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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Psychological and/or physical trauma can result in post-traumatic stress disorder (PTSD). Commonly, PTSD presents with sleep disturbances. However, little is known about the contribution of sleep to the acquisition and maintenance of PTSD symptoms. The main goal of this proposal was to assess the contribution of sleep to the development of PTSD. Using a rodent model of PTSD called Single Prolonged Stress (SPS), we performed sleep deprivation experiments to determine if sleep loss prior to SPS exposure is sufficient to exaggerate fear-associated memory impairments. Additionally, we assessed the effects of post-trauma optogenetic sleep enhancement on fear-associated memory function. The proposed experiments have concluded and have resulted in 2 published peer-reviewed papers (see attachments). We found that sleep deprivation did not exaggerate trauma-induced fear-associated memory impairments and that post-trauma-sleep enhancement was sufficient to alleviate fear-associated memory impairments typically found following trauma exposure in rats.

15. SUBJECT TERMS

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1. Introduction

The current research is focused on understanding the contribution of sleep to the acquisition and maintenance of post-traumatic stress disorder (PTSD). To achieve our proposed aims, we utilized a rodent model of PTSD called single prolonged stress (SPS) and focused on measuring trauma-induced fear-associated memory impairments in rats exposed to either sleep deprivation or optogenetic enhancement of sleep duration.

2. Keywords

Sleep, Post-Traumatic Stress Disorder, Sleep Deprivation, Stress

3. Accomplishments

Major Task 1: Perform Sleep deprivation/restriction and collect EEG/EMG recordings. These experiments have been completed and the data fully analyzed. The data obtained from these studies were recently published in PLOS ONE (Citation below, Full text in Attachments section).

Davis CJ, Gerstner JR, **Vanderheyden WM**. Single prolonged stress blocks sleep homeostasis and pre-trauma sleep deprivation does not exacerbate the severity of trauma-induced fear-associated memory impairments. PLOS ONE. 2021;16(1):e0243743. doi: 10.1371/journal.pone.0243743.

1) major activities: For Specific Aim1, I performed, 12 hr (acute) sleep deprivation experiments (n=9) and 18hr, 5day sleep restriction experiments (n=8). Sleep EEG and EMG data have been collected and are scored for behavioral state. Fear-associated memory tests were performed on these, and control animals. As shown previously, SPS results in impaired fear-associated memory as measured by increased freezing on the previously mentioned fear recall test. However, the addition of sleep deprivation did not further exaggerate the severity of the fear-associated memory task.

2) specific objectives: For Specific Aim 1, the objective of this aim was to assess whether sleep deprivation sensitized individual animals to trauma exposure. We hypothesized that sleep deprived animals would be show greater impairment on this fear-associated memory test.

3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative): For Specific Aim 1, these data suggest that sleep deprivation prior to trauma exposure is insufficient to increase the severity of PTSD symptoms.

Major Task 2: Use Optogenetic stimulation to improve sleep after trauma exposure. These experiments have also been completed and the data fully analyzed. The data obtained from these studies were recently published in Scientific Reports (Citation below, Full text in Attachments section).

Davis CJ, Vanderheyden WM. Optogenetic sleep enhancement improves fear-associated memory processing following trauma exposure in rats. Sci Rep. 2020;10(1):18025. Epub 2020/10/22. doi: 10.1038/s41598-020-75237-9. PubMed PMID: 33093538; PubMed Central PMCID: PMCPMC7581760.

1) major activities: For Specific Aim 2, I have completed all optogenetic stimulation experiments following trauma exposure. Control virus expression experiments are also complete.

2) specific objectives: For Specific Aim 2, I proposed that sleep restoration via optogenetic enhancement of sleep time would rescue trauma-induced fear-associated memory impairments.

3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative): For Specific Aim 2, I have found that the virus being used to express channelrhodopsin is expressed where it should be, and I am able to implant fiberoptic cables into the area where these cells are located. In addition, we now show that REM and NREM sleep is increased following opto stimulation compared to non-stimulation, in agreement with previously published data using this viral construct. 7 days of continuous optogenetic sleep enhancement significantly improved fear-associated memory outcomes suggesting that increasing sleep after trauma may be a potential therapeutic tool to stave off the negative impacts of trauma exposure.

Major Task 3: Preparation of Manuscripts. This task is complete and both manuscripts are accepted and in publication.

What opportunities for training and professional development has the project provided?
Nothing to Report.

How were the results disseminated to communities of interest?
WSU published a press release that was picked up by over 50 media outlets. The sleep research society highlighted my research and profile on their social media pages.

What do you plan to do during the next reporting period to accomplish the goals?
Nothing to Report, this is a final report.

4. Impact

What was the impact on the development of the principal discipline(s) of the project?
The data generated in Specific Aim 2 indicates that post-trauma sleep improvements are sufficient to ameliorate the negative consequences of trauma exposure. If translated to human populations, these data are likely to make a large impact on how deployed military personnel are treated following trauma-enhanced deployment. These data will also have an impact on trauma-exposed civilian populations.

What was the impact on other disciplines?
We have shown that sleep is sufficient to restore function in trauma-exposed animals. My lab has begun studies to examine if sleep can be used to modify addiction-related behaviors in opioid exposed animals. The contribution of sleep to opioid drug use and addiction is unstudied, however, there is a clear link between sleep and addiction and sleep enhancement may protect opioid exposed populations from developing addictions suggesting that sleep is therapeutic in these conditions just as it was for trauma-exposed populations.

What was the impact on technology transfer?
N/A

What was the impact on society beyond science and technology?
PTSD represents a massive societal burden. Developing sleep specific therapeutic tools to stave off the negative consequences of trauma exposure can have a huge impact on the health of individual trauma sufferers and on the economy.

5) Changes/Problems

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them:

The Optogenetics portion of the proposed experiments required more troubleshooting than anticipated; fabrication of the optical probes and verification of viral construct expression took longer than expected. However, all issues were resolved, and data collection was completed on schedule.

Some unexpected outcomes also resulted in delays. Faulty dental acrylic resulted in lost EEG headpieces. Some behavioral data was lost due to improper use of new software. Regardless, the experiments were completed on time and in full.

Changes that had a significant impact on expenditures:

Although delays in data collection occurred, expenditures remained on track and the award has been fully spent out.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents:

Vertebrate animals were handled according to ASAF #6263.

Significant changes in use of biohazards and/or select agents:

Nothing to Report.

6) Products

Publications, conference papers, and presentations

Two peer-reviewed papers were published during this Discovery Award. Both papers acknowledge support from the CDMRP/PRMRP Discovery Award. The two citations are as follows:

Davis CJ, Gerstner JR, **Vanderheyden WM**. Single prolonged stress blocks sleep homeostasis and pre-trauma sleep deprivation does not exacerbate the severity of trauma-induced fear-associated memory impairments. PLOS ONE. 2021;16(1):e0243743. doi: 10.1371/journal.pone.0243743.

Davis CJ, **Vanderheyden WM**. Optogenetic sleep enhancement improves fear-associated memory processing following trauma exposure in rats. Sci Rep. 2020;10(1):18025. Epub 2020/10/22. doi: 10.1038/s41598-020-75237-9. PubMed PMID: 33093538; PubMed Central PMCID: PMCPMC7581760.

Other publications, conference papers and presentations.

The data gathered during the Discovery Award has been presented at the Washington State University Research Showcase Event, as a discussion topic for a class in psychology at Oregon State University (at the invitation of Dr. Regan Gurung Ph.D.), as a discussion topic for a neuroscience, neuroplasticity, and sleep course at the University of St. Thomas (at the invitation of Dr. Roxanne Prichard), and were discussed in a WSU Spokane Oxidative Stress Journal Club. In Fall of 2021, the data will be presented at a University of Colorado, Boulder Journal Club.

Websites

N/A

Technologies

N/A

Inventions, patent applications, and/or licenses

N/A

Other Products

Biological Tissue: Brain tissue for future molecular studies (mRNA/Protein)

Viral construct: Control and channelrhodopsin expressing viral vectors sufficient to perform optogenetic sleep enhancement in additional 30 rats.

Optogenetic head caps: We fabricated 3D printed headcaps that mount LED lights and fiber optic cabling for optogenetic stimulation.

7) Participants & Other Collaborating Organizations

Name:	William Vanderheyden
Project role:	Principal Investigator
ORCID ID:	0000-0003-4124-8021
Month Worked:	12
Contribution to Project:	Performed surgery/behavior/data analysis/manuscript preparation.
Funding Support:	At the conclusion of the award period, Dr. Vanderheyden began working on R35 GM133440/GM/NIGMS (5 months)

Name:	Christopher Davis
Project role:	Co-Investigator
ORCID ID:	0000-0002-9613-928X
Month Worked:	1
Contribution to Project:	Performed data analysis/manuscript preparation.
Funding Support:	W81XWH-16-1-0319

8) Special Reporting Requirements

N/A

9) Appendices

Attached are the two manuscripts published during the Discovery Award.

scientific reports

Check for updates

OPEN

Optogenetic sleep enhancement improves fear-associated memory processing following trauma exposure in rats

Christopher J. Davis & William M. Vanderheyden✉

Sleep disturbances are commonly found in trauma-exposed populations. Additionally, trauma exposure results in fear-associated memory impairments. Given the interactions of sleep with learning and memory, we hypothesized that increasing sleep duration following trauma exposure would restore overall function and improve trauma-induced fear-associated memory dysfunction. Here, we utilized single prolonged stress, a validated rodent model of post-traumatic stress disorder, in combination with optogenetic activation of hypothalamic melanin-concentrating hormone containing cells to increase sleep duration. The goal of this work was to ascertain if post-trauma sleep increases are sufficient to improve fear-associated memory function. In our laboratory, optogenetic stimulation after trauma exposure was sufficient to increase REM sleep duration during both the Light and Dark Phase, whereas NREM sleep duration was only increased during the Dark Phase of the circadian day. Interestingly though, animals that received optogenetic stimulation showed significantly improved fear-associated memory processing compared to non-stimulated controls. These results suggest that sleep therapeutics immediately following trauma exposure may be beneficial and that post-trauma sleep needs to be further examined in the context of the development of post-traumatic stress disorder.

Abbreviations

PTSD	Post-traumatic stress disorder
SPS	Single prolonged stress
EEG	Electroencephalogram
EMG	Electromyogram
REM	Rapid eye movement sleep
NREM	Non rapid eye movement sleep
SSRI	Selective serotonin reuptake inhibitor

Post-traumatic stress disorder (PTSD) arises as a result of experiencing a physical or psychological trauma and is often accompanied by significant sleep disorders[1]. In fact, as many as 70–91% of PTSD patients have difficulty falling asleep and staying asleep through the night[2,3]. Furthermore, poor sleep has been linked to the exaggeration of PTSD symptoms such as anxiety, flashbacks, agitation, and fear[4–11]. Humans resilient to PTSD show increased theta activity during rapid eye movement (REM) sleep[10] and reduced autonomic nervous system activation as measured by changes in heart rate during quiescent periods[12,13]. These data suggest a reciprocal relationship between sleep and PTSD wherein trauma exposure impairs sleep and sleep disorders increase the severity of PTSD[14]. We hypothesize that sleep interventions designed to increase post-trauma sleep will break this vicious cycle and help stave off the development of PTSD.

Examining trauma-exposure-dependent sleep disturbances in humans is prohibitively difficult because the timing and intensity of trauma exposure is unpredictable. Therefore, to examine the molecular and systems biology components of trauma-exposure-dependent sleep disturbances, a pre-clinical animal model is required[15]. *Single prolonged stress* (SPS) is a rodent model of PTSD that consists of a combination of stressors that include physical restraint, a 20 min forced swim, exposure to ether vapors (a potent chemical activator of the stress

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system) until anesthetized, and seven days of social isolation[16]. SPS causes molecular, anatomical, and behavioral changes that resemble PTSD in humans[16–18]. Rats that have been exposed to SPS show fear-associated memory impairments including enhanced fear-renewal and deficits in extinction-retention for both contextual and cued fear[19,20]. These fear-associated memory impairments are used as a metric to determine the severity of the PTSD phenotype in rodents. Importantly, SPS results in quantifiable and robust sleep disturbances in rats[8,17], and has repeatedly shown good face validity to the human condition of PTSD[18]. Thus, SPS is an ideal animal model to assess the mechanisms regulating trauma-exposure-dependent sleep disorders. Further, fear-associated memory tests are a functional metric for confirmation of PTSD in this pre-clinical PTSD model.

In our previous work, we examined the effect of SPS exposure at Zeitgeber time (ZT) 0, when nocturnal rodents are predominantly asleep[17]. However, a gap in the literature exists to explain how the circadian system and the timing of trauma affects subsequent trauma-dependent sleep and fear-associated memory alterations. How SPS exposure alters sleep when animals receive SPS at the beginning of their predominantly awake period, ZT12, was assessed in our first set of experiments.

The precise role of sleep in mediating, exacerbating, or mitigating negative health outcomes in trauma exposed populations is unknown. Therefore, the main goal of this work was to assess the effects of post-trauma sleep increases on the development of PTSD phenotype in SPS exposed rats. Sleep manipulations, specifically increasing sleep duration, in rodents has become increasingly easier due to the development of optogenetic tools[21,22]. Optogenetic stimulation of melanin-concentrating hormone (MCH) cells within the lateral hypothalamus (LH) results in increased REM and non-rapid eye movement (NREM) sleep in mice[23,24] and rats[25]. Therefore, in our second set of experiments, we used post-SPS stimulation of MCH cells to increase sleep duration and then assessed fear-associated memory function to determine the contribution of sleep increases to the severity of the PTSD-like phenotype in control and optogenetically stimulated animals.

Methods

Animals. All animal procedures were carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approved by the *Washington State University Committee on the Use and Care of Laboratory Animals*.

Male, Long Evans rats (60–90 days old) were housed in temperature and humidity-controlled rooms and given ad libitum access to food and water. The animals underwent two surgeries, the first surgery was performed at 60–65 days old to stereotaxically inject the experimental or the control viral vector. Two weeks following the viral injection, animals underwent a second surgery to implant optogenetic stimulating fiber optic cable (optrodes) and electroencephalographic (EEG)/electromyographic (EMG) recording electrodes (described below). Animals were housed singly following surgical procedures.

Viral vector. We generated a plasmid containing the Channelrhodopsin-2 gene (ChR2) with an enhanced yellow fluorescent protein (EYFP) marker under the melanin-concentrating hormone (MCH) promoter sequence as previously published in Blanco-Centurion[5]. This MCH specific plasmid was packaged into a recombinant adeno-associated viral vector (rAAV, serotype 5) by the Vector Core at the University of North Carolina (Chapel Hill, NC) to create rAAV-MCH-ChR2-EYFP. Additionally, a control vector lacking the ChR2 gene (rAAV-MCH-EYFP) was constructed as previously published[26].

Viral transfection. Rats underwent surgery to inject viral vectors into the LH. Surgeries were performed with the aid of a digital stereotaxic apparatus (Stoelting, Wood Dale, IL), using aseptic conditions, under constantly monitored isoflurane anesthesia (1.5–2%). A midsagittal incision was made to reveal the skull which was cleaned and prepped using hydrogen peroxide solution and saline. Holes were drilled in the skull above the LH and rAAV viral vectors were microinjected bilaterally targeting the MCH neurons of the LH (AP = −2.9 mm, lateral = ± 1.2 mm, vertical = −7.8 mm). Virus (3 µl per hemisphere over 20 min) was delivered using microinjection pumps (Stoelting, Wood Dale, IL) and 10 µl Hamilton (Reno, NV) syringes. The injection needle was left in place for an additional 5 min before being removed. The incision was sutured closed at the conclusion of the surgery. Animals were treated with Dexamethasone (200 µg/kg), ciprofloxacin (5 mg/kg) and buprenorphine SR (1 mg/kg) to manage swelling, infection, and pain, respectively. Post-mortem studies were conducted to validate expression of EGFP as previously published[25]. Histological verification of expression of the viral construct was made by examining 4% paraformaldehyde perfused, frozen, and cryostat sectioned (40 micron) tissue using a Zeiss ApoTome fluorescent microscope. Histological analysis revealed similar anatomical expression to previously published data[25] and a representative image is shown in Figure S1.

Electrophysiology/optogenetics. Two weeks after the rAAV surgery, a second stereotaxic surgery was performed to implant the EEG/EMG sleep recording electrodes and stimulating optrode into the LH. As above, aseptic surgeries were performed under isoflurane anesthesia. A midsagittal incision was made on the top of the skull and the skin was retracted. After cleaning the surface of the skull, 2 holes were drilled through the cranium and screw electrodes (Plastics One, Roanoke, VA) were inserted bilaterally over the frontal area (± 2.5 mm lateral to midline, 2.5 mm anterior to Bregma) for EEG recordings. Two flexible wire electrodes were threaded through the dorsal neck muscles for EMG recordings. Gold pins were connected to the ends of each electrode then placed into a six-pin connector (Plastics One, Roanoke, VA) which was attached to the skull via light-curable dental acrylic. The holes in the skull that were previously used for injection of the rAAV were re-drilled to accommodate the insertion of a custom 3D printed head piece that housed guide cannula for placement of the optrode. The fiber optic unit was lowered through the guide cannula before finalizing the electronic connections through the six-pin connector to the Tucker Davis Technology (TDT) (Alachua, FL) electrophysiology recording device

and TTL controller. Rats were given at least 10 days to recover from surgery prior to beginning the experiment. Animal well-being was assessed daily during the surgical recovery period. Any sign of illness or pain, including decreased motility and responsiveness, vocalizations, lack of appetite, decreased grooming, etc. was noted and treated in consultation with veterinary staff.

Optogenetic stimulation. Blue light was delivered by bilateral head mounted blue-light-emitting LEDs (Broadcom, HSMR-C191-S0000) driven at 5v, at a frequency of 10 Hz for one minute during every 5 min period. The rate of stimulation was controlled by a TTL program developed in-house for the TDT system. Rats were stimulated for 7 consecutive days during the incubation period of SPS.

Sleep recording and analysis. As previously reported[17], following recovery from surgery, animals were housed individually and connected to the TDT recording system via a lightweight, flexible tether attached to a commutator (Sparkfun.com, Slip Ring) for free movement within the cage. The recording system was used to sample signals at 333 Hz, filtered between 0.1 and 100 Hz and amplified. Prior to analysis, signals were down-sampled to 250 Hz. The two EEG electrodes were differentially referenced to obtain one EEG channel. Two EMG channels were also differentially referenced to obtain the EMG signal. Animals were given 48 h to acclimate to the tethers prior to beginning baseline recordings. During the acclimation period, animals were supplied food to last the duration of the EEG/EMG experimental recordings. While connected to tethers, animals were monitored daily for food, water, and health via visual inspection and through the use of a video monitoring system to avoid disturbing the animals.

Collected data were transferred from the recording PC, stored onto disk, and scored off-line in 10-s epochs to determine sleep/waking state using Sleep Sign software (Nagano, Japan). Three vigilance states were assigned: Wake, REM sleep and NREM sleep. Wake consists of visible EEG theta activity and high EMG activity, REM sleep consists of clear, sustained EEG theta activity and phasic muscle twitches on a background of low EMG, NREM sleep consists of high amplitude, synchronized EEG and low EMG activity.

EEG and EMG signals were recorded for 24 h of baseline after which the animals were unhooked from the recording system and single prolonged stress (SPS, described below) was performed. Following SPS, animals were reconnected to the recording system and seven subsequent days were recorded and scored. Data collected after SPS were compared to the baseline recording day using one-way repeated measures ANOVA followed by Bonferroni post hoc comparisons of each day to the baseline. Sleep states were quantified as an average duration spent in state per hour (in seconds) over the Light Phase (ZT0-12) and Dark Phase (ZT12-24). Sleep architecture quantified the mean bout length and mean bout number per hour for each of the states discussed above and was averaged over the sleep/active phase and analyzed using one-way repeated measures ANOVA followed by Bonferroni post hoc comparisons of each day to the baseline.

Single prolonged stress. Single prolonged stress was performed as previously published[16,17]. Briefly, animals were exposed to 3 successive stressors at the start of the Dark Phase (lights off-ZT12). First, physical restraint was performed for 2 h in custom built plexi-glass restraining devices. Next, the animals were placed in a (25 × 17 × 16 i in.) plastic bin containing 21–24 °C water and were forced to swim in groups of 6–8 for 20 min. Following a 15-min recuperation period in a towel-lined bin, the animals were exposed to 60 ml of ether vapors in a 2000 cc isolation chamber until fully anesthetized (< 5 min). After which the animals were returned to their EEG/EMG recording-cage where they were isolated for the following seven days.

Fear conditioning, fear extinction, and extinction recall. At the conclusion of the EEG/EMG recording, fear conditioning experiments were conducted. Fear conditioning, extinction, and extinction recall were performed as previously published[17,20]. All fear conditioning/extinction/extinction recall experiments were performed in four identical Noldus fear conditioning chambers (Wageningen, the Netherlands) (12″W × 10″D × 12″H) containing a Shock Floor with current carrying metal bars, a wall-mounted speaker and in-chamber UV and white lighting. Test cages were housed in sound-attenuating boxes. Tones (2000 Hz, 80 dB) were delivered via speakers mounted in the housing of the test cages and controlled by data acquisition software (Noldus Ethovision). Ceiling mounted cameras recorded behavior for analysis and Noldus Ethovision software was used to quantify freezing levels.

Two unique contexts were created by manipulating olfactory and visual cues. Context A consisted of 50 ml of 1% acetic acid solution placed in a small dish next to the test cage using standard lighting conditions of the above mentioned housing boxes. Context B consisted of 50 ml a 1% ammonium hydroxide solution placed in a small dish above the test cage along with a checkerboard patterned paper placed on the chamber walls to alter the visual context.

Fear conditioned animals were exposed to five, 1 mA, 1-s foot-shocks paired with the cessation of a 10 s 80 dB tone in Context B. The first tone was presented 180 s after the animal was placed in the test cage and the subsequent tones occurred with a 60 s inter-tone interval. For all phases, baseline freezing was assessed for 180 s prior to the presentation of any tones, and the inter-tone interval was 60 s. 60 s after the last tone, animals were removed to their home cages. Fear extinction was conducted 24 h after fear conditioning and was performed in a distinctly different context (Context A). Fear extinction consisted of presentation of 30 tones (60 s inter-tone interval), without the paired foot-shock. Extinction recall was assessed 24 h after extinction and consisted of the animals being placed back into the fear extinction context (Context A) for 10 tones (60 s inter-tone interval), again without foot shock.

The percent time spent immobile (freezing) within each 60 s long block was calculated by Ethovision software. Statistical comparison of time spent freezing was made using two-tailed Students t-test via Graphpad

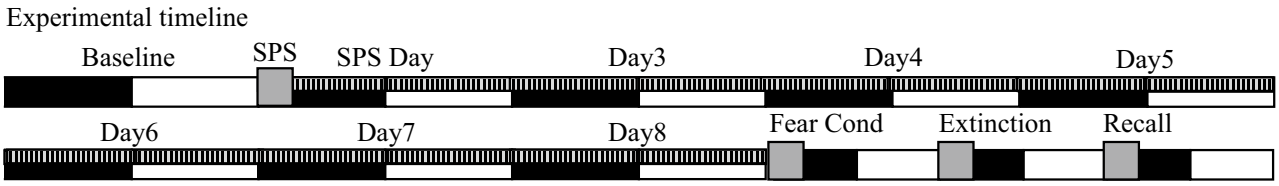

Figure 1. Experimental timeline. 24 h of baseline EEG/EMG activity was recorded on Baseline Day (12/12, Dark/Light schedule) in animals injected with rAAV-MCH-ChR2-EGFP. At the conclusion of the baseline recording (ZT12, the onset of the Dark Phase), SPS was delivered to all animals (gray box). Rats were immediately returned to the recording chambers and were either non-stimulated (represented by the continued LD schedule below), or optogenetically stimulated (vertical bars). Both groups of animals were recorded for the subsequent 7 days (SPS Day-Day8). At the conclusion of the recordings, all animals were unhooked and tested for fear-associated memory impairments using fear conditioning, extinction and recall (gray boxes).

Prism software statistical package. Average freezing levels between groups were compared on fear conditioning, extinction, and extinction recall days.

Experimental timeline. *Experiment 1.* Rats (n = 7) were injected with rAAV-MCH-ChR2-EGFP and tethered to stimulation/recording equipment baseline EEG/EMG signals were measured for 24 h from ZT12-ZT12 and followed by SPS (ZT12). After SPS, the animals were reconnected to the recording system and the optogenetic stimulation was not activated. Comparisons of duration in vigilance state were made vs. baseline (pre-trauma) recordings. Fear conditioning experiments proceeded at the conclusion of the EEG/EMG recording period (Fig. 1). Additional control experiments were performed at this time and compared baseline sleep in non-ChR2 expressing animals (rAAV-MCH-EYFP, optogenetically stimulated and non-stimulated) to non-AAV injected and ChR2 expressing animals (rAAV-MCH-ChR2-EYFP, optogenetically stimulated and non-stimulated) were made at this stage as per Blanco Centurion[25].

Experiment 2. As in Experiment 1, rats (n = 9) were injected with rAAV-MCH-ChR2-EGFP and tethered as described above. Baseline EEG/EMG signals were measured for 24 h from ZT12-ZT12, followed immediately by SPS (ZT12). After SPS, the animals were reconnected to the recording system and the optogenetic stimulation was activated. Comparisons of duration in vigilance state were made vs. baseline (pre-trauma) recordings. Fear conditioning experiments proceeded at the conclusion of the EEG/EMG recording period.

Results

Experiment 1. As a proof of concept of optogenetic stimulation increasing sleep time, same as in Blanco Centurion[25], we first made a comparison of baseline sleep duration from non-optogenetically-stimulated and stimulated rAAV-MCH-ChR2-EGFP injected animals to animals that received a control rAAV vector, and to non-virus injected animals. We found no difference in baseline sleep in either the control rAAV expressing or non-stimulated ChR2 expressing animals from non-virus injected animals, indicating that injection of virus alone had no effect on baseline sleep duration. Additionally, the control rAAV expressing animals showed no changes in sleep from their baseline when optically stimulated. Optogenetic stimulation of the rAAV-MCH-ChR2-EGFP expressing animals resulted in increased REM sleep during both the Dark and Light phase and increased Dark phase NREM sleep increases that replicate the results from previously published work with these two viruses[25]. As a result of these confirmatory control studies, we focused our subsequent efforts on determining the contribution of SPS with and without optogenetic stimulation in the ChR2 expressing animals.

SPS alters sleep and impairs fear-associated memory when given at "lights on" (ZT0)[17]. However, the impact of SPS on sleep and fear-associated memory processing when the trauma is presented at "lights off" (ZT12) was previously unexamined. Therefore, in order to determine the time-course of trauma-induced sleep alterations and fear-associated memory impairments, we exposed animals to SPS at the transition from "lights on-to lights off" (ZT 12). Our previous work showed that REM sleep was significantly increased in the Dark Phase during the 12 h after SPS trauma exposure and that NREM sleep duration was reduced during the Dark Phase 4–7 days after trauma[17]. Although time-of-day effects have been shown for other learning and memory processes, no work has been performed to determine the time-of-day effects of trauma exposure that leads to PTSD.

When delivered at ZT12, SPS resulted in a 43 min reduction in REM sleep during the Dark Phase following trauma, and 12 h later, during the subsequent Light Phase, SPS resulted in a 80 min increase in REM sleep (Fig. 2a) compared to their respective baseline measures. NREM sleep was reduced by 334.5 min during the Dark Phase immediately following SPS exposure and was unchanged from baseline for the remainder of the experiment (Fig. 2b). SPS exposure increased Wake duration by 380 min during the Dark Phase immediately following the trauma (Fig. 2c).

Sleep architecture was significantly altered by SPS exposure at ZT12. REM bout length increased by 25 min during the Light Phase on the SPS exposure day as compared with baseline (Fig. 3a). NREM and Wake bout length was unchanged compared to baseline at any point during the experiment (Fig. 3b,c). REM bout number increased day over day during the Dark Phase of the experiment, reaching statistical significance on Day 8 (Fig. 3d). SPS exposure resulted in a 38% and 36% reduction in the number of NREM and Wake bouts, respectively, during the Dark Phase compared to baseline (Fig. 3e,f).

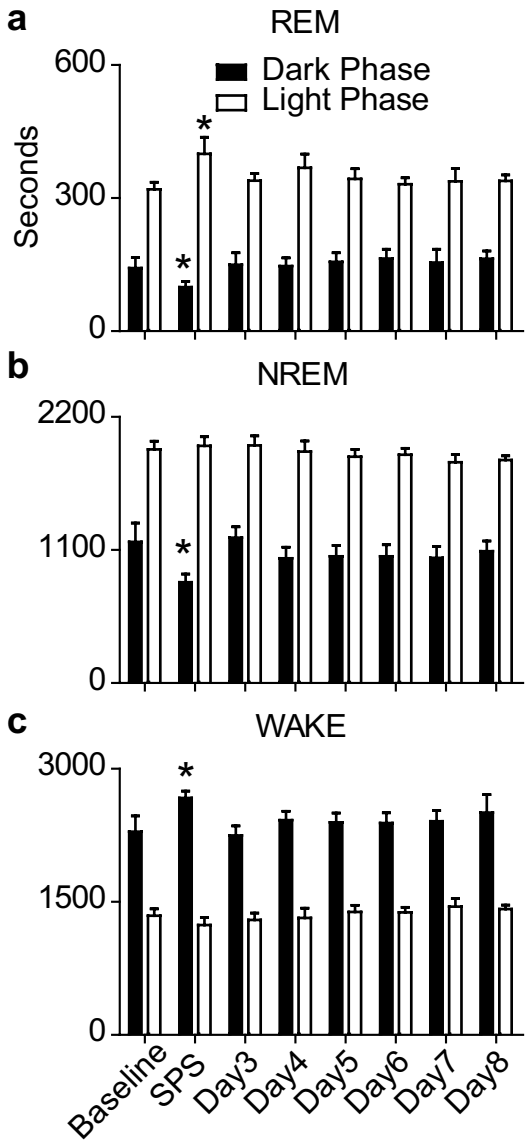

Figure 2. Sleep/Wake responses before and after SPS. Each bar represents the duration (seconds) spent in (**a**) REM, (**b**) NREM, and (**c**) Wake, averaged per hour over the 12 h Dark (or active) Phase (black bars), and the 12 h Light (or sleep) Phase (white bars) over the course of the 8 day experiment. One-way ANOVA was conducted to assess the effects of SPS on (**a**) REM, (**b**) NREM, and (**c**) WAKE duration over the Dark and Light phases. (**a**) REM sleep was significantly altered by SPS exposure during both the Dark Phase ($F(7,55) = 3.3$, $p = 0.007$) and Light Phase ($F(7,55) = 1.6$, $p = 0.05$). Bonferroni *post hoc* comparisons revealed that REM sleep duration was significantly reduced during the Dark Phase on the SPS day ($M = 99.1 \pm 13$ s) compared to baseline ($M = 142.0 \pm 23.6$ s). REM sleep during the Light Phase on the SPS day ($M = 399.9 \pm 37.2$ s) was significantly increased compared to baseline ($M = 319.5 \pm 16.4$ s). (**b**) NREM sleep was significantly altered by SPS exposure during the Dark Phase ($F(7,55) = 5.79$, $p < 0.0001$), but not during the Light Phase ($F(7,55) = 1.52$, $p = 0.19$). Bonferroni post hoc comparisons revealed that NREM sleep duration was reduced during the Dark Phase on the SPS day ($M = 833.5 \pm 67.5$ s) compared to baseline ($M = 1168.0 \pm 154.4$ s). (**c**) WAKE was significantly altered by SPS exposure during the Dark Phase ($F(7,55) = 2.74$, $p = 0.019$), but not during the Light Phase ($F(7,55) = 1.79$, $p = 0.12$). Bonferroni post hoc comparisons revealed that WAKE duration was significantly increased during the Dark Phase on the SPS day ($M = 2670 \pm 77.5$ s) compared to baseline ($M = 2290.0 \pm 177.2$ s) (*$p < 0.05$).

Experiment 2. The overarching goal of this work was to examine if increasing sleep via optogenetic stimulation of MCH containing cells would restore fear-associated memory functioning in SPS trauma-exposed rats. SPS with optogenetic stimulation (Opto Stim) resulted in a 75 min reduction in REM sleep during the Dark Phase following trauma. 12 h later, during the Light Phase, REM sleep increased 105 min compared to baseline. In addition, REM sleep showed daily increases during the Dark Phase of the experiment that reached statistical significance by Day 8 (Fig. 4a). NREM sleep was reduced by 392 min during the Dark Phase immediately fol-

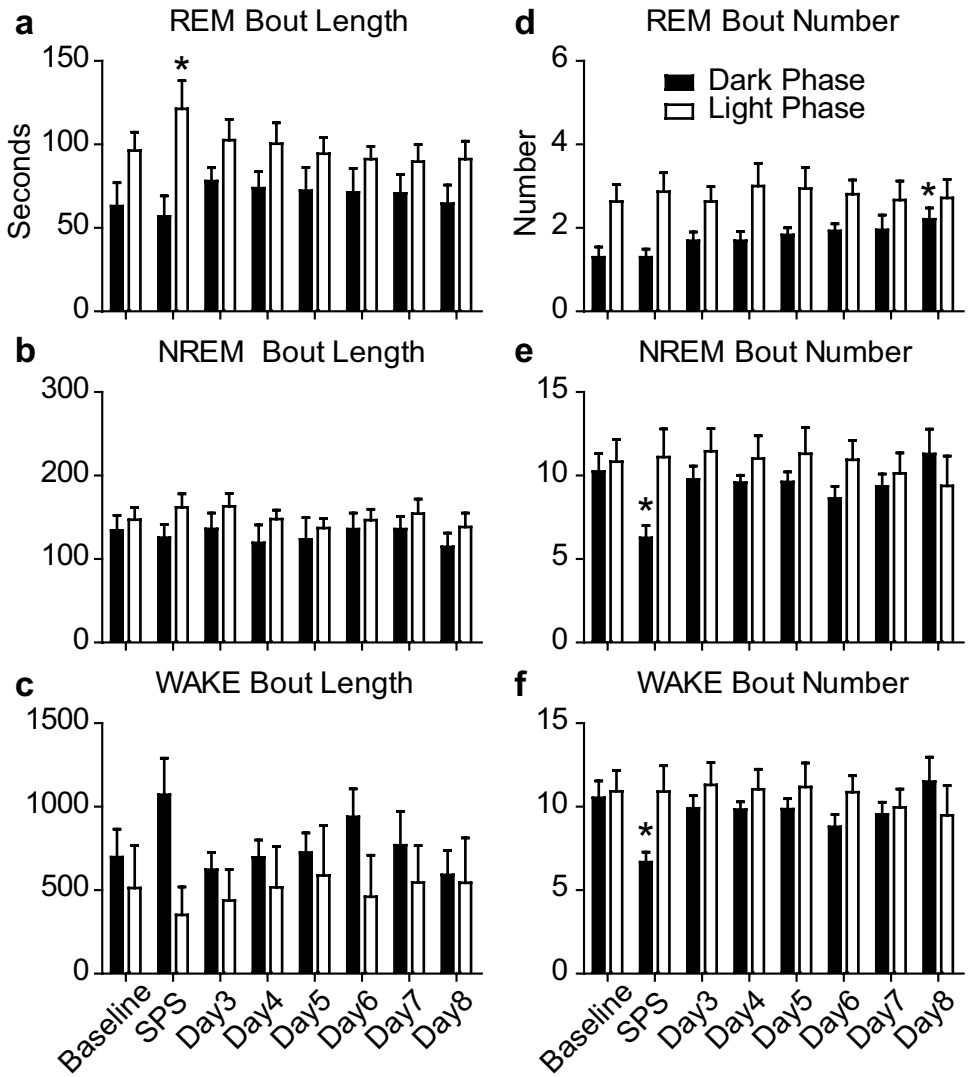

Figure 3. Sleep architecture before and after SPS. Each bar represents the average bout length (**a**–**c**) or average bout number (**d**–**f**) per hour over the 12 h Dark (or active) Phase (black bars), and the 12 h Light (or sleep) Phase (white bars) over the course of the 8 day experiment. One-way ANOVA was conducted to compare the effects of SPS on REM, NREM, and WAKE bout length and number over the Dark and Light phases. (**a**) REM bout length was significantly altered by SPS exposure during the Light Phase ($F(7,55) = 3.38$, $p = 0.006$) but not the Dark Phase ($F(7,55) = 0.65$, $p = 0.71$). Bonferroni post hoc comparisons revealed that REM sleep bout length was significantly increased during the Light Phase on the SPS day ($M = 121.3 \pm 16.8$ s) compared to baseline ($M = 96.3 \pm 10.9$ s). (**b**) NREM bout length was not significantly altered by SPS exposure during the Light Phase ($F(7,55) = 0.88$, $p = 0.53$) or Dark Phase ($F(7,55) = 0.90$, $p = 0.52$). (**c**) WAKE bout lengths were similarly not altered by SPS exposure during the Light Phase ($F(7,55) = 1.79$, $p = 0.11$) or Dark Phase ($F(7,55) = 1.75$, $p = 0.12$). (**d**) REM bout number was significantly altered by SPS exposure during the Dark Phase ($F(7,55) = 2.81$, $p = 0.017$) but not the Light Phase ($F(7,55) = 0.54$, $p = 0.80$). Bonferroni post hoc comparisons revealed REM sleep bout number was significantly increased during the Dark Phase on day 8 ($M = 2.2 \pm 0.22$) compared to baseline ($M = 1.3 \pm 0.24$). (**e**) NREM bout number was significantly altered by SPS exposure during the Dark Phase ($F(7,55) = 3.59$, $p = 0.004$) but not the Light Phase ($F(7,55) = 0.47$, $p = 0.85$). Bonferroni post hoc comparisons revealed NREM sleep bout number was significantly reduced during the Dark Phase immediately following SPS ($M = 6.3 \pm 0.72$) compared to baseline ($M = 10.24 \pm 1.1$). (**f**) WAKE bout number was significantly altered by SPS exposure during the Dark Phase ($F(7,55) = 3.39$, $p = 0.006$) but not the Light Phase ($F(7,55) = 0.54$, $p = 0.80$). Bonferroni post hoc comparisons revealed WAKE bout number was significantly decreased during the Dark Phase on the SPS day ($M = 6.68 \pm 0.6$) compared to baseline ($M = 10.54 \pm 1.0$) (*$p < 0.05$).

lowing SPS exposure and, similar to REM, showed daily increases over the Dark Phase that reached statistical significance on Day 8 (Fig. 4b). SPS exposure resulted in an increase of 468 min of Wake compared to baseline

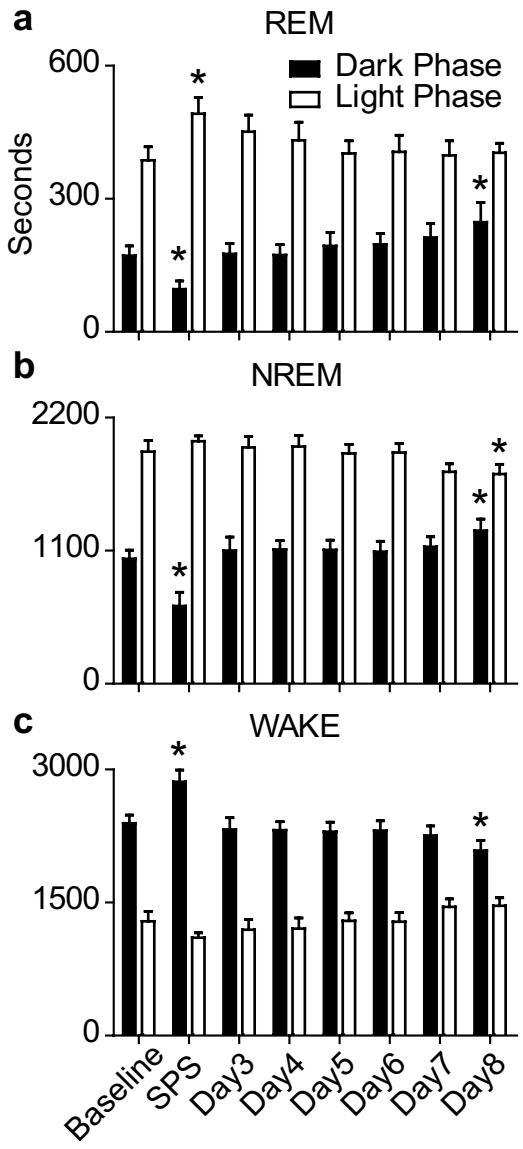

Figure 4. Sleep profile of SPS exposed animals with concurrent optogenetic stimulation of MCH containing cells of the lateral hypothalamus. Sleep/Wake responses before and after SPS with concurrent, 24 h, 10 Hz, 1 min on, 4 min off repeating optogenetic stimulation. Each bar represents the duration (seconds) spent in (**a**) REM, (**b**) NREM, and (**c**) Wake, averaged per hour over the 12 h Dark Phase (black bars), and the 12 h Light Phase (white bars) over the course of the 8 day experiment. One-way ANOVA was conducted to compare the effects of SPS and optogenetic stimulation on (**a**) REM, (**b**) NREM, and (**c**) WAKE duration over the Dark and Light phases. (**a**) REM sleep was significantly altered by SPS exposure and optogenetic stimulation during both the Light Phase ($F(7,71) = 2.31$, $p = 0.04$) and Dark Phase ($F(7,71) = 6.14$, $p < 0.0001$). Bonferroni post hoc comparisons revealed that REM sleep duration was significantly increased during the Light Phase on the SPS exposure day ($M = 491.7 \pm 37.2$ s) compared to baseline ($M = 386.7 \pm 30.5$ s). REM sleep during the Dark Phase was significantly decreased on the SPS exposure day ($M = 96.1 \pm 18.9$ s) compared to baseline ($M = 171.9 \pm 21.9$ s) and showed additional, post-SPS, daily increases in REM sleep duration that reached statistical difference from baseline on Day 8 ($M = 247.2 \pm 44.6$ s). (**b**) NREM sleep was significantly altered by SPS exposure and optogenetic stimulation during the Dark Phase ($F(7,71) = 12.14$, $p < 0.0001$), and Light Phase ($F(7,71) = 4.76$, $p = 0.0003$). Bonferroni post hoc comparisons revealed that NREM sleep duration was significantly decreased during the Dark Phase immediately following SPS ($M = 641.9 \pm 114.6$ s) compared to baseline ($M = 1034.0 \pm 70.6$ s) and showed increased NREM sleep time on Day 8 ($M = 1267.0 \pm 95.1$ s). NREM sleep duration was significantly decreased during the Light Phase on Day 8 ($M = 1733 \pm 62.3$ s) compared to baseline ($M = 1918.0 \pm 91.4$ s). (**c**) WAKE was significantly altered by SPS exposure and optogenetic stimulation during the Dark Phase ($F(7,71) = 14.27$, $p < 0.0001$), but not during the Light Phase ($F(7,71) = 18.12$, $p = 0.0002$). Bonferroni post hoc comparisons revealed that WAKE duration was significantly increased during the Dark Phase on the SPS exposure day ($M = 2862.0 \pm 131.2$ s) compared to baseline ($M = 2394.0 \pm 91.2$ s) and reduced by Day 8 ($M = 2086.0 \pm 114.0$ s). There were no significant changes from baseline during the Light Phase (*$p < 0.05$).

during the Dark Phase immediately following the trauma and a reduced duration spent awake on Day 8 that concurs with the increases in REM and NREM sleep duration on that day (Fig. 4c).

Sleep architecture was significantly altered by SPS exposure and concurrent optogenetic stimulation. REM bout length was significantly increased during the Dark Phase on the Day 8 (Fig. 5a). NREM bout length was significantly increased during the Light Phase on Day 4 above baseline (Fig. 5b). Wake bout length was unchanged compared to baseline at any point during the experiment (Fig. 5c). REM bout number decreased during the Dark Phase and increased during the subsequent Light Phase on the SPS exposure day (Fig. 5d). SPS exposure significantly reduced NREM and Wake bout number during the Dark Phase and resulted in a subsequent decrease in bout number on days 3–8 (Fig. 5e,f).

To visualize if optogenetic stimulation was effective at increasing sleep over the course of the experiment, we plotted the cumulative change in sleep for the Dark and Light Phases independently over the course of the experiment (as a change from baseline). Optogenetic stimulation was sufficient to cause daily increases in REM sleep during both the Dark and Light Phases as the slopes for both lines are significantly non-zero (Fig. 6a). NREM sleep was significantly increased over the course of the experiment only during the Dark Phase. The slope of the cumulative change in NREM sleep during the Light Phase was not different from zero (Fig. 6b). Similar to the NREM sleep duration, the effect of optogenetic stimulation of MCH cells resulted in a significant decrease in Wake over the Dark Phase of the experiment, but not during the Light Phase (Fig. 6c). Similarly, when optogenetically stimulated animals were cumulatively compared to control (non-optogenetically stimulated) SPS treated animals, a similar pattern of increased sleep emerged (Figure S2).

Fear-associated memory impairments are associated with SPS exposure and fear extinction recall impairments have been correlated to PTSD-like severity in this animal model. Therefore, given that optogenetic stimulation is sufficient to increase REM sleep during both the Light and Dark phase and NREM sleep during the Dark Phase following SPS we examined if this increasing sleep could rescue the cognitive deficits typically seen following SPS trauma exposure. At the conclusion of the EEG/EMG recordings, animals were unhooked from the recording tethers and exposed to the fear conditioning/extinction/recall protocol described in the methods to determine the effect of sleep restoration on the development of fear-associated memory impairments. Data were compared between control and optogenetically stimulated experimental animals using Students t-test. No differences were detected following the initial 5 tone/shock pairings in context B of fear conditioning (Fig. 7a) nor during the 30 trial, tone only presentations in context A of the extinction period (Fig. 7b). During the 10 trial, tone only presentations in context A of the Recall phase of the test (Fig. 7c), optogenetically stimulated animals showed a 33% reduction in freezing behavior compared to Control animals.

Discussion

The timing of trauma exposure is unpredictable and trauma-exposed human populations show variability in their resulting trauma-exposure-dependent sleep phenotypes[27–29]. Therefore, it is likely that the time-of-day plays a role in the manifestation of trauma-induced changes in sleep. Trauma-type or trauma-intensity may also be confounding factors in human studies. However, our use of SPS alleviates variability that may be introduced due to these factors; further solidifying a role for time-of-day in mediating trauma-exposure-dependent sleep phenotypes. The data presented from control animals (Figs. 2, 3) show an acute increase in REM sleep, 12 h after SPS exposure. This 12 h delay in increased REM sleep is a consistent phenotype across this study and our previous work[17]. However, sleep phenotypic differences remain between these data and our previous report. For instance, our previous work identified a significant SPS-induced, Dark Phase reduction of NREM sleep starting 4 days after SPS. These data also identified increased Wake activity during the Dark Phase concurrent with the decrease in NREM sleep. These results are lacking in the current experiments. It is possible that SPS results in acute and chronic sleep changes when the trauma is presented at ZT0 and only acute changes in sleep when presented at ZT12. A close examination of the data in Fig. 2 shows that NREM and Wake activity is trending towards those values seen when SPS is presented at ZT0, but the data are not statistically significant. It is possible that the development of this chronic sleep phenotype requires more time to develop when trauma occurs at ZT12. Sleep in the SPS model has not been examined past 7 days post-SPS and future studies should examine the long-term impact of SPS trauma exposure on sleep.

Consistent with a prior report, baseline sleep is not altered by expression of GFP control virus or ChR2 in the absence of stimulation[25]. Therefore, we chose to examine sleep and fear-associated memory in ChR2 expressing animals that were either stimulated or not stimulated. Non-virus expressing animals and GFP expressing control animals were therefore not examined over the course of the experiment. However, in our laboratory, optogenetic stimulation of MCH cells was sufficient to increase REM sleep following SPS during both the Light and Dark Phases of the circadian day (Figs. 4, 5, 6). Interestingly, NREM sleep showed significant cumulative increases from baseline only during the Dark Phase, but not during the Light Phase. Although curious, these data replicate findings by Blanco Centurion et al.[25] showing that 10 Hz optogenetic stimulation was effective to increase REM sleep during the Light and Dark Phase and NREM sleep only during the Dark Phase. This study examined optogenetic stimulation over 7 days and showed that although the increases in REM and NREM sleep from optogenetics may have been incremental, but were still sufficient to improve function on a fear-associated memory task. It is possible that long-term optogenetic stimulation may reach a ceiling where further stimulation does not enhance sleep any longer, but our data indicate that for at least 7 days it is effective. Further, although we did not verify expression of the viral construct in every animal tested, the behavioral changes we see in sleep and in response to fear recall are robust enough to suggest that expression of the channel was widespread and effective at improving fear-associated learning impairments.

For these studies, our primary outcome measure was to examine fear-associated memory impairment (as measured by freezing in response to foot shock and/or a conditioned stimulus tone) and compare optogenetically

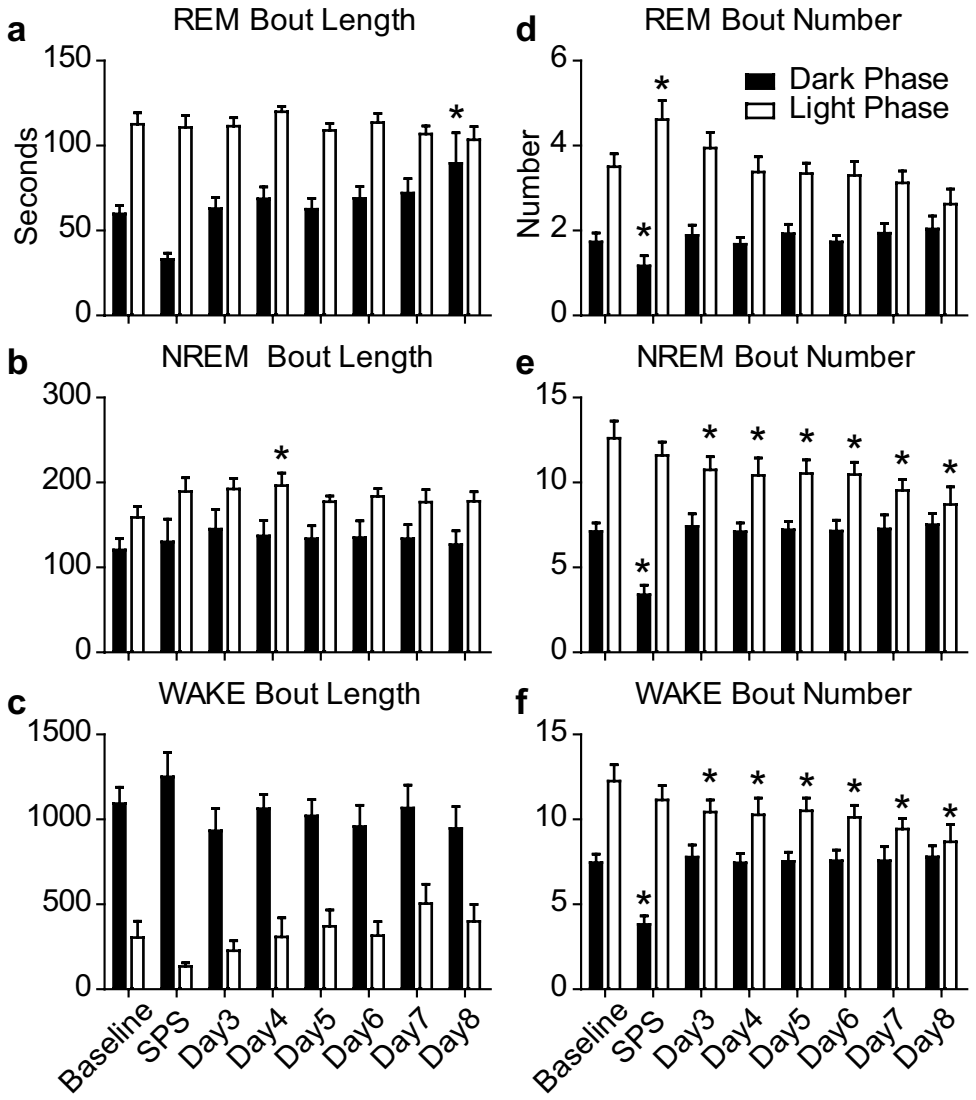

Figure 5. Sleep architecture alterations before and after SPS with combined optogenetic stimulation of MCH containing cells of the lateral hypothalamus. Each bar represents the average bout length (**a**–**c**) or average bout number (**d**–**f**) per hour over the 12 h Dark (or active) Phase (black bars), and the 12 h Light (or sleep) Phase (white bars) over the course of the 8 day experiment. One-way ANOVA was conducted to compare the effects of SPS and optogenetic stimulation on REM, NREM, and WAKE bout length and number over the Dark and Light phases. (**a**) REM bout length was significantly altered by SPS exposure during the Dark Phase ($F(7,71) = 4.65$, $p = 0.0004$) but not the Light Phase ($F(7,71) = 0.81$, $p = 0.58$). Bonferroni post hoc comparisons revealed that REM sleep bout length was significantly increased during the Dark Phase on the Day 8 ($M = 89.57 \pm 18.1$ s) compared to baseline ($M = 59.8 \pm 5.0$ s). (**b**) NREM bout length was significantly altered by SPS exposure during the Light Phase ($F(7,71) = 1.8$, $p = 0.01$) but not during the Dark Phase ($F(7,71) = 0.61$, $p = 0.74$). Bonferroni post hoc comparisons of light phase NREM bout lengths were significantly increased on Day 4 ($M = 196.3 \pm 14.9$) compared to baseline ($M = 158.9 \pm 12.9$). (**c**) WAKE bout lengths were altered by SPS exposure during the Light Phase ($F(7,71) = 4.9$, $p = 0.0002$) but not during the Dark Phase ($F(7,71) = 1.4$, $p = 0.21$). Bonferroni post hoc comparisons of Light Phase WAKE bout length revealed no significant changes from baseline. (**d**) REM bout number was significantly altered by SPS exposure during the Dark Phase ($F(7,71) = 3.9$, $p = 0.002$) and the Light Phase ($F(7,71) = 5.3$, $p = 0.0001$). Bonferroni post hoc comparisons revealed REM sleep bout number was significantly increased during the Light Phase on the SPS exposure day ($M = 4.6 \pm 0.45$) compared to baseline ($M = 3.5 \pm 0.31$) and REM sleep bout number was significantly reduced during the Dark Phase on the SPS exposure day ($M = 1.2 \pm 0.24$) compared to baseline ($M = 1.7 \pm 0.21$). (**e**) NREM bout number was significantly altered by SPS exposure during the Dark Phase ($F(7,71) = 5.9$, $p < 0.0001$) and the Light Phase ($F(7,71) = 9.9$, $p < 0.0001$). Bonferroni post hoc comparisons revealed NREM sleep bout number was significantly reduced during the Dark Phase immediately following SPS ($M = 3.4 \pm 0.56$) compared to baseline ($M = 7.1 \pm .49$) and during the Light Phase, NREM bout number was significantly reduced from baseline on days 3–8. (**f**) WAKE bout number was significantly altered by SPS exposure during the Dark Phase ($F(7,71) = 10.5$, $p = 0.0001$) and the Light Phase ($F(7,71) = 5.2$, $p = 0.0001$). Bonferroni post hoc comparisons revealed WAKE bout number was significantly decreased during the Dark Phase on the SPS day ($M = 3.8 \pm 0.5$) compared to baseline ($M = 7.5 \pm 0.5$) and significantly decreased from baseline on days 3–8) (* $p < 0.05$).

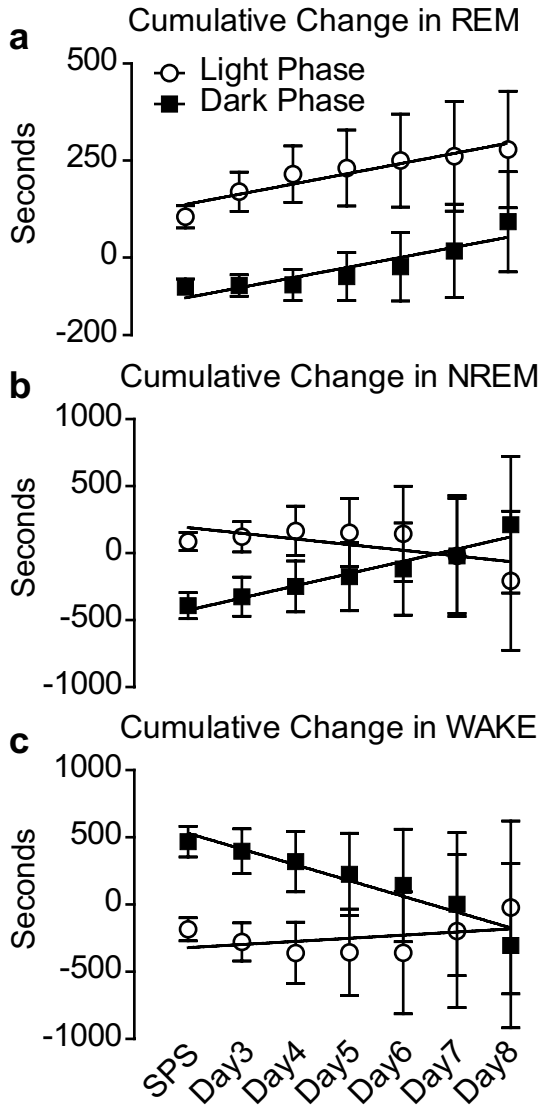

Figure 6. Continuous optogenetic stimulation of MCH containing cells of the LH following SPS results in cumulative changes in sleep/wake. The cumulative time spent in sleep/wake stage compared to baseline is shown for each day over the 12 h Dark Phase (black squares), and the 12 h Light (or sleep) Phase (white circles) over the course of the experiment. (**a**) Linear regression analysis revealed that REM sleep increased during both the Dark Phase ($r^2 = 0.83$, $p = 0.0045$) and the Light Phase ($r^2 = 0.89$, $p = 0.0013$) over the course of the experiment. (**b**) NREM sleep showed cumulative sleep time increases specific to the Dark Phase ($r^2 = 0.94$, $p = 0.0004$) whereas, NREM sleep time was unchanged during the Light Phase ($r^2 = 0.46$, $p = 0.09$). (**c**) Similar to the cumulative change in NREM, the cumulative changes in WAKE showed a significant reduction during the Dark Phase over the course of the experiment ($r^2 = 0.92$, $p = 0.007$) whereas WAKE was unchanged during the Light Phase of the experiment ($r^2 = 0.15$, $p = 0.37$).

stimulated animals to their non-stimulated controls. Freezing during the fear-conditioning trial was not different between the optogenetically stimulated and non-stimulated groups. Freezing on the extinction day, was again, not different between the optogenetically stimulated and non-stimulated groups. However, on the fear-recall day, optogenetically stimulated animals showed reduced freezing compared to their non-stimulated controls, indicating that they consolidated the memory of the extinction better and did not display the fear-extinction deficits that typify SPS exposure[17,20,30]. We interpret these results to mean that post-SPS sleep improvements were sufficient to improve subsequent fear-associated memory processing. These results are in line with findings in trauma exposed humans that show that poor sleep increases the likelihood of acquiring PTSD[31] and that improved sleep aids in the processing of emotional stimuli[32] and for the elimination of intrusive memories[33,34]. These results are interesting due to the well described contribution of sleep in memory processing[35–38] and in the molecular mechanisms that regulate memory[39]. Post-training sleep deprivation has been shown to impair memory consolidation for associative tasks[40], contextual fear memory processing[41], and fear avoidance[42,43]. In

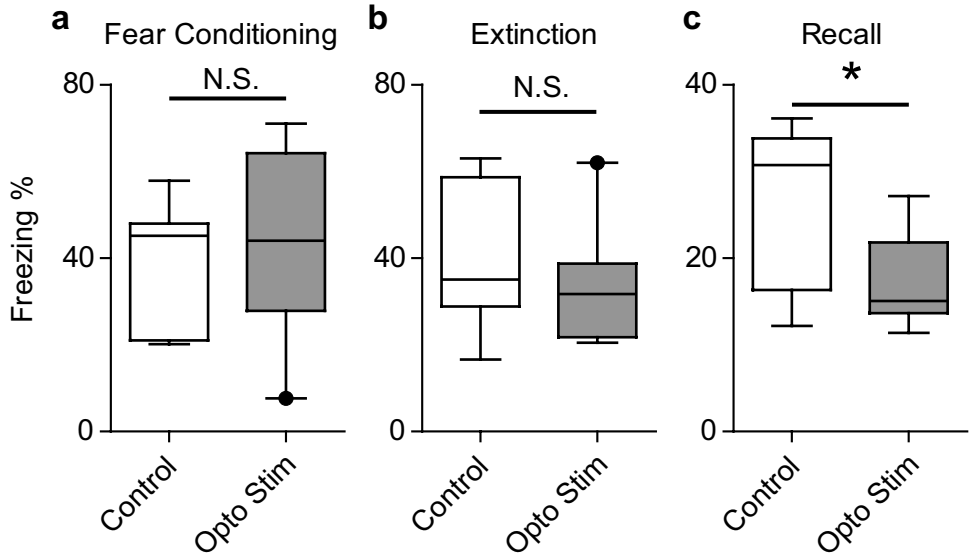

Figure 7. Fear-associated memory impairments are attenuated by optogenetic stimulation of MCH cells. Animals injected with rAAV-MCH-ChR2-EGFP were exposed to SPS and EEG/EMG recordings were made for 7 subsequent days. Control animals (those in Figs. 2 and 3) did not receive optogenetic stimulation, while another group (those in Figs. 4, 5, 6) received 7 days of optogenetic stimulation. At the conclusion of the stimulation, all animals underwent fear testing as described in the methods section. (**a**) After the fear conditioning day, optogenetically stimulated (Opto Stim) animals (M = 43.8, SEM = 6.9) and non-stimulated (Control) animals (M = 39.8, SEM = 5.3) showed no difference in freezing ($t(14) = 0.44$, $p = 0.67$). (**b**) 24 h later, fear memory extinction was examined and, again, Opto Stim (M = 32.6, SEM = 4.4) and control animals (M = 41.5, SEM = 6.8) had similar freezing levels ($t(14) = 1.14$, $p = 0.27$). (**c**) 48 h after initial fear conditioning, fear recall was examined and Opto Stim animals (M = 17.4, SEM = 1.8) showed significantly reduced freezing compared to non-stimulated Control animals (M = 26.0, SEM = 3.7) ($t(14) = 2.3$, $p = 0.04$) (*$p < 0.05$).

our model, we have improved sleep after SPS and have presumably consolidated the memory of the original trauma and yet have improved function on a subsequent fear-associated memory task. It is possible, therefore, that sleep following trauma exposure is performing a different function than sleep after a fear-associated task that does not result in PTSD-like phenotypes. Therefore, a distinction must be made between sleep alterations that accompany trauma exposure (SPS in this case) that results in long term impairment of neurobiological systems, and fear-associated memory tasks (fear conditioning) that are designed to probe how an animal deals with a stressor that has no long lasting impact on the underlying biology. In our studies, the optogenetic stimulation was discontinued prior to the fear-associated memory tasks and therefore the impact of post-fear-conditioning optogenetic stimulation on fear-associated memory processing remains unknown. However, our discontinuation of optogenetic stimulation at this time may have serendipitously revealed a window of time that is sufficient to improve subsequent fear-memory processing after trauma exposure since these animals did show reduced freezing on the recall day. Interestingly, there is also a trend towards better performance on the extinction task in optogenetically stimulated animals. Although not significant, these data suggest that extinction memory related molecular mechanisms tied to sleep need to be further explored. Additional future studies should include an examination of post fear-conditioning sleep and the subsequent result of post fear-conditioning sleep deprivation and/or optogenetic sleep improvements on fear-recall outcomes in trauma-exposed and control animals.

The optogenetically stimulated animals showed more REM sleep and better performance on a subsequent fear-associated memory task. These data are consistent with the *sleep to forget and sleep to remember* model of emotion processing posed by Walker et al.[44,45]. This model posits that REM sleep provides an optimal neurobiological setting for the reduction of affective tone for any given emotional memory while preserving the memory itself. This theory states that REM sleep is beneficial to cognitive processing of emotional memories and that REM sleep functions to reduce the affective tone of an emotionally enriched memory. Therefore, the optogenetic stimulation may have reset trauma exposed animals back to a normal level of functioning. Conversely, our previous data[17] showed that impairment on a fear-associated memory task was correlated to increased REM sleep immediately following trauma exposure. Additional human studies have also linked sleep to the preservation of emotional memories[46]. These are seemingly contradictory data, however, it is possible that timing and duration of sleep helps to determine specific outcomes. For example, increased REM immediately after trauma may increase memory consolidation of the traumatic event, while continued REM sleep increases help to remove the affective tone from that same experience (via *sleep to forget and sleep to remember* mechanisms). REM specific and total sleep deprivations following trauma exposure are required to fully assign function to this sleep state in improving subsequent fear-associated memory processing. Lastly, our current data show that NREM sleep increased over the Dark Phase of the experiment. It is possible that NREM sleep is the critical component in fear-associated

memory performance following traumatic stress. These data suggest that further work is required to isolate the components of REM or NREM sleep responsible for this behavioral restoration.

For years, researchers have argued that therapeutics designed to improve sleep may be required for the restoration of function in PTSD[2]. Yet, a primary treatment for PTSD remains the SSRI class of drugs which have been shown to reduce or abolish REM sleep altogether[47,48]. Many other drug classes are reported to decrease REM sleep (e.g., antiarrhythmics, alcohol, benzodiazepines, corticosteroids, diuretics and of course, caffeine), while only a handful are accompanied by REM sleep increases in humans. For example, in normal subjects, melatonin increased percent of time in REM and REM episode duration compared to their baseline[49]. This effect of melatonin administration was also confirmed in subjects with REM sleep disruptions[50]. Dopamine, norepinephrine and serotonin reuptake inhibitors (reserpine and bupropion), alpha adrenergic blockers (thymoxamine and mesoridazine) selective serotonin antagonists (nefazedone and ritanserine), and cholinergic agonists (carbachol and bethanechol) all increase REM sleep[51–56]. These therapeutics may provide promising ways to mitigate the adverse symptoms of PTSD.

Non-pharmacological approaches to decreasing REM are uncommon, while increasing ambient temperature and humidity increases REM sleep in rodents[57], this does not translate to humans and can disrupt NREM and REM sleep[58,59].

Increasing sleep was sufficient to rescue trauma-related fear-associated memory defects, however, the downstream physiological mechanisms for this result are unknown. Additionally, people with PTSD show impairment in memory recall for non-fear-based tasks that may also require sleep to function properly. Future studies are required to examine the contribution of sleep improvements on non-fear based cognitive function and to determine how optogenetic manipulation alters the underlying neuro-circuitry involved in fear, memory, and cognition.

Data availability

Data will be made available upon request.

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Author contributions

W.M.V. wrote the main manuscript text and figures. C.J.D. edited the manuscript and assisted with data analysis. All authors have reviewed the manuscript.

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RESEARCH ARTICLE

Single prolonged stress blocks sleep homeostasis and pre-trauma sleep deprivation does not exacerbate the severity of trauma-induced fear-associated memory impairments

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Abstract

Sleep is intimately linked to cognitive performance and exposure to traumatic stress that leads to post-traumatic stress disorder (PTSD) impairs both sleep and cognitive function. However, the contribution of pre-trauma sleep loss to subsequent trauma-dependent fear-associated memory impairment remains unstudied. We hypothesized that sleep deprivation (SD) prior to trauma exposure may increase the severity of a PTSD-like phenotype in rats exposed to single prolonged stress (SPS), a rodent model of PTSD. Rats were exposed to SPS alone, SD alone, or a combination of SPS+SD and measures of fear-associated memory impairments and vigilance state changes were compared to a group of control animals not exposed to SPS or SD. We found that SPS, and SPS+SD animals showed impaired fear-associated memory processing and that the addition of SD to SPS did not further exaggerate the effect of SPS alone. Additionally, the combination of SPS with SD results in a unique homeostatic sleep duration phenotype when compared to SD, SPS, or control animals. SPS exposure following SD represses homeostatic rebound and eliminates sleep-deprivation-induced increases in NREM sleep delta power. This work identifies a unique time frame where trauma exposure and sleep interact and identifies this window of time as a potential therapeutic treatment window for staving off the negative consequences of trauma exposure.

Introduction

According to the National Institutes of Health and Institute of Medicine, 50–70 million US adults suffer from a sleep disorder that effectively interferes with getting sufficient nighttime sleep. Lack of sleep impairs physical and cognitive functioning and sleep disturbances are often comorbid with and may exacerbate other mental or physical health impairments [1–4]. However, it is unknown if sleep disturbances contribute to increased susceptibility to psychiatric illness. For example, it is unclear if poor sleep prior to trauma exposure increases the

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likelihood of developing post-traumatic stress disorder (PTSD). PTSD occurs as the result of experiencing a physical or psychological trauma [5] and presents with hallmark traits of sleep disturbances including insomnia, nightmares, and sleep fragmentation [6–8]. Sleep disturbances are highly prevalent in PTSD, in fact, as many as 70–91% of PTSD patients have reported difficulty falling asleep and reduced sleep efficiency [6]. Poor sleep has been hypothesized to exaggerate PTSD symptoms creating a reciprocal relationship between sleep and PTSD wherein trauma exposure impairs sleep and trauma-induced sleep disorders increase the severity of PTSD [6, 9]. Still, the contribution of sleep loss prior to trauma exposure on the development or susceptibility to PTSD is unknown. Therefore, we examined whether sleep deprivation prior to trauma exposure alters subsequent sleep or the development of trauma-induced fear-associated memory impairments.

To achieve our goals, we utilized the single prolonged stress (SPS) rat model of PTSD. SPS is a combination of stressors that includes physical restraint, 20 min of forced swim, exposure to ether vapors until anesthetized, and 7 days of social isolation [10], and has consistently shown good face validity to the human condition of PTSD [10–13]. In this model, fear-associated memory impairments are used as a surrogate for the severity of the PTSD-like phenotype [14, 15] giving us a reliable metric to assess the contribution of pre-trauma sleep loss to the development of a PTSD-like phenotype.

In the present study, we compared measures on a fear-associated memory task in SPS exposed animals, sleep deprived (SD) animals, and SPS+SD treated groups to control animals. Sleep deprivation occurred during the animal's primary sleep phase (ZT0-12, lights on) and because SPS exposure occurred at ZT0 in our previously published work and due to circadian effects on learning and memory [16, 17], we also hypothesized that SPS exposure at ZT12, the onset of the predominantly active period in nocturnal rodents may have unique contributions to the effect of SPS trauma exposure on fear-associated memory.

Materials and methods

Animals

All animal procedures were carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approved by the *Washington State University Committee on the Use and Care of Laboratory Animals*.

Male, Long Evans rats were used for all experiments to eliminate the known impact of estrous cycle hormones on sleep and behavior [18–20]. Rats (60–90 days old) were housed in temperature (21˚C -24˚C) and humidity-controlled (30–70%) rooms on a 12:12 light-dark cycle and given *ad-libitum* access to food and water. The animals underwent one survival surgery to implant electroencephalographic (EEG) and electromyographic (EMG) recording electrodes (described below). Animals were housed singly following surgical procedures.

Experimental timeline

Animals were randomly assigned to the following 4 groups: control (no SPS, no SD, n = 9), SPS (SPS only, n = 9), SD (SD only, n = 9) or a combination of SPS and SD (SPS+SD, n = 14). All animals were recorded for a 24h baseline. Control animals were left untouched for the remainder of the experiment whereas, the sleep deprived animals (SD, and SPS+SD) experienced 12h of sleep loss during their primary sleep phase (ZT0-12, labeled SD in Fig 1). This was followed by SPS trauma exposure occurred at the transition from lights-on to lights-off (ZT12) in the SPS and SPS+SD groups (blue box in Fig 1 represents the time point of SPS treatment). After SPS, all groups were isolated for 7 more days. On the eighth day, all groups

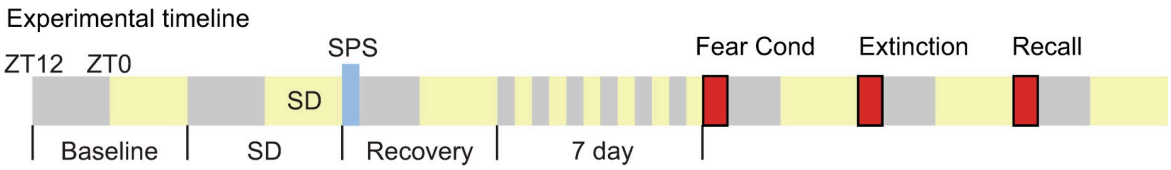

Fig 1. Schematic representation of experimental timeline. Baseline sleep was recorded for 24h. On the following day, SD animals underwent 12h of sleep deprivation from ZT0-ZT12 during their primary sleep phase while non-deprived animals were left alone. At the start of the 3rd day of recording, the SPS and SPS+SD groups were exposed to SPS at ZT12 (blue box) and then returned to their home cage for 7 subsequent days. At the conclusion of the 7-day SPS incubation period, all four groups of animals were exposed to the fear conditioning/extinction/recall paradigm (red boxes) as described in the methods section.

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underwent fear-associated memory testing which included one session each of fear conditioning, extinction and recall as described below (red boxes, Fig 1).

Electrophysiology

Survival stereotaxic surgery was performed to implant the EEG/EMG sleep recording electrodes as previously published [13, 21]. Aseptic surgeries were performed under isoflurane anesthesia. A midsagittal incision was made on the top of the skull and the skin was retracted. After cleaning the surface of the skull, 4 holes were drilled through the cranium and screw electrodes (Plastics One, Roanoke, VA) were inserted bilaterally over the frontal area (±2.5 mm lateral to midline, 2.5 mm anterior to Bregma) and over the hippocampal area (±2.5 mm lateral to midline, 3.5 mm posterior to Bregma) for EEG recordings. Two flexible wire electrodes were threaded through the dorsal neck muscles for EMG recordings. Gold pins were connected to the ends of each electrode then placed into a six-pin connector (Plastics One, Roanoke, VA) which was attached to the skull via light-curable dental acrylic. Electronic connections were finalized through the six-pin connector to a Tucker Davis Technology (TDT) (Alachua, FL) electrophysiology recording device. Rats were given at least 10 days to recover from surgery prior to beginning the experiment. Animal well-being was assessed daily during the surgical recovery period. Any sign of illness or pain, including decreased motility and responsiveness, vocalizations, lack of appetite, decreased grooming, etc. was noted and treated in consultation with veterinary staff.

Sleep recording and analysis

Following recovery from surgery, animals were housed individually and connected to the TDT recording system via a lightweight, flexible tether attached to a commutator (Sparkfun.com, Slip Ring) for free movement within the cage. The recording system was used to sample signals at 333 Hz, filtered between 0.1–100 Hz and amplified. Prior to analysis, signals were down sampled to 250 Hz. The two EEG electrodes were differentially referenced to obtain one EEG channel. Two EMG channels were also differentially referenced to obtain the EMG signal. Animals were given 48h to acclimate to the tethers prior to beginning baseline recordings. During the acclimation period, animals were supplied food to last the duration of the EEG/EMG experimental recordings. While connected to tethers, animals were monitored daily for food, water, and health via visual inspection by a video monitoring system to avoid disturbing the animals.

Collected data were transferred from the recording PC, stored onto disk, and scored offline in 10-second epochs to determine sleep/waking state using Sleep Sign software (Nagano, Japan). Three vigilance states were assigned: Wake, REM sleep and NREM sleep. Wake consists of visible EEG theta activity and high EMG activity, REM sleep consists of clear, sustained

EEG theta activity and phasic muscle twitches on a background of low EMG, NREM sleep consists of high amplitude, synchronized EEG and low EMG activity.

EEG and EMG signals were recorded for 24h of baseline and during the SD, after which the animals were unhooked from the recording system and single prolonged stress (SPS, described below) was performed. Following SPS, animals were reconnected to the recording system and seven subsequent days were recorded and scored. Data collected after SPS were compared to the baseline recording day using Graphpad Prism software by two-way repeated measures ANOVA followed by Bonferroni post hoc comparisons of each day to the baseline. Sleep states were quantified as an average duration spent in state per hour (in seconds) over the light phase (ZT0-12) and dark phase (ZT12-24).

EEG spectral analysis was performed using SleepSign Software and averaged in 12- and 24h artifact-free epochs across the experimental condition. Spectral power was examined by individual sleep state in half Hz bins in the 0–20 Hz range using a Hanning window filter. We also report the average spectral power of theta (5-8Hz) during REM sleep and delta (1-4Hz) during NREM sleep. Student's t-tests were performed on these average data between baseline and recovery days as indicated.

Sleep deprivation

Animals were sleep deprived for 12h (ZT0-12) by gentle handling as previously published [22]. Animals were stimulated by gently touching the tail, body, or nose of the animal with a wooden tongue depressor or small paintbrush when the animal appeared to fall asleep or when EEG/EMG indicated a transition to a sleep state. Instrumented animals remained connected to the EEG/EMG recording device during this manipulation.

Single prolonged stress

Single prolonged stress was performed as previously published [10, 13, 21]. Briefly, animals were exposed to 3 successive stressors at the start of the dark phase. First, physical restraint was performed for 2h in custom built plexi-glass restraining devices. Next, the animals were placed in a (63.5W x 43D x 40H cm) plastic bin containing 21–24˚C water and were forced to swim in groups of 6–8 for 20 min. Following a 15-min recuperation period in a towel-lined bin, the animals were exposed to 60ml of ether vapors in a 2000cc isolation chamber until fully anesthetized (<5 min). Ether exposure is a critical component in the development of the PTSD phenotype in rats. Substitution of an alternative anesthetic such as isoflurane for ether is insufficient to cause extinction retention deficits in fear-associated memory processing [14]. After which the animals were returned to their EEG/EMG recording-cage where they were isolated for the following seven days (as shown in Fig 1).

Fear conditioning, fear extinction, and extinction recall

At the conclusion of the EEG/EMG recording, fear-associated memory tests were conducted. Fear conditioning, extinction, and extinction recall, hereafter referred to as recall, were performed as previously published [13, 15, 21]. All fear conditioning, extinction, and recall experiments were performed in four identical Noldus fear conditioning chambers (Wageningen, the Netherlands) (30.5W x 25.5D x 30.5H cm) containing a Shock Floor with current carrying metal bars, a wall-mounted speaker and in-chamber UV and white lighting. Test cages were housed in sound-attenuating boxes. Tones (2000 Hz, 80 dB) were delivered via speakers mounted in the housing of the test cages and controlled by data acquisition software (Noldus Ethovision XT14). Ceiling mounted cameras recorded behavior for analysis and Noldus Ethovision software was used to quantify freezing levels. Freezing values were analyzed with

Graphpad Prism software by one-way and two-way ANOVA where appropriate, followed by Bonferroni post-hoc comparisons.

Two unique contexts were created by manipulating olfactory and visual cues. Context A consisted of 50 ml of 1% acetic acid solution placed in a small dish next to the test cage using standard lighting conditions of the above-mentioned housing boxes. Context B consisted of 50 ml a 1% ammonium hydroxide solution placed in a small dish above the test cage along with a checkerboard patterned paper placed on the chamber walls to alter the visual context.

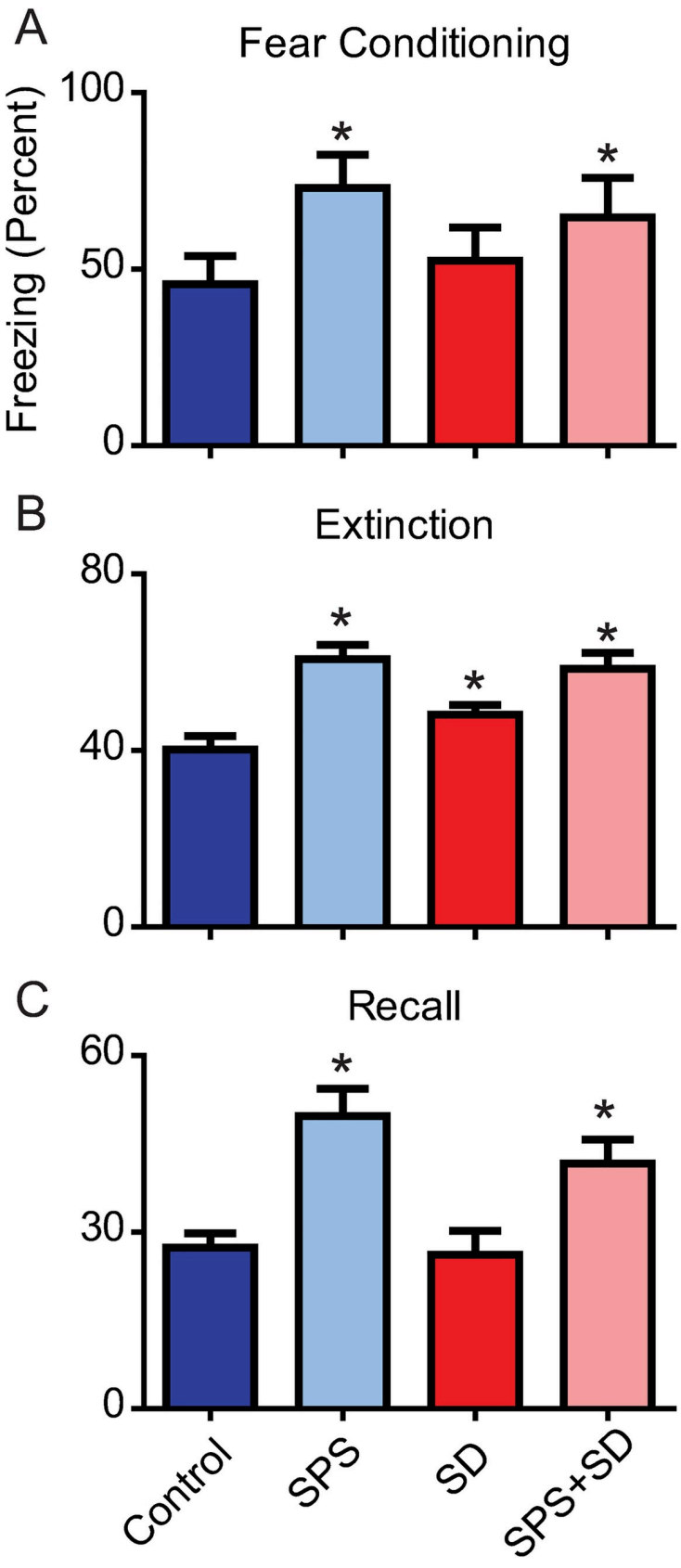
Fear conditioned animals were exposed to five, 1 mA, 1s foot-shocks paired with the cessation of a 10s80 dB tone in Context B. The first tone was presented 180safter the animal was placed in the test cage and the subsequent tones occurred with a 60sinter-tone interval. For all phases, baseline freezing was assessed for 180sprior to the presentation of any tones, and the inter-tone interval was 60s. 60s after the last tone, animals were removed to their home cages. Fear extinction was conducted 24h after fear conditioning and was performed in a distinctly different context (Context A). Fear extinction consisted of presentation of 30 tones (60sinter-tone interval), without the paired foot-shock. Recall was assessed 24h after extinction and consisted of the animals being placed back into the fear extinction context (Context A) for 10 tones (60sinter-tone interval), again without foot shock.

Results

The average percent time freezing during the fear conditioning training was significantly increased in SPS (72.99 ± 9.5%) and SPS+SD (64.6 ± 11.3%) exposed animals compared to controls (45.67 ± 8.0%). SD animals (52.4 ± 9.3%) did not differ from controls in response to fear conditioning (Fig 2A). During the extinction phase, the average percent time freezing was higher in the SPS exposed (60.7 ± 3.2%), SD (48.2 ± 2.1%) and SPS+SD (58.5 ± 3.6%) animals compared to controls (40.3 ± 3.0%) (Fig 2B). Finally, during the fear recall phase, the average percent time freezing was higher in the SPS exposed (49.7 ± 4.6%) and the SPS+SD (41.6 ± 4.1%) groups compared to controls (27.4 ± 2.4%). SD animals (26.1 ± 4.1%) did not differ from controls in response to fear recall (Fig 2C).

To further examine the time course of fear associated memory impairments, we examined group by time interactions on fear conditioning, extinction and recall in these 4 groups of animals. We found a significant group by time interaction on each of these days (Fig 3A–3C). The largest effect was seen on the fear conditioning day where, compared to control animals, the SPS exposed animals showed enhanced freezing at each of the 5 time points measured (* = $p < 0.05$, Fig 3A). The SPS+SD animals showed enhanced freezing at the 3–5 time points compared to controls (+ = $p < 0.05$). The SD group showed a significant increase in freezing at the 5^{th} time point of fear conditioning (# = $p < 0.05$). Although significant group x time interactions were detected on the extinction and recall day, Bonferroni post hoc analysis revealed that no individual time points reached statistical significance compared to control animals (Fig 3B and 3C).

Next, we examined the change in sleep duration in response to SPS, SD, and SPS+SD. Physiological EEG/EMG recordings were made over a baseline day, the sleep deprivation day, and finally on the trauma exposure/sleep recovery day (Fig 4A). SD and SPS+SD groups showed very little, if any, REM sleep on the deprivation day. In the 12h following sleep deprivation, the SD group showed a >250% increase in REM sleep duration compared to baseline. Interestingly, the SPS+SD group only showed a 150% increase in REM sleep duration suggesting that SPS suppresses normal REM sleep rebound. Additionally, REM sleep rebound in the SPS+SD group persisted in the subsequent 12h period afterwards. SPS alone was insufficient to cause a change in REM sleep duration immediately following trauma but resulted in increased REM


A
Fear Conditioning
Freezing (Percent)
100
50
0
*
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B
Extinction
80
40
0
*
*
*
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Recall
60
30
0
*
*
Control
SPS
SD
SPS+SD

Fig 2. SPS impairs fear-associated memory and sleep deprivation does not further exaggerate fear-associated memory impairments. Each bar represents the average freezing duration (percent) over the fear conditioning (A), extinction (B), and recall (C) phases of the fear-associated memory test. Control animals (dark blue bars) were compared to animals exposed to SPS (light blue bars), SD (dark red bars), or a combination of SPS+SD (light red bars). A one-way ANOVA was used to detect significant effects of treatment, followed by Bonferroni post-hoc analysis., A) A significant main effect of treatment ($F(3,19) = 12.95$, $P = 0.0005$) was found during the fear conditioning phase. Post-hoc analysis revealed that SPS (light blue) and SPS+SD (light red) animals froze significantly more than control animals (dark blue). B) A significant main effect of treatment was found during the extinction phase ($F(3,119) = 35.42$, $P < 0.0001$). Bonferroni post-hoc analysis revealed that the SPS exposed, SD, and SPS+SD groups froze significantly more than control animals. C) A significant main effect of treatment was found during the recall phase ($F(3,39) = 26.28$, $P < 0.0001$). Bonferroni post-hoc analysis showed that SPS treated (light blue) and SPS+SD (light red) treated animals froze significantly more than control animals (dark blue) * = $P < 0.05$.

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sleep duration 12h later (Fig 4B). These data are in agreement with previously published work that shows a 12h delay in increased REM sleep duration in response to trauma exposure [13, 21].

As with REM sleep, NREM sleep duration was also effectively reduced to zero in response to sleep deprivation in the SD and SPS+SD groups on the deprivation day. During the first 12h of the recovery sleep phase, NREM sleep rebounded about 150% in control animals but was completely blocked when SPS was presented at the conclusion of the sleep deprivation (Fig 4C).

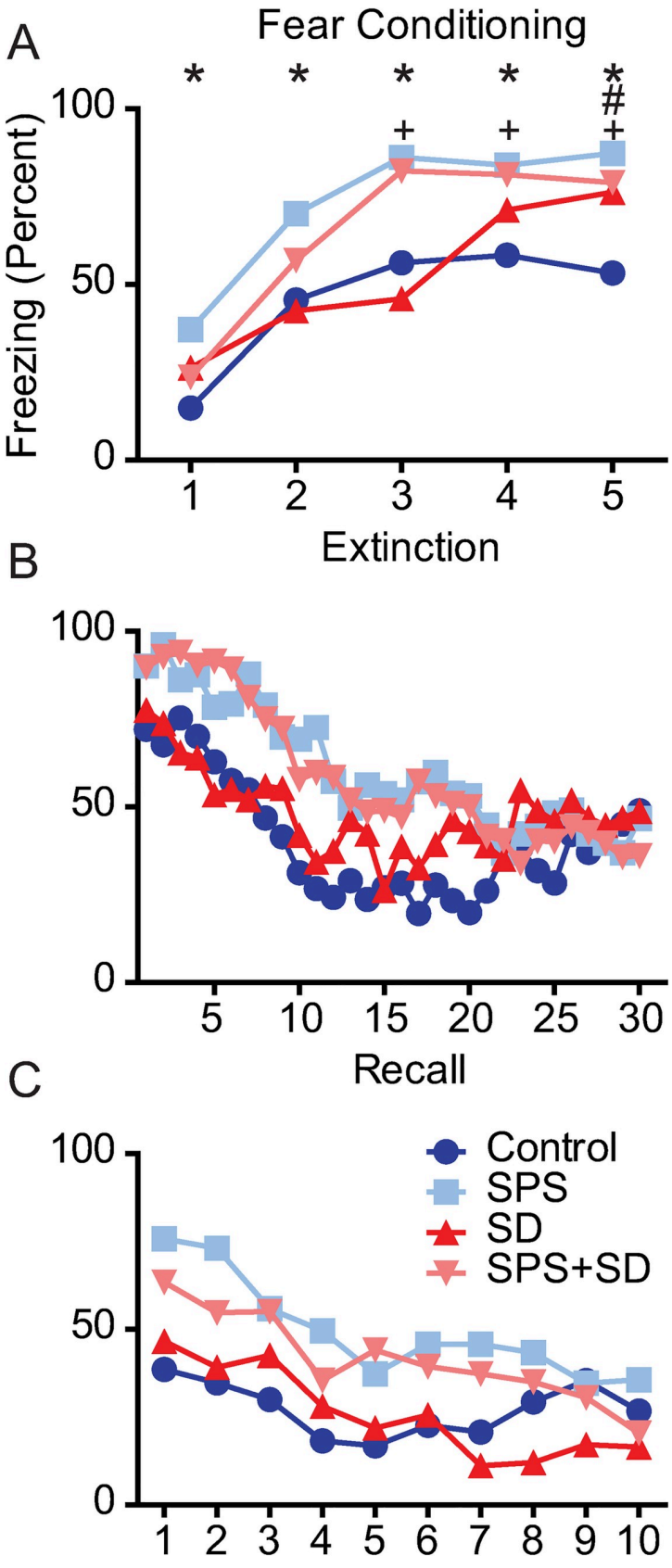
Sleep deprivation in the SD and SPS+SD groups resulted in increased waking (Fig 4D). In the subsequent 12h recovery time, the SD group showed a significant reduction in Wake duration, coinciding with increased sleep rebound. This reduction in Wake was not seen when SPS was performed at the conclusion of the sleep deprivation.

We examined EEG spectral power over the three days of the experiment. Control animals (Fig 5A and 5B) did not show any difference in NREM or REM spectral power over the 3 days. We found that SPS alone had no effect on NREM spectral power (Fig 5C) but resulted in reduced REM theta power in the 12h immediately after the trauma (Fig 5D, inset). In contrast, sleep deprivation alone was sufficient to increase NREM delta power (Fig 5E) and the combination of SPS+SD effectively eliminated the effects of each of these individual treatments (Fig 5G and 5H).

Discussion

The goal of this work was to examine if sleep deprivation prior to trauma exposure leads to increased severity of PTSD-like phenotype in SPS exposed rats. In previous studies, increased freezing on the recall day has served as an index of PTSD severity [13–15]. SPS typically results in animals with more severe extinction retention deficits that is manifest as increased freezing on the recall day. However, the data presented here show that sleep loss prior to trauma exposure did not further exaggerate the fear-associated memory impairments found by exposure to the trauma alone as there is no difference in freezing between the SPS and the SPS+SD group on the fear recall day.

However, the SPS exposed and SPS+SD animals showed significantly more freezing on the fear conditioning day and the extinction day compared to control and SD groups. This result was novel and unique to this set of experiments as previous groups showed no differences in acquisition or extinction of fear memory in SPS treated rats [13–15].Given that these studies were performed precisely as previously published, with the exception of the time of day that they were performed, leads to the suggestion that the timing of trauma exposure affects fear-associated memory acquisition and extinction processing in this model. To our knowledge, the effect of trauma exposure at different times of the day has not been systematically


A
Fear Conditioning
Freezing (Percent)
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50
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1
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Extinction
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Control
SPS
SD
SPS+SD

Fig 3. SPS exposure significantly alters the timeline of fear-associated memory training, extinction, and recall. Each line represents the freezing duration (percent) over each 1 min period of the fear conditioning, extinction, and recall phase of the fear-associated memory test. Each phase was analyzed with two-way ANOVA followed by Bonferroni post-hoc analysis that compared control animals (dark blue circles) to animals exposed to SPS (light blue squares), or SD (dark red triangles), or a combination of SPS+SD (light red triangles). A) two-way ANOVA revealed a significant group x time interaction ($F(12,148) = 3.14$, $P = 0.0005$) on the fear conditioning day, B) the extinction day, ($F(187,1073) = 1.54$, $P = 0.0015$) and, C) the recall day ($F(27,333) = 1.63$, $P = 0.027$). Bonferroni post-hoc comparisons revealed significant differences from (dark blue) control animals ($P < 0.05$) and are denoted by * for SPS (light blue), # for SD (red), and + for SPS+SD (light red) animals.

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investigated, while much effort has gone into examining the contribution of the circadian system on other learning and memory processes [16, 23–26]. Therefore, the primary findings of our study are two-fold, 1) we provide evidence that sleep loss prior to trauma exposure does not further exaggerate SPS-induced fear extinction retention deficits, and 2) we show that trauma exposure at ZT12 results in heightened SPS-induced fear-conditioning and fear-extinction acquisition freezing rates.

Using cued fear conditioning, we determine PTSD severity by comparing freezing on the fear recall day of the fear-associated memory task in SPS exposed and control rats. Fear conditioning, pairing an audible tone with a foot shock, typically results in increased freezing behavior that does not differ between SPS and control animals [13–15]. However, in these experiments, SPS treated animals and SPS+SD animals both show increased freezing in response to fear conditioning compared to control (and SD alone) animals (Fig 2A). These data indicate that trauma induced fear-associated memory processing may function differently at ZT12, than at ZT0. Identifying a mechanism or understanding this unique phenotype is an area of active research by our group and further examination is required to identify the brain regions and molecules by which this phenotype develops.

In the absence of SPS, sleep deprivation resulted in a robust REM and NREM homeostatic rebound. Interestingly, when SPS was added at the conclusion of the sleep deprivation (SPS +SD) REM sleep rebound was significantly attenuated (Fig 4B) and NREM sleep rebound was completely blocked. Remarkably, REM sleep was also increased in the subsequent 12h period after trauma exposure in both the SPS group and the combined SPS+SD group. The differences in sleep profile that emerges following these treatments may serve to identify critical windows-in-time for the application of sleep-specific interventions to stave off the negative consequences of trauma exposure. For instance, it may be possible to manipulate sleep time via optogenetics [27] or pharmacology during these critical time periods in order to help to assign function to each of these physiological sleep properties.

In addition to measuring sleep duration, we also examined changes in delta and theta spectral power during NREM and REM respectively. SPS reduces REM sleep theta band power while sleep deprivation increases NREM delta power in the 12h immediately after these manipulations. However, these spectral power phenotypes were abolished when SPS and SD were combined (SPS+SD). SPS is unique from other forms of stress and our application of it has identified a unique sleep signature that may be important for the development of PTSD.

Previous work has shown that stressful stimuli can change sleep [28–32]. This work has used a diverse collection of "stressors" (e.g., foot shock, predator scent, physical restraint) to change sleep. However, SPS is unique from these stressors in that it induces a PTSD-like phenotype [10] and creates long term changes in sleep architecture [13] and is different than an acute response to stress that does not induce a PTSD-like phenotype. For example, previous work showed social conflict stress, a stressor not known to induce PTSD, resulted in increased slow wave activity during NREM sleep [33]. Interestingly, when sleep deprivation was added

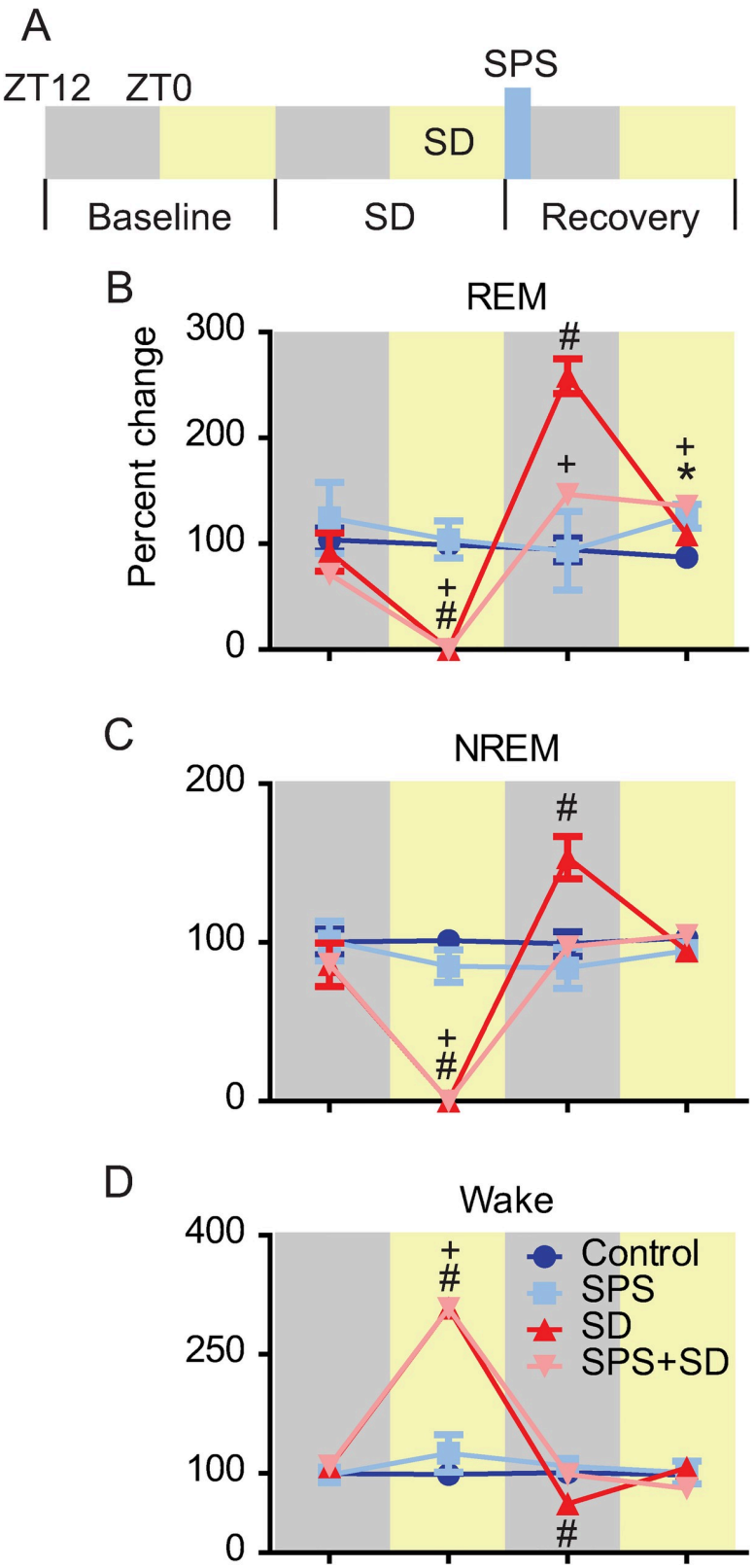

Fig 4. SPS exposure alters sleep-deprivation-induced rebound. A) Experimental timeline. 24h of (baseline) sleep was recorded for all groups of animals. On the following day, sleep deprived animals experienced 12h of sleep loss during the light phase (SD). On the final day of recording (recovery) SPS was performed at the transition from lights on to

lights off (blue bar). Each line represents the percent change in REM, NREM, and Wake time compared to baseline over the sleep deprivation and recovery day in control (blue), SPS (light blue), SD (red), and SPS+SD (light red) exposed animals. Each phase was analyzed with two-way ANOVA followed by Bonferroni post-hoc analysis. Significant differences from (dark blue) control animals ($P < 0.05$) are denoted by * for SPS, # for SD, and + for SPS +SD animals. B) REM sleep showed a significant group x time interaction ($F(9,51) = 12.24$, $P < 0.0001$). Bonferroni post-hoc analysis identified significant reductions in REM sleep on the SD day for the SD (red line) and SPS+SD. SD and SPS+SD animals showed a significant increase in REM sleep during the dark phase immediately after the sleep deprivation. The SD+SPS and SPS exposed groups showed increased REM sleep during the light phase after SPS. C) NREM sleep showed a significant group x time interaction ($F(9,51) = 19.99$, $P < 0.0001$). Bonferroni post-hoc analysis identified significant reductions in NREM sleep on the SD day for the SD and SPS+SD. SD animals showed a significant increase in NREM sleep during the dark phase immediately after the sleep loss. D) Wake duration showed a significant group x time interaction ($F(9,51) = 47.03$, $P < 0.0001$). Bonferroni post-hoc analysis identified significant increase in wake duration on the SD day for the SD and SPS+SD animals. SD animals showed a significant decrease in wake duration during the dark phase immediately after the sleep loss.

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to this social conflict stressor, slow wave activity was further increased above that of sleep deprived animals alone [34]. In our studies here, we find that NREM sleep rebound is completely abolished when SPS is added after sleep deprivation. That NREM sleep may be critical to recovery from trauma exposure and further work is required to examine the contribution of this NREM sleep on the development of PTSD. Therefore, our use of SPS to examine the contribution of sleep deprivation prior to trauma exposure on subsequent sleep time and sleep EEG spectral power is novel and may have better translational properties to the human condition of PTSD.

We live in a world where sleep deprivation is increasingly common. The Centers for Disease Control and Prevention estimates that up to a third of US adults get less than the required amount of sleep (www.cdc.gov/sleep). This sleep loss has severe consequences on emotional [35] and cognitive function [36, 37] and has resulted in a societal need to appreciate and respect sleep as vital to our functioning [38]. Sleep may be particularly relevant to trauma exposed populations who experience significant trauma-induced sleep disturbances [6, 9, 39]. This is especially true given that fixing trauma-induced sleep disturbances seems to assist in preserving function as our group has recently shown that optogenetically enhancing sleep in rats after SPS trauma exposure can significantly reduce trauma-induced fear-associated memory impairments [21]. Here we have used acute sleep deprivation to assess the impact of extended wakefulness near the time of trauma exposure on cognitive function. In the present study we find that acute sleep loss is ineffective to enhance fear-associated memory impairments. Future studies should compare the effects between circadian disruption and varying degrees of sleep deprivation pre- and post- trauma, as well as the consequences of mimicking sleep fragmentation and prolonged sleep restriction observed clinically by trauma-exposed individuals.

Understanding the role of sleep in PTSD is particularly relevant for military personnel, whose lifetime prevalence rates of PTSD range from 10–30% depending on the era of military deployment and whose sleep may suffer both while in combat zones and after tours of duty (www.ptsd.va.gov). In addition, 1–10% of non-military U.S. adults are also affected by PTSD and these cumulative populations account for an estimated $42 billion dollars per year in medical and prescription drug costs, lost wages, and mortality costs. PTSD and the associated sleep disturbances are a costly and immediate public health problem. This work has been foundational in improving our understanding of the interaction of trauma and sleep, however, further studies are required to aid in the development of sleep therapeutics, to identify sleep-related biomarkers to reduce PTSD prevalence, or to help currently affected individuals regain functioning.

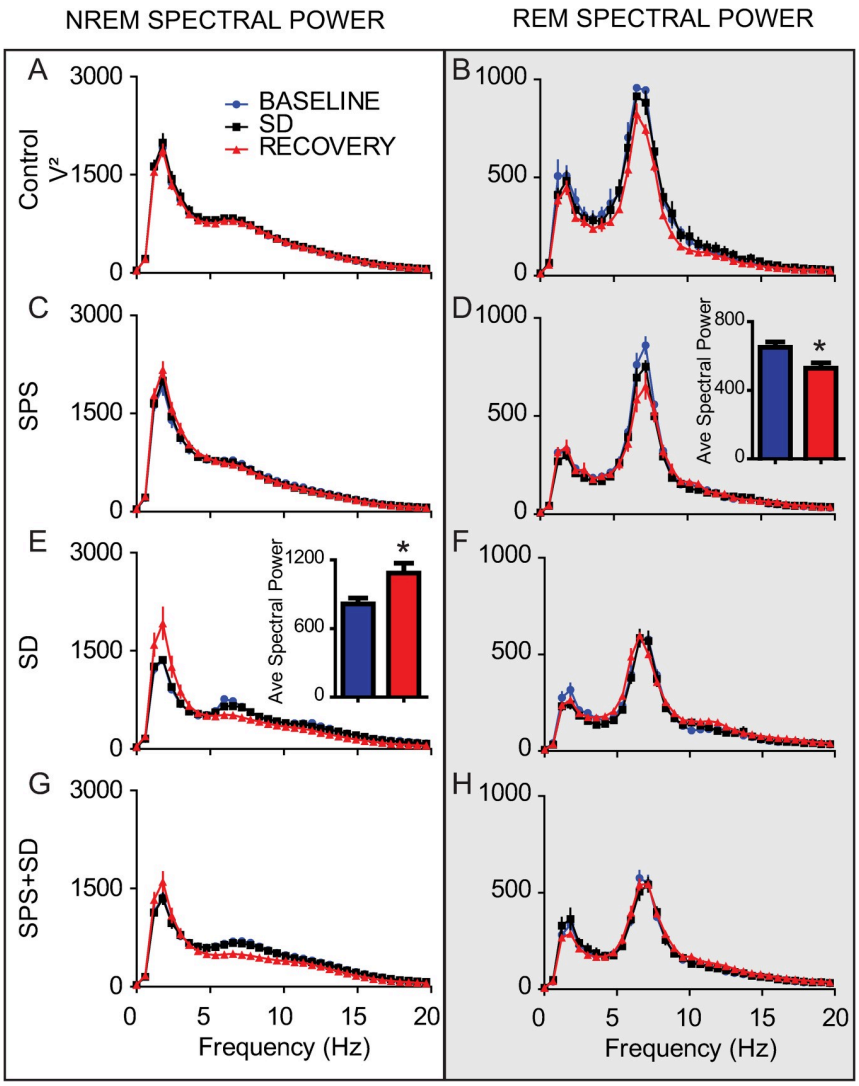

Fig 5. The combination of SPS and sleep deprivation ameliorates the increase of REM and NREM spectral power induced by either alone. Each line represents the average spectral power (ZT12-0) on the baseline, sleep deprivation and recovery day over the 0-20Hz frequency range during either NREM sleep (left column) or REM sleep (right column, shaded). Values are shown for control animals (A, B), animals exposed to SPS (C, D), or SD (E, F), or SPS+SD (G, H). Comparisons of differences from baseline over the theta range (4–8 Hz) and delta range (0-4Hz) were preformed using 2-tailed students T-test and are shown in the insets of D and E respectively. Significant differences and are denoted by * ($P < 0.05$).

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Supporting information

S1 Data. Compiled raw data for Figs 2–5.
(XLSX)

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Writing – original draft: William M. Vanderheyden.

Writing – review & editing: Christopher J. Davis, Jason R. Gerstner, William M. Vanderheyden.

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