**0.4673**	**0.0006**	**0.6035**	**<0.0001**				
ZT 2-4								
DW vs. SO	0.1189	0.1706	**0.2115**	**0.0057**	-SD-		-SD-	
DW vs. rD	**0.5139**	**<0.0001**	**0.5867**	**<0.0001**				
SO vs. rD	**0.3950**	**0.0004**	**0.3752**	**<0.0001**				
ZT 4-6								
DW vs. SO	**0.2990**	**0.0016**	**0.5068**	**<0.0001**	-SD-		-SD-	
DW vs. rD	**0.7990**	**<0.0001**	**0.8736**	**<0.0001**				
SO vs. rD	**0.5000**	**<0.0001**	**0.3668**	**<0.0001**				
ZT 6-8								
DW vs. SO	**0.5568**	**<0.0001**	**0.6849**	**<0.0001**	**-0.4293**	**<0.0001**	-0.4857	**<0.0001**
DW vs. rD	**1.1910**	**<0.0001**	**1.1520**	**<0.0001**	**0.5803**	**<0.0001**	0.4672	**<0.0001**
SO vs. rD	**0.6339**	**<0.0001**	**0.4671**	**<0.0001**	**1.0100**	**<0.0001**	0.9529	**<0.0001**
ZT 8-10								
DW vs. SO	**0.5808**	**<0.0001**	**0.8001**	**<0.0001**	**0.3579**	**<0.0001**	0.5250	**<0.0001**
DW vs. rD	**1.2770**	**<0.0001**	**1.2780**	**<0.0001**	**0.8371**	**<0.0001**	0.8757	**<0.0001**
SO vs. rD	**0.6959**	**<0.0001**	**0.4783**	**<0.0001**	**0.4792**	**0.0001**	0.3507	**<0.0001**
ZT 10-12								
DW vs. SO	**0.4971**	**<0.0001**	**0.8299**	**<0.0001**	**0.4644**	**<0.0001**	0.6680	**<0.0001**
DW vs. rD	**1.3150**	**<0.0001**	**1.4120**	**<0.0001**	**0.9473**	**<0.0001**	1.1580	**<0.0001**
SO vs. rD	**0.8178**	**<0.0001**	**0.5822**	**<0.0001**	**0.4829**	**<0.0001**	0.4900	**<0.0001**
ZT 12-14								
DW vs. SO	-0.0166	0.8499	-0.0079	0.9222	-0.0091	0.8665	0.0149	0.7957
DW vs. rD	**-0.8509**	**<0.0001**	**-0.5963**	**0.0006**	-0.0823	0.5456	0.0404	0.6876
SO vs. rD	**-0.8342**	**<0.0001**	**-0.5883**	**0.0008**	-0.0731	0.5897	0.0255	0.7965
ZT 14-16								
DW vs. SO	-0.0820	0.5992	-0.1543	0.3755	-0.0055	0.9514	0.0337	0.7117
DW vs. rD	-0.5406	0.0512	**-0.7472**	**0.0038**	-0.0057	0.9646	-0.0897	0.4874
SO vs. rD	-0.4586	0.1056	**-0.5929**	**0.0266**	-0.0002	0.9989	-0.1235	0.3108
ZT 16-18								
DW vs. SO	-0.3856	0.0546	**-0.6227**	**0.0062**	0.0021	0.9860	-0.1059	0.4280
DW vs. rD	**-1.3660**	**<0.0001**	**-1.2970**	**<0.0001**	-0.1178	0.4679	-0.1052	0.5295
SO vs. rD	**-0.9809**	**0.0007**	**-0.6744**	**0.0286**	-0.1200	0.4315	0.0007	0.9966
ZT 18-20								
DW vs. SO	**-0.4251**	**0.0338**	**-0.7918**	**0.0002**	0.0415	0.7183	-0.0573	0.6486
DW vs. rD	**-0.8464**	**<0.0001**	**-1.1610**	**<0.0001**	-0.2153	0.1384	**-0.4170**	**0.0083**
SO vs. rD	**-0.4214**	**0.0391**	-0.3697	0.0686	-0.2568	0.0617	**-0.3596**	**0.0185**
ZT 20-22								
DW vs. SO	-0.2810	0.0982	**-0.4795**	**0.0112**	0.1171	0.3224	-0.0430	0.7203
DW vs. rD	**-0.9006**	**<0.0001**	**-0.9988**	**<0.0001**	0.1046	0.3869	-0.0912	0.4760
SO vs. rD	**-0.6196**	**0.0003**	**-0.5193**	**0.0028**	-0.0124	0.9210	-0.0481	0.7097
ZT 22-24								
DW vs. SO	-0.2312	0.1442	**-0.3618**	**0.0253**	-0.0111	0.9268	-0.0652	0.5813
DW vs. rD	**-1.4230**	**<0.0001**	**-1.4550**	**<0.0001**	-0.3956	0.0289	**-0.6091**	**0.0004**
SO vs. rD	**-1.1920**	**<0.0001**	**-1.0930**	**<0.0001**	-0.3845	0.0329	**-0.5439**	**0.0011**

Supplementary Table 1. Multiple comparisons from 3-way mixed effects comparison. Significant differences among all three features exist across time and conditions, but the preponderance of discoveries are found in the late inactive phase (ZT 6-12). Comparisons during sleep deprivation were excluded for general lack of NREM sleep. Note that SO and DW is not reported during the 6-hour period of acute sleep deprivation (SD).



Supplementary Figure 5. Correlation matrix of delta phenotypes to behavioral markers of sleep pressure in mice. (Left) Exploration of potential linear relationships among delta features (rows) and behavioral features related to sleep pressure and sleep fragmentation (columns). Results of repeated spearman correlations are represented by shaded squares. BSLN = baseline condition; SD = Recovery condition; rD = relative delta power; DW = delta waves; SO = slow oscillations.



Supplementary Figure 6. Stereotypical NREM sleep behavior during baseline and recovery conditions.

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