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TITLE: The Role of Sleep in Mediating Post-Traumatic Stress Disorder

PRINCIPAL INVESTIGATOR: William M Vanderheyden PhD

CONTRACTING ORGANIZATION: Washington State University

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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 goal of this proposal is to first assess the contribution of sleep to the onset of PTSD. Using a rodent model of PTSD, we performed sleep deprivation experiments to determine if sleep loss prior to Single Prolonged Stress (SPS) trauma exposure is sufficient to exaggerate fear associated memory impairments. Experiments are ongoing, but preliminary data suggests that sleep loss prior to trauma does not enhance PTSD symptoms. Secondly, we assess the contribution of sleep to the maintenance of PTSD. For this, we are exposing animals to SPS followed by increasing sleep time via optogenetics. Experiments are ongoing, However, post-trauma-sleep restoration appears to be sufficient to alleviate fear associated memory impairments typically found following trauma exposure in rats.					
15. SUBJECT TERMS NONE LISTED					
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- 1. INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

The current research is focused on understanding the contribution of sleep to the acquisition and maintenance of post-traumatic stress disorder (PTSD). The purpose of this research is to identify whether sleep improvements or sleep disruptions contribute to the pathophysiology of PTSD.

- 2. KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

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

- 3. ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Major Task 1: Perform Sleep deprivation/restriction and collect EEG/EMG recordings. To date, I have collected EEG/EMG recording and behavioral recordings from all animals. The data collection portion of this Task is complete. This aim is complete. This work has resulted in 1 manuscript currently in submission at "PLOS 1".

Major Task 2: Use Optogenetics to improve sleep after trauma exposure. I have completed the "quantification of PTSD severity and sleep phenotypes" as proposed in my SOW. I have completed this Task and this work has resulted in 1 manuscript currently in submission at "Scientific Reports"

Major Task 3: Preparation of Manuscripts. I have 2 manuscripts in submission currently.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

1) major activities; For Specific Aim1 I performed SPS trauma exposure (or controls) at the conclusion of 12 hr of sleep deprivation. Control animals recieved either no sleep loss, or no trauma exposure (4 groups total) Sleep EEG and EMG data were collected and analyzed for vigilance state. Fear associated memory tests were performed all four groups of animals. The results of this aim can be found in the attached manuscript currently in submission at PLOS 1. Briefly, the results of this manuscript are that 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. Additionally, unique sleep profiles emerged to give a clearer picture of how sleep and trauma exposure interact.

2) specific objectives; 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); These data suggest that sleep deprivation prior to trauma exposure is insufficient to increase the severity of PTSD symptoms. These results are presented in the attached manuscript currently in submission at PLOS1.

1) major activities; For Specific Aim2, I have completed optogenetic stimulation experiments following trauma exposure. These studies are in revision at Scientific Reports and the data can be found in the attached manuscript.

2) specific objectives; For Specific Aim2 I proposed that sleep restoration via optogenetic enhancement of sleep time would rescue trauma associated fear memory impairments.

3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); 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 (Bright white MCH containing cells; See figure below). In addition, preliminary evidence suggests that REM and NREM sleep is increased following opto stimulation (blue lines) compared to non-stimulation (orange lines) in agreement with previously published data using this construct.

What opportunities for training and professional development has the project provided?

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. “Training” activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. “Professional development” activities result in increased knowledge or skill in one’s area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to report

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

If this is the final report, state “Nothing to Report.”

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

The experiments are complete and the results are being communicated in the literature.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

The findings on pre-trauma sleep not contributing to PTSD severity are very interesting and suggest that sleep before trauma is less important than sleep after trauma exposure and may, someday, determine how trauma exposed humans are treated for sleep interruptions immediately following a trauma.

The post-trauma sleep enhancement studies indicate that sleep can be used to treat PTSD.

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

We are working to expand these behavioral findings and further our molecular and anatomical understanding of these phenotypes.

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

These findings may impact how trauma exposed individuals are treated in many ways. For example, car crash victims may be allowed to rest after an accident instead of having their sleep disturbed while in the hospital. Additionally, individuals in the theatre of war may need to be removed to recover their sleep in a safe place.

- 5. CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:*

Nothing to Report.

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

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to report. all data are currently in submission.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report

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

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use of biohazards and/or select agents

none

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”*

- **Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume; year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

In preparation

1) Single Prolonged Stress Blocks Sleep Homeostasis and Pre-Trauma Sleep Deprivation Does Not Exacerbate the Severity of Trauma-Induced Fear-Associated Memory Impairments. PLOS 1 (in Review). Federal support acknowledged.

2) Optogenetic sleep enhancement improves fear-associated memory processing following trauma 1 exposure. Scientific Reports (in Revision). Federal support acknowledged.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of*

publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

None

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

None

- **Website(s) or other Internet site(s)**
List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

n/a

- **Technologies or techniques**
Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

n/a

- **Inventions, patent applications, and/or licenses**

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

n/a

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

n/a

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change”.

Example:

Name: Mary Smith

Project Role: Graduate Student

Researcher Identifier (e.g. ORCID ID): 1234567

Nearest person month worked: 5

Contribution to Project: Ms. Smith has performed work in the area of combined error-control and constrained coding.

Funding Support: The Ford Foundation (Complete only if the funding support is provided from other than this award.)

William M Vanderheyden
Christopher J Davis

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Feb 2020 marked the conclusion of active salary support from the Discovery award for Drs Vanderheyden and Davis. Dr. Vanderheyden is currently supported as key personnel on an R35 award from NIGMS (NIH. Gerstner Lab, WSU).

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:

Organization Name:

Location of Organization: (if foreign location list country)

Partner’s contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner’s facilities for project activities);*
- *Collaboration (e.g., partner’s staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner’s staff use each other’s facilities, work at each other’s site); and*
- *Other.*

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.*

9. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Single Prolonged Stress Blocks Sleep Homeostasis and Pre-Trauma Sleep Deprivation Does Not Exacerbate the Severity of Trauma-Induced Fear-Associated Memory Impairments

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research Article
Full Title:	Single Prolonged Stress Blocks Sleep Homeostasis and Pre-Trauma Sleep Deprivation Does Not Exacerbate the Severity of Trauma-Induced Fear-Associated Memory Impairments
Short Title:	Single Prolonged Stress Blocks Sleep Homeostasis in Rats
Corresponding Author:	William Martin Vanderheyden, Ph.D Washington State University - Spokane Spokane, WA UNITED STATES
Keywords:	Sleep; Stress; Trauma; Fear-conditioning; Cognition; Sleep Deprivation
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. Treatment-specific sleep phenotypes are shown for the SPS and SPS+SD animals when compared to SD and/or control animals. SPS exposure following SD represses homeostatic rebound and reduces NREM sleep delta power. This work identifies a unique time frame where trauma exposure and sleep may interact and identifies this window of time as a potential therapeutic treatment window for staving off the negative consequences of trauma exposure.
Order of Authors:	William Martin Vanderheyden, Ph.D Christopher John Davis Jason Robert Gerstner
Opposed Reviewers:	Gina Poe, PhD UCLA ginapoe@ucla.edu Post Doctoral Mentor Israel Liberzon, MD Texas A&M University College Station liberzon@tamu.edu Post Doctoral Mentor
Additional Information:	
Question	Response
Financial Disclosure Enter a financial disclosure statement that describes the sources of funding for the work included in this submission. Review the submission guidelines for detailed	The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

requirements. View published research articles from [PLOS ONE](#) for specific examples.

This statement is required for submission and **will appear in the published article** if the submission is accepted. Please make sure it is accurate.

Unfunded studies

Enter: *The author(s) received no specific funding for this work.*

Funded studies

Enter a statement with the following details:

- Initials of the authors who received each award
- Grant numbers awarded to each author
- The full name of each funder
- URL of each funder website
- Did the sponsors or funders play any role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript?
- **NO** - Include this sentence at the end of your statement: *The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*
- **YES** - Specify the role(s) played.

* typeset

Competing Interests

Use the instructions below to enter a competing interest statement for this submission. On behalf of all authors, disclose any [competing interests](#) that could be perceived to bias this work—acknowledging all financial support and any other relevant financial or non-financial competing interests.

This statement **will appear in the published article** if the submission is accepted. Please make sure it is accurate. View published research articles from [PLOS ONE](#) for specific examples.

The authors have declared that no competing interests exist.

NO authors have competing interests

Enter: *The authors have declared that no competing interests exist.*

Authors with competing interests

Enter competing interest details beginning with this statement:

I have read the journal's policy and the authors of this manuscript have the following competing interests: [insert competing interests here]

* typeset

Ethics Statement

Enter an ethics statement for this submission. This statement is required if the study involved:

- Human participants
- Human specimens or tissue
- Vertebrate animals or cephalopods
- Vertebrate embryos or tissues
- Field research

Write "N/A" if the submission does not require an ethics statement.

General guidance is provided below. Consult the [submission guidelines](#) for detailed instructions. **Make sure that all information entered here is included in the Methods section of the manuscript.**

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 (IACUC #6263).

Isoflurane was used for surgical anesthesia and ether exposure (5 minutes maximum exposure) was used as a chemical activator of the stress system in order to induce PTSD in rats. Alternative anesthesia is ineffective at inducing the PTSD phenotype and is a necessary step of the "single prolonged stress" experimental protocol used in this manuscript.

Format for specific study types

Human Subject Research (involving human participants and/or tissue)

- Give the name of the institutional review board or ethics committee that approved the study
- Include the approval number and/or a statement indicating approval of this research
- Indicate the form of consent obtained (written/oral) or the reason that consent was not obtained (e.g. the data were analyzed anonymously)

Animal Research (involving vertebrate animals, embryos or tissues)

- Provide the name of the Institutional Animal Care and Use Committee (IACUC) or other relevant ethics board that reviewed the study protocol, and indicate whether they approved this research or granted a formal waiver of ethical approval
- Include an approval number if one was obtained
- If the study involved *non-human primates*, add *additional details* about animal welfare and steps taken to ameliorate suffering
- If anesthesia, euthanasia, or any kind of animal sacrifice is part of the study, include briefly which substances and/or methods were applied

Field Research

Include the following details if this study involves the collection of plant, animal, or other materials from a natural setting:

- Field permit number
- Name of the institution or relevant body that granted permission

Data Availability

Authors are required to make all data underlying the findings described fully available, without restriction, and from the time of publication. PLOS allows rare exceptions to address legal and ethical concerns. See the [PLOS Data Policy](#) and [FAQ](#) for detailed information.

Yes - all data are fully available without restriction

A Data Availability Statement describing where the data can be found is required at submission. Your answers to this question constitute the Data Availability Statement and **will be published in the article**, if accepted.

Important: Stating 'data available on request from the author' is not sufficient. If your data are only available upon request, select 'No' for the first question and explain your exceptional situation in the text box.

Do the authors confirm that all data underlying the findings described in their manuscript are fully available without restriction?

Describe where the data may be found in full sentences. If you are copying our sample text, replace any instances of XXX with the appropriate details.

- If the data are **held or will be held in a public repository**, include URLs, accession numbers or DOIs. If this information will only be available after acceptance, indicate this by ticking the box below. For example: *All XXX files are available from the XXX database (accession number(s) XXX, XXX).*
- If the data are all contained **within the manuscript and/or Supporting Information files**, enter the following:
All relevant data are within the manuscript and its Supporting Information files.
- If neither of these applies but you are able to provide **details of access elsewhere**, with or without limitations, please do so. For example:

Data cannot be shared publicly because of [XXX]. Data are available from the XXX Institutional Data Access / Ethics Committee (contact via XXX) for researchers who meet the criteria for access to confidential data.

The data underlying the results presented in the study are available from (include the name of the third party

All relevant data are within the manuscript and its Supporting Information files.

<i>and contact information or URL).</i> • This text is appropriate if the data are owned by a third party and authors do not have permission to share the data. * typeset	
Additional data availability information:	



Elson S. Floyd College of Medicine

August 14, 2020

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Dear Editor and *PLOS ONE*:

Please find enclosed our manuscript entitled “Single Prolonged Stress Blocks Sleep Homeostasis and Pre-Trauma Sleep Deprivation Does Not Exacerbate the Severity of Trauma-Induced Fear-Associated Memory Impairments” for publication in *PLOS ONE*. This *research article* was previously submitted (Submission # PONE-D-20-24179). As requested, we have revised the methods section of the manuscript to include scientific justification for the use of Ether anesthetic in our animal model of PTSD (lines 192-195). We have additionally submitted a copy of our approved IUCUC protocol along with a letter from our Animal Welfare Program Assistant Director (Alan Ekstrand) confirming institutional review and acceptance of this IACUC protocol.

The attached manuscript describes the contribution of pre-trauma sleep loss to fear-associated memory impairments and alterations to sleep homeostatic systems.

In this manuscript, we show that:

- 1) Sleep deprivation, prior to trauma exposure, does not exaggerate subsequent fear-associated memory impairments.
- 2) We show that sleep homeostasis is attenuated by trauma exposure.
- 3) Sleep loss followed by trauma exposure eliminates the NREM delta power increases seen after sleep deprivation and the REM theta power increases seen after trauma exposure alone.

Thank you for considering our manuscript for publication in *PLOS ONE*.

Sincerely, 

Editor Suggestions: Vladyslav Vyazovskiy or Giorgio F Gilestro

Exclude Reviewers: Gina Poe (post-doctoral mentor) conflict of interest

Suitable Reviewers:

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Andrew Eagle aeagle@med.wayne.edu

Monica Fleshner monika.fleshner@colorado.edu

Single Prolonged Stress Blocks Sleep Homeostasis and Pre-Trauma Sleep Deprivation Does Not
Exacerbate the Severity of Trauma-Induced Fear-Associated Memory Impairments

Single Prolonged Stress Blocks Sleep Homeostasis in Rats

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24 Keywords

25 Sleep, Stress, Trauma, Fear-conditioning, Cognition, Sleep Deprivation

26

27 Abbreviations

28 Post-traumatic stress disorder PTSD

29 Single Prolonged Stress SPS

30 Sleep deprivation SD

31 Electroencephalogram EEG

32 Electromyogram EMG

33 Rapid eye movement sleep REM

34 Non rapid eye movement sleep NREM

35 Zeitgeber Time ZT

36

37 Funding Source

38 This work was supported by the Department of Defense, Congressionally Directed Medical

39 Research Program, Discovery Award # W81XWH-16-1-0319

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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. Treatment-specific sleep phenotypes are shown for the SPS and SPS+SD animals when compared to SD and/or control animals. SPS exposure following SD represses homeostatic rebound and reduces NREM sleep delta power. This work identifies a unique time frame where trauma exposure and sleep may 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 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 difficulty staying asleep through the night (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 minutes 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 (10-14). In this model, fear-associated

memory impairments are used as a surrogate for the severity of the PTSD-like phenotype (15, 16) 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 (17, 18), 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 (60-90 days old) were housed in temperature (21C-24C) 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 24-hour baseline. Control animals were left untouched for the remainder of the experiment whereas, the sleep deprived animals (SD, and SPS+SD) experienced 12 hours of sleep loss during their primary sleep phase (ZT0-12). 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. After SPS, all groups were isolated for 7 more days. On the eighth day, all groups underwent fear-associated memory testing (described below).

Electrophysiology

Survival stereotaxic surgery was performed to implant the EEG/EMG sleep recording electrodes. 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) for EEG recordings and over the hippocampal area (± 2.5 mm lateral to midline, 3.5 mm posterior to Bregma). 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 48 hours 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 off-line 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 24 hours 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 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.

EEG spectral analysis was performed using SleepSign Software and averaged in 12- and 24-hour 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 12 hours (ZT0-12) by gentle handling. 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, 14). Briefly, animals were exposed to 3 successive stressors at the start of the dark phase. First, physical restraint was performed for 2 hours 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 degree C water and were forced to swim in groups of 6-8 for 20 minutes. Following a 15-minute 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 minute). 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 (15). 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-associated memory tests were conducted. Fear conditioning, extinction, and extinction recall, hereafter referred to as recall, were performed as previously published (14, 16). 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.

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

Results

The average percent time freezing during the fear conditioning training was significantly increased in SPS ($M = 72.99 \pm 9.5$ percent) and SPS+SD ($M = 64.6 \pm 11.3$ percent) exposed animals compared to controls ($M = 45.67 \pm 8.0$ percent). SD animals ($M = 52.4 \pm 9.3$ percent) did not differ from controls in response to fear conditioning (Figure 1A). During the extinction phase, the average percent time freezing was higher in the SPS exposed ($M = 60.7 \pm 3.2$ percent), SD ($M = 48.2 \pm 2.1$ percent) and SPS+SD ($M = 58.5 \pm 3.6$ percent) animals compared to controls ($M = 40.3 \pm 3.0$ percent) (Figure 1B). Finally during the fear recall phase, the average percent time freezing was higher in the SPS exposed ($M = 49.7 \pm 4.6$ percent) and the SPS+SD ($M = 41.6 \pm 4.1$ percent) groups compared to controls ($M = 27.4 \pm 2.4$ percent). SD animals ($M = 26.1 \pm 4.1$ percent) did not differ from controls in response to fear recall (Figure 1C).

Figure 1. SPS impairs fear-associated memory. Each bar represents the average freezing duration (percent) over the fear conditioning, extinction, and recall phase of the fear-associated memory test. Control animals (dark blue bars) are compared to animals exposed to SPS (light blue bars), or SD (dark red bars), or a combination of SPS+SD (light red bars). A) During the fear conditioning phase, one-way ANOVA revealed a significant main effect of treatment ($F(3,19) = 12.95$, $P = 0.0005$). 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 ($F(3,119) = 35.42$, $P < 0.0001$). 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 ($F(3,39) = 26.28$, $P < 0.0001$). Post-hoc analysis showed that SPS treated (light blue) and SPS+SD (light red) treated animals froze significantly more than control animals (dark blue) $* = P < 0.05$.

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 (Figure 2A-C). 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 ($* = p < 0.05$, Figure 2A). 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 5th time point of fear conditioning ($\# = p < 0.05$). Although significant group x time interactions were detected on the extinction and recall day, post hoc analysis revealed that no individual time points reached statistical significance compared to control animals (Figure 2B,C).

Figure 2. SPS exposure significantly alters the timeline of fear-associated memory training, extinction and recall. Each line represents the freezing duration (percent) over each one-minute 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 ($F(27,333) = 1.63$, $P = 0.027$). 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.

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 (Figure 3A). SD and SPS+SD groups showed very little, if any, REM sleep on the deprivation day. In the 12 hours 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 sleep rebound. Additionally, REM sleep rebound in the SPS+SD group persisted in the subsequent 12-hour period afterwards. SPS alone was insufficient to cause a change in REM sleep duration immediately following trauma but resulted in increased REM sleep duration 12 hours later (Figure 3B). These data are in agreement with previously published work that shows a 12-hour delay in increased REM sleep duration in response to trauma exposure (14).

Figure 3. SPS exposure alters sleep-deprivation-induced rebound. A) Experimental timeline. 24 hours of (baseline) sleep was recorded for all groups of animals. On the following day, sleep deprived animals experienced 12 hours 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 $F(9,51)=12.24$, $P < 0.0001$. 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 $F(9,51)=19.99$, $P < 0.0001$. 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 $F(9,51)=47.03$, $P < 0.0001$. 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.

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 12 hours 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 (Figure 3C).

Sleep deprivation in the SD and SPS+SD groups resulted in increased waking (Figure 3D). In the subsequent 12-hour 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 (Figure 4A,B) did not show any difference in NREM or REM spectral power. We found that SPS alone had no effect on NREM spectral power (Figure 4C) but resulted in reduced REM theta power in the 12 hours immediately after the trauma (Figure 4D, inset). In contrast, sleep deprivation alone was sufficient to increase NREM delta power (Figure 4E) and the combination of SPS+SD effectively eliminated the effects of each of these individual treatments (Figure 4G,H).

Figure 4. 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 performed using 2-tailed students T-test and are shown in the insets of D and E respectively. Significant differences and are denoted by * ($P < 0.05$).

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, freezing on the recall day has served as an index of PTSD severity (14, 16). Accordingly, the data presented here show that sleep loss prior to trauma exposure does not further exaggerate the PTSD-like phenotype 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, suggesting that the timing of trauma exposure affects fear-associated memory processing in this model. To our knowledge, the effect of trauma exposure at different times of the day has not been systematically investigated, while much effort has gone into examining the contribution of the circadian system on learning and memory processes (19-21). 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 recall memory impairments, and 2) we show that trauma exposure at ZT12 results in heightened fear-conditioning and fear-extinction acquisition 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 (14, 16). 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 (Figure 1A). 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 (Figure 3B) and NREM sleep rebound was completely blocked. Remarkably, REM sleep was also increased in the subsequent 12-hour 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 (22) 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 12 hours 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 (23-27). 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 (14) 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 (28). Interestingly, when sleep deprivation was added to this social conflict stressor, slow wave activity was further increased above that of sleep deprived animals alone (29). 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.

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

412 and prescription drug costs, lost wages, and mortality costs. PTSD and the associated sleep
413 disturbances are a costly and immediate public health problem. This work has been foundational
414 in improving our understanding of how trauma alters sleep, but further studies are required to aid
415 in the development of sleep therapeutics, to identify sleep-related biomarkers to reduce PTSD
416 prevalence, or to help currently affected individuals regain functioning.

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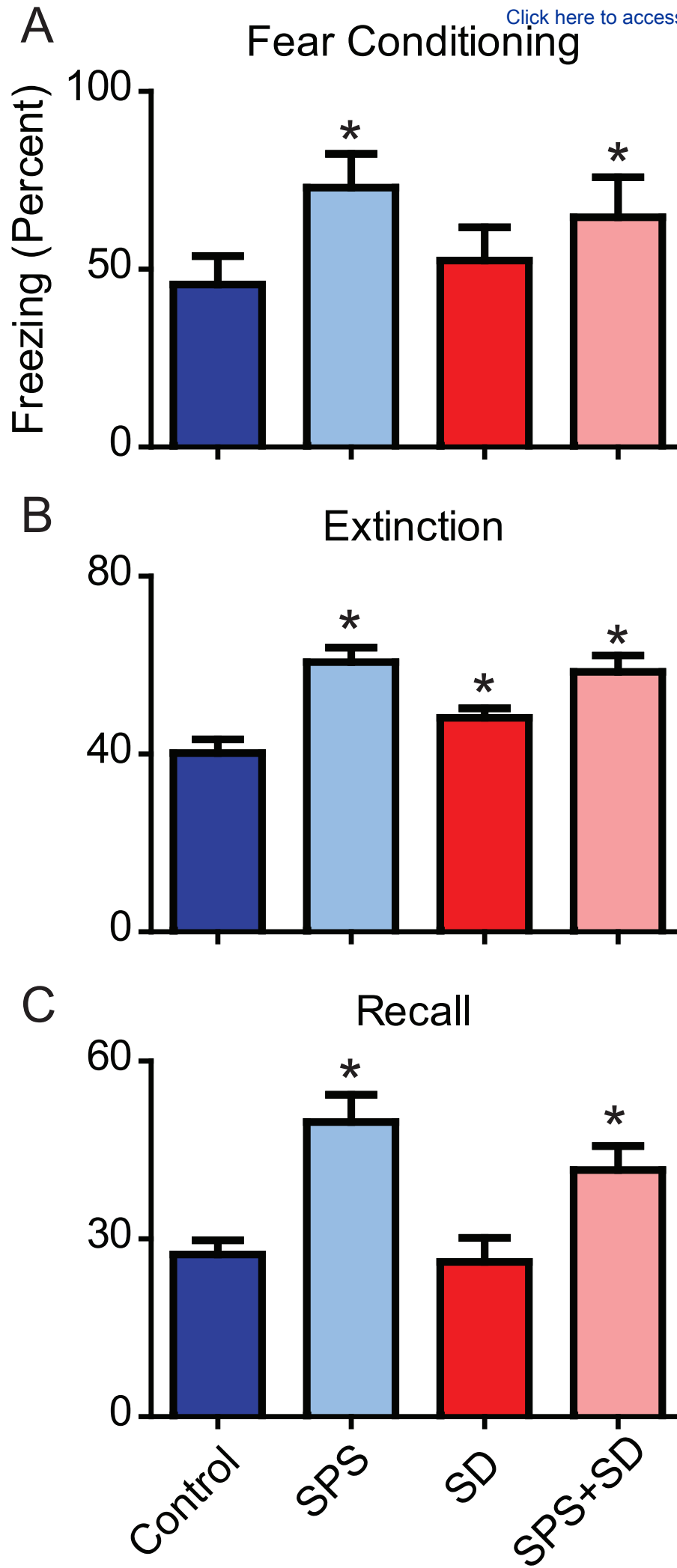
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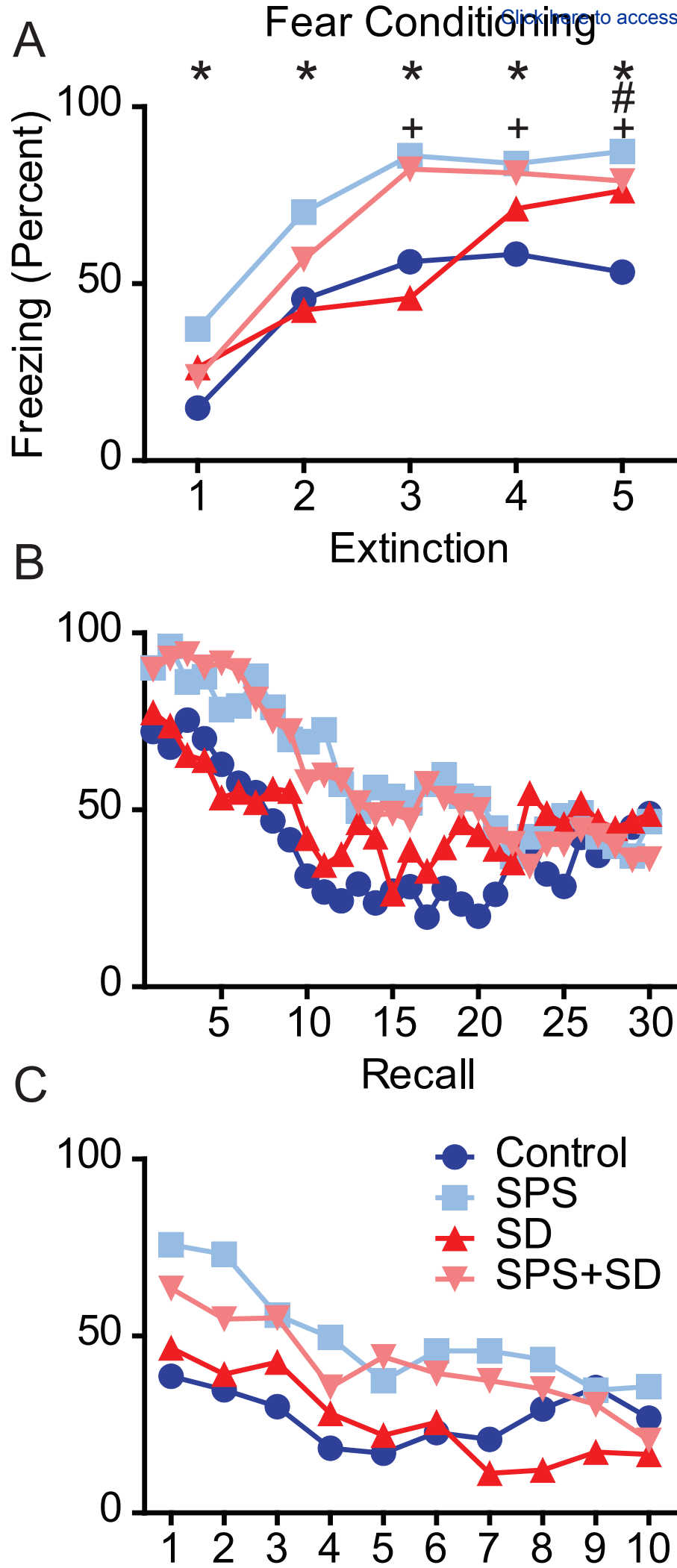
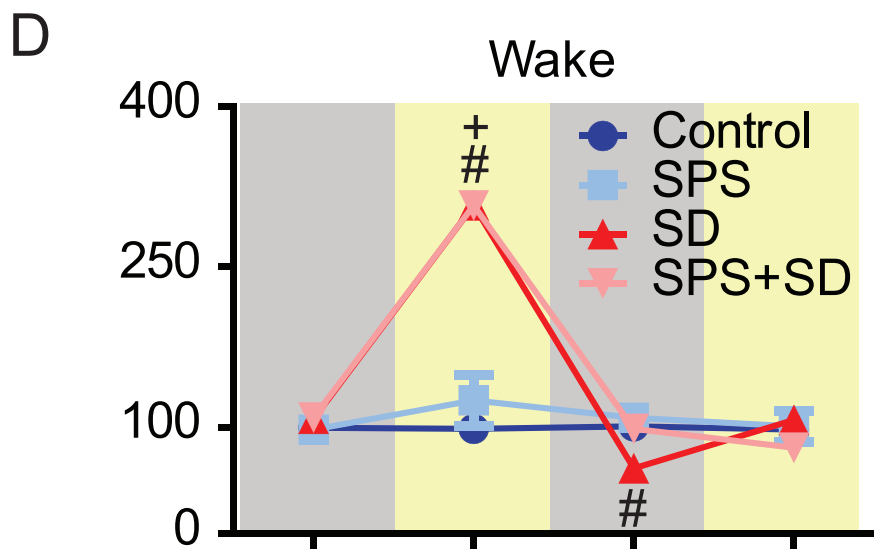
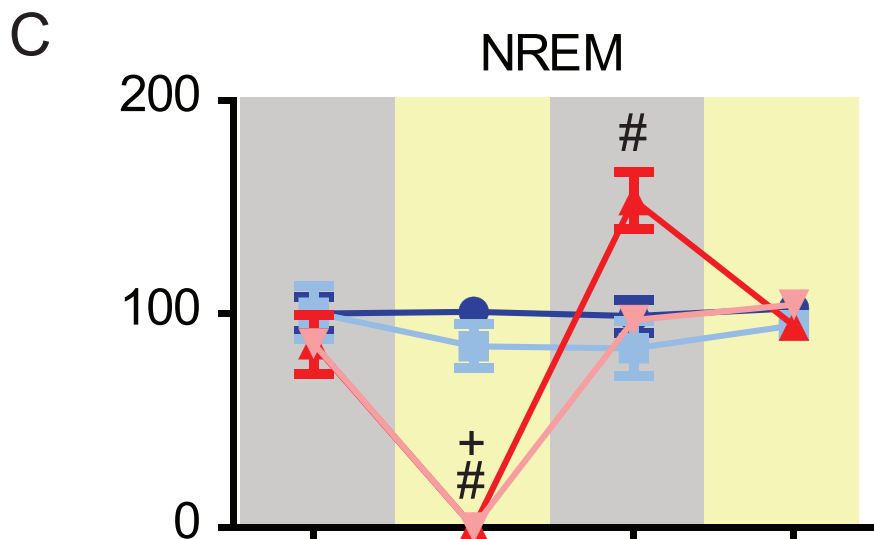
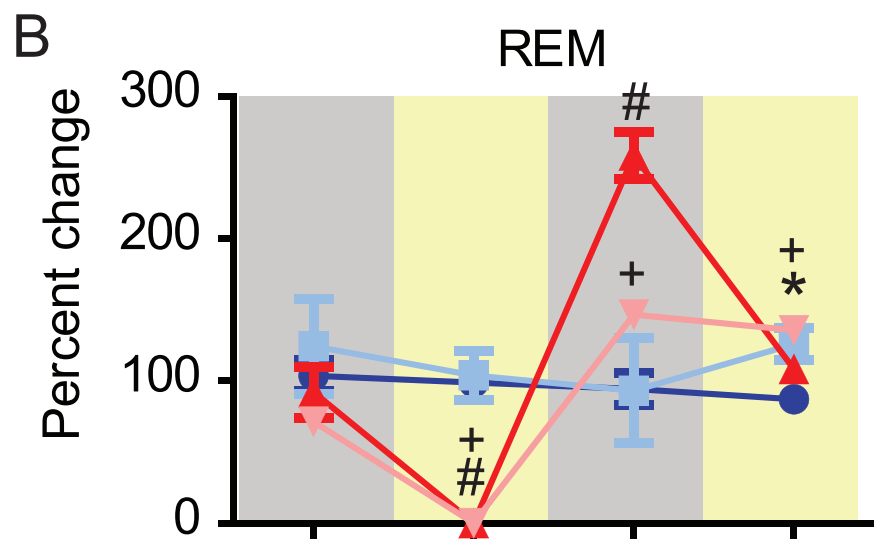
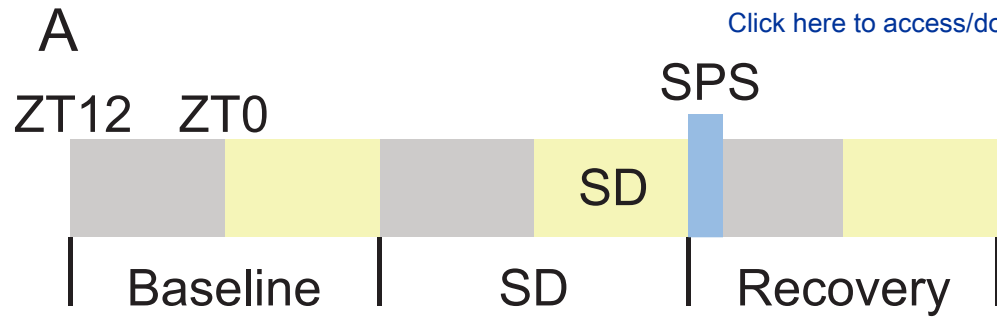
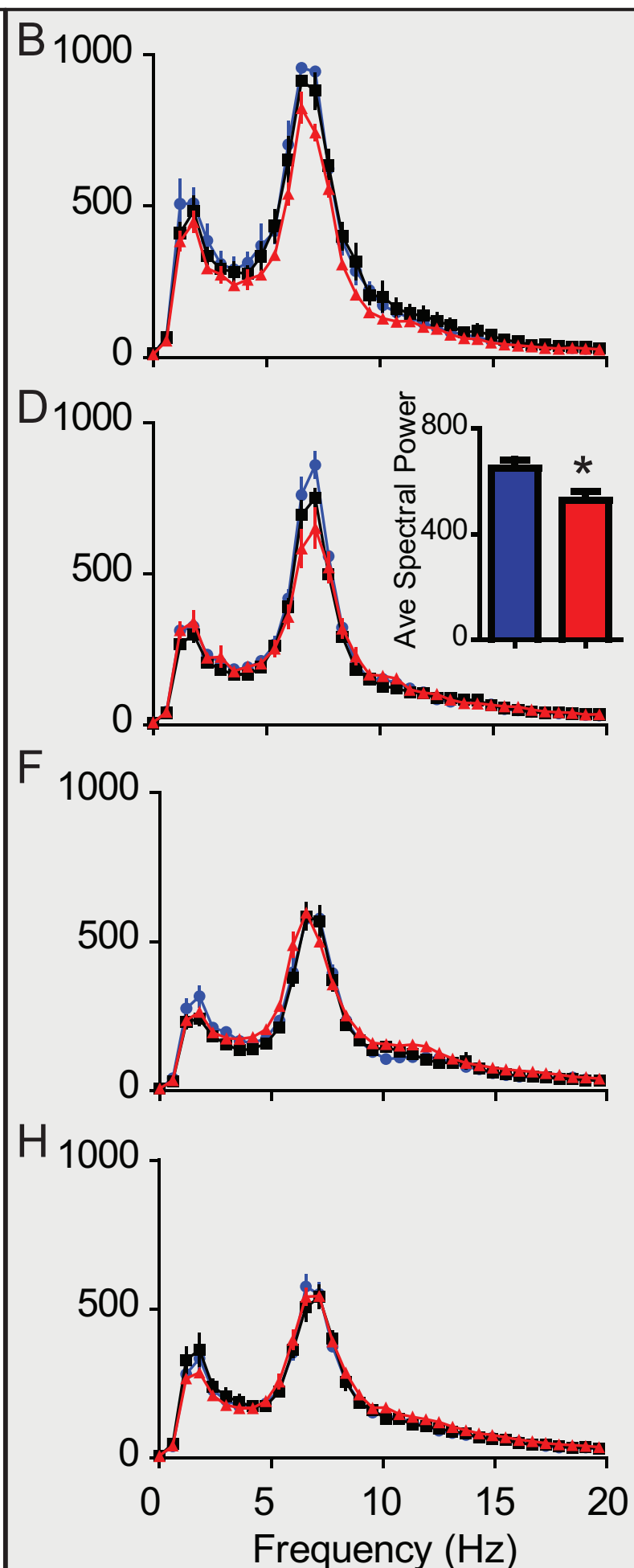
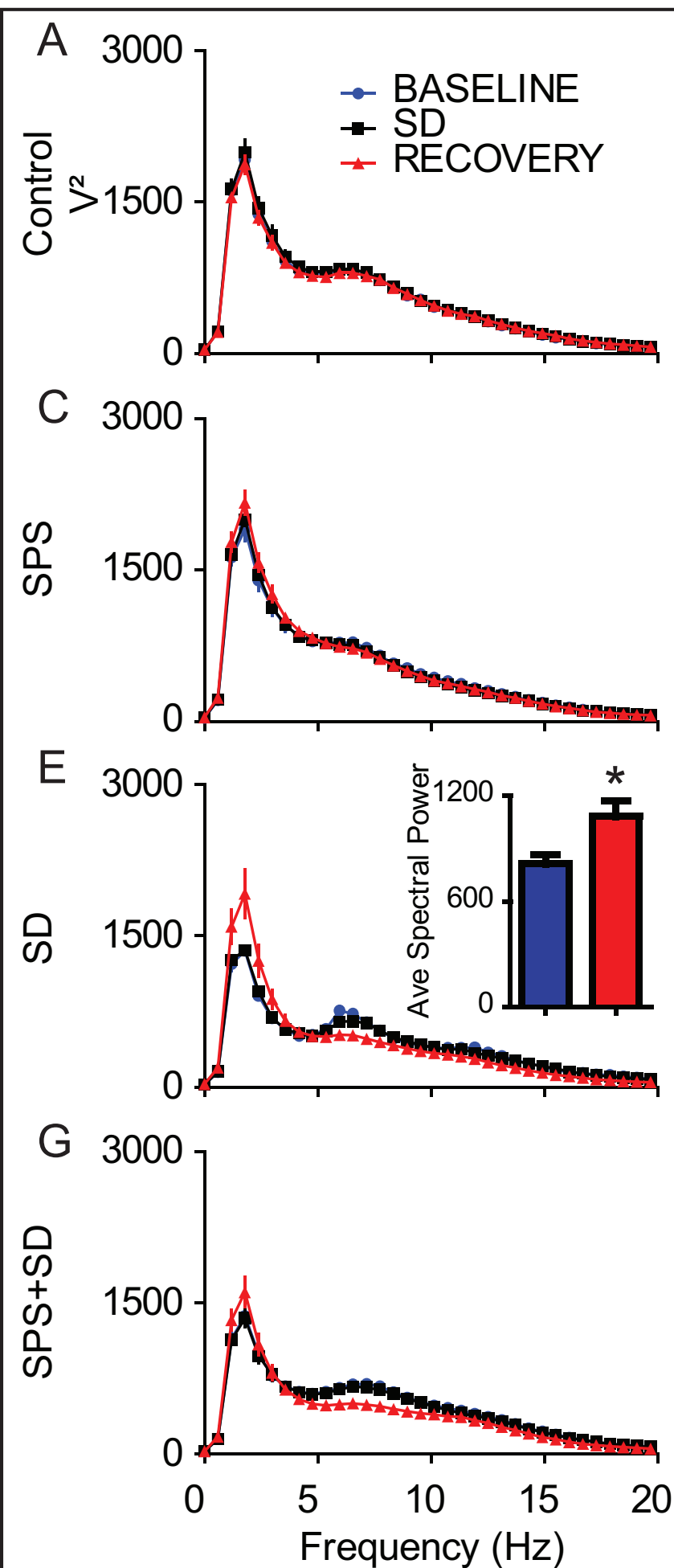


Figure3



NREM SPECTRAL POWER

REM SPECTRAL POWER





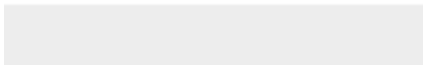
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Optogenetic sleep enhancement improves fear-associated memory processing following trauma exposure

Sleep improvements improve fear-associated memory

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Highlights

- Trauma exposure at ZT12 via Single Prolonged Stress (SPS) results in acute changes in sleep.

- Optogenetic stimulation of melanin-concentrating hormone cells of the lateral hypothalamus for seven days following trauma exposure results in increased REM and NREM sleep.

- Increasing sleep time via optogenetic stimulation significantly improves fear-associated memory processing.

- These data suggest that sleep may be therapeutic in resolving trauma-induced memory impairment and dysfunction in post-traumatic stress disorder.

Keywords

Sleep, Stress, Trauma, Fear-conditioning, Cognition, Optogenetics

Abbreviations

Post-traumatic stress disorder	PTSD
Single Prolonged Stress	SPS
Electroencephalogram	EEG
Electromyogram	EMG
Rapid eye movement sleep	REM
Non rapid eye movement sleep	NREM

46 Selective Serotonin Reuptake Inhibitor SSRI

47

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51

52 Competing Interests

53 The authors declare no competing interests.

54

55 Data Availability

56 Data will be made available upon request.

57 Abstract

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

Introduction

Post-traumatic stress disorder (PTSD) arises as a result of experiencing a physical or psychological trauma and is often accompanied by significant sleep disorders¹. 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 fear⁴⁻¹¹. Humans resilient to PTSD show increased theta activity during rapid eye movement (REM) sleep¹⁰ 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 PTSD¹⁴. 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 required¹⁵. *Single prolonged stress* (SPS) is a rodent model of PTSD that consists of a combination of stressors that include physical restraint, a 20 minute forced swim, exposure to ether vapors (a potent chemical activator of the stress system) until anesthetized, and seven days of social isolation¹⁶. SPS causes molecular, anatomical, and behavioral changes that resemble PTSD in humans¹⁶⁻¹⁸. 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 PTSD¹⁸. 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 asleep¹⁷. 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 rats²⁵. Therefore, in our second set of experiments, we used post-SPS stimulation of MCH cells to increase sleep

119 duration and then assessed fear-associated memory function to determine the contribution of
120 sleep increases to the severity of the PTSD-like phenotype in control and optogenetically
121 stimulated animals.

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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 stereotactically 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 by another lab²⁵. 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 published²⁶.

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 microliters per hemisphere over 20 minutes) was delivered using microinjection pumps (Stoelting, Wood Dale, IL) and 10 microliter Hamilton (Reno, NV) syringes. The injection needle was left in place for an additional 5 minutes 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 (1mg/kg) to manage swelling, infection, and pain, respectively. Post-mortem studies were conducted to validate expression of EGFP as previously published²⁵. 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 data²⁵ (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 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 10Hz for one minute during every 5 minute 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

202 Following recovery from surgery, animals were housed individually and connected to the TDT
203 recording system via a lightweight, flexible tether attached to a commutator (Sparkfun.com, Slip
204 Ring) for free movement within the cage. The recording system was used to sample signals at
205 333 Hz, filtered between 0.1-100 Hz and amplified. Prior to analysis, signals were down-
206 sampled to 250 Hz. The two EEG electrodes were differentially referenced to obtain one EEG
207 channel. Two EMG channels were also differentially referenced to obtain the EMG signal.
208 Animals were given 48 hours to acclimate to the tethers prior to beginning baseline recordings.
209 During the acclimation period, animals were supplied food to last the duration of the EEG/EMG
210 experimental recordings. While connected to tethers, animals were monitored daily for food,
211 water, and health via visual inspection and through the use of a video monitoring system to avoid
212 disturbing the animals.

213

214 Collected data were transferred from the recording PC, stored onto disk, and scored off-line in
215 10-second epochs to determine sleep/waking state using Sleep Sign software (Nagano, Japan).
216 Three vigilance states were assigned: Wake, REM sleep and NREM sleep. Wake consists of
217 visible EEG theta activity and high EMG activity, REM sleep consists of clear, sustained EEG
218 theta activity and phasic muscle twitches on a background of low EMG, NREM sleep consists of
219 high amplitude, synchronized EEG and low EMG activity.

220

221 EEG and EMG signals were recorded for 24 hours of baseline after which the animals were
222 unhooked from the recording system and single prolonged stress (SPS, described below) was
223 performed. Following SPS, animals were reconnected to the recording system and seven
224 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 hours in custom built plexi-glass restraining devices. Next, the animals were placed in a (25x17x16 inch) plastic bin containing 21-24 degree C water and were forced to swim in groups of 6-8 for 20 minutes. Following a 15-minute 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). 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 x 10"D x 12"H)

248 containing a Shock Floor with current carrying metal bars, a wall-mounted speaker and in-
249 chamber UV and white lighting. Test cages were housed in sound-attenuating boxes. Tones
250 (2000 Hz, 80 dB) were delivered via speakers mounted in the housing of the test cages and
251 controlled by data acquisition software (Noldus Ethovision). Ceiling mounted cameras recorded
252 behavior for analysis and Noldus Ethovision software was used to quantify freezing levels.

253

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

259

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

The percent time spent immobile (freezing) within each 60 second long block was calculated by Ethovision software. Statistical comparison of time spent freezing was made using one-way ANOVA and Bonferroni *post-hoc* comparisons via Graphpad 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 hours 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).

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 hours 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.

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 hours 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 minute reduction in REM sleep during the Dark Phase following trauma, and 12 hours later, during the subsequent Light Phase, SPS resulted in a 80 minute increase in REM sleep (Figure 2A) compared to their respective baseline measures. NREM sleep was reduced by 334.5 minutes during the Dark Phase immediately following SPS exposure and was unchanged from baseline for the remainder of the experiment (Figure 2B). SPS exposure increased Wake duration by 380 minutes during the Dark Phase immediately following the trauma (Figure 2C).

A comparison of baseline sleep from rAAV-MCH-ChR2-EGFP injected animals to animals that received a rAAV-MCH-EGFP control vector (no ChR2), and to non-virus injected animals revealed no significant alteration in baseline sleep and indicates that the virus alone had no effect on baseline sleep duration. Additionally, the animals injected with the rAAV-MCH-EGFP control vector showed no changes in sleep when optically stimulated (data not shown).

Sleep architecture was significantly altered by SPS exposure. REM bout length increased by 25 minutes during the Light Phase on the SPS exposure day as compared with baseline (Figure 3A). NREM and Wake bout length was unchanged compared to baseline at any point during the experiment (Figure 3B,C). REM bout number increased day over day during the Dark Phase of the experiment, reaching statistical significance on Day 8 (Figure 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 (Figure 3E,F).

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 minute reduction in REM sleep during the Dark Phase following trauma. 12 hours later, during the Light Phase, REM sleep increased 105 minutes compared to baseline. In addition, REM sleep showed daily increases during the Dark Phase of the experiment that reached statistical significance by Day 8 (Figure 4A). NREM sleep was reduced by 392 minutes during the Dark Phase immediately following SPS exposure and, similar to REM, showed daily increases over the Dark Phase that

reached statistical significance on Day 8 (Figure 4B). SPS exposure resulted in an increase of 468 minutes of Wake compared to baseline 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 (Figure 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 (Figure 5A). NREM bout length was significantly increased during the Light Phase on Day 4 above baseline (Figure 5B). Wake bout length was unchanged compared to baseline at any point during the experiment (Figure 5C). REM bout number decreased during the Dark Phase and increased during the subsequent Light Phase on the SPS exposure day (Figure 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 (Figure 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 (Figure 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 (Figure 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 (Figure 6C). Similarly, when optogenetically stimulated animals were cumulatively compared to control 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 (Figure 7A) nor during the 30 trial, tone only presentations in context A of the extinction period (Figure 7B). During the 10 trial, tone only presentations in context A of the Recall phase of the test (Figure 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 phenotypes²⁷⁻²⁹. 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 (Figure 2-3) show an acute increase in REM sleep, 12 hours after SPS exposure. This 12 hour delay in increased REM sleep is a consistent phenotype across this study and our previous work¹⁷. 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 Figure 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 day post-SPS and future studies should examine the long-term impact of SPS trauma exposure on sleep.

In our laboratory, optogenetic stimulation of MCH cells was sufficient to increase REM sleep duration during both the Light and Dark Phases of the circadian day (Figure 4-6). 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 10Hz 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.

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 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 interesting due to the well described contribution of sleep

in memory processing³¹⁻³⁴ and in the molecular mechanisms that regulate memory³⁵. Post-training sleep deprivation has been shown to impair memory consolidation for associative tasks³⁶, contextual fear memory processing³⁷, and fear avoidance^{38,39}. In 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* ^{40,41}. 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 ⁴². 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 PTSD². Yet, a primary treatment for PTSD remains the SSRI class of drugs which have been shown to reduce or abolish REM sleep altogether^{43,44}. 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 baseline⁴⁵. This effect of melatonin administration was also confirmed in subjects with REM sleep disruptions⁴⁶. Dopamine, norepinephrine and serotonin reuptake inhibitors (reserpine and bupropion), alpha adrenergic blockers (thymoxamine and mesoridazine) selective serotonin antagonists (nefazodone and ritanserine), and cholinergic agonists (carbachol and bethanechol) all increase REM sleep⁴⁷⁻⁵². 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 rodents⁵³, this does not translate to humans and can disrupt NREM and REM sleep^{54,55}.

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

498 sleep improvements on non-fear based cognitive function and to determine how optogenetic
499 manipulation alters the underlying neuro-circuitry involved in fear, memory, and cognition.
500

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642 Author Contribution Statement

643 Dr. Vanderheyden wrote the main manuscript text and figures. Dr. Davis edited the manuscript
644 and assisted with data analysis. All authors have reviewed the manuscript.

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Figure 1. Experimental Timeline. 24 hours 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).

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 hour Dark (or active) Phase (black bars), and the 12 hour 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 ($F(7,55) = 1.6$, $P = 0.05$). *Post hoc* comparisons revealed that REM sleep duration was significantly reduced during the Dark Phase on the SPS day ($M = 99.1 \pm 13$ seconds) compared to baseline ($M = 142.0 \pm 23.6$ seconds). REM sleep during the Light Phase on the SPS day ($M = 399.9 \pm 37.2$ seconds) was significantly increased compared to baseline ($M = 319.5 \pm 16.4$ seconds). 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 ($F(7,55) = 1.52$, $P = 0.19$). *Post hoc* comparisons revealed that NREM sleep duration was reduced during the Dark Phase on the SPS

day ($M = 833.5 \pm 67.5$ seconds) compared to baseline ($M = 1168.0 \pm 154.4$ seconds). 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 ($F(7,55) = 1.79$, $P = 0.12$). Post hoc comparisons revealed that WAKE duration was significantly increased during the Dark Phase on the SPS day ($M = 2670 \pm 77.5$ seconds) compared to baseline ($M = 2290.0 \pm 177.2$ seconds) ($* = P < 0.05$).

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 hour Dark (or active) Phase (black bars), and the 12 hour 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 ($F(7,55) = 0.65$, $P = 0.71$). *Post hoc* comparisons revealed that REM sleep bout length was significantly increased during the Light Phase on the SPS day ($M = 121.3 \pm 16.8$ seconds) compared to baseline ($M = 96.3 \pm 10.9$ seconds). 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 ($F(7,55) = 0.54$, $P = 0.80$). *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 ($F(7,55) = 0.47$, $P = 0.85$). *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 ($F(7,55) = 0.54$, $P = 0.80$). *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 ($M = 10.54 \pm 1.0$) (* = $P < 0.05$).

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 hour, 10 Hz, 1 minute on, 4 minute 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 hour Dark Phase (black bars), and the 12 hour 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 ($F(7,71) = 6.14$, $P < 0.0001$). *Post hoc* comparisons revealed that REM sleep duration was significantly increased during the Light Phase on the SPS exposure day ($M = 491.7 \pm 37.2$ seconds) compared to baseline ($M = 386.7 \pm 30.5$ seconds). REM sleep during the Dark Phase was significantly decreased on the SPS exposure day ($M = 96.1 \pm 18.9$ seconds) compared to baseline ($M = 171.9 \pm 21.9$ seconds) and showed additional, post-SPS, daily increases in REM sleep duration that reached statistical difference from baseline on Day 8 ($M = 247.2 \pm 44.6$ seconds). 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 ($F(7,71) = 4.76$, $P = 0.0003$). *Post hoc* comparisons revealed that NREM sleep duration was significantly decreased during the Dark Phase immediately following SPS ($M = 641.9 \pm 114.6$ seconds) compared to baseline ($M = 1034.0 \pm 70.6$ seconds) and showed increased NREM sleep time on Day 8 ($M = 1267.0 \pm 95.1$ seconds). NREM sleep duration was significantly decreased during the Light Phase on Day 8 ($M = 1733 \pm 62.3$ seconds) compared to baseline ($M = 1918.0 \pm 91.4$ seconds). 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 ($F(7,71) = 18.12$, $P = 0.0002$). *Post hoc* comparisons revealed that WAKE duration was significantly increased during the Dark Phase on the SPS exposure day ($M = 2862.0 \pm 131.2$ seconds) compared to baseline ($M = 2394.0 \pm 91.2$ seconds) and reduced by Day 8 ($M = 2086.0 \pm 114.0$ seconds). There were no significant changes from baseline during the Light Phase ($* = P < 0.05$).

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 hour Dark (or active) Phase (black bars), and the 12 hour 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 ($F(7,71) = 0.81$, $P = 0.58$). *Post hoc* comparisons revealed that REM sleep bout length was significantly increased during the Dark

Phase on the Day 8 ($M = 89.57 \pm 18.1$ seconds) compared to baseline ($M = 59.8 \pm 5.0$ seconds).

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 ($F(7,71) = 0.61$, $P = 0.74$). 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 ($F(7,71) = 1.4$, $P = 0.21$). 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 ($F(7,71) = 5.3$, $P = 0.0001$). *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 ($F(7,71) = 9.9$, $P < 0.0001$). *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 ($F(7,71) = 5.2$, $P = 0.0001$). *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) (* = $P < 0.05$).

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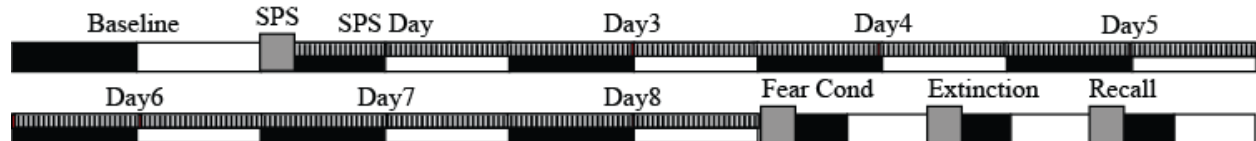
765 Figure 6. Continuous optogenetic stimulation of MCH containing cells of the LH following SPS
766 results in cumulative changes in sleep/wake. The cumulative time spent in sleep/wake stage
767 compared to baseline is shown for each day over the 12 hour Dark Phase (black squares), and the
768 12 hour Light (or sleep) Phase (white circles) over the course of the experiment. a) Linear
769 regression analysis revealed that REM sleep increased during both the Dark Phase ($r^2 = 0.83$, $P =$
770 0.0045) and the Light Phase ($r^2 = 0.89$, $P = 0.0013$) over the course of the experiment. b) NREM
771 sleep showed cumulative sleep time increases specific to the Dark Phase ($r^2 = 0.94$, $P = 0.0004$)
772 whereas, NREM sleep time was unchanged during the Light Phase ($r^2 = 0.46$, $P = 0.09$). c)
773 Similar to the cumulative change in NREM, the cumulative changes in WAKE showed a
774 significant reduction during the Dark Phase over the course of the experiment ($r^2 = 0.92$, $P =$
775 0.007) whereas WAKE was unchanged during the Light Phase of the experiment ($r^2 = 0.15$, $P =$
776 0.37).

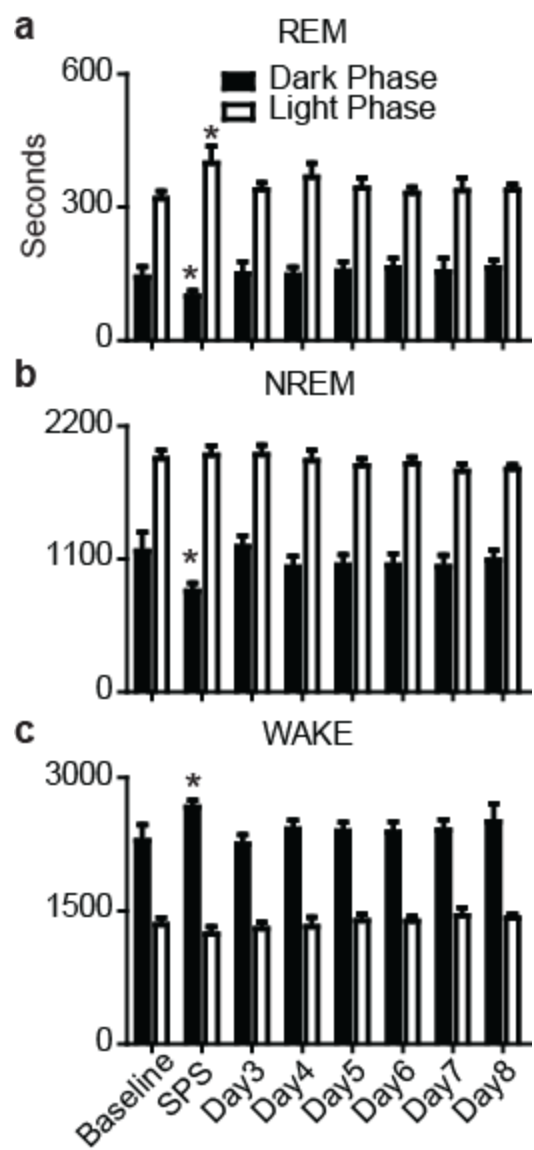
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778 Figure 7. Fear-associated memory impairments are attenuated by optogenetic stimulation of
779 MCH cells. Animals injected with rAAV-MCH-ChR2-EGFP were exposed to SPS and
780 EEG/EMG recordings were made for 7 subsequent days. Control animals (those in figure 2 and
781 3) did not receive optogenetic stimulation, while another group (those in figures 4-6) received 7
782 days of optogenetic stimulation. At the conclusion of the stimulation, all animals underwent fear
783 testing as described in the methods section. a) After the fear conditioning day, optogenetically
784 stimulated (Opto Stim) animals ($M = 43.8$, $SEM = 6.9$) and non-stimulated (Control) animals (M
785 $= 39.8$, $SEM = 5.3$) showed no difference in freezing ($t(14) = 0.44$, $p = 0.67$). b) 24 hours later,
786 fear memory extinction was examined and, again, Opto Stim ($M = 32.6$, $SEM = 4.4$) and control

787 animals ($M = 41.5$, $SEM = 6.8$) had similar freezing levels ($t(14) = 1.14$, $p = 0.27$). c) 48 hours
 788 after initial fear conditioning, fear recall was examined and Opto Stim animals ($M = 17.4$, SEM
 789 $= 1.8$) showed significantly reduced freezing compared to non-stimulated Control animals ($M =$
 790 26.0 , $SEM = 3.7$) ($t(14) = 2.3$, $p = 0.04$) (* = $P < 0.05$).

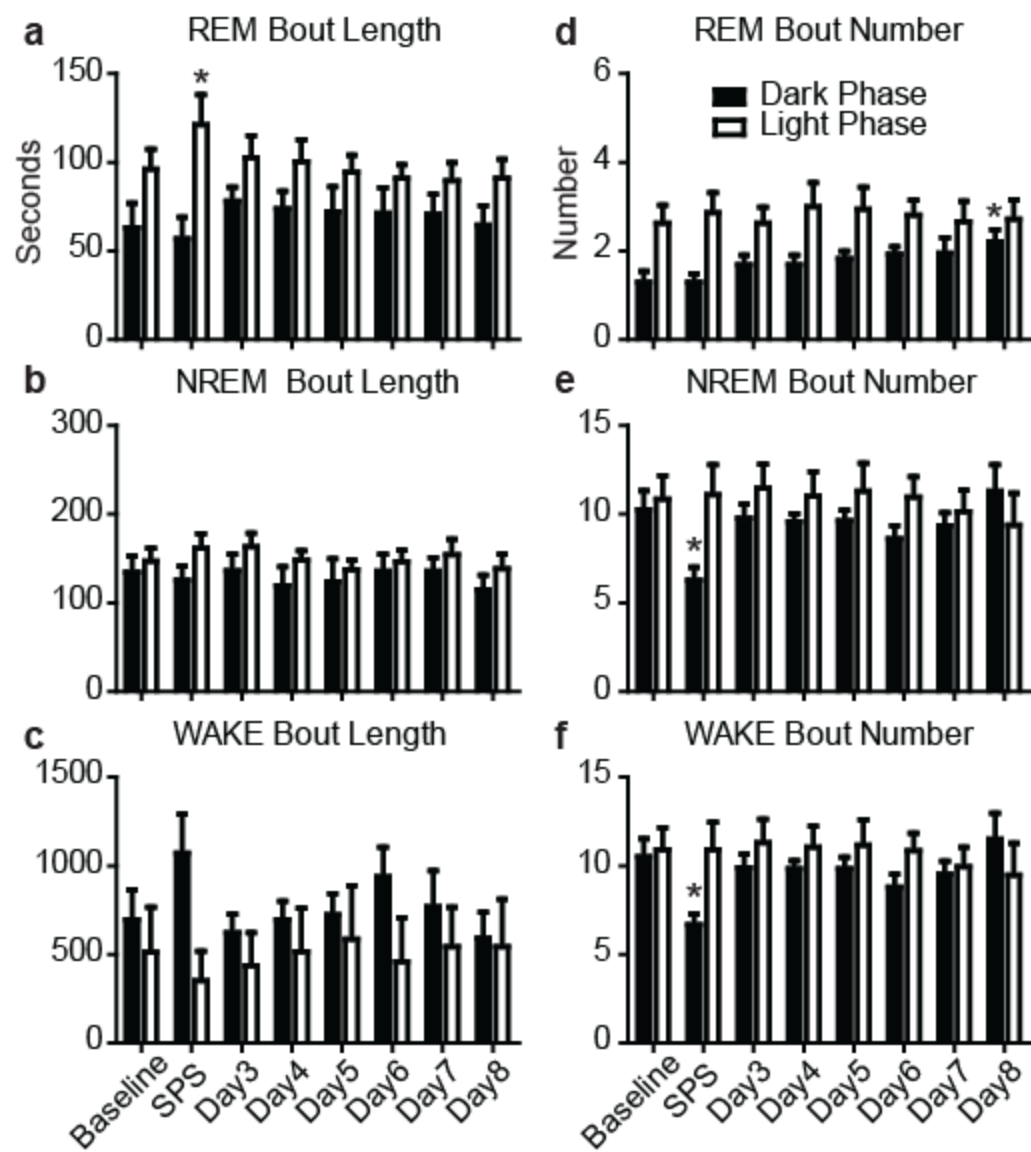
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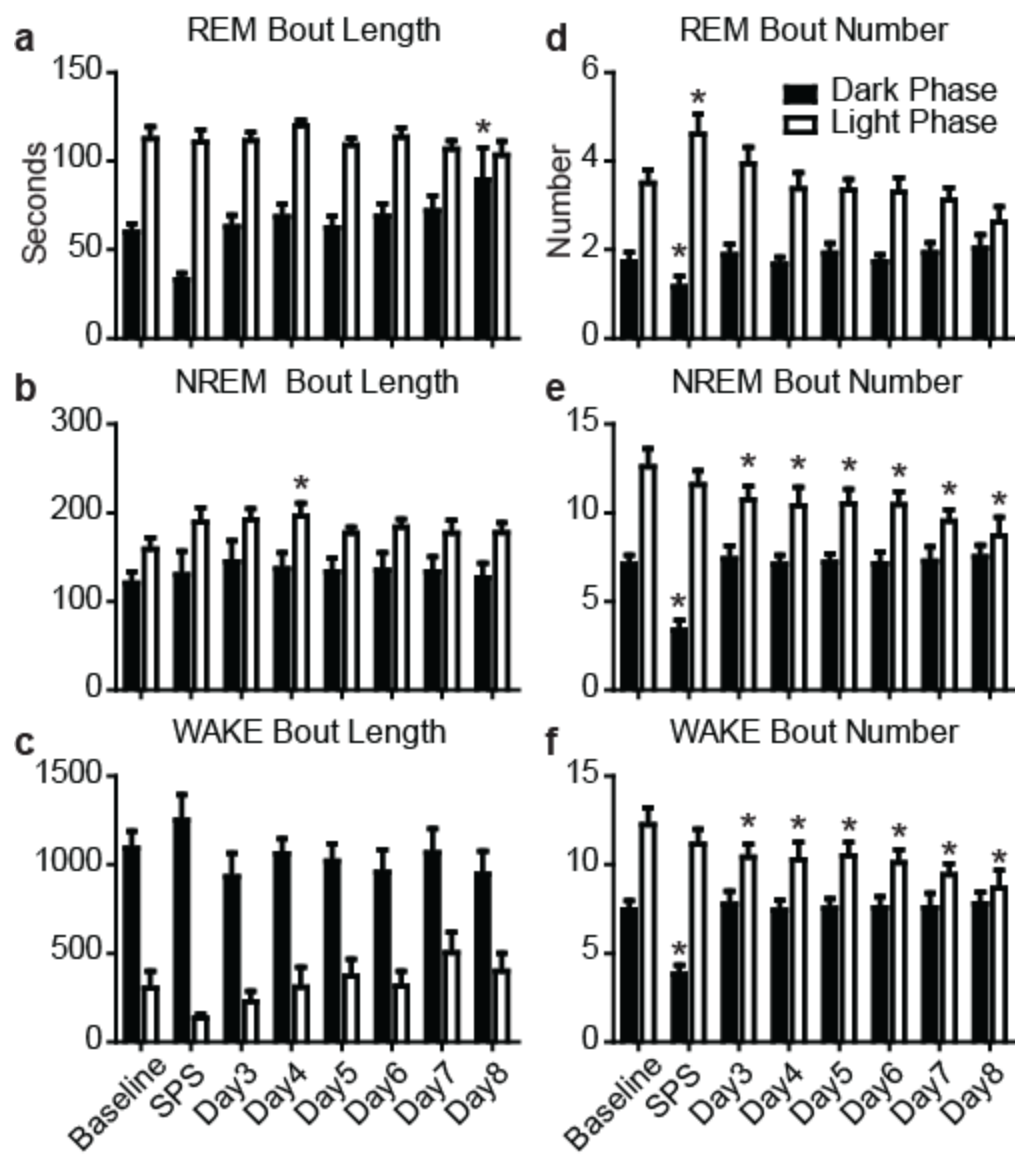




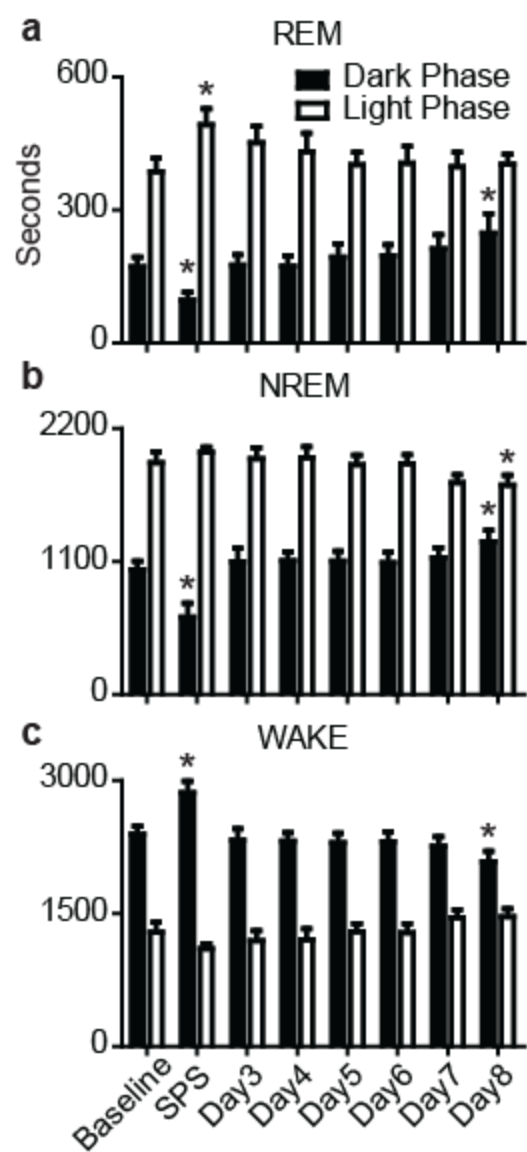
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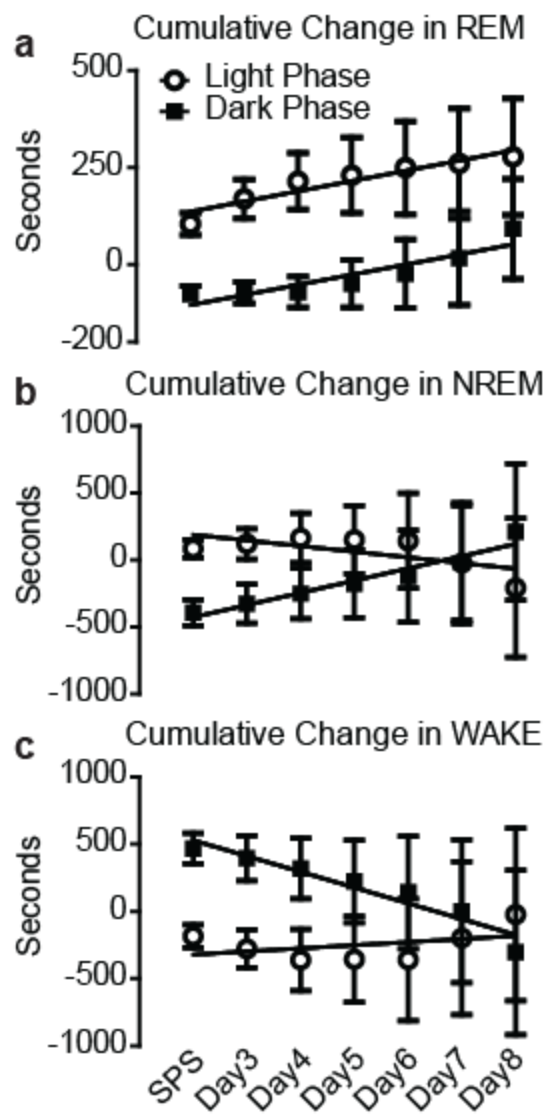


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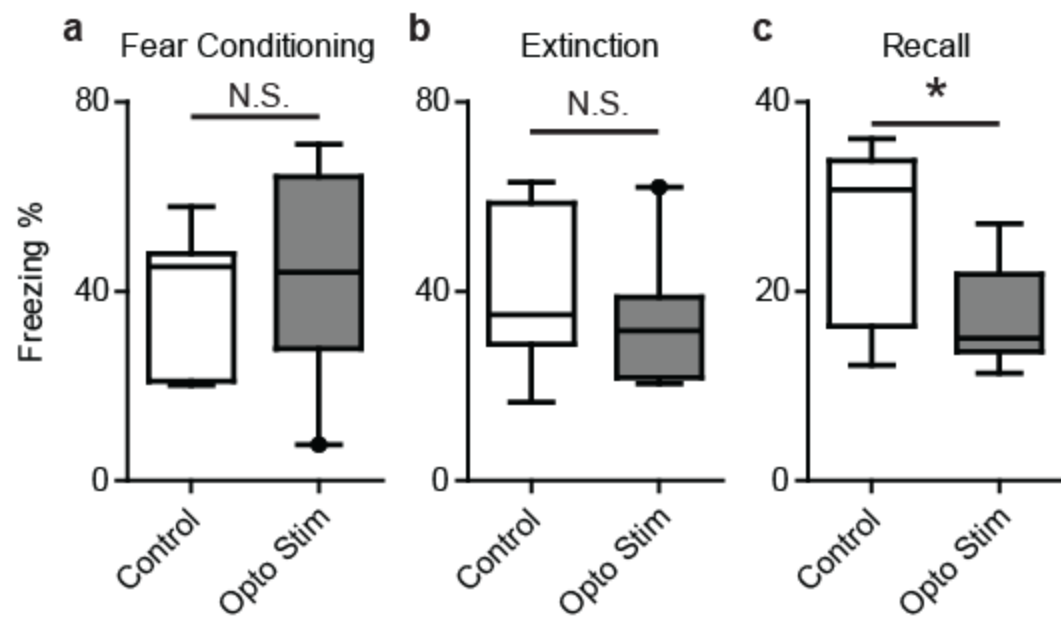
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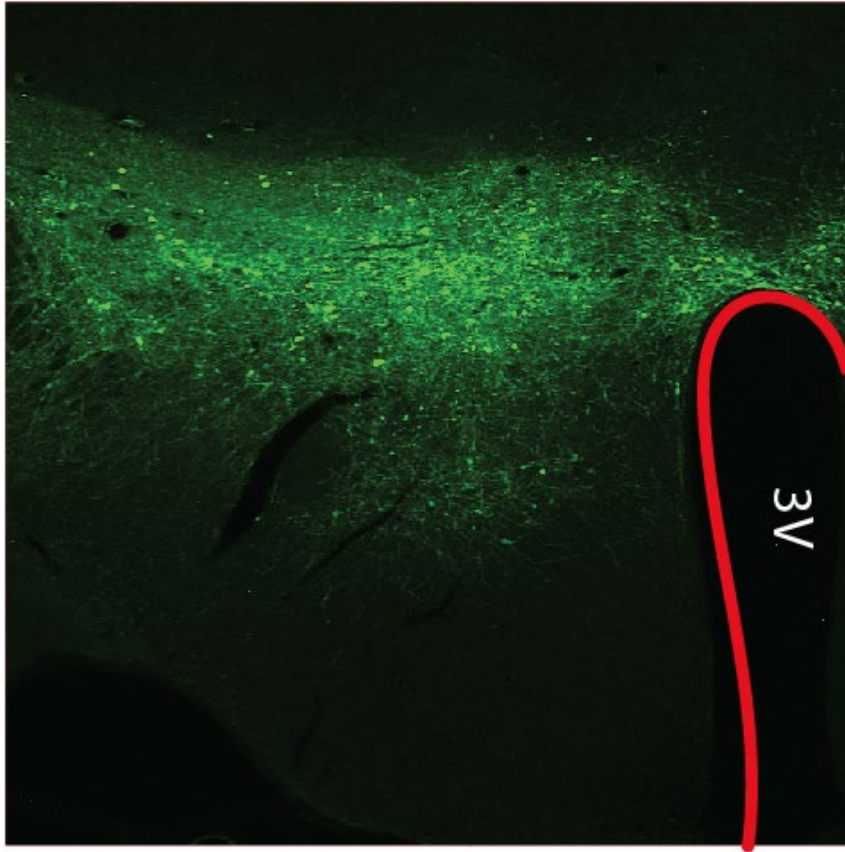


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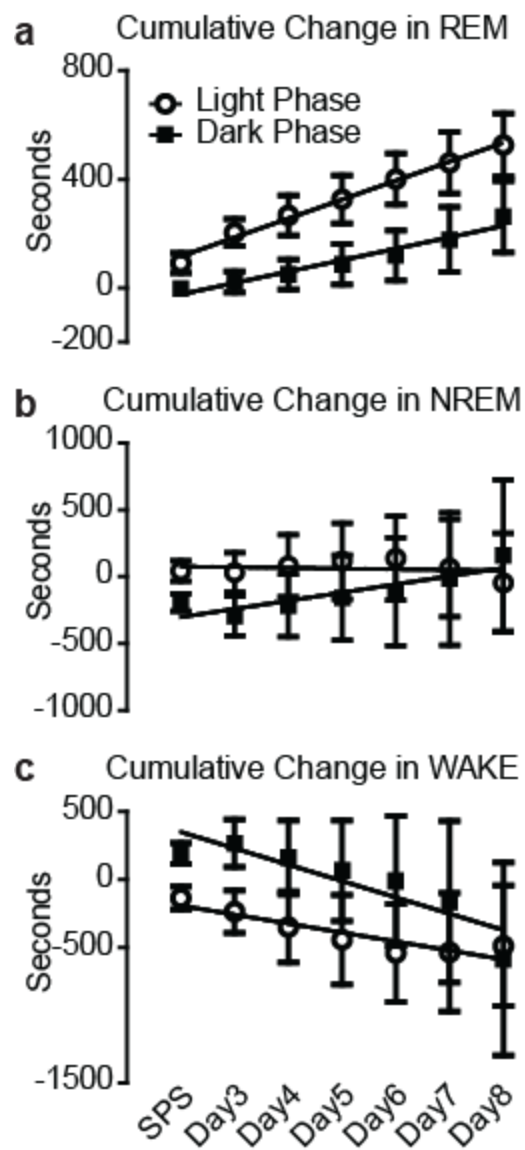
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