AWARD NUMBER: W81XWH-16-1-0313

TITLE: Metabolomics: A Window for Understanding Long-Term Physical Consequences of Disturbed Sleep and Hypothalamic-Pituitary-Adrenal Function in Posttraumatic Stress

PRINCIPAL INVESTIGATOR: Sabra Inslicht, Ph.D.

RECIPIENT: **NCIRE**
4150 Clement St.
San Francisco, CA 94121

REPORT DATE: APRIL 2020

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
APRIL 2020	Annual	31MAR2019 - 30MAR2020

4. TITLE AND SUBTITLE

Metabolomics: A Window for Understanding Long-Term Physical Consequences of Disturbed Sleep and Hypothalamic-Pituitary-Adrenal Function in Posttraumatic Stress

5a. CONTRACT NUMBER
W81XWH-16-1-0313

5b. GRANT NUMBER
PR152209

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Sabra Inslicht, PhD

E-Mail: sabra.inslicht@va.gov

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Northern California Institute
for Research and Education
4150 Clement Street (151NC)
San Francisco, CA 94121-1545

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Armv Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT: Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Post-traumatic stress (PTS) is a common psychiatric condition that may result after combat exposure and can have a profound effect on sleep and physical health conditions, such as metabolic syndrome. Sleep disturbances may lead to alterations in stress response hormones of the hypothalamic-pituitary-adrenal (HPA) axis that may increase metabolic risk. Women may be at particularly high risk for these health concerns, given an increased prevalence of PTS and metabolic conditions in women compared to men. The purpose of this study is to identify biological mechanisms using a broad-based study of metabolomics that may explain differences in PTS, sleep disturbances, and metabolic risk in men and women. This broad approach can reveal circulating small molecules that affect cell and physiological function and will be used to identify biochemical pathways involved in PTS, sleep disturbances, and health. Metabolomic analysis will be performed on pre-collected plasma samples from a study that had a two-group cross-sectional design in which main comparisons were with medically healthy medication-free male and pre-menopausal female subjects with chronic PTS (N= 44) and trauma-exposed, age-matched controls (N= 44). Previously collected measures, including sleep EEG and metabolic markers (e.g., fasting glucose, insulin response to oral glucose tolerance test (OGTT)), fasting lipids, and leptin, will also be examined.

15. SUBJECT TERMS
Adrenocorticotropic hormone; Lipids; Hypothalamic-Pituitary-Adrenal; Kynurenine; Metabolomics; Neurosteroids; Posttraumatic Stress; Polyunsaturated Fatty Acids; Sex Differences; Sleep

16. SECURITY CLASSIFICATION OF:	17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
			USAMRMC

a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified	12	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

1. INTRODUCTION:

Post-traumatic stress (PTS) is a common psychiatric condition that may result after combat exposure and can have a profound effect on sleep and physical health conditions, such as metabolic syndrome. Sleep disturbances may lead to alterations in stress response hormones of the hypothalamic-pituitary-adrenal (HPA) axis that may increase metabolic risk. Women may be at particularly high risk for these health concerns, given an increased prevalence of PTS and metabolic conditions in women compared to men. The purpose of this study is to identify biological mechanisms using a broad-based study of metabolomics that may explain differences in PTS, sleep disturbances, and metabolic risk in men and women. This broad approach can reveal circulating small molecules that affect cell and physiological function and will be used to identify biochemical pathways involved in PTS, sleep disturbances, and health. Metabolomic analysis will be performed on pre-collected plasma samples from a study that had a two-group cross-sectional design in which main comparisons were with medically healthy medication-free male and pre-menopausal female subjects with chronic PTS (N= 44) and trauma-exposed, age-matched controls (N= 44). Previously collected measures, including sleep EEG and metabolic markers (e.g., fasting glucose, insulin response to oral glucose tolerance test (OGTT)), fasting lipids, and leptin, will also be examined.

2. KEYWORDS:

Adrenocorticotropic hormone; Lipids; Hypothalamic-Pituitary-Adrenal; Kynurenine; Metabolomics; Neurosteroids; Posttraumatic Stress; Polyunsaturated Fatty Acids; Sex Differences; Sleep

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Major Study Goals	Timeline (months)	Percentage Complete
1. Study Start Up and Approvals	1-3	100%
2. Coordinate Study Staff for Sample Analysis	1-6	100%
3. Assay Biological Samples	7-30	85%
4. Data Analysis	18-35	85%
5. Finalize study requirements, prepare for future funding, and dissemination of findings	31-42	25%

What was accomplished under these goals?

The study started on September 30th 2016. This report describes accomplishments to date. All study start up activities were completed on schedule, including regulatory paperwork and approvals and hiring and coordination of study staff for sample analysis. Biological samples were organized, procedures for sample shipping and receiving was developed, a tracking system was created, and biological samples were shipped for processing. Three metabolite panels have been assayed to date, including primary amino acid metabolites, steroids and complex lipids. Initial group analyses have been performed. Specialized analyses (e.g., enrichment analysis with subsequent regression analyses) are underway to identify the subgroups of amino acid, steroid, and lipid metabolites that significantly differ by group with follow up on metabolic pathway analysis. A second set of sample analysis is required to be performed to confirm preliminary steroid values. The shipment of samples is awaiting the reopening of the San Francisco VA Laboratory. We have coordinated with Data Management team to monitor data entry and quality. Preliminary data was presented at the American College of Neuropsychopharmacology in 2018 and 2019 meetings, at Sex Differences, Dimorphisms, Divergences: Impact on brain and behavior in health and disease; Sicily, Italy, May 2019; and was accepted for presentation at Biological Psychiatry in May, 2020. Preliminary data was used as pilot work in a grant application for a Discovery Award that was awarded by the DoD. Our detailed accomplishments to date include:

Major Task 1: Study Start Up and Approvals	**Timeline**
Subtask 1: Prepare regulatory documents and submit for IRB approval	Completed
Develop IRB application and other regulatory documents	Completed
Submit IRB application to UCSF IRB and obtain full committee review	Completed
Review by SFVAMC regulatory personnel	Completed
Review by HRPO	Completed
Prepare IRB reports for continuing review approvals	Annually
Milestone Achieved: IRB approval from UCSF, VA, and HRPO	Complete
Major Task 2: Coordinate Study Staff for Sample Analysis	**Timeline**
Subtask1: Hiring and Training of Study Staff	
Coordinate with NCIRE to prepare job description and advertisement	Complete
Interview research staff candidates	Complete
Coordinate with SFVAMC for candidate approval and required trainings	Complete
Training of research staff on study procedures and biospecimen storage, shipping, and receiving	Complete
Milestone Achieved: Research staff hired and trained	Complete
Subtask 2: Coordinate with laboratory personnel for sample shipments	
Contact staff at receiving laboratories	Complete
Develop procedures manual for sample shipping and receiving	Complete
Develop sample tracking system	Complete
Schedule batched shipments	Complete
Milestone Achieved: Sample shipment protocol established	Complete

Subtask 3: Build database for incoming data	
Work with Data Manager to establish data extraction protocol and build database	Complete
Establish logistical plan for data quality check	Complete
Milestone Achieved: Database built	Complete
Major Task 3: Assay Biological Samples	**Timeline**
Subtask 1: Ship stored samples to the receiving laboratory and acquire data	
Package and ship stored samples to UC Davis	Complete
Milestone Achieved: 1st batch of samples shipped for assay	Complete
Milestone Achieved: Final batch of samples shipped for assay	In process
Subtask 2: Receive data from laboratory	In process
Milestone Achieved: All data acquired	In process
Milestone Achieved: All Assays complete	In process
Major Task 4: Data Analysis	**Timeline**
Subtask 1: Enter data and maintain database	Complete
Perform quality checks on incoming data	In process
Enter all data and maintain database	In process
Subtask 2: Aim 1: To ascertain the neurosteroid (including glucocorticoid) metabolite profile in plasma of male and female patients with PTSD, and in healthy controls	
Clean and process incoming data and prepare for analysis	In process
Subtask 3: Aim 2: To ascertain the primary amino acid and lipid metabolite profiles in plasma of male and female PTSD patients, and in healthy controls	
Clean and process incoming data and prepare for analysis	In process
Major Task 5: Finalize study requirements, prepare for future funding, and dissemination of findings	**Timeline**
Subtask 2: Prepare grant application for DOD or VA Merit Award funding for a clinical trial based on study findings	Complete

Preliminary Findings:

BACKGROUND: A metabolomics analysis has the potential to explain the regulation of metabolic pathways and networks of physiologically relevant interactions that lead to increased health risks in PTSD. As an untargeted approach, metabolomics identifies changes in circulating small molecules that affect cell and physiological function and can provide a more comprehensive examination of the broad range of physiological pathways that may be missed with more traditional, targeted approaches. **METHODS:** Metabolomic analyses were performed on fasting plasma samples collected from a cross-sectional study (PTSD/control) involving 90 medically healthy, non-obese, non-medicated male and follicular phase female adults who were participating in a 3-night sleep study in an inpatient sleep laboratory. Participants included 44 PTSD subjects (49% female) and 46 controls (42% female). Ages were 19-39 years (Mean Age = 30). The Clinician Administered PTSD Scale was used to determine PTSD status. The Structured Clinical Interview for DSM-IV was used to the presence of other psychiatric diagnoses. Biochemical assays included the identification of the following: 1) **Lipid metabolites:** Ceramides, sphingomyelins, cholesteryl esters, oxysterols, lyso- and phospholipids, mono-, di- and triacylglycerols, galactosyl- and glucuronyllipids were assayed using Agilent Technologies 1290 UHPLC with Agilent 6530 QTOF mass spectrometer; 2) **Steroids:** Targeted mode using UPLC and quadrupole linear ion trap mass spectrometry (UPLC-QTRAP MS/MS).

STATISTICAL ANALYSES: Statistical analyses were performed using R (version 3.5.1). Metabolomic data were transformed to log base 10 scale. Missing values were replaced by 1/2 of detected minimum value for each lipid. Mann-Whitney U tests and logistic regression were performed to identify compounds that were significantly associated with PTSD. P-values were adjusted for multiple testing using Benjamini-Hochberg procedure.

Chemical Set Enrichment Analysis (ChemRICH) (4) were performed on metabolites to identify chemical classes significantly altered in PTSD compared to control samples in males and females separately. This technique is a statistical enrichment approach based on chemical similarity. It provides an alternative to pathway analysis that relies on sparse biochemical knowledge annotations. The method uses structure similarity and chemical ontologies to map known metabolites and name metabolic modules and it yields study-specific, non-overlapping sets of all identified metabolites. An advantage of ChemRICH is that sets have self-contained size: p-values do not rely on size of background database.

Preliminary Results:

Complementing our earlier findings of hyperlipidemia in PTS [8], the metabolomics approach allowed for identification of 217/414 lipid metabolites, 61/104 primary metabolites, and 2/53 steroids that were mostly upregulated in PTS (See Figure 1). There were 67 known lipid metabolites that differed by sex (p's<.05). Interestingly, the greatest majority of lipid alterations in PTS occurred in men, but not in women. In dividing the sample by sex, 138 lipid metabolite (8 out of 11 lipid clusters) alterations in PTS were found in men, whereas only 42 (4 clusters) were altered in women (p's<.05).

Figure 1. Heatmap indicating the distribution of lipid alterations according to sex and PTSD status.

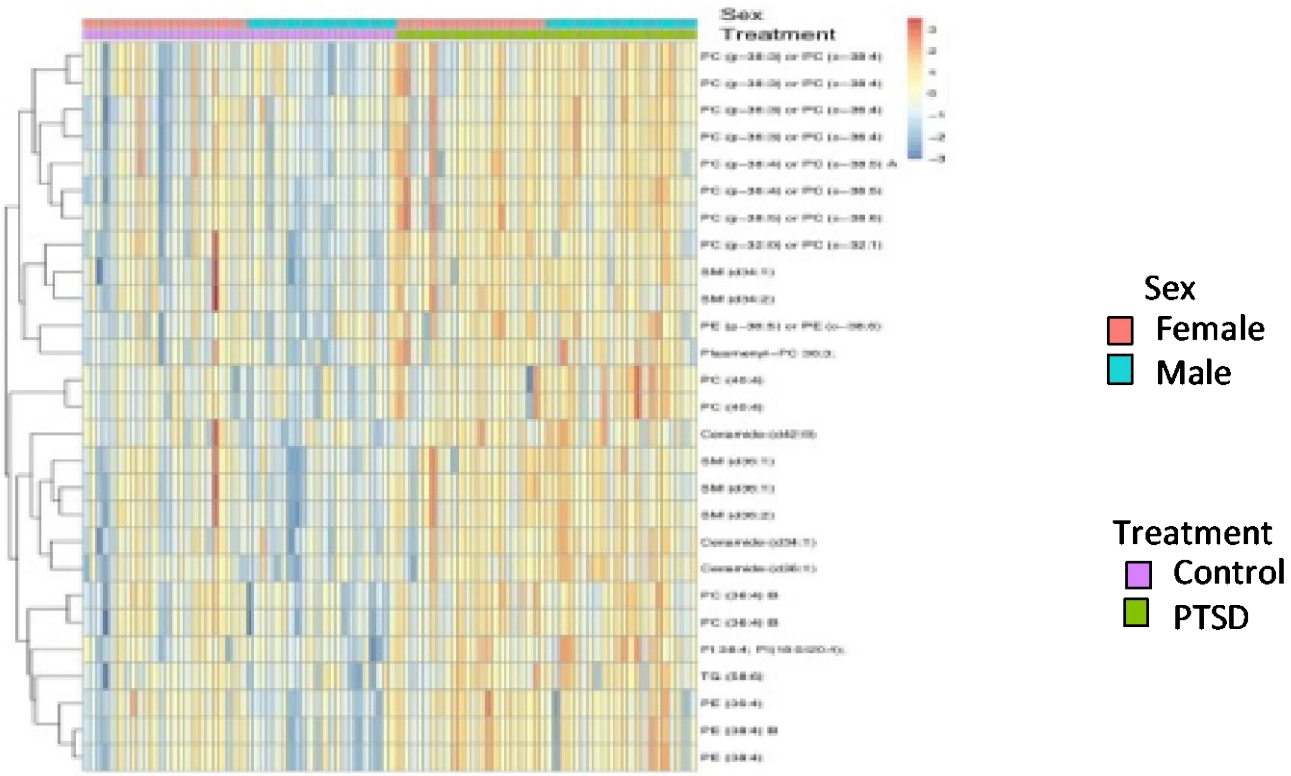
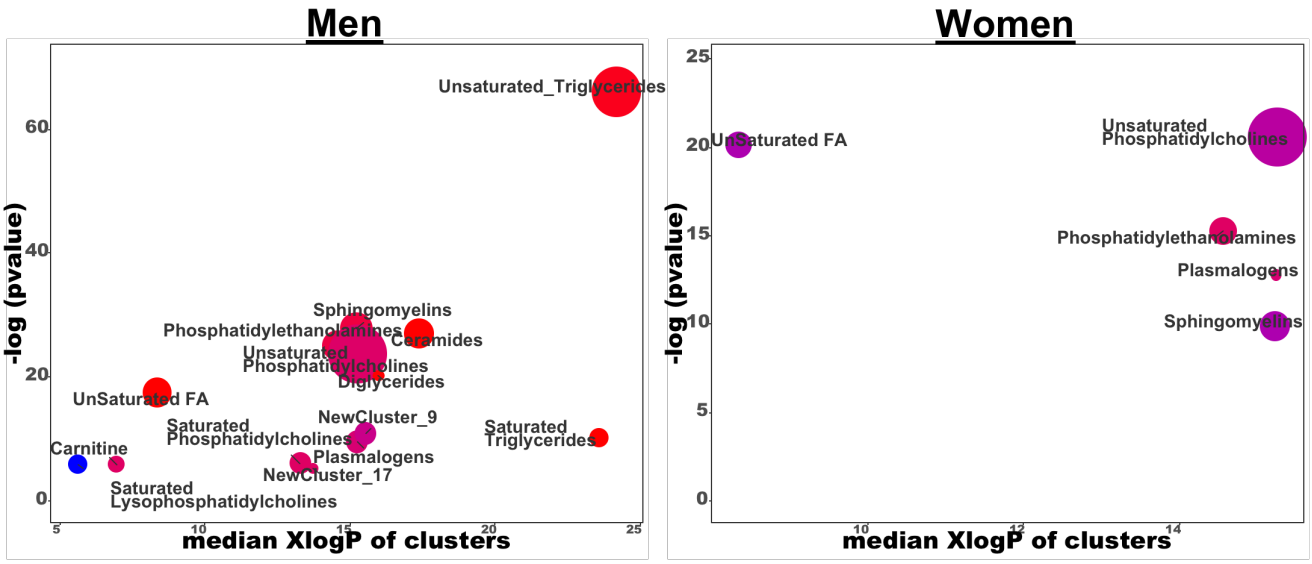


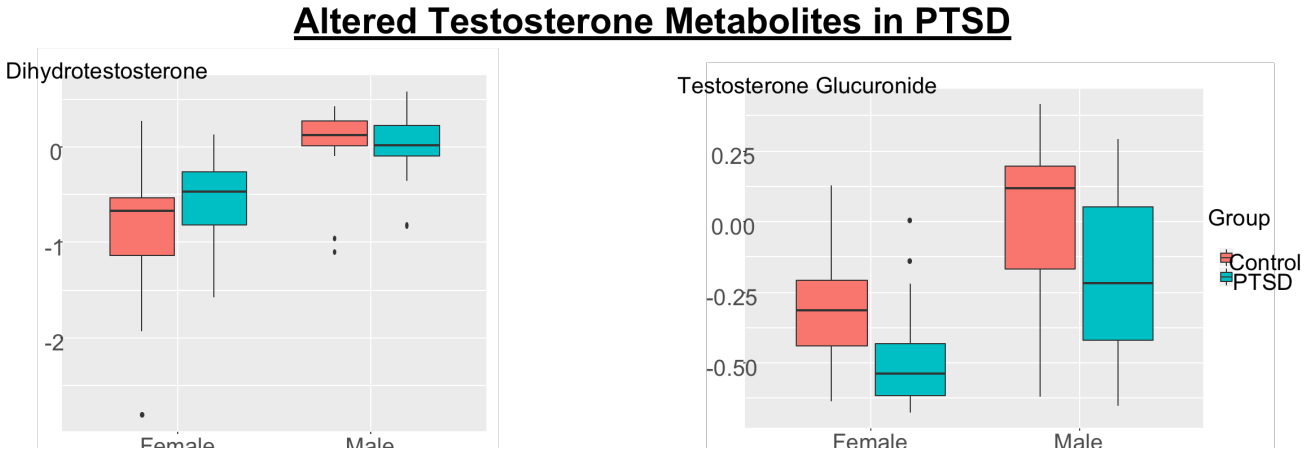
Figure 2. ChemRICH Analysis: Lipid Metabolite Modules Altered in PTSD



ChemRICH set enrichment statistics plot. Each node reflects a significantly altered cluster of metabolites. Enrichment *p*-values are given by the Kolmogorov–Smirnov-test. Node sizes represent the total number of metabolites in each cluster set. The node color scale shows the proportion of increased (red) or decreased (blue) compounds in PTSD to controls. Purple-color nodes have both increased and decreased metabolites. **PTSD was associated with alterations in 8 out of 11 lipid clusters in men, but only 4 in women.**

Multiple linear regression analyses were performed within each sex controlling for sex steroids that were found to be altered in PTSD to determine their potential influence on lipid alterations (Figure 3). Among women, 381 lipids (vs. 238) were significantly associated with PTSD when controlling for testosterone glucuronide. Among men, 781 lipid compounds (vs 813) were significantly associated with PTSD, when controlling for testosterone glucuronide. These findings suggest that testosterone glucuronide accounted for the relationship between PTSD and lipid alterations, in women in particular.

Figure 3. Testosterone Glucuronide Accounts for Lipid Metabolite Alterations in women with PTSD



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

The PI has participated in the following scientific meetings:
2016 American College of Neuropsychopharmacology Annual Meeting
2017 American College of Neuropsychopharmacology Annual Meeting
2017 Biological Psychiatry Annual Meeting
2018 VA PTSD Psychopharmacology Initiative
2018 VA NCPTSD Women Veteran's Health Summit
2019 American College of Neuropsychopharmacology Annual Meeting
2019 VA Research Week
2019 Sex Differences, Dimorphisms, Divergences: Impact on brain and behavior in health and disease meeting
2020 Biological Psychiatry

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

Plans until next reporting period:

1. Assay remaining samples for steroids and confirm preliminary steroid findings.
2. Finalize data analysis tasks: We are currently working with Dr. Fiehn and the Biostatisticians to conduct chemRich analyses on significant metabolites and conduct correlational analyses with health outcomes (Task 4).
3. Continue dissemination of study findings and finalize study closeout requirements (Task 5).
    a. Submit 2 manuscripts
    b. Present data on sex differences in lipids at American College of Neuropsychopharmacology Annual Meeting
    c. Submit data for presentation at Biological Psychiatry Annual Meeting, April 2021
    d. Apply for additional future funding

Major Task 4: Data Analysis	**Timeline**
Subtask 2: Aim 1: To ascertain the neurosteroid (including glucocorticoid) metabolite profile in plasma of male and female patients with PTSD, and in healthy controls	
Work with Biostatistician to conduct analyses	In progress
Share output and findings with co-investigators	In progress
Milestone Achieved: Aim 1	In progress
Subtask 3: Aim 2: To ascertain the primary amino acid and lipid metabolite profiles in plasma of male and female PTSD patients, and in healthy controls	
Work with Biostatistician to conduct analyses	In progress
Share output and findings with co-investigators	In progress
Perform the integration of the metabolite data with the health outcome data that we have collected, including measures of HPA axis function, sleep EEG, triglycerides, blood glucose, and body fat content.	In progress
Milestone Achieved: Aim 2 addressed	*42*
Milestone Achieved: Data analysis complete	*42*
Subtask 4: Share output and findings with co-investigators and with the greater community	
Conduct the background research to interpret the results and the implications of these findings and to write manuscripts to disseminate the findings.	In progress
Dissemination of findings (abstracts, presentation, publications, DOD)	In progress
Milestone Achieved: Report results from data analyses	*31-42*
Milestone Achieved: Characterize the metabolomic profile associated with glucocorticoid regulation mediating sleep and metabolic disturbances associated with PTS	*31-42*
Milestone Achieved: Identify specific metabolites associated with PTS for future clinical trial	*31-42*
Perform the integration of the metabolite data with the health outcome data that we have collected, including measures of HPA axis function, sleep EEG, triglycerides, blood glucose, and body fat content.	31-42
Major Task 5: Finalize study requirements, prepare for future funding, and dissemination of findings	**Timeline**
Subtask 1: Dissemination of findings (abstracts, presentation, publications, DOD)	In progress
Subtask 3: Complete final report.	42
Milestone Achieved: Report results from data analyses	*42*
Milestone Achieved: Submit grant proposal for clinical trial to examine changes in specific metabolites and related inflammatory and metabolic processes on limbic responses in an fMRI paradigm.	*42*

4. IMPACT:

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

Nothing to report

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

The study has obtained a no-cost extension to 3/30/2021. Due to COVID-19, we experienced laboratory closures at the San Francisco VA Health Care System laboratory and at West Coast Metabolomics Center. We were therefore unable to organize and ship the remaining blood samples to WCMC for the final assays for steroids required to validate and confirm our preliminary findings. WCMC is the only laboratory with the necessary equipment and technical expertise to perform the assays needed to answer the specific research aims: to ascertain the primary amino acid and lipid metabolite profiles in plasma of male and female PTS patients, and in healthy controls. We expect to send the final shipment for assay upon reopening of the laboratories. We will then be able to complete the final statistical analysis and finalize the study results.

The initial findings on PTSD effects on metabolites was presented at the following conferences:

Inslicht S.S., Bhargava, A., Olshen A., Lujan, C., Neylan, T.C. The Lipidome in PTSD. The American College of Neuropsychopharmacology Annual Meeting, December 9-13, 2018, Hollywood, FL

Bhargava, A., Fan, S., Lujan, C., Feihn, O., Neylan, TC, Inslicht, S.S. Lipidome Analysis in Men and Women with Posttraumatic Stress Disorder. Sex Differences, Dimorphisms, Divergences: Impact on brain and behavior in health and disease; Sicily, Italy, May 2019.

The findings on sex differences in lipid metabolites were presented at the following conferences:

Inslicht S.S., Bhargava, A., Olshen A., Lujan, C., Neylan, T.C. Sex Differences in Lipid Metabolism in PTSD. The American College of Neuropsychopharmacology Annual Meeting, December 8-11, 2019, Orlando, FL.

Inslicht S.S., Bhargava, A., Olshen A., Lujan, C., Neylan, T.C. Sex Differences in Lipid Metabolism in PTSD. Poster accepted at the 75th Annual Meeting for the Society for Biological Psychiatry, April 30 - May 2, 2020, New York, NY.

A related manuscript on secondary variables from this dataset has been published:

Inslicht, S.S., Rao, M.N., Richards, A., Gibson, C., Metzler, T.J., Neylan, T.C. Sleep and HPA Axis Responses to Metyrapone in Posttraumatic Stress Disorder. Psychoneuroendocrinology. 2017 Dec 7; 88:136-143. PMID: 29268182

A grant was funded based on these preliminary findings:

Discovery Award, Peer Reviewed Medical Research Program 2019 (PR192475).
Sex differences in stress-related cardiometabolic risk in PTSD (PI: Inslicht).
This project proposes to examine the impact of PTS on female biology and increased CVD risk. For this Discovery Award, we propose to analyze data that were collected and stored in a previous study of sex differences in stress-related psychophysiological responses in men and women with chronic PTS and trauma-exposed healthy controls. Blood was drawn at 4 timepoints, providing multiple measures across the menstrual cycle. We will to assay these stored samples to address the following specific aims: Aim 1. To examine lipid metabolites that associate with CVD risk and PTS in women (across the menstrual cycle) relative to men. Aim 2. To examine which sex steroids modulate the relationship between CVD risk, PTS and lipid metabolism in women relative to men.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	**Thomas Neylan**
Project Role:	Co-Investigator
Nearest person month worked:	*1 person month*
Contribution to Project:	Dr. Neylan has extensive expertise in the biology of PTSD, sleep, metabolic function, clinical trials, and laboratory-based psychophysiological research. He provides onsite support to Dr. Inslicht on the conduction of the proposed project, interpretation of sleep and HPA axis data, and will be involved in data analysis and manuscript preparation.

Name:	**Aditi Bhargava**
Project Role:	Co-Investigator

Name:	**Sabra Inslicht**
Project Role:	Principal Investigator
Nearest person month worked:	1 person month
Contribution to Project:	Dr. Inslicht has expertise in psychophysiology and the neuroendocrinology of PTSD. Dr. Inslicht assumes overall scientific and administrative responsibility for this project, ensuring that research goals are met in a timely manner with scientific integrity. She has designed and is implementing each phase of the research plan. She is working with the study coordinator to oversee human subjects regulatory documentation and compliance, coordination of personnel involved in this protocol, the coordination of assay completion, as well as the development of a data tracking system to manage participant information, biological samples, and assay data. Over the next reporting period, Dr. Inslicht will work with the statistician to conduct data analyses and will prepare manuscripts and disseminate findings.

Nearest person month worked:	*1 person month*
Contribution to Project:	Dr. Bhargava is molecular biologist with extensive research experience in the area of neuroendocrinology, including pain, stress, and inflammation. Dr. Bhargava is responsible for design, execution, data analysis, and manuscript preparation. She is also responsible for conduction of assays in collaboration with colleagues at the UC Metabolomics Core.

Name:	**Callan Lujan**
Project Role:	*Study Coordinator/Staff Research Associate*
Nearest person month worked:	*6 person months*
Contribution to Project:	Ms. Lujan prepares all regulatory submissions to the IRB and VA Research and Development Committee and oversees compliance. Ms. Lujan supervises and coordinates study personnel, assists with sample organization and shipping, and the coordination of assay completion. Ms. Lujan has been working with the SFVA Stress and Health program data manager to develop a data tracking system to manage participant information, biological samples, and assay data.

Name:	**Olga Mayzel**
Project Role:	Database Manager
Nearest person month worked:	1 person month
Contribution to Project:	Ms. Mayzel has created a database for tracking biological samples, participant information, and metabolomics data collection. The database manager oversees database operations and will maintain all computer equipment including a main data server.

Name:	**Thomas Metzler**
Project Role:	Biostatistician
Nearest person month worked:	1 person month

Contribution to Project:	Mr. Metzler will continue to work closely with the investigators to complete correlate measure analyses.

Name:	**Ritu Roy**
Project Role:	Biostatistician
Nearest person month worked:	1 person month
Contribution to Project:	Dr. Roy has provided bioinformatics support for the metabolomics data and will continue to provide statistical support to complete metabolite analyses.

Name:	**Oliver Fiehn, PhD**
Project Role:	Consultant
Nearest person month worked:	1 person month
Contribution to Project:	Dr. Fiehn is the director of the West Coast Metabolomics Center at UC Davis. He is currently reviewing the outputs from the completed assays and will determine if additional assays are required.

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

Nothing to report

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS
COLLABORATIVE AWARDS: Nothing to Report

9. APPENDICES: N/A